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Effects of Arbuscular Mycorrhizal Fungi on Growth and Nutrient Uptake of Apple Rootstocks in Calcareous Soil

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Abstract
The effects of three Arbuscular Mycorrhizal Fungi (AMF) species (*Glomus versiforme, Claroideoglomus etunicatum* and *Rhizophagus intraradices*) were studied on the growth parameters and nutrient uptake of three apple rootstocks (M.9, M.7 and MM.106). The soil medium contained a high level of calcium carbonate (38.65%). The results showed that AMF inoculation could increase almost all growth parameters. Plants inoculated with *G. versiforme* had the highest shoot height, stem diameter, leaf size, and biomass compared to other AMF species. Rootstocks also varied substantially in their growth. MM.106 exhibited the highest growth in general, whereas the M.9 had the lowest. Plants inoculated with mycorrhizal fungi contained more N, P, Ca, Mg, Zn, and Fe compared to those of Non Mycorhizal (NM) control plants. However, AMF inoculation did not influence concentrations of K, Cu, and Mn in the leaf. Rootstocks exhibited various nutrient concentrations in their leaves and the highest concentrations of K, Ca, Mg, and Zn were measured in MM.106. The highest leaf concentration of N, Fe, Mn, and Cu occurred in M.9, while the M.7 accumulated the highest amount of P in its leaves. Here we demonstrate the beneficial effects of symbiosis between apple rootstocks and AMF species in calcareous soil with a very high level of lime concentration.

Keywords: Apple rootstocks, fungi, growth parameters, nutrient concentrations.

Abbreviations: AMF, Arbuscular Mycorrhizal Fungi; **NM**, non mycorrhizal; **P**, Phosphorus; **Fe**, Ferrous; **Zn**, Zinc; **Cu**, Copper; **Mn**, Manganese, **Ca**, Calcium; **Mg**, Magnesium; **N**, Nitrogen; **K**, Potassium.

Introduction

Apple (*Malus* × *domestica* Borkh.) is the 3rd most important fruit crop worldwide, after citrus and banana (FAOSTAT, 2013). In 2013, the total apple production and harvest in the world was estimated at 80.8 million tons and 5.2 million hectares, respectively. Iran with nearly 2.8 % of the total harvestable area (~130,000 ha) and 2.2% of the total production (1.7 million tons), is the 7th largest apple producer in the world after China, the USA, Turkey, Poland, India, and Italy (FAOSTAT, 2013). Approximately 55% of all commercial apple production in Iran comes from four provinces, namely, West Azerbaijan, East Azerbaijan, Fars, and Khorasan-e Razavi. There are commercial apple productions also in Isfahan, Teheran, Alborz, Zanjan, and Ardebil provinces (Ardestani, 2015). Most apple orchards in Iran are located on calcareous soils

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characterized by high pH associated with high carbonate. Under such conditions, plant growth is limited by several factors including low concentrations of several micronutrient elements such as Fe, Mn, Zn (Malakouti, 2006). As a consequence, large input of acidic fertilizers and high value nutrient chelates are required, making apple production costs in Iran higher than countries with normal soils.

The arbuscular mycorrhizal (AM) symbiosis is an association between the roots of higher plants and soil fungi that promotes plant development, especially under suboptimal growth conditions (Koltai *et al.*, 2010). In this symbiosis, the fungal partner provides some vital nutrients such as phosphorus (Harrison & van Buuren, 1995), nitrogen (Hodge *et al.*, 2001), and other nutrients (Liu *et al.*, 2000) to the host plant. Through photosynthesis, the plant partner supplies portions of the fixated carbon to the fungus in exchange (Graham, 2000). AMF can improve biomass production (Smith *et al.,* 2009) and pathogen resistance (Vigo *et al.*, 2000) of the host plant. In addition, AMF mitigate various kinds of plant stresses such as drought, salinity and cold (Aroca *et al.*, 2007; Hoda *et al.*, 2011), and heavy metal toxicity (Hildebrandt *et al.*, 1999) and can protect plants against root-eating herbivores (Gange, 2001).

The effects of AMF on the growth, development and productivity of many horticultural crop plants have been studied. Interestingly, fruit crops have received more attention than vegetables or ornamentals (Kolati *et al.,* 2010). Several reports have shown that the arbuscular mycorrhizal association is very efficient in promoting growth in apple plants. Inoculation of apple cuttings with *Glomus macrocarpum* had beneficial effects on their growth and nutrient uptake in a soil fertilized with various P levels (Gnekow and Marschner, 1989). Morin *et al.* (1994) studied the efficiency of inoculation via three species of vesicular-arbuscular mycorrhizal fungi on four cultivars of apple rootstocks (M.26, Ottawa 3, P.16, and P.22) in soil containing high levels of extractable phosphorus. They found that mycorrhizal plants were taller, produced more biomass, and had a higher P concentration in the leaf, compared to the control plants. Geddeda *et al.* (1984) showed that inoculation with AMF isolates can result in increased plant height and biomass in the apple plant compared with non-inoculated plants. They also reported a negative correlation between soil P levels and root colonization rates, reflecting the consequences of adding fertilizers to the soil. Miller *et al.* (1985) also reported that plants growing in soils which do not receive the P fertilizer treatment are the only plants that are responsive to the mycorrhizal association. Enhanced growth and P uptake has been reported in micropropagated apple rootstocks inoculated with *Glomus mosseae* at the transplant stage (Schubert and Lubraco, 2000). Inoculation of apple rootstock with selected fungal isolates during the acclimatization stage represents a useful strategy for producing micropropagated apples that can withstand acidic soil conditions (Cavallazzi *et al.*, 2007). Sharma *et al.* (2012) reported significant improvement in the vegetative growth parameters of the apple saplings by using single and/or dual application of soil inoculation of AM fungi and Azotobacter strains at nursery stage under reduced inorganic fertilization.

Dwarfing rootstocks are widely used in the apple industry throughout the world to reduce the strong vigor of scions, allowing them to be grown as dwarf, closely planted trees that are easy and inexpensive to manage. A very large range of clonal rootstocks is now available for use by nurserymen and fruit growers. Rootstocks providing vigor control similar to that of M.27 are often referred to as super dwarfing and those similar to M.9 as dwarfing. Similarly, rootstocks with vigor similar to M.26 are classified as semi-dwarfing and those similar to MM.106 as semi-vigorous. The vigorous rootstocks are those similar to

M.25 or to seedlings (Webster and Wertheim, 2003). The majority of Iran's apple production is practiced in traditional and low density orchards by using seedling rootstocks. However, a slow shift from low density orchards to semi-intensive and intensive orchards is occurring in recent years, whereby semi-vigorous and dwarfing rootstocks are used respectively. The MM.106, M.9, M.26, and MM.111 are among the frequently used rootstocks for establishing new orchards. It is well known that rootstocks can influence mineral concentrations in the leaves of scion cultivars (Fallahi *et al.,* 1994; Kucukyumuk *et al.,* 2011). Therefore, the appropriate choice of rootstock can, in certain circumstances such as adverse soil conditions, make a difference by optimizing the general profitability and preventing the loss of the apple orchard.

Knowledge of the capacity of rootstocks in nutrient uptake is important in determining how much nutrient is required for a special rootstock-cultivar combination. Since AMF symbiosis results in improved plant growth and health, it is expected that through this association, agronomic practices will be able to lessen their chemical fertilizer and pesticide input. The specific aim of this research was to integrate the use of native AMF isolates with the frequently used apple rootstock so as to alleviate the hazards of calcareous soils. Specifically, we used native AMF isolates. The soil also was sampled from a commercial apple orchard, the calcareous nature of which represents a common restriction on apple growing areas.

Materials and Methods

Experimental site and treatments

The study was conducted over a whole growing season, from March to September 2012. Under greenhouse conditions, a factorial experiment was used based on a completely randomized design with three replications in order to evaluate the effects of three AM fungus species (*Glomus versiforme*, *Rhizophagus intraradices* (synonym = *G. intraradices*), and *Claroideoglomus etunicatum* (synonym = *G. etunicatum*)) on three clonal apple rootstock cultivars (M.9, M.7 and MM.106). Plant materials were obtained from a commercial nursery in Neyriz, Iran. They included one-year-old uniform layers of three apple rootstocks which had been propagated in a sandy-loam soil substrate via the mound layering method. Apple rootstock cultivars were either not inoculated (control) or were inoculated with either of the three AMF-species. An experimental unit (each treatment of each replication) consisted of 3 observations (Each part contains 3 plants).

Inoculum sources

The fungal spores were prepared in the department of Soil Science, Shiraz University. *R. intraradices* and *G. versiforme* were taken from a non-contaminated area of the Anguran Mine in Zanjan, Iran (Zarei *et al.,* 2008a) and *C. etunicatum* was collected from Tabriz University, Tabriz, Iran. These fungi are found abundantly in Iranian soils (Aliasgharzadeh *et al.,* 2001; Kariman *et al.,* 2005). Mycorrhizal inocula were prepared via the trap culture of maize (*Zea mays* L.). The trap culture medium was composed of autoclaved soil/quartz-sand (< 1 mm) (1: 4, v/v) (Liu and Wang, 2003). The potential efficacy of inoculants was measured based on the described methods of Zarei *et al.* (2008b), for spore extraction, counting, and evaluation of root colonization. The spore numbers were 9-11 g^{-1} of substrate and the rate of root colonization was 75-85%.

Soil preparation and its physic-chemical analysis

The soil samples that were used for the pot experiment were collected from a commercial apple orchard located in Kudian region of Fars province. Some physical and chemical properties of the soil are presented in Table 1.

Table 1. Some physical and chemical properties of the studied soil.

Physical properties				Chemical properties									
Texture	Sand (%)	Clay (%)	Silt (%)	Organic matter (%)	$CaCO_3$ (%)	pH	EC (dS m^{-1})	Olsen-P (mg kg^{-1})	DTPA-Fe (mg kg^{-1})	DTPA-Cu (mg kg^{-1})	DTPA-Zn (mg kg^{-1})	DTPA-Mn (mg kg^{-1})
Sandy clay loam	58.00	30.80	11.20	1. 80	38.65	7.80	0.40	12.80	6.30	8.40	1.80	10.60

Rootstocks planting and AMF inoculation procedure

The 5.6 kg plastic pots were filled with soil that had been 4mm-sieved and air-dried. In order to be close to real field conditions, the soil samples were not sterilized. In mycorrhizal plants, 50 g of AM inoculants (containing spore numbers of 9-11 g-1 substrate and a root colonization of 75-85%) were added to the pots just below the root zone of the rootstocks at the time of planting. The non mycorrhizal (NM) control received an equal amount of autoclaved inoculum plus 1 ml of filtrated suspension of inoculum. Immediately after planting, rootstocks were headed back (3 cm above soil), and new growth was allowed to begin. Plants were irrigated every three days, with small amounts of tap water to avoid leaching. No additional fertilizers were applied.

Growth and root colonization measurements

At the end of the experiment, but before harvest, and chronologically 5 months after mycorrhizal inoculations, plant height and diameter were measured using the ruler and a digital calliper device, respectively. The leaf size of each plant was measured by a planimeter based on five fully expanded mature leaves of the same position on plants (Sokkisha, model KP-90N, made in Japan). Plants were harvested. Roots and stems were separated, weighed, and then dried at 65 °C for 72 h and weighed once again. To assess the rate of RC, 2 grams of fresh root samples per plant (sampled before drying) were fixed in formalin/acetic acid/alcohol solutions (FAA). After washing the roots in 10% KOH and staining them with 0.01% acid fuchsin and lactoglycerol, the grid-line intersect method was used to measure the percentage of the AM-root colonization (Kormanik and McGraw, 1982). All measurements were done separately in the 3 observations of each experimental unit. Measurements were then averaged and used in statistical analysis.

Nutrients assessment

Composite subsamples of dried leaves from each experimental unit were grinded and burnt to ash for mineral analysis. The concentration of Nitrogen (N) was determined using the Kjeldahl's method (Bremner, 1996). The concentration of Phosphorus (P) was measured using the ammonium molybdate vanadate method (Chapman and Pratt, 1961). The potassium (K) concentration was measured using the flame photometer (Model PFP 7, made by JENWAY Ltd., UK). The concentrations of Ferrous (Fe), zinc (Zn), copper (Cu), manganese (Mn), calcium (Ca) and magnesium (Mg) were measured via atomic absorption spectrometry (Shimadzu AA-670 G, Made in Japan) (Wu *et al.,* 2011). Concentrations of the macronutrients (N, P, K, Ca and Mg) were expressed as percentages of dry weight, while those of micro-nutrients (Fe, Zn, Cu and Mn) were expressed as µg g^{-1} dry weight.

Statistical analysis

A test for the normality of data was used to check normal distribution in Minitab software (v.14). All of the measured data had normal distribution and consequently the original data for all traits were used to be analyzed. The data were analyzed based on a factorial experiment and a completely randomized design in SAS software (V. 9.0). A Least Significant Difference (LSD α= 0.05) test, using General Linear Model (GLM) was used for mean comparisons. Based on ANOVA results, both of the main factors (rootstocks and AMF) were significant for all the measured traits, but the interaction effects were significant in only some of the measured traits. Consequently, we present the main effects for all of the traits and also the interaction effects just in the case of their significance.

Results

Root colonization

Root colonization was significantly higher in mycorrhizal plants, compared to those of non-mycorrhizal control plants. Amongst the AMF species, *G. versiforme* was successful in colonizing apple rootstocks more than the other two AMF species. The highest and the lowest root colonization were found in M.7 and M.9 rootstocks, respectively (Table 2). Considering the interaction effects of AMF species and rootstocks, the highest root colonization on M.7 and MM.106 were observed following the inoculation with *G. versiforme*. On the other hand, there were no significant differences among inoculation treatments on the M.9 rootstock (Table 3).

Growth parameters

As compared to non-mycorrhizal plants, mycorrhizal inoculation increased almost all of the measured growth parameters. Amongst AMF species, *G. versiforme* proved to be significantly superior to the other two species, thereby improving almost all of the growth parameters. Among rootstocks, the MM.106 showed significantly higher values for many of the growth parameters, including stem diameter, fresh and dry weights of the stem, and also fresh and dry weights of the root. Furthermore, the M.7 had the highest shoot height while M.9 showed the largest leaf size (Table 2).

Considering the interaction effects of rootstocks and mycorrhizal treatments (Table 3), the highest shoot heights of M.7 and M.9 were observed following their inoculation with *G. versiforme*. However, there were no significant differences among inoculation treatments on the MM.106 rootstock. However, the largest leaf size in MM.106 was seen following the inoculation with *G. versiforme*. The largest leaves in the M.7 resulted from separate inoculations with *G. versiforme* and *R. intraradices*, and no significant differences were observed between the two cases. The largest leaves in the M.9 were observed when inoculated with *G. versiforme* and *C. etunicatum* as separate treatments. No significant differences occurred between these two treatments, either. Inoculation treatments did not influence shoot fresh weight of MM.106 and M.9, but significant differences were observed on shoot fresh weight of M.7 plants following the inoculations with different fungi. Differences in shoot biomass among rootstocks, caused by AMF inoculations, are illustrated in Figure 1.

Nutrients concentrations

AMF inoculation significantly affected leaf concentrations of N, P, Ca, Mg, Zn, and Fe in apple rootstocks compared to the NM control plants. However, leaf concentrations of K, Cu, and Mn were not affected by mycorrhizal treatments. There were significant differences in the nutrient uptake of P, Mg, and Fe as a result of inoculation with the various AMF species (Table 4). The plants colonized by *G. versiforme* showed the highest concentrations of P, Mg, and Fe in their leaves (Table 4).

Rootstocks also varied in their leaf accumulation of all measured nutrients.

The MM.106 accumulated higher concentrations of K, Mg, Ca, and Zn in its leaves while the M.9 showed more N, Cu, Mn, and Fe in its leaves. The M.7 accumulated P more efficiently than the other two rootstocks (Table 4).

Table 2. Mean comparison of simple effects of rootstocks and AMF inoculation on root colonization, growth, and biomass of apple rootstocks 21 weeks after inoculation

Treatments	Root colonization (%)	Shoot height (cm)	Stem diameter (mm)	Leaf area (cm^2)	Fresh weight ($g\ plant^{-1}$)		Dry weight ($g\ plant^{-1}$)	
					Shoot	Root	Shoot	Root
AMF Species								
Control	5.55^{c}	47.22^{c}	5.56^{c}	32.36^{d}	27.63^{b}	18.34^{c}	13.39^{c}	10.20^{c}
G. versiforme	72.77^{a}	57.55^{a}	7.23^{a}	37.25^{a}	34.20^{a}	29.06^{a}	17.64^{a}	15.06^{a}
R. intraradices	67.55^{b}	54.55^{b}	6.63^{b}	35.62^{b}	32.94^{a}	26.76^{b}	16.67^{ab}	13.05^{b}
C. etunicatum	67.44^{b}	52.77^{b}	6.83^{b}	34.38^{c}	33.05^{a}	25.57^{b}	15.61^{b}	12.80^{b}
ANOVA	**	**	**	**	**	**	**	**
Rootstocks								
MM.106	54.08^{b}	54.66^{b}	7.48^{a}	33.41^{b}	35.50^{a}	28.78^{a}	17.87^{a}	14.60^{a}
M.7	55.83^{a}	59.08^{a}	5.72^{c}	31.91^{c}	28.77^{c}	21.45^{c}	15.02^{b}	10.93^{c}
M.9	50.08^{c}	45.33^{c}	6.74^{b}	39.39^{a}	31.59^{b}	24.56^{b}	14.60^{b}	12.80^{b}
ANOVA	**	**	**	**	**	**	**	**

Means followed by different letters are significantly different at the adjacent ANOVA level (* = $P \leq 0.05$ and ** = $P \leq 0.01$) according to LSD. ns = non-significant.

Table 3. Mean comparison of interaction effects of rootstocks and AMF inoculation on root colonization, shoot height, leaf size, and shoot fresh weight of apple rootstocks 21 weeks after inoculation

Rootstocks	AMF species	Root Clonization (%)	Shoot Height (cm)	Leaf size (cm^2)	Shoot fresh weight ($g\ plant^{-1}$)
MM.106	Control	7.00^{e}	52.33^{ef}	31.32^{f}	32.79^{bc}
	G. versiforme	75.33^{a}	56.33^{cd}	36.38^{c}	37.01^{a}
	R. intraradices	67.66^{cd}	54.00^{de}	33.6^{d}	35.31^{ab}
	C. etunicatum	66.33^{d}	56.00^{cd}	32.32^{e}	36.90^{a}
M.7	Control	7.33^{e}	49.00^{fg}	28.92^{g}	22.54^{e}
	G. versiforme	76.00^{a}	66.66^{a}	34.10^{d}	33.80^{b}
	R. intraradices	69.33^{bc}	66.00^{a}	34.22^{d}	30.85^{c}
	C. etunicatum	70.66^{b}	58.66^{bc}	30.38^{f}	27.90^{d}
M.9	Control	2.33^{f}	40.33^{h}	36.82^{c}	27.56^{d}
	G. versiforme	67.00^{cd}	49.66^{fg}	40.06^{a}	31.78^{bc}
	R. intraradices	65.66^{d}	47.66^{g}	39.04^{b}	32.66^{bc}
	C. etunicatum	65.33^{d}	43.66^{h}	40.42^{a}	34.36^{b}
ANOVA		*	**	**	*

Means followed by different letters in the column are significantly different at the adjacent ANOVA level (* = $P \leq 0.05$ and ** = $P \leq 0.01$) according to LSD.

Fig. 1. Root volume and morphology of different rootstocks inoculated with different AMF species, 21 weeks after inoculation

Table 4. Mean comparison of simple effects of rootstocks and AMF inoculation on leaf mineral concentration (of dry weight) of apple rootstocks 21 weeks after inoculation

Treatments	N (%)	P (%)	K (%)	Mg (%)	Ca (%)	Zn (μg g^{-1})	Cu (μg g^{-1})	Mn (μg g^{-1})	Fe (μg g^{-1})
AMF Species									
Control	1.93^{b}	0.22^{c}	1.33^{a}	0.34^{c}	1.21^{b}	15.78^{b}	11.57^{a}	55.77^{a}	124.11^{c}
G. versiforme	2.13^{a}	0.36^{a}	1.34^{a}	0.42^{a}	1.39^{a}	17.53^{a}	11.54^{a}	54.66^{a}	131.22^{a}
R. intraradices	2.11^{a}	0.32^{b}	1.34^{a}	0.41^{a}	1.37^{a}	16.93^{a}	11.68^{a}	54.55^{a}	129.33^{b}
C. etunicatum	2.10^{a}	0.31^{b}	1.34^{a}	0.36^{b}	1.36^{a}	17.20^{a}	11.61^{a}	54.11^{a}	130.00^{c}
ANOVA	**	**	ns	**	**	**	ns	ns	**
Rootstocks									
MM.106	1.97^{b}	0.31^{b}	1.40^{a}	0.43^{a}	1.40^{a}	19.34^{a}	11.08^{b}	50.00^{b}	127.75^{b}
M.7	1.98^{b}	0.33^{a}	1.34^{b}	0.34^{c}	1.36^{b}	15.91^{b}	10.66^{b}	49.66^{b}	124.00^{c}
M.9	2.26^{a}	0.27^{c}	1.27^{c}	0.39^{b}	1.24^{c}	15.33^{b}	13.06^{a}	64.66^{a}	134.25^{a}
ANOVA	**	**	**	**	**	**	**	**	**

Means followed by different letters are significantly different at the adjacent ANOVA level (* = $P \leq 0.05$ and ** = $P \leq 0.01$) according to LSD. ns = non-significant

The rootstock cultivar × inoculation interaction effects were evident in leaf concentrations of P, Mg, Zn, and Fe but not for N, K, Mn, and Cu (Table 5). However, the highest leaf concentrations of P in all of the rootstocks were observed following the inoculation with *G. versiforme*. It should be noted that the leaf concentration of P in M.9 was significantly lower than those of the other two rootstocks. Regarding the MM.106, there were no significant differences in the leaf concentration of P following the inoculations with *R. intraradices* and *C. etunicatum.* Similar results were obtained in M.7 after inoculation with *G. versiforme* and *R.*

intraradices and in M.9 after inoculation with *R. intraradices* and *C. etunicatum*. Likewise, the highest leaf concentrations of Mg in all of the rootstocks were observed following the inoculation with *G. versiforme*. Leaf concentrations of Mg in M.7 were significantly lower than those of the other two rootstocks. The MM.106 exhibited no significant differences in the leaf concentration of Mg among most mycorrhizal treatments. An exception was the inoculation with *R. intraradices* which resulted in a significantly lower leaf concentration of Mg in M.7 and M.9. This lower concentration is to be compared to those of the other two mycorrhizal fungi. The highest leaf concentration of Zn in MM.106 was observed as a result of inoculation with *G. versiforme* and this had significant differences with those of the other two fungi treatments. However, there were no significant differences in the leaf concentration of Zn among mycorrhizal treatments when considering the M.7 and M.9. The highest leaf concentration of Fe in MM.106 and M.7 were observed as a result of inoculation with *G. versiforme* and *C. etunicatum*, although with no significant differences. Similar results in M.9 were observed after inoculation with *G. versiforme* and *R. intraradices,* again without any significant differences.

Table 5. Mean comparison of interaction effects of rootstocks and AMF inoculation on leaf mineral concentration (of dry weight) of apple rootstocks 21 weeks after inoculation

Rootstocks	AMF species	P (%)	Mg (%)	Zn ($\mu g\ g^{-1}$)	Fe ($\mu g\ g^{-1}$)
MM.106	Control	0.253^{f}	0.41^{b}	17.23^{c}	123.30^{e}
	G. versiforme	0.390^{a}	0.44^{a}	21.43^{a}	131.00^{c}
	R. intraradices	0.310^{cde}	0.44^{a}	19.33^{b}	127.30^{d}
	C. etunicatum	0.316^{cd}	0.43^{ab}	19.36^{b}	129.33^{c}
M.7	Control	0.230^{g}	0.28^{f}	15.67^{d}	119.00^{g}
	G. versiforme	0.390^{a}	0.38^{c}	15.46^{de}	126.00^{d}
	R. intraradices	0.373^{a}	0.36^{cd}	16.03^{cd}	124.00^{e}
	C. etunicatum	0.340^{b}	0.33^{e}	16.50^{cd}	126.60^{d}
M.9	Control	0.190^{h}	0.36^{de}	14.44^{e}	130.00^{c}
	G. versiforme	0.323^{bc}	0.44^{a}	15.71^{d}	136.60^{a}
	R. intraradices	0.290^{e}	0.43^{ab}	15.43^{de}	136.30^{a}
	C. etunicatum	0.300^{de}	0.34^{de}	15.73^{d}	134.00^{b}
ANOVA		**	**	**	**

Means followed by different letters are significantly different at the adjacent ANOVA level (* = $P \leq 0.05$ and ** = $P \leq 0.01$) according to LSD. ns = non-significant.

Discussion

Low rates of root colonization in non-inoculated plants proved that the endemic AMF population is not high enough to colonize apple rootstocks effectively. Among the AMF species, *G. versiforme* managed to colonize apple rootstocks more than the other two AMF species. Morin *et al.* (1994) showed that *G. versiforme* produced more external mycelium which explains why this species is the best isolated fungi in this context, compared to *R. intraradices* and *G. aggregatum*. Considering the interaction effects, the AMF species generated varied colonization rates in different apple rootstocks. This variation among fungi species could stem from the differences in their mycelium type and abundance. The degree of root colonization by AMF species varied among the rootstocks. The highest and the lowest root colonizations were found in M.7 and M.9 rootstocks, respectively. In relation to this, Morin *et al.* (1994) studied four apple rootstocks (P.22, O.3, M.26 and P.16) and found that the most undersized one (P.22) was the least colonized rootstock which harmonizes with the results of our study. Variations in the degree of colonization in apple rootstocks could be due to their root morphology, root exudates, and their compatibility with AMF species (Schubert and Lubraco, 2000; Cavallazzi *et al.*, 2007).

From the results of the present study, it is obvious that most of the evaluated traits were observed to be significantly more favorable in rootstocks inoculated with mycorrhiza, as compared with their non-mycorrhizal counterparts. It has been well-established that AM symbiosis increases the growth of the host plant due to the improved nutrient absorption and better water uptake via external hyphae in inoculated roots (Hodge *et al.*, 2010; Kolati *et al.*, 2010).

The results proved that among the AMF treatments, *G. versiforme* was the most efficient species to improve growth parameters. This finding is in agreement with those of Morin *et al.* (1994), who reported that *G. versiforme* is the best AMF for growth promotion of apple rootstocks. However, Wu *et al.* (2011) reported that among the three AMF species they used (*G. mosseae*, *G. versiforme* and *Paraglomus occultum*), the *G. mosseae* was the most efficient and *G. versiforme* ranked as second in improving growth parameters of peach cultivars.

It is not surprising to observe that the M.9 produced the shortest plant among other rootstocks. It is well documented that the M.9 is considered as one of the most important dwarfing apple rootstocks. From this point of view, the M.7 and MM.106 are classified as semi-dwarfing and semi-vigorous rootstocks respectively (Webster and Wertheim, 2003).

Even though AMF symbiosis offers a wide range of benefits to the host plant, the most common cited benefit is the enhancement of nutrients acquisition, especially where plants are facing adverse soil conditions (Hodge *et al.*, 2010). In this study, all the AMF treatments contained higher leaf concentrations of N, P, Mg, Ca, Zn, and Fe compared to non-mycorrhizal plants. However, K, Mn, and Cu concentrations remained statistically similar to that of the control plants. The reason why AMF did not influence the concentrations of K, Mn, and Cu could be due to the fact that mycorrhizal hyphae are only responsible for the uptake of these nutrient elements, but are not involved in their translocation (Wu *et al.*, 2011). There are studies suggesting that AMF symbiosis may have little effects on improving or even hampering K, Cu, and Mn uptake (Arines *et al.*, 1989; Cavallazzi *et al.*, 2007).

Increased P absorption is one of the best known responses of host plants to AMF inoculation. Our results emphasize that the P concentration of apple leaves is greatly dependent on the species of mycorrhizal fungi, probably because the absorbing surface of plant root systems goes on to be well extended (Bolan *et al.*, 1991). Rootstocks are also different in their ability to accumulate P in their leaves; therefore, responsiveness to mycorrhizal colonization is a characteristic that could be considered in the evaluation of apple rootstocks. Furthermore, Zarei *et al.* (2006) reported that AMF may dissolve insoluble inorganic forms of P via the production of organic and inorganic acids and also via chelating agents.

All AMF species in this study significantly increased the uptake of Zn and Fe which is an important achievement since these elements are found in limited amounts in calcareous soils. Generally, elements with low mobility in the soil, such as P, Zn, and Fe can be absorbed in higher amounts by mycorrhizal plants (Yano-melo *et al.*, 1999). It has been proved that mycorrhizal symbiosis can improve Zn acquisition as a secondary consequence of P uptake (Liu *et al.*, 2000).

It is considered that mycorrhizal fungi increase nutrient uptake and transport by producing a variety of siderophores and chelating agents (Caris *et al.*, 1998).

Higher nutrient uptake by plants inoculated by AMF could also be ascribed to the fact that fungal hyphae penetrate into the root and soil, thereby increasing the surface areas of roots and thus acquiring more elements beyond the depletion zone (Schnepf *et al.*, 2011).

Regarding the MM.106 and M.7 rootstocks that were more vigorous, all of the measured macroelements were absorbed in higher amounts, except for N. This is in comparison to the dwarfing (M.9) rootstock. However, the M.9 was more efficient than the other two rootstocks in absorbing N and also in absorbing many of the micro elements apart from Zn (Table 4). Several researches have shown that more vigorous rootstocks have higher mineral concentrations (Aguirre *et al.*, 2001, Fallahi *et al.*, 2002; Kucukyumuk and Erdal, 2011). Similarly, Amiri and Fallahi, (2009) found that cultivars grafted on MM.106 and on seedling rootstocks accumulate the highest amounts of P and K in their leaves. Dwarf rootstocks such as the M.9 have coarser root systems and fewer fine roots than vigorous rootstocks (Neilsen *et al.*, 1997). Therefore, this can be a major reason why dwarf rootstocks have lower amounts of nutrients compared to other rootstocks.

High concentrations of N in M.9 rootstocks can be due to their lower rates of growth. This makes the M.9 more able to accumulate high concentrations of minerals in the leaves (Amiri and Fallahi, 2009). Dong *et al.* (1998) also observed higher N uptake in the M.9 EMILA rootstocks. The differences among the nutrient uptake of rootstocks could be explained by the different nutrient absorption capacities through the roots (Kayan, 2008). It can also be a result of the structure of root systems, root cation exchange capacities, rhizosphere pH, and characteristics of root exudates (Kucukyumuk and Erdal, 2011).

Enhanced growth and nutrient uptake of apple rootstocks following inoculation with mycorrhizal fungi were reported previously in the presence of high levels of phosphorus (Morin *et al.*, 1994) and acidic pH (Gavallazzi *et al.*, 2007). Here we approved the beneficial effects of a symbiosis relationship between apple rootstocks and AMF species in calcareous soils with very high levels of lime concentration. Particularly, the increased leaf concentration of Fe and Zn in apple rootstocks through symbiosis with AMF is of paramount importance. Varied leaf concentrations of these two trace elements among rootstocks means that the selection of suitable rootstocks can serve as part of the solution to alleviating the Fe and Zn deficiencies in calcareous soils. Since the majority of Iranian soils are calcareous, inoculation of apple rootstocks with suitable AMF species can enhance the growth and productivity of apple orchards. This practice could also lead to reduced fertilizer applications in the soil, which is important from the standpoint of economy and environmental concerns.

Conclusions

The results of this study show that AMF inoculation generally enhanced the growth parameters of apple rootstocks in calcareous soils. Nevertheless, there was noticeable variation among the rootstock cultivars in response to symbiosis with AMF. The fungus *G. versiform* was the most efficient in alleviating the adverse effects of calcareous soil. The overall performance of apple rootstocks varied in the calcareous soil. Among them, the MM.106 rootstock benefited to the greatest extent as a result of the symbiosis. Based on the results of this study, it could be advisable to inoculate the MM.106 rootstock with AMF, especially with *G. versiforme* so as to overcome parts of the problematic restrictions associated with apple orchards in calcareous soils - a common misfortune for Iranian soil. We demonstrated that AMF can stimulate the growth of plant biomass and assist in the uptake of nutrients by apple trees which, in this study, grew on calcareous soils under greenhouse conditions. Further work is needed to investigate the capabilities of the same AMF isolates directly under field conditions.

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