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Effects of arbuscular mycorrhizal inoculation on the growth, photosynthetic pigments and soluble sugar of *Crocus sativus* (saffron) in autoclaved soil

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Abstract

The beneficial soil microorganisms as arbuscular mycorrhizal fungi (AMF) form the mutualistic relationship with plant roots and act as bio fertilizers for saffron (*Crocus sativus* L.). In the present study, the plant growth, AMF colonization and nutrient uptake of *C. sativus* evaluated in earthen pots filled with sterile soil. *C. sativus* seedlings with or without AMF spores of the *Glomus* species, were cultivated for six months in autoclaved sediment medium. The results of the first year showed a significant increase of the above and below ground growth of saffron plant. The fresh and dry weight content indicated in higher levels of inoculated group that the value of biomass was 4.05 (gr) and 0.42 (gr) than non-inoculated group, respectively. The photosynthetic pigments and soluble sugar content increased in the mycorrhiza infected as compared to the non-inoculated ones with rate of 36.69% and 43.1%, respectively. In addition, the mycorrhizal dependency (MD) of *C. sativus* to AMF reached a maximum of 38.18% under AMF inoculation treatment, which was significant ($p < 0.05$).

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Introduction

The mutualistic symbiosis between arbuscular mycorrhizal fungi (AMF) and plant roots are of great ecological significance as they can form with most terrestrial plant species (Smith and Read, 2010). Soil is considered to be the store house of nutrients for organisms and plants. The organisms convert the nutrients into those forms that plant can easily absorb. Symbiotic associations AMF in the vicinity of roots can ultimately influence health, vigor and productivity of the plant (Wang and Qui, 2006).They can cause improvement of macro- and micro-nutrients status to plants such as N, K, Mg, Cu, Zn and especially P present in soil in soluble form, (Clark and Zeto, 2000; Goussous and Mohammad, 2009; Cozzolino *et al*., 2010). Incidence of AM fungal colonization has been reported in corms of *C. sativus* (Lone, 2014), that it is a sterile triploid (2n = 3x = 24) (Brighton, 1977; Mathew, 1982) and is vegetatively propagated via its corms. Although the phenology of the vegetative development is well established (Botella *et al*., 2002; Kafi, 2006), literature about photosynthesis in saffron and other *Crocus* is also lacking. Saffron has low water requirements and is typically cultivated under rained conditions because it is well adapted to the rainfall pattern of diverse Mediterranean areas (Alizadeh, 2006). While gaining soluble carbohydrates and other growth substances from the host plant such as *C. sativus*, AMF hyphae can absorb water and mineral nutrients from the soil and transport them to the root, acting as a living bridge between soil and plants. The levels of purity, homogeneity, health and improvement of nutrition status of saffron corms influence vegetative development and the production of daughter corms in rhizosphere region (European Saffron White Book, 2006). Present study is based on a simple premise whether or not the AM fungi have any constitutive association with saffron corms which involve in the growth and development of the saffron plant. It investigates the changes in leaf photosynthetic efficiency, plant growth and soil nutrition status in saffron during vegetative development and to know the importance of mutualistic symbiosis. Understanding mycorrhizal symbiosis-related practices will help better to develop the agriculture and natural habitats for this crop.

Material and methods

Sample collection

Soil was collected from Khalil Abad natural reserve, Khorasan Razavi province, North eastern Iran, in May and December 2011. The area (33◦11_N, 58◦72_E) is characterized by a subtropical climate, with an annual mean temperature and Altitude of 22.2◦C and 1210 m, respectively. The sampling site mainly consists of a regrowth and mature *Crocus sativus* (saffron) community. Roots and rhizosphere soils of the subsurface layer (5–30 cm) were collected from representative adult individuals of saffron plant. For the corm sample, only the nutritive corms attached to the plant were collected. The soil that remained adhered to the corm after gentle shaking (i.e. the rhizosphere soil) was also collected for AMF identification.

AMF identification and trap culture

The wet sieving and decantation methods (Gerdemann and Nicolson, 1963) were used to isolate spores of AMF from corm-associated soil. Then the AMF spores were identified from sporemorphology by reference to type descriptions of the species (Schüßler and Walker, 2010). Three AMF species were found to be dominant in Khalil Abad farms, which were: *Glomus aggregatum* (Koske), *Glomus mosseae* (Nicol & Gerd) and *Glomus etunicatum* (W.N.Becker & Gerd.). Those three major types of AMF spores were selected and propagated together to prepare the AMF inocula for the pot experiment. Trap culture experiments were conducted using autoclaved (120◦C, 0.2 MPa, 20 min) sand and commercial peat soil (pH = 7.7) (v:v = 1:1) as the culture medium and *Zea mays* as the host plant. After the successful trapping process, the mixtures containing AMF spores, mycelium, sandy soil and mycorrhizal corm fragments were used as the inocula. Every 100 g of the prepared inocula contained about 400 propagules, and the proportion of those three AMF species was maintained at 1:2:1 to mimic the natural environment.

Pot experiment

Plant growth conditions

Five pots of each treatment (non-inoculated group (NG) and inoculated group (IG)), with a diameter of 15 cm and a depth of 20 cm were used to grow *C. sativus* seedlings. These plastic pots were filled with sterilized soil including peat soil, clay and sand (1:1:1 v:v:v). For the inoculated group (IG), 100 g of prepared inocula were thoroughly mixed with the soil, while the same amount of sterilized inocula were mixed in non-inoculated group (NG). Every three corms of *C. sativus* were planted in a cultivation pot and grown in a sunlit greenhouse with natural light, day/night temperature of 25/15 ◦C. Plants were watered with deionized water during the growing period.

Soil analysis

The soil samples of non-inoculated group (NG) and inoculated group (IG) were air-dried and passed through a 0.25 mm sieve for determining total N (TN), total P (TP) and total K (TK) amount. These analysis were based on the standard method described by Page (1982) .Total N (digested with H_2SO_4–H_2O_2) were measured by a Kjeldahl apparatus (Kjeltec, Foss 2003, Denmark). Total P (digested with 65% HNO_3) were measured by Molydenum-antimony-Diso-ascorbic-acid-colorimetry. Total K was quantified by atomic absorption spectrophotometry after extraction with ammonium acetate 1 M, pH 7.0.

Evaluation of AMF root colonization

Plants of *C. sativus* were harvested after 6 months of growth. Leaves, shoots and roots were sampled separately. Sub-samples of fresh nutritive roots were taken to assess mycorrhizal colonization. Rinsed root samples were cleared with 10% KOH at 90◦C for 60 min and soaked with 1% HCl for 5 min, and then stained with lactophenol cotton blue (LPCB) (Phillips & Hayman, 1970). The stained roots segments suspended in lactoglycerin were then spread in a petri dish marked with 1cm grid to facilitate scanning, and viewed under a stereomicroscope at 12 to 50* (Bierman and Linderman, 1981). The proportion of the length of each root segment which contained the any of the endophytic elements - hyphae, coils, vesicles and arbuscules - was taken as evidence of mycorrhization and estimated to the nearest 10%. The percentage of the root length with mycorrhiza endophytes in the sample was then calculated from the frequency distribution. Percentage root colonization (PRC) was determined as the proportion of the total number of root segments with hyphae, coils, vesicles and arbuscules by use of the same root segments as in the previous measurements.

Measurement of plant growth

Plant height of *C. sativus* was measured with a tapeline before harvest. Remaining roots and other parts of the plant were rinsed with deionized water three times. Fresh weight of total leaves, shoots and roots were measured and then dried at 70◦C in an oven for 48 h to constant weight. The percentage water content of remaining roots and total fresh root weight were used to estimate total root dry weight. The mycorrhizal dependency (MD) was calculated based on the following formula (Plenchette *et al.*, 1983): Mycorrhizal dependency (MD) = (dry weight of mycorrhizal plants – dry weight of non-mycorrhizal plants)/dry weight of mycorrhizal plants × 100%.

Carotenoid and Chlorophyll Analysis

Fresh shoot tissues from non-inoculated group (NG) and inoculated group (IG), weighing 150 mg, were extracted in 80% acetone. The debris were removed by filtration using Whatman filter paper and the contents of Chl a, Chl b and carotenoids were determined in the filtrates by recording absorbance at 664, 648 and 470 nm using a spectrophotometer [Shimidzu-ultraviolet–visible-1601-pc]. Contents of Chls and carotenoids were calculated according to Lichtenthaler (1987)

Soluble Sugar Analysis

Reducing sugar concentration was analyzed using the phenol–sulphuric acid Method (Dubois *et al.*, 1956). The phenol reagent was prepared by adding 10mL of distilled water to 90mL of 90% phenol solution. Dry

weight of root (150 mg) mixed with 70% ethanol. Then the phenol reagent and sulphuric acid were added and the solutions were incubated at room temperature for 30min and the UV absorbance was read at 485nm. Glucose was used as the standard for this analysis.

Data analysis

The experiment was laid out as a completely randomized design, with five replicates of each treatment. Data were analyzed using a statistical package, SPSS version 16.0. One-way analysis of variance (ANOVA) followed by the t-test (honestly significant difference, $p < 0.05$) were carried out to determine differences between means. Correlation analysis was carried out by two-tailed Pearson test, with $p < 0.05$ as the correlation degree. All data were plotted using Origin 8.0 software.

Results

AMF root colonization

Our investigation indicated no AMF structures in non-mycorrhizal inoculation treatments. In the AMF inoculation treatment, all plant roots showed associations with AMF. Intracellular hyphae and arbuscular were the dominant structures, and the arbuscular structure was 'Arum' type (Fig. 1). The PRC of the inoculated AMF (including *Glomus etunicatum, G. mosseae, and G. aggregatum*) calculated 39% ($p < 0.05$).

Table 1. Effect of non-inoculated and inoculated soils on chlorophyll and carotenoids ($mg.g^{-1}FW$) of *C. sativus* plant in pot experiment.

Group	No. of pots	Chl.a	Chl.b	Carotenoid	Chl.a + Chl.b	Chl.a /Chl.b	Chl.a + Chl.b/ Carotenoid
Non-inoculated (NG)	5	0.752	0.371	0.385	1.123	2.027	2.917
Inoculated (IG)	5	1.12	0.654	0.576	1.774	1.713	3.08

Enhanced plant growth response

Significant increase in rate and total emergence of *C. sativus* seedling in soil treated with AMF inoculation were observed (Fig. 2). Sizes of emerged plant in soil infested with AMF inoculation were more uniform than those of control plant. In addition, the plant height of *C. sativus* differed significantly under AMF inoculation ($p < 0.05$). In AMF inoculation treatment group, plant height reached a maximum of 27.61 cm, which was 35.8% higher than the control.

Biomass and MD of C. sativus

The results of pot study showed that inoculation of *C. sativus* seedling with AMF affected root and shoot fresh weight (Fig. 3). Total Shoot fresh weight significantly increased by inoculation in sterile soil, which was 3.14 g higher than the control (5.02 g) ($p<0.05$). Shoot dry weights were significantly affected by AMF inoculation treatment and increased 34.7% over the control. Furthermore, inoculation with AMF had significant effect on the root fresh and dry weight. The results revealed that infested soil caused an increase of 62.7% and 55.5% for root fresh and dry weight compared to control, respectively. The mycorrhizal dependency (MD) of *C. sativus* to AMF reached a maximum of 38.18% under AMF inoculation treatment, which was significant ($p < 0.05$)

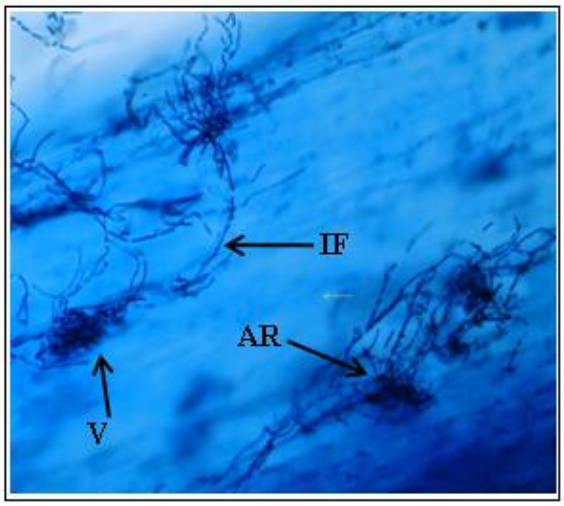

Fig. 1. Typical AMF structure in roots of *C.sativus*. AR stands for arbuscular. V stands for vesicle, and IF stand intercellular hyphae.

N-P-K in pot soil of C. sativus

As shown in Figure 4, the significant difference was found among essential elements of growth including N, P, and K in non-inoculated and inoculated soils of *C. sativus*. The N amount of inoculated soil increased 56.5% than non-inoculated soil ($p < 0.05$). Total content of P increased significantly in inoculated soil with AMF (66.04%) over the non-inoculated soil. In addition, K content was much higher in inoculated soil (77.7 %) than the non-inoculated soil ($p < 0.05$).

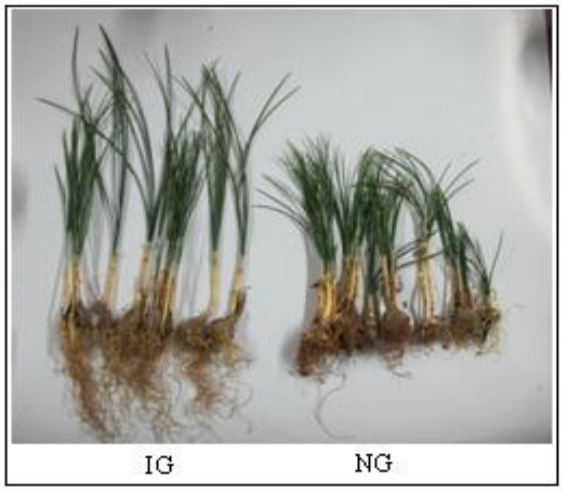

Fig. 2. Plant growth of *C. sativus* in the non-inoculated group (NG) and inoculated group (IG).

Total carotenoid and chlorophyll content

Application of inoculated soil with AMF for plant growth of *C. sativus* showed the highest concentration of chl.a, chl.b and chl.a+chl.b as well as total carotenoids in the pots experiment (Table 1). Our studies indicated that total chlorophyll significantly increased in inoculated group (IG) (36.69%) than non-inoculated group (NG) ($p < 0.05$). In addition, Table 1 show the spectrophotometric measurement of total carotenoids based at the sample Abs at 470 nm. The results revealed that the IG are the most rich in these components. The amount of total carotenoids in NG presented 33.15% lower than IG. Therefore, the promotion in total chlorophyll and carotenoids exhibited positive correlation with the increase of PRC (39%) in IG ($p < 0.05$).

Soluble sugars content

The inoculated soil with AMF in pot experiments significantly increased soluble sugar contents in mycorrhizal plants (Fig. 5). Soluble sugar contents in mycorrhizal plants increased (43.1%) in comparison with non-mycorrhizal plants (1.61 $mg.g^{-1}$ DW) that, there was a significant ($P< 0.05$) interaction of AMF inoculation and soluble sugar content of plants.

Discussion

AMF symbiosis with C. sativus roots

Plant–microbe interactions in rhizosphere can be defined as any volume of soil specially influenced by the plant roots or in association with the roots and plant-produced material and often extending a few mm from the root surface (Bringhurst *et al.*, 2001; Smith and Read, 2010). Arbuscular mycorrhizal fungi (AMF) are endo-mycorrhizae that are classified in the fungal phyllum Glomeromycota (Schüssler *et al.*, 2001). They live symbiotically, as obligate biotrophs, in the roots of about 80% of plant species (Wang and Qiu, 2006). Saffron corms produce both fibrous roots and contractile roots. The fibrous roots emerge from a single ring at the base of the corms. This type of roots enable corms to dig into the ground, so corms rest in optimum depth and position in the soil (Chio-Sang, 1996). In our study, the PRC differed a lot under the inoculated AMF treatment. Compared to non-mycorrhizal plants, we observed increases in pot experiment of inoculated AMF treatment (39%; $p < 0.05$). However, the mixed AMF inocula applied in this study successfully colonized *C. sativus* roots, and intercellular hyphe, arbuscular, and vesicle structures were present in all inoculated treatments. The tested AMF species were dominant indigenous species selected from Khalil Abad regions including *Glomus etunicatum, G. mosseae, and G. aggregatum*. These species have developed adaptation characteristics to semi-arid climate.

Effects of inoculated AMF on the growth of *C. sativus*

Mycorrhiza helps plants with such as shallow sparse root system to increase absorption of nutrition and metabolic level (Ganesan and Mahadevan, 1998). It is now vastly reported that the mycorrhizal colonization improves plant growth by facilitating mineral nutrition and progressive water relations which lead to large plant size and higher yield (Auge, 2001; Xie *et al.*, 2014). In this study, inoculated soil alone

significantly promoted the height of *C. sativus*, which was 35.8% higher than the non-inoculated soil in pot experiment. When inoculated with AMF, the total biomass of *C. sativus* increased significantly with enhancing the plant height. Studies on onion and saffron demonstrated that fresh and dry biomass is more in inoculated AMF plants than non-inoculated plants (Kianmehr, 1981; Shuab *et al.*, 2014). The analysis of MD value confirmed that AMF inoculation make a greater biomass of *C. sativus* than non-inoculated plants. MD value depended on P amount and significantly increased with high P in rhizosphere that resulted in optimal growth promotion effect (Xie *et al.*, 2014). Therefore, our data indicated that the higher P treatment can create by AMF in *C. sativus* roots.

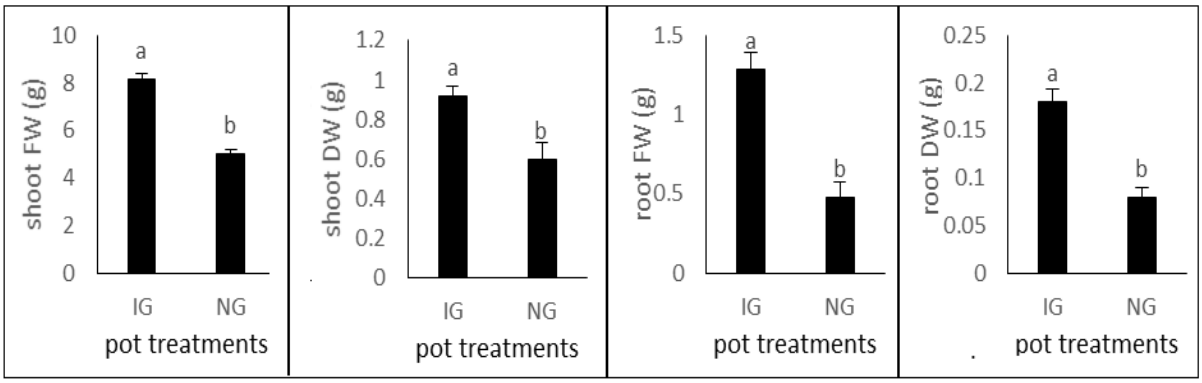

Fig. 3. Fresh and dry weight of shoot and root of non-inoculated group (NG) and inoculated group (IG) of *C. sativus*. Statistical analysis and the mean ± SD were performed in $p < 0.05$ level on the three different samples.

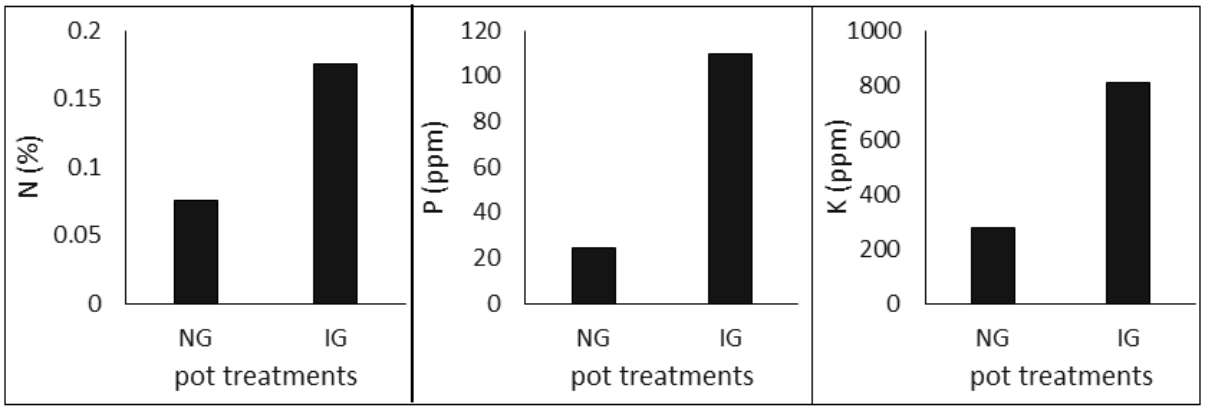

Fig. 4. Nitrogen (N), Phosphorus (P), and Potassium (K) amount and content in non-inoculated group (NG) and inoculated group (IG) of *C. sativus*.

N-P-K in pot soil of C. sativus

The growth of plants closely related to absorption of water and nutrients from the environment. The rhizosphere microbial communities are vigorously associated with the biogeochemical cycling of nutrient elements (Cardoso and Freitas, 1992). The improvement of plant nutrition through mycorrhizal symbioses and the molecular bases of nutrient transfer are currently well studied for phosphorus (Javot *et al.*, 2007; Plassard and Dell, 2010) and nitrogen (Müller *et al.*, 2007; Jin *et al.*, 2012) and potassium (Benito and Gonzalez-Guerrero, 2014). In our study, P promoted the growth and vitality of the root in inoculated soil with AMF and increased significantly (66.04%) over the non-inoculated soil. As an essential macronutrient, P is absorbed by plants in the form of inorganic phosphate anion (Pi). The AMF could produce the enzymes that hydrolyzed organically bound P into Pi to enter the plant root (Javot *et al.*, 2007). N as the principle component of protein in stem and leaf growth increased 56.5% in inoculated soil than non-inoculated. The contribution

of this mutualistic symbiosis to the enhancement of plant K^+ nutrition will lead to benefits for the plant in inoculated soil (77.7 %) over the non-inoculated soil. Although the role of K^+ is still poorly investigated in mycorrhizal studies, it appears that plant K^+ nutrition is clearly improved by mycorrhization (Garcia and Zimmermann, 2014). N-P-K accumulation in inoculated plants with AMF can positively correlate with PRC and plant biomass, suggesting which led to greater MD.

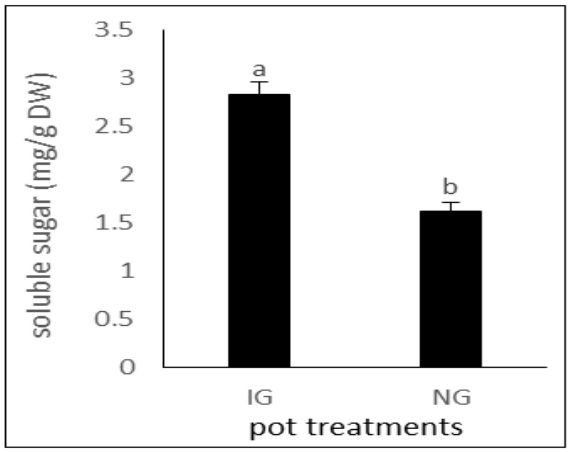

Fig. 5. Soluble sugars in non-inoculated group (NG) and inoculated group (IG) of *C. sativus*. Statistical analysis and the mean ± SD were performed in $p < 0.05$ level on the three different samples.

Effects of inoculated AMF on photosynthetic pigments and soluble sugar

AMF colonization enhances the concentration of photosynthetic pigments than non mycorrhizal ones (Morte *et al.*, 2000, Giri *et al.* 2003). Therefore, significant differences in concentrations of chlorophyll-a, chlorophyll-b and carotenoids observed in *C. sativus* plant may be attributed to plant-microbe interaction in leaves of AM-inoculated and non-mycorrhizal plants in pot experiment. The high amount of chlorophyll in mycorrhizal inoculated plants increased the rate of photosynthesis and so as to increase the rates of photosynthetic storage and export at the same time (Haneef *et al.*, 2013). The enhancement of the soluble sugar content studied in AM plants of *M. tetraphylla* (Yooyongwech *et al.*, 2013), AM treated pigeon pea (Qiao *et al.* 2011) and AM treated lettuce cultivars (Baslam and Goicoechea, 2011) that might generally act as an osmoprotectant to stabilize the plant organelles when subjected to water deficit stress.Our data indicated that mycorrhizal plants had higher soluble sugar content in the roots of *C. sativus* in pot experiment, compared with non-mycorrhizal plants. This may be due to the sink effect of the AM fungus-demanding sugar from leaves (Porcel and Ruiz-Lozano, 2004). AM fungi regulate growth under water deficit and may lead to high accumulation of soluble carbohydrates.

Conclusion

Based on the symbiotic relationships of saffron and AMF, this research investigated the eco-physiological function of AMF on saffron plant growth and nutrient uptake. The inoculated AMF successfully infected *C. sativus* roots, developed intercellular hyphae, arbuscular (Arum-type), and vesicle structures. Present communication for the first time, reports the cultivable AMF spores of the *Glomus* species such as *G. aggregatum*, *G. mosseae* and *G. etunicatum,* present in rhizosphere of Saffron (grown in Khalil Abad, Khorasan Razavi province, Iran). The AMF colonization improved positively the overall growth and development of saffron plant. In addition, the photosynthetic pigments and soluble sugar content too were found higherin AMF inoculated than control.

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