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Micropropagation and Evaluation of Genetic Stability of Wild *Curcuma* Species and *C. longa* Morphotypes Using Random Amplified Polymorphic DNA

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Abstract

Species specific responses in micropropagation of 10 wild species of *Curcuma* and 15 morphotypes of *C. longa* were observed. Five to eight shoots were produced from a single rhizome bud explant of *C. aeruginosa*, *C. amada*, *C. amarissima* and six morphotypes of *C. longa* (M1, M4, M7,M9,M14 and M16) in Murashige and Skoog (MS) medium containing 5.0 mg dm-3 6-Benzyl Adenine (BA). *C. aurantiaca*, *C. caesia*, *C. zeodaria* and four *C. longa* morphotypes (M2, M8, M10 and M11) produced six to eleven shoots in MS medium containing 0.1 mg dm-3 Indole-3 acetic acid (IAA) and 2.5 mg dm-3 BA. *C. brog*, *C. latifolia*, *C. malabarica*, *C. raktakanta* and five *C. longa* morphotypes (M3, M15, M17, M18 and M19) developed five to seven shoots in MS medium containing 0.1 mg dm-3 IAA and 5.0 mg dm-3 BA. Initiated shoots were multiplied and rooted in the same medium. Rooted plantlets were transferred to field and 95% of the plantlets survived during acclimatisation. Genetic stability of in- vitro raised plants was tested using 30 Random Amplified Polymorphic DNA (RAPD) primers during each stage of subculture. Twenty primers produced 275 bands out of which 249 were polymorphic exhibiting 90.53% polymorphism for the species and morphotypes establishing their wide genetic basis. In-vitro cultures were stable in their genetic makeup until fifth or sixth passage of subculture depending on the species and showed variation in RAPD banding pattern after these cycles. These results indicates that exposure to long term in-vitro conditions resulted in variation in the DNA in almost all the species and morphotypes of *Curcuma*.

Keywords: *Curcuma*, turmeric, RAPD, in vitro culture, genetic stability.

INTRODUCTION

The genus *Curcuma* comprises of more than 80 species and about 40 of them are indigenous to the subcontinent (1). Based on the morphological characteristics 550 accessions of *C. longa* were grouped as 21 distinct morphotypes of six taxonomic groups (2). Vegetative propagation of turmeric is mainly by underground rhizomes and improvement of the genus by breeding is not yet reported due to the inability of the seeds to develop into complete plantlets. A large number of wild and cultivated accessions of Curcuma are conserved in field gene banks; which suffer serious destruction due to the high susceptibility to soft rot caused by Pythium myriotylum and P. graminicolum and bacterial wilt caused by Pseudomonas solanacearum. Tissue culture methods are used for mass propagation of important spices crops though, in-vitro establishment of most of the *Curcuma* species are impeded due to inborn bacterial and fungal contamination (3). Still, tissue culture methods are developed for in-vitro establishment and conservation of turmeric (4). Forced axillary shoot development is not expected to induce genetic variation since their ontogeny is through lateral meristems, however, the development of adventitious buds or regeneration from callus and long-term exposure to various chemicals is more prone to genetic variation (5,6).

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Prolonged exposure to in-vitro conditions can lead to genetic variations thus raising serious concern about the genetic fidelity of the tissue culture raised plants necessitating reliable methods to assess these variations. Tissue cultured plants, developed from pre-existing meristems or through organogenesis, exhibits epigenetic or heritable variations due to changes in growth environment and exposure to different growth regulators and various chemicals (7,8). Explant source, culture initiation period, levels of subculture, ploidy of the cultured cells in the parent plant and genetic mosaics are capable of inducing variability in in vitro cultures (9).Variation may also arise due to the insertion or deletion of chromosome complement, methylation pattern, laggard formation, genome rearrangement or transpositions which may be advantageous or deleterious (10). Assessment of genetic variability in micropropagated plants during each stage of subculture is necessary to reduce the chances of genetic instability (11).

Morphological, karyological and biochemical methods are used for the assessment of genetic variations but have limitations. Morphological markers are environment dependant and cannot be used as a reliable marker system. Karyological methods are unable to reveal small changes in the chromosome (12). Biochemical markers like isozymes are limited in number, mainly co-dominant and dependent upon the ontogenic factors and DNA coding for soluble proteins expressed as isoforms can only be analysed. Random primer based polymerase chain reactions are used as reliable methods for detecting changes in the genetic makeup and also intra and inter specific variations in plant species (13,14). This technique has been used for assessing the genetic stability of tissue culture raised plants and is a reliable and affordable method (15,16).

Present study was undertaken to develop a suitable in vitro regeneration system for ten wild *Curcuma* species and 15 morphotypes of *C.longa* and assess the genetic stability of regenerated plants during each subculture period by using RAPD.

MATERIALS AND METHODS

Culture Initiation

Ten wild species of Curcuma, viz., *C. aeruginosa, C. amada, C. amarissima, C. aurantiaca, C. brog, C. caesia, C. latifolia, C. malabarica, C. raktakanta, C. zedoaria* and 15 C. longa morphotypes (M1, M2, M3, M4, M7, M8, M9, M10, M11, M14, M15, M16, M17, M18 and M20) were collected from different geographical locations and maintained in the botanical garden of University of Calicut, Kerala, India. The newly emerged rhizome buds (2-3 cm) were excised and thoroughly washed under running tap water to remove adhered soil particles. Outer scales were removed with a scalpel and the buds were immersed in distilled water containing 2 or 3 drops of Tween-20 for 20 min and washed 7 or 8 times with distilled water and used as explants for initiation of cultures. The buds were surface sterilized with 0.1% (m/v) mercuric chloride and 2 or 3 drops of Tween-20 for 10-15 min and thoroughly washed 7 or 8 times with sterilized distilled water to remove traces of mercuric chloride. The sterilized buds were cultured on MS medium (17) containing 2.5 mg dm-3 BA and subcultured to fresh media containing BA 2.5 mg dm-3 or 5.0 mg dm-3 either alone or in combination with IAA 0.1 mg dm-3 to obtain multiple shoots and compared species specific responses of different growth regulators.

Media and culture conditions

The pH of all the media containing 3% (m/v) sucrose was adjusted to 5.8 with 0.1N NaOH prior to the addition of agar-agar (0.75% m/v, Hi-media, India) and approximately 20 ml of the medium was dispensed into culture tubes (25 x 150 mm, Borosil, India). Each culture tube received one explant. The media were autoclaved at 121oC temperature and 15 psi pressure for 20 min. Cultures were incubated at 25±2oC, a light intensity of 140µEm-2 s-1 provided by cool white fluorescent lamps (Philips, India) and a photoperiod of 16 h/d. All the chemicals used were of analytical grade (Hi-media, India and Sigma, U.S.A.). One or two shoots from the regenerated shoot clumps were subcultured to fresh medium after excising the roots, and the remaining plantlets were hardened in plastic pots (15 cm. dia.) containing a mixture of horticultural grade pelrite with Irish peat moss and vermiculite (Kelpelrite, Bangalore, India) moistened with 1/10 MS major salt solution. The plantlets were kept covered for 10-15 days to maintain humidity and exposed to ex vitro conditions and subsequently hardened in earthen pots (30 cm. dia.) containing soil mixture. Leaves from the hardened plants and from tissue cultured plants were used to isolate DNA for assessing genetic stability during each subculture cycle.

Each tissue culture experiments comprised of 21 cultures and repeated 3 times. Data were recorded at periodic interval of 40 days for number of shoot per culture and presented as mean value of 63 cultures. The experiments were conducted in completely randomised block design. Data were subjected to analysis of variance and Duncan's Multiple Range Test (DMRT, 18) to test the significance of differences.

DNA extraction

Extraction of DNA was done from the youngest upper leaves of three sets of materials, 1) mother plants, 2) tissue cultured plants during each subculture, 3) hardened plants during each phase of subculture. The leaves collected from the mother plants, six random in vitro regenerated plants and hardened plants were washed thoroughly by using tap water, swabbed with detergent teepol and stored in liquid nitrogen. DNA was extracted according to modified CTAB method (19). The DNA thus obtained was subjected for RNase A treatment (60µg of RNaseA for 1 cm-3 of DNA solution). The quantity and quality of DNA was assessed by reading the absorbance at 260 nm and 280 nm by using a nanodrop spectrophotometer. Purified DNA samples were diluted to 25µg cm-3 for RAPD analysis.

PCR amplification

Thirty arbitrary random decamer operon primers (Operon technologies Inc. Alameda, California) were used for PCR reaction. Each 0.025µcm-3 reaction mixture contained 0.0025 µcm-3 10X reaction buffer, 100 µM dNTPs (Sigma), 10 nmoles of each primer, 2.0mM MgCl2 and 0.5 units of Taq DNA polymerase (Genei, Bangalore,India). 25 ng of genomic DNA was added to each reaction mixture and the control received double distilled water in the place of DNA. PCR reactions were done in a thermal cycler (Eppendorf mastercycler Gradient S) programmed with the first cycle comprising of 92o C for 5 min followed by 35 cycles comprising of 92o C for 1min, 37o C for 1 min and 72o C for 1 min. The final extension step was conducted at 72oC for 10 min.

The amplified products were visualised in 1.5% (m/V) agarose gel containing 0.5µg ethidium bromide along with 100bp DNA ladder as marker (Invitrogen) in 1X TAE buffer and documented using cell-bio science chemiluminescence system. The reactions were repeated thrice to ensure repeatability of the banding pattern. The electrophoretic bands which were not intense and clear were not considered as markers and considered ambiguous.

RESULTS

Culture initiation

Rhizomes buds of all the species and morphotypes developed new shoots within a period of 9-11 d. The cultures which showed contamination with endogenous bacteria were re-sterilised twice and cultured onto fresh initiation medium to eliminate the bacterial contamination. The initiated shoots were sub-cultured to multiplication medium and the regenerated shoots were used to study the efficacy of multiplication and assessment of genetic stability. Five to eight shoots were regenerated from a single shoot explant in the multiplication medium within a period of 17-21 days; however, the quantitative response of shoot formation was species and growth hormone dependant. Rhizome buds of *C. aeruginosa*, *C. amada, C. amarissima and C. longa* morphotypes M1, M4, M7, M9, M14 and M16 produced a maximum of 5-6 shoots in MS medium containing 5.0 mg dm-3 BA. *C. aurantiaca, C. caesia, C. zedoaria* and morphotypes M2, M8, M10 and M11 produced six or seven shoots in MS medium containing 0.1 mg dm-3 IAA and 2.5 mg dm-3 BA. *C. brog, C. latifolia, C. malabarica, C. raktakanta* and morphotypes M3, M15, M17, M18 and M20 produced a maximum of six to eight shoots in MS medium containing 0.1 mg dm-3 IAA and 5.0 mg dm-3 BA. The shoot regeneration potential of the species and morphotypes studied with respect to different growth regulators is summarised in Table-1. The initiated cultures were maintained in the same multiplication medium without deterioration in the growth

characteristics. The cultures grew profusely for the initial 21-25 d and further increase in number of shoots were not observed. The subculture periods varied with species and morphotypes and were in a range of 55- 70 d. The in vitro raised plants were hardened and planted in the soil mixture. More than 30,000 plants were transferred to field conditions. Morphological variations or decrease in the number of offshoots formed were not observed though, an increase in the number of shoots was observed. The hardened plants have the same morphological features during each subculture period. The plants regenerated until the 35th passage of subculture transferred to field conditions were observed no morphological variations warranting the assessment of genetic fidelity as exposure to tissue culture conditions may induce genetic variability.

Table-1.No.of shoots (±S.E) developed from rhizome bud explants of different *Curcuma* species and morphotypes cultured in MS medium containing different growth regulators.

Species/Morphotypes	BA (2.5mg/l) (5.0mg/l)	IAA (0.1 mg/l)+ BA (2.5mg/l) (5.0mg/l)
C. aeruginosa	3.0±0.2^{bc} 5.2±0.7^{ba}	2.2±0.4^{cd} 4.2±06^{cb}
C. amada	2.5±0.5^{cd} 6.4±0.8^{ab}	2.7±0.5^{cd} 3.4±0.8^{dc}
C. amarissima	2.8±0.5^{cd} 7.4±0.7^{a}	1.8±0.7^{d} 2.4±0.6^{d}
C. aurantiaca	4.3±0.6^{cd} 5.1±0.6^{c}	8.9±0.7^{a} 6.8±0.3^{ab}
C. brog	2.7±0.2^{cd} 3.2±0.6^{cb}	4.2±0.3^{cb} 5.4 ±0.9^{ba}
C. caesia	2.5±0.2^{d} 3.8±0.7^{d}	6.8±0.3^{ab} 4.3±0.6^{cb}
C. latifolia	1.9±0.2^{ba} 2.2±0.3^{dc}	3.6±0.3^{cd} 4.4±0.5^{cb}
C. malabarica	3.4±0.4^{cd} 3.8±0.2^{dc}	4.3±0.4^{cb} 5.8±0.6^{ba}
C. raktakanta	2.9±0.2^{a} 3.2±0.4^{ba}	3.9±0.5^{dc} 5.3±0.7^{ba}
C. zeodaria	4.9±0.6^{a} 5.8±0.5^{ab}	10.4±0.6^{a} 7.2± 0.5^{a}
M1	4.2 ±0.4^{a} 5.2±0.7^{ba}	3.1±0.4^{dc} 3.8±0.9^{dc}
M2	3.5±0.6^{ba} 4.8±0.6^{cd}	6.2±0.6^{cd} 5.2±0.5^{cb}
M3	3.2±0.4^{c} 5.4±0.4^{ba}	6.3±0.4^{ab} 7.2±0.7^{ab}
M4	3.2±0.5^{c} 4.3±0.8^{cb}	2.9±0.3^{dc} 3.8±0.5^{dc}
M7	3.4±0.5^{bc} 5.1±0.5^{ba}	3.6±0.5^{cd} 4.2±0.5^{cb}
M8	4.4±0.2^{a} 5.0±0.3^{ba}	6.7±0.2^{ab} 5.8±0.5^{bc}
M9	4.3±0.5^{a} 5.3±0.4^{ba}	3.6±0.3^{cd} 4.6±0.4^{cb}
M10	2.3±0.8^{cd} 4.5±0.5^{cb}	5.3±0.6^{ba} 4.8±0.2^{cb}
M11	3.6±0.5^{ba} 4.7±0.6^{cb}	6.4±0.5^{ab} 5.8±0.4^{ba}
M14	4.5±0.4^{a} 5.2±0.6^{ba}	3.6±0.9^{cd} 4.6±0.3^{cb}
M15	3.8±0.4^{ba} 4.3±0±.5^{cb}	5.4±0.4^{ba} 6.9±0.6^{ab}
M16	3.6±0.6^{ba} 5.8±0.2^{ab}	2.9±0.3^{cd} 4.1±0.4^{cb}
M17	3.8±0.6^{ba} 4.2±0.5^{cb}	5.4±0.3^{ba} 7.3±0.6^{a}
M18	4.3±0.2^{a} 5.2±0.3^{ba}	5.6±0.3^{ba} 7.6±0.4^{a}
M20	3.1±0.6^{bc} 4.8±0.3^{bc}	6.2±0.4^{ab} 6.9±0.8^{ab}

Means having the same letter in a column were not significantly different at P<0.001 on the basis of Duncan's Multiple Range Test

Monitoring of Genetic Stability

The protocol described for DNA extraction worked for all the species and morphotypes of Curcuma used for the study. Better yield of DNA was obtained from the younger leaves as compared to the mature leaves. Out of 30 random decamer primers used 20 produced polymorphic bands and other 10 generated 4-8 monomorphic bands in all the species and morphotypes. The polymorphic primers produced a total of 275 bands of which 249 were polymorphic exhibiting 90.53% polymorphism amongst the species and morphotypes studied. The number of polymorphic bands developed and percentage polymorphism shown by each species with respect to the primers is shown in Table-2. Primers OPA1, OPA3, OPA5, OPB5, OPC3, OPC4, OPC5, OPC10, OPC15, OPC18, OPD2, OPD6, OPD7, OPD10, OPD18, OPG2, OPH1, OPI1, OPK1, and OPM 14 produced 1-18 scorable and reproducible bands which ranged from a molecular weight of 0.3 kb to 2kb and the primers OPA10, OPD 5, OPD 9, OPD12, OPD13, OPD14, OPD 19,OPH6, OPH 9 and OPM1 produced monomorphic bands ranging from the molecular weight 0.3kb to 1kb.

Table:2. Sequences of RAPD primers, number of polymorphic bands produced by each primer and percentage polymorphism observed in all the species and morphotypes of *Curcuma*.

Primer	Sequence (5'-3')	Number of polymorphic bands	Polymorphism (%)
OPA01	CAGGCCCTTC	13	93.6
OPA03	AGTCAGCCAC	10	95.0
OPA05	AGGGGTCTTG	16	95.1
OPB05	TGCGCCCTTC	11	99.1
OPC03	GGGGGTCTTT	10	88.0
OPC04	CCGCATCTAC	12	94.2
OPC05	GATGACCGCC	10	88.8
OPC10	TGTCTGGGTG	17	85.0
OPC15	GACGGATCAG	10	85.2
OPC18	TGAGTGGGTG	18	95.6
OPD02	GGACCCAACC	15	94.0
OPD06	ACCTGAACGG	08	100
OPD07	TTGGCACGGG	13	88.6
OPD10	GGTCTACACC	10	84.4
OPD18	GAGAGCCAAC	18	85.1
OPG02	GGCACTGAGG	13	83.9
OPH01	GGTCGGAGAA	12	90.3
OPI 01	ACCTGGACAC	11	83.7
OPK 01	CATTCGAGCC	12	89.0
OPM 14	AGGGTCGTTC	10	92.0

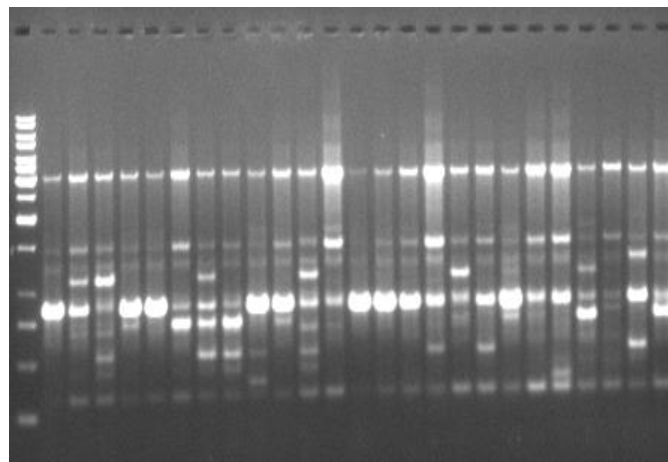
Homologous RAPD profiles were observed in tissue cultured and hardened plants and their parent plants until the 5th subculture. However, from the 6th subculture onwards, the RAPD profile of primers OPA5, OPC5, OPD7 and OPH1 showed considerable variation in the number of bands in almost all the species and morphotypes of both TC and hardened plants when compared to mother plants. In case of OPA 5 *C. brog* produced 3 additional bands (one band each at 2kb, 1kb and 0.6 kb) during the 6th subculture

than the 5th subculture and all the other species and morphotypes did not show any variation. In case of RAPD with OPC 5 primer all the species except *C. raktakanta* and all the morphotypes except M8 produced more number of bands in the tissue culture raised plants during the sixth subculture. In OPD 7 the banding pattern was entirely different during the fifth and sixth subculture in the tissue cultured and hardened plants (Fig-1). The primer OPH1 showed variation in the banding pattern during the sixth subculture when compared to the plants from fifth subculture in both TC and TC hardened plants and mother plants. Total number of bands produced by the tissue culture raised plants during the fifth subculture and sixth subculture for all the species and morphotypes are shown in Table-3. The RAPD profile of plants from in vitro cultures of fifth subculture and the same after three months of hardening showed exactly the same banding pattern confirming the change in genetic constitution of the plants. These cultures showed profuse growth during sixth subculture onwards with increase in shoot production from the base of the initial inoculum, but when the plants were transferred to the field did not show any morphological variations. In this report, we demonstrate that RAPD can be used to appraise the genetic stability of in vitro grown cultures in large number of accessions conserved in gene banks as well as mass propagation agencies. It was observed, necessary to check the genetic constitution of the tissue culture raised and hardened plants to that of their parent plants during each passage of subculture to limit the propagation to minimum subcultures.

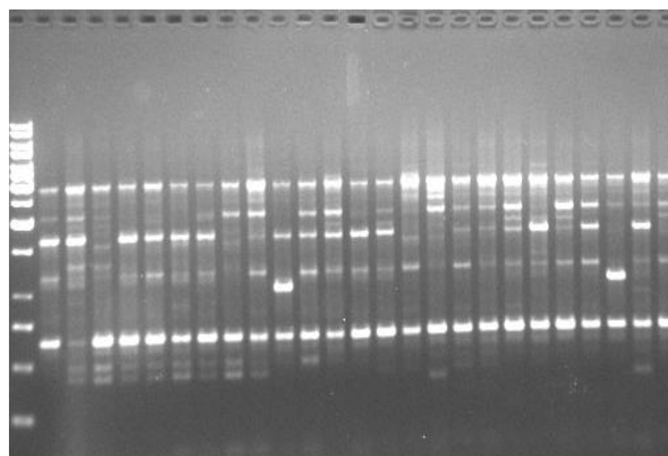
Table-3 Variability in the number of RAPD bands produced in different species and morphotypes of *Curcuma* after 5th and 6th subculture

Species/ Morphotype	OPA 05- 5th /6th subculture	OPC- 05 5th /6th subculture	OPD- 07 5th /6th subculture	OPH- 01 5th /6th subculture
C. aeruginosa	2/2	2/5	2/5	2/5
C. amada	2/2	5/9	4/6	1/3
C. amarissima	2/2	7/10	8/7	0/5
C. aurantiaca	1/1	7/6	3/4	4/9
C. brog	2/5	5/6	2/4	4/9
C. caesia	4/4	4/7	5/4	3/5
C. latifolia	3/3	5/7	6/5	5/2
C. malabarica	3/3	6/7	5/6	3/9
C. raktakanta	1/1	6/6	2/6	6/2
C. zeodaria	3/3	5/4	5/4	5/3
M1	5/5	8/5	8/6	9/6
M2	6/6	7/5	5/6	2/9
M3	2/2	3/4	2/4	5/8
M4	2/2	4/5	4/7	3/9
M7	4/4	4/6	3/5	4/9
M8	2/2	5/5	5/6	3/9
M9	2/2	5/6	6/5	5/8
M10	1/1	5/6	5/6	3/6
M11	2/2	6/7	3/7	2/7
M14	4/4	6/7	4/6	1/5
M15	7/7	5/6	5/6	2/5
M16	5/5	6/5	6/5	1/6
M17	2/2	6/4	4/5	3/9
M18	8/8	5/6	4/5	2/6
M20	5/5	4/5	4/5	5/7

M1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25



M1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25



M- 100 bp DNA ladder 1.*C.aeruginosa*,2.*C.amada*,3.*C.amarisima*, 4.*C. aurantiaca*, 5.*C.brog*,6.*C.caesia*, 7.*C.latifolia*,8.*C.malabarica*, 9.*C. raktakanta*, 10.*C.zeodaria*,11-14. M1-M4, 15-19. M7-M11, 20-24. M14-M18 and 25. M20.

Fig.1- Representative RAPD profile of *Curcuma* species and morphotypes generated by primer OPD- 07 during 5th (A) and 6th (B) subculture

DISCUSSION

Establishment of in vitro cultures of *Curcuma* species is always a problem due to the large number of microbial flora present underground rhizomes. Serial subculturing and sterilisation with mercuric chloride until contamination free cultures are established is the routine practice in eliminating the microbes. Serial sterilisation of the in vitro rhizomes was practiced for establishing contamination free cultures from the rhizomes of *Curcuma* species (20). Auxins and cytokinins either alone or in combination produced multiple shoots; however, the quantitative response was hormone dependant. These results are in agreement with the effects of different cytokinins on shoot regeneration and conservation of 8 wild species of *Curcuma* (4). Monitoring the genetic stability of in vitro cultures is essential in order to ascertain whether the plantlets produced are genetically stable. Various techniques are used to assess the genetic stability of in vitro cultures (21). RAPD is a technique which is considerably cheaper and consistent and used to reveal polymorphism among different spices crops (22). Comparative RAPD analysis of the species and morphotypes revealed a high polymorphism in the banding pattern proving the diversity within and among the species of *Curcuma* (23). Exposure to various chemicals and culture conditions emanates vast amount of adventitious branching in most

of the perennial plants and in all the species and morphotypes of *Curcuma* studied. Similar observation on growth rate were made in sugarcane when grown in BA, and presumed that methylation of DNA and increased subculture interval also influence the genetic stability/instability of the in vitro cultures (24). This is in contrary to the reports that during meristem culture of Zingiberaceous members the genetic constitution of the in vitro raised plants remained the same (25). In Populus deltoides and Begonia, RAPD variation was observed among 23 tissue culture raised plants questioning the feasibility of the technique (26,27), however, the effects of subculture period on the genetic stability/instability of the cultures was not highlighted in these studies. Callus cultures of *Curcuma* were shown to have polymorphic bands in single species, which may be due to the imbalance in the multiplication as well as the effect of growth regulators on the cell division (28). The present study underlines the need of periodic monitoring of genetic stability of in vitro conserved germplasm in in vitro repositories and laboratories.

CONCLUSION

Successful micropropagation methods were developed for the wild species and morphotypes of *Curcuma* and by assessing the genetic stability during each subculture phase stressed the importance of assessing the genetic stability of in vitro cultures during each phase of subculture in in vitro repositories and gene banks.

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