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GENETIC FIDELITY OF MICROPROPAGATED *OROXYLUM INDICUM* (L.) VENT. BY RAPD MARKER

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Abstract

The present work was undertaken to assess the genetic fidelity of micropropagated plant as well as mother plant using random amplified polymorphic DNA (RAPD). Apical buds and axillary buds were used as explant for mass multiplication. Fourteen arbitrary decamer primers generated clear and distinct bands. Total genomic DNA extracted from fresh leaves of *in vitro* regenerated as well as mother plants. No genetic variation was found within the efficacy of the protocol developed for the production and conservation of the selected plant.

Keywords: *Oroxylum indicum*, Genetic fidelity, RAPD, Molecular marker.

Introduction

Oroxylum indicum (L) Vent. which belongs to the family Bignoniaceae, is one of the most important plant, distributed throughout India and other country like Sri Lanka, Malaysia, China, Thailand, Philippines and Indonesia (Anonymous, 1972). This plant has been used in Ayurvedic formulation like Dashmularisht and Chyawanprash (Yasodha *et al.*, 2004). It is also important ingredient of Ayurvedic formulation such as Amartarista, Dantyadyarista, Narayana Taila, Dhanawantara Ghrita, Brahma Rasayana, Awalwha (Anonymous, 1998).

The natural population of *Oroxylum indicum* is decreasing in its habitat due to high demand in many pharmaceutical industries, its indiscriminate collection, over exploitation and uprooting of whole plants it has been categorized as vulnerable in Karnataka and Andhra Pradesh and endangered in Kerala, Maharastra, M.P. and Chhatisgarh (Darshan and Ved, 2003; Jayaram and Prasad, 2008) . Its low seed viability and poor vegetative propagation is also a reason.

Therefore, to overcome all these problems, plant tissue culture is an alternative method for mass multiplication. However, there is possibility of developing somaclonal variation in *in vitro* cultures (Bindiya and Kanwar, 2003; Martins *et al.*, 2004). Apart from morphological observation, the genetic stability of the *in vitro* regenerated plant should be assessed by using molecular markers. A number of molecular markers can be used to detect genetic fidelity of *in vitro* developed plant. Out of these Random Amplified Polymorphic DNA (RAPD) is the cheapest yet reliable and could be a powerful tool for the detection of genetic variability in plants (Fernando *et al.*, 1996; Cassells *et al.*, 1997). This technique does not require any prior knowledge of DNA sequence for primer design (Fang and Roose, 1997). Based on this fact, the present work was carried out for the assessment of genetic fidelity of micropropagated plants using DNA based markers RAPD. RAPD marker study on this plant was seen by Jayaram and Prasad (2008), but they have been reported genetic diversity of this selected plant from different geographic region. Hence, the present study was undertaken to study genetic fidelity of *in vitro* raised plant and mother plant.

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Materials and Methods

A. Plant materials

The seeds from mature fruits were collected from selected plant during January-February, 2012-2013 at Hemchandracharya North Gujarat University, Patan, Gujarat, India. Collected seeds were pre-treated with aqueous solution of different growth regulators like Gibberellic acid (GA_3), Kinetin (Kn), 6-Benzylaminopurine (BAP) and Zeatin. The seeds were surface sterilized in 0.01% (v/v) Tween – 20 for 2 minutes. Followed by 0.1% (w/v) $HgCl_2$ for 2 minutes. Each treatment was followed by repeated washing minimum of 3 times in autoclaved distilled water. Surface sterilized seeds were inoculated in glass tubes containg Murashige and Skoog (1962) (MS) Media supplemented with 30 gl^{-1} sucrose and 0.8% agar without any plant growth regulators. Apical bud and axillary bud were excised from

5-6 weeks old *in vitro* raised seedlings for *in vitro* propagation.

B. Culture medium

Each explants were cultured aseptically in MS medium with different concentration and combination of BAP and Kn with IAA for shoot multiplication. For root induction, these plantlets (3-4 cm long) were cultured on half strength MS medium with different concentration of IBA, IAA and NAA.

C. Acclimatization

Well developed rooted plantlets were transferred to cup with sterile garden Soil: Sand: vermicompost in the ratio of 2:1:1: for a month and subsequently transferred to pots. Later these *in vitro* developed plants were established in the field.

D. RAPD analysis

a. DNA extraction

DNA was extracted from fresh leaves of *in vitro* raised plant and mother plant by using cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some modification. For this purpose, 6 *in vitro* raised plants were randomly selected and compared with mother plant. fresh leaves (~ 500 mg) were freezed using liquid nitrogen. Frozen samples were grounded thoroughly in to fine powder in a pre-chilled mortar and pestle. To this 2mg Polyvinyl pyrrolidine (PVP) was added. the mixture was transferred to centrifuge tubes containing 4 ml preheated extraction buffer (Containing EDTA (50 mM), Tris-HCl (100mM), NaCl (5M), 2% CTAB, 1 % PVP, 2 mercaptoethanol 1% Solution). Then the tubes were incubated at 65 ºC for 60-90 minutes with occasional shaking. After removing from water bath, add equal volume of Chloroform: Iso-amyl alcohol (24:1, v/v) and mixed by inversion for 15 minutes. After that centrifuged at 10,000 rpm for 10 min. Collect the supernatant, add equal volume of Iso-propanol and sodium acetate (2mM) was added. It was incubated at -20^{0}C overnight. DNA precipitate was collected by centrifugation at 10,000 rpm for 10 minutes at 4^{0}C. Pellet was washed with 70% ethanol, by centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The pellet was resuspended in 0.5 ml TE buffer and also 2 µl RNase A Solution for Purification purpose incubated for 1 hour at 37^{0}C. Equal volume of mixture of chloroform: isoamylalcohal (24:1) was added. It was centrifuged at 12,000 rpm for 10 min at 4^{0}C. The aqueous phase was collected and mixed cold absolute chilled 100% ethanol for precipitation of DNA. After centrifugation, at 12,000 rpm for 10 min. at 4^{0}C. Finally, DNA pellet was dissolved in 50 µl of 1X TE buffer and stored at -20 ºC for future analysis.

After DNA extraction, quantity of DNA was estimated by using spectrophotometer Nanodrop N.D.1000 (Software V.3.3.0) at absorbance ratio 260/ 280 nm. The quality of the extracted DNA was tested by running the DNA in 0.8% agarose gel.

b. PCR amplification

Sixty arbitrary 10-base RAPD primers (Operon technologies, USA) were used for PCR amplification, following the protocol of Williams et al (Williams *et al.*, 1990) with minor modification. Amplification reaction were performed in 25 µl volumes contained 2.5µl of 10 × assay buffer, 10 mM dNTPs (Thermo Scientific, India), Taq DNA polymerase (5U/ µl),(Thermo scientific, India), random primers (5 pMole/µl), template DNA (30 ng). The amplification reaction was carried out in DNA thermal cycler (Eppendrof Gradient, Germany). Reaction program were set at 94 ^{0}C for Initial DNA denaturation for 4 minutes. Followed by 37 cycles of 94 ^{0}C for 1 minute, 1 minute for primer annealing at 38 ^{0}C and 2 minute extension at 72 ^{0}C, final extension at 72 ^{0}C for 5 minute. After amplification, the products were analyzed on 1.5% agarose gel in 1 × TAE buffer, stained with ethidium bromide. The gels were documented with gel doc system (BioRad, USA). Bands were estimated by comparing them to standard DNA ladder (Bangalore Genei, India).

c. Data Scoring

Only clear and scorable bands were considered for data analysis.

Results and Discussion

The seeds of *Oroxylum indicum* germinated within 3-4 weeks of culture in nutrient medium. GA_3 had best effect on shoot length, root length, fresh and dry weight (Rami and Patel, 2014). Among the various concentrations maximum multiplication of shoots were found in MS media supplemented with 2.0 mg/l BAP in combination with 0.2 mg/l IAA. On this medium, the maximum number of shoots (8.14 ±1.13) shoot length (8.26 ± 0.79 cm). Similar results were obtained in *Bacopa monneri* (L.) (Tejavathi and Shailaja, 1999). Well developed shoots were transferred to the half strength MS media supplemented with IAA, IBA and NAA. Maximum numbers of healthy roots (8.3 ± 0.69) were found with 1.0 mg/l NAA and 1.0 mg/l IAA (Data not shown).

DNA extraction from different plant samples is the most important step. DNA can be extracted either from fresh preserved or dried samples. Due to some contamination like protein, polysaccharide and RNA there are some difficulties to get plant DNA. Quantity of DNA samples was assessed by taking absorbance at 260/280 nm ratio by nanodrop spectrophotometer as well as visual assessment of band intensity using agarose gel. A ratio of absorbance at 260/280 nm from various samples, which ranged from 1.7-1.9 showed that DNA was free from contamination.

Table 1: List of RAPD primers.

S. No.	Primer Series	Sequence
1	OPA-05	5‘-AGGGGTCTTG-3‘
2	OPA-13	5‘-CAGCACCCAC-3‘
3	OPA-20	5‘-GTTGCGATCC-3‘
4	OPE-04	5‘-GTGACATGCC-3‘
5	OPE-06	5‘-AAGACCCCTC-3‘
6	OPF-02	5‘-GAGGATCCCT-3‘
7	OPF-03	5‘-CCTGATCACC-3‘
8	OPF-04	5‘-GGTGATCAGG-3‘
9	OPF-05	5‘- CCGAATTCCC-3‘
10	OPF-10	5‘GGAAGCTTGG-3‘
11	OPF-12	5‘-ACGGTACCAG-3‘
12	OPF-13	5‘-GGCTGCAGAA-3‘
13	OPF-16	5‘-GGAGTACTGG-3‘
14	OPF-19	5‘- CCTCTAGACC-3‘

For RAPD analysis sixty primers from Operon Technologies (Alameda, California,USA) were screened to generate DNA fingerprint profile of selected plant. Out of sixty random primers used for 7 samples, 14 primers showed amplified scorable band pattern while 46 primers did not produce any amplification. The details of different primers amplification are given in table- 1.

These fourteen RAPD primers generated 104 amplicons in total, ranging from 166-4493 bp in size. The maximum numbers of bands (11) were obtained with the marker (OPF-13). The result showed different primers generated different fragments numbers and length of DNA. The number of bands in the selected primers varied from 4 to 12, with an average of 7.4 bands per RAPD primer (table- 2, figure-2).

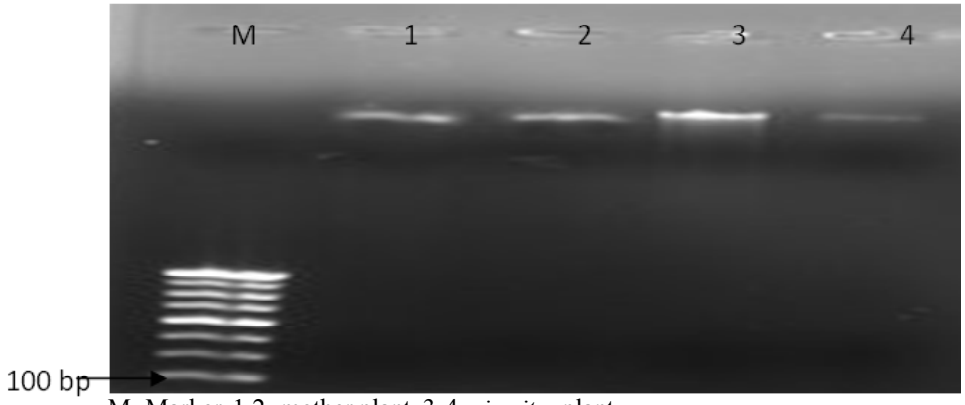
Molecular tool are more reliable observation for evaluating variations. RAPD technique has been used for the assessment of genetic relationship and variation. In present study, RAPD profiles generated by 14 primers indicated a uniform pattern among the mother plant and

Table 2: RAPD analysis of *Oroxylum indicum.*

Primer	Range of fragment size (bp)	Total No. of fragments	No. of polymorphic Fragments
OPA-05	524-2194	7	2
OPA-13	230-1581	8	0
OPA-20	312-1768	7	0
OPE-04	215-1640	5	0
OPE-06	335-1429	4	1
OPF-02	232-1193	8	1
OPF-03	166-1697	8	1
OPF-04	192-1484	9	0
OPF-05	358-1637	9	2
OPF-10	182-1486	10	0
OPF-12	569-1444	6	0
OPF-13	286-2795	11	0
OPF-16	555-2677	7	0
OPF-19	622-2365	6	0

micropropagated plant**.** Genetic assessment of micropropagated plants by RAPD markers has been reported by many workers in different plant group (Rani *et al.*, 1995; Rout and Das, 2002; Rout, 2002; Sanghmitra and Satyabrata, 2008; Gupta *et al.*, 2009; Mohan *et al.*, 2012). Some researchers found variability in RAPD profiles among the *in vitro* derived plantlets in different plants (Bhowmik *et al.*, 2009; Chen *et al.*, 1998; Khoddamzadeh, 2010). Shoot multiplication through axillary bud proliferation maintains genetic stability in tissue culture generated plants rather than the plants regenerated through adventitious bud explant (Balachandran *et al.*, (1990); Shenoy and Vasil (1992); Joshi and Dhawan (2007). However, occasionally there is still a chance of obtaining somaclonal variation through adopting this approach (Rani and Raina (2000).

The present study provides the first report on the genetic fidelity of micropropagated *Oroxylum indicum* (L.) Vent. obtained using RAPD. No variability was



M- Marker, 1,2- mother plant, 3-4 – *in vitro* plant

Fig.1: Electrophoresis of genomic DNA from mother plant and *in vitro* plant

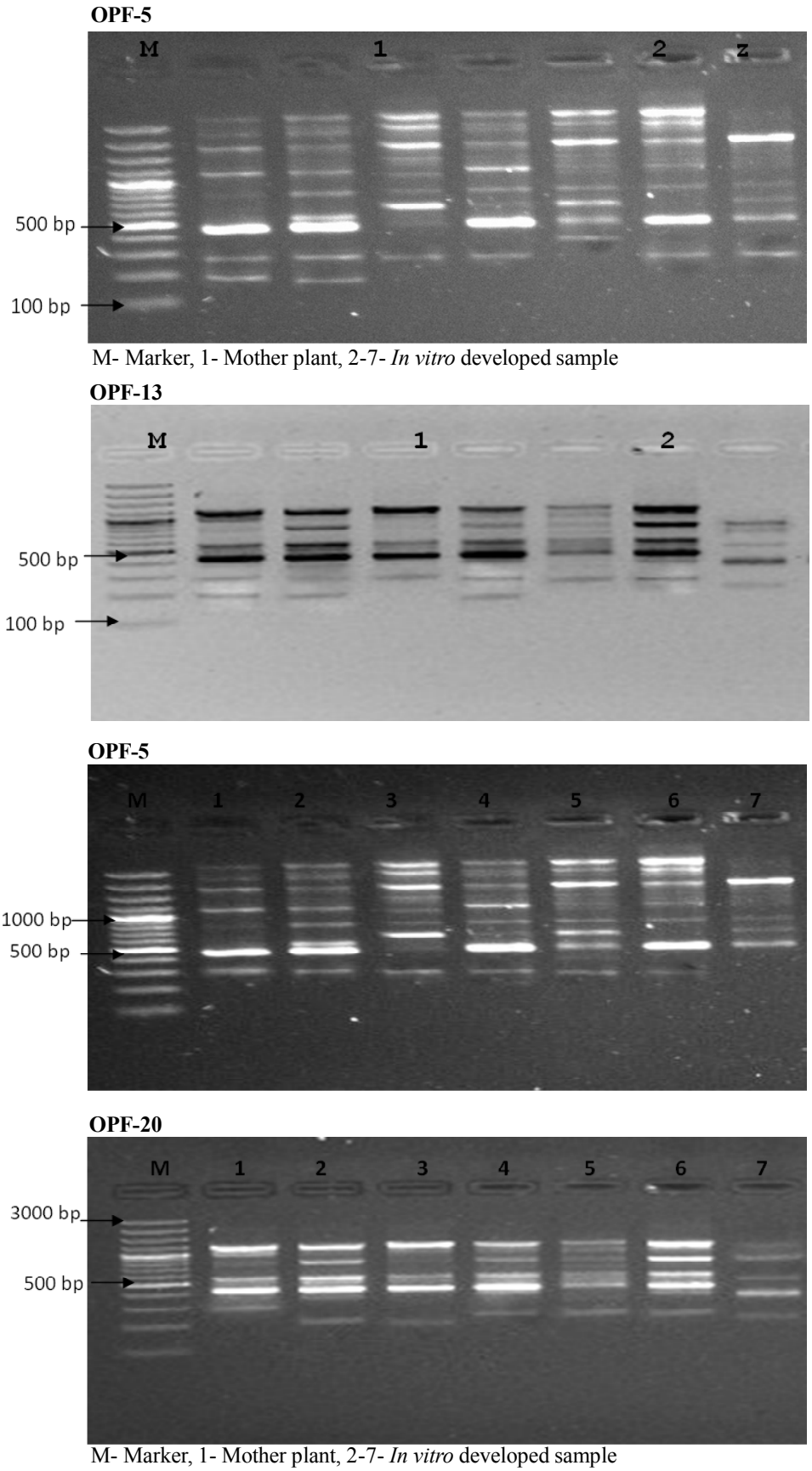


Fig. 2: RAPD analysis of mother plant and *in vitro* plant

detected among the tissue cultured plantlets and mother plant hence it was concluded that the protocol developed could be effectively used for rapid micropropagation without risk of genetic instability.

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