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IN VITRO MICROPROPAGATION OF *VANDA ROXBURGHII*: A MEDICINAL ORCHID OF THE WESTERN GHATS AND DETERMINATION OF GENETIC FIDELITY USING RAPD MARKERS

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ABSTRACT

Objective: An competent *in vitro* protocol was developed for rapid multiplication of medicinally and commercially important rare orchid, *Vanda roxburghii* R.Br., cost effective method was adapted for acclimatization and fidelity test to confirmed genetic similarities. **Methods**: Different types of media fortified with 10% coconut water (CW) and casein hydrolysate (CH) were used for seed germination as well as PLB formation. MS medium with different auxins i.e. 2,4-D and NAA alone were used for callus induction from leaf explant. Callus were subcultured on Terrestrial Orchid Media (TOM) supplemented with cytokinins and activated charcoal (AC) will yield PLBs and shoot formation. For root development, shoots were subcultured on TOM fortified with BAP along with different concentration of NAA and IAA were analyzed. **Results**: The maximum seed germination (87%) and PLBs was observed in MS medium fortified with 10% CW and CH 3.28M. Well-developed callus formation was achieved on MS supplemented with 2,4-D (2.27 µM). The PLBs and shoots were observed from the callus on TOM with BAP (8.88 µM) and AC (8.3M). Well-developed root with shoots were observed on TOM fortified with BAP at 8.88 µM with 8.05 µM NAA. The novel brick base method adopted for primary acclimatization and 95% survival frequency were observed. The genetic uniformity was confirmed through RAPD profile. **Conclusion:** The present investigation provides a commercially viable protocol for this species with adopting novel technique for acclimatization process and field trials. The RAPD results confirms similarity of genome *in vitro* plants.

KEYWORDS: Acclimatization, indirect regeneration, orchid seed germination, protocorm like bodies, *Vanda roxburghi*

INTRODUCTION

Vanda roxburghii R.Br. is a least concern orchid epiphytic in nature, growing up to 60 cm in height on trees in dry and intermediate zone. The plant is widely used in indigenous medicine systems such as Ayurveda and some local traditional medical practices. The leaf juice is used for the treatment of certain inflammatory conditions, and the paste is applied to the body to bring down fever.[1,2] In the Unani system of medicine, root is used as tonic to the liver and brain; and is used in the treatment of bronchitis, piles, lumbago toothache, heals and fractures.[3]

The absence of endosperm and the undeveloped state of embryo causes poor seed germination in natural habitat. In addition, the habitat destruction and over exploitation for medicinal purpose are the two important factors threatening its survival in nature. Hence there is an urgent need to conserve this rare taxon. At present plant tissue culture is a potentially useful technique for ex situ multiplication and restoration of affected taxa. Hence, in the present study, an attempt was made to multiply *V. roxburghii* through *in vitro* seed germination, indirect regeneration from leaf and acclimatization were successfully established.

Plant tissue culture leading to somaclonal variation has been considered as one of the possible sources of inducing genetic fidelity in *in vitro* derived plants. Some circumstance the occurrence of genetic defects arising via somaclonal variation in the regenerates can seriously limits like agronomic traits, yield, quality and seed setting. Therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility.

At present molecular techniques are powerful and valuable tools used in analysis of genetic fidelity of *in vitro* propagated plants, presently RAPD technique is employed.[4]

MATERIALS AND METHODS

Plant material and pod setting

The *V. roxburghii* plants were collected from Kulasekaram forest, Kerala and Tamil Nadu border, India (N8° 19.755' E77° 18.835'), at an altitude of 60 MSL and maintained in the R&D polyhouse as a source material. This plant flowers regularly, it fails to set pods. This may be due to the lack of pollinating process. As a result, the flowers get dried and withered out. Hence, artificial hand pollination technique was employed for pod setting. The mature pods were harvested and subjected to staining technique by 2,3,5-triphenyl-2-H-tetrazolium chloride (TTC) to confirm seed viability[5] and percentage were calculated.

Surface sterilization

The mature pods and young leaves were surface-sterilized with running tap water for 30 to 45 minutes to remove dust particle. Further, the explants were sterilized with liquid detergent (Labolene 5% v/v) for 10 to 15 minutes, and rinsed five to six times with distilled water. The pods and young leaves were then surface-sterilized with 0.1% mercuric chloride and 70% alcohol for 5 minutes and rinsed five to six times with sterile distilled water to remove the traces of surface sterilizing agents.

Culture media and growth condition

The chief components of medias are macro nutrients, micro nutrients, vitamins, iron source and sucrose (w/v). The pH of the media was adjusted to 5.6 to 5.8 by using 0.1N HCl/NaOH before addition of agar (0.8%) and then autoclaved at 121 °C for 20 minutes. The autoclaved medium was stored at room temperature for 24 hours to check the contamination. The cultures were maintained in the culture room at a temperature of 25 ± 2 °C under built-in white fluorescent light at a photon flux density of 30 to 50 $\mu Em^{-2}s^{-1}$ under a photoperiodic regime of 16 hour light and 8 hour dark cycles.

Seed germination

The mature seeds were inoculated on Vacin and Went medium (V&W)[6], Knudson C medium (Kn'C),[7] MS medium,[8] and TOM[9] fortified with coconut water 10% and casein hydrolysate 3.28 M to study their germination percentage. The PLBs obtained from seeds were maintained through regular sub culturing at 4-week intervals on fresh medium with the same composition to produce more number of PLBs. Percentage of regenerated PLBs were recorded at the end of regeneration period. The tiny green PLBs obtained from seeds were transferred to TOM fortified with different concentrations of cytokinins which are 6-benzyl amino purine (BAP) (4.44, 6.66, 8.88, 11.11, 13.32 µM) and Kinetin (Kn) (4.64, 6.96, 9.28, 11.06, 13.92 µM) independently for development of multiple shoots.

Indirect regeneration

Sterilized leaves sections were cultured on two different sets of MS medium fortified with auxins such as 2,4-Dichlorophenoxyacetic acid (2,4-D) (2.27, 4.52, 6.78, 9.04, 11.30 µM) and α-naphthaleneacetic acid (NAA) (2.68, 5.36, 8.05, 10.74, 13.42 µM) to find the rate of callus induction (data not shown). The obtained callus were sub cultured on TOM supplemented with cytokinins such as BAP (4.44, 6.66, 8.88, 11.11, 13.32 µM) and Kn (4.64, 6.96, 9.28, 11.06, 13.92 µM) with 8.3 M activated charcoal (AC) to get PLBs. The PLBs obtained from callus were maintained through regular subculturing at 4-week intervals on TOM fortified with BAP (8.88 µM) in order to obtain shoots. For root development, shoots were subcultured on TOM fortified with BAP (8.88 µM) as constant concentration along with different concentration of NAA and IAA were analyzed.

Economical aspect and acclimatization

The well-developed plantlets were placed on different types of supporting media such as burnt brick pieces, paper bridge and plastic net pot in liquid TOM fortified with BAP (8.88 µM) and NAA (8.05 µM) for primary acclimatization in *in vitro* condition. The rooted plants were taken out from the culture bottles and washed thoroughly to remove the traces of media and transplanted to clay pots in a potting mixture of burnt bricks and charcoal. The pots were watered twice a day and fertilized at weekly intervals with foliar spray and NPK (1:1) and maintained in the green house as well as orchidarium with controlled temperature, light and relative humidity.

RAPD analysis

For RAPD analysis, fresh leaf samples of *in vitro* derived plants and mother plant were collected from The Himalaya Drug Company campus. The samples were washed with running tap water with a drop of soap oil for 10 min and rinsed with double distilled water to remove surface contaminants. The cleaned leaves of both samples (100 mg each) were frozen separately in liquid nitrogen and ground in a morter to become a fine powder. DNA was extracted from both the leaves as per the manufacturer's instruction manual of NucleoSpin® plant II (Macherey-Nagel). The DNA concentration and purity were analysed by 0.8% agarose gel electrophoresis with ethidium bromide staining and spectrometric method.

Polymerase chain reaction (PCR) amplification was carried out in 25 µL volume using 10 different decamer primers. The reaction mixture comprises of 1U of Taq DNA polymerase (Bangalore Genei), 0.25 µL each of dNTP (10 mM), 5 pmole of decanucleotide primer (Sigma Aldrich), 1× polymerase buffer (2.5 µL), 0.25 mM $MgCl_2$ and 50 ng of DNA sample. The amplification was performed using a thermal cycler (ProFlex™ PCR System, Thermo Fisher Scientific). The program consisted of initial denaturation at 93 °C for 4 min followed by 40 cycles of denaturation at 93 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 2 min, and at a final extension cycle of 8 min at 72 °C. The amplified products were checked in a 2% agarose gel

stained with 10 mg/mL of ethidium bromide and documented by a gel documentation system (Pharmacia Biotech).

Data analysis

All the cultures were examined periodically, each experiment was repeated three times, and subculturing was carried out at 4-week intervals. The mean number of shoots per culture, mean number of roots per shoot and root lengths were recorded. The data were analyzed statistically using one-way ANOVA with analysis of variance to determine the variation between the treatments and critical difference to determine variations within the treatments.

RESULTS

Hand pollination greatly enhanced capsule formation with a frequency up to 100%. After fertilization, the ovary will remain as a part of the raceme and begin to enlarge into a pod. The mature pods were then utilized for *in vitro* seed germination. TTC staining was used to distinguish viable seeds from the population of seeds generated. Seeds with intact embryos turned red due to the conversion of TTC to tetrazolium formazone (TF) and the seed coat became pale green due to malachite green indicating 95% viability (Fig. 1). The unorganized embryos in nonviable seeds failed to turn red, but the seed coat turned green. This will be helpful for analysing the viable seeds in population.

Formation of PLBs from seeds

The seeds started germinating after 7 to 8 days of culture and the embryo developed into tiny green PLBs after 25 days of culture. The maximum germination (87%) was observed in MS medium fortified with 10% CW and CH 3.28 M (Fig. 2). The germination frequency of the seeds ranged between 60 and 72% in all other media tried with varied culture duration (60 - 70 days) (Table 1). Well-developed PLBs were observed after 75 days of culture on MS medium. Further, the shoots were obtained on TOM fortified with BAP (8.88 µM) and it was observed that the maximum number of shoots (8.0 ± 1.00) with a mean length of (3.67 ± 0.28 cm) as achieved with-in 2 to 3 subcultures on the same medium. Whereas in other variable concentrations of BAP and Kn could not produce any remarkable shoot formation from PLBs (Table 2).

Indirect regeneration

The callus was obtained on MS medium fortified with auxins. The different frequency level of initiation of callus was observed after 15 days of culture in all the concentrations of 2,4-D and NAA. The highest frequency of callus initiation (25 g fresh weight/culture) was observed on MS medium supplemented with 2,4-D (2.27 µM) (Fig. 3). However, lowest percentage of callus (5 g fresh weight/culture) was obtained on MS medium fortified with NAA (2.68 µM). After 25 to 30 days, the callus started producing PLBs on TOM fortified with two different cytokinins (BAP, Kn) with AC (8.3 M) showed varying frequency of response. The highest frequency of PLBs formation was observed on TOM supplemented with BAP (8.88 µM) with AC (8.3 M) (Fig. 4), and the lowest frequency of response on TOM fortified with Kn (4.64 µM) with AC (8.3 M). The PLBs were subcultured on TOM fortified with BAP (8.88 µM) to get multiple shoots (Fig. 5).

Keeping the constant concentration of BAP at 8.88 µM with all concentrations of NAA (2.68 - 13.42 µM) and IAA (2.85 - 14.25 µM) in TOM showed positive response towards development of roots. From these two tested combinations highest number of roots and its length were noticed in BAP with NAA (8.05 µM) and BAP with IAA (8.55 µM). Among these, NAA (8.05 µM) has significant effect on producing roots when compared with IAA (8.55 µM). However, on TOM supplemented with BAP and NAA (8.05 µM) showed highest mean number (7.0 ± 0.50) and mean length (5.1 ± 0.83 cm) of roots among all the combinations tested (Fig. 6). Whereas lowest mean number (2.0 ± 0.77) and length (1.3 ± 0.44 cm) were observed in BAP (8.88 µM) with IAA (2.85 µM) (Table 3).

Economical aspect and hardening

The aim of the investigation was to develop cost-effective protocol during primary acclimatization. Autoclaved burnt brick pieces, paper bridge and plastic net pot were used as the substitute for gelling agent in primary acclimatization. The well-developed plants were transferred to TOM containing BAP (8.88 µM) and NAA (8.05 µM) with all substitute for gelling agents. It was noticed that the TOM with autoclaved burnt brick pieces method was suitable for better growth of shoots, roots and primary acclimatization when compared with other tested methods (Fig. 7). The use of burnt brick pieces was not only a substitute source for gelling agent but also minimized the quantity of liquid medium required for its growth and constant supply of nutrients in-to the rooting system. The acclimatized plants were further transferred to clay pots in a potting mixture of burnt brick pieces and charcoal. The pots were watered regularly and fertilized at weekly intervals with a foliar spray and NPK (1:1). The acclimatized plants were maintained in the green house and also orchidarium with controlled temperature, light and relative humidity with 95% survival frequency (Fig. 8).

RAPD analysis

In the present study, genetic similarities were determined in *in vitro* derived hardened field grown plant and mother plant with the help of RAPD technique. To our knowledge, no report was available on the comparative genetic stability of *in vitro* derived plant and mother plant of *V. roxburghii* by using RAPD markers. In the present study 5 RAPD primers were used to screen somaclonal variations. These 5 RAPD primers gave rise to a total of 34 scorable bands and the size ranging from 300 to 1300 bp (Fig. 9). In this way, more than 95% monomorphic bands were obtained from *in vitro*

hardened plant and mother plant (Table 4). Based on RAPD analysis, no somaclonal variations was observed in tissue cultured raised plants with respect to mother plant.

Table 1: Effect of different nutrient media on seed germination and protocorm like bodies (PLB's) of *Vanda roxburghii.*

Type of Media	Culture Duration (in days)	Germination Frequency of PLBs (%)
MS + CM (10%) + Casein hydrolysate 3.28 M	25	87
TOM + CM (10%) + Casein hydrolysate 3.28 M	60	72
V&W + CM (10%) + Casein hydrolysate 3.28 M	70	65
Kn'C + CM (10%) + Casein hydrolysate 3.28 M	65	60

Table 2: Effects of different concentrations of cytokinins for initiation and multiplication of shoots from PLBs of *Vanda roxburghii.*

Medium	Cytokinin combinations	No. of Shoots per Explant $X^* \pm SE$	Shoot length (Cm) $X^* \pm SE$
TOM + BAP (µM)	4.44	1.0 ± 0.60*	1.02 ± 0.32*
	6.66	3.0 ± 0.89**	1.57 ± 0.50***
	8.88	8.0 ± 1.00****	3.67 ± 0.28****
	11.11	6.0 ± 0.97***	1.43 ± 0.24**
	13.32	3.0 ± 0.90**	0.16 ± 0.62
TOM + Kn's (µM)	4.64	1.0 ± 0.48*	0.46 ± 0.21
	6.96	2.0 ± 0.70*	0.67 ± 0.32
	9.28	6.0 ± 0.83***	1.77 ± 0.65***
	11.06	4.0 ± 0.70**	1.00 ± 0.43*
	13.92	2.0 ± 0.70*	0.14 ± 0.24

Note: Number of stars indicate the significant level compared with lower concentration according to one-way ANOVA with Tukey's Multiple Comparison post hoc test.
****: most significant level: P<0.0001

Table 3: Effects of different concentrations of auxins for initiation and elongation of roots of *Vanda roxburghii.*

Basal Medium	Constant Concentration of Cytokinin	Auxins Combinations	No. of Roots Per Explant $X^* \pm SE$	Root Length (cm) $X^* \pm SE$
TOM + BAP + NAA (µM)	8.88	2.68	3.0 ± 0.77*	1.6 ± 0.48*
		5.36	5.0 ± 0.77**	2.6 ± 0.91*
		8.05	7.0 ± 0.50****	5.1 ± 0.83****
		10.74	6.0 ± 0.77***	3.1 ± 0.94***
		13.42	5.0 ± 0.50*	2.3 ± 0.64*
TOM + BAP + IAA (µM)	8.88	2.85	2.0 ± 0.77	1.3 ± 0.45
		5.70	3.0 ± 1.26*	2.3 ± 0.45*
		8.55	4.0 ± 0.83**	4.3 ± 0.64**
		11.40	3.0 ± 0.74*	2.9 ± 0.70*
		14.25	3.0 ± 0.74*	2.2 ± 0.60*

Note : Number of stars indicate the significant level compared with lower concentration according to one-way ANOVA with Tukey's Multiple Comparison post hoc test.
**** : most significant level : P<0.0001

Table 4: The number of amplification products generated of RAPD primers in the analysis of *Vanda roxburghii.*

Sl. No.	Sequence (5'-3')	Oligo primers name	No. of bands obtained	Size of the amplified bands (bp)	No. of Monomorphicbands
1.	5'GCGTTGACTG 3'	HD-2	3	700-1100	3
2.	5'AGGGCAAACC3'	HD-3	11	300-1200	8
3.	5' CCCGTTACAC3'	HD-4	5	500-1300	5
4.	5'CATACGCCTC 3'	HD-5	8	400-1300	7
5.	5'CCGAAGACAC3'	HD-6	7	500-1000	6

Figures.

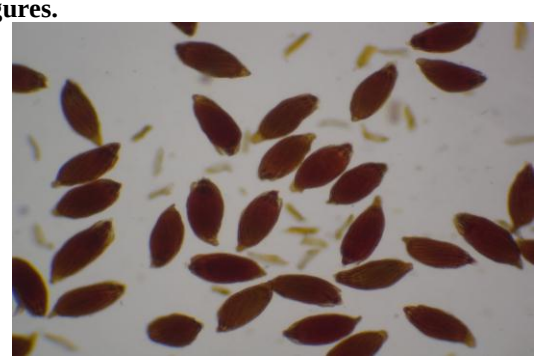

Figure 1: Mature seeds treated with TTC for viability test.

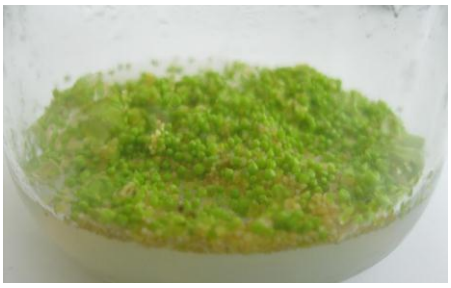

Figure 2: Initiation of PLBs after 25 days of culture on MS medium fortified with coconut water (CW) 10% and casein hydrolysate (CH) 3.28 M.

Figure 3: Well developed callus on MS medium supplemented with 2.27 µM 2,4-D.

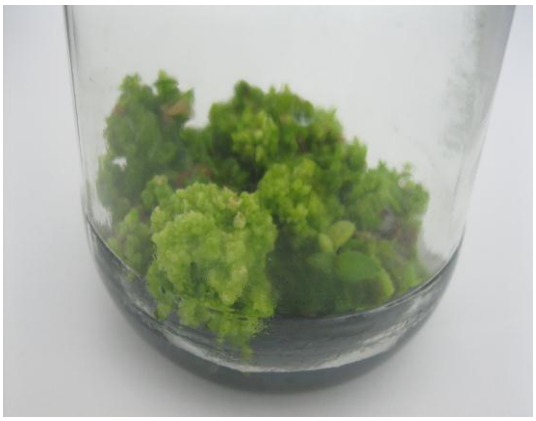

Figure 4: Callus showing PLBs on TOM fortified with BAP 8.88 µM and AC 8.3 M.

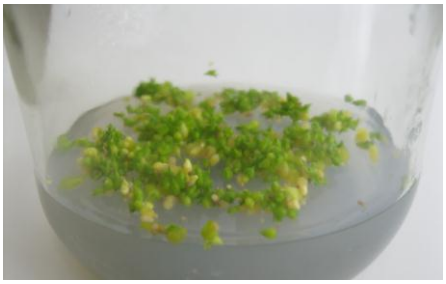

Figure 5: Regeneration of shoots from PLBs on TOM with BAP 8.88 µM.

Figure 6: The Well developed rooted plantlets on TOM with BAP 8.88 µM and NAA 8.05 µM.

Figure 7: Primary acclimatized plants on TOLM contain autoclaved burnt brick pieces.

Figure 8: Plantlets transferred in pots for secondary hardening.

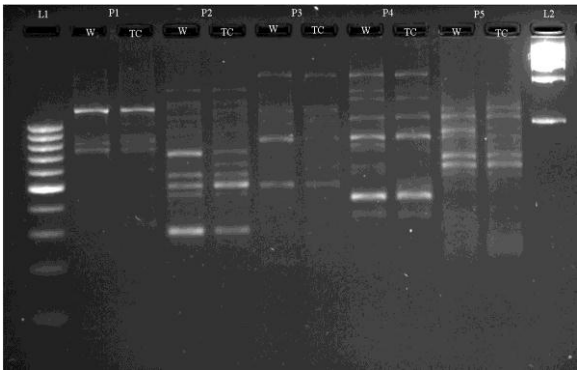

Figure 9: RAPD profile generated by Oligo primers HD 2-6. L1: Marker (100 bp); P1: Primer HD2 (W: wild, TC: tissue cultured); P2: Primer HD3 (W: wild, TC: tissue cultured); P3: Primer HD4 (W: wild, TC: tissue cultured); P4: Primer HD5 (W: wild, TC: tissue cultured); P5: Primer HD6 (W: wild, TC: tissue cultured); L2: Marker (1kb).

DISCUSSION

The tissue culture techniques have tremendous advantages in floriculture, agriculture, horticulture and other economically important plant systems. Plant tissue culture is the only powerful technique for the multiplication and conservation of orchid plants.[10,11,12] In our study, we focused on developing an efficient protocol for the large-scale multiplication of important medicinal orchid *V. roxburghii* through *in vitro* seed germination and indirect regeneration from leaf explants. Further, an attempt was made to reduce the cost of *in vitro* micropropagation of *V. roxburghii* orchid through novel acclimatization technology.

In the Orchidaceae family, insect pollination is very difficult because of the complex morphological structure of the flowers. Hence, hand pollination technique was attempted for pod setting and mature seeds used for *in vitro* multiplication.[13]

The selection of appropriate nutrient medium is also important for the success of all experimental systems in plant tissue culture. In the present study, MS, V&W, Kn'C and TOM basal media were selected to screen for *in vitro* responses. According to Bhojwani and Razdan,[14] in order to formulate a suitable medium for new plant system it would be better to select a popular MS basal medium than others. This agrees with the findings of above work.

Selection of media for orchid seeds propagation is a very crucial step because rate of regeneration is high in matured seeds compared with immature seeds. This statement was seen in the studies of Bonga[15] and Madhulika Singh et al.[16] In our study, mature seeds were inoculated on V&W, Kn'C, MS and TOM basal media to get PLBs. This is further supported by the evidence of Abraham et al.[17] where they obtained PLBs from matured seeds of *Coelogyne nervosa* on MS medium by *in vitro* technique. In our study, MS medium supplemented with 10% CW and CH (3.28 M) was suitable for seed germination and proliferation of PLBs from mature seeds. In general, MS medium has highest concentrations of nitrogen and total ionic concentration when compared with V&W, Kn'C and TOM.[18] The *in vitro* development of *Vanda roxburghii*, *Habenaria macroceratitis*, and *Cattleya* spp. requires inorganic nutrition, which influences the development of seed germination.[19,20] This will support our finding MS as best media for seed germination. Further, addition of CW, CH, yeast extract and peptone is considered to be good sources of carbohydrates, vitamins, amino acids and organic additives that are usually required for orchid seed germination and PLBs formation.[21,22,23,24]

For indirect regeneration, the young leaves were selected during the months of October and November because the plants were in preparation for their vegetative growth and meristematic activity was maximum after dormancy period of summer.[25] This result was coincide with our findings**.** However, callus induction from orchid leaves was very difficult due to its slow growth and high dormancy condition.[26] Hence, in our study we were able to produce callus successfully on MS medium fortified with 2,4-D (2.27 µM) with-in a span of 30 days. Whereas high concentrations of 2,4-D we could not get any remarkable results. These findings are in agreement with Chen et al. and Kuo et al.[27,28] where they found higher concentration of 2,4-D suppresses callus formation in *Oncidium* and *Phalaenopsis* spp.

The TOM containing AC significantly influenced the formation of PLBs since the main function of AC is adsorption of toxin and phenolic exudations from explants, which suppress PLBs formation. This concept in documented by Egyptians findings during 1550 B.C.[29]. *V. roxburghii* explant is rich in phenolic compounds, and this was controlled by addition of AC to the medium which in turn facilitated the activation of endogenous auxin/cytokinin level during incubation. Many researchers succeeded in obtaining somatic embryos and PLBs by using AC with PGR, and they found AC to be greatly helpful in their investigation, such as *Castanea dentate*,[30] *Vitis* spp.,[31] *Myrciaria aureana*.[32] Cytokinin and auxin significantly influenced the development of shoot and root formation from PLBs. It is believed that media containing BAP was sole responsible for development of shoots of *V. roxburghii* and addition of low concentrations of NAA induced better root formation. Similar observations were made in studies on *Oncidium tigrinum*[33] and *Encyclia mariae*.[34]

As alternative to these gelling agents, in our study we used autoclaved burnt brick pieces. This is a new technique and is being used for the first time. Autoclaved burnt brick pieces are cost-effective gelling agent for primary *in vitro* hardening and acclimatization of *V. roxburghii*. In addition to being cost-effective, brick pieces are reusable and eco-friendly as well.

The RAPD technique is simple, quick to perform, requires little quantity of plant material for somaclonal detection and cost effective.[35] Many researches showed that the authenticity of RAPD was best to detect genetic changes in some of tissue culture derived plants such as sugarcane[36] and wheat.[37] This will further strengthen our RAPD data reveals there is no evidence of somoclonal variations between *in vitro* raised hardened field grown plants and mother plants of *V. roxburghii*.

CONCLUSION

The present investigation provides a commercially viable protocol for large scale multiplication of *V. roxburghii* through seeds and indirect regeneration. Adopting in vitro cost reduction technique for primary acclimatization process and field trials. The genetic fidelity of the micropropagated plants were analyzed using RAPD markers.

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