Indian J. Hort. 67(Special Issue), November 2010: 37-42

Standardization of embryo rescue technique for grape hybrids under sub-tropical conditions

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ABSTRACT

The development of new high yielding cultivars of good quality with large berry size is the main objective of seedless grape breeding programme. Seedless grapes suffer from stenospermocarpy so, technique of embryo rescue employing *in-ovulo* culture has been adopted in grape breeding in western world which not only produce superior seedless cultivars but also curtails 7-8 years from entire breeding cycle. With respect to mean ovule age (days after pollination) and ovule-embryo growth (2.13 mm^2), culturing of ovules 24 days after pollination was found to be the best. The concentration of IAA (4 mg/l) + GA_3 (0.5 mg/l) proved to be most effective for germination (13.84 %). Among various media used for culturing, ovules on MS medium (1/2 macro + 1/1 micro) registered highest germination (13.75%). Chilling treatment was proved to be one of the important factors for embryo maturation and 60 days of chilling treatment at 4°C was found better than 45 days. With respect to the effect of different growth regulators on various rooting and shooting parameters IBA (1.0 mg/l) and NAA (1.5 mg/l) were found superior. While comparing different hardening strategies, glass jar with polypropylene cap (GPP) was found to be most effective as far as hardening success (66.67%) was concerned.

Key words: Embryo rescue, grape, sub-tropical region.

INTRODUCTION

Grapes are one of the most important fruit crops of the world. Grape cultivation is a remunerative farming enterprise in India and has undoubtedly become the most lucrative fruit crop of the world with substantial increase in area under 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 is a polyphenol antioxidant and has been positively linked to inhibiting cancer, heart disease, degenerative nerve disease, viral infections and mechanisms of Alzheimer's disease. The development of new high yielding cultivars of good quality and large berry size are major objectives of grape breeding programme in order to meet the emerging interest in seedless grapes. Conventional breeding through hybridization to obtain seedless progenies using seeded cultivars as female parents is of limited use due to low proportion of seedless progeny, not higher than 10-15 per cent. However, due to highly heterozygous nature of this crop, identification of a desirable hybrid is a complicated task. In seedless grape fertilization takes place but embryo and / or endosperm development stops soon after anthesis and seed aborts at different stages of growth, which mainly depends upon the genotypes in question, this phenomenon is referred as stenospermocarpy (Cain *et al.*, 1). The technique of embryo rescue employing *in-ovulo* culture has been adopted in grape breeding in western world and results show that a mean 85 per cent of the progenies from seedless × seedless crosses can be seedless. Furthermore, the role of embryo rescue is important particularly under subtropical conditions where 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. Therefore, an attempt has been made in this study to standardize *in ovulo* embryo rescue technique for grapes under subtropical conditions.

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MATERIALS AND METHODS

Following cultivars of grape vine 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 orchard of the Division of Fruits and Horticultural Technology, were selected for hybridization and work related to tissue culture was carried out at CTCL, IARI, New Delhi.

For hybridization of grape cultivars, a day before emasculation and pollination, the healthy panicles were selected and bagged with perforated butter paper bags. Emasculation was done on the following day from 7.00

to 10.00 a.m. in the flowers when calyptra (corolla) was detached easily followed by pollination. Flowers from male parents (Perlette and Pusa Seedless) at balloon stage were collected in petridishes and exposed to the sun for anthesis. The butter paper bags were removed after 4 days of pollination. Young developing immature fruitlets (berries) at 16, 20, 24 and 28 days after pollination (DAP) were collected for ovule excision. The fruitlets were washed in 0.1% liquid soap solution for 15 minutes followed by agitation in tap water and rinsed with distilled water before shifting to laminar-air flow. Surface sterilization was done with $HgCl_2$ (0.1%) for 10 min. followed by 3-4 washings with sterile distilled water. Using sterile scalpels and forceps, fruitlets were cut longitudinally and the ovules were excised without any damage and ovules were then inoculated on the establishment media. Care was taken to minimize damage to the ovule as it results in excessive phenolic exudation and following media namely, MS (1/2 macro + 1/1 micro), B5, and NN supplemented with various phytohormones (IAA, IBA, NAA and GA3) were used at different stages of *in ovulo* culture and regeneration of plantlet.

For culture establishment and maturation, medium comprising of MS (1/2 macro + 1/1 micro) basal medium and GA_3 (0.2 and 0.5 mg/l) + IAA (2.0, 4.0, 8.0 and 10.0 mg/l) along with 2% sucrose, 2 per cent activated charcoal (AC) and 0.8 per cent agar-agar were used. The germination medium was also same and after establishment ovules were then stratified (4^0C) for forty five and sixty days for maturation Rooting medium comprised of MS basal medium along with IBA (0.5, 1.0, 1.5 mg/l) or NAA (0.5, 1.0 and 1.5 mg/l) + 200 mg/l AC followed by hardening of rooted seedlings in glass jar filled with peat + soilrite (1:1) moistened with MS macro salts.

RESULTS AND DISCUSSION

Data on effect of ovule age at culturing on *in vitro* germination (%) have been portrayed in Table 1 and Fig. 2. It reflected that the maximum mean *in vitro* germination (%) was obtained in hybrid PU x PS (10.08) which turned out to be statistically significant over the values obtained in other hybrids, namely, BS x PS (8.50), BS x P (7.25) and PU x P (6.42). Observations on the effect of ovule age (days after pollination) on *in vitro* germination (%) showed that the maximum mean *in vitro* germination was seen when ovules were cultured at 28 days after pollination (12.67%) which was significant over the values obtained in other three stages, *viz.,* 16 days after pollination (3.08), 20 days after pollination (5.00) and 24 days after pollination (11.50). When the effect of interaction between S x H was taken into consideration

Table 1. Effect of ovule age at culture on *in vitro* germination (%).

Hybrid	Ovule age (days after pollination)				
	16	20	24	28	Mean
BS x PS	4.67 (12.43)*	5.67 (13.77)	12.33 (20.50)	11.33 (19.67)	8.50 (16.59)
BS x P	3.33 (10.37)	5.00 (12.87)	10.00 (18.37)	10.67 (19.03)	7.25 (15.16)
PU x PS	4.33 (11.97)	5.00 (12.87)	14.33 (22.23)	16.67 (24.10)	10.08 (17.79)
PU x P	0.00 (0.00)	4.33 (11.97)	9.33 (17.73)	12.00 (20.23)	6.42 (12.48)
Mean	3.08 (8.69)	5.00 (12.87)	11.50 (19.71)	12.67 (20.76)	
		CD at 5%			
Stage		1.1238			
Hybrid		1.1238			
S x H		2.2476			

it was found that the hybrid PU x PS at 28 DAP showed maximum germination (16.67) and the value was significantly superior over other duration and hybrid interactions.

Under *in vitro* conditions the ovules after isolation require simulated environment like maternal tissue (endosperm etc.) which nurture it until maturity. These specific requirements vary with genotype and stage of ovule taken for culture (Narayanaswamy, 4). During maturation the difference in the rate of growth of embryo may be due to the genotypes as the effect of maternal tissue, *i.e*. embryo-endosperm, interaction is very critical sometimes (Weaver and Pool, 14).

Data on effect of ovule age on *in vitro* ovule-embryo growth (mm^2) have been presented in Table 2 (Fig. 2). It showed that the maximum mean *in vitro* ovulo-embryo growth was seen in hybrid BS x PS (2.15) which was significant over the values obtained in other progenies, *viz*., BS x P (1.73) and PU x P (1.86). The maximum

Table 2. Effect of ovule age on *in vitro* ovule-embryo growth (mm^2).

Hybrid	Ovule age (days after pollination)				
	16	20	24	28	Mean
BS x PS	1.77	2.27	2.37	2.20	2.15
BS x P	1.57	1.83	1.90	1.60	1.73
PU x PS	1.73	2.20	2.27	2.33	2.13
PU x P	1.63	1.87	1.97	1.97	1.86
Mean	1.68	2.04	2.13	2.03	
		CD at 5%			
Stage		0.1451			
Hybrid		0.1451			
S x H		0.2902			

mean *in vitro* ovule- embryo growth was obtained when ovules were cultured at 24 days after pollination (2.13) stage, which was found statistically at par with 20 days after pollination (2.04) and 28 days after pollination (2.03) but statistically better over the value obtained when ovules were cultured at 16 days after pollination (1.68). When interaction between stage and hybrid was studied, it was found that the hybrid BS x PS at 24 DAP registered highest ovule growth (2.37) which was non-significantly followed by PU x PS (2.33) and BS x PS (2.27) at 28 and 20 DAP, respectively. In the present studies, the ideal sampling time for ovule culture ranges from 16 to 28 days post pollination. The result corroborates with findings of Singh and Brar (10). However, depending upon genotype, the ideal age for embryo rescue vary widely *viz.*, 14-101 days (Cain *et al.*, 1), 60-70 days (Spiegel-Roy *et al.*, 11), 40-60 days (Gray *et al.*, 3), 60-70 days (Emmershard *et al.*, 2), 52-66 days (Tsolova, 12), 60 days (Tsolova and Atanassov, 13). The above reports were mainly from the temperate region where growing season is longer compared to sub-tropical region.

The effect of different growth regulators concentrations (mg/l) on germination (%) as depicted in Table 3, revealed that the maximum mean germination was seen in hybrid PU x PS (14.10) which came out to be statistically at par with BS x PS (12.25) and BS x P (11.17) and statistically superior over hybrid PU x P (10.00). The concentration of IAA (4.0 mg/l) + GA_3 (0.5 mg/l) proved to be most effective for germination (13.84 %) but treatments IAA at 8 mg/l + GA_3 at 1.0 mg/l (13.17 %) and IAA at 10 mg/l + GA_3 at 1.5 mg/l (13.25 %) also found at par. When the interaction between growth regulator and hybrid was studied it was found that hybrid PU x PS (16.00%) on medium supplemented with IAA (4.0 mg/l) + GA_3 (0.5 mg/l) registered the highest germination. The effect of different growth regulators concentrations on ovule growth (mm^2) as shown in Table 4, reflected that the maximum mean ovule growth was obtained in hybrid PU x PS (2.20) which was statistically at par with the value obtained in BS x PS (2.14) and significant over the values seen in hybrids PU x P (1.84) and BS x P (1.91). The maximum ovule growth (2.27) was obtained with concentration of IAA (8.0 mg/l) + GA_3 (1.0 mg/l) which was statistically at par with the value obtained with IAA at 4mg/l + GA_3 at 0.5 mg/l (2.19), but significant over the value obtained with IAA at 2 mg/l + GA_3 at 0.2 mg/l (1.62).

Table 3. Effect of different growth regulator concentrations on embryo germination (%).

Hybrid	Growth regulators concentrations (mg/l)				
	IAA(2)+ GA_3(0.2)	IAA(4)+ GA_3(0.5)	IAA(8)+ GA_3(1.0)	IAA(10)+ GA_3(1.5)	Mean
BS x PS	9.33 (17.70)*	13.67 (21.60)	13.33 (21.40)	12.67 (20.83)	12.25 (20.38)
BS x P	6.33 (11.97)	13.00 (21.13)	12.33 (20.57)	13.00 (21.10)	11.17 (18.69)
PU x PS	10.00 (18.37)	16.00 (23.60)	15.33 (23.07)	15.00 (22.80)	14.10 (21.96)
PU x P	3.33 (6.13)	12.67 (20.83)	11.67 (20.00)	12.33 (20.53)	10.00 (16.88)
Mean	7.25 (13.54)	13.84 (21.79)	13.17 (21.26)	13.25 (21.32)	
		CD at 5%			
GR		3.2391			
Hybrid		3.2391			
GR x H		6.4781			

*Transformed values

Table 4. Effect of different growth regulator concentrations on ovule growth (mm^2).

Hybrid	Growth regulators concentrations (mg/l)*			
	IAA (2) + GA_3 (0.2)	IAA (4) + GA_3 (0.5)	IAA (8) + GA_3 (1.0)	Mean
BS x PS	1.67	2.37	2.40	2.14
BS x P	1.57	2.00	2.17	1.91
PU x PS	1.83	2.43	2.33	2.20
PU x P	1.40	1.97	2.17	1.84
Mean	1.62	2.19	2.27	
		CD at 5%		
GR		0.1354		
Hybrid		0.1563		
GR x H		0.2708		

* IAA(10 mg/l) + GA_3 (1.5 mg/l) = Callusing

The results of present investigation supported that the combined application of GA_3 and IAA significantly affected the establishment per cent and ovule growth of all hybrids. The treatment 0.5 mg/l GA_3 + 4.0 mg/l IAA gave the highest germination success in MS (1/2 macro + 1/1 micro) medium for all hybrids. The increased efficiency of IAA alone or along with in culture establishment of grape ovules has been reported by Tsolova (12), and Singh *et al.* (9) and it seems to have some synergistic interaction between IAA and GA_3 when used in combination. Data furnished by Table 5 exhibited the superiority of NN medium as far as culture establishment (%) was concerned (68.33), The B5 medium also produced results at par with the NN medium, however, MS (1/2 macro + 1/1 micro) was significantly inferior to the best medium with respect to culture establishment (66.67). Irrespective of various media tried, genotype BS x PS showed the highest culture establishment (68.66). Although, genotype BS x

Table 5. Effect of media composition on culture establishment (%).

Hybrid	MS (1/2 macro + 1/1 micro)	B_5	NN	Mean
BS x PS	67.67 (55.40)*	68.33 (55.80)	70.00 (56.40)	68.66 (55.87)
BS x P	70.00 (56.83)	65.66 (54.20)	69.00 (54.80)	68.22 (55.28)
PU x PS	64.00 (53.20)	67.00 (55.00)	67.66 (56.83)	66.22 (55.01)
PU x P	65.00 (53.80)	68.66 (56.00)	66.66 (56.23)	66.77 (55.34)
Mean	66.67 (54.81)	67.41 (55.25)	68.33 (56.07)	
		CD at 5%		
Medium		1.3789		
Hybrid		1.5922		
M x H		2.7578		

P (68.22) was at par to the best hybrid but genotypes PU x PS (66.22) and PU x P (66.77) were significantly lagging behind in culture establishment. While studying interaction between M x H it was found that ovules of BS x P on MS medium (1/2 macro + 1/1 micro) and BS x PS on NN medium showed highest culture establishment (70.00) and this was non-significantly followed by BS x P on NN medium (69.00). Regarding the effect of media composition over germination (%), as revealed by Table 6, ovules on MS medium (1/2 macro + 1/1 micro) exhibited highest germination (13.75) which

Table 6. Effect of media composition on value germination (%).

Hybrid	MS (1/2 macro + 1/1 micro)	B_5	NN	Mean
BS x PS	14.33 (22.27)*	12.67 (20.83)	13.67 (21.70)	13.56 (21.60)
BS x P	13.67 (21.70)	12.00 (20.30)	12.67 (20.83)	12.67 (20.94)
PU x PS	14.67 (22.53)	13.67 (21.70)	14.33 (22.27)	14.22 (22.17)
PU x P	12.33 (20.57)	11.33 (19.67)	12.00 (20.30)	11.89 (20.18)
Mean	13.75 (21.77)	12.42 (20.63)	13.17 (21.28)	
CD at 5%				
Medium		0.4343		
Hybrid		0.5015		
M x H		0.8686		

*Transformed values

was significantly higher than the ovules cultured on NN medium (13.17) and B5 medium (12.42). Among various genotypes, ovules of PU x PS (14.22) showed highest germination and was significantly better over other hybrid genotypes. While studying interaction between M x H, it was found that ovules of PU x PS on MS medium (1/2 macro + 1/1 micro) showed highest germination (14.67) and this was non-significantly followed by BS x PS with same medium (14.33) and PU x PS on NN medium (14.33). Out of three media and their modifications tested for ovule culture in grape, modified MS medium (1/2 macro + 1/1 micro) was ascertained most responsive followed by NN medium. Superiority of MS and NN media over other media for ovule/embryo culture has been observed by several workers (Gray *et al.,* 3; Tsolova, 12; Singh and Brar, 10; Norstog, 6).

Effect of chilling treatment duration on days to germination is evident in Table 7. Hybrid PU x PS was the best performer (87.17 days) and the result was significantly better over other hybrids as far as days to

Table 7. Effect of chilling treatment duration on germination (%).

Treatment	BS x PS	BS x P	PU x PS	PU x P	Mean
45 days	14.00 (21.87)*	13.00 (21.03)	14.67 (22.50)	13.33 (21.33)	13.75 (21.68)
60 days	16.00 (23.50)	13.67 (21.60)	16.33 (23.77)	14.00 (21.87)	15.00 (22.68)
Mean	15.00 (22.68)	13.34 (21.32)	15.50 (23.13)	13.67 (21.60)	
		CD at 5%			
Hybrid		3.285			
Treatment		2.3229			
H x T		4.6457			

germination was concerned. 45 days of chilling treatment took significantly lesser days to germinate if counted from days after inoculation (85.25). While studying interaction data, it was found that the hybrid PU x PS after 45 days of chilling treatment took minimum days to germinate (81.33), which was non-significantly followed by BS x PS at same chilling duration (84.67). Phenomenon of shoot bud sprouting was highly influenced by various growth regulators and their concentrations (Table 8). Shoot bud sprouting was earliest on medium supplemented with IBA at 1.0 mg/l (8.50 days), this value was significantly better over all concentration of NAA, however, ovules cultured on media supplemented with IBA at 0.5 mg/l and 1.5 mg/l took non-significantly higher number of days for shoot bud sprouting than the best one. Irrespective of growth regulators and their concentrations, hybrids BS x PS

Table 8 . Effect of growth regulators (mg/l) on shoot bud sprouting.

Treatment	Days to shoot bud sprouting				
	BS x PS	BS x P	PU x PS	PU x P	Mean
IBA (0.5)	9.00	9.33	8.67	11.00	9.50
IBA (1.0)	8.00	8.67	8.00	9.33	8.50
IBA (1.5)	8.33	8.67	8.33	9.67	8.75
NAA (0.5)	12.00	12.33	12.00	13.00	12.33
NAA (1.0)	11.33	12.00	11.67	12.33	11.83
NAA (1.5)	11.67	12.00	11.67	12.67	12.00
Mean	10.06	10.50	10.06	11.33	
		CD at 5%			
Hybrid		0.9062			
Treatment		1.1098			
H x T		2.2197			

and PU x PS were earliest to sprout (10.06 days) and took significantly lesser days to sprout than PU x P (11.33). Interaction studies between H x T revealed the superiority of PU x PS and BS x PS on medium supplemented with IBA at 1mg/l (8.00).

As shown in Table 9 (Fig. 2), the effect of growth regulators was very much evident on rooting of rescued plantlets. Micro-shoots on medium supplemented with IBA at 1 mg/l had taken significantly minimum days to root (6.50) as compared to other treatments. Hybrid BS x P took minimum number of days to root (8.39) as compared to the other hybrids. When interaction between T x H was taken into account, hybrid PU x P (6.00) and BS x P (6.33) on medium supplemented with IBA at 1.0 mg/l were found to be the best and gave at par results.

Data furnished by Table 10, shows the effect of different growth regulators on average root length (cm), the maximum average root length was seen in hybrid PU x P (2.59 cm) which was at par with the values

Table 9. Effect of growth regulators (mg/l) on rooting of micro-shoots.

Treatment	Days to rooting				
	BS x PS	BS x P	PU x PS	PU x P	Mean
IBA (0.5)	10.67	10.33	12.00	10.33	10.83
IBA (1.0)	7.00	6.33	6.67	6.00	6.50
IBA (1.5)	7.67	7.00	7.33	7.33	7.33
NAA (0.5)	12.00	11.67	13.07	11.33	12.02
NAA (1.0)	8.33	7.67	8.67	8.33	8.25
NAA (1.5)	8.67	7.33	8.33	8.33	8.17
Mean	9.06	8.39	9.34	8.61	
		CD at 5%			
Hybrid		0.6856			
Treatment		0.8397			
H x T		1.6794			

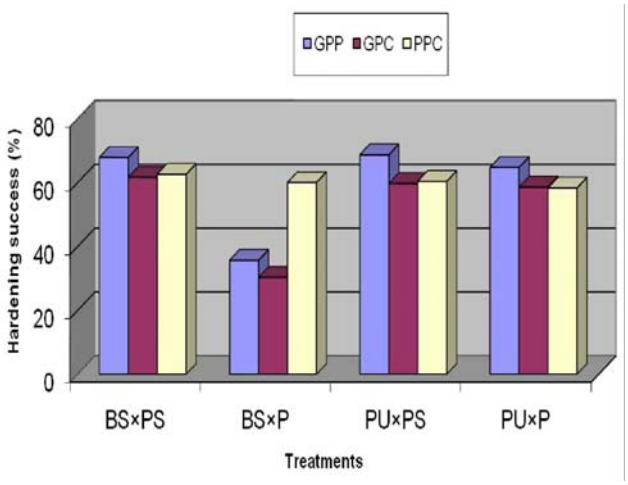

Fig. 1. Effect of different strategies on *in vitro* hardening of grape hybrid progenies.

Table 10. Effect of growth regulators (mg/l) on average shoots length.

Treatment	Average shoot length (cm)				
	BS x PS	BS x P	PU x PS	PU x P	Mean
IBA (0.5)	2.47	2.37	2.30	2.37	2.38
IBA (1.0)	2.77	2.73	2.77	2.80	2.77
IBA (1.5)	2.50	2.53	2.57	2.77	2.59
NAA (0.5)	2.40	2.37	2.30	2.37	2.36
NAA (1.0)	2.50	2.53	2.57	2.63	2.56
NAA (1.5)	2.63	2.67	2.63	2.60	2.63
Mean	2.54	2.53	2.52	2.59	
		CD at 5%			
Hybrid		0.1616			
Treatment		0.1979			
H x T		0.3957			

obtained in other hybrids, namely, BS x PS (2.54), PU x PS (2.52) and BS x P (2.53). With respect to the effect of different growth regulators, the maximum average root length was obtained with the application of IBA at 1.0 mg/l (2.77). The role of IBA and NAA in different rooting parameters in tissue culture was earlier reported by Singh *et al.* (7).

Fig. 1 & 2 represented different strategies adopted for enhancing hardening of *in vitro* raised plantlets; the maximum success was achieved by using glass jar with polypropylene cap (66.76 %), followed by plastic pot with polythene cover (60.35 %). Hardening success was highest in hybrid progeny of BS x PS (63.90) followed closely by PU x PS (62.95). Similar results were reported by Singh *et al.* (7), as the glass being transparent allowed early hardening, faster leaves expansion and early cuticle development.

Fig. 2. Different stages of *in ovulo* embryo rescue in sub-tropical grapes. (a)Cultured green ovules of hybrids, (b) Mature ovules after chilling treatment, (c) Germinated embryos, (d) Shoot elongation, (e) Rooted embryo rescued plantlets, (f) Hardening of rooted plants.

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Received: March, 2008; Revised: July, 2010
Accepted: August, 2010