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Standardization of embryo rescue technique and bio-hardening of grape hybrids (*Vitis vinifera* L.) using Arbuscular mycorrhizal fungi (AMF) under sub-tropical conditions

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Summary

***In-ovulo* embryo rescue in grape breeding programme assures breeding efficiency by curtailing 6 to 8 years in the development of seedless grape cultivars. Effect of different growth regulators, culture media, ovule age, cultivars and mycorrhizal strains on successful *in-ovulo* embryo rescue were studied in this experiment. With respect to mean ovule age (days after pollination), maximum ovule-embryo growth (2.13 mm^2) were obtained when ovules were cultured at 24 days after pollination (DAP) but maximum germination (12.67 %) was obtained when ovules were cultured at 28 DAP. The concentration of IAA (4 mg) + GA3 (0.5 mg) proved to be most effective for germination (13.84 %). Among various media used for culturing ovules on modified MS medium (1/2 macro + 1/1 micro) required minimum days to germinate (96.67) and registered highest germination (13.75 %). Chilling treatment was proved to be one of the important factors for embryo maturation and 60 d of chilling treatment at 4 °C improved embryo germination. With respect to the effect of different growth regulators on various rooting and shooting parameters IBA (1.0 mg·L^{-1}) and NAA (1.5 mg·L^{-1}) were found superior. While comparing different hardening strategies, a glass jar with polypropylene cap (GPP) was found to be most effective as far as hardening success (66.67 %) was concerned. One of the serious impediments in the success of *in vitro* rescued plantlets is very high field mortality, field survival of rescued plantlets can be effectively increased by using different mycorrhizal strains (AMF) as bio-hardening agents. Among the various strains used for hardening of rescued plantlets T3 inoculated plantlets registered highest survival percent (88.00).**

K e y w o r d s : *In ovulo*, embryo rescue, seedless grapes.

Introduction

Grapes are one of the most important fruit crops of the world. It has undoubtedly become the most lucrative fruit crop of the world with substantial increase in area of its cultivation. Grape is now bred for different purposes *viz*., table or dessert type, juice, wine, raisin, etc. besides these grapes also contain ample amount of antioxidants like resveratrol, which has been positively linked to inhibiting cancer, heart disease, degenerative nerve disease, viral infections and mechanisms of Alzheimer's disease. So, development of high yielding cultivars of good quality and large berry size are major objectives of grape breeding programmes in order to meet the emerging interest in seedless grapes.

In seedless grape fertilization takes place but embryo and / or endosperm development stops soon after anthesis and seeds abort at different stages of growth, which mainly depends upon the genotypes in question. This phenomenon is referred as stenospermocarpy (Cain *et al.* 1983). The technique of embryo rescue employing *in-ovulo* culture has been adopted in grape breeding in the western world and results show that a mean 85 per cent of the progenies from seedless × seedless crosses can be seedless. The role of embryo rescue is more important particularly under subtropical conditions where the grape breeding season is very short as compared to temperate regions and hence different factors are to be standardized for its breeding through *in-ovulo* technique. Thus, by adopting *in-ovulo* embryo rescue in grape breeding programmes about 7-8 years can be curtailed for developing superior seedless cultivars. Furthermore, one of the serious impediments in the success of *in vitro* rescued plantlets is very high field mortality, field survival of rescued plantlets can be effectively increased by using different Arbuscular mycorrhizal fungi (AMF) strains, known to form symbiosis with many fruit crops including grape. Symbiotic association of microbes with roots of plants is beneficial as it stimulates growth and development in plants, increase absorption of water and plants immobile nutrients, increase drought tolerance and minimize disease incidence.

Therefore, an attempt has been made in this study to standardize *in ovulo* embryo rescue technique for grapes under subtropical conditions and to screen out suitable AMF strain for bio-hardening of grape hybrids.

Material and Methods

Following cultivars of grapevine *viz*., Female parents ('Pusa Urvashi' and 'Beauty Seedless') and male parents ('Pusa Seedless' and 'Perlette') were chosen for hybridization. Seven to ten years old healthy vines maintained in

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the Grape Germplasm Block of the Division of Fruits and Horticultural Technology, were selected for hybridization.

For hybridization of grape, a day before emasculation and pollination, the healthy panicles were bagged with finely perforated butter paper bags. Emasculation was done on the following day from 7.00 to 10.00 a.m. when calyptra (corolla) was detached easily followed by pollination. Pollination was carried out from 9.30 to 11.30 a.m. followed by rebagging and tagging of panicles. Developing immature fruitlets at 16, 20, 24 and 28 d after pollination (DAP) were collected for ovule excision. The ovules were inoculated on the establishment media namely, MS (1/2 macro + 1/1 micro), B5, and NN separately, supplemented with various phytohormones (IAA, IBA, NAA and GA_3) at different stages of *in ovulo* culture and regeneration of the plantlets. Chilling treatment at 4 °C for 60 d was employed for embryo maturation (Figure B).

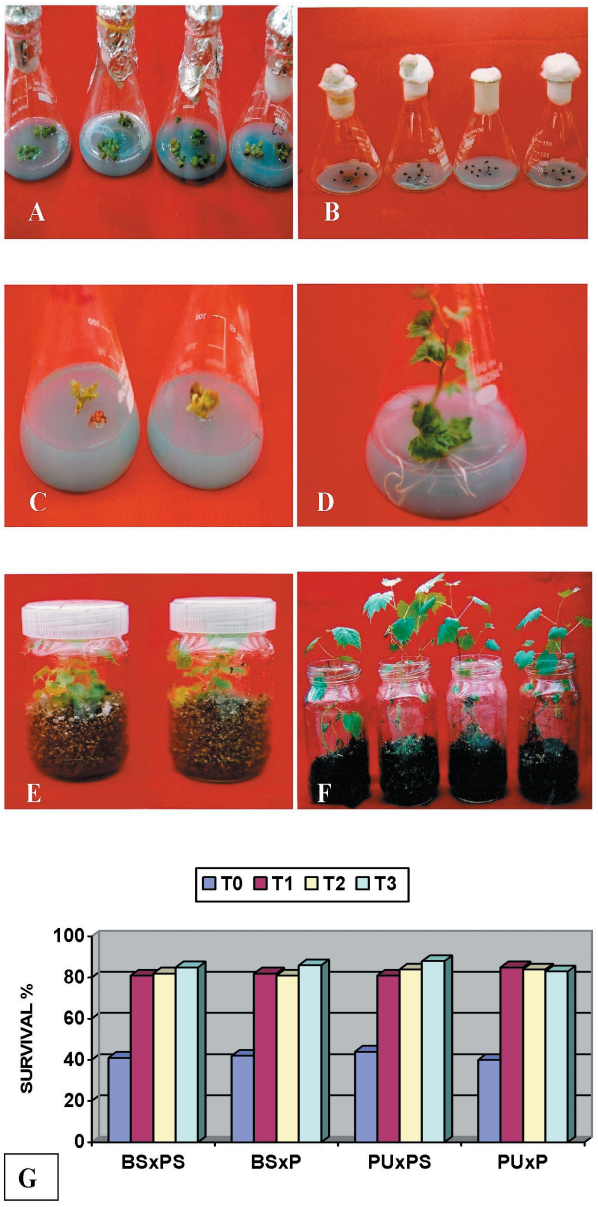

Figure: **A**: Cultured green ovules of hybrids. **B**: Mature ovules after chilling treatment. **C**: Initiation of embryo germination. **D**: Germinated embryos of different hybrids. **E** and **F**: *In vitro* raised plantlets. **G**: Effect of AMF inoculation on *ex vitro* survival.

In vitro raised rooted plants (45 d after root initiation) were taken for Arbuscular mycorrhizal fungi Inoculation (AMF). Three AMF strains were used for bio-hardening of embryo rescued grape plantlets namely, *Glomus mossae* (T1), *Glomus manihotis* (T2) and mixed AMF strain (T3). The rooted plantlets were transferred to plastic pots filled with sterile soil, sand and FYM (2:2:1) mixture. Approximately 20 g of chopped Bahia grass roots having pure AMF strains (Bahia grass is used to maintain pure culture of AMF) were mixed with soil near the root zone of transferred plantlets. The plantlets after inoculation were immediately watered with sterile water and maintained under controlled glasshouse conditions.

Results and Discussion

Effect of ovule age on *in vitro* ovule-embryo growth (mm^2) have been studied in this experiment, the maximum mean *in-ovulo* embryo growth (2.13) was obtained when ovules were cultured at 24 d after pollination stage. In the present study, the ideal sampling time for ovule culture ranged from 16 to 28 d post pollination (Figure A). This result corroborates with findings of Singh and Brar (1992). However, depending upon genotype, the ideal age for embryo rescue vary widely *viz*., 14-101 d (Cain *et al.* 1983), 55 d (Nookaraju *et al.* 2007) and 40-60 d (Gray *et al.* 1990). Relationship between ovule age at culture and *in vitro* germination (%) revealed that the maximum mean *in vitro* germination (%) was seen when ovules were cultured at 28 days after pollination (12.67). Under *in vitro* conditions the ovules after isolation required simulated environment similar to maternal tissue (endosperm, etc.) which nurture it until maturity. These specific requirements vary with genotype and stage of the ovule taken for culture (Narayanaswamy 1994). The maximum mean ovule growth (2.27) was obtained on medium supplemented with IAA (8.0 $mg \cdot L^{-1}$) + GA_3 (1.0 $mg \cdot L^{-1}$). TANG *et al.* (2009) also reported the role of different growth regulators on the development of ovule and subsequent embryo rescue of stenospermocarpic grape. Observations related to growth regulator concentrations on germination, revealed that the concentration of IAA (4.0 $ml \cdot L^{-1}$) + GA_3 (0.5 $mg \cdot L^{-1}$) proved to be most effective for germination with a value of 13.84 % (Figure C). However, shoot bud sprouting was earliest on medium supplemented with 1.0 $mg \cdot L^{-1}$ IBA (8.50 d). The combined application of GA_3 and IAA significantly affected the establishment per cent and ovule growth of all hybrids. The increased efficiency of IAA alone or along with GA_3 in culture establishment of grape ovules has been reported by Tsolova (1990) and Singh *et al.* (1991). NN medium found to be the best for culture establishment (68.33 %), B5 medium also produced results at par with the NN medium, however, ovules on modified MS medium (1/2 macro + 1/1 micro) showed the highest germination (13.75 %). Superiority of MS and NN media over other media for ovule/embryo culture has been observed by several workers (Tsolova 1990, Singh and Brar 1992, Yang *et al.* 2007). Role of growth regulators was very much evident on rooting of rescued plantlets.

Varying concentrations of IBA (0.5, 1.0 and 1.5 mg·L^{-1}) and NAA (0.5, 1.0 and 1.5 mg·L^{-1}) had differing response on rooting of micro-shoots (Figure D). Micro-shoots on medium supplemented with 1.0 mg·L^{-1} IBA had taken minimum days to root (6.50). It is obvious from the studies that IBA is the best auxin for most of the rooting factors and has a definite role in root elongation but the role of NAA in increasing the number of roots is also well documented (Nas and Reed 2004, Singh *et al*. 2004). Among different strategies adopted for hardening of *in vitro* raised plantlets, the maximum success (66.76 %) was achieved by the use of a glass jar with polypropylene cap (Figure E and F). Similar results were shown by Singh *et al.* (2007), who proposed that the glass being transparent material allows better hardening, faster leaves expansion and early cuticle development.

Ex vitro survival of rescued hybrid progenies was highly enhanced by AMF inoculation (Figure G). PU x PS when inoculated with strain T3 showed highest survival (88.00 % each). Higher survival percentage of AMF inoculated micropropagated plants was earlier reported by Puthur *et al*. (1998) and Krishna *et al.* (2006) in different fruit crops. Higher survival rates of mycorrhizae inoculated plantlets might be due to the development of strong root system (Elemeskoui *et al.* 1995), improved uptake of plant immobile nutrients and improved water uptake (Yamashita *et al.* 1998). Beneficial association of AMF with grapes has also been reported by Balestrini *et al*. (2010).

Phenol content is correlated with resistance and tolerance of the plants against stresses and AMF inoculation was found to increase the phenol content (µg·g^{-1} fresh wt) in rescued plantlets (Table). Among various AMF strains used for hardening of rescued plantlets, T2 was found to be the most responsive irrespective of hybrid and duration (9.10). As AMF inoculation enhances uptake of different nutrients, among which some are part of chlorophyll structure hence its content (mg/g fresh weight) was significantly enhanced by AMF inoculation (Table). T1 inoculated hybrid plantlets synthesized significantly higher amount of chlorophyll (3.24) than other AMF strains. The increased phenolic content might be attributed to enhanced polyphenol oxidase activity and increased chlorophyll content could be attributed to enhanced uptake of Mg, Fe and Cu, which are essential for synthesis of chlorophyll (Mathur and Vyas 1995).

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T a b l e

Effect of AMF inoculation on total phenol and total chlorophyll content

Hybrid	Stage	Total phenol content (µg·g^{-1} fresh weight)					Total chlorophyll content (mg·g^{-1} fresh weight)				
		Treatment					Treatment				
		T0	T1	T2	T3	Mean	T0	T1	T2	T3	Mean
BS X PS	30 DAT*	2.63	8.57	8.67	8.03	6.98	2.27	3.47	3.03	2.93	2.93
	45 DAT	3.60	9.73	9.93	9.60	8.22	2.20	3.50	3.07	3.07	2.96
	Mean	3.12	9.15	9.30	8.83	7.60	2.24	3.49	3.05	3.00	2.95
BS X P	30 DAT	2.80	8.70	8.77	8.07	7.09	2.33	3.40	3.07	3.03	2.96
	45 DAT	3.13	9.10	9.17	8.43	7.46	2.43	3.20	3.00	2.93	2.89
	Mean	2.97	8.90	8.97	8.25	7.27	2.38	3.30	3.04	2.98	2.93
PU X PS	30 DAT	2.70	8.20	8.37	8.10	6.84	2.80	3.07	2.63	2.63	2.78
	45 DAT	3.57	9.13	9.27	8.73	7.68	3.10	3.27	2.93	2.90	3.05
	Mean	3.16	8.67	8.82	8.42	7.27	2.95	3.17	2.78	2.77	2.92
PU X P	30 DAT	2.90	8.93	9.13	8.90	7.47	2.83	3.07	2.83	2.77	2.88
	45 DAT	3.37	9.30	9.50	8.87	7.76	2.37	2.97	2.67	2.73	2.69
	Mean	3.14	9.12	9.32	8.89	7.62	2.60	3.02	2.75	2.75	2.78
Pooled Mean		3.09	8.96	9.10	8.59		2.54	3.24	2.90	2.87	

	SEm±	CD at 5 %		SEm±	CD at 5 %
Hybrid	0.0526	0.1511	Hybrid	0.0439	0.1261
Duration	0.0372	0.1069	Duration	0.0310	0.0892
H x D	0.0744	0.2137	H x D	0.0621	0.1783
Treatment	0.0526	0.1511	Treatment	0.0439	0.1261
T x H	0.1052	0.3022	T x H	0.0878	0.2522
T x D	0.0744	0.2137	T x D	0.0621	0.1783
T x H x D	0.1488	0.4274	T x H x D	0.1242	0.3566

* DAT: Days after transfer

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