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Standardization of micropropagation protocol for grape rootstock Dog Ridge

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ABSTRACT

In the present study, attempt was made to standardize *in vitro* micropropagation protocol of grape rootstock Dog Ridge. Among two explants compared (axillary bud and shoot tip), axillary buds registered superior shoot proliferation response (75.81%) compared to shoot tips (40.38%) during January to March in Coimbatore conditions. Full strength Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) 2.0 mg l^{-1} produced the highest bud sprouting response of explants. Addition of 0.2 mg l^{-1} 1-Naphthaleneacetic acid (NAA) along with BAP 2.0 mg l^{-1} was found effective for producing maximum number of lengthier multiple shoots within short time and MS medium fortified with BAP 2.0 mg l^{-1} and gibberellic acid (GA_3) 0.5 mg l^{-1} resulted in elongated shoots. Half-strength MS medium with 2.0 mg l^{-1} Indole-3 butyric acid (IBA) and 200 mg l^{-1} activated charcoal resulted in the maximum *in vitro* root induction of 83.00% within 11.74 days. For hardening of *in vitro* rooted plantlets, a combination of pot mix (1:1:1 of sand +soil +farm yard manure) and vermicompost in 1:1 proportion was found to be optimum (86.67% *ex vitro* survival).

Key words: *Vitis champini,* shoot, proliferation, rooting, acclimatization.

INTRODUCTION

Significance of rootstock as a 'hidden half' to protect against biotic and abiotic stresses along with influence on canopy and fruit quality associated traits in grapes is a well acknowledged fact worldwide. Additional bottlenecks including increasing soil linked problems like salinity, drought and nematode infestation as well as poor fruitfulness of varieties has pressurized the requirement of ideal rootstocks in Indian viticulture. Consequently, several rootstock species and their hybrids of *Vitis rupestris*, *V. riparia*, *V. berlandieri*, *V. champini* and *V. vinifera* were introduced to Indian vineyards and widely used for grape cultivation under salinity and drought prone areas (Chadha and Sikhamany, 4). Among these rootstocks, Dog Ridge, a seedling selection from *Vitis champini* has adapted well to Indian soils and offered tremendous potential as a hardy rootstock for one of the most economical grape variety, namely, Thompson Seedless. Due to its adaptability in marginal saline soils and ability to induce high vigour in the grafted vine, this rootstock aids in production of quality fruit under sandy and nematode infested soils. However, some of the intrinsic traits of Dog Ridge such as high polyphenol content in the cuttings have severely hampered root formation in semi hardwood cuttings, making its commercial multiplication very cumbersome. Moreover, delayed attainment of suitable grafting thickness with slow rate of multiplication poses an additional problem in commercial exploitation of this potential rootstock. *In vitro* micropropagation, a biotechnological opportunity can serve as an alternative. Further, a reliable and reproducible protocol is required for conducting additional research on thrust area of *in vitro* grafting involving this rootstock species in future.

In vitro regeneration of different grape rootstocks has been attempted in recent past, but availability of explant and the requirement of growth regulator combinations may vary from each species according to its growing environment (Alizadeh *et al*., 1). Keeping these issues in consideration, an attempt was made in the present study to standardize micropropagation protocol for Dog Ridge rootstock. The main objectives were identification of the most viable explant and time of explant collection, optimization of culture media with growth regulator requirements for *in vitro* regeneration, multiplication and rooting followed by acclimatization of *in vitro* derived plantlets.

MATERIALS AND METHODS

Healthy, three-year-old mother plants of Dog Ridge rootstock maintained in the propagation chamber at the College Orchard, Tamil Nadu Agricultural University, Coimbatore were used for the study. The explants were collected from mother plant by excising newly emerged two to six-week-old shoots containing dormant buds throughout different seasons by pruning the matured wood. Immediately, the collected shoots were taken to the tissue culture laboratory in clean polyethylene bags and washed under running tap water. The leaves and other unwanted parts in the collected shoots were removed

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and the single nodal axillary bud (2 cm long) and shoot tip (1-2 cm long) explants were prepared by trimming with sterile blades. Then the explants were washed under running tap water thoroughly. The washed explants were taken in sterile bottles containing two to three ml of Teepol® in 100 ml of distilled water and constantly stirred for 10 min. followed by rinsing thrice with distilled water. The remaining surface sterilization steps were followed according to previously standardized surface sterilization protocol of Theivanai *et al*. (15). MS medium with sucrose as carbon source and agar as the gelling agent was employed for studying the *in vitro* response. After adding required stocks and growth regulators in 600 ml of distilled water the final volume of 1 litre was made and pH was adjusted between 5.6-5.8. Prepared media was dispersed into test tubes and sterilized at 15 psi with 121°C temperature for 20 min. IBA is thermo liable and hence it was filter sterilized by passing through 0.2 µm Millipore filters and added to the media in laminar air flow chamber after it had cooled down to lukewarm temperature following autoclaving. The explants after sterilization were inoculated on MS medium fortified with different growth regulator combinations to find their effect on shoot regeneration, proliferation and multiplication. The cultures were maintained at temperature of 25 ± 2°C and 70 to 80% relative humidity. White fluorescent lamps were used as light source with the intensity of 2500 to 3000 Lux for 16 h light and 8 h darkness period. Different combinations of growth regulators, *viz.,* cytokinin alone (BAP 1 and 2 mg l^{-1}) or in combination with auxin (NAA 0.1 and 0.2 mg l^{-1}) were used. Subculturing was done on to fresh medium with similar composition to facilitate shoot proliferation after three weeks of initial culture. During subculture, apical portions of shoots were removed to arrest apical dominance and facilitate induction of multiple shoots. For elongation, individual shoots were transferred to elongation media containing the best treatment of shoot regeneration and proliferation experiment with GA_3 at different concentrations (0.25, 0.50, 0.75 and 1.00 mg l^{-1}). Elongated shoots from both explants were transferred to rooting media. Half strength MS (½ MS) medium supplemented with IBA alone (1, 2 and 3 mg l^{-1}) or IBA with activated charcoal (200 mg L^{-1}) was used for root induction. The rooted plantlets were carefully taken out of the rooting medium without causing any damage to the roots and after washing them free of the adhering agar, they were planted in micro pots filled with different kinds of hardening media as listed in Table 3.

The data obtained from the various experiments were subjected to statistical analysis as per the standard procedures of Panse and Sukhatme (11). The experiment was laid out as completely randomized block design. Observations recorded as percentage were subjected to angular transformation (Snedecor and Cochran, 14). Analysis was carried out with AGRES software package.

RESULTS AND DISCUSSION

Standardization of *in vitro* mass multiplication protocol for Dog Ridge rootstock was carried out using pre-existing active meristem containing explants namely axillary buds and shoot tips in order to avoid genetic variation. Since the institute is situated under mild tropical climate, getting explant was not a serious concern except in winter months (mid November to December), during which Dog Ridge vines were under physiological dormancy. New growth was continuously obtained throughout the year except winter by regular pruning of selected branches of the mother plant. Among the different seasons, young shoots collected between second week of January to last week of March gave early response upon culturing besides proliferated well. Explants collected during the remaining seasons took longer time to respond. Further, growth and multiplication vary even under similar culture conditions and media composition (data not included). Work conducted at IARI, New Delhi with axillary buds of different rootstocks from north western India recorded better response during April (Alizadeh *et al*., 1). This dissimilarity may be due to the prevailing agro climatic variations. The early dormancy breaking under south Indian conditions compared to north India may be attributed to the milder winter prevalent in South India.

The data and results presented in this study represent *in vitro* culture response of Dog Ridge explants during January to March. Among the explants, axillary buds proved better and required lesser number of days for bud break than the time taken by shoot tips to put forth new growth. This may be due to the injury caused by the surface sterilization chemicals on the soft and tender dividing tissues of shoot tips than the well organized, hardy and dormant axillary buds. Full strength MS medium was used for shoot proliferation, multiplication and elongation and half strength MS medium was used for *in vitro* rooting. The positive response of Dog Ridge rootstock to *in vitro* regeneration may be attributed to the higher salt concentration in MS medium. Several earlier works on successful micropropagation of different grape cultivars with MS medium supported the present findings (Chowdhury *et al*., 5).

Data on the effect of growth regulators on days taken for positive response indicated that full strength MS medium supplemented with BAP 2.0 mg l^{-1} took the least number of days required for positive response

(3.77 days for bud break in axillary buds and 11.91 days new growth in shoot tips) and also produced more number of leaves in axillary buds (5.00) and shoot tips (4.33), compared to all other treatments (Tables 1& 2). These results are in agreement with the findings of Alizadeh *et al*. (1) in grape rootstocks and

Table 1. Effect of growth regulators on days taken for positive response, shoot proliferation percentage and number of shoots per explant of grape rootstock Dog Ridge.

Treatment	Days taken for positive response			Shoot proliferation percentage			Number of shoots per explant		
	Axillary bud	Shoot tip	Mean	Axillary bud	Shoot tip	Mean	Axillary bud	Shoot tip	Mean
T_1	6.49	19.42	12.96	40.00 (39.21)	20.00 (26.45)	30.00 (32.83)	1.00	1.00	1.00
T_2	5.51	15.46	10.49	72.33 (58.28)	31.67 (34.15)	52.00 (46.22)	2.10	2.11	2.11
T_3	3.77	11.91	7.84	87.00 (68.88)	53.33 (46.92)	70.17 (57.90)	4.72	3.76	4.24
T_4	5.65	15.38	10.52	74.00 (59.35)	33.00 (35.06)	53.50 (47.21)	2.27	2.22	2.25
T_5	5.84	15.30	10.57	75.67 (60.45)	34.33 (35.87)	55.00 (48.16)	2.35	2.34	2.35
T_6	3.88	12.26	8.07	90.67 (72.22)	54.33 (47.49)	72.50 (59.86)	4.77	3.89	4.33
T_7	3.96	12.30	8.13	91.00 (72.56)	56.00 (48.45)	73.50 (60.51)	4.91	3.93	4.42
Mean	5.01	14.58	9.80	75.81 (61.56)	40.38 (39.20)	58.10 (50.38)	3.16	2.75	2.96
Source of variation	SEd	CD (P=0.05)		SEd	CD (P=0.05)		SEd	CD (P=0.05)	
Explant	0.11	0.23		0.69	1.41		0.02	0.05	
Treatment	0.21	0.43		1.29	2.64		0.05	0.09	
Explant × Treatment	0.30	0.618		1.82	3.73		0.06	0.13	

T_1 - Control; T_2 - Basal MS medium + BAP (1 mg L^{-1}); T_3 - Basal MS medium + BAP (2 mg L^{-1}); T_4 - Basal MS medium + BAP (1 mg L^{-1}) + NAA (0.10 mg L^{-1}); T_5 - Basal MS medium + BAP (1 mg L^{-1}) + NAA (0.20 mg L^{-1}); T_6 - Basal MS medium + BAP (2 mg L^{-1}) + NAA (0.10 mg L^{-1}); T_7 - Basal MS medium + BAP (2 mg L^{-1}) + NAA (0.20 mg L^{-1}).
Note: Numbers in parentheses are arcsine transformed values.

Table 2. Effect of growth regulators on length of microshoots, number of leaves per shoot and days taken for multiple shoot induction of grape rootstock Dog Ridge.

Treatment	Length of microshoots (cm)			No. of leaves per shoot			Days taken for multiple shoot induction		
	Axillary bud	Shoot tip	Mean	Axillary bud	Shoot tip	Mean	Axillary bud	Shoot tip	Mean
T_1	1.19	0.52	0.86	1.33	1.00	1.17	50.35	56.87	53.61
T_2	2.89	1.32	2.11	3.33	3.00	3.17	44.20	48.86	46.53
T_3	4.54	2.08	3.31	5.00	4.33	4.67	36.46	36.82	36.64
T_4	2.93	1.38	2.16	2.67	2.33	2.50	45.07	46.88	45.98
T_5	3.25	1.40	2.33	2.33	2.00	2.17	46.11	44.69	45.40
T_6	4.69	2.14	3.42	4.67	3.67	4.17	34.45	35.61	35.03
T_7	4.73	2.28	3.51	4.33	2.67	3.50	32.81	34.89	33.85
Mean	3.46	1.59	2.53	3.38	2.71	3.05	41.35	43.52	42.44
Source of variation	SEd	CD (P=0.05)			CD (P=0.05)		SEd	CD (P=0.05)	
Explant	0.02	0.04			0.39		0.18	0.38	
Treatment	0.04	0.08			0.79		0.35	0.71	
Explant × Treatment	0.05	0.11			NS		0.49	1.01	

Note: NS = non significant.

Table 3. Effect of hardening media on *ex vitro* plantlet survival percentage, days for emergence of 1st new leaf and number of new leaves during *in vitro* derived plantlets of grape rootstock Dog Ridge.

Hardening media combinations	*Ex vitro* plantlet survival %	Days for emergence of 1st new leaf	No. of new leaves
H_1 - Pot mix (1:1:1 of sand : soil : FYM)	53.33 (46.91)	17.08	2.77
H_2 - Cocopeat	36.67 (37.26)	18.28	1.90
H_3 - Cocopeat + sand + soil	78.33 (62.29)	18.04	2.13
H_4 - Pot mix + Cocopeat	66.67 (54.75)	15.62	3.10
H_5 - Pot mix + Vermicompost	86.67 (68.67)	12.63	6.15
Mean	64.33 (53.98)	16.33	3.21
SEd	1.59	0.06	0.08
CD (P=0.05)	3.55	0.03	0.18

Note. Numbers in parentheses are Arc Cine transformed values

Barreto *et al*. (3) in different grape cultivars. Earlier, Wong (18) also found that medium supplemented with BAP resulted in the highest number of leaves per explant in Dog Ridge rootstock. The unique finding of this experiment is that axillary buds of Dog Ridge cultured during January to first fortnight of February responded earlier (within three days) than the explants collected during the second fortnight of February and March. This observation emphasizes the positive influence of well balanced endogenous hormonal level with exogenously supplied hormonal and media composition upon stimulation of growth response.

Mean shoot proliferation rate was recorded higher with axillary buds than the shoot tips. Addition of NAA (0.2 mg l^{-1}) along with BAP 2.0 mg l^{-1} resulted in enhanced shoot proliferation percentage in both axillary bud (91.00%) and shoot tip (56.00 %) explants (Table 1). Result was statistically on par with addition of BAP 2.0 mg l^{-1} either alone or with NAA 0.1 mg l^{-1}. The same composition was found to register the highest number of microshoots per culture (4.91 in axillary buds and 3.93 in shoot tips) and the longest microshoot (4.73 cm in axillary buds and 2.28 cm in shoot tips) two months after culture initiation (Table 1 & 2). Multiple shoot induction was found earlier in axillary buds than the shoot tips. Least number of days required for multiple shoot induction in axillary bud (32.81) and shoot tip (34.89) was recorded on MS medium supplemented with BAP 2.0 mg l^{-1} + NAA 0.2 mg l^{-1}, whereas MS medium without growth regulator recorded more number of days for shoot multiplication on both axillary bud (50.35 days) and shoot tip (56.87 days) (Table 2). Alizadeh *et al.* (1) tested BAP ≥ 2 mg l^{-1}in combination with NAA for promotion of shoot multiplication in different grape rootstocks, but in the present study, BAP ≤ 2 mg l^{-1} in combination with NAA was tested for Dog Ridge. In both the studies, BAP 2 mg l^{-1} resulted in promotion of shoot proliferation and multiplication. Itoo *et al.* (7) reported that axillary buds and shoot tips of Perlette cultivar cultured on MS medium supplemented with BAP and NAA responded well to shoot proliferation. Similar results were also reported by Barreto *et al*. (3) with respect to length of shoots and early induction of multiple shoots in grape cultivar Red Globe. The positive effect of cytokinin (BAP) on shoot proliferation, multiplication may be due to induction of axillary bud proliferation by cell division, their absence correlated with impaired synthesis of proteins involved in formation and function of the mitotic spindle apparatus (Sachs and Thiman, 12; Jouanneau, 8). It is evident that auxins do not promote shoot proliferation and are required in culture medium to promote growth of shoots by counteracting suppressive effect of high cytokinin. Plant hormones do not function in isolation within the plant body, instead function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue.

Elongation of primary microshoots is one of the essential steps during *in vitro* regeneration. In the present study, it could be observed that MS medium supplemented with BAP 2 mg l^{-1} and GA_3 0.50 mg l^{-1} can induce lengthier (8.68 cm) microshoots with more number of leaves (6.47) (Fig. 1). Since the endogenous natural phyto hormones present in explant are highly sensitive to culture environments, exogenously supplied synthetic growth regulator analogs can compensate the effect and stimulate the growth of cultures. Moreover, shoot elongation phase is sensitive to higher concentration of growth regulators. The observations made on the effect of

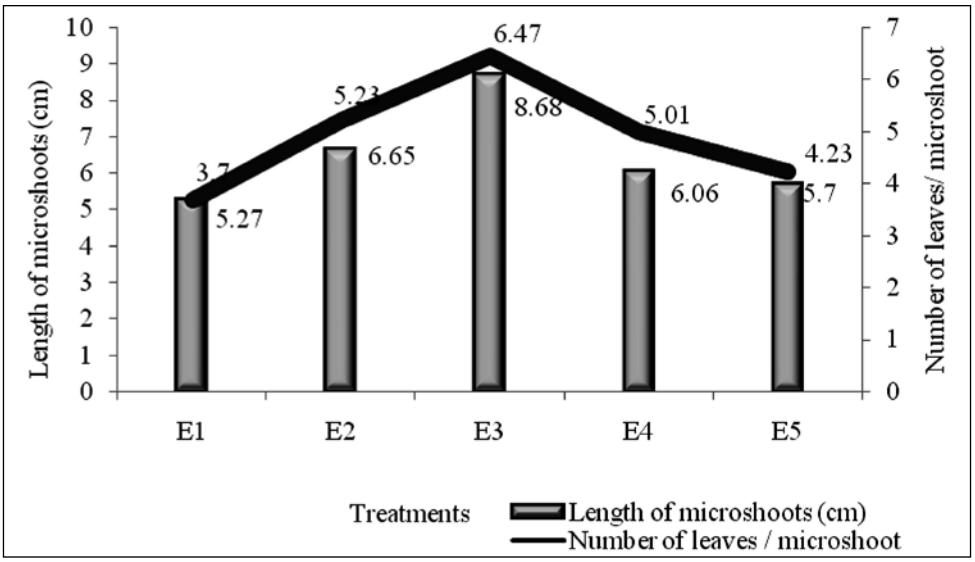

Fig. 1. Effect of elongation media on length of microshoots (cm) and number of leaves in grape rootstock Dog Ridge.

E_1 - Control (Basal MS medium + BAP (2 mg L^{-1}) (without GA_3), E_2 - Basal MS medium + BAP (2 mg L^{-1}) + GA_3 (0.25 mg L^{-1}), E_3 - Basal MS medium + BAP (2 mg L^{-1}) + GA_3 (0.50 mg L^{-1}); E_4 - Basal MS medium + BAP (2 mg L^{-1}) + GA_3 (0.75 mg L^{-1}), E_5 - Basal MS medium + BAP (2 mg L^{-1}) + GA_3 (1 mg L^{-1})

combination of cytokinin and gibberellin, *i.e*. BAP and GA_3 on number of leaves and shoot length revealed that there was a significant difference in microshoot length and leaves produced among the different treatments. Similar studies in peach rootstock added further insights to our studies that BAP in combination with low level GA_3 has a positive effect on shoot elongation whereas higher concentration of GA_3 may inhibit the growth and cause deleterious side effects such as shoot tip drying. The combination of BAP and GA_3 significantly influenced shoot length due to their combined effect on cell division and expansion (Gordon and Letham, 6).

In vitro rooting is a key step to establish a viable regeneration system in plant tissue culture because the development of physiologically active roots could be an important criterion for better field performance. Results of the *in vitro* rooting experiment of present study showed that half MS medium supplemented with 2 mg l^{-1} IBA + activated charcoal at 200 mg l^{-1} initiated more number (4.92) of lengthier (7.58 cm) roots in a shorter duration (11.74 days) with maximum percentage (83.00%) of root induction when compared to other treatments (Fig. 2 & 3). The balance between endogenous and exogenously applied auxins may be crucial for root initiation and development.

Grapevine, being a woody plant, represents a highly complex system in which endogenous hormone levels, transport, dormancy, storage and inhibitory compounds influence adventitious root growth and all these depend on genetic nature of the species (Wilson, 17). Among different auxins, IBA has been very effective in inducing rooting in most species including grapes as reported by many workers. Earlier investigations have shown that *in vitro* rooting could be successfully achieved by reducing salt concentrations in the media, particularly in high salt media like MS and its derivatives and half-strength media devoid of BAP but supplemented with auxin (Singh *et al*., 13). Activated charcoal is often used in tissue culture to improve cell growth and development. The results of rooting experiment of the present study are in conformity with the results of Alizadeh *et al*. (1, 2) and Motha *et al*. (9) in four different grape rootstocks and Singh *et al.* (13) and Itoo *et al*. (7) in different grape cultivars. This may be due to the result of an indole molecule (auxin), which induces master signal to initiate chain of events ultimately leading to *in vitro* root development, especially cell elongation and root initiation. Rhizogenesis during *in vitro* culture of grapevines seems to be strongly genotype dependent and root regeneration in *Vitis* tissues showed some difficulty, due to the existence of compounds that inhibit root formation. The stimulatory effect of activated charcoal on *in vitro* rhizogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the accumulation of toxic metabolites, phenolic exudation and brown exudates in the culture medium (Thomas, 16).

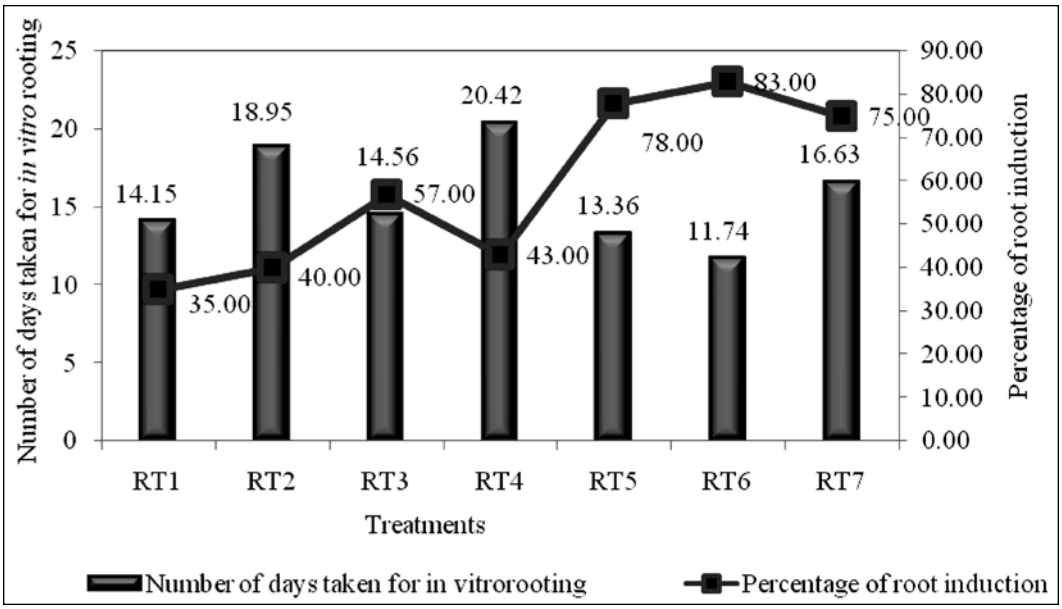

Fig. 2. Effect of rooting media on number of days taken for *in vitro* rooting and percentage of root induction in grape rootstock Dog Ridge.

RT_1 - Half MS (control); RT_2 - Half MS + IBA (1 mg l^{-1}); RT_3 - Half MS + IBA (2 mg l^{-1}); RT_4 - Half MS + IBA (3 mg l^{-1}); RT_5 - Half MS + IBA (1 mg l^{-1}) + Activated Charcoal (200 mg l^{-1}); RT_6 - Half MS + IBA (2 mg l^{-1}) + Activated Charcoal (200 mg l^{-1}); RT_7 - Half MS + IBA (3 mg l^{-1}) + Activated Charcoal (200 mg l^{-1}).

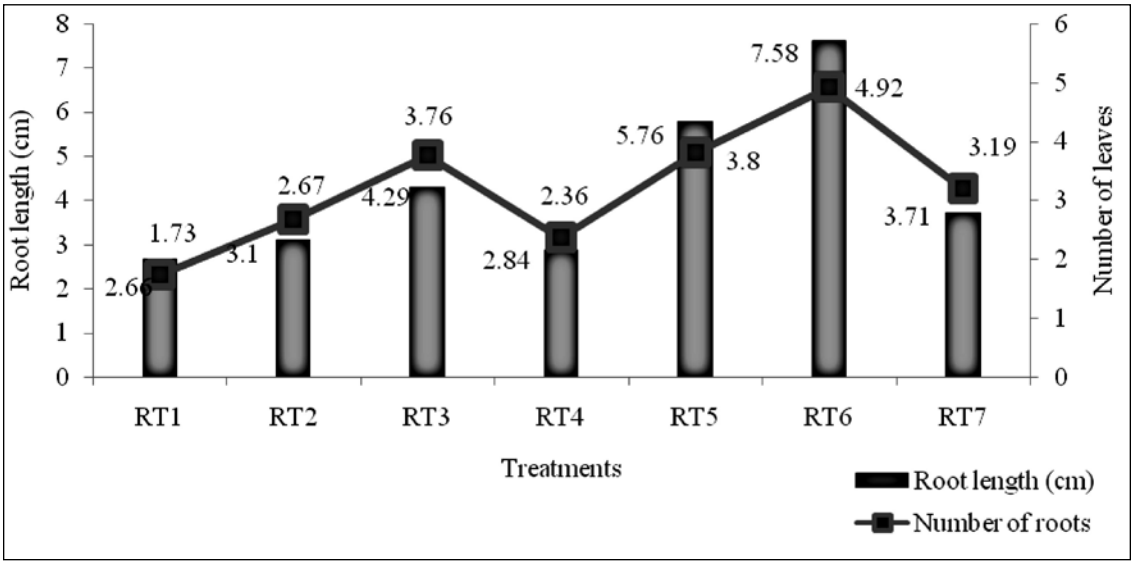

Fig. 3. Effect of rooting media on root length (cm) and number of roots in *in vitro* cultures of grape rootstock Dog Ridge.

The ultimate success of micropropagation depends on the ability to transfer plants out of culture on a large scale with high survival rates. The *in vitro* culture conditions results in the plantlets with altered morphology, anatomy and physiology which generally render field survival of the plantlets a difficult task. In the present study, it was observed that the *in vitro* propagated plantlets transferred to the hardening media containing pot mixture and vermicompost (1:1) and protected with polythene covers recorded the highest *ex vitro* survival (86.67%) and more number of leaves (6.15) within 12.63 days (Table 3). This may be attributed to the optimum conditions favoured by rich supply of nutrients, good water retention, good aeration and good drainage provided by the medium and these various factors may have attributed for the successful establishment of the plantlets under *ex vitro* condition. These results are in corroboration with

the observations of Muhammad *et al*. (10) in grape cultivar Perlette.

It could be inferred from the present study that utilization of axillary bud as explant for grape rootstock Dog Ridge during January to March on MS medium containing adequate level of growth regulators can favour *in vitro* multiplication, elongation and rooting. The total time taken for plant development worked out to 129 days from axillary buds and 145 days from shoot tips. In one subculture, axillary buds can yield 1:5 and shoot tips can yield 1:3 proportion of microshoots. Among the two explants, axillary buds proved superior to shoot tips with respect to early proliferation and multiplication. Hence, micropropagation using axillary bud explants collected during January to March can be a viable propagation technique for grape rootstock Dog Ridge.

DECLARATION

The authors declare no conflict of interest.

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