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In vitro Clonal Multiplication of Two Grape (*Vitis* spp.) Rootstock Genotypes

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Abstract

Single node segments were used to initiate *in vitro* cultures in two grape rootstocks namely, Dogridge (*Vitis champini*) and H-144 (*Vitis vinifera* × *V. labrusca*). Culture establishment was enhanced using different growth regulators, while BAP was found essential for culture initiation in both genotypes. Less success (38.31%) was obtained in culture establishment of H-144 but it exhibited better vegetative growth and rooting and *ex vitro* performance as compared to Dogridge. Higher shoot multiplication rate (12 micro-cuttings per culture) was recorded in H-144 while only 9 micro-cuttings per subculture were registered in Dogridge. Addition of activated charcoal to the rooting medium was found beneficial with enhancement of rooting and reduction in time to root initiation in both genotypes. The results suggested that multiplication of these two grape rootstock genotypes can be carried out efficiently by means of direct *in vitro* regeneration using nodal segments. *In vitro* performance of these two genotypes was also compared during different stages of micropropagation.

Introduction

Modern fruit growing creates an ever-increasing demand for new cultivars and rootstocks. The importance of rootstocks is widely recognized, which in terms of their influence on yield and productivity are not less important than the grafted scions. On the other hand, a large number of commercial micropropagation ventures were reported so far all over the world (Lakshmi et al. 1982, Shekhawat et al. 1998) and several of them have targeted rootstocks owing to robustness of this technique (Webster 1995). Besides mass multiplication and production of highly uniform plantlets, it allows screening genotypes for various *in vitro* induced stresses which are also vital aspects. The potential for integration of

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micropropagation to viticulture is numerous. Several reports have elucidated micropropagation of *V. vinifera* (Chee and Pool 1982, Reisch 1986, Singh et al. 2004) as well as muscadine grapes (Lee and Wetzstein 1990, Gray and Benton 1991, Sudarsono and Goldy 1991, Thies and Graves 1992, Torregrosa and Bouquet 1995, Roubelakis-Angelakis 2001). However, different species may vary in their performance under *in vitro* conditions (Poudel et al. 2005, Hassan and Zayed 2018). Thus the protocol developed for one cultivar or species may not be equally applicable to another cultivar or species (San Perdro et al. 2017). Hence, present authors studied the *in vitro* culture ability and micropropagtion of two diverse grape rootstock genotypes.

Materials and Methods

The field-grown, 5-year-old mother plants of the two grape rootstocks, *viz.* Dogridge (*Vitis champini*) and H-144 (*Vitis vinifera* × *V. labrusca*) were selected from the Main Orchard, Division of Fruits and Horticultural Technology, IARI, New Delhi. Besides proper maintenance, the mother plants were sprayed thrice with carbendazim (Bavistin® @ 2 g/l) at three days intervals prior to explant collection.

In vitro culture was initiated using single-node segments (1.5 - 2 cm long) procured from fresh mature canes in the month of March. The explants were thoroughly washed with tap water (30 min) and agitated in a solution of carbendazim (Bavistin® @ 2 g/l) along with 8-HQC (200 mg/l) for 1 hr. These were then transferred to laminar air-flow hood, where surface sterilization was done using $HgCl_2$ (0.1 %) for 7 min followed by at least three times rinsing with autoclaved double distilled water. Explants were then inoculated in test tubes (25 × 150 mm) containing culture initiation medium. MS was used as basal medium for culture initiation as well as shoot proliferation/rooting medium. A combination of BAP (2.0 - 4.0 mg/1), Kin (2.0 - 4.0 mg/1) individually or along with 0.2 mg/1 NAA was used for culture initiation medium. However, shoot proliferation/rooting medium was supplemented with IBA (2.0 or 4.0 mg/1) either singly or in combination with activated charcoal (200 mg/l). All media used in the study were supplemented with 30 g/1 sucrose and solidified with 8.0 g/1 Agar-Agar. The pH was adjusted to 5.8 prior to autoclaving (1.05 kg/cm^2 for 15 min.)

Shoots developed on established cultures were excised from original explants as two-node micro-cuttings and inoculated either for rooting or further proliferation.

The *in vitro* rooted 21-day-old plantlets were transferred to glass jars with polypropylene cap containing coco peat: perlite: vermiculite (2: 1: 1). The potting

mixture was moistened with half strength of MS macro- and micronutrients devoid of organics and subjected to autoclaving prior to plantlet transfer. The glass jars were then shifted to cool white fluorescent lights (227 µmol/m^2/sec) with controlled photoperiod (16/8 hrs) and 26 ± 1^0C) temperature. The polypropylene caps were loosened gradually after three weeks and finally removed completely. The growing plantlets were then misted with sterile distilled water containing 0.1% carbendazim (w/v) at regular intervals. One hardened plantlets were transferred to *ex vitro* glasshouse conditions during 6 to 7th week after transfer. Plantlets cultured in plastic pots were filled with sand: soil: FYM (2: 1: 1) and irrigated with normal tap water. In addition, the experimental plantlets were weekly sprayed with one fourth strength MS salts solution (pH 5.8).

Results and Discussion

The present investigation was carried out *in vitro* multiplication of two grape rootstock genotypes through nodal segments following direct regeneration pathway. The effect of different plant growth regulators on culture establishment and time taken to bud sprouting is presented in Table 1. Culture initiation was achieved successfully for both genotypes but the results were less satisfactory for H-144 genotype and about 60% of its explants died during the first two weeks after inoculation. Some explants remained green but without bud sprouting for long period, which coincided with medium dehydration or incidence of microbial contamination. It can be attributed to the morphology of axillary buds in H-144 genotype which is bigger in size compared to Dogridge. This situation may lead to getting higher injury during surface sterilization with $HgCl_2$. However, the genotypic effect also can be considered as one of the reasons in grape micropropagation studies (Chee and Pool 1983, Novak and Juvova 1982/83, Singh et al. 2004, Poudel et al. 2005). Culture establishment was enhanced using different growth regulators and BAP was found essential for culture initiation in both genotypes. Though, BAP at higher concentration (4 mg/l) enhanced culture establishment and bud sprouting of this genotype but compared to Dogridge the success was still lower. BAP has been reported to be effective in enhancing axillary bud proliferation in several *Euvitis* species with optimum levels of PGR between 5 and 10 µM (Goussard 1981, Gray and Fisher 1985, Harris and Stevenson 1982, Reisch 1986).

Besides good culture establishment, Dogridge showed early bud sprouting (Fig. 1) and more than 70% explants showed sprouting within the first week following inoculation (data not shown). Data (Table 1) suggest that MS supplemented with BAP (2.0 mg/1) along with 0.2 mg/1 NAA was the best

combination in order to achieve a higher culture establishment and minimum time to bud sprouting. In this treatment Dogridge sprouted significantly earlier (6.3 days) compared to H-144 in which sprouting took 14.3 days.

Table 1. Effect of different growth regulators on culture initiation in two grape rootstock genotypes.

Treatment (mg/l)	Culture establishment (%)			Time taken to bud sprouting (days)		
Genotype	Dogridge	H-144	Mean	Dogridge	H-144	Mean
BAP (2.0)	36.0 (36.87)*	31.6 (34.20)*	33.8 (35.55)*	8.7	16.4	12.55
BAP (4.0)	50.1 (45.00)	42.6 (40.74)	46.35 (42.88)	9.4	15.1	12.25
BAP (2.0) + NAA (0.2)	68.7 (55.98)	38.3 (38.23)	53.5 (47.01)	6.3	14.3	10.3
BAP (4.0) + NAA (0.2)	53.4 (46.95)	41.2 (39.93)	47.3 (43.45)	6.7	16.1	11.4
Kin (2.0)	32.4 (34.70)	34.1 (35.73)	33.25 (35.18)	7.3	15.8	11.55
Kin (4.0)	30.8 (33.71)	36.7 (37.29)	33.75 (35.4)	8.6	17.2	12.9
Kin (2.0) + NAA (0.2)	43.5 (41.27)	40.8 (39.70)	42.15 (40.46)	9.6	14.8	12.2
Kin (4.0) + NAA (0.2)	45.3 (42.30)	41.2 (39.93)	43.25 (41.09)	10.1	16.4	13.25
Mean	45.02 (42.05)	38.31 (38.05)		8.46	15.76	
Treatment (T)		CD at 5%	1.10			0.40
Genotype (G)			1.64			0.57
T × G			3.29			1.15

* ArcSin √ % transformed values.

Application of Kin for culture establishment was also studied (Table 1). Two genotypes exhibited shoddier response to Kin than BAP. Kin (2 mg/l) gave minimum culture establishment (34.1%) in H-144 genotype, but interestingly, high culture establishment (40.8%) was achieved using the same treatment along with NAA (0.2 mg/l). These results suggest that irrespective of the cytokinin source, addition of low level of auxin is necessary in order to attain a higher culture establishment. This essential role of NAA was evident in all treatment combinations.

Table 2. Effect of IBA and activated charcoal on rooting of grape micro-shoots.

Treatment (mg/l)	Rooting (%)			Time to root initiation (days)			Number of roots per microshoot		
Genotype	Dogridge	H-144	Mean	Dogridge	H-144	Mean	Dogridge	H-144	Mean
Control	61.0 (51.35)*	69.4 (56.42)*	65.2 (53.85)*	24.1	20.4	22.25	2.1	4.1	3.1
IBA (2.0)	85.0 (67.21)	98.2 (82.29)	91.6 (73.15)	11.0	8.6	9.8	6.1	12.6	9.35
IBA (4.0)	76.8 (61.21)	80.4 (63.72)	78.6 (62.44)	16.4	12.6	14.5	4.1	10.1	7.1
IBA (2.0) + AC (200)	97.8 (81.47)	99.0 (84.26)	98.4 (82.73)	9.6	7.8	8.7	4.4	11.5	7.95
IBA (4.0) + AC (200)	86.8 (68.70)	91.8 (73.36)	89.3 (70.91)	10.1	8.2	9.15	5.4	9.8	7.6
Mean	81.4 (65.90)	87.7 (71.30)		14.2	11.5		4.4	9.6	
Treatment (T)	CD at 5%		1.96			1.65			0.80
Genotype (G)			2.20			1.47			0.72
T × G			4.40			3.30			1.61

*ArcSin √ % transformed values.

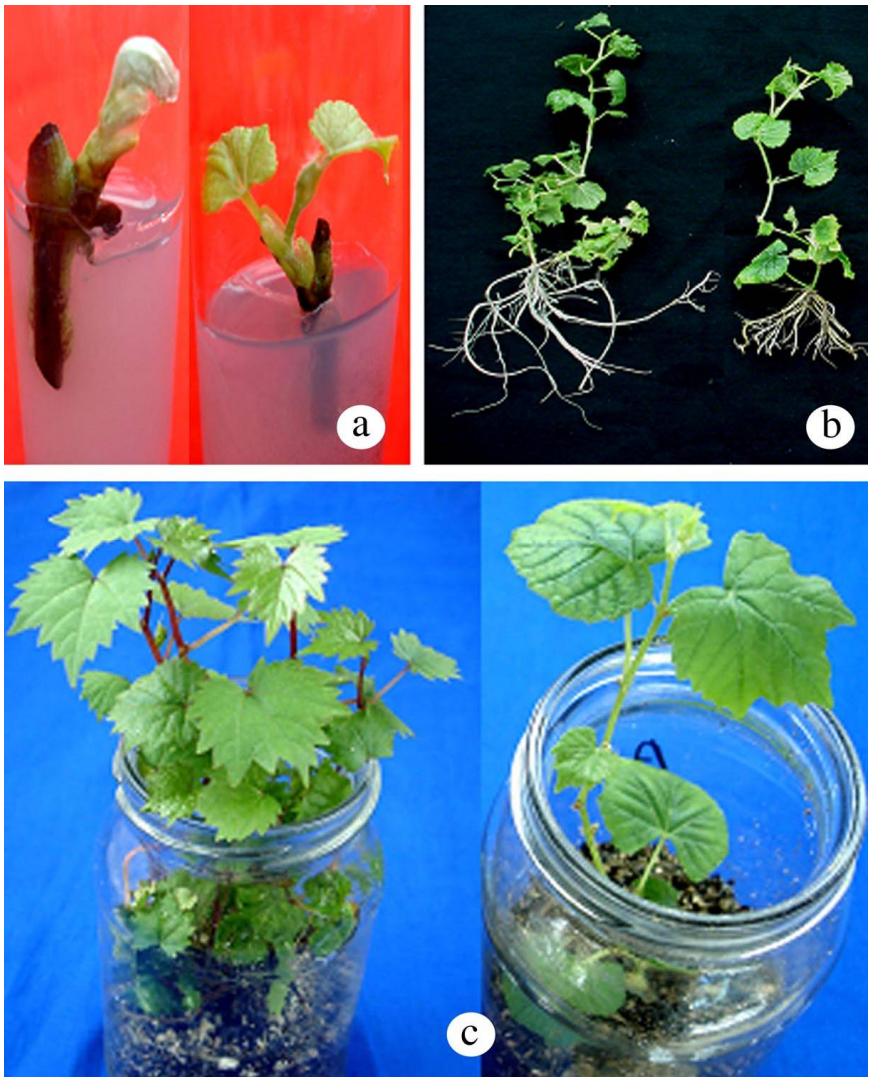

Fig. 1. *In vitro* performance of H-144 (left) and Dogridge genotypes during different stages of micropropagation. (a) Culture initiation 20 DAI (days after inoculation), (b) Rooting and shoot proliferation 35 DAI and (c) *In vitro* hardening 45 DAI.

The effectiveness of Kin for culture establishment of grapevine has been previously reported from Japan (Poudel et al. 2005). They demonstrated that Kin was more effective than 2-ip and BAP for culture establishment in a wild grape genotype. However, earlier studies recommended BAP as an effective cytokinin for grapevine culture establishment (Novak and Juvova 1982/83, Singh et al. 2004). Sudarsono and Goldy (1991) on the other hand, reported that thidiazuron (TDZ) alone or in combination with BA or Kin was effective for *in vitro* culture

establishment in *Vitis rotundifolia.* Present experiments suggest that low concentrations (2 mg/l) of BAP or Kin may be used for culture initiation of these two grape rootstocks. However, an addition of low level of NAA (0.2 mg/l) is indispensable.

When single-node microcuttings were used for subsequent subculturing, the rate of mortality and explant injury during transfer to fresh medium increased. Besides, more time was needed for bud sprouting as well as root initiation (data not shown). Therefore, to avoid these problems, two-node microcuttings were used either for rooting or shoot proliferation throughout the study.

IBA has been reported to have favoured root induction in several species including kiwifruit (Monette 1986), walnut (Tang et al. 2000) and grape (Mhatre et al. 2000, Singh et al. 2004, Barreto and Nookaraju 2007). Two IBA concentrations (2 and 4 mg/l) used in this study, differed in their effects on the rooting parameters. Both concentrations alone or in combination with activated charcoal (200 mg/l) significantly enhanced the rooting percentage of the two grape genotypes as compared to the hormone-free medium.

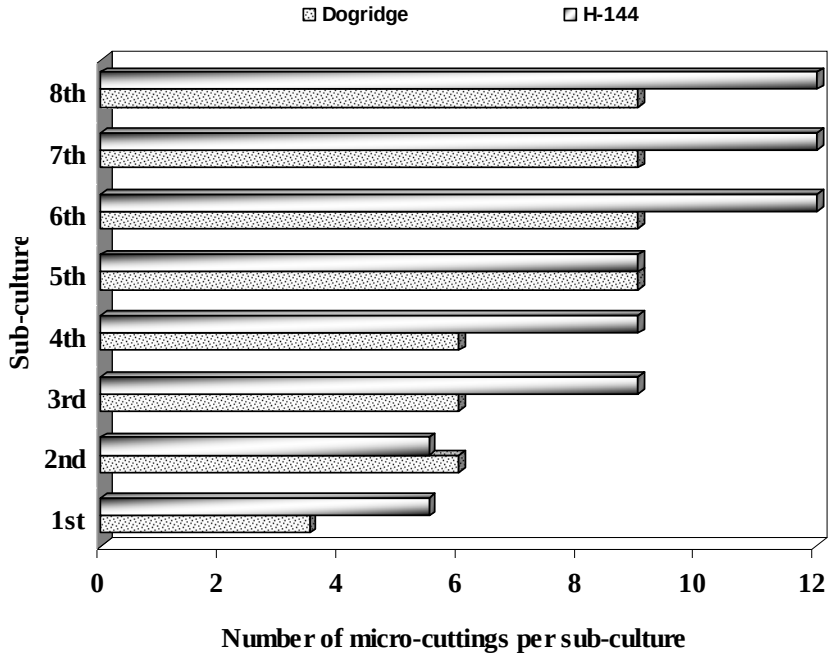

Fig. 2. Average number of microcuttings recovered per subculture (Multiplication rate).

Despite low response in culture establishment of H-144, once the culture is established vegetative growth and rooting were found superior to Dogridge (Fig. 1). The genotype showed early root initiation (11.5 days), also the mean number

of roots per inoculated micro-shoot was recorded higher (9.6) than Dogridge (4.4) (Table 2). That was considered as a desirable character especially to attain a higher plantlet survival during *ex vitro* transfer or hardening stage.

Addition of activated charcoal was found beneficial in order to enhance rooting ability. Media supplemented with activated charcoal showed higher rooting for both the genotypes (Table 2). Since activated charcoal leads to darkening of the media (Proskauer and Berman 1970), it provides an environment conducive to the accumulation of photo-sensitive auxin or cofactors (Druart et al. 1982). Some reports have also emphasized that activated charcoal absorbs agar impurities (Kohlenbach and Wernicke 1978) and phenols (Johnsson 1983) and as a result induces root initiation. In the present study, the main objective for integration of activated charcoal to the medium was minimization of phenolics exuded from grapevine tissues to avoid media browning or necrosis of explants. However, stimulative effect of this compound on rooting can be considered as a positive point in micropropagation of woody species including grapevine.

Cobalt chloride, activated charcoal and PG (1, 3, 5-trihydroxy benzene) along with IBA treatment had beneficial effect on rooting of *in vitro*-derived shoots of *Decalepis hamiltonii.* These two compounds were found useful for hardening and survival of *in vitro*-derived plants upon transfer to field (Reddy et al. 2001).

The average number of microcuttings recovered per subculture in the two genotypes increased gradually during eight successive subcultures (Fig. 2). The H-144 plantlets showed a higher vegetative growth and as a result **a** higher number of microcuttings (11 to 13) were recovered as compared to Dogridge (8 - 10). The number of microcuttings produced per subculture became stabilized following 3rd subculture and was found to be almost constant after 5th subculture.

Poor acclimation and establishment of plantlets in the greenhouse has been a hindrance noticeable complication toward commercial production of grapevine (Swartz and Lindstrom 1986). In this study, both the genotypes were satisfactorily transferred to the glasshouse using glass jars with polypropylene cap (Fig. 1). However, the level of success was higher in H-144 genotype and 87.75% of the plantlets survived. The survival rate of Dogridge plantlets was lower (72.35%) and more time was also needed for plantlets in *ex vitro* transfer, i.e. 45.8 days. Recently, similar strategy has been used for acclimation of two *V. vinifera* grape varieties (Singh et al. 2004). They observed that tissue culture raised plantlets grew vigorously compared to plants derived from cuttings. It may be due to the carryover effect of plant growth regulators.

Monitoring vegetative growth on 60 days after transfer to glasshouse, demonstrated that micropropagated plantlets of H-144 attained significantly longer vine length and a greater number of leaves even under glasshouse conditions (Table 3).

Table 3. Hardening of grape plantlets using glass jars with polypropylene cap.

Genotypes	Plantlet survival (%)	Time to *ex vitro* transfer (day)	Mean vine length (cm)**	No. of leaves**
Dogridge	72.35 (58.24)*	45.8	23.3	26.8
H-144	87.75 (69.47)	42.8	18.3	19.6
Mean	80.05 (63.44)	44.3	20.8	23.2
CD at 5%	5.82	2.51	1.92	2.67

*ArcSin √ % transformed values. **sixty days after transfer to glasshouse.

The results suggested that multiplication of these two grape rootstocks can be carried out efficiently by means of direct shoot proliferation using nodal segments from field grown vines. The procedure can be used for other tissue culture based techniques, i.e. micrografting, *in vitro* screening for different biotic and abiotic stresses, induced *in vitro* mutation studies, etc. Furthermore, commercial application of this technique would aid in providing a large number of healthy rootstocks for grafting any desired scion variety.

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