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In vitro PLANT REGENERATION OF GRAPE cv. 'RED GLOBE' THROUGH DIRECT ORGANOGENESIS

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ABSTRACT

A study was conducted to develop a protocol for *in vitro* plant regeneration in grape cv. 'Red Globe' *via* nodal segments and shoot tips. The surface sterilization treatment-combination of 0.5% carbendazim (30 min) + 0.5% NaOCl (10 min) + 70% ethanol (30 sec) was most effective for nodal segments with highest survival rate of 68.33%. For shoot tips the treatment with 0.3% carbendazim (30 min) + 0.25% NaOCl (10 min) + 70% ethanol (30 sec) was found favourable. MS medium supplemented with 2.0 mg L^{-1} BAP was optimum for initial culture establishment of both the explants. However, MS medium supplemented with 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA resulted in the highest proliferation rate in both explants and the treatment was used for subsequent subcultures for shoot elongation. For *in vitro* rooting, 2 mg L^{-1} IBA + 200 mg L^{-1} activated charcoal was optimum with highest response of 85.96% and 5.1 roots $shoot^{-1}$. The highest *ex vitro* survival percentage of plantlets (72.84%) was obtained with a hardening media of pot mix and vermicompost (1:1).

Keywords: Grapes, nodal segments, plant regeneration, Red Globe, shoot tips

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is grown worldwide for a variety of purposes including fresh fruits and processed products *viz.*, juice, jam, jelly, wine and raisins, etc. Grape cultivation has economic and social importance due to its use in winery and raisin production. Amongst the commercial cultivars, 'Red Globe' is an important grape cultivar widely cultivated in India, Australia, USA, Chile and China (Jogaiah *et al*., 2021). The cultivar is well known for its very large size, seeded red colour berries with firm flesh, used mainly for table purpose. Any genetic improvement of grapes against various biotic and abiotic stresses through biotechnological approaches demands a standard micro-propagation protocol. A direct adventitious shoot formation without the intervention of callus is a reliable method for clonal propagation, mutation and ploidy breeding of grapes (Nuzzo *et al*., 2022).

Grape being a woody perennial is highly recalcitrant to *in vitro* culture. However, successful regeneration protocol has been developed for various explants *viz*., axillary buds (Lazo-Javalera *et al*., 2016), nodal and internodal segments (Kurmi *et al*., 2011; de Carvalho *et al*., 2011), shoot apices (Mezzetti *et al*., 2002) and microcuttings (Kryukov *et al*., 2022). Different culture media *viz*., MS, ½ MS, WPM (woody plant medium) and NN (Nitsch and Nitsch) were used to develop *in vitro* propagation protocols for *Vitis* spp. (Mozafari *et al*., 2016, Pedro *et al*., 2017, Nuzzo *et al*., 2022). In grapes, the type and concentration of cytokinins in growth medium play a key role in shoot organogenesis and it varies within the *Vitis* species and different cultivars of grapes. Thidiazuron (TDZ) has proved been found more effective for Muscadine grapes (*V. rotundifolia*) (Sudarsono and Goldy, 1991); whereas, Gray and Benton (1991) reported BAP as a better cytokinin for the same

species. Mhatre *et al*. (2000) reported that for shoot multiplication of *V. vinifera*, BAP was ideal. Even within the two cultivars of Muscadine grape, BAP @ 1.13 mg L^{-1} was better for cv. 'Carlos', while a 4-fold higher concentration of BAP (4.5 mg L^{-1}) was required for cv. 'Fry' (Gray and Benton, 1991). The reports from Sudarsono and Goldy (1991), Mhatre *et al*. (2000) and Alizadeh *et al.* (2018) suggested that the protocols for *in vitro* propagation of *Vitis* species might be genotype- or cultivar- or species-specific and the degree of success at each stage of *in vitro* culture was found genotype dependent and was influenced by specified culture conditions (Alizadeh *et al.,* 2018). For this reason, the effect of different plant growth regulators on *in vitro* shoot proliferation and rooting of grape cv. 'Red Globe' was evaluated with the objective to develop an efficient and reliable protocol for its micro-propagation *via* direct organogenesis from nodal segments and shoot tips.

MATERIALS AND METHODS

Plant material

Actively growing young shoots of grape cv. 'Red Globe' were collected from a mature healthy vine, maintained in the vineyard of college orchard, Horticultural College and Research Institute, TNAU, Coimbatore (India). The collected shoots of 1.5-2.0 cm length were excised by removing all the leaves and separated as nodal segments and shoot tips. To standardize the sterilization treatment, the explants were treated 2-3 times with aqueous solution of a liquid detergent Tween 20 and then treated with a broad-spectrum systemic fungicide carbendazim (0.3 and 0.5%) against fungal contaminants for 30 min, followed by thorough washing with running tap water. Under aseptic conditions, the explants were surface sterilized by immersing in 70% ethanol for 30 sec followed by sodium hypochlorite treatment (NaOCl @ 0.25 and 0.5% for 5, 10 and 15 min) with occasional swirling and finally washing three times with sterilized distilled water for 5 min each.

Culture establishment and growth conditions

The sterilized explants were inoculated in individual 25 × 150 mm culture tubes containing 25 mL medium. The inorganic salts and organic ingredients used for culture media preparation and growth regulators used for the study were procured from Hi-Media, Mumbai, India and Sigma Chemicals, Bengaluru, India. To study the response of culture establishment and shoot proliferation, MS medium supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar and BAP alone (2 mg L^{-1}) or in combination with NAA (0.1, 0.2, 0.3 and 0.4 mg L^{-1}) were used. The concentration of BAP (2 mg L^{-1}) was fixed based on the preliminary observation made with different concentration of BAP, where minimum days to positive response of explants was recorded in 2 mg L^{-1}; and also the earlier reports suggested the use of BAP instead of other cytokinins. The pH of culture medium was adjusted to 5.7 before autoclaving at 1.05 kg cm^{-2} pressure (121 ℃) for 15 min. After inoculation, the cultures were incubated in a culture room at 25 ± 2℃ with a 16 h photoperiod.

Shoot growth and proliferation

During early subculture phase (initial two subcultures), *in vitro* cultures were sub-cultured on modified media [½ MS with one and half times of $CaCl_2$ (6.8 g L^{-1}) and BAP (1 mg L^{-1})] to alleviate shoot tip necrosis and later revert to normal levels of Ca in MS media to obtain better results. After sub-culturing twice with modified media, the *in vitro* shoots were further sub-cultured on normal MS media with BAP (2 mg L^{-1}) alone or in combination with NAA (0.1, 0.2, 0.3 and 0.4 mg L^{-1}) to induce multiple shoots and shoot elongation. The cultures were maintained under conditions as mentioned previously.

In vitro rooting

For *in vitro* rooting, the excised shoots from cultures were transferred onto fresh ½ MS media supplemented with 0, 1, 2 or 3 mg L^{-1} IBA (indole-3-butyric acid) alone and/or in combination with activated charcoal (200 mg L^{-1}).

Plantlet acclimatization

The rooted plantlets were carefully taken out of rooting medium without damage and thoroughly washed under running tap water to remove the adhering agar. These were then transferred to wide mouthed bottle containing quarter-strength liquid MS medium (without sucrose). After two weeks, the plantlets were transferred to small plastic pots containing different media combinations [H_1 - pot mix (1:1:1 of sand: soil: FYM), H_2 - cocopeat, H_3 - cocopeat + sand + soil, H_4 - pot mix + cocopeat, H_5 - pot mix + vermicompost] and shifted to net house with 50% shade by covering each pot with transparent polythene to maintain high humidity. After 15-20 days, the polythene covers were removed during new leaf emergence to reduce excessive humidity.

Observations and statistical analysis

The data on percent contamination, mortality and survival of explants were recorded until first subculture. The days to positive response *i.e.* days taken for bud break and formation of new shoots in nodal segments and shoot tips were recorded during culture initiation. Observations on multiple shoot induction, shoot length and number of new shoots explant^{-1} were recorded as per treatment during 3rd sub-culture. The number of days taken to rooting, root length and root induction were recorded after root initiation from the day of transfer onto rooting media. All the experiments were conducted in a completely randomized design with each treatment replicated three times. Data were analysed using one-way analysis of variance, and least significant difference was used to ascertain the significant differences between means with the help of a statistical software, SPSS v.24. Data recorded in percentage were subjected to arcsine transformation before analysis (Panse and Sukhatme, 1967).

RESULTS AND DISCUSSION

Surface sterilization

Surface sterilization is an essential pre-requisite in tissue culture, and the viability of any protocol is based on the efficiency of surface sterilization. In present study, explants were exposed to surface sterilants *viz*., carbendazim and NaOCl in varied concentration and duration, and the best surface sterilization treatment was determined on the basis of low contamination percentage with high survival of explants. Contamination percentage decreased as the dosage and duration of exposure to carbendazim and NaOCl increased in both the explants. The lowest contamination of 8.33% was observed in nodal segments treated with 0.5% carbendazim for 30 min and surface sterilization with 70% ethanol for 30 sec followed by 0.5% NaOCl for 10 min (T_{11}), while the highest contamination (90%) was found in control (washing with sterile water). Similarly, in shoot tips the lowest contamination (5%) was observed in treatment of 0.5% carbendazim for 30 min, followed by surface sterilization with 70% ethanol for 30 sec and 0.5% NaOCl for 15 min (T_{13}); whereas the highest contamination was found in control (86.67%) (Fig. 1a). The highest survival for nodal segments (68.33%) was observed by treating with 0.5% carbendazim for 30 min followed by surface sterilization with 70% ethanol for 30 sec and 0.5% NaOCl for 10 min (T_{11}) and for shoot tips, 0.3% carbendazim for 30 min and surface sterilization with 70% ethanol for 30 sec, followed by 0.25% NaOCl for 10 min (T_4) which recorded the higher survival of 56.67% (Fig. 1b).

Carbendazim is a broad-spectrum systemic fungicide that affects the fungi present on explant surface and inactivate them (Theivanai *et al*., 2020a; Kiruthikadevi *et al*., 2022). NaOCl is a bleaching agent widely used as surface sterilant @ 0.1-10% depending upon the explants, and it acts as bactericide (Singh *et al.,* 2011). In present study, due to variable tissue sensitivity of the explants used, the concentrations of surface sterilant also varied. It may be concluded that the nodal segments can be regenerated with least contamination and mortality percentage with highest survival rate in treatment 0.5% carbendazim for 30 min, followed by 70% ethanol for 30 sec and 0.5% NaOCl for 10 min. These results are in line with Abido *et al*. (2013) in grapes and Theivanai *et al.* (2020a) in 'Dog Ridge'

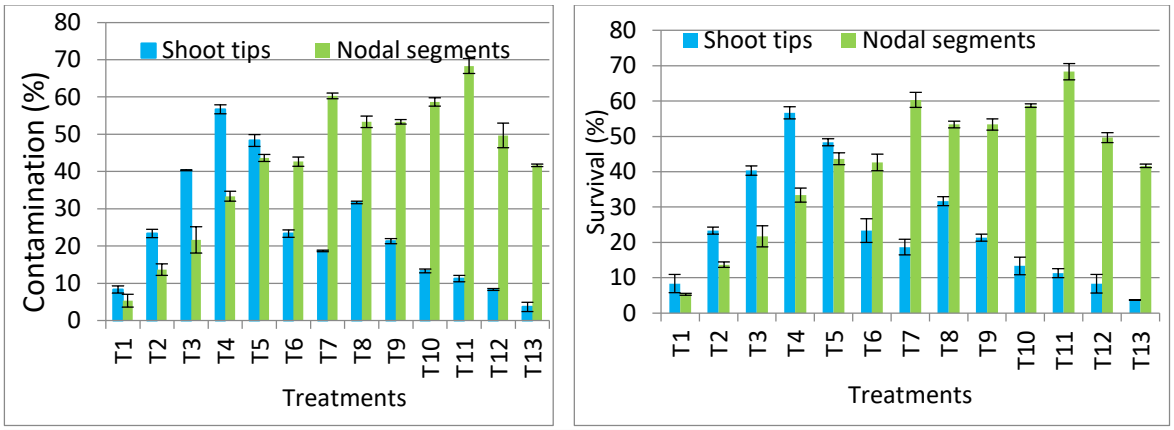

Fig. 1: Effect of surface sterilants on a) contamination (%) and b) survival (%) in the explants of grape cv. 'Red Globe'; Treatment details: T_1 = Washing with sterile distilled water (control), T_2 = 0.3% Carbendazim (30 min) + NaOCl 0.25% (5 min), T_3 = 0.3% Carbendazim (30 min) + NaOCl 0.5% (5 min), T_4 = 0.3% Carbendazim (30 min) + NaOCl 0.25% (10 min), T_5 = 0.3% Carbendazim (30 min) + NaOCl 0.5% (10 min), T_6 = 0.3% Carbendazim (30 min) + NaOCl 0.25% (15 min), T_7 = 0.3% Carbendazim (30 min) + NaOCl 0.5% (15 min), T_8 = 0.5% Carbendazim (30 min) + NaOCl 0.25% (5 min), T_9 = 0.5% Carbendazim (30 min) + NaOCl 0.5% (5 min), T_{10} = 0.5% Carbendazim (30 min) + NaOCl 0.25% (10 min), T_{11} = 0.5% Carbendazim (30 min) + NaOCl 0.5% (10 min), T_{12} = 0.5% Carbendazim (30 min) + NaOCl 0.25% (15 min), and T_{13} = 0.5% Carbendazim (30 min) + NaOCl 0.5% (15 min)

rootstock. In case of shoot tips, 0.3% carbendazim, followed by 70% ethanol for 30 sec and 0.25% NaOCl for 10 min was best surface sterilization treatment. It is clear that shoot tips are more susceptible to tissue injury as compared to the nodal segments. This can be inferred based on the reduced concentration of sterilants required for higher survival rate (Theivanai *et al.*, 2020a).

Culture establishment, shoot growth and proliferation

In present study, MS medium supplemented with 2 mg L^{-1} BAP proved optimum for initial culture establishment. The least number of days for bud break in nodal segments (7 days) and new shoot initiation in shoot tips (18 days) were observed in MS medium supplemented with 2 mg L^{-1} BAP. This was followed by MS medium supplemented with 2 mg L^{-1} BAP + 0.1 mg L^{-1} NAA (Fig. 2a). Nodal segments exhibited early response of 7.67 days as compared to the shoot tips (18 days) for bud break. The results revealed that cytokinin is important for *in vitro* shoot development of grapevine (Barreto *et al*., 2006; Theivanai *et al*., 2020b) and the higher concentration of cytokinin requirement is in line with Theivanai *et al*. (2020b). Contrarily, Novak and Juvova (1982) reported that BAP (10 µM) [10

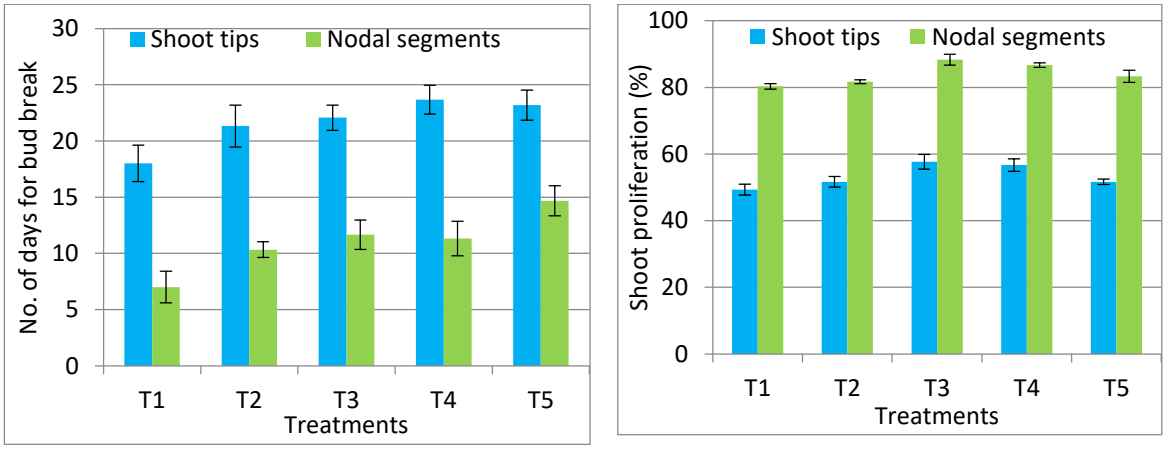

Fig. 2: Effect of BAP and NAA on a) days taken for budbreak, and b) shoot proliferation (%) in explants of grape cv. Red Globe; Treatment details: T1 = 2 mg L^{-1} BAP; T2 = T1 + 0.1 mg L^{-1} NAA; T3 = T1 + 0.2 mg L^{-1} NAA; T4 = T1 + 0.3 mg L^{-1} NAA; T5 = T1 + 0.4 mg L^{-1} NAA

times lesser concentration used in this experiment] in MS medium was optimum for adventitious bud formation on shoot apex fragments in grapes. A low auxin level along with cytokinin is reportedly beneficial for shoot growth and proliferation of *in vitro* cultures (Theivanai *et al*., 2020b). With this background BAP with varied concentration of NAA were tested for shoot growth and proliferation. The treatment MS medium supplemented with 0.2 mg L^{-1} NAA and 2 mg L^{-1} BAP gave higher shoot proliferation, number of leaves and shoot length in nodal segments and the same combination yielded highest number of microshoots along with highest shoot proliferation and more leaves in shoot tips. The improvement may be attributed to the cytokinin-induced stimulation of both cell division and shoot growth (Gray *et al*., 2005; Theivanai *et al*., 2020b). It may be inferred that NAA and BAP used in appropriate combination are rewarding in many fruit tree species (Theivanai *et al*., 2020b). For multiplication rate, cytokinins were effective when used in combination with an auxin. Higher shoot proliferation in both nodal segment (88.33%) and shoot tip (57.67%) explants were observed in MS medium supplemented with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (T_3) and in shoot tips, treatment T_4 (2 mg L^{-1} BAP + 0.3 mg L^{-1} NAA) was on par with treatment T_3 (Fig. 2b). For nodal segments, treatments T_3 [MS medium + BAP (2.0 mg L^{-1}) + NAA (0.2 mg L^{-1})] and T_5 [MS medium + BAP (2.0 mg L^{-1}) + NAA (0.4 mg L^{-1})] induced higher multiple shoots (4.33 explant^{-1}) and took least number of days for multiple shoot induction (27.66 days). However, treatment T_3 [MS medium + BAP (2.0 mg L^{-1}) + NAA (0.2 mg L^{-1})] produced higher number of leaves microshoot^{-1} and longest microshoots in nodal segments. Shoot tips also gave maximum number of microshoots (3.33) and number of leaves (2.33) in MS medium supplemented with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, whereas the longest shoots (3.33 cm) and lowest days to induce multiple shoots (40.66 days) were observed in MS medium supplemented with 2 mg L^{-1} BAP and 0.4 mg L^{-1} NAA (T_5). As MS medium supplemented with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (T_3) induced higher number of new shoots explant^{-1}, it caused optimum shoot proliferation in grape cv. 'Red Globe' using nodal segments and shoot tips. The shoot proliferation depends upon the balance of cytokinins and auxins. The addition of low concentration of NAA was beneficial in the initiation of grapevine *in vitro* (Itoo *et al*., 2013).

The first positive response observed during *in vitro* culture was bud break in nodal segments or growth initiation and sprouting in shoot tips. Nodal segments showed early positive response along with other shoot proliferation parameters as compared to the shoot tips. Shoot tips during shoot proliferation stage required higher concentration of NAA (0.4 mg L^{-1}) with BAP as compared to the nodal segments. The positive mode of action of NAA may be because of its capacity to control various distinctive processes such as promotion of stem elongation and growth (George *et al*., 2008). Among the two explants used, nodal segments proved superior to shoot tips as it performed better with respect to some important parameters including days taken for positive response, shoot proliferation, number of leaves, length of microshoots, days for multiple shoot induction and number of multiple shoots explant^{-1} (Table 1). The use of nodal segments as propagules is common for *in vitro* multiplication of

Table 1: Effect of BAP and NAA on days taken for multiple shoot induction (DTMSI), number and length of microshoots, and number of leaves in explants of grape cv. 'Red Globe'

Treatments	Nodal segments				Shoot tips			
	DTMSI	Microshoots		No. of leaves	DTMSI	Microshoots		No. of leaves
		No.	Length (cm)			No.	Length (cm)	
2 mg L^{-1} BAP	31.00	3.33	4.40	3.33	48.67	2.00	2.77	2.67
2 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	28.67	3.67	4.47	3.67	47.67	2.67	2.83	2.67
2 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	28.33	4.33	6.50	5.00	44.00	3.33	3.27	2.33
2 mg L^{-1} BAP + 0.3 mg L^{-1} NAA	28.00	4.00	6.00	4.67	42.00	3.33	3.30	2.33
2 mg L^{-1} BAP + 0.4 mg L^{-1} NAA	27.67	4.33	6.10	4.67	40.67	3.33	3.33	2.33
Mean	28.73	3.93	5.49	4.27	44.60	2.93	3.10	2.47
SEd	0.745	0.38	0.124	0.380	0.796	0.46	0.109	0.365
$CD_{0.5}$	1.719*	ns	0.286*	0.876*	1.835*	ns	0.251*	ns

*Significant at 5% level**;** ns **-** Non significant

different species and varieties of grapevine, since they provide genetic stability and only require the growth induction of the preformed buds, which contain all the elements of a shoot in miniature (Wong, 2009). The observations may be attributed to the better status of endogenous food reserves and hormones, and relatively less injury caused to the tissues during exposure of sterilizing agents in nodal segments.

In vitro rooting

The *in vitro* rooting of microshoots was tried with various concentrations of IBA with activated charcoal in half strength MS medium. The various concentration of IBA tested with activated charcoal showed significant improvement in rooting. The results showed that 2 mg L^{-1} IBA with 200 mg L^{-1} activated charcoal in half strength MS medium was most effective for *in vitro* rooting of grape cv. 'Red Globe' as it induced significantly higher rooting, number of roots $shoot^{-1}$ and root length (Table 2). It may be inferred that the addition of auxin is essential for normal rooting of microshoots. The results are in agreement with Sanjay *et al*. (2004) in 'Pusa Urvashi' and 'Pusa Navrang' wherein early rooting, and higher root number and length were recorded in half strength medium supplemented with activated charcoal and IBA. The auxin (IBA) used is an indole molecule, which induces master signal to initiate chain of events ultimately leading to the *in vitro* root development. Auxins are known to promote cell elongation and root initiation (Omar *et al*., 2004). In present study, the addition of activated charcoal appears to induce rooting, as observed in other woody plants (Thomas, 2000; Theivanai *et al*. 2020b). The rooting in present study is in conformity with Itoo *et al*. (2013), Motha *et al*. (2017) and Alizadeh *et al*. (2018) in other grape rootstocks/cultivars. The various stages of *in vitro* propagation of grape cv. 'Red Globe' is given in Fig, 3.

Table 2: Effect of IBA and activated charcoal on *in vitro* rooting of microshoots of grape cv. 'Red Globe'

Treatments	Root induction (%)	NDTR**	Root length (cm)	No. of roots
1/2 MS (Control)	48.23 (43.97)	17.12	2.45	2.18
1/2 MS + IBA @ 1 mg L^{-1}	63.25 (52.66)	19.56	4.09	3.41
1/2 MS + IBA @ 2 mg L^{-1}	75.23 (60.12)	17.42	6.53	4.25
1/2 MS + IBA @ 3 mg L^{-1}	58.23 (49.71)	23.65	2.98	3.15
1/2 MS + IBA @ 1 mg L^{-1} + activated charcoal @ 200 mg L^{-1}	79.23 (62.88)	15.78	8.15	4.70
1/2MS + IBA @ 2 mg L^{-1} + activated charcoal @ 200 mg L^{-1}	85.96 (67.97)	14.89	10.23	5.10
1/2 MS + IBA @ 3 mg L^{-1} + activated charcoal @ 200 mg L^{-1}	56.23 (48.55)	20.87	3.84	3.96
SEd	0.73	0.35	0.08	0.06
$CD_{0.05}$	1.59*	0.76*	0.18*	0.13*

*Significant at 5% level; **No. of days taken for rooting

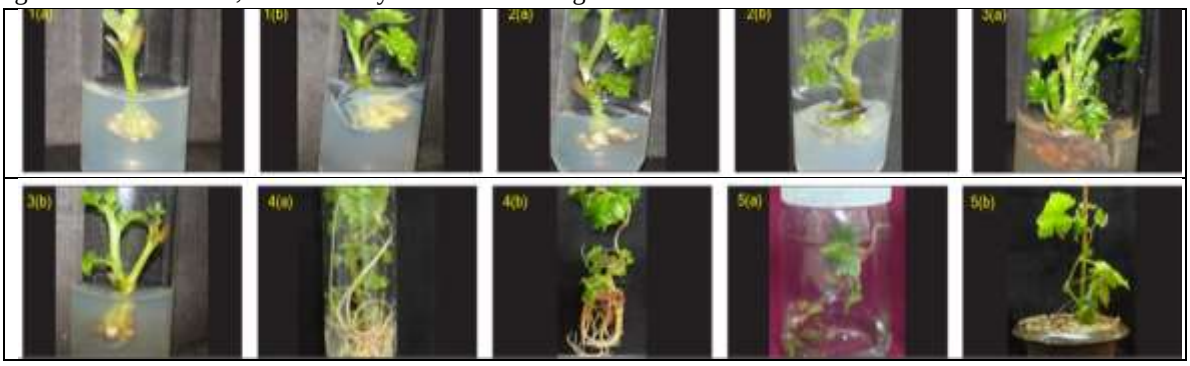

Fig. 3: Various stages of *in vitro* propagation of grape cv. 'Red Globe': 1a) Bud break from nodal segment and b) Shoot tip 2a) Shoot development from nodal segment and b) from shoot tip 3a) Multiple shoots formed from nodal segments and b) shoot tips 4a) & 4b) Shoot with roots formed 5a) Hardening of plantlet in wide mouthed bottle 5b) Established plant in net house after 25 days of transfer from hardening to mixture of soil, sand and FYM (1:1:1) in plastic pot.

Table 3: Effect of hardening media on *ex vitro* plantlet survival percentage, days for emergence of first new leaf and number of leaves in *in vitro* derived plantlets of grape cv. 'Red Globe'

Treatments	*ex vitro* plantlet survival percentage	Days for emergence of first new leaf	No. of leaves in *in vitro* derived plantlets
H_1 – Pot mix (1:1:1 sand: soil: FYM)	45.31 (39.25)	17.08	2.57
H_2 – Cocopeat	31.25 (36.57)	18.28	1.98
H_3 – Cocopeat + sand +soil	64.25 (56.47)	14.04	2.32
H_4 – Pot mix + cocopeat	55.87 (47.82)	15.62	3.28
H_5 – Pot mix + vermicompost (1:1)	72.84 (63.31)	12.56	5.15
SEd	3.20	0.60	0.38
$CD_{0.05}$	7.10*	1.21*	0.80*

* Significant at 5% level; Numbers in parentheses are arcsine transformed values

Acclimatization and pot transfer of plantlets

Acclimatization of plantlets to field conditions is a crucial step in tissue culture. A successful tissue culture protocol must result in high frequency of re-establishment of tissue culture derived plants in soil. Present study revealed that *in vitro* propagated plantlets transferred to the hardening medium containing pot mixture and vermicompost (1:1) showed highest *ex vitro* survival (72.84%); while the lowest survival (31.25%) was in found in cocopeat medium (Table 3). Optimal supply of nutrients, good water retention, good aeration and good drainage provided by the hardening medium may have attributed to the establishment of plantlets under *ex vitro* condition. The results are in corroboration with Muhammad *et al.* (2008) in grape cv. 'Perlette' and Theivanai *et al*. (2020b) in 'Dog Ridge'.

Conclusion: The present study established the *in vitro* propagation protocol for grape cv. 'Red Globe', with high shoot multiplication rate. The protocol developed could be useful for large-scale clonal propagation and *in vitro* breeding. For initial culture establishment, the MS medium supplemented with 2.0 mg L^{-1} BAP was found optimum for further multiplication, while MS medium supplemented with 2.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA was found good. Half strength MS medium with IBA (2 mg L^{-1}) and activated charcoal (200 mg L^{-1}) was optimum for yielding highest rooting response and number of roots $shoot^{-1}$. The acclimatized plantlets were established in pot mixture and vermicompost (1:1) under shade net house conditions.

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