Vitis **51** (4), 175–182 (2012)

In vitro propagation of four Iranian grape varieties: Influence of genotype and pretreatment with arbuscular mycorrhiza

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Summary

There is a great demand for table grape saplings, mainly for commercial varieties indicating that micropropagation could be an effective method for their mass propagation. Internal contamination in woody plant species is an important problematic issue and arbuscular mycorrhizal fungi (AMF) have been known as potential plant biological protectors. In the present study, the glasshouse grown mother plants of four grape varieties ('Asgari', 'Khalili', 'Keshmeshi', and 'Shahroudi') were inoculated with AMF as pre-treatment. The fungi strains were *Glomus mosseae*, *G. fasciculatum*, *G. intraradices* and a mixture of all three species. The comparative *in vitro* performance of these genotypes was evaluated following optimization of *in vitro* growth conditions for each genotype. Furthermore, the positive effect of AMF inoculation of stock plant on micropropagation process was studied. Changes in biochemical features (total chlorophylls, total phenols and total sugars), growth parameters (root length and total leaf area) and *in vitro* behavior of AMF pretreated as well as control explants were recorded. The mycorrhizal association with grapevine roots was confirmed following root staining and evaluation of colonization rate. The results revealed a distinct difference and clear genotypic effect on various *in vitro* parameters of studied grape genotypes. The utilized inocula were found to have the capability of mycorrhizal association with grapevine roots, leading to enhancing phenolics as a defense mechanism, increasing sugars and chlorophyll and finally growth of whole plant corresponding to the grape variety and AMF strain. These results confirmed that health and physiological conditions of the stock plants are important parameters for *in vitro* grape culture establishment and suggest the integration of mycorrhizal technology with tissue culture to accomplish better results.

K e y w o r d s : micropropagation, contamination, arbuscular mycorrhizal fungi, grapevine.

Introduction

In vitro vegetative propagation is a commercial technique which is used for several plants all over the world (Razdan 2003). Iran is the origin of commercial grape growing and there are numerous varieties of Persian origin (Winkler 1974). 'Asgari', 'Keshmeshi', 'Shahroudi' and 'Khalili' are considered as important varieties with appropriate commercial traits (Fatahi *et al.* 2003, Najafi *et al.,* 2006). 'Keshmeshi' and 'Asgari' are known to be the most important Iranian seedless grapes which are consumed as fresh table grapes as well as raisin production (Fatahi *et al.* 2003, Nikkah *et al*. 2010). 'Kishmish' ('Keshmesh') means raisin in several countries in the Middle East and specially Iran (Fatahi *et al.* 2003, Nikkah *et al*. 2010). For example, the grape called 'Thompson Seedless' in California is known as 'Oval Kishmish' in Turkey, Iran and some other Asian countries; 'Sultana' in Australia, South Africa; and sometimes, 'Sultanina' in the Near East (Winkler 1974). 'Shahroudi', a very late-ripening and 'Khalili' an early-ripening variety are also table grapes of economic importance both producing seeded fruits (Krochmal and Nawabi 1961, Fatahi *et al.* 2003, Najafi *et al.*2006). In an attempt to characterize Iranian grape varieties using molecular markers, high genetic overlap was found among some Iranian and European grape varieties (Najafi *et al.*2006). For more supplying and introducing these commercial varieties to the world, breeding and hybridization efforts as well as mass scale propagation are necessary that require an efficient tissue culture and mainly micropropagation technique (Neumann *et al.* 2009).

Previous studies on *in vitro* propagation of *Vitis* genus have shown that the rate of succession in each culture stage depends on genotype (Bajaj 1986, Peros *et al.* 1998, Smerea *et al*. 2010). However, contamination is considered as a serious problem in commercial micropropagation that if occurs in advance stages of production causes irrecoverable damages (Bhojwani and Razdan 1996). It has been believed that *in vitro* cultures are rarely completely lacking contamination. Slowly growing bacteria along with explant might firstly remain concealed and be detected in latter stages (Bhojwani and Razdan 1996, Razdan 2003). Different contamination factors do not make much difficulties when they are on the surface of the plant but pathogens can enter into the plant tissue through natural stomata or injuries or carriers (like insects) (Kumar 2005). Reported disinfection methods are not mostly responsive in tree species as they inhibit external contaminations but removing internal ones is problematic. Removing contamination entered the host tissue which could appear even after 3 to 4 months of inoculation is so complicated and affects the *in vitro* tissue growth. If the disinfectant concentration and the treatment time are increased, it will conversely affect the explant tissue. Overcoming this problem is the most important bottleneck in tissue culture particularly in perennial tree species. Because these plants grow in the natural environment

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through several years and are exposed to the attack of various fungal and bacterial pathogens. These pathogens may not be able to harm the plant health but when the explant is excised from field-grown plant, removing the external pathogens is so difficult too. However success in establishing sterile cultures has been reported in lots of tree species. Hence, it is necessary to track internal microorganisms of stock plants through advanced methods. Growing stock plants in sanitary conditions is a solution (Chandra and Mishra 2003). Moreover, increasing mother plants resistance to pathogens is possible through the positive role of AMF (arbuscular mycorrhizal fungi) inoculation. The induced local or systemic resistance observed in plant body especially through phenol synthesis following mycorrhization, inoculation of stock plants with different AMF strains particularly, ones compatible with each special kind of plant could be greatly helpful. Furthermore, AMF produce plant hormone (Barea and Azcon-Aguilar 1982), so *in vitro* development and rooting of explants collected from inoculated plants may be improved. Although it is difficult to achieve non-theoretical results due to complication of microorganism-plant system and certain effects of predominant environmental conditions, but finding out proper combination of fungus strain and plant genotype for maximizing AMF beneficiary appears to be possible. Therefore, the present study aimed to investigate the effect of AMF symbiosis with mother plants on *in vitro* performance of explants assaying some *ex vitro* growth and biochemical parameters under the effect of AMF inoculation and *in vitro* performance of grapevines and ultimately the effect of explant source conditions (field and glasshouse) on direct regeneration of four grape varieties. To our knowledge, this is the first complied report with respect to the effect of AMF inoculation of stock plant on *in vitro* micropropagation of grapevine. The present study evaluates the role of mycorrhizal biotechnology in management of stock plants in stage zero of micropropagation.

Material and Methods

Plant material and application of AMF: Mature vines of four Iranian table grape (*Vitis Vinifera* L.) varieties 'Asgari', 'Khalili', 'Keshmeshi' and 'Shahroudi' (Fatahi *et al.* 2003, Najafi *et al.*2006) were selected based on unity in vine growth and cultivation conditions in vineyards at the Shahroud Agricultural Research Center, Semnan province (latitude 35º 34´ N, longitude 53º 23´ E, altitude 1130 m), Iran. The grape hard wood cuttings were harvested by mid March and inserted in a presoaked sawdust medium to induce rooting without any hormonal treatment.

Four strains of arbuscular mycorrhizal fungi (AMF), namely, *Glomus mosseae, G. fasciculatum, G. intraradices* and a mixture of all three species procured from Turan Biotech Co., Shahroud, Iran, were utilized. The rooted vines were transplanted in 8 L plastic pots in fine sand: leaf mold (1:1). While transplanting, inoculation was performed by incorporating 100 g expanded clay containing spores, mycelium and infected/colonized *Trifolium repens* root fragments just beneath the root system of each plantlet. Control plants did not receive the aforementioned inoculums. Plantlets were maintained in glasshouse under 35/25 °C day/night temperatures, RH 80-85 % and natural photoperiod (10 to 14.5 h light). Pots were hand-watered 2-3 times a week.

Evaluation of mycorrhizal colonization: The root samples were harvested 90 d after AMF inoculation in order to assess fungal colonization. The AMF root colonization was confirmed through staining fresh root segments, according to Phillips and Hayman (1970) and calculated using the formula proposed by Nicolson (1995).

Ex vitro growth and biochemical analyses: Morphological parameters of glasshouse grown stock plants; *viz*., root length (RL) and total leaf area (TLA) were recorded 90 days after AMF inoculation. Vines TLA were calculated through a model (Eftekhari *et al.* 2011) constructed between leaf area (*LA*), length (*L*) and width (*W*): $LA = a + b\,(L \times W) + c\,(L \times W)^2$ (R^2 = 0.926) or $LA = a + b\,(L + W) + c\,(L + W)^2$ (R^2 = 0.920).

Biochemical analyses were also made 90 d after inoculation on glasshouse grown stock plants prior to micropropagation. Leaf total chlorophyll contents were assessed following the method suggested by Barnes *et al*. (1992). Fully matured leaf samples were cut and dipped in dimethyl sulphoxide (DMSO) and incubated at 70 ºC for 4 h. The absorbance of the solution was then read against blank (solvent) at 645, 663 and 480 nm using a spectrophotometer. Total phenol content of leaf tissues were estimated using a slightly modified method proposed by Malik and Singh (1980). Immature leaves were collected and dried in a hot oven (40 °C for 72 h until constant weight) and approximately 500 mg dry matter of each sample was extracted with 80 % methanol using a shaker (120 rpm for 24 h) followed by filtering through filter paper. The supernatant was collected and evaporated to dryness. Residues were dissolved in distilled water. Folin-Ciocalteau reagent and Na_2CO_3 solution (20 % w/v) were added, mixed thoroughly and placed in a hot water bath exactly for 1 min. Then it was cooled down and the absorbance was read at 650 nm. Estimation of total soluble sugars was carried out according to the method described by Hedge and Hofreiter (1962). 100 mg of fresh leaf samples were hydrolyzed by HCl in boiling water bath for 3 h and then it was neutralized with sodium carbonate and centrifuged. Thereafter Anthrone reagent was added and heated for 8 min in a boiling water bath. Then it was cooled down immediately and finally, the absorbance was measured at 630 nm.

In vitro culture procedures: The *in vitro* culture establishment was initiated 90 d after mycorrhizal inoculation ensuring root colonization. The previously standardized micropropagation protocol developed by Alizadeh *et al*. (2007) was found to be highly responsive in our selected grape genotypes (Eftekhari 2011). Glasshouse grown shoots as well as newly emerged flush of field-grown grapevines were harvested. Samples were wrapped immediately in moist cloth and transferred to the laboratory for the *in vitro* studies. Single node explants (2-4 cm length) were then prewashed in a solution contained

0.1-0.2 % commercial detergent (Pril, Iran) followed by stirring in Mancozeb (2 $g \cdot l^{-1}$, Mahan, Iran) solution for 45 min. The explants were surface disinfected using 60 % (v/v) NaOC1 solution (5 % available chlorine) for 30 min. After 4-5 rinses in sterile distilled water, single node cuttings (2-4 cm) were inoculated in test tubes (20 × 150 mm) containing 20 ml MS (Murashige and Skoog 1962) medium supplemented with 2.0 $mg \cdot L^{-1}$ of 6-benzyl amino purine (BAP) and 0.2 $mg \cdot L^{-1}$ α-naphthalene acetic acid (NAA) and 200 $mg \cdot L^{-1}$ activated charcoal (AC). The pH was adjusted to 5.8 prior to the addition of 0.8 % agar, and the media were autoclaved at 121 °C and 15 PSI for 15 min. The cultures were incubated at 25 ± 2 °C under continuous light (50 $\mu mol \cdot m^{-2}\ s^{-1}$). Days to bud sprouting (DBS) of single node explants were recorded and after 4 weeks of growth, the percent of contaminated cultures (CC) and established cultures (EC) were evaluated. Established cultures were subcultured 4 weeks after inoculation and proliferation rate was calculated as the number of double node microcuttings (NDNM) taken from each grownup explant, number of roots (NR), number of leaves (NL), days to root initiation (DRI), shoot length (SL) and root length (RL) recorded 4 weeks after subculture.

Statistical analysis: The experiments were conducted as complete randomized block design with factorial arrangement including four replications with respect to morphological and biochemical measurements and at least three replications in micropropagation tests. Data were analyzed by analysis of variance using the GLM procedure in SAS software (SAS Institute 2003) and mean values were compared using the Least Significant Difference (LSD) test ($P < 0.05$). Student's T-test was used for comparing the performance of *in vitro* explants excised from glasshouse and field-grown plants. Data were transformed where it was necessary using $\sqrt{x + 0.5}$ before analysis but presented as original untransformed data.

Results

Root colonization percent: The highest root colonization was estimated in Shahroudi variety followed by Keshmeshi with high significant difference ($p < 0.01$) to other varieties (Tab. 1). Furthermore, among fungi species, *G. fasciculatum* showed maximum root colonization (Tab. 2). In the present study, natural non-sterile leaf mold was used as a part of potting mixture, thus some levels of colonization also was observed in control plantlets.

Effect of mycorrhizal inoculation on growth and biochemical status: The highest total leaf area was estimated in Shahroudi variety followed by Asgari. The TLA in 'Khalili' and 'Keshmeshi' varieties were found to be significantly lesser than two other varieties (Tab. 1). The longest root was detected in 'Asgari' vines followed by 'Shahroudi'. The 'Keshmeshi' roots were significantly shorter than others ($p < 0.01$) (Tab. 1). On the other hand, there was a significant variation for RL by different examined AMF treatments. *G. fasciculatum* and *G. intraradices* followed by mixed strain were found to be significantly ($p < 0.05$) superior over the rest (Tab. 2). There were insignificant differences among AMF strains with regard to changes in TLA.

Table 1

Influence of grape variety on arbuscular mycorrhizal fungi (AMF) colonization of root and some growth factors (90 d after AMF inoculation)

Grape variety	Colonization (%)	Total leaf area (cm^2)	Root length (cm)
Asgari	67.01 [bc]	551.75 [a]	23.1196 [a]
Khalili	62.40 [c]	327.11 [b]	17.3084 [b]
Keshmeshi	72.20 [a]	288.50 [b]	12.9689 [c]
Shahroudi	73.05 [a]	649.75 [a]	21.1225 [ab]

Values followed by the same letter in each column show insignificant differences ($p < 0.05$).

Table 2

Root colonization of arbuscular mycorrhizal fungi (AMF) and its effect on root length and total phenols of grapevines

AMF treatment	Colonization (%)	Root length (cm)	Total phenols ($mg \cdot 100\ g^{-1}$ dw)
G. intraradices	72.4 [a]	20.7 [a]	6.34 [ab]
G. mosseae	69.9 [a]	18.4 [ab]	5.29 [c]
G. fasciculatum	75.1 [a]	20.8 [a]	6.02 [abc]
Mixed strain	71.2 [a]	18.6 [a]	6.78 [a]
Control	54.7 [b]	14. 0 [b]	5.61 [bc]

Values followed by the same letter in each column show insignificant differences ($p < 0.05$).

Present results revealed that total chlorophyll content were considerably different ($p < 0.01$) among *G. mosseae* and *G. fasciculatum* inoculated 'Asgari' vines, mixed strain inoculated Khalili variety and *G. intraradices, G. mosseae* and *G. fasciculatum* inoculated 'Keshmeshi' variety with non-inoculated control plants of the same varieties but there was no significant difference among various AMF treatments of Shahroudi variety (Fig. 1). Total sugar accumulation in grape varieties varied under the effect of AMF so that 'Asgari' and 'Khalili' varieties inoculated with *G. mosseae* contained the highest total sugars. Although there was no significant difference among various AMF treatments of 'Keshmeshi' and 'Shahroudi' varieties on total sugars content, however the most total sugars percent in 'Shahroudi' variety plants was related to *G. mosseae* treatment (Fig. 1). Among four studied AMF strains, mixed strain inoculated grapevines showed significantly ($p < 0.05$) more total phenol content as compared to control plants (Tab. 2).

Effect of genotype on *in vitro* performance: The comparative *in vitro* performance of explants collected from four different varieties showed distinct difference and clear genotypic effect on various *in vitro* parameters (Tab. 3). The 'Keshmeshi' explants were so early in bud sprouting; hence least DBS of nodal explants was recorded in the same variety. The least CC % was estimated in nodal culture of 'Shahroudi' which was significantly ($p < 0.05$) lower than 'Asgari'. Although there was no significant difference among various varieties, but also the most percent of culture establishment was evaluated in 'Shahroudi' (Tab. 3). Among different measured parameters, NR was affected significantly by genotype and its most quantity was evaluated in 'Asgari' with high

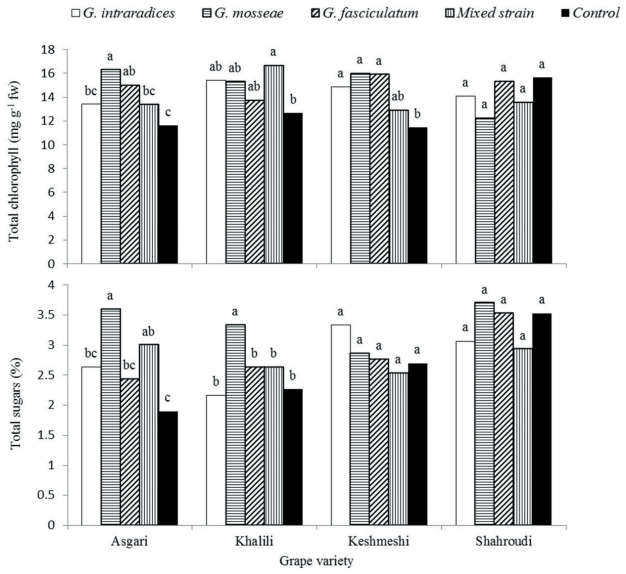

Fig. 1: Interaction effect of grape variety and AMF strain on total chlorophylls and total sugar contents.

significant ($p < 0.01$) difference to other genotypes (Tab. 3). There was also significant difference ($p < 0.05$) among varieties for NS and DRI. The most NS was evaluated in 'Keshmeshi' which had significant difference only with 'Shahroudi'. Furthermore, root initiation was first observed in 'Asgari' microshoots. The DRI in 'Shahroudi' microshoots was found to be longer than the rest ones with significant difference to 'Asgari'. According to the present results, the best RL, SL and NL were detected in 'Keshmeshi' and the most NDNM was recovered following subculture of 'Asgari' *in vitro* shoots (Tab. 3).

Effect of AMF pretreatments of stock plants on nodal in vitro performance: Besides data presented in Tab. 3 for genotypic effect on *in vitro* performance of different grape varieties, it was found that inoculation of stock plants with mycorrhizal fungi may improve their *in vitro* behavior (Tab. 4). For example, the best DRI was detected in the mixed strain inoculated grapevines. Also, the same treatment could enhance the CE % and NR of inoculated explants but it was not statistically considerable. The effect of AMF inoculation on the other *in vitro* measured parameters was found to be insignificant (Tab. 4).

Irrespective of the AMF effect, explants collected from glasshouse grown stock plants responded better than those of field-grown plants to primary culture establishment. T-test results (Tab. 5) about 'Asgari', 'Khalili' and 'Shahroudi' revealed that DBS was significantly improved ($p < 0.01$). Furthermore, CC % in explants collected from glasshouse grown plants was significantly ($p < 0.05$) less than field-grown ones. However, it may be stated that the similar results were not observed in case of 'Keshmeshi' explants. Generally it can be claimed that, despite good primary CE and lower CC of glasshouse derived explants, they exhibited poor *in vitro* performance as compared to those prepared from field grown plant. For example; NS, SL, NR in 'Asgari', NL in 'Khalili', NS, NL, NR, DRI, RL in 'Keshmeshi' and NL, SL, NDNM in 'Shahroudi' variety were significantly inferior than explants collected from field grown stock plants.

In order to realize the influence of AMF treatments on *in vitro* performance of grape varieties, the response of explants collected from mycorrhizal plants grown in glasshouse were compared to those of field grown non-inoculated grapevines (Tab. 6). Inoculation of 'Asgari' grapevines with mixed AMF strain significantly ($p < 0.05$) enhanced DRI and decreased NR but CE % and DBS were improved significantly ($p < 0.01$) by mixed strain and *G. fasciculatum* respectively. Statistical analysis showed that other measured parameters in this variety were not affected by AMF treatments (Tab. 6). Regarding to 'Khalili', NDNM ($p < 0.05$) and SL ($p < 0.01$) were decreased in plants inoculated with *G. mosseae* and DRI ($p < 0.05$) was enhanced following inoculation with mixed strain.

Table 3

Effect of grape genotype on *in vitro* response of single node explants

RL (cm)	NR	DRI	NL	SL (cm)	NS	NDNM	CE (%)	CC (%)	DBS	Variety
3.90 ^{a}	10.4 ^{a}	5.69 ^{b}	3.94 ^{a}	3.07 ^{a}	0.92 ^{ab}	2.19 ^{a}	17.6 ^{a}	29.2 ^{a}	12.7 ^{a}	Asgari
2.77 ^{a}	3.20 ^{b}	6.63 ^{ab}	3.85 ^{a}	2.27 ^{a}	0.61 ^{ab}	1.74 ^{a}	17.0 ^{a}	23.5 ^{ab}	14.8 ^{a}	Khalili
5.41 ^{a}	3.89^{b}	8.14 ^{ab}	4.90 ^{a}	3.36 ^{a}	1.27 ^{a}	2.16 ^{a}	17.7 ^{a}	25.8 ^{ab}	6.00 ^{b}	Keshmeshi
3.64 ^{a}	3.04 ^{b}	9.04 ^{a}	2.75 ^{a}	2.93 ^{a}	0.56 ^{b}	2.10 ^{a}	28.3 ^{a}	21.2 ^{b}	17.8 ^{a}	Shahroudi

Values followed by the same letter in a column show insignificant differences ($p < 0.05$). DBS, days to bud sprouting; CC, culture contamination; CE, culture establishment; NDNM, number of double node microcuttings; NS, number of shoots; SL, shoot length; NL, number of leaves; DRI, days to root initiation; NR, number of roots; RL, root length.

Table 4

The effect of four AMF species on *in vitro* growth of grape nodal explants

RL (cm)	NR	DRI	NL	SL (cm)	NS	NDNM	CE (%)	CC (%)	DBS	AMF
1.90 ^{a}	1.40 ^{a}	13.0 ^{ab}	1.90 ^{a}	0.20 ^{a}	0.05 ^{a}	1.57 ^{a}	33.7 ^{a}	6.68 ^{a}	3.5 ^{b}	*G. mosseae*
1.09 ^{a}	1.40 ^{a}	17.6 ^{a}	1.72 ^{a}	0.0041 ^{a}	0.0041 ^{a}	1.35 ^{a}	27.9 ^{a}	6.05 ^{a}	4.47 ^{a}	*G. intraradices*
1.96 ^{a}	0.94 ^{a}	15.3 ^{ab}	1.66 ^{a}	0.0041 ^{a}	0.0041 ^{a}	1.21 ^{a}	18.7 ^{a}	13.7 ^{a}	3.26 ^{b}	*G. fasciculatum*
1.35 ^{a}	1.75 ^{a}	11.1 ^{b}	1.81 ^{a}	0.0041 ^{a}	0.05 ^{a}	1.54 ^{a}	39.3 ^{a}	10.1 ^{a}	3.46 ^{b}	Mixed strain
1.63 ^{a}	1.72 ^{a}	16.7 ^{a}	1.90 ^{a}	0.11 ^{a}	0.05 ^{a}	1.54 ^{a}	18.3 ^{a}	8.03 ^{a}	3.78 ^{ab}	Control

Values followed by the same letter in a column show insignificant differences ($p < 0.05$). DBS, days to bud sprouting; CC, culture contamination; CE, culture establishment; NDNM, number of double node microcuttings; NS, number of shoots; SL, shoot length; NL, number of leaves; DRI, days to root initiation; NR, number of roots; RL, root length.

Table 5

Comparison of measured micropropagation factors in two nodal explants collected from grapevines grown in the greenhouse and in the field

Variable sources	DBS	CC (%)	CE (%)	NDNM	NS	SL(cm)	NL	DRI	NR	RL (cm)
Asgari										
Field	13.4 ± 1.9	29.5 ± 2.5	19.3 ± 4.8	2.30 ± 0.4	1.00 ± 0.2	3.65 ± 1.0	4.30 ± 0.9	6.10 ± 1.1	11.5 ± 2.04	4.07 ± 0.6
Greenhouse	4.94 ± 0.9	6.67 ± 4.7	23.3 ± 10.3	1.75 ± 0.5	0.00 ± 0.0	0.00 ± 0.0	3.25 ± 1.1	12.7 ± 5.1	2.50 ± 0.9	2.90 ± 1.2
T	4.47	4.54	-0.38	0.84	4.74	3.72	0.67	-1.26	2.68	1.00
Pr > t	0.0001	0.0027	0.7126	0.4196	0.0011	0.0048	0.5171	0.2906	0.0199	0.3381
Khalili										
Field	15.2 ± 1.5	24.6 ± 5.4	17.1 ± 1.2	1.90 ± 0.4	0.70 ± 0.2	2.70 ± 0.8	4.20 ± 0.9	6.80 ± 0.8	3.60 ± 0.9	3.20 ± 0.9
Greenhouse	3.70 ± 0.4	4.20 ± 2.9	38.9 ± 14.2	1.25 ± 0.2	0.25 ± 0.2	0.75 ± 0.7	0.75 ± 0.5	8.00 ± 0.0	2.75 ± 0.2	2.10 ± 0.3
T	7.25	3.10	-1.53	0.86	1.20	1.44	2.25	-1.50	0.91	1.20
Pr > t	< 0.0001	0.0174	0.2216	0.4074	0.2546	0.1745	0.0437	0.1679	0.3822	0.256
Keshmeshi										
Field	7.10 ± 2.3	26.1 ± 2.7	19.0 ± 5.1	2.20 ± 0.2	1.30 ± 0.1	3.55 ± 0.6	5.00 ± 0.5	8.70 ± 1.6	4.45 ± 1.1	5.69 ± 0.8
Greenhouse	4.18 ± 0.8	38.9 ± 14.2	3.33 ± 23.6	2.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	3.00 ± 0.4	29.7 ± 1.2	0.25 ± 0.2	0.50 ± 0.5
T	1.18	-0.88	-0.59	0.86	8.51	1	2.21	-7.6	3.61	6.03
Pr > t	0.2632	0.438	0.5918	0.4074	< 0.0001	0.3434	0.047	< 0.0001	0.0049	< 0.0002
Shahroudi										
Field	18.0 ± 1.4	21.4 ± 2.4	28.7 ± 3.5	2.30 ± 0.4	0.70 ± 0.3	3.40 ± 0.9	3.00 ± 0.6	9.10 ± 0.5	3.20 ± 0.5	4.23 ± 1.0
Greenhouse	3.76 ± 0.6	6.67 ± 4.7	16.7 ± 11.8	1.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	1.50 ± 0.2	24.2 ± 6.7	2.50 ± 1.0	2.35 ± 1.0
T	-9.61	-2.96	-1.09	-2.9	-2.33	-3.64	-2.45	2.24	-0.66	-1.08
Pr > t	<0.0001	0.0211	0.3123	0.0176	0.4445	0.0054	0.0325	0.1102	0.5192	0.3015

Data represents mean ± standard deviation. DBS, days to bud sprouting; CC, culture contamination; CE, culture establishment; NDNM, number of double node microcuttings; NS, number of shoots; SL, shoot length; NL, number of leaves; DRI, days to root initiation; NR, number of roots; RL, root length.

Table 6

Comparison of measured micropropagation factors in two grape nodal explants collected from grapevines inoculated with AMF and non-inoculated grapevines grown in the field

Variable sources	DBS	CC (%)	CE (%)	NDNM	SL(cm)	DRI	NR	RL (cm)
Asgari								
Field	13.4 ± 1.9	30 ± 21.2	19.3 ± 4.8	2.30 ± 0.3	3.65 ± 1.0	6.10 ± 1.1	11.5 ± 2.04	4.07 ± 0.6
Greenhouse	4.29 ± 0.7	29.5 ± 2.5	66.6 ± 6.8	1.5 ± 0.2	1.00 ± 1.0	13.2 ± 2.8	2.75 ± 1.4	2.13 ± 1.2
T	4.57	-0.02	-5.84	1.29	1.56	-2.93	2.56	1.66
Pr > t	< 0.0007	0.9821	0.0006	0.2199	0.1440	0.0125	0.0248	0.1235
Khalili								
Field	15.2 ± 1.5	24.6 ± 5.4	17.1 ± 1.2	2.20 ± 0.2	3.55 ± 0.6	6.80 ± 0.8	3.60 ± 0.9	3.20 ± 0.9
Greenhouse	4.00 ± 0.0	15.3 ± 6.9	57.5 ± 17.9	1.25 ± 0.2	0.37 ± 0.4	11.5 ± 2.8	2.25 ± 0.2	3.89 ± 1.3
T	-7.37	-1.09	-2.25	2.67	3.24	-2.24	1.45	0.42
Pr > t	< 0.0001	0.3106	0.1087	0.0205	0.0017	0.0446	0.1769	0.6833
Keshmeshi								
Field	7.30 ± 2.6	26.1 ± 2.7	19.0 ± 5.1	2.20 ± 0.2	3.55 ± 0.6	8.70 ± 1.6	4.45 ± 1.1	5.69 ± 0.8
Greenhouse	4.35 ± 0.3	15.3 ± 6.9	31.1 ± 8.2	1.25 ± 0.2	0.37 ± 0.4	11.5 ± 6.6	1.50 ± 0.6	1.12 ± 1.1
T	-1.14	-1.60	-1.31	2.67	3.24	-0.41	1.57	-3.16
Pr > t	0.2867	0.1529	0.2318	0.0205	0.0017	0.7049	0.1427	0.0082
Shahroudi								
Field	18.0 ± 1.4	21.4 ± 2.4	28.7 ± 3.5	2.30 ± 0.4	3.40 ± 0.9	9.10 ± 0.5	3.20 ± 0.5	4.23 ± 1.0
Greenhouse	4.18 ± 1.3	22.2 ± 15.7	24.1 ± 10.2	0.50 ± 0.3	0.00 ± 0.0	13.0 ± 6.0	1.50 ± 0.6	3.25 ± 1.9
T	-7.01	0.05	-0.47	-2.41	-3.64	0.65	-1.80	-0.49
Pr > t	<0.0001	0.9635	0.6497	0.0328	0.0054	0.5636	0.0971	0.6311

Data represents mean ± standard deviation. DBS, days to bud sprouting; CC, culture contamination; CE, culture establishment; NDNM, number of double node microcuttings; NS, number of shoots; SL, shoot length; NL, number of leaves; DRI, days to root initiation; NR, number of roots; RL, root length.

Furthermore, DBS was improved in explants treated with *G. fasciculatum*. In case of other characteristics there was no considerable difference as compared to AMF treatments in each grape variety (Tab. 6). The explants of 'Shahroudi' inoculated with *G. mosseae* was found to have a shorter SL ($p < 0.01$) and less amount of NDNM ($p < 0.05$) but similar to 'Khalili' explants, DBS ($p < 0.01$) was improved due to *G. fasciculatum* (Tab. 6). There was no considerable positive response in explants of 'Keshmeshi' variety treated with AMF strains. It must be stated that SL and NDNM were decreased in 'Keshmeshi' explants due to *G. mosseae*. In addition, they produced shorter RL following inoculation with *G. fasciculatum* (Tab. 6).

Discussion

Successful *in vitro* culture establishment is prerequisite for micropopagation since fail in this critical stage, will make the following stages impossible. Explant resource is a key factor which has considerable effect on different biochemical parameters (Krishna *et al*. 2008) and this itself will affect explant *in vitro* contamination and performance. Plant material selection and preparation of disinfected explant is vital in this process because plant tissue should remain alive in primary culture to be established and proliferate well. To attain this goal, considering environmental conditions and physiological status of stock plant is essential.

Effect of mycorrhizal inoculation on growth and biochemical status: Variable capability of AMF strains in improving morphological, physiological and biochemical status of plant species and specially grape have been studied extensively (Krishna *et al*. 2005, Camprubí *et al*. 2008, Eftekhari *et al*. 2010) and their effectivity depends on fungus strain and plant genotype (Bleach *et al*. 2008) which in turn might refer to the percentage of mycorrhizal colonization. Successful development of grapevine highly depends on proper selection of AMF strain and in result determining the best mycorrhiza for different plant varieties is necessary. Mycorrhizal benefits for host plants are abundant and may be stated as a single parameter however, these benefits are not essentially correllated (van der Heijden 2001). Observed growth difference among AMF inoculated plants can be also attributed to relative mycorrhizal dependence of different varieties (Jaizme-Vega *et al*., 2003).

As it is evident in Fig. 1, there is no significant difference among different AMF treatments in 'Shahroudi' variety for total chlorophyll content indicating that probably there is incompatibility between this genotype and AMF strains. Especially in mixed strain treatment of 'Shahroudi' variety owing to minimum amount of chlorophyll, this incongruity is more apparent and may show antagonistic effect of AMF strains to each other in inoculation with 'Shahroudi' variety. However, in 'Khalili' variety, treatment of mixed strain resulted in the highest amount of chlorophyll biosynthesis which probably indicates a synergistic effect of AMF strains together in inoculation with this variety (Tiwari and Adholeya 2003). The positive effect of AMF on chlorophyll content is in agreement with the results reported on other grape genotypes (Krishna *et al*. 2005) and other plants such as *Capsicum annum* L. (Kaya *et al*., 2009).

As it is obvious in Fig. 1, the highest levels of chlorophylls and sugars were estimated in Asgari variety plants inoculated with *G. mosseae*. Despite this positive relationship, chlorophyll and sugar synthesis in 'Shahroudi' variety were not affected by the AMF treatments. The AMF colonized root is a strong sink of absorbing carbohydrate and the vigor of mycorrhizal sink affects total plant carbohydrate balance (Wright *et al*. 1998), hence, in some AMF treatments in spite of increasing chlorophylls, a decline in total sugars could be found.

Synthesis of phenols correlates to microorganism pressures, which suggests they have a protective role in preventing bacterial or fungal infections (Duval *et al.* 1999). Phenolic compounds occur naturally in plant system and owing to their antimicrobial properties inhibit fungal spore germination and toxin production by pathogens (Vidhyasekaran 1973). The increased level of total phenols in the present research by mixed AMF strain suggests increased resistance in inoculated plants against internal contamination, which led to decreased *in vitro* culture contamination.

Effect of genotype on in vitro performance: All four studied varieties were successfully established on MS basal medium. However, different *in vitro* responses were observed among them. Such genotypic effect in grape micropropagation was also previously reported (Harris and Stevenson 1982, Chee and Pool, 1983, Alizadeh *et al*. 2010). Furthermore, Peros *et al*. (1998) compared micropropagation reactions of several grape varieties and found high significant differences among their number of roots, stem length and number of nodes. In several plant species, it has been suggested that differences of *in vitro* responses among genotypes may be related to differences in endogenous content of hormones (Looney *et al.* 1988, Alvarez *et al*. 1989, Grönroos *et al*. 1989). The same assumption may be adopted to explain the great variability among *V. vinifera* varieties (Peros *et al*. 1998). The variability among grape genotypes with regard to their *in vitro* performance is in accordance with findings reported by Novak and Juvova (1982), Chee and Pool (1983) and Reisch (1986). Also, Clog *et al*. (1990) found that plant regeneration capacity of *Vitis* species is controlled by the genotype of explant resource. Phillips (2004) reported that specific genes are involved in plant regeneration *in vitro*. In some genotypes, genes involved in shoot organogenesis may be suppressed due to inappropriate culture condition. The recorded deviations in the regenerative capacity of 11 grapevine genotypes on three culture media were dependent primordially on genotype (Smerea *et al*. 2010).

Effect of AMF pretreatments of stock plants on nodal *in vitro* performance: Mixed AMF strain could improve root initiation rate of grape varieties which can be ascribed to the more internal phyto-hormones produced in AMF inoculated plants (Barea and Azcon-Aguilar 1982).

As it has been pointed out in results (Tabs 5 and 6), most of *in vitro* characteristics of glasshouse grown plants declined that was probably due to decrease in growth vigor as a result of low nutrition reserve of glasshouse grown plants to the field ones (Figs 2 and 3). However, the surprising results obtained for DBS, CC % and CE % would be very interesting and may encourage further research to find out the logical justification.

Total evaluation of T-test results (Tabs 6 and 5) showed that regardless of AMF inoculation, CC % decreased significantly in all varieties except 'Keshmeshi' indicating that growing of stock plant in glasshouse has significant ($p < 0.05$) effect on reducing *in vitro* culture contamination.

As stated before, selection of appropriate explant for culture initiation is of paramount importance because it will

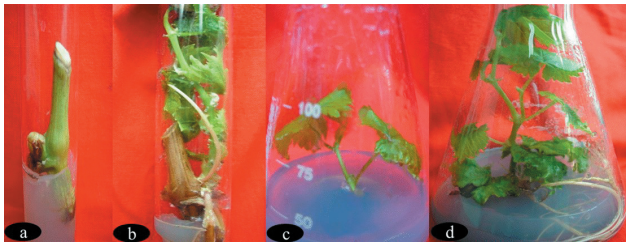

Fig. 2: Micropropagation stages of 'Asgari' grape variety using single node explants collected from farm-grown plants, **a**) single node microcutting, **b**) sprouted and grown up microcutting a month after inoculation, **c**) subcultured double node explant, **d**) subcultured double node microcutting after a month.

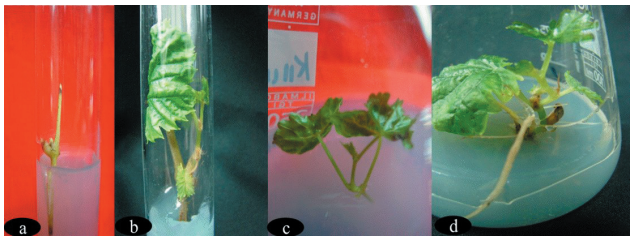

Fig. 3: Micropropagation stages of 'Keshmeshi' grape variety using single node explant collected from AMF inoculated plants, **a**) single node microcutting, **b**) sprouted and grown microcutting a month after inoculation, **c**) subcultured double node explant, **d**) subcultured double node microcutting after a month.

determine the subsequent culture response. Explant source has also considerable effect on different plant biochemicals. Working with mango, Krishna *et al*. (2008) observed that glasshouse grown mango seedlings survived more than explants provided from field-grown stock plants. They stated that explants collected from glasshouse grown seedlings, irrespective of mycorrhizal inoculation, responded better than field-grown ones as they overcame on culture shock and started growing in a short period of time. However regenerative responses of mycorrhizal mango plants were not much surprising compared to non-inoculated ones. Macadamia explants originated from field-grown trees showed characteristics like those collected from grafted seedlings in glasshouse but responses varied according to the genotype (Gitonga *et al*. 2010). Kibbler *et al*. (2003) attributed differences among bud sprouting of explants collected from glasshouse grown cuttings and field-grown stock plants to the difference of physiological or ontogenic age. In research of Krishna *et al*. (2005) mycorrhizal plants showed lower *in vitro* contamination percent which was ascribed to more total phenols of *in vivo* treated plants.

Several studies were performed on mycorrhizal local bio-protection from pathogens but some reports of mycorrhizal systemic protection also may be observed in the literature (Cordier *et al*. 1998, Pozo *et al*. 2002). Therefore, lower contamination observed in explants derived from mycorrhizal plants in the present study can be attributed to the systemic protection created in stock plants which is mainly due to phenolics role (Cordier *et al*. 1998, Pozo *et al*. 2002).

Apart from abiotic factors affective on efficiency of AM fungi, such as temperature, moisture and soil P content, bio-control protection effect against pathogens appear to depend on several biotic factors such as host genotype, fungal strain and degree of colonization (Pozo *et al*. 2002). *In vitro* contamination rate estimated in different grape varieties following inoculation with AMF in this research may not be directly attributed to each above mentioned factors since the relationship between host genotype and AMF strain is so complicated and needs more precise studies. Hence, in the present paper only some main reasons of these differences *i.e.* host plant genotype and AMF strain may be discussed. It has been suggested that AMF development and its effect on host plant is at least partially under the control of host genetics. Furthermore, the host genome appears to be involved in protective effect of AMF as it seems that host genotype responses to mycorrhizal relationship with different bio-protective reaction (Vierheilig *et al*. 2008). The AMF make a sink for carbon resources and therefore mycorrhizal host plants need to increase their assimilation (Smith and Read 1997). Competence for carbon between host and fungus is strong. This competence could result in decrease on mycorrhizal plant growth compared to non-mycorrhizal ones especially under light deficit or other photosynthetic restricting conditions. It is suggested that some mechanisms are involved in mycorrhization bioprotectivity against pathogens but information are not available adequately. Some of these mechanisms are included: variation in plant nutrition and growth and biochemical and molecular changes in mycorrhizal plants which stimulates resistance to pathogens. Finally, it has been believed that bio-protection is the result of several mechanisms and not a single mechanism (Pozo *et al*. 2002, Fritz *et al*. 2006).

Conclusion

- Response to mycorrhizal inoculation depends on plant genotype and fungi strains.
- Evaluated inoculums were able to colonize studied grape varieties and increased total phenols as defense factor, sugar and chlorophyll content of the plant tissues and resulted to improved vegetative growth.
- Four Iranian table grape varieties responded positively to a standard tissue culture protocol however variations observed among them indicates that efficiency of *in vitro* techniques highly depends on genotype.
- The micropropagation protocol tested here could be used for large-scale clonal propagation of these valuable table grapes which in turn could be utilized for grafting or cultivation and would be an effective tool for grape breeders.
- Growing stock plants in glasshouse and their proper pretreatment such as mycorrhizal inoculation can considerably decrease *in vitro* contamination and increase bud sprouting rate however, organogenesis will probably vary with attention to the nutrient reserve and explant vigor.

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Received May 24, 2012