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An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers

G.R. ROUT* and G. DAS

Plant Biotechnology Division, Plant Tissue Culture Laboratory, Regional Plant Resource Centre, Bhubaneswar-751 015, Orissa, India

Abstract

Clones of *Plumbago zeylanica* were micropropagated using nodal culture. The application of random amplified polymorphic DNA (RAPD) in assessing the genetic integrity of the micropropagated plants was evaluated by polymerase chain reaction. Twenty arbitrary decamers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material to assess the genetic fidelity. All RAPD profiles from micro-propagated plants were monomorphic and similar to those of field grown mother plants. No polymorphism was detected within the micropropagated plants.

Additional key words: genetic stability, *in vitro*, polymerase chain reaction.

Introduction

In vitro culture technique has long been recognised as an efficient tool for rapid clonal multiplication. However, the occurrence of somaclonal variation among micropropagated plants has expanded the application of this technique to crop improvement (Skirvin 1978). Plant regeneration *in vitro* and re-introduction into the original or favourable habitats is one strategy for conservation of important plant species. Monitoring the degree of genetic integrity between *in vitro* raised plants using molecular markers is desirable to reduce the chances for inclusion of variable genotypes. There are a number of molecular markers used for such assessment, however, random amplified polymorphic DNA (RAPD) is the cheapest and appear to be powerful tool for the detection of genetic variability in plants (*e.g.*, Williams *et al.* 1990, Cassells *et al.* 1997).

Plumbago zeylanica L. (*Plumbaginaceae*) is an important medicinal plant, used in the treatment of dyspepsia, piles, diarrhoea and skin diseases. The roots of this plant are the main source of an alkaloid, plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) used as anticancer drug (Krishnaswamy and Purushothaman 1980). Preservation of genetic stability in germplasm collections and *in vitro* raised plants is of the utmost importance. In this paper, we describe the rapid micropropagation of *Plumbago zeylanica* through meristem culture and evaluate the genetic homogeneity using RAPD technique.

Materials and methods

Plumbago zeylanica L. were collected from Chandaka Reserve Forest, Bhubaneswar, Orissa, India. Internodal segments (3 - 4 cm long) with leaves were collected and washed with 2 % (v/v) detergent *Teepol* (*Qualigen*, Bombay, India) and rinsed with running tap water. The explants were surface sterilized in 0.1 % (m/v) aqueous mercuric chloride solution for 15 min followed by washings with sterile distilled water. The internodal segments further cut into 0.5 - 1.0 cm pieces having one node in each segment. Each segment was cultured in

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Abbreviations: BA - 6-benzylaminopurine; bp - base pair; IAA - indole-3-acetic acid; IBA- indole-3-butyric acid; Kin - kinetin; NAA - 1-naphthaleneacetic acid; MS medium - Murashige and Skoog (1962) medium; RAPD - random amplified polymorphic DNA.

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*Address for correspondence; fax: (+91) 674 550274, e-mail: grrout@usa.net

Murashige and Skoog (1962; MS) medium supplemented with different concentration and combinations of 6-benzylaminopurine (BA) or kinetin (Kin) (0.0, 0.25, 0.5, 1.0, and 1.5 mg dm^{-3}) and indole-3-acetic acid (IAA; 0.0, 0.01, 0.1, and 0.25 mg dm^{-3}) for bud proliferation and multiplication. For induction of rooting, the excised microshoots (1 - 2 cm) were cultured on half-strength MS basal medium supplemented with different concentrations of IAA or indole-3-butyric acid (IBA) (0.0, 0.05, 0.1, 0.25, and 0.5 mg dm^{-3}) and 2 % (m/v) saccharose. The pH of the media was adjusted to 5.8 using 0.1M NaOH before autoclaving. The MS medium was gelled with 0.8 % (m/v) agar (*Qualigen*, Bombay, India). Routinely, 25 cm^3 molten media was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheese cloth and sterilized at 121 °C and 1.06 kg cm^{-2} pressure for 15 min. All cultures were incubated at temperature of 25 ± 2 °C and irradiance of 55 μmol $m^{-2}s^{-1}$ (cool, white fluorescent lamps) in 16-h photoperiod. The cultures were subcultured at 4-week intervals to fresh medium with the same composition.

Rooted plantlets were removed from culture vessels, washed gently under running tap water and planted in earthen pots (5 cm in diameter) containing a mixture of sand, soil and well rotted cow-dung manure (1:1:1; v/v/v). The plantlets were kept in the greenhouse for acclimatization before subsequent transfer to the field.

DNA was extracted from fresh leaves derived from both micropropagated and field grown mother plants by the cetyltrimethyl ammonium bromide (CTAB) method (Bousquet *et al.* 1990). Approximately, 500 mg of fresh leaves were ground to a powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 25 cm^3 sterile polypropylene tube with 10 cm^{-3} of CTAB buffer. The extraction buffer consisted of 2 % (m/v) CTAB, 1.4 M NaCl , 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 0.2 % (v/v) β-mercaptoethanol. The homogenate was incubated at 60 °C for 2 h, extracted with an equal volume of chloroform:isoamyl alcohol (24:1), and centrifuged at 10 000 *g* for 20 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10 000 *g* for 10 min., the DNA pellet was washed with 70 % ethanol, air dried, and resuspended in 10 mM Tris pH 8.0, 0.1 mM EDTA buffers. DNA quantity was estimated spectrophotometrically (*V-530*, *Jasco*, Japan) by measuring the absorbance at 260 nm.

Twenty arbitrary 10-base primers (*Operon Technologies*, Alameda, USA) were used for Polymerase Chain Reaction (PCR). Amplification reactions were performed in volumes of 0.025 cm^{-3} containing 0.002 cm^{-3} of 1.25 mM each of dNTP's, 15 ng of the primer, 1x Taq polymerase buffer, 0.5 U of Taq DNA polymerase (*Genei*, Bangalore, India) and 20 ng of genomic DNA. DNA amplification was performed in a PTC-100 DNA Thermal Cycler (*M.J. Research*, Watertown, USA) programmed for 45 cycles as follows: 1st cycle of 3.5 min at 94 °C, 1 min at 37 °C and 2 min at 72 °C; followed by 44 cycles each of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C followed by one final extension cycle of 7 min at 72 °C. Amplified products were electrophoresed in a 1.0 % (m/v) agarose (*Sigma*, St. Louis, USA) gels with 1x TAE buffer, stained with ethidium bromide, and photographed under UV light. The sizes of the amplification products were estimated for a 100-bp (100-bp to 3.0 kb) ladder (*MBI Fermentas*, Amherst, USA), All the reactions were repeated at least thrice.

Amplified DNA markers were scored as present or absent both in the regenerated and the mother plants. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

Fifteen cultures were used per treatment and each experiment was repeated three times. All cultures were examined periodically and visual observations of any morphological changes were recorded. The data were analysed statistically by the Duncan's multiple range test (Harter 1960).

Results and discussion

The axillary buds proliferated within 5 - 6 d of culture on MS medium containing 0.5 - 1.0 mg dm^{-3} BA. The axillary shoots proliferated and elongated to 1.0 - 1.5 cm within 2 weeks of culture (Fig. 1*a*). Shoot multiplication was achieved on medium containing 1.0 mg dm^{-3} BA with 0.01 mg dm^{-3} IAA and a maximum of 8.8 shoots were produced per culture within 4 weeks of culture (Table 1). The multiplication ability was maintained up to the eight subculture period on MS medium having 1.0 mg dm^{-3} BA and 0.01 mg dm^{-3} IAA by regular subculture every 4 weeks (Rout *et al.* 1998). Kin alone or in combination with IAA also promoted shoot multiplication but in less frequency as compare to BA or BA in combination with IAA. The percentage of shoot multiplication was better in the media of BA + IAA (90.8 %) as compared to in media having Kin alone (58.8 %). The frequency of shoots per culture varied from 3.0 - 8.8 % in medium containing BA alone or BA + IAA and 2.6 - 4.2 % in Kin or Kin + IAA, respectively (Table 1). The differential response in varying concentrations of the growth regulators used in the medium as in agreement with the previous results (*e.g.*, Chen *et al.* 1995, Sharma and Singh 1997).

Microshoots derived from axillary buds were rooted on half-strength MS media containing either IAA or IBA with 2 % (m/v) saccharose (Table 2). A optimum

percentage of shoots rooted in the medium containing half-strength MS basal salts supplemented with 0.25 mg dm^{-3} IBA; within 7 - 8 d of transfer without an intervening callus (Fig. 1*b*). Rooted plantlets were acclimatized in the greenhouse and 90 % plants survived 4 weeks after transfer (Fig. 1*c*). The plants grew normally in the greenhouse and no gross morphological variation was noticed.

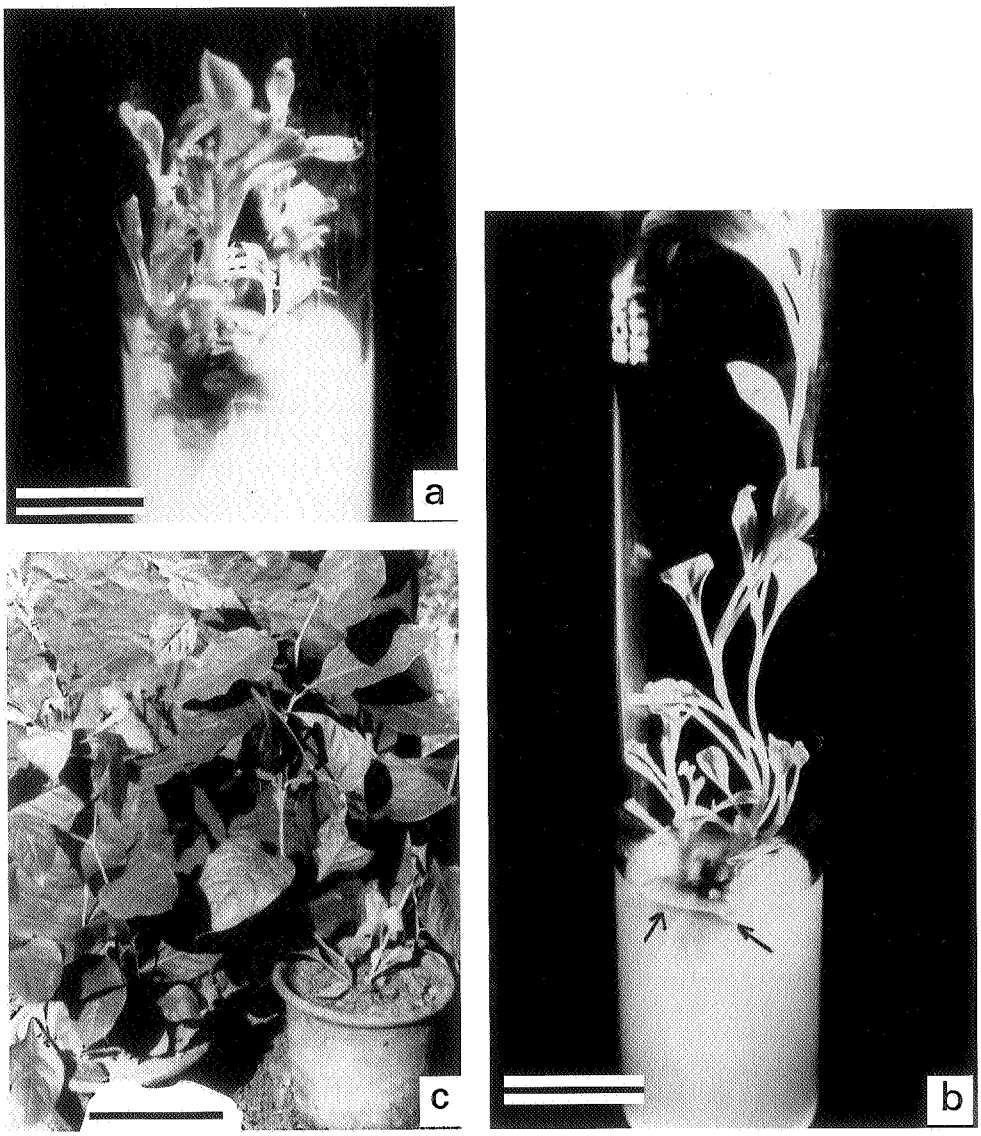

Fig. 1. *a* - shoot proliferation from nodal explants of *Plumbago zeylanica* cultured on MS medium supplemented with 1.0 mg dm^{-3} BA + 0.01 mg dm^{-3} IAA after 2 weeks of culture (*bar* = 10 mm); *b* - induction of roots (*arrow*) from microshoots of *P. zeylanica* cultured on half strength basal MS medium supplemented with 0.25 mg dm^{-3} IBA + 2 % (m/v) saccharose after 7 - 8 d of culture (*bar* = 20 mm); *c* - plants established in soil (*bar* = 0.25 cm).

Of the twenty random primers tested, six were selected on the basis of the clarity of the banding patterns. The result showed that the primers OPA-01, OPA-11, OPA-16, OPN-13, OPN-19 and OPN-20 produced amplification products which were monomorphic across all micropropagated plants. No variation was detected in the micropropagated plants derived from the axillary shoots (Table 3). The size of the monomorphic DNA fragments, produced by OPA-01 and OPN-20 primers ranged from 800 to 2730 bp for OPA-01 and 600 to 5400 bp for OPN-20 (Figs. 2*A,B*). The variation of monomorphic bands in micropropagated plants by using different primers has been reported earlier (Potter and Jones 1991, Nehra *et al.* 1994, Angel *et al.* 1996, Rout

et al. 1998). Shenoy and Vasil (1992) reported that the micropropagation through meristem culture is generally associated with low risk of genetic instability because the organised meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* condition. The DNA amplification products, which represent one allele per locus, could result from changes in either the sequence of the primer binding sites or changes which alter the size and present the successful amplification of target DNA. In the present study, the amplified products exhibited monomorphisms among all the *in vitro* raised plants and were similar to those from parent plants. The method is simple and the results are reproducible. Because only micro-amounts of material are necessary, this approach can be used to assess tissue at several stages of *in vitro* culture. Large sample sizes can be handled rapidly, and the technique lends itself to automation (Williams *et al.* 1990). Furthermore, the genome is most probably randomly sampled without the influence of ontogeny. However, only major fragments genetically characterized through segregation analysis should be used as markers. Minor fragments, which tend to be unstable in staining intensity, are unreliable and should be considered. Many authors using RAPD markers have failed to observe intraclonal variations in various species including *Picea mariana*, *Saccharum* spp. and *Fragaria* × *ananassa* (Isabel *et al.* 1993, Chowdhury and Vasil 1993 and Kumar *et al.* 1999). Karp (1995) reported that there are several factors such as explant source, duration of culture and growth regulators influencing the variation in the culture condition.

In conclusion, an efficient protocol on mass propagation of *P. zeylanica* has been developed. The results demonstrate that RAPD markers can be applied to rapid evaluation of genetic fidelity of micropropagated plants for the conservation of genetic richness.

Table 1. Effect of BA, Kin and IAA [mg dm^{-3}] on shoot multiplication from nodal explants of *Plumbago zeylanica* after 4 weeks of culture on MS medium. Mean ± SE of 15 replicates per treatment; repeated thrice (+ - callusing at the basal end). Means having the same letter in a column were not significantly different by Duncan's multiple comparison test ($P < 0.05$).

BA	Kin	IAA	Cultures with shoots [%]	Number of shoots [explant^{-1}]
0	0	0	0	0
0.25	0	0	64.2 ± 1.0i	5.6 ± 0.6g
0.50	0	0	68.8 ± 0.8j	4.8 ± 0.7e
1.00	0	0	76.2 ± 0.6k	5.1 ± 0.8f
1.50	0	0	50.6 ± 0.8f	4.4 ± 0.6d+
0	0.25	0	42.6 ± 0.4d	4.3 ± 0.5d
0	0.50	0	58.8 ± 0.8h	4.0 ± 0.6c
0	1.00	0	50.6 ± 1.1f	3.1 ± 0.7b
0	1.50	0	38.4 ± 0.7b	2.6 ± 0.8a+
0.50	0	0.01	78.6 ± 1.1 l	5.8 ± 0.6g
1.00	0	0.01	90.8 ± 0.7n	8.8 ± 0.8i
1.00	0	0.10	82.4 ± 0.8m	7.2 ± 0.6h
1.00	0	0.25	43.5 ± 0.4d,e	3.2 ± 0.7b+
1.50	0	0.10	52.4 ± 0.6g	3.0 ± 0.6b+
0	0.50	0.10	40.2 ± 0.5c	3.2 ± 0.8b
0	1.00	0.10	44.8 ± 1.1e	3.8 ± 0.6c
0	1.00	0.25	36.2 ± 0.6a	2.8 ± 0.7a

Table 2. Effect of IBA and IAA [mg dm^{-3}] on rooting of excised shoots of *Plumbago zeylanica* cultured on half strength MS medium supplemented with 2 % (m/v) saccharose. Means ± SE of 15 replicates per treatment in three repeated experiments (c - basal callusing at the cut end.

IBA	IAA	Shoot rooted [%]	Rooting [d]
0	0	0	0
0.05	0	44.6 ± 0.8	10 - 11
0.10	0	56.2 ± 1.2	8
0.25	0	92.6 ± 1.3	7 - 8
0.50	0	42.8 ± 0.6c	10 - 11c
0	0.05	36.2 ± 0.8	9 - 10
0	0.10	48.8 ± 1.0	8 - 9
0	0.25	77.4 ± 0.7	9 - 10
0	0.50	30.6 ± 0.8c	10 - 11c

Table 3. RAPD analysis of the micropropagated and field grown mother plants of *Plumbago zeylanica*.

Primer	Sequence	Number of amplified fragments	Fragment size range [bp]
OPA-01	5`-CAGGCCCTTC	8	800 - 2730
OPA-11	5`-CAATCGCCGT	10	432 - 2800
OPA-16	5`-AGCCAGCGAA	12	400 - 2000
OPN-13	5`-AGCGTCACTC	10	300 - 2000
OPN-19	5`-GTCCGTACTC	11	600 - 1500
OPN-20	5`-GGTGCTCCGT	10	600 - 5400

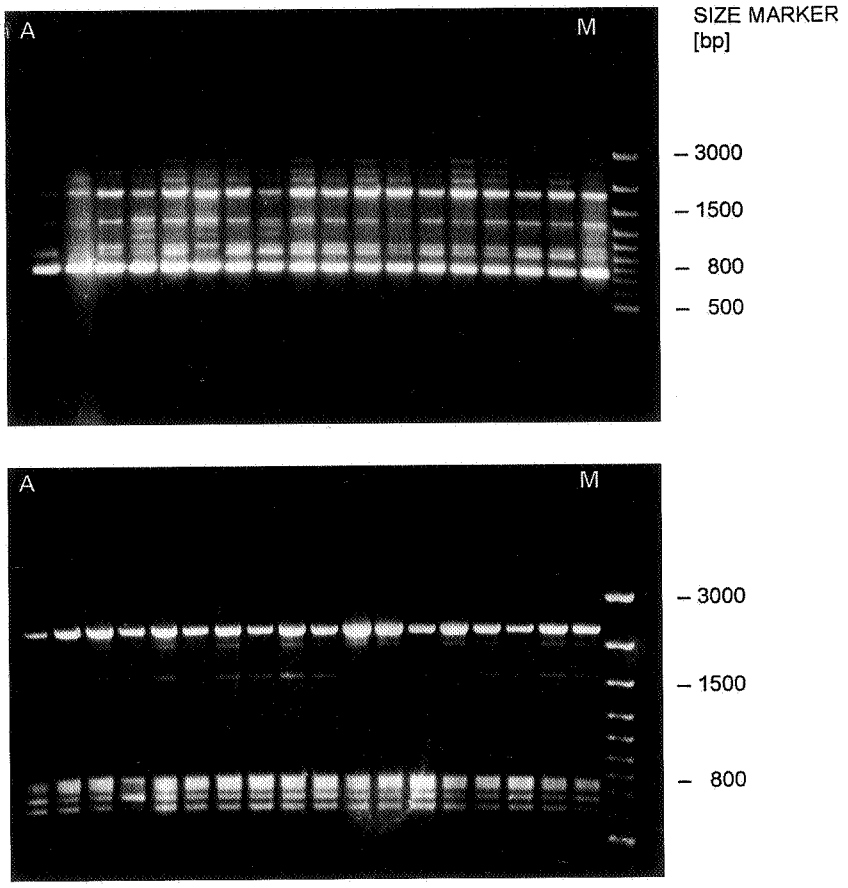

Fig. 2. RAPD profiles of micropropagated and field grown mother plants of *P. zeylanica* generated by primer OPA-01 (5`-CAGGCCCTTC-3`) (*A*) and OPN-20 (5`-GGTGCTCCGT-3`) (*B*). M show RAPD bands from the field grown mother plant. *Arrow* indicates the size of the marker.

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