In vitro multiplication of *Oroxylum indicum* (L.) through apical and axillary buds and determination of genetic fidelity using RAPD markers

Dhananjaya Sasalu Panchaksharaiah[1]*, Gururaj Chalageri[2], Uddagiri Venkanna Babu[2]

ABSTRACT

Present study involves to develop a protocol for large scale multiplication of *Oroxylum indicum* L. plants through apical and axillary buds using *in vitro* techniques. The apical and axillary buds were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of 6-benzylaminopurine (BAP) and kinetin. The maximum number of shoots were observed on MS medium supplemented with 8.88 µM of BAP. Rooting was observed in half-strength MS medium supplemented with 8.88 µM of BAP and 7.38 µM of Indole-3-butyric acid. The well-developed plants were hardened in peat and perlite (1:1) and acclimatized in green house and 98% survival rate was observed and then shifted to field for cultivation. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic fidelity of the micropropagated plants. Ten different decamer primers were used to amplify genomic DNA from *in vitro* field grown plants and the mother plant to assess the genetic stability. The RAPD profile analysis revealed that micropropagated plants has no somaclonal variations with the mother plants. These results suggest that the micropropagation protocol developed by this study for rapid *in vitro* multiplication is appropriate and applicable for clonal mass propagation of *Oroxylum indicum* which helps in silviculture developments.

Key words: Genetic fidelity, *In vitro*, Micropropagation, *Oroxylum indicum* L, RAPD marker

1. INTRODUCTION

Oroxylum indicum is one of the important medicinal plant, belonging to the family Bignoniaceae. It is found in India, Sri Lanka, South - East Asia, Philippines and Indonesia up to an altitude of 1200 m [1]. This plant has been used as a single drug or as a component of certain poly-herbal drug preparations in ayurvedic system of medicine. It is an active ingredient of well known ayurvedic formulations like Chyavanprash and Dashmularista [2].

Most parts of *O. indicum* contain bioactive compounds such as carbohydrates, phenolics, terpenoids, carotenoids, anthocyanins and lipids [3]. Root and stem bark contain major flavonoids such as baicalein, oroxylin A and chrysin. Due to these phytoconstituents it has rich therapeutic importance resulting in over exploitation and unsustainable harvesting for commercial purpose. Naturally this plant is propagated by seeds. However, seeds are very thin, papery with broad silver wings and seed dispersal is transpire through anemochory and many seeds may reach unfavourable habitats resulting in poor seed germination. The above two important factors threaten its survival in nature. The flora of this species become vulnerable in Karnataka, Andhra Pradesh and endangered in Kerala, [4, 5].

Various problems related its propagation can be addressed by an emerging skill to conserve this taxon. At present, the plant tissue culture is a potentially useful technique for *ex situ* multiplication and restoration of affected taxa in a short period of time. Thus, an attempt has been made to multiply *O. indicum* through *in vitro* technique. Further genetic fidelity test would support our protocol for true to type clones, since some times *in vitro* raised plantlets might exhibit somaclonal variation by numerous factors such as prolonged culture period or genetic mutation [6, 7]. Major disadvantage of somaclonal variation occurs in plants not suitable for complex agronomic traits like yield, quality and further its seed setting. Normally, morphological, biochemical and molecular analysis have been developed to assure the genetic fidelity of *in vitro* derived clones. However, most of these methods have their own limitations [8]. Polymerase chain reaction (PCR)-based techniques like random amplified polymorphic DNA (RAPD) is very useful in establishing the genetic stability of *in vitro* regenerated plantlets in many crop species [9, 10]. This method is considered to be sensitive enough to detect the variations or genetic relationship among individuals between and within species.

Quite a number of researchers have been published on *in vitro* multiplication of *O. indicum* however, these studies have not attempted fidelity test on *in vitro* raised hardened plants [11, 12, 13].

Nevertheless RAPD marker studies on *O. indicum* were reported by differentiating the genetic diversity of this plant from different geographic locations [14]. Henceforth, the present investigation emphasizes the development of plants through *in vitro* technique and analysis of the genetic fidelity through molecular markers.

2. MATERIALS AND METHODS

2.1 Plant material and surface sterilization

Apical and axillary buds of *O. indicum* were collected from near Alsoor lake, Bangalore, India and used as a source material. The collected specimens were identified using *The Flora of the presidency of* Madras [14]. These freshly collected apical and axillary buds were washed with running tap water containing soap oil for 30 min to remove the dust particles. The explants were then washed twice with Tween 20 for 2 to 3 min and rinsed five to six times with distilled water. For surface sterilization, the explants were immersed in 5.0% Bavistin (Carbendazim) for 1h to eliminate fungal contamination followed by rinsing with autoclaved doubled-distilled water. Further, the explants were sterilized with 70% alcohol for 2 to 3 min and 0.1% mercuric chloride for 2 to 3 min and rinsed three to four times with sterile distilled water under aseptic conditions in a laminar air flow chamber.

2.2 Culture media and growth condition

The Murashige and Skoog's (MS) basal medium, ~~and~~ supplemented with different concentrations of growth regulators like 6-benzylaminopurine (BAP), kinetin (Kn) and with 3% sucrose (w/v) as a carbon source were prepared [15]. The pH of the media was adjusted to 5.6 to 5.8 by using 0.1N HCl/NaOH, before addition of 0.8% agar (w/v) and then autoclaved at 121°C for 20 min. The autoclaved medium was stored at room temperature for 24h to check microbial contaminations. The cultures were maintained in a culture room at a temperature 25 ± 2°C under built-in white fluorescent light at a photon flux density of 30-50 $\mu Em^{-2}s^{-1}$ under a photoperiodic regime of 16 h of light and 8 h of dark cycles.

2.3 Regeneration of shoots and roots

For shoot regeneration, apical and axillary buds (Fig. 1) were cultured on MS medium fortified with different concentrations of cytokinins like BAP (1.11 – 11.11 µM) and Kn (1.16 – 11.62 µM) individually. Percentage of regenerated shoots and the number of shoots per explants were recorded at the end of regeneration period. The shoots were subcultured on half strength MS medium fortified

with BAP 8.88 µM and different concentrations of auxins such as α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) with 2% sucrose (w/v) in all the combinations for root development. The mean number of roots and its length were examined periodically up to 4 weeks of culture.

2.4 Hardening and acclimatization

Hardening and acclimatization is the most important steps in the micropropagation process, where it provides plants to tolerate and surviving in the external environmental conditions. The rooted plants were taken out from the culture bottles and washed thoroughly to remove the traces of agar medium and immersed in Bavistin (5%) solution for 5 min to protect from fungal infection. Then plantlets were transplanted in to pot containing sterilized peat and perlite (1:1) and were kept in green house to maintain high humidity and watered at 2-day intervals**.** Survival rate of the hardened plants were observed before transferring to the field.

2.5 Genetic fidelity testing of *in vitro* raised plants and mother plant

Fresh leaf samples of *O. indicum (*obtained from *In vitro)* were collected from The Himalaya Drug Company campus and mother plants from near Alsoor lake, Bangalore. The samples were washed with running tap water in addition of soap oil for 10 min and rinsed with double distilled water to remove surface contaminants. These cleaned leaves of both samples (100 mg each) were frozen separately in liquid nitrogen and ground in a mortar and pestle to become a fine powder. Total DNA was extracted from the leaves as per the manufacturer's instruction manual of Gen Elute^{TM} Plant Genomic Kit (Sigma Aldrich). 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 consisted 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 (PTC-100TM, MJ Research. Inc., USA). 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 ethidium bromide and documented by a gel documentation system (Pharmacia Biotech).

2.6 Data analysis

All the cultures were examined periodically. Each experiment was repeated 3 times, and subculturing was carried out at 4-week intervals. The mean number of shoots per culture and mean number of roots per shoot and their lengths were recorded. The data were analysed statistically by one-way ANOVA with Tukey's multiple comparison post hoc tests. The minimum level of significance was fixed at $p<0.05$ with two tailed p value. Statistical analysis was performed using GraphPad Prism version 6.0 for windows (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1 Shoot regeneration

Apical and axillary buds did not show any response for shoot initiation on MS basal medium. Different types and concentrations of growth hormones are used for shoot formation as shown in Table 1. Shoot initiation was observed in all the six BAP combinations; however highest initiation response was observed on MS medium fortified with BAP (8.88 µM) after 4 days of culture. After 30 days shoots were ready for first subculture, the shoots attained length (3 - 4 cm) and formed clusters. The maximum number of multiple shoots (9.5 ± 0.10 shoots/explants) and shoot length (6.9 ± 0.44 cm) were observed after second subculture (60 days) on the same medium (Fig. 2). Further, increasing the concentration of BAP showed decline in multiplication rate, in addition MS media with Kn failed to induce shoot proliferation in all tested combinations (Table 1).

3.2 Rooting

In vitro rooting of multiple shoots was achieved on half strength MS medium enriched with different concentrations of auxins (IBA, NAA, IAA) with cytokinin (BAP 8.88 µM) and 2% sucrose. The significant induction of roots (7.5 ± 0.14) per shoot with an average root length (3.9 ± 0.20 cm) was found in culture containing 8.88 µM of BAP with 7.38 µM of IBA within 20 to 25 days of subculture (Fig. 3), whereas, number of root formation ~~were~~ declined with respect to its length in lower concentration of IBA. Subsequently, NAA in combination of BAP showed moderate development of roots with late development of initiation (45-50 days) when compared to earlier concentrations. However IAA with BAP combinations ~~were~~ failed to produce roots in tested media. As an inference, IBA is referred to as the sole auxin to induce rooting in *in vitro* condition (Table 2).

3.4 Hardening of plantlets

The rooted plantlets were transferred to pots containing sterilized peat and perlite (1:1) and kept in the greenhouse for hardening (Fig. 4). After four weeks of incubation 98% survival rate was noticed. ~~in the greenhouse.~~ Later the plants were transferred to a normal environment (Fig. 5).

3.5 RAPD and genetic fidelity analysis

The evaluation of the genetic fidelity of tissue culture derived plants is ~~an~~ essential for detection of true-to-type clones. In the present study, homogeneity of genetic materials of *in vitro* derived plants was compared to mother plant with the help of RAPD technique. To our knowledge, no report was available to ~~ill~~ date on the comparative genetic stability of *in vitro* hardened plants and mother plant of *O. indicum* by using RAPD markers. Based on RAPD analysis, no somaclonal variations was observed in tested plants (Figs. 6, 7, 8). In the present study, total number of bands observed was 73 out of which 31 bands were monomorphic in nature (16.6 % - 50%). The number of amplification products obtained by these primers are summarised in Table 3. The size of the monomorphic DNA bands produced by the tested primers ranged from 250 bp to 2000 bp.

4. DISCUSSION

In the present study, we standardized the protocol for multiple shoot formation from apical and axillary bud of *O. indicum*. Six different concentrations of growth regulators (BAP and Kn) were tested for shoot proliferation. The maximum number of shoots with significant shoot length was observed in MS medium fortified with 8.88 µM of BAP after second subculture. Out of two cytokinins BAP was identified as significant growth hormone for shoot initiation and multiple shoot formation. Earlier reports have shown that MS with BAP media stimulate the regeneration of plants and gives more shoot regeneration than Kn [16, 17]. The present finding is also agreement with earlier study [18].

The significant development of roots was observed in half strength MS medium containing 8.88 µm of BAP and 7.38 µM of IBA with 2% sucrose. In the present investigation, IBA is used as the sole exogenous auxin to produce rooting in *in vitro* regenerated shoots. The similar kind of success of IBA in promoting efficient root induction has been reported for *Clitoria ternatea* L., *Murraya koenigii* L. Spreng, and *Ocimum basilicum* L. [19, 20, 21].

In vitro regenerated plants do not survive without acclimatization, because they have to adjust external environmental conditions like light, humidity and temperature. However, *in vitro* raised plants need gradual changes in environmental conditions to avoid desiccation, water relations and

photoinhibition. In the present study, micropropagated plants were acclimatized [22, 23, 24] in primary hardening chamber and transferred to green house where 98% survival rate was found.

The evaluation of the genetic stability of tissue culture derived plants is an essential step to assess true-to-type clones [25]. The present study confirms the homogeneity of micropropagated plantlets compared with that of the mother plant by using RAPD technique. In general, the *in vitro* raised plants obtained from apical meristems and axillary meristems do not alter any genetic makeup, hence this mode of propagation is considered as a safest method to multiply the plantlets [26, 27]. This will further strengthen our RAPD data reveals there is no evidence of somaclonal variations between *in vitro* raised hardened plants and mother plants of *O. indicum*. Hence, this is an efficient protocol for the regeneration of *O. indicum* through apical and axillary buds. However, the present protocol will help the plant breeders and foresters able to generate disease-free, uniform parental clones with genetic fidelity to generating the population.

5. CONCLUSION

The present investigation is focused on establishment of mass multiplication of *O. indicum* through micropropagation tool. Researchers who have performed various studies ~~have deduced~~ used protocols to obtain plant-lets through high concentration of growth regulators~~,,~~ whereas, the present study provides a protocol to develop multiple shoots, roots and hardened plants in least concentration of growth regulators with maximum number of shoots and roots followed by well developed plants that are propagated in a normal environment successfully. The assay of genetic fidelity through RAPD markers is a very important aspect for analyzing the somaclonal variation in *in vitro* propagated plants. Plants developed in this study have not shown any variation in the genetic makeup compared with the mother plant. This kind of investigation is carried out for the first time for *O. indicum* to ascertain its stability and this protocol could be an important source for forest crop breeders, species conservation and herbal industries

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COMPETING INTERESTS

This manuscript has not been published and is not under consideration for publication elsewhere. The authors declare that there is **"No conflict of interest**".

AUTHORS CONTRIBUTIONS

DSP and GC were designed the study and draft the manuscript. UVB has analyzed the draft and made it into final format. All authors read and approved the final manuscript

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Tables

Table 1.Effect of different concentrations and combinations of cytokinin on development of multiple shoots in *Oroxylum indicum*

Growth regulators concentration in MS media (μM)		Mean number of shoots/culture ± SE	Mean length of shoots (cm) ± SE
BAP	Kn		
1.11	-	8.5 ± 0.08	3.4 ± 0.15
2.22	-	9.0 ± 0.11*	4.1 ± 0.22*
4.44	-	9.2 ± 0.11**	5.1 ± 0.24**
6.66	-	9.3 ± 0.11***	5.8 ± 0.30***
8.88	-	9.5 ± 0.10****	6.9 ± 0.44****
11.11	-	8.6 ± 0.09**	5.3 ± 0.33***
-	1.16	-	
-	2.32	-	
-	4.65	-	
-	6.97	-	
-	9.30	-	
-	11.62	-	

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 2 - Effect of plant growth regulators for development of roots

Growth regulators concentration in MS media (μM)		Mean number of roots	Mean length of roots
BAP	NAA		
8.88	1.35	6.2 ± 0.13	3.0 ± 0.21
8.88	2.70	6.4 ± 0.14**	3.2 ± 0.19***
8.88	5.40	6.8 ± 0.10***	3.3 ± 0.17***
8.88	8.10	5.8 ± 0.14*	2.7 ± 0.13**
8.88	10.80	5.3 ± 0.11	2.2 ± 0.20
	IAA		
8.88	1.42	-	-
8.88	2.85	-	-
8.88	5.71	-	-
8.88	8.21	-	-
8.88	11.42	-	-
	IBA		
8.88	1.23	5.0 ± 0.12	2.0 ± 0.26
8.88	2.46	5.6 ± 0.15*	2.6 ± 0.12**
8.88	4.92	6.5 ± 0.14**	3.0 ± 0.18***
8.88	7.38	7.5 ± 0.14****	3.9 ± 0.20****
8.88	9.84	6.8 ± 0.11***	2.0 ± 0.26

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 - The number of amplification products generated of RAPD primers in the analysis of *Oroxylum indicum* regenerants.

Sl. No.	Sequence (5'-3')	Oligo primers name	No. of bands obtained	Size of the amplified bands (bp)	No. of Mono-morphic bands	Mono-morphic (%)
1.	5'AGAGGCTCAC 3'	HD-1	7	390-2000	3	42.85
2.	5'GCGTTGACTG 3'	HD-2	4	400-600	2	50
3.	5'AGGGCAAACC3'	HD-3	8	250-1300	4	50
4.	5' CCCGTTACAC3'	HD-4	6	600-1300	2	33.3
5.	5'CATACGCCTC 3'	HD-5	12	300-1800	6	50
6.	5'CCGAAGACAC3'	HD-6	6	290-500	2	33.3
7.	5'GCAGTACGAG 3'	HD-7	7	350-1000	3	42.85
8.	5'GCAGTACGTG 3'	HD-8	10	300-1900	5	50
9.	5'CGAGGAGACC3'	HD-9	6	300-1900	1	16.6
10.	5'GTTCCCAGAC3'	HD-10	7	550-1200	3	42.85

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

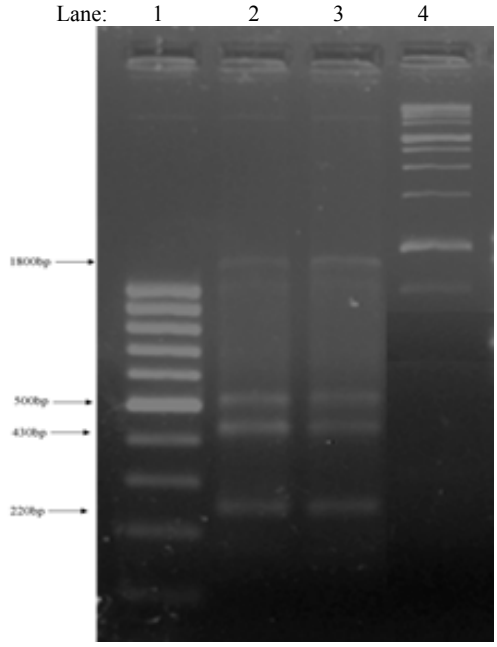

Figure 6

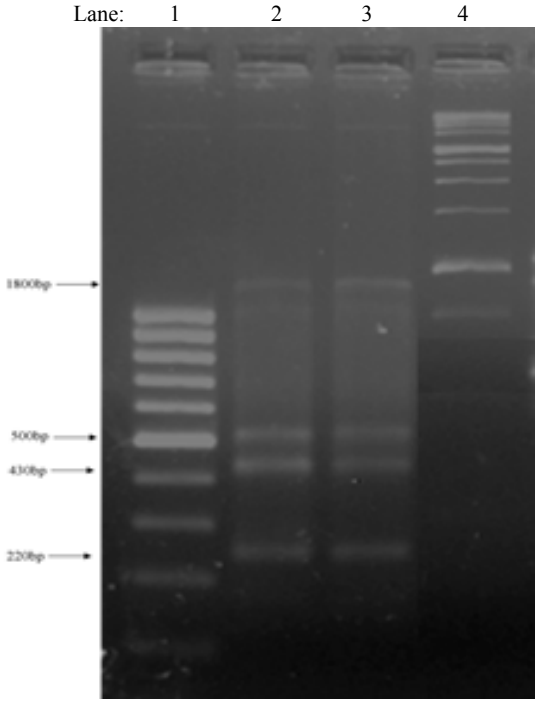

Figure 7

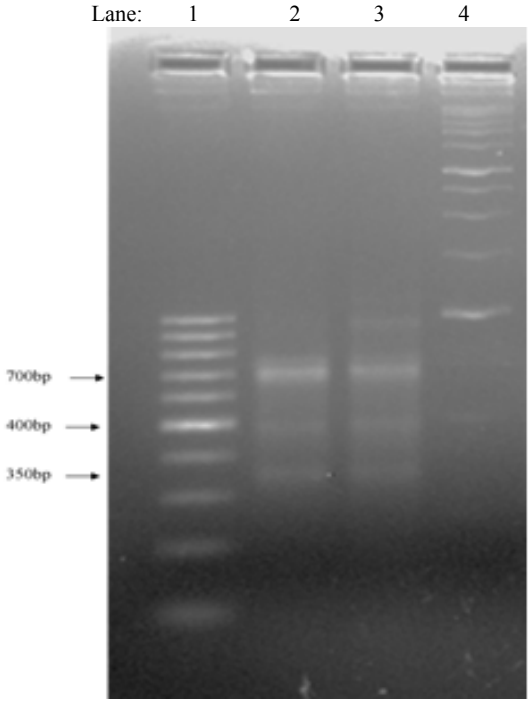

Figure 8

Figures legends

Fig 1 - Apical meristems inoculated in medium

Fig 2 - Regeneration of multiple shoots observed in MS medium contain BAP 8.88 µM

Fig 3 - Development of roots in half MS medium with 2% sucrose and IBA (4.92µM)

Fig 4 - Hardened plantlets of *Oroxylum indicum* in green house

Fig 5 - In vitro raised *Oroxylum indicum* growing in a normal environment

Fig 6 - A RAPD profile generated by Oligo primer HD 3. Lane 1: Marker (100 bp); Lane 2: Mother plant; Lane 3: *In vitro*-raised hardened plant; Lane 4: Marker (1kb)

Fig 7 - RAPD profile generated by Oligo primer HD 5. Lane 1: Marker (100 bp); Lane 2: Mother plant; Lane 3: *In vitro*-raised hardened plant; Lane 4: Marker (1kb)

Fig 8 - RAPD profile generated by Oligo primer HD 7. Lane 1: Marker (100 bp); Lane 2: Mother plant; Lane 3: *In vitro*-raised hardened plant; Lane 4: Marker (1kb)