INTEGRATION OF ARBUSCULAR MYCORRHIZAL FUNGI TO GRAPE VINE (VITIS VINIFERA L.) IN NURSERY STAGE

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ABSTRACT

The Arbuscular Mycorrhizal (AM) association is being considered as the commonest Mycorrhizal type involved in grape community. Low population density of these useful fungi in vineyard soil suggests the need for manual inoculation of grapevine plantlets at the nursery stage. The influence of three commercial Arbuscular Mycorrhizal fungi strains (Glomus Intraradious, G. Mosseae, G. Fasciculatus and a mixture of them) on growth and biochemical status of four grapevine varieties (Shahroodi, Asgari, Keshmeshi and Khalili) was investigated under greenhouse conditions. Rooted plantlets derived from hard-wood cuttings were transplanted in pots containing leaf mold and sand (1:1) followed by inoculation with different fungal inoculums. Various physiological and biochemical parameters were measured at 30 days intervals. The percentage of root colonization was found to be slightly different amongst inoculated vines but it was found to be significantly different with non-inoculated, control plants. Most growth related parameters (vine length, shoot length and leaf area) were enhanced following Mycorrhization but root length and number of leaves were not significantly affected by any fungal intervention. Treated plants typically showed more obvious modifications in their biochemical status. The chlorophyll content (especially "b" and total), total root and shoot phenols were raised in treated plants. The chlorophyll "a" and total soluble sugars were not statistically different in inoculated and control plants. Overall results of the present study suggest that AM fungi can be manually applied, as an easy and economical approach during nursery production, to boost the physiological and biochemical status of the treated plants and production of high quality healthy plantlets.

Keywords: Arbuscular Mycorrhizal fungi, Vitis Vinifera L., Growth, Biochemical Analysis
INTRODUCTION

The roots of most plant species show symbiosis with a kind of soil microorganisms. Approximately 70% of all plant families contain species that develop specialized Endomycorrhizae called Vesicular-Arbuscular Mycorrhizae (VAM) or just Arbuscular Mycorrhizae (AM) on their roots (1). This kind of symbiosis has been known to increase plant growth in a very wide variety of plant species including several crops and trees (2). The effects of AM fungi on the growth and development of horticultural plants have been well documented (3, 4 & 5). It has been known for over a century that grapevines (Vitis spp.) form symbiotic associations in their roots with such micro-organisms (6). Mycorrhizal colonization of grafted grapevines was studied during early establishment of an experimental rootstock vineyard to determine rootstock variability forming functional association (7). There are also some reports on the role of AM fungi as an aid to hardening in micro-propagated grape plantlets to reduce transplantation shock and alleviation of stresses in weaning stage, the process which is commonly known as bio-hardening (5 & 8). According to Aguin et al. (9), population of AM fungi in field may be low or rare (in fumigated soils), suggesting the need for AM inoculation of grapevine plants at the nursery stage. Hence, addition of AM fungi inoculums to rooting substrate could be an effective strategy for the nursery production of Mycorrhizal plants. Differential growth of Mycorrhizal field-inoculated grapevine rootstocks in replant soils was also recently studied (10). Owing to extension activities held by private and governmental institutes, integration of AM fungi to horticulture and particularly vineyard management is recently getting popular in this area. Hence, the present investigation was designed to examine the influence of three AM fungal species on growth and other morpho-physiological parameters of grape hard-wood cuttings during nursery production.

MATERIALS AND METHODS

Plant materials

Hard-wood stem cuttings of four table grape (Vitis Vinifera L.) varieties namely, Shahroodi, Asgari, Keshmeshi and Khalili were collected from a well maintained vineyard at the Shahrood Agricultural Research Center, Semnan province (latitude 35°34´ N, longitude 53°23´ E, altitude 1130m), by mid March. The cuttings were further dissected and pruned to at least four buds (about 30 cm long) and the same were inserted in a pre-soaked saw dust medium to induce rooting without any hormonal treatment. These were raised in a glasshouse under normal day length (12 hours) and an average temperature of 25ºC. Root emergence was observed in all varieties within three weeks following insertion.

Inoculum preparation and application

Three AM fungi species namely, Glomus Mosseae, G. Fasciculatum and G. Intraradices were used. Mycorrhizal Inocula were procured from a commercial laboratory (Turan Biotech Co., Shahroud Iran). These consisted of soil, spores (spore density of 150/100 g dry soil), Mycelium, and infected/colonized host root fragments. The rooted grape cuttings were transplanted in plastic pots (three per pot) containing natural decomposed forest leaf mold mixed with fine sand (1:1 v/v). For Mycorrhizal inoculation, each pot was inoculated with 100 g soil based inoculums (1:50) from above mentioned strains, just distributed beneath the Rhizosphere (root zone area) to facilitate root colonization. An additional treatment also was used as mixed species (combination of all three strains). The transplanted plants were irrigated about 80% of field capacity and kept under glasshouse conditions for further growth and evaluation. The non-inoculated pots were filled with the same potting mixture (without inoculum) and were used as control.

Assessment of root colonization

Root colonization percentages were measured 60 days after inoculation (DAI) through modified method proposed by Phillips and Hayman (11). Fresh root segments were stained with 0.01% Trypan
blue in lactic acid. The stained roots were distributed in a glass petri-dish in which a grid with 0.5 × 0.5 inch squares was affixed to the base (Fig. 1). Total number of intersects between lines and roots (R1) and total number of intersects where the root was Mycorrhized (R2) were recorded using an inverted microscope equipped with a digital camera. Percentage of AM infection was calculated using the following formula proposed by Nicolson (12):

\[
\text{Percent root colonization} = \left( \frac{R2}{R1} \right) \times 100
\]

Fig. 1: Percent of AMF root colonization in four grape varieties, 90 DAI. Columns with the same letter(s) are not significantly different. A=Asgari, H=Khalili, K=Keshmeshi, S=Shahroodi, C=control, I=I. Intraaradices, M=Glomus Mosseae, F=G. Fasciculatum, IMF=Mixed Strains. The data are the means ± standard errors of means (n=48).

**Growth parameters and measurement of biochemical status**

Morphological parameters; viz., vine length (VL), root length (RL), number of shoots (SN) and leaves (LN) and total leaf area were recorded at 30, 60 and 90 DAI.

Biochemical analyses were made 90 DAI. The leaf chlorophyll contents (a, b and total) were assessed following method suggested by (13). Fully matured leaf samples were cut and dipped in Dimethyl Sulphoxide (DMSO) and incubated at 70 °C for 4 h. The absorbance of the solution was then read against blank (solvent) at 645, 663 and 480 nm using spectrophotometer. Total phenol contents present in leaf (LTP) and root (RTP) samples were assayed using slightly modified method proposed by Malik and Singh (14). Immature leaves/root tips (10 cm in length) were collected. Foliar and root samples were dried in an oven (40º C for 72 h) and approximately 500 mg dry matter of each sample was extracted with 80% Methanol by means of a shaker (120 RPM for 24h) followed by filtering through filter paper. The supernatant was collected and evaporated to dryness. Residues were dissolved in distilled water. Folin-Ciocalteau reagent and Na₂CO₃ 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 (TSS) was carried out according to the method described by (15). 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.

**Statistical analysis**

The experiment was carried out as complete randomized block design with factorial arrangement including four replications. The average values obtained from three plants per each pot were used for
INTEGRATION OF VITIS VINIFERA L. IN NURSERY STAGE

M. EFTEKHARI et al

analysis. Data were analyzed by analysis of variance using the GLM procedure in SAS software (16) and mean values were compared using the Least Significant Difference (LSD) test (P < 0.05).

RESULTS

Root colonization

Results of root fragment staining and microscopy observations (Fig. 2) revealed that the highest root colonization occurred in Keshmeshi grape inoculated with mixed AM strains (82.6%) followed by ‘Shahroodi’ inoculated with G. Fasciculatum (82.4%). The least percentage of root colonization was recorded in non-inoculated ‘Khalili’ (49.9%). The percentage of root colonization was found to be slightly different amongst inoculated vines but it was found to be significantly different with non-inoculated control plants.

![Fig. 2: Squared petri-dish utilized for measurement of root colonization (a), stained root segments distributed in Petri-dish (b), root colonization occurred in Shahroodi variety plants growing on leaf-mould (c) and inoculated with G. Mosseae (b) 90 days after inoculation](image)

Growth and Morpho-physiological parameters

According to ANOVA (Table 1), significant differences observed among the three different stages of sampling (30, 60 and 90 DAI) with respect to various growth parameters. Furthermore, interactions of AM Inoculum and plant variety were also statistically different for the same parameters. Mean values of recorded different characters are shown in Table 2. ‘Keshmeshi’ plantlets inoculated with G. Mosseae attained minimum height (10.6 cm) that was not significantly different with control (12.2 cm) as well. However, ‘Shahroodi’ plantlets inoculated with the same AM strain revealed highest length (32.3 cm). Total leaf area in treated plants was either different among AM strains (Khalili and Keshmeshi varieties) or it was not influenced by symbiosis (Shahroodi and Asgari varieties). Some treatments increased number of shoots (Table 2). Changes in number of leaves were found to be more relevant in case of ‘Asgari’ and ‘Keshmeshi’. Though higher number of leaves and longer roots were produced in cuttings inoculated with AMF strains as compared to control but, overall it can be perceived that these traits were not significantly affected by any fungal interventions.
### Table 1: ANOVA table (Pr > F values) for the effects of variety, AM Inoculum, and their interaction on AM root colonization, physiological and biochemical parameters.

( PRC-Percent root colonization, SN-shoot number, TLA-total leaf area, LN-leaf number, VL-vine length, RL-root length, TS-total sugars, Cha-chlorophyll a, Ch b-chlorophyll b, TCh-total chlorophyll, LTP-root total phenol, RTP- root total phenol).

<table>
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<th>Treatment</th>
<th>PRC*</th>
<th>Cha</th>
<th>Chb</th>
<th>TCh</th>
<th>LTP</th>
<th>RTP</th>
<th>TS</th>
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<th>SN</th>
<th>TLA</th>
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<td>0.3412</td>
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<td>0.6625</td>
<td>0.0864</td>
<td>0.9956</td>
<td>0.8917</td>
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</tbody>
</table>

Asgari  | G. Mosseae  | 1.11 ± (0.1) bcd | 560.2 ± (55.7) a | 14.7 ± (2.2) abc | 20.3 ± (3.0) cdef | 12.4 ± (2.6) ab |
| G. Infracirradianus  | 1.78 ± (0.2) a | 460.0 ± (30.3) abcd | 17.2 ± (2.1) a | 16.6 ± (2.4) def | 13.1 ± (2.6) ab |
| G. Fasciculatus  | 1.67 ± (0.2) a | 469.7 ± (44.3) abcd | 17.0 ± (2.2) ab | 19.2 ± (2.7) cdef | 15.5 ± (3.0) ab |
| Mixed AMF  | 1.42 ± (0.1) ab | 526.2 ± (59.6) a | 15.0 ± (2.3) abc | 19.1 ± (3.2) cdef | 13.3 ± (3.0) ab |
| Control  | 1.33 ± (0.2) ab | 488.8 ± (76.4) abc | 14.9 ± (2.7) abc | 14.0 ± (2.7) def | 10.6 ± (2.7) abc |

Khallili  | G. Mosseae  | 1.30 ± (0.2) abc | 189.7 ± (31.0) g | 10.5 ± (2.0) bcd | 16.0 ± (2.1) def | 12.1 ± (1.9) abc |
| G. Infracirradianus  | 1.61 ± (0.1) ab | 242.7 ± (23.8) fg | 14.7 ± (1.6) abc | 22.3 ± (2.3) bcde | 16.1 ± (2.2) a |
| G. Fasciculatus  | 1.47 ± (0.1) ab | 316.0 ± (28.9) defg | 14.7 ± (1.8) abc | 24.9 ± (2.8) abcd | 14.8 ± (1.8) ab |
| Mixed AMF  | 1.58 ± (0.2) ab | 323.7 ± (41.4) efgh | 15.8 ± (2.3) ab | 22.6 ± (2.6) bcde | 13.6 ± (1.7) ab |
| Control  | 1.61 ± (0.2) ab | 275.6 ± (46.4) efgh | 13.6 ± (2.2) abcd | 19.7 ± (2.9) cdef | 11.9 ± (1.7) abc |

Keshmeshi  | G. Mosseae  | 0.86 ± (0.2) cd | 213.5 ± (42.5) fg | 7.94 ± (1.6) d | 10.6 ± (2.4) f | 8.25 ± (2.2) bc |
| G. Infracirradianus  | 1.08 ± (0.1) bcd | 397.7 ± (51.0) bcde | 11.3 ± (1.8) abcd | 16.9 ± (3.4) def | 9.26 ± (2.3) abc |
| G. Fasciculatus  | 1.36 ± (3.0) abc | 359.9 ± (53.6) cdef | 12.2 ± (0.2) abcd | 18.2 ± (3.0) cdef | 10.2 ± (2.4) abc |
| Mixed AMF  | 0.86 ± (0.1) cd | 296.8 ± (43.2) efgh | 9.03 ± (1.3) cd | 15.0 ± (2.9) ef | 9.62 ± (2.2) abc |
| Control  | 0.67 ± (0.1) d | 335.9 ± (76.6) defg | 8.06 ± (1.9) d | 12.2 ± (3.6) f | 5.20 ± (2.0) c |

Shahroodi  | G. Mosseae  | 1.50 ± (0.1) ab | 565.7 ± (36.2) a | 13.9 ± (1.6) abcd | 32.3 ± (3.5) a | 16.6 ± (2.1) a |
| G. Infracirradianus  | 1.47 ± (0.1) ab | 579.9 ± (41.3) a | 13.8 ± (1.5) abcd | 31.4 ± (3.3) ab | 14.1 ± (1.3) ab |
| G. Fasciculatus  | 1.30 ± (0.1) abc | 465.0 ± (37.9) abcd | 10.8 ± (1.3) abcd | 25.7 ± (3.1) abcd | 13.0 ± (1.8) ab |
| Mixed AMF  | 1.30 ± (0.1) abc | 498.8 ± (47.2) abc | 12.3 ± (1.5) abcd | 27.6 ± (3.1) abc | 12.7 ± (1.6) ab |
| Control  | 1.11 ± (0.06) bcd | 589.2 ± (46.8) a | 11.8 ± (1.3) abcd | 29.9 ± (3.4) ab | 14.3 ± (1.8) ab |

Table 2: Morphological parameters of grape cuttings following Inoculation with different AMF strains.

[SN: shoot number; TLA: total leaf area; LN: leaf number; VL: vine length; RL: root length. The data are the mean values ± standard errors (n=48)].
**Table 3:** Biochemical changes of grape cuttings following inoculation with different AMF strains.
[Cha: chlorophyll a; Ch b: chlorophyll b; TCh: total chlorophylls; TSS: total soluble sugars; LTP: leaf total phenols; RTP: root total phenols. The data are the mean values ± standard errors (n=48)].
Biochemical changes

The results of the present study clearly revealed the significant variations among different grape genotypes inoculated with AMF species for their biochemical modifications (Table 3). However, the content of chlorophyll 'a' was estimated to be analogous amongst all studied plantlets. It can be stated that, the leaf chlorophyll content (a, b and total) recorded in the Mycorrhizal plants was typically higher than their respective non-treated control. Khalili plantlets inoculated with AM mixed strains yielded the highest amount of total chlorophylls (17.0 mg/g FW). While non-treated Keshmeshi plants gained least amount (11.3 mg/g FW). The content of chlorophyll ‘a’ was not significantly affected by any AMF treatments.

As compared to other biochemical traits, a minor difference was observed in total sugars present in grape varieties following AM fungi inoculation. It means, ‘Khalili’, ‘Keshmeshi’ and ‘Shahroodi’ grape varieties were not found to be significantly different in their total sugars. However, the latter accumulated the highest amount of sugars among different screened (3.52 %). Asgari plantlets inoculated with mixed AMF treatments showed least amount of sugars (1.9 %). The total phenols present in both leaf and root parts were also affected due to microbial intervention (Table 3). ‘Khalili’ plants received AMF mixed strains showed the highest amount of phenolics in their foliage (71.3 mg/100 g FW). In case of root samples, the highest phenol level was recorded in Shahroodi plants treated with G. Fasciculatus (36.2 mg/100 g DW).

DISCUSSION

The AMF association being the commonest Mycorrhizal type involved in agricultural systems (3) and the variability of AM species in their ability to improve the growth of different plant species has been largely demonstrated (17). It is necessary to determine the best Mycorrhiza corresponding special plant varieties. It has been recognized that the creation of a permanent relationship between host and fungus is in result of identification and approval of molecular signals by both symbionts, which consequence in genome expression of both organisms and it can be understood that the percentage root colonization is under control of plant genotype (3, 18). It might be for the same reason that, in the present study, different grape genotypes colonized with a varying degree following inoculation with selected Mycorrhizal strains. Such variations in root colonization among genotypes of a species have been already confirmed in some grapevine rootstocks (5, 8) and some other plants namely, wheat (19), corn (20) and citrus (21). In the majority of these studies, the sterile media and/or fumigated soils were used but we have used a natural, non-fumigated mixture (commonly used medium for grape propagation in Iranian nurseries) and as a result some amount of colonization were also observed in non-inoculated, control plantlets that was actually due to presence of native fungal strains.

Since, in the present study, clonally propagated plant materials (i.e. hard-wood cuttings) were used, the uniform growth pattern might be expected, hence, any morphological amelioration could be attributed to the fungal interference. However, irrespective of any fungal intervention, the overall measured growth parameters suggested that ‘Shahroodi’ was the most vigorous variety followed by ‘Khalili’, ‘Asgari’ and ‘Keshmeshi’. It is obvious that in our morphological data (Table 2), integration of AM fungi to nursery bed simply enhanced the growth parameters. However, this improvement was considerably different with regard to the type of fungal strain used, for example, all inoculated plantlets generally gained higher length than control, but given a particular strain, different vine lengths were observed in four inoculated grape varieties. However, irrespective of the statistical aspects, an elevated trend could be observed for vine length as well as other morphological characters following microbial treatments. The inoculation resulted in higher growth rate of the Mycorrhizal plants but the degree of enhancement was limited to host-fungi interaction.

Although, our results obtained for number of leaves per vine is in incongruity with the findings of (22) on pepper plants that AM-inoculated plants showed the lowest number of leaves, the same is in agreement with the results obtained on Chrysanthemum (23), Guava plantlets (24), Salix repens (25) and olive (26).
Furthermore, it was found that *Glomus Fasciculatum* had most effect on ‘Keshmeshi’ grapevine growth which was corresponded by the works of Bheemareddy and Lakshman (19) on some *Glomus* species. Considering morphological characters (Table 2), Mycorrhizal inoculation did not increase significantly the growth rate of ‘Shahroodi’ plants, but according to Schiavo et al., (27), AMF inoculation increased the height of *Acacia* sp. and *Sesbania* sp. as compared to control under glasshouse conditions.

Different AMF strains varied in their efficacy to increase the synthesis of different biochemicals, thereby improving the plant growth. These differences may depend on the genetically controlled physiological characters of the fungal strains (18).

In this study, increased total chlorophyll and chlorophyll ‘b’ in plants of ‘Asgari’ and ‘Keshmeshi’ grapevines is similar to the findings of Bavaresco and Fogher (28) on the effect of *G. Mossea*. However, in case of ‘Khalili’ grape, the mixed AM strain treatment lead to highest biosynthesis of chlorophylls. The positive effect of AM symbiosis on chlorophyll content was also reported in Maize (29), *Sesbania* (30), *Lotus* (31) and Zucchini (32). Furthermore, Estrada-Luna (24) indicated that leaf chlorophyll may vary according to light conditions (or other factors such as mineral status of the plants, in which N, Mg, Cu and Fe have important roles). Increased chlorophyll content after AM inoculation has also been reported by Mathur and Vyas (33, 34); Krishna et al., (5, 8). Who suggested that, the higher chlorophyll content in Mycorrhizal plants may be due to the higher concentrations of Mg, Fe and Cu in foliar tissues thereby influencing chlorophyll synthesis. Mycorrhizae regulate not only uptake, but also the relative abundance of available and transportable nutrients in the tissue concentration of essential micronutrients like Cu and Zn. Siderophores are formed by Mycorrhizal fungi that enable the fungus to take up Fe from solutions in low amounts (17).

Phenols are important components of plant defense mechanism against the diseases. Phenolic compounds occur naturally in plant system and owing to their antimicrobial properties inhibit fungal spore germination and toxin production by pathogens (35). In the present study, inoculated grape cuttings accumulated higher phenolic compounds in their root as well as foliage. The mixed AM inoculum was found the most efficient one in enhancing leaf phenolics in ‘Khalili’. Earlier, Tang et al., (36) reported that a significant increase in the level of phenolic compounds in the bark of AM-inoculated poplar plants. The increased level of total phenols suggests higher resistance of inoculated plants against diseases, which led to increased plant survival under nursery or glasshouse as well as field conditions (36). Furthermore, organic grown tomatoes had increased total phenolic contents in their fruits as a result of the AMF treatment (37), Devi and Reddy (38); Kapoor (39) showed that AM inoculation induced quantitative and qualitative changes in phenolics of groundnut and tomato, respectively. In addition, inoculated *in vitro* grown grapevine plantlets had the higher phenols during their hardening period (5, 8).

In conclusion, taking overall account of the results obtained in the present study, it can be stated that AM fungi can be manually applied in the nursery, where moderate amounts of colonization are often naturally achieved so that following transplanting to the vineyard they could colonize and enhance plant growth and production. Future research works must be undertaken on the effects of these fungi on the performance of such developed cuttings under field conditions and upon fruiting.

**ACKNOWLEDGEMENT**

The help and co-operation received from the Turan Biotech Co. is fully acknowledged.

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INTEGRATION OF VITIS VINIFERA L. IN NURSERY STAGE

M. EFTEKHARI et al


INTEGRATION OF VITIS VINIFERA L. IN NURSERY STAGE

M. EFTEKHARI et al


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