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Biomass allocation, leaf gas exchange and nutrient uptake of hazelnut seedlings in response to *Trichoderma harzianum* and *Glomus intraradices* inoculation

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Abstract

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Mycorrhizal fungi form mutualistic symbioses with the roots of 80% of plants which increase growth and nutrient uptake for the host plants. This research was conducted to determine the effect of individual *Glomus intraradices* Schenck & Smith and *Trichoderma harzianum* Rifai species on the root colonization, biomass allocation, physiological characteristics and nutrient uptake of hazelnut (*Corylus avellana* Linnaeus) seedlings in the nursery. The results showed that both *G. intraradices* and *T. harzianum* improved biomass, physiological characteristics and nutrient uptake of hazelnut seedlings as well as simultaneous root colonization. However, the growth rate for *G. intraradices* treatment was significantly higher than that for *T. harzianum* treatment. The highest leaf dry mass (2.66 g), root dry mass (3.39 g), root volume (11.31 cm^3), total plant dry weight (11.20 g) were detected in seedlings inoculated with *G. intraradices*. Inoculation with *G. intraradices* and *T. harzianum* increased net photosynthesis (64 and 26%), stomatal conductance (66.1 and 31.4%) and water use efficiency (50 and 22%). Both *G. intraradices* and *T. harzianum* showed increased nutrient accumulation. The *G. intraradices* treatment resulted in the most efficient nutrient absorption with increases of 58.4% (N), 85.2% (P) and 83.2% (K) in plants. It can be deduced that although *G. intraradices* in comparison with *T. harzianum* more favourably affected the growth and leaf gas exchange as well as nutrient uptake of hazelnut seedlings, it can be suggested that the inoculation of hazelnut roots with both arbuscular mycorrhizal fungi is a proper measure to produce the healthy and strong seedlings of this species in the nursery.

Keywords: *Corylus avellana*; fungi; growth rate; leaf nutrients; nursery; stomatal conductance

Soil microorganisms, such as arbuscular mycorrhizal fungi (AMF), represent a key link between plants and soil mineral nutrients. Thus, they receive growing interest as natural fertilizers (Berruti et al. 2016) which can form mutualistic symbioses with the roots of about 80% of plant species

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(Smith, Smith 2011). The arbuscular mycorrhizal symbiosis can establish extraradical mycelia which disperse outside the roots to have access to a greater quantity of water and soil minerals for the host plants (Smith et al. 2010). It is well demonstrated that AMF facilitate higher water absorption and nutrient uptake in plants which in turn help to combat various diseases and stress resistance of host plants and enhance plant growth (Chanway 1997; Domínguez-Núñez et al. 2013).

In a research in the micropropagated plants of *Corylus avellana* Linnaeus potted into polypropylene containers with a peat and perlite mixture (3:1 v/v) and inoculated with *Glomus intraradices* Schenck & Smith after one year, their colonized roots had greater biomass compared to the control (Mirabelli et al. 2009). Previous studies showed that AMF stimulated growth and increased yield in several forest species such as *Prunus cerasifera* Ehrhart (Berta et al. 1995), *Olea europaea* Linnaeus (Estaun et al. 2003), *Rhamnus lycioides* Linnaeus and *Retamaspha erocarpa* (Caravaca et al. 2005), *Pinus densiflora* Siebold & Zuccarini (Choi et al. 2005), *Dalbergia sissoo* Roxburgh (Bisht et al. 2009). It has been reported that the inoculation of mulberry (*Morus alba* Linnaeus) seedlings with *Glomus mosseae* (Nicolson & Gerd) Gerdemann & Trappe, *G. intraradices* and *G. mosseae* + *G. intraradices* significantly affected the growth in greenhouse conditions, and the *G. intraradices* treatment caused the most efficient nutrient absorption (Mirzaei 2014). In another study, the inoculation of *Cercis griffithii* Boissier seedlings showed the root colonization rate of seedlings via *G. mosseae* and *G. intraradices* was higher than that of *Glomus gigantea*. The height, fresh and dry weight of the roots and the shoots of seedlings inoculated with *G. mosseae* were higher than those inoculated with other fungi. Also, the AMF increased P and N uptake in leaf plants (Lu et al. 2015).

Trichoderma spp. are free-living fungi growing vigorously in soil and plant root ecosystems (Harman et al. 2004; Hermosa et al. 2012). In a research the inoculation of crack willow (*Salix fragilis* Linnaeus) saplings with *Trichoderma harzianum* Rifai increased the length of shoots and roots by 40% compared to that of the control (Adams et al. 2007). Likewise, the inoculation of blue pine (*Pinus wallichiana* A.B. Jackson) seedlings with *T. harzianum* in a nursery had a significant effect on the plant growth and caused a considerable increase in N, P and K uptake (Ahangar et al. 2012). In another research the inoculation of carob tree (*Ceratonia siliqua* Linnaeus) seedlings with AMF and *T. harzianum* significantly affected the plant growth. Moreover, the frequency (98%) and the intensity (73%) of mycorrhization were higher on the level of the roots of seedlings inoculated only with AMF (Talbi et al. 2016).

Hazelnut is one of the world's major nut crops. Its distribution extends from the Mediterranean coasts of North Africa and northward to the British Isles and the Scandinavian Peninsula, and eastward to the Ural Mountains of Russia, the Caucasus Mountains, Iran, and Lebanon (Bombeli et al. 2002). It plays a major role in human nutrition and health because of its specific composition of oleic acid, protein, carbohydrates, dietary fibre, vitamins, minerals and antioxidant phenolics (Alasalvar et al. 2003). In nurseries of northern Iran produced seedlings of hazelnut (for reforestation purposes) do not reach the plantation size within the first year. On the other hand, because of the generous fertilizer and pesticide use to promote rapid initial growth, the seedling root system is devoid of beneficial symbiotic mycorrhizal fungi. Since the appropriate mycorrhizal fungi can improve the seedling growth in nurseries by facilitating nutrient and water availability (Ortega et al. 2004; Quoreshi, Khasa 2008), the present study is going to evaluate the potential of *G. intraradices* and *T. harzianum* on growth parameters such as allocation of aboveground and belowground biomass, as well as nutrient uptake and leaf gas exchange, in *C. avellana* seedlings.

MATERIAL AND METHODS

Plant material and experimental conditions. This investigation was carried out from March 11 to October 25, 2015 (one growing period) in the forest nursery of Fandoglou, located 24 km of East Ardabil, Ardabil province, Iran (48°36'E, 38°19'N, at 1,380 m a.s.l.). The average annual precipitation is 430 mm, average annual minimum temperature 3.6°C and average annual maximum temperature 25.1°C. The average annual relative humidity of the respective meteorological stations ranges from 54 to 70%.

Seedling preparation for inoculation with fungi. 200 seeds of *C. avellana* were collected from a local provenance (Ardabil Fandoglou Forest, Ardabil province, Iran). The seeds were soaked in a solution of sodium hypochlorite for surface sterilization for 10 min and were placed in 45 × 20 × 8 cm plastic containers filled with sieved and sterilized sand. Then, the seeds were subjected to cold stratification

in a refrigerator at 4°C for 4 months. At the end of the stratification period, seeds were sown in 200 plastic pots containing 4 kg of sterilized soil. The properties of the soil were: clay 36%, silt 26%, sand 38%, pH 6.34, electrical conductivity 0.386 $dS \cdot m^{-1}$, total N 0.23%, P 9.44 ppm, K 174 ppm, organic matter 1.21%. Pots were kept under natural photoperiod in the forest nursery of Fandoglou.

The fungi *Glomus intraradices* (250 propagules per gram) and *T. harzianum* Rifai (60 propagules per gram) were received from the microbial collection of the Soil Microbiology Department of Soil and Water Research Institute, Iran. One month after the start of experiment, healthy and uniform-sized seedlings were selected for inoculation. Then, 20 g fresh weight of the inoculum was placed into the middle of seedling roots (5 cm depth) for fungal treatment (Wu et al. 2011). The experimental plan comprised 3 treatments: (*i*) control (without fungi), (*ii*) *G. intraradices*, (*iii*) *T. harzianum*. The seedlings were irrigated regularly with a special container to fulfil the plant needs depending on climate and to ensure that the water would not be a limiting factor during the experimental period.

Biomass allocation measurements. Seedlings were destructively harvested and the soil adhering to the root system was gently cleaned and the aboveground parts and roots were separated at the root collar. The roots were washed and the root volume was determined by immersion in a graduated test tube and measurement of the displaced water volume. Root, shoot and leaf were dried at 70°C for 48 h and weighed to determine the average root and shoot dry weights.

Leaf gas exchange. At the end of the growing period, the net photosynthetic rate – A (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), transpiration rate – E (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$) and stomatal conductance – g_s (mol $CO_2 \cdot m^{-2} \cdot s^{-1}$) of the seedlings were measured with a portable photosynthesis system (ADC BioScientific, Ltd., UK). The water use efficiency (WUE_i) was calculated by dividing the photosynthetic rate by the transpiration rate (Zhang et al. 2005), as Eq. 1:

$$WUE_i = \frac{A}{E} \quad (1)$$

The parameters were measured on the uppermost, fully expanded leaves of each plant from 09:00 to 11:00 AM under bright sunlight on a clear, cloudless day (Yu et al. 2014). Three leaves were prepared for each seedling. Leaf chlorophyll content was measured using a chlorophyll meter model SPAD 502 (Minolta Co., Ltd., Japan) (Mielke, Schaffer 2010).

Nutrient uptake. Fully developed mid-shoot leaves were sampled in August to determine the content of minerals. The samples were oven-dried at 68°C for 48 h and then ground. An amount of 0.2 g of dried materials was used to analyse their nutrient concentrations and added to a 100 ml Kjeldahl flask containing 5 ml of concentrated sulfuric acid. The mixtures were gently shaken and then heated until turned brown-black. After cooling, 5 ml of 30% (w/v) H_2O_2 was added to the solution. The mixtures were then gently shaken and heated again for 20 min. The last step was repeated until the liquid became clear, and the flasks were heated for 10 min until H_2O_2 was eliminated. Distilled water was then added to each flask to a final volume of 100 ml. Each solution was analysed for N, P and K, and expressed in mg per plant on the basis of dry weight. The total nutrient content was determined using the Kjeldahl method (Bradstreet 1965), P by the phosphovanadomolybdate method (Jackson 1973) and total K was detected using ammonium acetate extraction-flame photometry (Nelson 1982).

Mycorrhizal colonization. The entire root systems were carefully washed, fifty thin fragments of roots, each 1 cm in length, were taken from the entire root system in each treatment, cut into 1 cm long segments, cleared in 10% KOH at 90°C for 20 min, acidified in 2% HCl for 5 min, and stained with 0.01% acid fuchsine (Phillips, Hayman 1970). Mycorrhizal colonization rate was measured using the gridline intercept method (Giovannetti, Mosse 1980).

Experimental design and statistical analysis. The experiment was done based on a completely randomized design with four replicates per treatment, as each replicate consisted of eight plants. Normality and homogeneity were confirmed using Kolmogorov-Smirnov and Levene's tests, respectively. The data were analysed using one-way ANOVA. Differences between means were analysed by Duncan's multiple range test at $P \leq 0.05$. Statistical analyses were performed with the SAS statistical software (Version 9.3, 2011).

RESULTS

Colonization and biomass allocation

Corylus avellana seedlings were colonized after all treatments involving inoculation with fungi. The non-inoculated samples showed no colonization. However, colonization rates between the two treat-

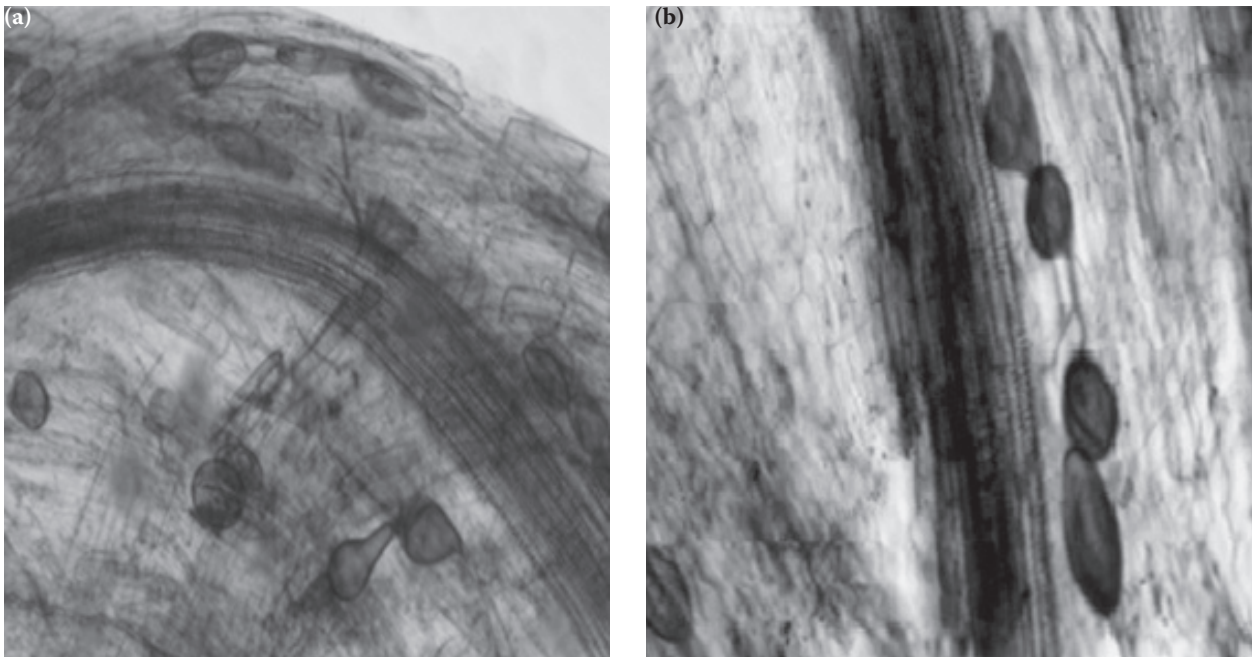

Fig. 1. Arbuscular mycorrhizal fungi colonization of *Corylus avellana* Linnaeus seedling roots: *Trichoderma harzianum* Rifai (a), *Glomus intraradices* Schenck & Smith (b)

ments showed significant differences ($P \leq 0.05$). The colonization rates were: *G. intraradices* (48.8%) and *T. harzianum* (35.5%) (Figs 1 and 2).

The effect of inoculation with *G. intraradices* and *T. harzianum* strains on biomass allocation of *C. avellana* seedlings is presented in Table 1. The inoculation with *G. intraradices* or *T. harzianum* significantly ($P \leq 0.05$) enhanced all biomass parameters compared to the controls. Stem dry mass increased by 30 and 14% by *G. intraradices* and *T. harzianum*, respectively (Table 1). Similarly, the inoculation of *G. intraradices* caused a 21% increase in the stem dry mass of seedlings compared to uninoculated ones. All biomass parameters in plants inoculated with *T. harzianum* were significantly lower than those in plants inoculated with *G. intraradices*. *G. intraradices* had a significant effect on root dry mass (Table 1). The application of *G. intraradices* also increased root volume by 75.6%. Likewise, the highest total dry mass (11.20 g) was observed in seedlings inoculated with *G. intraradices* (Table 1).

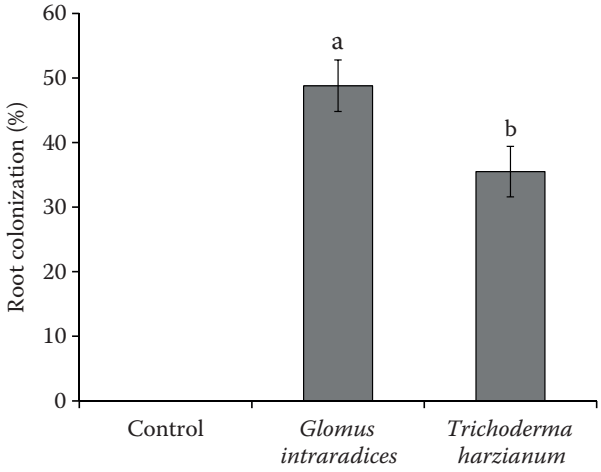

Fig. 2. Root colonization rates of *Corylus avellana* Linnaeus seedlings. Means ± SE on bars followed by different letters are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

Effects of inoculation on photosynthetic capacity of hazelnut seedlings

Higher photosynthetic and transpiration rates were observed in plants dually inoculated with *G. intraradices* or *T. harzianum*. Inoculation with *G. intraradices* and *T. harzianum* enhanced net photosynthetic rate (63.6 and 24.4%), stomatal conductance (66.1 and 31.4%) and water use efficiency (48.8 and 22.4%) compared to controls (Table 2). Similarly, inoculation with *G. intraradices* or *T. harzianum* promoted the chlorophyll content of hazelnut seedlings by 105.19 and 62.82%, respectively (Fig. 3).

Nutrient uptake

Both *G. intraradices* and *T. harzianum* improved nutrient accumulation. *G. intraradices* resulted in the most efficient nutrient absorption (Table 3),

Table 1. Biomass allocation of *Corylus avellana* Linnaeus seedlings in response to fungi treatments

Parameter	Uninoculated	*Glomus intraradices* Schenck & Smith	*Trichoderma harzianum* Rifai
Leaf dry mass (g)	2.05 ± 0.21	2.66 ± 0.18	2.38 ± 0.21
Stem dry mass (g)	4.27 ± 0.81^{b}	5.17 ± 0.91^{a}	4.58 ± 0.76^{b}
Root dry mass (g)	2.22 ± 0.26^{c}	3.39 ± 0.23^{a}	2.72 ± 0.41^{b}
Total dry mass (g)	8.54 ± 0.76^{b}	11.20 ± 0.93^{a}	8.72 ± 0.81^{b}
Root volume (cm^{3})	6.44 ± 1.03^{c}	11.31 ± 1.13^{a}	8.85 ± 1.08^{b}

means ± SE in a row followed by different letters are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

Table 2. Effect of inoculation with *Glomus intraradices* Schenck & Smith and *Trichoderma harzianum* Rifai on the net photosynthetic rate, stomatal conductance, transpiration rate and water use efficiency of *Corylus avellana* Linnaeus seedlings

Parameter	Control	*G. intraradices*	*T. harzianum*
Net photosynthetic rate (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)	7.26 ± 0.91^{c}	11.88 ± 1.90^{a}	9.18 ± 1.77^{b}
Transpiration rate (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$)	2.96 ± 0.28^{b}	3.23 ± 0.13^{a}	3.01 ± 0.14^{b}
Stomatal conductance)mol $CO_2 \cdot m^{-2} \cdot s^{-1}$	0.121 ± 0.07^{b}	0.201 ± 0.09^{a}	0.159 ± 0.06^{ab}
Water use efficiency (μmol $CO_2 \cdot mmol^{-1}$ H_2O)	2.45 ± 0.21^{c}	3.68 ± 0.62^{a}	3.00 ± 0.27^{b}

means ± SE in a row followed by different letters are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

Table 3. Influences of *Glomus intraradices* Schenck & Smith and *Trichoderma harzianum* Rifai on nutrient uptake in leaves of *Corylus avellana* Linnaeus seedlings

Parameter	Control	*G. intraradices*	*T. harzianum*
N (mg per plant)	3.22 ± 0.22^{c}	5.10 ± 0.13^{a}	4.11 ± 0.21^{b}
P (mg per plant)	0.34 ± 0.10^{c}	0.63 ± 0.09^{a}	0.50 ± 0.04^{b}
K (mg per plant)	1.31 ± 0.19^{b}	2.40 ± 0.34^{a}	2.04 ± 0.17^{ab}

means ± SE in a column followed by different letters are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

with increases of 58.4% (N), 85.2% (P) and 83.2% (K) in plants. *T. harzianum* only slightly affected leaf nutrient contents compared to *G. intraradices*. The inoculation of seedlings with *T. harzianum* increased N, P and K uptake by 27.6, 47 and 83.2%, respectively (Table 3).

DISCUSSION

In the present study, two fungal strains, *G. intraradices* and *T. harzianum*, were evaluated for improving morphological and physiological parameters of hazelnut seedlings under nursery conditions. In reality, *G. intraradices* and *T. harzianum* had the ability of colonizing on the roots of hazelnut seedlings after 7 months. The colonization rate for the *G. intraradices* treatment was 48.8%, which is significantly higher than that for the *T. harzianum* (35.4%) treatment. In fact, the colonization rates for the two inoculation treatments significantly differed. This might be due to different status of hosting in hazelnut seedlings (Estaun et al. 2003; Mirabelli et al. 2009). Similarly, several authors demonstrated that the highest colonization rate was caused by *G. intraradices* (Adams et al. 2007; Mirzaei 2014). It can be stated that the coloniza-

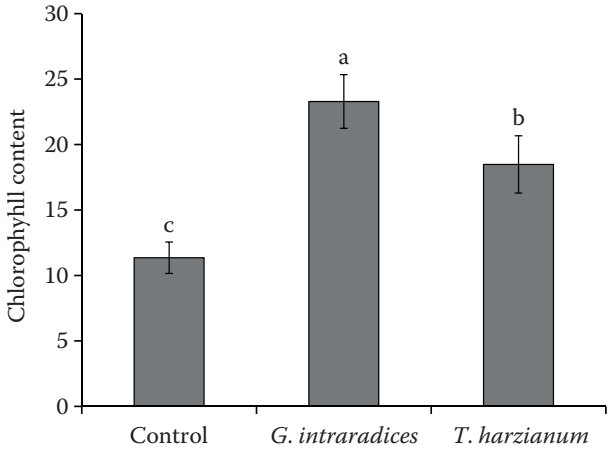

Fig. 3. Effects of inoculation with *Glomus intraradices* Schenck & Smith and *Trichoderma harzianum* Rifai on the chlorophyll content of *Corylus avellana* Linnaeus seedlings measured by chlorophyll meter model SPAD 502 (Minolta Co., Ltd., Japan). Means ± SE on bars followed by different letters are significantly different according to Duncan's multiple range tests at $P \leq 0.05$

tion rate of plants by fungi depends mainly on the host plant, fungal species, and on environmental conditions, such as nutrient levels, light intensity and temperature (Smith, Smith 2011).

In our study, both *G. intraradices* and *T. harzianum* also stimulated the growth of hazelnut seedlings in all growth parameters over the uninoculated seedlings. Several authors suggested that mycorrhizal fungi produced growth hormones that change root growth, development and physiology (Ek et al. 1983; Smith, Reid 1997). Some growth characteristics enhanced by fungi in hazelnut seedlings are fully explicable from this experiment. It appeared to be directly related to the production of relatively efficient root systems as mycorrhizal seedlings had significantly higher root biomass compared with the control. This is in agreement with many studies on plants, such as Quoreshi and Khasa (2008), who reported that the increase in the height and shoot dry weight of *Populus tremuloides* Michaux seedlings by 46.7 and 186.5%, respectively, was due to *G. intraradices* after 8 months of inoculation. Likewise, *P. wallichiana* seedlings inoculated with *T. harzianum* produced the significantly higher biomass of needles, trunks and roots than uninoculated plants (Ahangar et al. 2012).

It is known that AMF stimulate plant growth through a range of mechanisms that include improved ability to absorb water, nutrients and to combat various diseases (Sharma et al. 1992; Artursson et al. 2006). The same as Alguacil et al. (2006) in our findings, leaf gas exchange parameters were remarkably improved by fungal strains. Inoculated seedlings with *G. intraradices* increased net photosynthetic rate and water use efficiency by 63.6 and 48.8%, respectively, which could also explain higher nutrient content in the shoots of plants grown in these media. This is also in agreement with Querejeta et al. (2003), who demonstrated that inoculation with *G. intraradices* significantly enhanced leaf gas exchange parameters of *O. europaea* seedlings, so that the rate of photosynthesis was 48% higher than that in the *G. intraradices* inoculated plants. The rate of photosynthesis may also have been differentially stimulated by the increased sink strength arising from the additional carbon requirements of the mycorrhizal fungus colonizing the roots (Wright et al. 1998).

The *T. harzianum* application in our study only slightly improved photosynthetic and transpiration rates compared to *G. intraradices*. Changes in transpiration could cause a change in the rate of photosynthesis changing the supply of carbohydrates to the fungus. Alternatively, higher nutrient uptake due to higher transpiration rates could be due to the mass flow of nutrients towards the roots (Sharma et al. 1992). In our findings, inoculation of the two strains improved the chlorophyll content of hazelnut seedlings as compared with uninoculated seedlings. The increase in levels of chlorophyll content in fungus treated seedlings has been reported due to the direct consequence of symbiotic association which led to a higher uptake of water and nutrients resulting in higher biosynthesis (Dutt et al. 2013).

The same as Ahangar et al. (2012), in our research findings in spite of the root colonization, the growth, physiological parameters and nutrient uptake in hazelnut seedlings inoculated with *T. harzianum* were lower than those with *G. intraradices*, but the inoculated seedlings showed an increase in growth and physiological characteristics compared to the control. Correspondingly, the findings of Talbi et al. (2016) on *C. siliqua*, Roohbakhsh and Davarynejad (2013) on *Ziziphus jujuba* Miller indicated that *T. harzianum* had a significant effect on the growth of seedlings. It can be confirmed that *Trichoderma* Persoon species produce hormone-like metabolites and release nutrients from soil or organic matter, thereby facilitating better plant growth (Windham et al. 1986).

In line with Ahangar et al. (2012), in our findings, nutrient (N, P, and K) uptake was significantly improved by fungal inoculants. Although *T. harzianum* inoculation had a smaller effect on the seedling nutrient concentration, it can be suggested that mycorrhizal symbiosis improves the utilization of the absorbed nutrients. Similarly, Lu et al. (2015) reported that container-grown mulberry seedlings infected by *G. intraradices* increased the internal concentration of N, P and K in seedlings. Quoreshi and Khasa (2008) also showed that the effects of *G. intraradices* on P uptake in *P. tremuloides* seedlings were mostly within the range of the effects exerted by the respective single species.

CONCLUSIONS

In our investigation both *G. intraradices* and *T. harzianum* improved the growth and physiological characteristics of hazelnut seedlings and simultaneous root colonization, when the growth rate induced by *G. intraradices* was significantly higher than that induced by *T. harzianum*. However, even though *G. intraradices* compared with *T. harzianum* more properly influenced the characteristics of growth, gas exchange and leaf nutrients in

seedlings of hazelnut, the inoculation of hazelnut roots with both AMF can be advised to produce the healthy and strong seedlings of this species in the nursery.

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