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The Usefulness of Mycorrhizal Fungi to Mitigate Weaning Stress in Micropropagated Grapevine Plantlets

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Abstract
Large-scale mortality of *in vitro* raised plantlets occurred during acclimatization, *i.e*. glasshouse hardening, and later at field transfer still has remained as a significant bottleneck in micropropagation. The usefulness potential of some arbuscular mycorrhizal fungi (AMF) to minimize mortality rate of three commercial grape rootstock genotypes during glasshouse acclimatization was studied. Inoculation was carried out using soil based inocula added below the root system per each plantlet. Of the three different AMF strains used, *Glomus manihotis* showed the highest survival rate (80.15%) in the inoculated plants during hardening. All the inoculated plantlets exhibited morphological alterations such as higher vine length, leaf area and root length over the control. Beneficial effect of AMF inoculation on shoot growth resulted about 1.6 times taller vines compared to the non-inoculated control plantlets. The biochemical analyses revealed that the amount of total chlorophylls in leaves and phenols in vines were increased significantly in the mycorrhizal plants. Though total phenolic compounds were significantly increased in the treated plants following inoculation, but inoculated plants with *G. monosporum* and *G. manihotis* showed higher phenols in their foliage. The present investigation revealed the integration of AMF association in tissue culture as a helpful strategy to minimize weaning stress and mortality rate of microplants during *ex vitro* acclimatization and may be utilized in commercial laboratories after verification of these results by the complementary experiments.

Keywords: Hardening; Mycorrhizal inoculation; Rootstocks; Stress; Vitis

Introduction

While the development of the science of plant tissue culture is historically linked to more than 200 years ago (Razdan 2003) but large-scale mortality of *in vitro* raised plantlets through this technique occurred during acclimatization, *i.e.* glasshouse hardening, and later at field transfer still has remained as a significant bottleneck (Dami and Hughes 1997; Gribaudo *et al.* 2001; Chandra *et al*. 2010). Very often desiccation and wilting are the main causes of low survival. Micropropagation on a large scale can be successful only when high plantlet survival rates could be achieved and the cost involved in the process is low. The use of beneficial plant-microbe interactions in the rhizosphere can be a potent approach to mitigate transplantation shock (stress) of such plantlets. An effective strain can aid in large scale plantlet survival during acclimatization phase in a wide range of plant species including several horticultural crops where there is huge demand for planting material (Krishna *et al.* 2006). The usefulness of Arbuscular Mycorrhizal Fungi (AMF) for agricultural production systems, per se and for the enhancing survival of several high-value micro-propagated plants has been the subject of several studies on many fruit crops such as kiwifruit (Schubert *et al.* 1992), apple (Granger *et al.* 1983), cherry (Pons *et al.* 1983), strawberry

(Hrselova *et al.* 1989), grape (Krishna *et al.* 2006) etc. These studies have shown that AMF inoculation can improve the growth, survival rate and plant quality during weaning period and even reduce the microbial attack, a major problem encountered during Stage IV of micropropagation cycle. The previous studies suggested mycorrhization to be an effective aid for improving plantlet survival of in vitro raised grape plantlets (Singh *et al.* 2004; Krishna *et al*. 2006). Furthermore, AMF inoculation induced biochemical changes were also reported in micropropagated grape plantlets which are directly or indirectly are associated with the better plantlet survival (Krishna *et al*. 2005). However, in the above studies they targeted grape varieties as experimental materials. It is evident that, evaluation of bio-hardening agents such as AM fungi to minimize plantlet mortality during acclimatization for different grape rootstock genotypes would open new vistas in viticulture and rootstock improvement, so that commercial integration of these micro-organisms to viticulture would help in providing large numbers of healthy rootstocks which is required to be adopted for grafting any desired scion variety. Therefore, the present investigation was conducted to evaluate the effectiveness of mycorrhizal inoculation on four different grape rootstock genotypes during *ex vitro* transfer for stress alleviation, enhanced plant survival and better establishment upon field transplant.

Materials and Methods

Four grape rootstocks of different genetic origin namely, Dogridge (*Vitis champini*), SO4 (*V. riparia* × *V. berlandieri*), H-144 (*V. vinifera* × *V. labrusca*) and 3309C (*V. riparia* × *V. rupestris*) maintained at Grape Germplasm Block at the main experimental orchard, IARI, New Delhi, were selected for the study. Aseptic cultures were initiated following an *in vitro* multiplication protocol standardized by Alizadeh *et al.* (2010). The rooted plantlets were subjected to a short acclimatization period (45 to 55 days depending on the genotype) using glass jars with polypropylene caps explained in the same protocol. These plantlets were then transplanted in plastic pots and were subjected to bio-hardening using different arbuscular mycorrhizal fungi (AMF) strains *viz.*, *Glomus manihotis* (T1), *G. monosporum* (T2) and Pusa mixed strain (T3). The last treatment, a randomly mixed soil based inocula of both *G. manihotis* and *G. monosporum* strains, was procured from the Division of Microbiology, IARI, New Delhi and rest of the strains were the pure strains maintained on Bahia grass (*Paspalum notatum*) host grown on sterile potting mixture and maintained in the controlled glasshouse conditions. The potting mixture was composed of soil: farm yard manure (FYM): sand (2:1:1) that was sterilized by application of formalin (5% aqueous solution) in polyethylene bags for fortnight followed by two weeks of air-drying on polyethylene sheet and exposed to the sun till formalin vapor was completely ceased. Plastic pots were filled one quarter with this mixture and then about 25 g soil based inocula (rhizosphere soil of Bahia grass containing spores, mycelia, arbuscules and root segments) was added just below the roots per each grape plantlet. Inoculated plants were mildly irrigated with

autoclaved tap water and the pots kept under control glasshouse conditions [27 ±1°C with 16/8 h light and dark photoperiod gained from cool white fluorescent tubes (630 µmol m^{-2} s^{-1}) and relative humidity of 80-85% maintained with mist system]. The plantlet survival and/or mortality along with morpho-physiological characters were measured under glasshouse conditions at 30, 45 and 60 days after inoculation (DAI). The dried-up plantlets that were not able to successfully acclimatize were removed from the experiment and considered as dead. The percentage of number of dead plantlets from the total number of inoculated samples in each treatment was considered as the mortality rate. The total chlorophyll contents of the leaves were measured following the method as suggested by Barnes *et al*. (1992). The method proposed by Malik and Singh (1980) was employed for quantification of total phenols. The estimation of leaf area was undertaken using graph papers. Ten approximately average size leaves from each treatment were traced out on a graph paper. Then, the leaf area was estimated through the calculation of graph paper total area. The experiments were conducted as completely randomized design with three replications using 45 units per treatment. The percentage data was transformed using angular transformation (Arc Sin√ %) before carrying out the analysis of variance. Means were compared with the LSD test, also called critical difference (CD)

Results and Discussion

One of the major impediments to the success of micropropagation is high mortality rate of tissue culture derived plantlets either during acclimatization phase or at transfer to the field conditions (Mathur and Vyas 1999). This problem can be obviated by combining the micropropagation technique with 'mycorrhization' during hardening (Singh *et al.* 2004). In the present study, mortality rate was minimized in inoculated grape microplants over control and microbial inoculation effectively increased plant survival of *in vitro* derived plantlets (Table 1). *Glomus manihotis* was found more effective in improving *ex vitro* survival of Dogridge, H-144 and 3309C plantlets (80.4, 86.7 and 80.1% respectively), while SO4 responded better to Pusa mixed AMF strain (74.2%). Thus, the data suggested that various genotypes responded differently to each AMF strain. Earlier, Varma and Schuepp (1994; 1995) reported that the endomycorrhizal root colonization is affected by the host-fungus combination in micropro pagated strawberry, raspberry and hortensia and survival rate following their acclimatization was found to be 100%. Our data on *ex vitro* grape plantlet survival is consistent to those reported by Krishna *et al.* (2006) who reported that *ex vitro* survival rates almost would be doubled following AMF inoculation. Furthermore, working with some micropropagated fruit tree rootstocks, Monticelli *et al*. (2000) also obtained the encouraging results with marked improvement in *ex vitro* explant survival.

Mycorrhizal inoculation is a promising, sustainable technique to enhance plant growth (Lovato *et al.* 2006). AMF inoculated plantlets exhibited apparently higher mean vine length over

Table 1. Effect of AMF inoculation on plantlet survival of *in vitro* raised grape plantlets during hardening (60 days after inoculation)

Treatment / Genotype	Plantlet survival (%)				
	Dogridge	SO4	H-144	3309C	Mean
Control (T0)	43.7 (41.3)*	40.8 (39.7)	46.1 (42.7)	38.7 (38.4)	42.32
Glomus manihotis (T1)	80.4 (63.7)	73.4 (58.9)	86.7 (68.6)	80.1 (64.1)	80.15
G. monosporum (T2)	65.2 (53.8)	67.6 (55.3)	69.7 (56.6)	62.3 (52.1)	66.2
Pusa mixed strain (T3)	72.4 (58.3)	74.2 (59.4)	81.2 (64.2)	75.4 (60.2)	75.8
Mean	87.2	64.0	70.9	64.1	
LSD at 5%	Treatment (T)= 1.21; Genotype (G)= 0.69; T× G= 1.72				

*Transformed data: ArcSin √ %

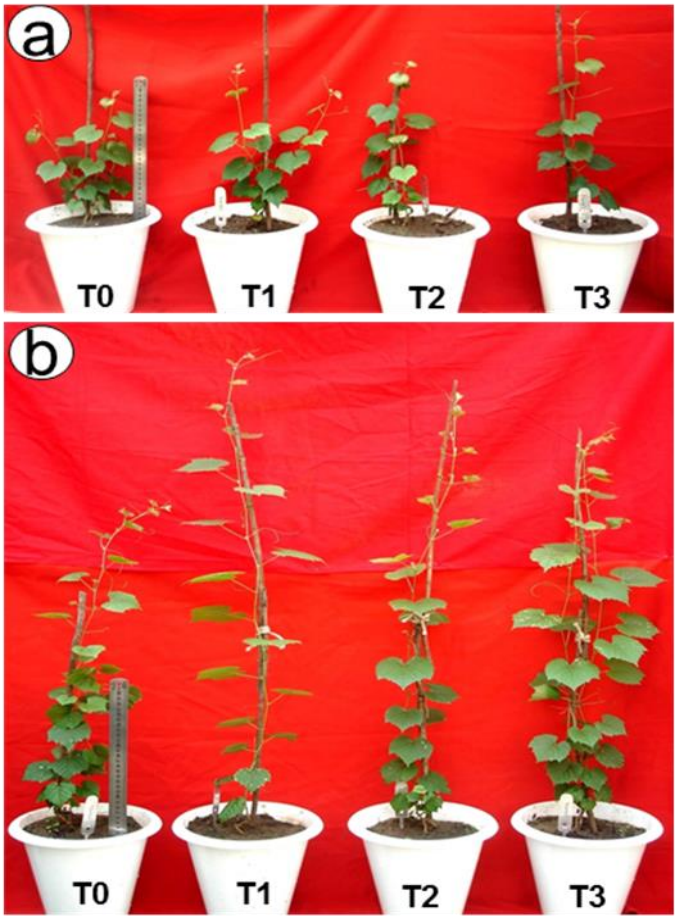

Figure 1. Four randomly selected 3309C grapevine plantlets inoculated with AMF strains. (a) 15 days after inoculation (DAI); (b) 60 DAI. T0, T1, T2, T3 = control, *Glomus manihotis*, *G. monosporum* and Pusa mix AMF strain, respectively.

the control (Figure 1). In addition, number of leaves and leaf area were found to be positively affected by AMF inoculation (Table 2).

Generally speaking, the beneficial effect of AMF inoculation on shoot growth resulted about 1.6 times taller vines compared to the non-inoculated control plantlets. The increase in plant height may be attributed to the multifaceted role

of mycorrhiza including better nutrient uptake (Menge *et al*. 1980). On the other hand, Allen *et al*. (1980) suggested that probably additional factors other than nutrition, such as hormonal balance modifications, are induced by the AMF symbiosis which leads to marked improvement in plant growth and other physiological and biochemical characters. Though all the inoculated plantlets showed higher leaf area over the control, but the difference between *Glomus monosporum* and Pusa mixed strain inoculated plantlets was found to be non-significant. However, *Glomus manihotis* was more effective than other strains with regard to leaf area enhancement. Significant increase in leaf area of grape rootstocks in the present study is comparable to those reported for three micropropagated pineapple varieties inoculated with five different VAM fungi (Guillemin *et al.* 1992).

The root length also was monitored in the present investigation (Table 3). Owing to endomycorrhizal inoculation, the micropropagated plants have changed root length. It caused increase in lateral root number (data not shown) and length. Higher number of roots was produced following AMF inoculation. However, there was no significant difference of *Glomus monosporum* inoculated plantlets with either *Glomus manihotis* or Pusa mixed AMF strain treated plantlets. The three AMF strains led to the equal increase in root length in mycorrhizal plants and as a result, irrespective of the genotype, the difference in root length was not found significant. The positive effect of AMF strains on root length of apple rootstocks has also been reported by Sbrana *et al.* (1994). Biricolti *et al.* (1997) observed overall improvement in root and shoot growth of grape rootstock inoculated with *Glomus mosseae* and *G. deserticola.* Inoculation with AMF, showed positive effects on plant growth, particularly root development, compared with the control in micropropagated *Scutellaria integrifolia* plants (Joshee *et al.* 2007).

Besides morphological changes, microbial inoculation had considerable effects on photosynthetic pigments and *in vivo* phenol production (Table 3). *Glomus manihotis* and *G. monosporum* were more efficient with respect to enhancement of leaf total chlorophyll. The increased chlorophyll content of leaves could be attributed to enhanced uptake of Mg, Fe and Cu, which are essential elements for synthesis of chlorophyll (Krishna *et al*. 2006).

Phenolic compounds are widely distributed in the plant kingdom. Plant tissues may contain phenolic compounds up to several grams per kilogram. External stimuli such as microbial infections, ultraviolet radiation and chemical stressors induce their synthesis (Alizadeh 2007). It has been reported that tissue cultured plantlets inoculated with AM fungi had a higher ortho-dihydric phenol in the root tissue. Higher phenolic content increases the defence mechanism of the host plant and thereby imparts resistance to various diseases and stresses (Sivaprasad and Sulochana 2005). In the present study, *in vivo* phenol content of AMF inoculated plantlets were enhanced (Table 3). Plantlets inoculated with *Glomus manihotis* and *G. monosporum* exhibited the higher phenol contents. The phenol content in all the genotypes was higher as the time after inoculation progressed. These findings are mainly

Table 2. Effect of AMF inoculation on mean vine length, number of leaves and leaf area of *in vitro* raised grape plantlets during hardening (60 days after inoculation).

Treatment / Genotype	Vine length (cm)					Number of leaves					Leaf area (cm^2)				
	D	S	H	C	Mean	D	S	H	C	Mean	D	S	H	C	Mean
T0	23.4	25.3	23.3	24.2	24.0	19.6	19.1	26.8	17.5	20.7	31.4	32.7	35.8	28.4	32.0
T1	36.4	29.4	39.4	37.4	35.6	23.4	22.5	34.9	22.2	25.7	38.6	38.7	41.4	34.1	38.2
T2	29.3	23.2	34.9	29.4	29.2	16.4	16.2	25.6	23.2	20.3	34.4	32.9	37.8	31.5	34.1
T3	30.9	33.4	32.9	28.3	31.3	21.7	21.8	32.7	24.0	25.0	32.8	34.4	35.9	32.9	34.0
Mean	30.0	27.8	32.6	29.8		20.2	19.9	30.0	21.7		34.3	34.6	37.7	31.7	
CD at 5%	Treatment (T)= 0.34; Genotype (G)= 0.69; T× G= 1.72					T= 0.32; G= 0.08; T× G= 0.31					T= 0.44; G= 0.61; T× G= 1.03				

D= Dogridge; S= SO4; H= H-144; C= 3309C

Table 3. Leaf total chlorophylls, vine phenols contents and root length as affected by AMF inoculation during hardening in four grape rootstocks (60 days after inoculation)

Treatment / Genotype	Total chlorophylls (mg/g f.w)					Total phenols (mg/g f.w)					Root length (cm)				
	D	S	H	C	Mean	D	S	H	C	Mean	D	S	H	C	Mean
T0	7.6	9.6	11.4	10.2	9.7	3.1	5.2	3.6	5.0	4.2	11.4	12.3	17.4	13.4	13.6
T1	8.8	9.4	12.1	10.7	10.2	21.2	31.2	18.8	28.0	24.8	14.5	13.1	22.0	16.7	16.5
T2	8.4	8.9	11.2	9.4	9.4	14.2	33.1	18.3	31.1	24.1	13.6	16.1	21.8	16.4	16.9
T3	8.7	8.4	10.8	8.2	9.0	12.7	19.7	16.4	21.7	17.6	14.2	14.2	18.9	14.2	15.3
Mean	8.3	9.0	11.3	9.6		12.8	22.3	14.2	21.4		13.4	13.9	20.0	15.2	
CD at 5%	Treatment (T)= 0.03; Genotype (G)= 0.10; T× G= 0.26					T= 2.82, G= 20.4; T× G= 1.41					T= 0.84, G= 0.74; T× G= 1.11				

D= Dogridge; S= SO4; H= H-144; C= 3309C

corroborated with those reported by Singh *et al.* (2004) and Krishna *et al.* (2005) in different grapevine cultivars.

The present investigation revealed the integration of AMF association in tissue culture as a useful strategy to minimize mortality rate of microplants during *ex vitro* hardening and transfer. However, root colonization data will be taken in a complementary study to ensure the root-microbe interaction as well as to prove the usefulness of a specific fungal strain in mitigation of weaning stress. Furthermore, it is clear that rootstock has a key role in establishing the fruit orchard and while using *in vitro* derived materials, microbial inoculation would be able to enhance the quality of the planting materials, survival rate and their further performance and subsequently production of large number of healthy plants for commercialization.

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