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Research Article

Mycorrhizal Sainfoin (*Onobrychis sativa* L.) Plant Responses to Water Deficit Stress

Sakineh Abdi[1] and Alireza Pirzad[2]

[1]*Ahar Faculty of Agriculture and Natural Resources, University of Tabriz, Iran*
[2]*Department of Agronomy, Urmia University, Iran*

***Corresponding author**
Sakineh Abdi, Ahar Faculty of Agriculture and Natural Resources, University of Tabriz, Iran, Tel: 009-8914-4263-179; Fax: 009-8414-4237-718; Email: s.abdi@tabrizu.ac.ir

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Abstract

Water stress and the deficiency of mineral nutrients are major constraints that limit forage legumes production, particularly in arid and semi-arid regions. To evaluate the role of arbuscular mycorrhizal fungi (AMF) in alleviating the aforementioned condition, a greenhouse factorial experiment on sainfoin plant (*Onobrychis sativa* L.) was conducted. The experiment was based on the completely randomized design (CRD) with three replications in 2014. The treatments were carried out on species of fungi (*Faneliformis mosseae*, *Rhizophagus intraradices*, *Claroideoglomus claroideum*, *Funneliformis caledonius*, *Glomus versiforme*, and non-inoculated control) and irrigation (irrigation at 80% (well watering) and 50% (water deficit) field capacity (FC)). The highest root colonization (66%) occurred with *G. versiforme* in well-watered plants. The mycorrhizal (*F. mosseae* and *Rh. intraradices*) sainfoin leaf chlorophyll index (SPAD) increased as a result of water deficit stress. The leaf osmolytes (proline and total soluble sugars) increased in all mycorrhizal plants. Mycorrhizal species produced taller plants than those produced under non-inoculated control. The highest leaf phosphorus content was obtained from plants inoculated with *F. mosseae* (294.0 mg / 100 g dry weight). In well-watered sainfoin plants, the highest percent of leaf N (4.73%) belonged to *G. versiforme*. Leaf dry weight showed a significant decrease in irrigation at 50% FC, but the mycorrhizal symbiosis compensated it to more than well-watered plants. In this research work, all mycorrhizas species were significantly enhanced the leaf properties due to highly root colonization caused in water and phosphorus uptake.

INTRODUCTION

Sainfoin (*Onobrychis sativa* L. Scop, Fabaceae family) is an important perennial forage legume preferred by farmers due to high palatability, high nutritional value properties, and its ability to improve soil fertility [1,2]. Today, in many arid and semi-arid regions of the world, drought stress and deficiency of available mineral nutrients - especially phosphorus (P) - limits agricultural production [3]. Furthermore, the growth and productivity of legumes are reduced under water-deficit conditions [4]. The use of Arbuscular Mycorrhizal Fungi (AMF), as relievers of the drought stress effects on plants, has been studied for many years. AMF symbiosis with plant roots can improve crop production under irrigated and drought-stressed conditions [5,6]. Its mycelium can enlarge the absorption range of the roots, which helps accelerate the absorption of water and nutrients. This is more pronounced in the case of P element, which moves 10 times faster in mycelium than in roots [7-10].

In the absence of AMF, much higher amounts of P fertilizer are required (as a part of several plant structure compounds and as a catalyst in the conversion of numerous biochemical reactions) to obtain the same level of productivity as procured by plants, which are mycorrhizal [11]. AMF symbiosis of plants helps to tolerate water deficit by improving hydraulic conductivity, water absorbing capacity, changing water relations, expanding root system, improving plant nutrition, and increasing plant metabolism [12]. Osmolytes are associated with the stability of turgor pressure [13]. Therefore, in the water deficit conditions, the total soluble sugar [14] and proline accumulation [15-17], have been observed in mycorrhizal plants in comparison with non-mycorrhizal plants. Mung bean plants that inoculated with *G. intraradices* exhibited further colonization percentage and leaf phosphorus under water-deficit conditions [18,19]. The benefits of AMF inoculation depend on the genotypic host-fungus combinations and also on the type of inoculums used [20].

Iran is located in arid and semi-arid zones (73% of the country) of the world. Therefore, the main objective of this project was to study AMF species including *F. mosseae*, *Rh. intraradices*, *C. claroideum*, *F. caledonius,* and *G. versiforme* on the forage yield. It also aimed to examining the physiological responses of sainfoin plants under water deficit conditions. However, we have very few works in the literature on the second objective of our study.

MATERIALS AND METHODS

Experimental location

The greenhouse study (in open space) was done to evaluate the effects of mycorrhizal symbiosis of fungi species in alleviating of water deficit in the *Onobrychis sativa* plants at the University of Tabriz, Ahar Faculty of Agriculture and Natural Resources

(38º28' N, 47º4' E and 1391 altitude). Environmental conditions of the experimental site, including the highest and the lowest temperatures, humidity, and the total time spent under the sun are shown in Table 1. Some physicochemical properties of soil, used in pots, were determined (Table 2).

Experimental design

The experiment was arranged as factorial based on completely randomized design with three replications (four pots in each replicate, two pots for forage harvest and two pots for grain harvest). The treatments included five species of mycorrhizal fungi (*F. mosseae* (synonym = *Glomusmosseae*), *Rh. Intraradices* (synonym = *G. Intraradices*), *C. claroideum* [synonym = *G. claroideum*), *F. caledonius* (synonym = *G. caledonium*), *G. versiforme,* and non-inoculated plant as control) and two levels of irrigation (irrigation at 80% (well watering) and 50% (water deficit) of field Capacity (FC)). The mycorrhizal inoculum, produced on maize host plants, was a mixture of sterile sand, mycorrhizal hyphae, spores (20 spores g^{-1} inoculum), and colonized root fragments, provided by the Urmia University (Dr. Y. Rezaee Danesh, Department of Plant Protection). Seeds of sainfoin plants were sown in pots (22cm depth and diameter) 2cm deep on May 14, 2014. In each pot, 20grams of the appropriate inoculum was placed below the seeds and covered with soil, and irrigated immediately. For non-inoculated control, plants were sown in untreated pots. When the seedlings grew, those were thinned out leaving three plants per pot. The irrigation treatments (irrigation at 80% and 50% of Field Capacity) started when plants were well-established (at three primary leaf stage on June 5). Daily records of soil moisture were taken to define the irrigation time. The pots were irrigated to get FC during the growing period of the plants.

Measurements

To measure the leaf phosphorus, leaf nitrogen, and leaf dry weight, plants were harvested in the early stages of flowering (10% flowering on July 24). The leaf samples (three plants per pot) were washed and dried in the oven (70 ºC for 48 hours) and the dry leaf weight was recorded as an average of three plants per pot. To measure leaf phosphorus content, dried leaves were milled, digested, and analyzed using the method described by Ohnishi et al. [37]. The method described for phosphorus content involves drying, homogenization, and combustion (4 hours at 500ºC) of the sample. The plant ashes (5mg) were digested in 1mL of HCl, filtered, and the total P was determined as PO_4^- using the ascorbic acid method. The amount of PO_4^- in the solution was determined colorimetrically at 882nm [21]. Leaf nitrogen was measured based on the Kjeldahl method, using Kjeltec Analyzer Unit 2000 system [22].

At maturity, AMF colonization of sainfoin plant roots was recorded on three plants per pot. The roots were cleared with 10% KOH and stained with 0.05% trypan blue in lacto-phenol, as described by Phillips and Hayman [23]. The percentage of root colonization was estimated by microscope and the gridline intersection method [24]. In the end of the growing season, height of the plant was measured as an average of three plants per pot.

Proline and the total soluble sugar were measured in leaves in the early stages of flowering. Leaf proline content was measured according to Bates et al. [25]. To do that, the samples were homogenized in 3% sulpho-salicylic acid, and proline was assayed by the acid ninhydrin method. The absorbance was estimated by spectrophotometer at 515nm. To measure the total soluble sugar, we used the method described by Dubois et al. [26]. In order to evaluate chlorophyll index (SPAD), five leaves with chlorophyll meter (Minolta- SPAD-502) per plant per pot were selected (healthy and mature leaves from three different points of per plant) and measured. Following that, the average was calculated. In the end of the growing season (September 3), the height of the plant was measured as an average of three plants per pot. To evaluate the grain characteristics, plants were harvested after the grain filling stage on September 3.

Statistical analysis

Data obtained were analyzed for variance using the MSTATC software. The Duncan Multiple Range Test (DMRT) method was used to find out the significant difference of data ($P \leq 0.01$).

RESULTS AND DISCUSSION

Bio characteristics

The analysis of variance indicated the significant ($P \leq 0.01$) interaction effects of irrigation × mycorrhiza on root colonization, plant height, leaf proline, leaf Total Soluble Sugar (TSS), leaf chlorophyll index (SPAD), leaf phosphorus, leaf nitrogen, and leaf dry weight (Table 3).

In well-watered sainfoin plants (Figure 1A), the highest percentage of root colonization (65.83%) belonged to *G. versiforme*, which was similar in the case of stressed plants (64.00%). In the case of *Rh. intraradices*, root colonization was increased by water deficit stress, while colonization of *F. mosseae*, *C. claroideum,* and *F. caledonius* were respectively reduced by 4.4, 34.1, and 5.1% under stressed condition. Lowest root colonization was observed in non-inoculated control plants in both the irrigation levels (Figure 1A). Habibzadehet al. [18], reported that the colonization of mung bean root was variable from 27-49% for *Rh. intraradices* and 22-41% for *F. mosseae.* They showed that increasing water stress caused a reduction in root colonization of *G. intraradices.* The root colonization of *G. intraradices*was found to be lesser than that of *G. mosseae*. Wu & Xia [27], found the significant reduction in *Citrus tangerine* root colonization with *G. versiform* under drought condition. Despite the differences of mycorrhizal fungi species, AMF symbiosis was improved under drought stress [28].

The plant height (Figure 1B) was taller in mycorrhizal plants than non-inoculated control plants in two irrigation (well watering and water deficit stress) levels. The tallest sainfoin plant (85.61cm) was obtained from well-watered mycorrhized plants by *G. versiforme*. Under water-deficit condition, inoculation with *F. mosseae*, *Rh. intraradices*, *C. claroideum*, *F. caledonius,* and *G. versiforme* respectively improved plant height by 60%, 67%, 57%, 37%, and 84%, as compared with non-inoculated plants. In the case of well irrigation, the above mentioned improvements were 35%, 24%, 25%, 9%, and 42%. The reduction in plant height might be related to the declining cell enlargement and cell growth. The reduction occurred because of low turgor pressure and more leaf senescence, under water-deficit conditions.

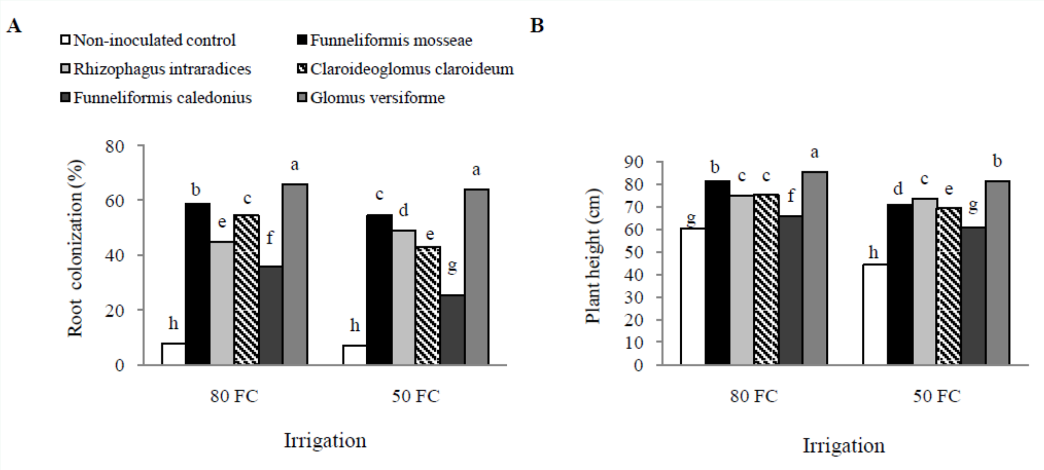

Figure 1 Percentage of mycorrhizal root colonization (A) and plant height in sainfoin plants non-inoculated or inoculated with different species of mycorrhizal fungi and irrigated with an amount of water equivalent to 80% (80FC) or 50% (50FC) of the field capacity (FC). Histograms represent means (n = 3). Different letters indicate significant differences between values (P ≤ 0.01).

However, mycorrhizal relation enhances the status of the leaf water [29]. In this study, the efficiency of AMF on sainfoin plant height was found in the following order from the highest to the lowest: *G. versiforme*, *Rh. intraradices*, *F. mosseae*, *C. claroideum,* and *F. caledonius* under both the irrigation treatments. Wu et al. [30], showed that *F. mosseae* and *G. geosporum* colonizations were more efficient in order to grow plant (*Citrus tangerine*) height than *G. versiforme,* regardless of the status of water.

Leaf osmolytes

Leaf proline increased under stressed condition, with higher values in mycorrhizal sainfoin plants, in the following order: *G. versiforme, Rh. intraradices, C. claroideum, F. mosseae,* and *F. caledonius*. However, the highest leaf proline occurred in stressed plants, inoculated with *G. versiforme* (51.79µmol/ g fresh weight), and the lowest (1.81µmol/ g fresh weight) belonged to well-watered non-inoculated (control plants) (Figure 2A). Accumulation of leaf proline, as an osmoprotectant, plays adaptive (acclimatized) roles in a plant stress tolerance capability [31,32]. There were contradictory reports on proline accumulation by exposing mycorrhizal plants to water-deficit stress. The colonization of plant roots by AMF induced proline accumulation under water-deficit conditions. It seems that the reduced proline oxidase might be the reason for increase of proline accumulation [33]. The previous studies on inoculated plants exhibited increasing trends of leaf proline accumulation. Water stress increased proline contents in mycorrhizal *Vigna subterranean* [34] and *Vigna radiate* [19] plants.

The total soluble sugar (Figure 2B) showed a similar trend of leaf proline. In other words, the increase in leaf TSS under stressed condition was more in mycorrhizal sainfoin plants. The highest leaf TSS (83.35mg/ g fresh weight) was obtained from the plants inoculated with *G. versiforme,* followed by *Rh. intraradices, C. claroideum, F. mosseae*, and *F. caledonius* treatments, respectively, in both the irrigation levels. The lowest leaf TSS was observed in well-watered non-inoculated controlled plants (28.69mg/ g fresh weight). In the present study, all species of mycorrhiza caused more TSS than non-inoculated plants under water-deficit conditions (Figure 2B). These results were supported by higher rates of photosynthesis in response to higher carbon requirement by its allocation to roots 30 [54], as it uptakes water and nutrient from soil especially under water-stressed conditions [18,19]. Increased photosynthetic activity or water use efficiency has been reported in AMF plants growing under drought stress. This was attributed to mycorrhizal enhancement of the plant water status and directly influences the efficiency of photosystem II [35,36].

Leaf characteristics

The lowest leaf phosphorus (122.8mg/ 100g dry weight) was obtained from non-inoculated stressed sainfoin plants (Figure 3A). However, the leaf P of mycorrhizal stressed plants increased in all fungi species symbiosis. Therefore, the highest leaf phosphorus content was obtained from plants inoculated with *F. mosseae* (294.0mg/ 100g dry weight) followed by *G. versiforme* (285.0mg/ 100g dry weight), *Rh. intraradices*, *C. claroideum*and *F. caledonius* under water-deficit condition, respectively. Lu et al. [37], stated that AMF hyphae are mighty in absorbing phosphorus from soil that is not available to the plant roots. In mycorrhizal mung bean [18], and flax seed [38], plants, root colonization and yield improved and the leaf phosphorus increased. Increasing phosphorus uptake in mycorrhizal sorghum was also reported more than the non-inoculated plants, under drought condition [38].

In mycorrhizal plants, leaf N increased in the inoculation of all fungi species. However, in well-watered sainfoin plants, the highest percentage of leaf N (4.73%) belonged to *G. versiforme,* which witnessed a decrease by 4% under water-deficit conditions (Figure 3B). The most important sources of N for plants are nitrate (NO_3^-) and ammonium (NH_4^+) ions. Nitrates are predominantly found in agricultural soils and can be easily absorbed by plants. Nevertheless, some eco-physiological situations, such as drought stress, may interfere with the movement of the nutrient to the root surface [40]. Mycorrhizal associations are important in

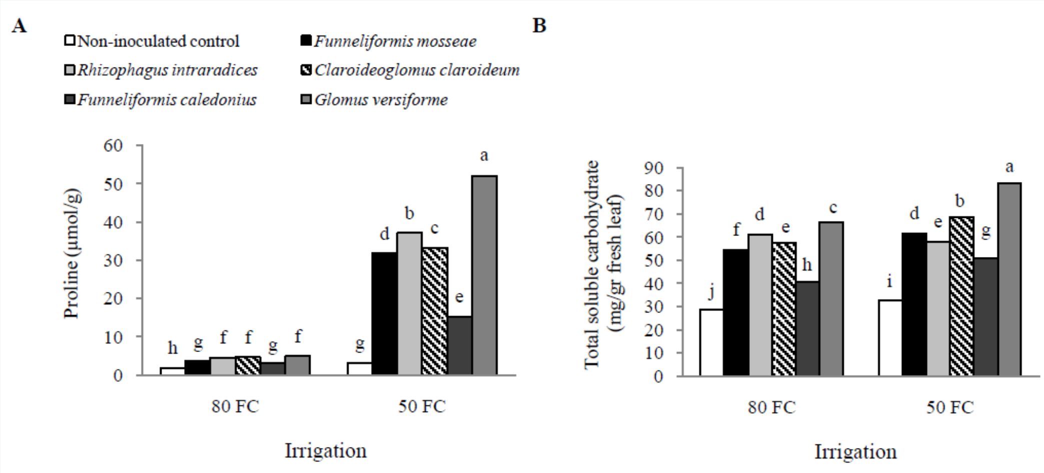

Figure 2 Leaf osmolytes; proline (A) and total soluble carbohydrate (B) in sainfoin plants non-inoculated or inoculated with different species of mycorrhizal fungi and irrigated with an amount of water equivalent to 80% (80FC) or 50% (50FC) of the field capacity (FC). Histograms represent means (n = 3). Different letters indicate significant differences between values (P ≤ 0.01).

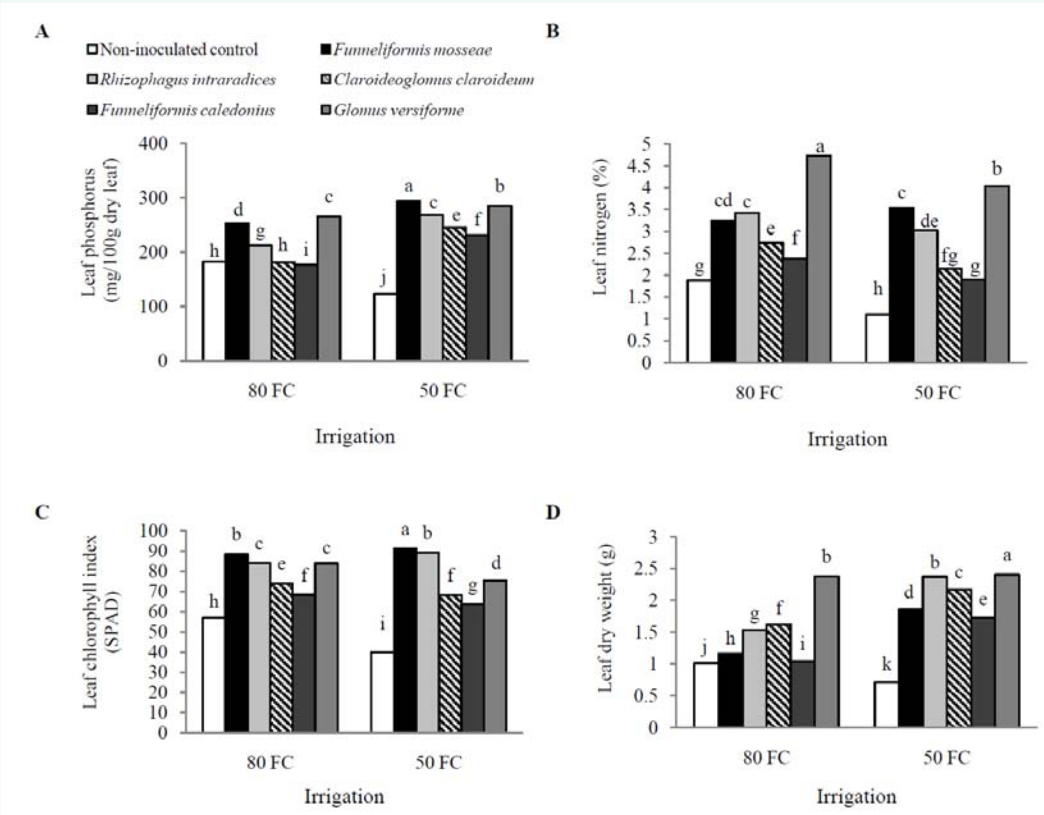

Figure 3 Leaf characteristics, leaf phosphorus (A), leaf nitrogen (B), leaf chlorophyll index (C) and leaf dry weight (D) in sainfoin plants non-inoculated or inoculated with different species of mycorrhizal fungi and irrigated with an amount of water equivalent to 80% (80FC) or 50% (50FC) of the field capacity (FC). Histograms represent means (n = 3). Different letters indicate significant differences between values (P ≤ 0.01).

nitrate uptake under water-deficit conditions 40 [13]. Sainfoin can establish symbiosis with nitrogen fixing bacteria (*Rhizobium*). The dual symbiosis with mycorrhiza and rhizobium revealed synergistic effect [41]. The highest percentage of leaf N in plants inoculated with *G. versiforme* showed that synergistic interaction of this species with *Rhizobium* was the best and had the potential to enhance the percentage of leaf N in sainfoin plants.

Water deficiency led to a significant decrease in the chlorophyll index (Figure 3C) in sainfoin (mycorrhizal and non-inoculated) plants. However, this was an exception in the

Table 1: Environmental conditions at the experimental site during May to September, 2014.

Parameter	May	June	July	August	September
Highest temperature (°c)	31.4	37.4	33.4	37.0	32.0
Lowest temperature (°c)	6.4	7.4	12.8	13.8	8.0
Average relative humidity (%)	54	49	46	36	56
Sum of sunny hours (no.)	271	299	309	336	267

Table 2: Some of soil Physicochemical characteristics.

Soil texture	Electrical Conductivity (ds m^{-1})	pH	Organic Carbon (%)	Phosphorus (mg kg^{-1})	Potassium (mg kg^{-1})
Silty clay	0.52	7.8	0.78	9.8	324

Table 3: Means square for analysis of variance (ANOVA) effect of irrigation and mycorrhizal species on the root colonization and some physiological traits of sainfoin plant.

Source of variation	df	Means square						
		Root colonization	Plant height	Leaf proline	Leaf TSS	Leaf chlorophyll index	Leaf phosphorus	Leaf dry weight
Mycorrhiza(M)	5	2298.97**	719.58**	141.79**	994.33**	1399.70**	12673.5**	1.610**
Irrigation(I)	1	286.18**	461.18**	5974.52**	732.61**	186.87**	13650.0**	1.870**
M×I	5	48.34**	42.37**	104.23**	70.47**	97.58**	1382.6**	0.115**
Error	24	1.87	0.41	0.04	0.32	0.41	3.7	0.0001
CV (%)		4.37	2.91	2.12	3.00	2.87	3.83	3.04

** Significant at the 1% probability level.

case of inoculated plants with *F. mosseae* (91.37 SPAD) and *Rh. intraradices* (89.30 SPAD), where it showed a little increase (up to 6%). The lowest chlorophyll index (40.02 SPAD) belonged to non-inoculated plants under water-deficit condition (Figure 3C). Increasing chlorophyll index was reported in *Dioscorea rotundota* [42], and *Cicer arietinum* [43]. Drought stress caused damage in macromolecules, such as chlorophyll, resulting in the loss of photosynthetic activity [44-46]. The increase in chlorophyll content of mycorrhizal plants even under water-stressed conditions might be as a result of nutrients uptake [47,48]. Findings by Demir [49], showed an increase in photosynthesis in pepper plants inoculated with mycorrhiza, resulting from improved chlorophyll content under water-stressed conditions. Manoharan et al. [50], reported that the symbiotic efficacy of *F. mosseae* raised the plant chlorophyll content in *Erythrina variegate,* subjected to different water-stressed conditions.

Water deficit-induced reduction in leaf weight was improved by mycorrhizal inoculation under drought stress (Figure 3D). Even the plants inoculated with *G. versiforme* showed the highest leaf weight (2.403g/ plant). Increase in the leaf dry weight of the stressed plants inoculated with *F. mosseae*, *Rh. intraradices*, *C. claroideum*, *F. caledonius,* and *G. versiforme* was recorded at about 83%, 133%, 141%, 70%, and 137 % respectively, which was found to be more than those under well-watered conditions. Leaf area flexibility was an important criterion, which helped the plant control the water usage under stressed condition [51]. The effect of water stress on the leaf dry weight could be the result of cell small size and a reduction in cell division. This led to a reduction in leaves growth. It was a compatible way for the plant survival under such condition [52,53]. However, a higher value of leaf dry weight, obtained from mycorrhizal stressed plants, could be the result of growing chlorophyll content in inoculated plants (Figure 3C). This consequently enhanced the photosynthesis efficiency. Furthermore, this could be attributed to increase water and nutrient uptakes of AMF hyphae [50]. Rahimzadeh and Pirzad [54], reported the enhancement of mycorrhizal fungi species in flax seed (improvement of seed and oil yield) due to physiological (osmolytes and antioxidants) acclimation [38]. It is remarkable that sainfoin plants growing in soil without AMF inoculation had leaves with lowest dry weight (Figure 3D) and chlorophyll content (Figure 3C). However, these values were increased by mycorrhizal inoculation. These results show that under some environmental conditions, AMF is essential for plant growth and development [55]. This could be due to reducing the leaf phosphorus content.

CONCLUSIONS

According to the results of this study, inoculation with different species of arbuscular mycorrhizal fungi showed a significant increase in the root colonization of sainfoin plants under both well-watered (irrigation at 80% FC) and water-deficit (irrigation at 50% FC) conditions. It should be noted that the root colonization with *G. intraradices* and *G. versiform* was higher in stressed plants, whereas other AMF species (*Glomusmosseae, G. claroideum,* and *G. caledonium*) showed high colonization under well-watered conditions. In water deficit condition, an increase in leaf proline and total soluble sugar against reduced chlorophyll index was the highest in AMF inoculation. Despite an increase in the height of mycorrhizal plants, they were taller in well-watered plants than the plants under water-stressed conditions. Leaf phosphorus demonstrated the enhancement of mycorrhiza for P uptake even under water deficit condition.

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