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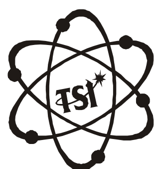

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In vitro true to type propagation of *Curcuma caesia* Roxb. (Zingiberaceae) and assessment of its genetic fidelity using RAPD marker

Arghya Ghosh[1,2], Gautam Saha[2], Padma Chatterjee[1], Parthadeb Ghosh[2]*
[1]Plant Biochemistry, Molecular Biology & Advance Plant Physiology Research Laboratory, Department of Botany, University of Kalyani, Kalyani 741235, Nadia, West Bengal, (INDIA)
[2]Cytogenetics & Plant Breeding Section, Department of Botany, University of Kalyani, Kalyani 741235, Nadia, West Bengal, (INDIA)
E-mail: pdgbot@yahoo.co.in

ABSTRACT

An efficient plant propagation system through rhizomal explants was established in *Curcuma caesia* Roxb., a medicinally important herbaceous annual herb belonging to the family Zingiberaceae. Here we report a rapid and reliable method for high fidelity micro-propagation. Rhizomal explants from two months old seedlings were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of N6-benzyladenine (BA) (0.5 - 5.0 mg/l), Napthalenic acetic acid (NAA) (0.5 - 5.0 mg/l) and Indole 3 butyric acid (IBA) (0.5 - 5.0 mg/l). During the first culture on 1.5 mg/l of 6-benzylamino purine (BAP) and 1 mg/l of Napthalenic acetic acid (NAA) maximum 15.40±0.40[a] shoots with an average shoot-length of 8.46±0.06[a] were produced. The elongated shoots produced a maximum of 12.00±0.00[a] roots on half-strength MS liquid medium supplemented with 1 mg/l of Indole 3 butyric acid (IBA). The plantlets were acclimatized by transferring them first to peat moss: compost (1:1) mixture followed by sand: soil (2:1) mixture, recording 95% survival.
Genetic fidelity was assessed by DNA fingerprinting using random amplified polymorphic DNA (RAPD) of *in vitro* and *in vivo* plants. Five arbitrary decamers displayed same banding profile showed no genomic alterations, indicating homogeneity among the tissue culture regenerates and genetic uniformity with that of donor plants. The present study provides high genetic fidelity micropropagated system for efficient and rapid micropropagation protocol of this important medicinal plant and great use in conserving without risk of genetic instability.
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KEYWORDS

Micropropagation;
Multiple shoot;
Rhizomal explant;
Genetic fidelity.

INTRODUCTION

The genus *Curcuma* L. of the family Zingiberaceae is well known as the turmeric genus, because Of *Curcuma longa*. *C. longa* is the most investigated species of this genus, although there are over 100 others in this

genus[1,2]. Most of the species of this genus are perennials and grow well in tropics and subtropics where it requires a hot and moist climate and a fairly light soil. *Curcuma* species are native to the countries of the Southeastern Asia and extensively cultivated in Bengal (Bangladesh and India), China, Taiwan, Sri Lanka, Indonesia, Peru, Australia, and the West Indies. Although turmeric is quite common due to its frquent usage as spices, most of the other species of *Curcuma* including *Curcuma caesia* as well as turmeric posses some active medicinal constituents. Medicinal plants are important to the global economy, as well as source of income for rural people in developing countries. Generally 70% to 80% of the ayurvedic medicine was derived from plants[3]. Herbal medicines are produced from field-grown plants and are used to cure several diseases that are caused by pathogenic bacteria, fungi, and insects[4]. It is difficult to ensure the quality control as the medicinal preparations are multiherb preparations and also difficult to identify and quantify the active constituents[5]. Due to the absence of side effects in herbal medicines the demand of herbal drugs are increasing day by day[6]. So people have to take the synthetic drugs and antibiotics to get cured from these diseases. Plants provide a major contribution to the pharmaceutical industry[7]. The major cause of inadequacy of inavialability of herbal medicines are due to excessive human exploitation, non-regulated collection, unresolved inherent problems of seed viability and seed germination, and this priority many species have become threatened or endangered[8,9].

Curcuma caesia Roxb. is a native of northeast India extending up to the present day Bastar region of Chattisgarh. The rhizome has a deep bluish black or grayish black color. It is used in native medicines. This species occurs mainly in the northeastern and west coastal regions of India, extending to the hills. It has also been cultivated in earlier times for extracting arrowroot powder and for the production of Abir. Rhizome is sometime pale yellow or colorless. It is ground to a powder, which is purified by repeated washing and dried. Dried powder is mixed with a decoction of sappan wood (*Cesalpinia sappan*) when the red color is obtained. It is used in traditional and local medicines as a stimulant and carminative. This was reported to have cosmetic properties. Species such as *Curcuma aeruginosa*, *Curcuma caulina*, *Curcuma leucorrhiza*, *Curcuma pseudomontana*, and *Curcuma rubescens* are also used as sources of arrow root powder and in local and tribal medicines. *Curcuma caesia* was in use earlier as a dye, and now as a cosmetic, often as a substitute for *Curcuma aromatica*. Perhaps, India is the only country where there is a strong R & D base for turmeric. However, research on *Curcuma caesia*, especially in the area of pharmacology, is being pursued by many workers in many countries). The first ever research on *Curcuma caesia* in India was initiated at Udayagiri in Orissa in 1944 under the Imperial Council for Agriculture Research. However, organized research programs were initiated in independent India during the first Five-Year Plan. Based on a recommendation of the Spices Enquiry Committee (1953), turmeric research was started in Kandaghat (Punjab), Targaon (Maharashtra), and Thodupuzha and Ambalavayal (Kerala). A scheme for turmeric research was initiated in 1955 at Andhra Pradesh (at Peddapalem). However, the real impetus for turmeric research was received with the organization of the All India Coordinated Spices and Cashew Improvement Project. In 1975, research programs were started in two centers, Coimbatore (Tamil Nadu Agriculture University — TNAU) and Pottangi (Orissa University of Agricultural and Technology, High Altitude Research Station). *In vitro* culture techniques represents an excellent option for the study and conservation of rare, threatened or endangered medicinal plants[10,11], as well as tool for efficient rapid clonal propagation of important plants allowing production of genetically stable and *true to true* type progeny[12]. Therefore, the interest in using these techniques for rapid and large-scale propagation of medicinal and aromatic plants has been significantly increased[13,14]. Till now, there is no report on micropropagation and assessment of genetic fidelity of *Curcuma caesia* Roxb. In this study we represent for the first time an efficient protocol for rapid large scale regeneration of plantlets *in vitro* from rhizomal explants of *Curcuma caesia* Roxb. with maintaining stable gene pool fidelity of the regenerants as assessed by RAPD.

MATERIALS AND METHODS

Explant preparation

Young disease free rhizomal explants (rhizomal buds

of 2.5 - 3 cm) were collected from 2 months old plant growing in the medicinal and aromatic plant garden of the Department of Botany, University of Kalyani, Kalyani, India. Explants were washed thoroughly under running tap water and then treated with 5% (m/v) Teepol (Qualigen, Mumbai, India) for 20 min, followed by rinsing three to five times in sterile double distilled water. The rhizomal segments were then surface disinfested with 70% alcohol for 5 min followed by immersion in 0.1% (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 - 6 min and finally rinsed with sterile double distilled water (five to six times) in a flow chamber. The surface sterilized explants were trimmed at cut ends and about 1.2-1.5 cm prior to inoculation on culture media.

Culture media and conditions

Surface sterilized rhizomal segments (1.2 - 1.5 cm) were cultured on MS[15] basal medium containing 3% (w/v) sucrose (Himedia, Mumbai, India) for culture initiation and served as explant sources for subsequent experiments. The pH of the medium (Supplemented with respective growth regulators) was adjusted to 5.8 with 1N NaOH or 1N HCl before gelling with 0.8% (w/v) agar (Himedia, Mumbai, India). In all the experiments, the chemicals used were of analytical grade. The explants initially were implanted vertically on the culture medium in test tube (150 × 25 nm) and plugged tightly with non-absorbent cotton. All the cultures were kept under cool fluorescent light (16 h photo period 40 μmol·m–2 s–1, Philips, India at 25°C ± 2°C) and 60% - 70 % relative humidity (RH).

Induction of multiple shoots

For initial multiple shoot induction, the explants were cultured on Murashige and Skoog's medium supplemented with various concentrations of BA (0.5 - 5.0 mg/l) in combination with NAA (0.1 - 5.0 mg/l). The induced shoots were allowed to grow for 27 days. After 27 days it was found that maximum number of shoots (15.40 ± 0.40^a) was obtained in the MS medium supplemented with 1.5 mg/l of BA and1 mg/l of NAA.

Rooting of shoots

Small micro shoots grown on subculture medium were transferred to half and full strength MS media separately, supplemented with various concentrations of IBA (0.5 – 5 mg/l) for root developement. Number of roots (12.00 ± 0.00^a) developed from micro shoots with an average length of 4.00 ± 0.11^{cd} were higher in half strength of MS medium supplemented with 1 mg/l of IBA. Where as the number of roots produced in full strength MS medium supplemented with 2.5 mg/l of IBA was 7.40 ± 0.24^d with an average length of 5.36 ± 0.15^a. IBA was filter sterilized and added to the medium after autoclaving under the sterilized environment of laminar air flow cabinet. Data were recorded on the percentage of rooting, the mean number of roots per shoot and the root length after four weeks of transfer onto the rooting medium.

Acclimatization of regenerated plants

The complete rooted plantlets with 6 - 8 fully expanded leaves were removed from the culture medium and the roots were washed gently under running tap water to remove agar. The plantlets were transferred to plastic pots (5 cm diameter) containing a mixture of sterilized garden soil and vermiculite in the ratio 2:1 and covered with transparent plastic bags to ensure high humidity. Each was irrigated with 1/6 MS basal salt solution devoid of sucrose and inositol every 4 days for 2 weeks. The growth chamber was maintained at 26°C ± 1°C, 80% - 85% relative humidity with light intensity of 50 μmol·m–2·s–1 on a 16 h photoperiod inside the culture room conditions. The relative humidity was reduced gradually and after 30 days the plantlets were transferred to pots (25 cm diameter) containing garden soil and kept under green house for another 2 weeks for further growth and development. Well acclimatized *in vitro* raised plants were transferred finally to its original habitat for its survivalability. There are no changes in respect to morphology, growth characteristics and floral features etc in between tissue culture regenarate plants and naturally grown field plants.

DNA extraction and pcr amplification

Genomic DNA was extracted from young leaves of *in vitro* raised and field grown plants of *Curcuma caesia* Roxb. and mother plant by Cytl trimethyl ammonium bromide (CTAB) procedure[16] with minor modifications. Quality and quantity of DNA was checked on 0.8% agarose gel and also from values obtained by 260/280 nm UV absorbance ratio[17].

Twelve arbitrary decamer RAPD primers (Bengalore Genni Pvt. Ltd., India) were used for polymerase chain reaction (PCR) for DNA amplification. DNA finger printing profiles were compared to evaluate clonal fidelity and genetic stability. Amplification was performed in 25 μL using PCR mixture of consisting of 2.5 μL Taq buffer, 1 μL dNTPs, 0.5 μL Taq polymerase, 2 μL DNA (approximate 50 ng/μL), 1.0 μL primer (10 pmol), 2.5 μL $MgCl_2$, 1 μL oil and 14.5 μL Mili Q water. The PCR reaction conditions were: preheating for 5 min at 94°C; 40 cycles of 25 sec at 94°C, 20 sec at 40°C and 1.25 min at 72°C and elongation was completed by a final extension of 6 min at 72°C. After amplification, the PCR product was resolved by electrophoresis in 1.4% agarose gel (Himedia, Mumbai, India) and stained with ethidium bromide (0.5 μg/ml). 2.0 - 23.1 kb λ DNA di-gested Hind III was used as the DNA marker, and bands were visualized under UV light and photographed using the Gel Doc equipment (Bio Rad). All the PCR reaction was repeated for thrice.

Statistical analysis

Experiments were set up in completely randomized block design. Each experiment was repeated three times with 10 - 12 replicates. Data were analyzed by one way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test $P \leq 0.05$[18] on the statistical package of SPSS (Version 10).

RESULTS AND DISCUSSION

In vitro establishment

For the primary establishment of *in vitro* culture from field grown plants surface sterilization of the explants was essential because of the microorganism can live or survive in the vascular tissue of the plants, therefore contamination attached to the surface of the explants. The main contaminants that affect tissue cultured plants are bacteria and fungi. Particularly hairy plants are a problem because bubble of air became entrapped in the explants and prevent good contact with the disinfesting agent. Evenly storage water and its included microorganism can be a source of some of the internal (endogenous) contamination observed *in vitro*[19]. The duration of exposure of the explants of the sterilizing agent is most important for any tissue culture studies. Therefore, to overcome contamination problem, surface sterilization of explants was done with 0.1% aquous solution of Mercuric chloride ($HgCl_2$) for 2, 4, 6, 8 and 10. Mercuric chloride ($HgCl_2$) is a very strong sterilant[20]. When the explants sterilization was done with 0.1% aquous solution of $HgCl_2$ for less than 5 min, 65% of the explants get contaminated. Whereas, when the duration of exposure of 0.1% aquous solution of $HgCl_2$ was above 5 min contamination frequency was significantly reduced to 10%. These explants remained green and showed healthy growth and proliferation of auxillary shoots. But when the duration of exposure was above 6 min, a death rate of the explant increases significantly (70%). All of the explants died when the explants were treated with 0.1% aquous solution of $HgCl_2$ for 7 - 10 min.

Effect of cytokinin and auxin combination on shooting

The morphogenetic responses of rhizomal segment explants to various cytokinin (BA) and auxin (NAA) are summarized in TABLE 2 and Figure 1C, 1D, 1E. When Explants were cultured on basal MS medium or, MS medium contains solely cytokinin (BA), or solely auxin (NAA) failed to produce shoots even after 4 weeks of inoculation, but when the explants cultured on MS medium supplemented with different concentrations and combinations of cytokinins and auxins with showed variation in the regeneration percentage and number of shoots formed. Rhizomes cultured on half strength MS basal medium showed no response. Actually there was a greater need of nitrogen and potassium containing compounds which induce greater amount of new proteins[21]. These components are deficit in half strength MS basal medium compared to full strength MS basal medium. Initial induction of shoots was noted after 10 - 12 days of inoculation (Figure 1B, 1C). Observations on different growth parameters from different treatments were recorded after 4 weeks of culture initiation following repeated subculturing after 7 days intervals. Among the different combinations of cytokinin and auxin tested, the best response (78.22±0.12 %) was obtained in the presence of 1.5 mg/l BA and 1 mg/l NAA (Figure 1E, 1F). The maximum number of multiple shoots was obtained in the same medium con-

taining same concentrations of BA and NAA. The number of shoots developed in this medium was 15.40±0.40 after 3 weeks. The average length of shoot in this medium was 8.46±0.06. The BA and NAA concentrations higher than 1.5 mg/l BA and 1 mg/l NAA, the number of shoots as well as percent response was reduced (TABLE 2). Reduction in the number of shoots generated from each bud of rhizome at BA concentration higher than the optimal level was also reported for several medicinal plants[13,22]. BA was reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation[23]. The stimulating effective of BA and NAA on multiple shoot formation has been reported earlier for several medicinal plant species including *Ocimum basilicum L.*, *Feronia limonia* L., *Mentha piperita L.*[14,24-28].

TABLE 1 : Standardization of $HgCl_2$ treatment period for surface sterilization of the explants.

Treatment Duration (min) with 0.1% HgCl2	Number of explants	Rate of Contamination (after day of treatment) 2	Rate of contamination after day of treatment) 3	Rate of Contamination (after day of treatment) 4	Rate of Contamination (after day of treatment) 5	Rate of Contamination (after day of treatment) 6	Rate of Contamination (after day of treatment) 7	Percentage of Contamination free explants after 10 days
2	12	3	6	7	9	12	12	11
4	12	1	4	5	6	6	8	28
6	12	0	0	0	0	0	1	60
7	12	0	0	2	All explant becomes dead***	All explant becomes dead***	All explant becomes dead*	85
8	12	1	2	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	97
10	12	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	All explant becomes dead*	100

"*" indicates explant death due to tissue killing, "**" indicates explant death due to microbial contamination.

TABLE 2 : Effect of cytokinin and auxin combination on shooting.

Concentrations of growth Regulators (mg/l)	Percentage of shoot proliferation	Number of shoots/ explant	Average Shoot Length (cm)
MS (Control)	$0.00±0.00^{i}$	0.00±0.00g	0.00±0.00f
MS+BA+NAA (mg/l)			
0.5+0.5	$67.22±0.16^{c}$	$7.20±0.20^{ef}$	$6.48±0.10^{b}$
0.5+1	$72.40±0.10^{b}$	$8.00±0.31^{e}$	$6.42±0.07^{b}$
1+1	$56.28±0.28^{f}$	$12.00±0.31^{cd}$	$5.42±0.13^{cd}$
1.5+1	$78.22±0.12^{a}$	$15.40±0.40^{a}$	$8.46±0.06^{a}$
2+1	$64.40±0.34^{d}$	$14.00±0.63^{b}$	$5.66±0.10^{c}$
2.5+1.5	$63.88±0.14^{d}$	$12.20±0.73^{cd}$	$6.54±0.08^{b}$
3+2	$67.80±0.50^{c}$	$13.40±0.60^{bc}$	$6.30±0.07^{b}$
3.5+2	$57.52±0.65^{e}$	$12.60±0.40^{bcd}$	$6.32±0.16^{b}$
4+2.5	$57.26±0.10^{ef}$	$11.20±0.58^{d}$	$5.22±0.08^{d}$
4.5+3	$44.82±0.50^{g}$	$7.60±0.40^{ef}$	$5.26±0.10^{d}$
5+4	$28.80±0.48^{h}$	$6.20±0.58^{f}$	$4.46±0.15^{e}$

Values are means ± SE. n = 10 - 12 (in triplicate); Means followed by same does not differ significantly according to Duncan's Multiple Range Test (p d" 0.05).

Effect of auxin on rooting

Healthy elongated shoots (4 - 9 cm in length) were excised and placed on full and half strength MS basal medium supplemented with different concentrations of auxin (IBA) at the range of 0.5 - 5.0 mg /l for induction of roots (TABLE 3). The effects of these auxins on root induction as well as the length of the roots were examined after 15 days of inoculation in root induction medium. In the preliminary experiments conducted, no rooting was observed when the shoots were culture on basal (Control) MS medium. Full strength MS medium containing auxins showed very poor response in rooting, but well developed roots were achieved on half strength MS medium supplemented with IBA (1 mg/ml) with increase sucrose concentration (4%) gave us well developed roots within 15 - 20 days. This foundation was supported by reports in various species like *Lavandula vera*[29], *Ocimum kilimandscharicum*[26], rooting frequency was higher when shoots were inoculated on half strength MS medium. The cause behind the favorable effect of reduced macronutrient concentration is that the increasing concentration of nitrogen ions reduced root development. So rooting is favourable in much lower nitrogen ions concentration than that are required for shoot developement[30]. IBA was more effective for root induction than any other auxins in both (half & full strength MS) types to media. The possible reason could be that IBA is more stable than any other auxins like IAA etc. to chemical degradation in tissue culture media, both during auto-claving and at room

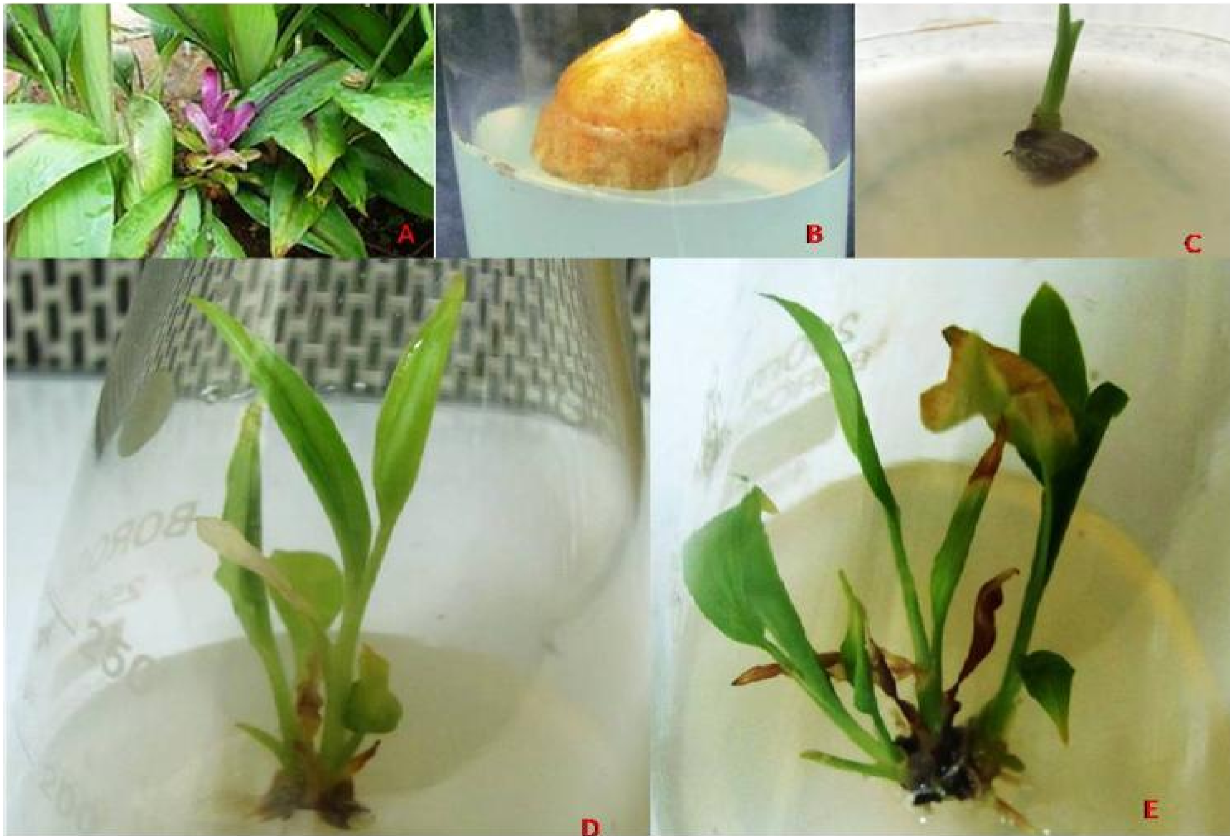

Figure 1 : (A) Field grown *Curcuma caesia* Roxb. plant; (B) Shoot induction from rhizomal explant on MS medium supplemented with 1.5 mg/l BA and 1 mg/l NAA after 10-12 days of inoculation; (C) Shoot proliferation from rhizomal explant on MS medium supplemented with 1.5 mg/l BA and 1 mg/l NAA after two subculture; (D) Induction of shoot multiplication on MS medium supplemented with1.5 mg/l BA and 1 mg/l NAA after repeated subculture; (E) More shoot multiplication on the same medium by repeated subculture.

temperature[31]. However, NAA nd IAA formed slender roots in half & full strength MS media. Similar response was observed by other species[32,33]. Among various concentrations of IBA tested, 1 mg /l of IBA proved to be the best in eliciting the highest frequency of root formation. Shoot formed roots at a high frequency (90.06±0.19 %) on medium containing 1 mg/l of IBA. In this medium a maximum number of 12.00±0.00 roots attaining a average length of 5.36 ± 0.15 cm were obtained. Further increase in the IBA concentration to 1.5 - 5 mg/l reduces root initiation. It has also been reported that shoots contain high level of endogenous auxins and the addition of exogenous auxin caused the inhibition of root development, thus resulted in callusing at the base of the shoots[34]. The stimulatory effect of IBA for root formation has also been reported in many medicinal plant species, including *Ocimum basilicum* L.[26], *Mentha piperita* L.[28]; *Ocimum gratissimum* L.[35], *Tylophora indica*[36].

Acclimatization and field establishment

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in soil. The well developed rooted plantlets were taken out gently from the test tubes and thoroughly washed with sterile water to remove adhered agar and traces of the medium to avoid contamination. 60 plantlets were transferred to plastic pots containing potting a mixture of (2:1) soil and vermiculite (Figure 1I). In the first week of transplantation, plantlets kept covered in a polythene tent for providing the condition of high humidity and sufficient light. The polythene cover was removed periodically and progres-sively whenever leaves appeared water soaked. Polythene covers were completely withdrawn after 4 - 5 weeks of hardening. After 5 weeks plants were then transferred to larger potted filled soil with organic manure kept under green house for further growth and development. Finally the acclimated plants were then shifted to the field conditions having 85%

TABLE 3 : Effect of auxin on rooting.

Concentrations of growth regulators (mg/l)	Percentage of Rooting	Number of roots/shoot	Average root length (cm)	Concentrations of growth regulators (mg/l)	Percentage of Rooting	Number of roots/shoot	Average root length (cm)
MS ½ strength	0.00±0.00^{p}	0.00±0.00^{j}	0.00±0.00^{g}	MS full strength	0.00±0.00^{p}	0.00±0.00^{j}	0.00±0.00^{g}
MS½ +IBA (mg/l)				MS+IBA (mg/l)			
0.5	19.46±0.12^{m}	6.20±0.58^{e}	4.24±0.11^{bc}	0.5	2.88±0.34^{o}	2.00±0.00^{i}	4.54±0.08^{b}
1.0	90.06±0.19^{a}	12.00±0.00^{a}	5.36±0.15^{a}	1.0	15.84±0.32^{n}	2.40±0.24^{i}	4.68±0.11^{b}
1.5	59.46±0.12^{d}	10.80±0.20^{b}	4.32±0.27^{bc}	1.5	56.46±1.12^{e}	4.40±0.24^{f}	4.54±0.06^{b}
2.0	64.18±0.03^{c}	10.60±0.40^{b}	4.16±0.19^{bc}	2.0	53.96±0.21^{fg}	6.20±0.20^{e}	4.46±0.14^{bc}
2.5	53.20±0.05^{g}	8.40±0.24^{c}	4.48±0.10^{bc}	2.5	74.62±0.18^{b}	7.40±0.24^{d}	4.00±0.11^{cd}
3.0	45.42±0.08^{h}	9.00±0.31^{c}	3.48±0.10e	3.0	74.88±0.30^{b}	5.40±0.24^{e}	4.38±0.09^{bc}
3.5	42.54±0.22^{i}	5.60±0.24^{e}	3.54±0.28^{e}	3.5	39.34±0.18^{j}	5.40±0.24^{e}	3.38±0.10^{de}
4.0	54.70±0.48^{f}	7.60±0.40^{d}	2.92±0.27^{f}	4.0	53.10±0.53^{g}	3.60±0.24^{fgh}	3.54±0.12^{de}
4.5	38.42±0.14^{j}	5.80±0.37^{e}	2.80±0.27^{f}	4.5	34.10±0.78^{k}	3.40±0.24^{gh}	2.60±0.08^{f}
5.0	27.36±0.79^{l}	4.00±0.31^{fg}	2.54±0.32^{f}	5.0	26.08±1.27^{l}	2.80±0.37^{hi}	2.50±0.13^{f}

Values are means ± SE. n = 10 - 12 (in triplicate); Means followed by same does not differ significantly according to Duncan's Multiple Range Test (p d" 0.05).

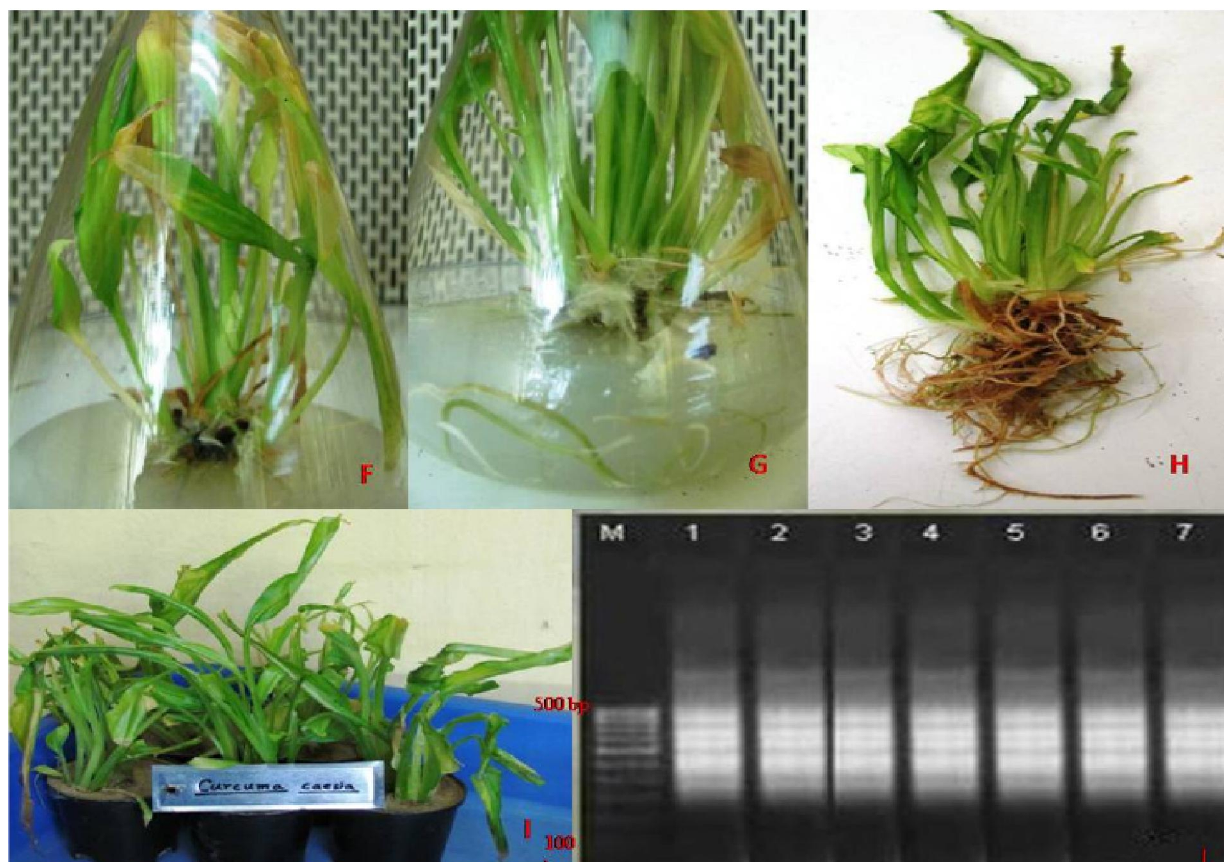

Figure 1 : (F) Root induction from micropropagated shoots on full strength MS medium supplemented with 1 mg/l and 3% sucrose concentration after 25-35 days of inoculation; (G) Rapid root induction from micropropagated shoots on ½ strength MS medium supplemented with 1 mg/l and 4% sucrose concentration after 15 days of inoculation; (H) Mature micropropagated plant having profuse roots; (I) Acclimatization of micropropagated plants; (J) RAPD marker analysis of *in vitro* raised field grown plants and mother plant of *Curcuma caesia* Roxb.: *lane* M corresponding to λ digested with *EcoRI* and *HindIII* as molecular weight marker (100-500bp), *lane* 1,2,3 DNA randomly selected regenerated plantlets, *lanes* 4 and 5 DNA from mother plant.

survived. The growth characteristics of *in vitro* raised plants did not show any significant morphological differences from those of natural occurring field plants.

Molecular analysis

Genetic uniformity is one of the most important prerequisites for the successful micropropagation of any crop species. Nevertheless, a major problem encountered in cells grown *in vitro* is the occurrence of genetic variation due to change in either DNA sequences, in chromosome structure (duplications, translocations) or in chromosome number (leading to polyploidy). Furthermore, abnormalities in tissue culture particularly growth regulators (in particular BA, IBA etc)[37], and in the plants produce from them often increase in frequency with increasing culture passages[38].

The PCR based RAPD technique does not require DNA sequence information and species specificity and hence it is being conveniently used for assessing genetic stability and clonal fidelity of micropropagated plants in a number of genera. There are many reports on molecular characterization of micropropagated plants by the RAPD technique especially to confirm the clonal fidelity and genetic stability among tissue culture grown plants and donor[39]. Because RAPD analysis is particularly well suited to high output system required for plant breeding, it is easy to perform, fast, reliable and of relatively low cost[39]. Keeping this perspective in mind, in this paper we performed the genetic integrity of *in vitro* regenerated plants from rhizomal explants and respective naturally occurring field grown donor plant of *Curcuma caesia* Roxb.

Total 12 primers were initially screened and finally 5 primers produce clear and scorable amplified bands ranging from 2 - 5 bands per primer (TABLE 4). Each primer produced a unique set of amplification products ranging in size from 100 bp - 3 kb (Figure 1J with primer 5' TGGCTCGGTA 3'). All 5 primers produced a total of 17 bands with an average of 3.40 fragments. All the 17 scorable bands were monomorphic in nature, indicating homogeneity among the culture regenerates and genetic uniformity with that of the donor plants. The possible reason may be multiple shoot bud differentiation without intervening callus phase is least vulnerable to genetic changes. However, no differences were observed between mother plant and plantlets regenerated from rhizomal segments by any five primers tested in present RAPD study.

TABLE 4 : Number of amplification products generated with the use of RAPD primers to assess genetic fidelity of micropropagated and field grown plants.

SL. No.	Primer Code	Nucleotide sequence (5'-3')	Number of generated bands
1	RAPD1	GTCCTACTCG	-
2	RAPD2	GTCCTTAGCG	2
3	RAPD3	CGGGATCCGC	-
4	RAPD4	CTTCCGGCAG	3
5	RAPD5	GGTATTACTT	4
6	RAPD6	TGGCTCGGTA	5
7	RAPD7	CTTCGGCAGA	-
8	RAPD8	GGTATTACTT	-
9	RAPD9	GACAATGGTA	-
10	RAPD10	TTAGCTTAGG	-
11	RAPD11	CTCTCCGCCA	-
12	RAPD12	GCACGCCGGA	3

CONCLUSION

In conclusion, the present study, we established an efficient and reliable micropropagation protocol for *in vitro* regeneration of *Curcuma caesia* Roxb. from rhizomal explant, which can ensure large scale propagation, as well as protocol can also be used for raising genetically uni-form plants, which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm. As all the micropropagated plants are genetically true to type with naturally occurring plants so there reduced chance of genetic manipulations in micropropagated plants. As either no or reduced chance of genetic manipulation occurs, so there is also reduced chance of variability of secondary metabolite contents in micropropagated plants with that of naturally occurring plants. Our results also indicate that multiple shoot induction, rooting of shoots and ultimately regeneration of *Curcuma caesia* Roxb. regulated by appropriate cytokinin and auxin concentration and combinations. As the micropropagated plants are acclamatized to survive in natural environment and all the micropropagated plants are genetically *true to type*, so this protocol can be used in industry to make large scale plant production. Further, our results demonstrate that RAPD markers

can be applied to evaluate the genetic stability of regenerants for the *ex situ* conservation of this important aromatic and medicinal herb.

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