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In vitro plant regeneration of grape cv. Perletle through axillary bud and shoot tip explants

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ABSTRACT

An efficient, rapid and reproducible *in vitro* plant regeneration protocol was developed for *Vitis vinifera* L. cv. Perletle using axillary bud and shoot tip explants on Murashige and Skoog (MS) medium fortified with different combination of cytokinins and auxins. The surface sterilization was done with 0.1% $HgCl_2$ 5-7 min. for getting pure cultures. The problem of browning was minimized by addition of ascorbic acid (50 mgl^{-1}) in to the culture medium. The best combination for fast and efficient proliferation and multiplication was MS medium supplemented with BAP 2.0 mgl^{-1} + NAA 0.2 mgl^{-1}, whereas the best rooting was obtained on ½ MS medium supplemented with IBA 2.0 mgl^{-1} + NAA 0.2 mgl^{-1} except root length, which was maximum on IBA 2 .0 mgl^{-1} + activated charcoal 200 mgl^{-1}. Regeneration potential of axillary bud was found better than shoot tip explant. The regenerated plantlets were successfully established in sterile + soilrite soil (1:1) for hardening.

Key words: *Vitis vinifera* L., *in vitro* plant regeneration, growth regulators, axillary bud, shoot tip.

INTRODUCTION

Grape is a perennial deciduous woody vine cultivated in the temperate, tropical and subtropical regions of the world. Micropropagation represents an efficient method of plant regeneration and rapid propagation through organogenesis and embryogenesis of any valuable genotype obtained by non-conventional methods (Mederos, 7; Stamp *et al.*, 16).

Although tissue culture protocols were available for grapes, the degree of response is highly dependent on the particular genotype, as various *Vitis* species, cultivars or hybrids respond differently to certain culture conditions (Qiu *et al.*, 11). Tissue culture of selected grape genotypes can be carried out by the culture of intact or fragmented shoot apical meristems (Gray and Fischer, 4), micro-cuttings (Barlass and Skene, 2) or through adventitious bud formation (Heloir *et al.*, 6). Thus, this study aims to develop an improved, efficient protocol for rapid multiplication of grape cv. Perlette.

MATERIALS AND METHODS

The study was carried out at Department of Horticulture, Institute of Agricultural Sciences, BHU, Varanasi. In this study, axillary bud and shoot tip explants were taken for culture. The explants were collected from field grown grapevines.The explants were washed thoroughly under running tap water for 30 min. followed by soaking in Teepol® (5%) for 5 min. to remove adherent dirt followed by rinsings with double-distilled water. The surface sterilization was done with $HgCl_2$ (0.1%) for 5-7 min. followed by 70% ethanol for 30 sec. under aseptic conditions. The explants were again washed 4-5 times with double-distilled water to remove every trace of the sterilant. The explants were then inoculated on to MS medium (Murashige and Skoog, 9) with 3% sucrose and 0.8% agar supplemented with different plant growth regulators. The pH was adjusted 5.8 before autoclaving of media containing vessels at 121^0C at 1.06 kg cm^{-2} for 20 min. The problem of phenolic browning was minimized by the addition of ascorbic acid (50 mg l^{-1}) into the culture medium.

The shoot proliferation experiment was conducted on MS medium supplemented with cytokinins (BAP and kinetin) and auxin (NAA) either singly or in combination and a set of basal MS medium without growth regulators served as control (Tables 1 & 2). The proliferated shoots were subcultured on the same fresh medium after every 4 weeks. The micro-shoots measuring 6-7 cm were transferred to half-strength MS medium supplemented with various combinations of auxins (IBA and NAA) with or without activated charcoal for rooting (Table 3). All the cultures were maintained at 25 ± 2°C with 16/8 h photoperiod at a photon flux intensity of 50-70 E m^2s^{-1}.

Plantlets with well developed shoot and roots were removed from the culture medium, washed gently under running tap water followed by washing with carbendazim (1%) solution and transferred to sterile soilrite: soil (1:1) mixture for hardening. The plantlets were incubated in culture room and were covered with transparent polythene bags to ensure high humidity

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Table 1. Effect of cyokinins and auxin on bud sprouting and days to shoot proliferation in grape.

Plant growth regulators (mg l^{-1})			Bud sprouting (%)		Days to shoot proliferation	
BAP	NAA	KIN	Axillary bud	Shoot tip	Axillary bud	Shoot tip
0.0	0.0	0.0	18.31 (25.39)^{*a}	15.97 23.61)^{*a}	28.32^{ij}	31.23^{jkl}
1.0	0.1	0.0	42.24 (40.63)^{ghi}	38.95 (38.71)^{hi}	23.59^{ef}	26.01^{fgh}
1.0	0.2	0.0	46.26 (42.86)^{ij}	42.56 (40.72)^{ij}	22.47^{def}	24.72^{efg}
1.5	0.1	0.0	48.24 (44.10)^{jk}	44.49 (41.94)^{jk}	20.94^{cde}	23.10^{def}
1.5	0.2	0.0	52.10 46.09)^{kl}	47.81 (43.64)^{kl}	19.03^{bc}	20.94^{bcd}
2.0	0.1	0.0	71.47 (57.86)^{o}	65.92 (54.42)^{o}	17.36a^{b}	19.14^{ab}
2.0	0.2	0.0	82.48 (65.42)^{p}	76.07 (60.86)^{p}	15.32^{a}	17.20^{a}
2.5	0.1	0.0	58.22 (49.73)^{mn}	53.56 (47.04)^{mn}	18.90^{bc}	20.84^{bcd}
2.5	0.2	0.0	61.28 (51.65)^{n}	56.52 (48.87)^{n}	22.09^{def}	19.26^{ab}
3.0	0.1	0.0	54.13 (47.37)^{lm}	49.80 (44.89)^{lm}	17.47^{ab}	24.30^{efg}
3.0	0.2	0.0	56.41 (48.68)^{lm}	52.03 (46.28)^{lmn}	21.88^{def}	24.07^{fg}
1.0	0.0	0.5	30.38 (33.45)^{b}	21.74 (27.79)^{b}	33.29^{l}	33.29^{l}
1.0	0.0	1.0	32.37 (34.68)^{bc}	24.36 (29.50)^{bc}	31.96^{kl}	31.96^{kl}
1.0	0.0	1.5	34.72 (36.10)^{bcd}	27.57 (31.68)^{cd}	30.65j^{kl}	30.65^{jkl}
1.0	0.0	2.0	36.64 (37.25)^{cdef}	30.28 (33.38)^{def}	29.20^{ijk}	29.20^{ijk}
1.5	0.0	0.5	39.66 (42.47)^{efgh}	34.62 (36.04)^{fgh}	26.67^{ghi}	26.67^{ghi}
1.5	0.0	1.0	45.59 (46.73)^{ij}	43.38 (41.19)^{ijk}	24.21^{fg}	24.21^{efg}
1.5	0.0	1.5	53.02 (41.41)^{l}	54.24 (47.43)^{mn}	19.90^{bcd}	19.90^{abc}
1.5	0.0	2.0	43.75 (39.39)^{hij}	40.65 (39.61)^{ij}	22.50d^{ef}	22.50^{cde}
2.0	0.0	0.5	40.27 (38.26)^{fgh}	35.52 (36.58)^{gh}	24.74^{fgh}	24.74^{efg}
2.0	0.0	1.0	38.35 (37.18)^{defg}	32.72 (34.79)^{efg}	27.22^{hi}	27.22^{ghi}
2.0	0.0	1.5	36.52 (36.27)^{cdef}	30.10 (33.27)^{def}	28.78i^{j}	28.78^{hij}
2.0	0.0	2.0	35.00 (36.27)^{cde}	27.96 (31.92)^{cde}	31.96^{kl}	31.96^{kl}

Means followed by the same letters within the columns are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

*Figures in parentheses show Arc Sine transformation value.

which were gradually perforated after 4 weeks and were removed after 8 weeks in order to acclimatize plants to field conditions. The plantlets were watered every 3-4 days with 1/10 of MS salt solution for two weeks during incubation.

All the experiments had four replicates per treatment and 20 units were maintained for each replication. The results were analyzed statistically using SPSS ver.16 (SPSS Inc., Chicago, IL., USA). The significance of differences among means was carried out using Duncan's Multiple Range Test at $P = 0.05$.

RESULTS AND DISCUSSION

Regeneration potential of axillary buds was found better over shoot tips. Explants remained fresh green and showed poor response of shoot initiation on growth regulator free MS medium (control). The phenolic browning was minimized by the addition of ascorbic acid (50 mg l^{-1}) into the medium. The occurrence of higher levels of polyphenols and tannins in grape has been reported by Roubelakis-Angelaris (13). Effect of different plant growth regulators supplemented to MS medium on bud sprouting and days to shoot proliferation. Both the explants cultured on to MS medium fortified with cytokinins alone or in combination (BAP + Kin) induced shoot proliferation to lesser extent as compared to combination of cytokinin and auxin (BAP + NAA) for per cent bud sprouting, days to shoot proliferation, number of shoots and shoot length. Treatment BAP 2.0 mgl^{-1} + NAA 0.2 mgl^{-1} showed the highest bud sprouting (82.48 & 76.07%), early bud break (15.32 & 17.20 days), maximum number of shoots (4.03 and 3.72), maximum shoot

Table 2. Effect of cyokinins and auxins on number of shoots and shoot length in grape.

Plant growth regulators (mgl^{-1})			No. of shoots/ explant		Shoot length (cm)	
BAP	NAA	KIN	Axillary bud	Shoot tip	Axillary bud	Shoot tip
0.0	0.0	0.0	1.03^{a}	1.01^{a}	1.36^{a}	1.09^{a}
1.0	0.1	0.0	2.47^{ij}	2.27^{ijk}	2.89^{efg}	2.37^{efg}
1.0	0.2	0.0	2.69^{j}	2.47^{kl}	3.29^{gh}	2.69^{ghi}
1.5	0.1	0.0	2.98^{k}	2.75^{mn}	3.68^{h}	3.02^{i}
1.5	0.2	0.0	3.08^{kl}	2.83^{mn}	4.37^{ij}	3.57^{j}
2.0	0.1	0.0	3.72^{m}	3.43^{p}	6.10^{l}	5.01^{n}
2.0	0.2	0.0	4.03^{n}	3.72^{q}	6.78^{m}	5.57^{o}
2.5	0.1	0.0	3.13^{kl}	2.89^{no}	5.34^{k}	4.40^{lm}
2.5	0.2	0.0	3.35^{l}	3.09^{o}	5.64^{k}	4.64^{m}
3.0	0.1	0.0	2.39^{hi}	2.20^{hijk}	4.36^{ij}	3.58^{j}
3.0	0.2	0.0	2.49^{ij}	2.29^{jk}	4.60^{j}	3.78^{jk}
1.0	0.0	0.5	$1.17a^{b}$	1.17^{ab}	1.61^{ab}	1.61^{b}
1.0	0.0	1.0	1.38^{bc}	1.38^{bc}	1.81^{bc}	1.81^{bc}
1.0	0.0	1.5	1.58^{cd}	1.58^{cd}	1.94^{bc}	1.94^{bcd}
1.0	0.0	2.0	1.73^{def}	1.73^{def}	2.21^{cd}	2.21^{def}
1.5	0.0	0.5	2.01^{fg}	2.01^{ghi}	2.77^{ef}	2.77^{hi}
1.5	0.0	1.0	2.18^{gh}	2.18^{hij}	3.46^{h}	3.46^{j}
1.5	0.0	1.5	2.59^{ij}	2.59^{lm}	4.56^{j}	4.56^{m}
1.5	0.0	2.0	2.39^{hi}	2.39^{jkl}	4.10^{i}	4.10^{kl}
2.0	0.0	0.5	1.99^{fg}	1.99^{fgh}	$2.96f^{g}$	2.96^{i}
2.0	0.0	1.0	1.91^{efg}	1.91^{efg}	2.48^{de}	2.48^{fgh}
2.0	0.0	1.5	1.83^{def}	1.83^{defg}	2.20^{cd}	2.20^{def}
2.0	0.0	2.0	1.66^{de}	1.66^{de}	2.05^{cd}	2.05^{cde}

Means followed by the same letters within the columns are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

length (6.78 and 5.57 cm) in axillary bud and shoot tip explants, respectively (Tables 1 & 2; Fig. 1). Cytokinin BAP has been reported the most commonly used cytokinin in grape tissue culture (Thies and Graves, 18; Mhatre *et al*., 8; Singh *et al*., 16). The beneficial effect of BAP on multiple shoots induction in grape has been reported by Alizadeh *et al.* (1). The axillary bud was found better explant than shoot tip might be due to better status of endogenous food reserves and hormones (Hartmann and Kester, 5; Sundarsono and Goldy, 17). This may also be due relatively less injury to the tissues by sterilizing agents coupled with less inhibitory effects of lower phenolic exudates from axillary buds. The regeneration response declined with increased cytokinin concentration beyond the optimal level in both the explants. BAP has been known for breaking apical dominance and enhancement of axillary branching, but higher levels may sometimes suppress growth and is even toxic to the tissues (Blomstdt *et al.*, 3).

The role of BAP in combination of NAA for shoot multiplication in different grape rootstocks has been reported by Alizadeh *et al.* (6). Auxins do not promote shoot proliferation but are required in culture medium to promote growth of shoots by counteracting suppressive effect of high cytokinins.The combination IBA 2.0 mgl^{-1} + NAA 0.2 mgl^{-1} in half-strength MS medium recorded the highest rooting (92.71%), number of roots (9.12) with early root initiation in 14.65 days (Table 3; Fig. 1). The higher rooting in dual auxin treatment may be attributed to synergistic effect of both the auxins (Rana and Singh, 12). In grape, addition of low level of auxins has been reported for *in vitro* rooting (Torregrossa and Boquet, 19; Mhatre *et al*., 8). The root length was maximum (9.05 cm) on ½ MS supplemented with IBA 2.0 mgl^{-1} + Activated

Charcoal (AC) 200 mgl^{-1} (Table 3; Fig. 1). Activated charcoal alone or in combination with IBA did not improve rooting frequency of *Vitis ficifolia* var. *ganebu* and its inter-specific hybrid but significantly increased root length in both the genotypes (Poudel *et al.*, 10). Auxin IBA is the most effective in most of the cases apparently because it is not being destroyed by IAA oxidase or other enzymes.

The leaves of *in vitro* raised plants are generally photosynthetically not competent because they are

Table 3. Effect of auxins and activated charcoal on *in vitro* rooting in grape.

Treatment (mg l^{-1})		Activated charcoal (mgl^{-1})	Days to root initiation (days)	Rooting (%)	Root length (cm)	No. of roots per shoot
IBA	NAA					
0.0	0.0	0.0	28.12^{g}	22.32 (28.26)*^{a}	2.25^{a}	1.13^{a}
1.0	0.1	0.0	19.19^{def}	63.37 (52.89)^{b}	4.12^{b}	4.29^{c}
1.0	0.2	0.0	18.34^{cde}	68.14 (55.50)^{bcd}	4.23^{b}	5.11^{d}
1.5	0.1	0.0	17.69^{bcde}	72.48 (58.51)^{cd}	4.46^{b}	5.24^{d}
1.5	0.2	0.0	17.11^{bcd}	76.41 (61.09)^{de}	4.57^{bc}	6.28^{e}
2.0	0.1	0.0	15.86^{ab}	86.27 (68.59)^{fg}	5.19^{cd}	7.44^{f}
2.0	0.2	0.0	14.65^{a}	92.71 (74.52)^{g}	6.37^{f}	9.12^{g}
1.0	0.0	150	21.11^{f}	60.84 (51.26)^{b}	5.64^{de}	2.92^{b}
1.0	0.0	200	19.81^{ef}	65.44 (54.00)^{bc}	5.92^{ef}	3.47^{b}
1.5	0.0	150	19.46^{def}	69.58 (56.53)^{bcd}	6.15^{ef}	3.41^{b}
1.5	0.0	200	18.48^{cde}	74.12 (59.42)^{cd}	6.44^{f}	4.08^{c}
2.0	0.0	150	17.45^{bcde}	83.68 (66.17)^{ef}	7.27^{g}	4.46^{c}
2.0	0.0	200	16.12^{abc}	90.86 (72.40)^{fg}	9.05^{h}	5.47^{d}

Means followed by the same letters within the columns are not significantly different (P = 0.05) using Duncan's Multiple Range Test; *Figures in parentheses show Arc Sine transformation value

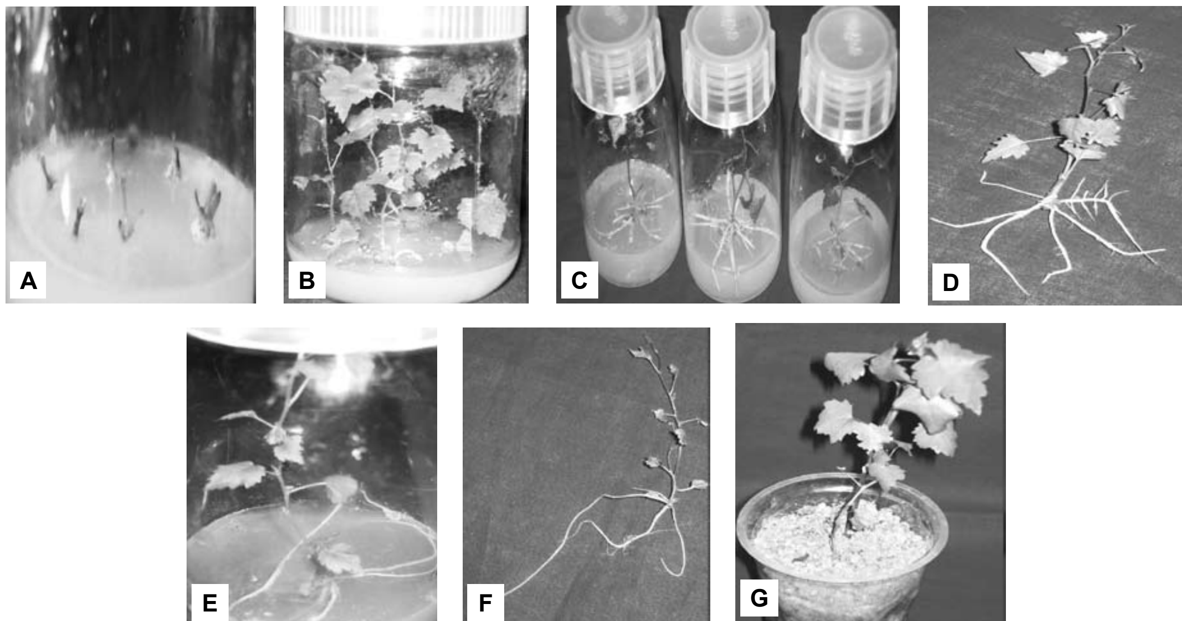

Fig. 1. *In vitro* plant regeneration in *Vitis vinifera* L. cv. Perlette.
(A) Sprouting of shoots on MS + BAP 2.0 mgl^{-1} + NAA 0.2 mgl^{-1}. (B) Elongation of shoots on MS + BAP 2.0 mgl^{-1} + NAA 0.2 mgl^{-1}. (C&D) *In vitro* rooting on ½ MS + IBA 2.0 mgl^{-1} + NAA 0.2 mgl^{-1}. (E&F) *In vitro* rooting on ½ MS + IBA 2.0 mgl^{-1} + AC 200 mgl^{-1}, and (G) Acclimatized plant in soilrite: soil (1:1).

growing in sucrose rich medium but are active in transpiration and respiration, which may be the reason for low establishment of plantlets during hardening (Singh and Pandey, 14). The rooted plantlets were successfully hardened inside the culture room in sterile soilrite: soil (1:1). There was no detectable variation among the potted plants with respect to morphological and growth characteristics. The protocol described here is efficient, reproducibleand provide a rapid technique for mass multiplication of this potential grape variety.

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