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Comparative performance of *in vitro* multiplication in four grape (*Vitis* spp.) rootstock genotypes

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Abstract

The magnitude of demand for planting materials in grape, mainly for rootstock genotypes indicates that micropropagation is inevitably necessary for their mass scale propagation. Therefore, the studies on micropropagation of four genetically different grape rootstocks namely Dogridge (*Vitis champini*), SO4 (*V. riparia*× *V. berlandieri*), H-144 (*V. vinifera* × *V. labrusca*) and 3309 C (*V. riparia* × *V. rupestris*) were conducted to develop an optimized protocol and to compare *in vitro* behavior of these genotypes. Culture establishment using nodal segements was enhanced using different growth regulators. Though culture establishment increased using either BAP (Benzyl amino purine) or KIN (Kinetin) but the treatment, 2.0 mgl^{-1} BAP + 0.2 mgl^{-1} NAA (Naphthalene acetic acid) was most effective with regard to enhancement in culture establishment and reduction in time to bud sprouting. Least success (38.31%) in culture establishment was observed for H-144 but it exhibited better vegetative growth and rooting among genotypes, i.e. higher shoot multiplication rate (12 micro-cuttings per culture), highest rooting (87.7%) and early root initiation (11.52 days). Addition of activated charcoal to the rooting medium was found beneficial with respect to enhancement of rooting and minimizing time to root initiation in different genotypes. Among the rootstock genotypes, 3309 C was found most responsive in terms of higher *ex vitro* plantlet survival (84.95%) during hardening and shorter duration required for *ex vitro* transfer. These results indicate that multiplication of these grape rootstocks can be performed efficiently by means of direct shoot proliferation using nodal segments from field grown vines. The influence of different factors like culture medium and genotype on the overall micropropagation of grape rootstocks is discussed.

Keywords: Grapevine; *In vitro*; Micropropagation; Rootstocks; *Vitis*

Introduction

From commercial viticulture perspective, grafting on a proper rootstock is a standard vineyard practice in most parts of the world. The majority of commercial plantations presently evade direct planting of selected grape varieties due to susceptibility of cultivated varieties to pests, i.e. microbes, mites, insects, nematodes and more importantly *Phylloxera,* leading to decline. The key for successful commercial cultivation is to focus on

accessibility to suitable rootstock planting materials in large numbers. Micropropagtion is an important alternative to conventional methods of plant propagation. Indeed, the order of magnitude in demand for planting materials indicates that micropropagation will inevitably be necessary for mass propagation in different horticultural crops. Besides generating uniform clonal rootstocks within a short time independent of season, the technique also has value in application of other tissue culture based techniques, i.e. induced mutation, *in vitro* screening, genetic engineering and germplasm exchange (Webster, 1995). The use of *in vitro* techniques for propagation of various *Vitis vinifera* cultivars has been well documented (Chee and Pool, 1982; Reisch, 1986; Singh et al., 2000; Mhatre et al., 2000; Singh et al., 2004). Protocols have also been reported for muscadine grape (Lee and Wetzstein, 1990; Gray and Benton, 1991; Sudarsono and Goldy, 1991; Thies and Graves, 1992; Torregrosa and Bouquet, 1995; Roubelakis-Angelakis, 2001) and some wild grapes (Poudel et al., 2005; Zhang et al., 2006). It is evident that there is little empirical data on the *in vitro* cloning and performance of several new grape rootstocks. Hence, in the present study, we examined the *in vitro* behavior of various *Vitis* rootstocks during different micropropagation stages and multiplication protocols and different media factors were optimized for four diverse rootstock genotypes.

Materials and Methods

Selection and preparation of the mother plants

Four grape rootstocks of different genetic origin namely, Dogridge (*Vitis champini*), SO4 (*V. riparia* × *V. berlandieri*), H-144 (*V. vinifera* × *V. labrusca*) and 3309 C (*V. riparia* × *V. rupestris*) were selected for the present study. The canes for explants were collected from the five years old vines maintained at Grape Germplasm Block, Main Experimental Orchard, IARI, New Delhi, India, where the genotypes have been maintained in big sized cement pots (50 × 50 × 45 cm). The stock plants were hand sprayed with Carbendazim at the rate of 2 g l^{-1} thrice at three-day intervals, prior to explant collection to reduce microbial load and likely internal infections.

Initiation of aseptic culture

In vitro performance of different grape genotypes was examined following direct shoot regeneration from single node stem micro-cuttings. Newly emerged, just mature canes (about 2-month-old) were collected during April and immediately transferred to laboratory in polybags. Shoots were further dissected to remove leaves and/or tendrils and made into nodal segments (1.5 to 2 cm long each containing a single axillary bud). The segments situated on the middle portion of the shoot were selected, i.e. above the second to third node from the base and below forth to fifth node from tip portion. These were washed thoroughly with normal tap water (30 min.) and agitated (1h) in a solution of 200 mgl^{-1} 8-hydroxy quinnoline citrate (8-HQC) and 2 gl^{-1} Carbendazim on a horizontal shaker (120 rpm) followed by rinsing with autoclaved doubled-distilled water. These pre-treated explants were then subjected to $HgCl_2$ solution (0.1%) for 7 min. followed by 70% ethyl alcohol for 5 sec. inside a laminar air-flow hood. Segments were washed in sterile double-distilled

water for at least three times and inoculated individually in test tubes (150 × 25 mm) on solid MS (Murashige and Skoog, 1962) medium supplemented with Benzyl amino Purine (BAP) or Kinetin (KIN) (2.0 or 4.0 mgl^{-1}) either singly or in combination with low concentration of NAA (Naphthalene acetic acid) (0.2 mgl^{-1}). The inoculated tubes were incubated at 25 ± 1 °C with 16/8 h light and dark photoperiod from cool white fluorescent tubes (47 µmol $m^{-2}s^{-1}$).

Shoot multiplication and rooting

Two-node micro-shoots procured from sprouted explants were then excised and multiplied on proliferation *cum* rooting medium comprising full-strength MS medium supplemented with 2.0 or 4.0 mgl^{-1} indole-3-butyric acid (IBA) with or without 200 mgl^{-1} activated charcoal (AC). Cultures were continuously maintained during entire experimental period to produce large number of plants for hardening or additional studies. Different media (culture initiation and proliferation) were solidified using agar-agar (8 gl^{-1}, Qualigens Fine Chemicals, Mumbai) with pH adjusted at 5.8 prior to autoclaving (1.05 $kgcm^{-2}$ for 20 min.).

Acclimatization and ex vitro transfer

The rooted plantlets, 3 weeks after inoculation on rooting medium (Figure 3 b), were subjected to hardening using two strategies, i.e. glass jars with polypropylene (PP) caps or plastic pots with polythene cover. Medium for both hardening methods was a pre-sterilized mixture of peat: vermiculite: perlite (2:1:1) moistened with half-strength MS inorganic salts. The rooted plantlets were washed in sterile double-distilled water containing 0.1% Carbendazim and then transferred individually to each container. Cultures were kept under controlled photoperiod and temperature condition as previously described for nodal cultures. During 3rd week the PP caps were loosened and gradually removed or the polythene covers punctured to reduce the humidity. During this stage the plantlets were irrigated regularly with one-forth MS salts solution minus organics. Hardened plants were then transferred to plastic pots filled with sand, soil and F.Y.M (Farm Yard Manure) (2:1:1) in a glasshouse during 6-8th week of hardening depending up on the genotype and hardening strategy.

Experimental design

All the sub-experiments were conducted using 45 units (each explant was considered as a single unit) per treatment and the same was repeated three times to confirm the findings. The percentage data was transformed using angular transformation (Arc Sin√%) before carrying out ANOVA.

Results and Discussion

The *in vitro* multiplication protocol applied to these four grapevine rootstock genotypes showed different levels of success in the present study. The explants obtained from middle portion of cane was found most responsive materials for culture initiation because those procured from tip portion got easily injured during surface sterilization and others situated on the basal region showed poor or delayed bud sprouting (data not shown). Culture establishment was enhanced using $HgCl_2$ either singly or in combination of 70% ethyl alcohol. For surface sterilization treatment, the best combination was found to be 0.1% $HgCl_2$ for 7 min. followed by quick dip ethyl alcohol for 5 sec. When treatment duration exceeded 7 min. the culture establishment declined mainly owing to explant necrosis (data not shown). Effect of different plant growth regulators supplemented to MS medium on culture establishment and time to bud sprouting is shown in Table 1. Though culture establishment was enhanced using BAP alone or with NAA in combination as compared to KIN but among the different combinations tried, 2.0 mgl^{-1} BAP + 0.2 mgl^{-1} NAA showed better response (Table 1). It enhanced culture establishment and minimized the time to bud sprouting. Benzyl amino purine has been the most commonly used cytokinin in grape tissue culture media (Chee and Pool, 1982; Harris and Stevenson, 1982; Reisch, 1986; Martinez and Tizio, 1989; Thies and Graves, 1992; Mhatre et al., 2000; Singh et al., 2004). In all genotypes BAP treatments either singly or in combination with a low level of NAA registered better response than individual or combined kinetin treatments (Table 1). Reisch (1986) reported a small but significant negative response with regard to shoot bio-mass by increasing the kinetin levels in 'Concord' variety but shoot proliferation rate did not vary significantly to the treatments. Combination of 1 mgl^{-1} BAP + 0.5 mgl^{-1} GA_3 was found most useful for *in vitro* culturability of seven different grape varieties (Martinez and Tizio, 1989). In contrary to our results, Poudel et al. (2005) reported the effectiveness of KIN on culture establishment of two wild grapes. This may be due to difference in genotypes utilized; because different plant growth regulators at varying concentrations show considerable variability for *in vitro* regeneration of different species or cultivars. Plant growth regulator (s) effective for one species may not be equally effective for another cultivar or species (Poudel et al., 2005; Singh et al., 2004).

The genotype 3309 C showed the highest culture establishment (54.5%) followed by Dogridge (45.02%) and SO_4 (40.12%). The genotype H-144 was found to give poor response in this regard and only (38.31%) culture establishment could be achieved. On the other hand, established cultures of this genotype also showed late bud sprouting (15.76 days) compared to other three genotypes, while, Dogridge was found to give early bud sprouting and during first week of inoculation more than 70% of the explants sprouted (Table 1).

When the sprouts attained 3.0 cm length (at least two nodes), they were excised from original explant and sub-cultured onto proliferation *cum* rooting medium. Prolonged culture on proliferation medium led to high incidence of microbial contamination and in media where 4 mgl^{-1} BAP was supplemented, occurrence of hyperhydricity (vitrification) was common e.g. in SO4 genotype (Figure 1). This phenomenon has already been reported by some workers (Morini et al., 1985; Heloir et al., 1997) and it was found to appear mainly during proliferation phase. Furthermore, they have stressed that low level of BAP

concentration was necessary to maintain a good proliferation rate and also to reduce the incidence of vitrification. Recently, Lai et al. (2005) reported that the hyperhydricity in shoot cultures of *Scrophularia* could be minimized by progressively ventilating the vessels, i.e. Parafilm® sealing without affecting sterile conditions. In our study, the culture vessels were sealed with cotton plugs, where sufficient gas exchange can be one of the reasons to minimize this phenomenon. Few cultures of SO4 which showed symptoms of vitrification (data not shown) were immediately sub-cultured on to proliferation cum rooting medium which recovered within 14 days after sub-culture.

Table 1. Effect of different plant growth regulators on culture establishment and time to bud sprouting in four grape rootstock genotypes.

Treatment(mgl^{-1})	Culture establishment (%)					Days to bud sprouting				
	Dogridge	SO4	H-144	3309C	Mean	Dogridge	SO4	H-144	3309C	Mean
2.0 BAP	36.0 (36.8)*	34.7 (36.0)*	31.6 (34.2)*	50.5 (45.2)*	38.2 (38.1)*	8.7	9.1	16.4	9.2	10.85
4.0 BAP	50.1 (45.0)	41.8 (40.2)	42.6 (40.7)	65.4 (53.9)	49.9 (44.6)	9.4	8.4	15.1	6.5	9.85
2.0 BAP+0.2 NAA	68.7 (55.9)	49.0 (44.4)	38.3 (38.2)	82.2 (65.0)	59.5 (38.9)	6.3	7.8	14.3	7.5	9.22
4.0 BAP+0.2 NAA	53.4 (46.9)	42.3 (40.5)	41.2 (39.9)	78.7 (62.5)	53.9 (47.4)	6.7	8.4	16.1	11.4	10.65
2.0 KIN	32.4 (34.7)	36.7 (37.2)	34.1 (35.7)	29.6 (32.9)	33.2 (35.1)	7.3	9.2	15.8	12.4	11.17
4.0 KIN	30.8 (33.7)	41.7 (40.2)	36.7 (37.2)	31.6 (34.2)	35.2 (36.3)	8.6	8.1	17.2	12.2	11.52
2.0 KIN+0.2 NAA	43.5 (41.2)	46.7 (43.1)	40.8 (39.7)	49.7 (44.8)	45.1 (42.2)	9.6	8.4	14.8	12.5	11.32
4.0 KIN+0.2 NAA	45.3 (42.3)	39.6 (39.0)	41.2 (39.9)	48.3 (44.0)	43.6 (41.2)	10.1	9.6	16.4	13.8	12.47
Mean	45.02 (42.0)	41.5 (40.1)	38.31 (38.0)	54.5 (47.8)		8.46	8.62	15.76	10.68	
			CD at 5%					CD at 5%		
Treatment(T)			1.10					1.18		
Genotype(G)			1.64					1.66		
T×G			3.29					3.33		

*Values in parentheses show ArcSin √% transformed data.

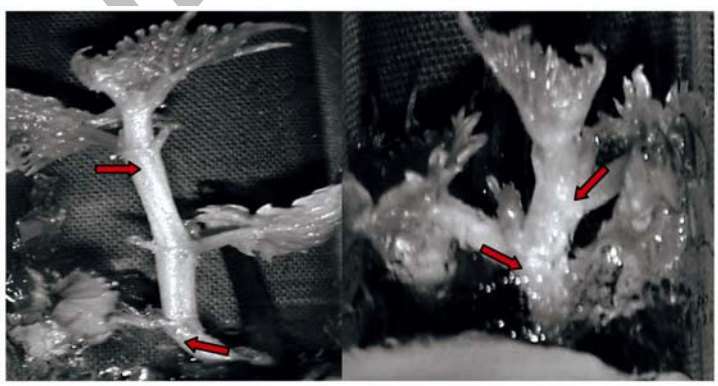

Figure 1. Vitification (Hyperhydricity) in SO4 genotype micro-shoots appeared on MS medium supplemented with 4.0 mgl^{-1} BAP.

The multiplication rate (average number of micro-cuttings recovered per sub-culture) was monitored during eight successive sub-cultures in healthy and established cultures. Lower multiplication rate was observed till 3rd sub-culture which gradually increased thereafter in all the genotypes. Rootstock H-144 yielded higher number of micro-cuttings per subculture (10 to 12) followed by Dogridge (8 to 10). The other two genotypes produced 6 to 8 micro-cuttings per sub-culture concurrently (Table 2).

Table 2. Average number of micro-cuttings recovered per sub-culture (Multiplication rate) in four grape rootstock genotypes.

Genotype	Sub-culture No.							
	1st	2nd	3rd	4th	5th	6th	7th	8th
Dogridge	3-4 (3.5)*	5-7 (6.0)	5-7 (6.0)	5-7 (6.0)	8-10 (9.0)	8-10 (7.0)	8-10 (9.0)	8-10 (9.0)
SO4	3-4 (3.5)	4-5 (4.5)	5-7 (6.0)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)
H-144	5-6 (5.5)	5-6 (5.5)	8-10 (9.0)	8-10 (9.0)	8-10 (9.0)	11-13 (12.0)	11-13 (12.0)	11-13 (12.0)
3309C	2-3 (2.5)	3-4 (3.5)	3-4 (3.5)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)	6-8 (7.0)

*Values in parentheses show average data.

Despite lower success achieved for culture establishment in H-144, its growth (Table 2) and rooting performance were found to be considerably better during multiplication phase (Table 3 and Figure 2). This genotype showed highest rooting (87.7%) and also the higher number of roots per explant (data not shown) which were higher than all genotypes (Figure 2). This response was considered as a desirable behavior and as a result *ex vitro* survival of tissue culture raised plantlets was enhanced. Early root initiation was registered in H-144 (11.52) and 3309 C (10.58) micro-shoots that was significantly different from other two genotypes.

Table 3. Effect of IBA and AC on time taken to root initiation and rooting in four grape rootstock genotypes in four grape rootstock genotypes.

Treatment	Days to root initiation					Rooting (%)				
Genotype	Dogridge	SO4	H-44	3309C	Mean	Dogridge	SO4	H-144	3309C	Mean
Control	24.1	26.1	20.4	16.1	21.67	85.0 (67.2)*	44.8 (42.0)*	69.4 (56.4)*	64.8 (53.6)*	60.0 (31.1)*
IBA (2 mgl^{-1})	11.0	12.3	8.6	9.3	10.30	76.8 (61.2)	55.3 (48.0)	98.2 (82.2)	76.2 (60.8)	78.6 (64.5)
IBA (4 mgl^{-1})	16.4	14.2	12.6	11.2	14.10	97.8 (81.4)	69.4 (56.4)	80.4 (63.7)	72.3 (58.2)	74.7 (59.0)
IBA(2 mgl^{-1})+AC (200 mgl^{-1})	9.6	10.4	7.8	8.2	9.00	86.8 (68.7)	94.6 (76.5)	99.0 (84.2)	84.2 (66.5)	93.9 (77.2)
IBA(4 mgl^{-1})+AC (200 mgl^{-1})	10.1	9.6	8.2	8.1	9.00	85.0 (67.2)	86.7 (68.6)	91.8 (73.3)	80.4 (63.7)	86.4 (68.5)
Mean	14.24	14.72	11.52	10.58		81.4 (65.9)	70.1 (58.5)	87.7 (71.3)	75.6 (60.5)	
Treatment (T)			CD at 5% 1.65					CD at 5% 1.96		
Genotype (G)			1.47					2.20		
T×G			3.30					4.40		

*Values in parentheses show ArcSin √% transformed data.

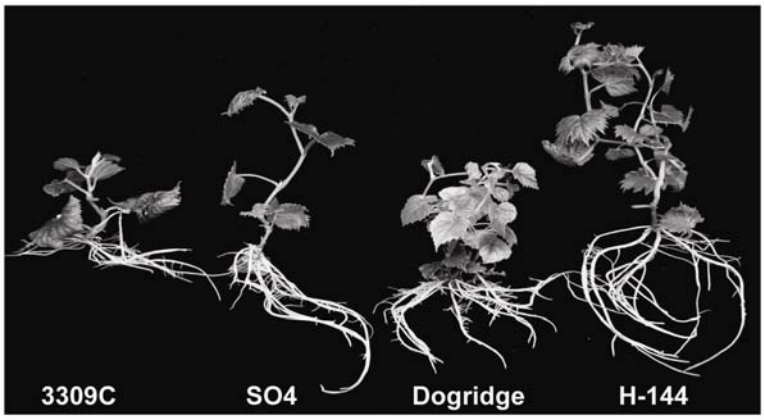

Figure 2. Comparative vegetative growth and rooting of different grape rootstock genotypes on MS medium supplemented with 2.0 mg l^{-1} IBA and 200 mg l^{-1} activated charcoal (45 days after inoculation).

The genotype SO4 did not respond in the rooting stage. Besides, there was delayed root initiation (14.72 days) (Table 3) and less number of roots per explant in all treatments (data not shown). Some of the explants, though remained green, failed to initiate root during the entire evaluation period. The genotypes Dogridge and 3309 C registered high rooting percentages (81.4% and 75.6% respectively) and were categorized in the intermediate rank. Since auxin stimulates root initiation but may inhibit subsequent root growth (George, 1993), the appropriate concentration is critical which helps in better rooting.

During the course of *in vitro* growth and development, plant tissues not only deplete the nutrients that are supplemented to the medium, but also release substances that can accumulate in the cultures. These substances, such as phenols, may have detrimental physiological effects on the cultured tissues. Like other woody species, grapevine tissues exhibit high levels of polyphenols and tannins (Roubelakis-Angelakis, 2001). In the present study, activated charcoal was incorporated into the culture initiation as well as rooting medium to minimize such problems. Addition of 200 mgl^{-1} activated charcoal to the IBA supplemented medium improved rooting (Table 3).

Activated charcoal alone or with IBA did not improve rooting frequency of *Vitis ficifolia* var. *ganebu* and its interspecific hybrid but significantly increased root length in both the genotypes (Poudel et al., 2005). Addition of activated charcoal in the rooting medium of *Pinus pinaster* improved the overall rooting capacity of mature explants to an average of 78% (Dumas and Monteuuis, 1995). Similarly, IBA in combination with activated charcoal was found to induce rooting faster than activated charcoal alone in banana (Aziz et al., 1992). The addition of activated charcoal enhanced root development, particularly root branching in walnut plantlets (Tang et al., 2000). Contrary to its effect on rooting performance of explants, addition of charcoal to the proliferation medium was not advantageous in case of SO4 genotype as some of the explants though remained alive for a long period but did not proliferate. Hence, the charcoal concentration when reduced to half (100 mgl^{-1}) resumed shoot proliferation. Apart from activated charcoal and IBA treatments, this genotype was considered as poor performer in terms of rooting parameters compared to

other rootstocks utilized in this investigation (Figure 2). Application of activated charcoal was also beneficial for inducing early rooting, easy and early detection of contaminated cultures since its addition to the medium yield a dark color in contrast to fungi or bacterial colonies.

In vitro raised plantlets were successfully transferred to the glasshouse after a short period of acclimatization. Use of glass jars with PP caps was clearly better strategy as compared to plastic pots with polythene cover. *In vitro* hardening of H-144 plantlets using glass jars is presented in Figure 3 (a-h). Highest level of success was achieved with 3309 C plantlets inoculated in glass jars (Table 4). This genotype showed the highest plantlet survival (89.65%) compared to other genotypes. Plantlets of H-144 could be transferred to glasshouse in a shorter duration (42.8 days). Similar strategy has earlier been reported by Singh et al. (2004) for *Vitis vinifera* cultivars. However, they used a mixture of peat: soilrite (1:1) moistened with ¼ strength MS salts minus organics.

Table 4. Effect of different hardening strategies on plantlet survival and time taken to glasshouse transfer in four grape rootstock genotypes

Treatment	Plantlet survival (%)					Days taken to *ex vitro* transfer				
	Dogridge	SO4	H-144	3309C	Mean	Dogridge	SO4	H-144	3309C	Mean
Glass jar with PP cap	72.35 (58.24)*	78.27 (62.17)*	87.75 (69.47)*	89.65 (71.19)*	82.00 (64.90)*	48.5	52.5	42.8	46.7	45.1
Plastic pots with polythene cover	62.31 (52.12)	68.00 (55.55)	73.44 (58.95)	80.25 (63.58)	71.00 (57.42)	58.2	60.4	46.7	52.8	52.1
Mean	67.33 (55.12)	73.13 (58.76)	80.59 (63.79)	84.95 (67.13)		53.3	56.4	44.75	50.2	
Treatment (T)	CD at 5% 8.04					CD at 5% 6.41				
Genotype (G)	8.04					6.57				
T×G	17.02					13.25				

* Values in parentheses show ArcSin √% transformed data

Lower plantlet survival was noted in plastic pots used for hardening; besides, delayed *ex vitro* transfer by about one week. Most of hardening techniques are based on controlling the ambiance of the developing plantlets in terms of humidity, temperature and nutrition in a pre-sterilized potting medium (Bhojwani and Razdan, 1992).

The variability observed in *in vitro* performance of the four grape rootstocks indicates that the efficiency of *in vitro* multiplication technique is strongly genotype dependent, allowing the selection of genotypes with high performance. In the present study, H-144 genotype had the highest multiplication rate. Therefore, the overall results indicate that multiplication of these grape rootstocks can be performed efficiently by means of direct shoot proliferation using nodal segments from field grown vines on a commercial scale.

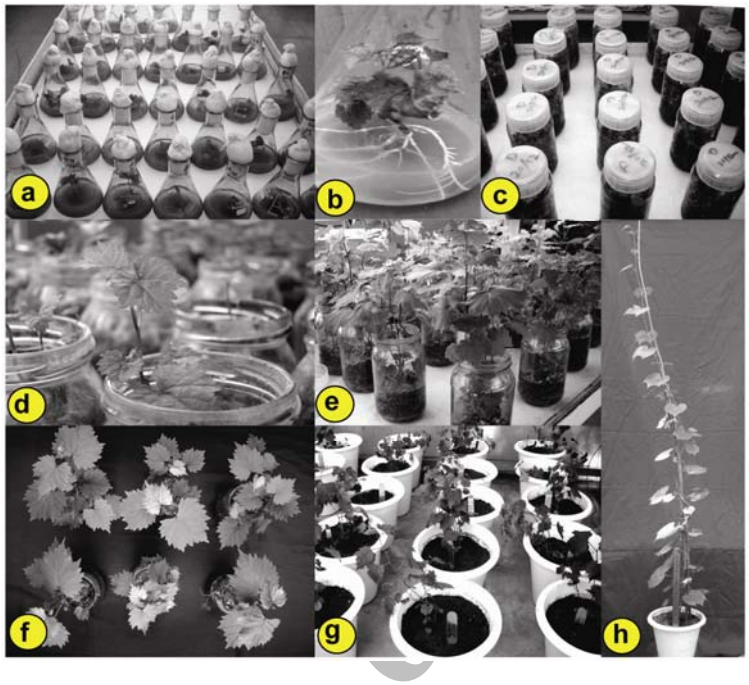

Figure 3. Rooting and acclimatization of H-144 plantlets using glass jars with PP caps. (a) Plantlets sub-cultured on rooting medium, (b) a rooted plantlet 3 weeks after inoculation on MS medium supplemented with 2.0 mgl^{-1} IBA + 200 mgl^{-1} AC, (c) Glass jars containing plantlets incubated in culture room, (d) PP caps removed, (e, f) Hardened plantlets ready for *ex vitro* transfer, (g) Potted plantlets in glasshouse, (h) A potted plantlets 90 days after acclimatization.

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