Plant regeneration in *Curcuma* species and assessment of genetic stability of regenerated plants

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Abstract

An efficient plant regeneration protocol was developed from rhizomes of two *Curcuma* species *C. longa* and *C. amada*. Response was highly dependent on the season, with above 69 % of culture developing adventitious shoots during spring. Greatest regeneration and multiplication was observed in modified Murashige and Skoog (MS) medium supplemented with 13.31 μM benzyladenine and 2.68 μM α-naphthalene acetic acid (NAA) in *C. longa* or 2.46 μM indolebutyric acid in *C. amada*. Effect of sugars and agar at different concentrations were also studied and 2 % maltose and 0.7 % agar were found optimum for shoot multiplication and regeneration. Most plantlets developed roots simultaneously but others formed roots when subcultured in ½ MS medium supplemented with 2.68 μM NAA. Plants were successfully hardened in greenhouse with 80 % survival. The genetic purity of micropropagated plantlets was analyzed using RAPD and protein profiles.

Additional key words: axillary bud, micropropagation, RAPD, SDS-PAGE, *Zingiberaceae*.

Introduction

Curcuma amada Roxb. and *Curcuma longa* L. are the two commercially important plants of the family *Zingiberaceae*. They are propagated vegetatively by underground rhizomes at a slow rate. *In vitro* clonal multiplication of *Curcuma* species through rhizome buds has been reported (Sit and Tiwari 1997, Salvi *et al.* 2002, Prakash *et al.* 2004) but the difficulty has been to establish good regenerating material in a suitable medium. Even attempts at using axillary bud meristem have faced problem at the multiplication stage. Another inherent problem with *Curcuma* rhizome is to establish a good axenic culture. A rapid multiplication method is therefore necessary to provide disease-free planting material in enough quantity especially for endemic species *C. longa* that are available in limited quantities.

The establishment of genetic stability of *in vitro* regenerated plants will be an essential requisite for large scale multiplication. Despite the advantages of the *in vitro* propagation, genetic instability has been observed in micropropagated species hence it is necessary to establish a system that produces genetically stable and identical plants. Several strategies can be used to assess the genetic stability of *in vitro* derived plants such as karyological analysis and isozyme markers, but they have their own limitations (Isabel *et al.* 1993). Bimolecular analytical techniques like polypeptide and DNA polymorphisms profiling facilitates direct and reliable measurements to detect culture-induced variation at the DNA and protein level (Cloutier and Landry 1994). Of several molecular markers used for assessment, random amplified polymorphic marker DNA (RAPD) is the simplest, cheapest and appears to be useful for the analysis of genetic fidelity of *in vitro* propagated plants and have been well established in many plants (Williams *et al*. 1990, Salvi *et al.* 2001, Panda *et al.* 2007, Hussain *et al.* 2008, Tyagi *et al.* 2010). Polypeptide profiling (SDS-PAGE) is a useful biochemical marker system, which has been applied, *e.g.*, to seeds analysis for diagnostics and estimation of outcrossing rates (Ferreira *et al.* 2000).

In the present communication we report an efficient regeneration from mature rhizomes for two *Curcuma* species from Northeast India with the aim to multiply, conserve and establish a disease free material for further

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Abbreviations: BA - benzyladenine; bp - base pairs; IBA - indolebutyric acid; KIN - kinetin; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; RAPD - random amplified polymorphic DNA; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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studies. We investigated the effect of modified Murashige and Skoog (1962; MS) media with different hormone combination for multiplying *Curcuma* species. In addition, we performed RAPD and SDS-PAGE analysis of the micropropagated plants for genetic purity assessment.

Materials and methods

Mature rhizomes of *Curcuma amada* Roxb. and *Curcuma longa* L. collected from different districts of Assam (Kamrup, Barpeta, Jorhat and Sivasagar) having axillary buds were used as starting material. Outer scales were removed and buds were cut into small pieces (1 - 2 cm long) and washed under running tap water to remove dust and adhering soil particles. The explants were treated with 0.1 - 0.2 % *Bavistin* solution along with (2 % m/v) *Tween 20* for 5 min and rinsed with water. Explants were pretreated with 70 % ethanol for 2 min before surface sterilizing with 0.1 % (m/v) mercuric chloride for 10 min followed by five rinses with sterile double distilled water.

Both ends of the explants were trimmed and incubated in culture tubes (*Borosil*, Mumbai, India) containing the standard MS medium and modified MS medium (MSR) containing additional supplements like yeast extract (300 mg dm^{-3}) and casein hydrolysate (100 mg dm^{-3}) with 0.8 % agar (*Himedia*, London, UK) and 3 % sucrose. The pH of the media was adjusted to 5.8 and tubes were autoclaved at 121 °C for 15 min. Cultures were incubated at temperature of 25 ± 2 °C, 16-h photoperiod and irradiance of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Different concentrations of benzyladenine (BA) and kinetin (KIN) were tried singly and in combination with NAA and IBA for shoot multiplication and maintenance (MM medium).

To study the effect of gelling concentration on shoot multiplication, shoot tips were cultured on MM medium solidified with 0.6, 0.7 and 0.8 % agar. The effect of sugars at varying concentrations on *in vitro* shoot multiplication was also carried out by supplementing MM medium with 1, 2 or 3 % sucrose. Subculturing was carried out after 3 and 8 weeks of culture, the number of shoots per explant, shoots length, number of roots per shoot and root length were recorded.

Rooted plantlets were removed from culture tubes and washed thoroughly with tap water to remove the adhering medium and planted in poly-bags containing sand and clay at the ratio of 1:4, and kept in the mist chamber. Plants were hardened under a 16-h photoperiod, irradiance of 55 μmol m^{-2} s^{-1}, 28 ± 2 ºC and relative humidity of 80 % in the greenhouse. Intermittent mist was supplied for 30 s at 15-min intervals. The percentage survival was determined after two months.

For RAPD analysis, 10 regenerated plants of *C. amada* and *C. domestica* collected from 60-d-old plantlets selected randomly and mother plant were analyzed. Total genomic DNA was extracted from fresh tender leaves using SDS protocol with slight modifications (McCouch 1992). Leaf tissue (1 g) was ground in liquid nitrogen and suspended in 10 cm^3 of extraction buffer (100 mM Tris, 0.5 M NaCl, 50 mM EDTA) containing 1 % β-marcaptoethanol. The suspension was incubated at 65 °C for 30 min, extracted with 5 M potassium acetate and centrifuged at 2 795 *g* at 4 °C for 30 min. The aqueous phase was precipitated with isopropanol and again centrifuged at 2 795 *g* at 4 °C for 20 min. The pellet was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and treated with RNAse. DNA was purified by ethanol precipitation. The quality and quantity of the extracted DNA was confirmed to be consistent both spectrophotometrically and by running the extracted DNA on 1.0 % agarose gels containing 0.5 μg cm^{-3} of EtBr.

PCR amplification of the genomic DNA was carried out using 10 arbitrary decamer oligonucleotide primers (*Operon Technologies*, Almeda, USA). Each reaction mixture of 0.02 cm^3 contained 50 μg cm^{-3} of template DNA, 1× assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1 % gelatin), 0.2 mM each dNTPs (*Banglore Genei*, Bangalore, India), 5 pmol of each primer and 0.05 U of *Taq* polymerase (*Banglore Genei*). The reaction was performed in 0.2 cm^3 microfuge tubes (*Dialabs*, Austria). PCR amplification was carried out in a mini thermal cycler (*Applied Biosystems*, CA, USA) programmed for 40 cycles. The first amplification cycle consisted of initial denaturation step of 5 min at 94 °C. This was followed by 40 cycles of 45 s at 94 °C, annealing for 1 min at 32 °C, and extension at 72 ºC for 2 min. An additional cycle of 10 min at 72 ºC was used for primer extension. The amplification products were electrophoresed in 1.3 % agarose gels in 1× TAE (50× stock contained 2 M Tris, 0.5 M EDTA and glacial acetic acid). The gels were visualized and photographed under UV radiation by a gel documentation system (*BioRad*, Hercules, USA). The size of amplification products was estimated using λ DNA marker (*Banglore Genei*). PCR amplification was repeated twice and only primers producing reproducible bands were considered for analysis.

Protein extraction was carried out for the same regenerated plants from which DNA was extracted. Young leaves were homogenized in 10 mM Tris-EDTA buffer (pH 8.0) containing 10 % SDS, 5 mM β-mercapto-ethanol and 0.1 mg cm^{-3} phenylmethanesulphonyl fluoride (PMSF). After centrifugation at 10 000 *g* for 10 min (4 ºC), the supernatant was boiled for 10 min. Protein content was measured by Bradford method (1976) using bovine serum albumine as a standard. Proteins were separated using discontinuous SDS-PAGE (10 % running gel, pH 8.8 and 5 % stacking gel, pH 6.8) at 4 ºC according to Laemmli (1970). After electrophoresis, gels

were stained overnight with 0.25 % Coomassie Brilliant Blue R250, destained, fixed and photographed. Molecular mass of polypeptides was determined according to the mobility of the standard proteins.

Three replicates (10 explants per replicate) were inoculated per treatment to test the effects of medium on multiplication rate. All experiments were carried out independently and repeated thrice. The data were analyzed using one-way *ANOVA* (*SPSS 16.0* version, 2008) and significant differences between treatment means were assessed using Duncan's multiple range test (DMRT) at a 5 % probability level ($P < 0.05$). Amplified DNA and polypeptide fragments were scored as present or absent both in the regenerated and in mother plants. Electrophoretic bands of low visual intensity that could not be readily distinguished as present or absent were considered as ambiguous and were not scored.

Results

Establishment of the *in vitr*o cultures in *Curcuma* species posed considerable problems with contamination in primary cultures, which reappeared even after repeated subculturing. The problem was overcome by treating the explants with 0.2 % (m/v) *Bavistin* for 2 h and then with 70 % ethanol for 2 min.

No organogenic response was observed after 4 weeks, in the excised meristem cultured *in vitro* on MS medium supplemented with vitamins and carbon source but devoid of growth regulators. The addition of BA and KIN (4.43 µM each) to the basal medium induced axillary buds sprouting (Fig. 1*A*). Multiple shoots were induced from *in vitro* raised explants on MS and MSR media with varying concentrations and combinations of cytokinins and auxins. The greatest response in *C. amada* were recorded after 2 weeks in MS (93.33 %) and MSR medium (100 %), both supplemented with 13.31 µM BA + 2.46 µM IBA (Table 1).

Rhizome buds of *C. amada* when cultured on MSR medium containing 13.31 µM BA + 2.46 µM IBA started to proliferate producing new shoots within 10 d whereas in MS medium with the same hormone combination, buds started to proliferate only after 3 weeks of culturing. The highest mean number of buds per explant (4.3 in MS and 8.9 in MSR medium) was recorded at the same hormone combination (Fig. 1*B*). This medium also showed the highest length of the longest shoot (Table 1). The buds of *C. longa* showed a regeneration frequency of 80 and 100 % in MS and MSR medium, respectively (Table 1). Response was 100 % in MSR medium with 13.31 µM BA + 2.46 µM IBA, but with a lesser average number of shoot per explants. The highest mean number of shoots per culture (8.2) was in MSR medium supplemented with 13.31 µM BA + 2.68 µM NAA (Fig. 1*C*), showing greater response than in MS medium having the same hormone concentration (4.2). The highest length of the longest shoot in MSR medium was also recorded in the same medium (Table 1). BA was found to be a more effective than KIN for multiple shoot regeneration when used alone. Subsequent subculturing on the optimal multiplication medium repeatedly for two or three cultures did not increase the multiplication rate.

When different concentrations of agar were tested, 0.7 % was found to be better than the others for shoot

Table 1*A*. Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma amada* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [µM]	MS medium response [%]	shoot number [explant^{-1}]	shoot length [cm]	MSR medium response [%]	shoot number [explant^{-1}]	shoot length [cm]
BA	4.43	0	-	-	30.0 ± 10.0	1.80 ± 0.11b	1.47 ± 0.05a
	13.31	43.3 ± 6.67	1.33 ± 0.13a	2.70 ± 0.85c	56.6 ± 6.67	2.40 ± 0.15cd	2.68 ± 0.07c
	22.19	16.6 ± 3.33	1.00 ± 0.00a	2.10 ± 0.17b	63.3 ± 3.33	1.50 ± 0.11ab	2.01 ± 0.06b
KIN	2.32	0	-	-	53.3 ± 3.33	1.50 ± 0.12ab	1.66 ± 0.08e
	4.64	46.6 ± 6.67	1.50 ± 0.13abc	2.60 ± 0.08c	70.0 ± 0.00	2.00 ± 0.16abc	2.90 ± 0.06d
	9.29	56.6 ± 3.33	1.16 ± 0.34cd	1.60 ± 0.08a	56.6 ± 6.67	1.30 ± 0.11a	2.60 ± 0.12b
BA + NAA	13.31 + 2.68	73.3 ± 6.67	3.40 ± 0.19f	4.00 ± 0.10e	93.3 ± 3.33	7.60 ± 0.25g	4.96 ± 0.07f
	13.31 + 5.37	60.0 ± 0	2.40 ± 0.14de	3.10 ± 0.09d	56.6 ± 6.67	2.10 ± 0.12bc	3.23 ± 0.08e
KIN + NAA	4.64 + 2.68	53.3 ± 3.33	2.30 ± 0.13de	2.50 ± 0.09c	90.0 ± 5.78	3.20 ± 0.15e	3.18 ± 0.07e
	4.64 + 5.37	50.0 ± 0	1.90 ± 0.18bcd	1.70 ± 0.08a	66.6 ± 3.33	2.40 ± 0.13cd	2.03 ± 0.11b
BA + IBA	13.31 + 2.46	93.3 ± 6.67	4.30 ± 0.29g	4.90 ± 0.09 f	100.0 ± 0.00	8.96 ± 0.43h	5.56 ± 0.10g
	13.31 + 4.92	53.3 ± 3.33	2.30 ± 0.12de	2.64 ± 0.81c	83.3 ± 8.82	3.90 ± 0.16f	2.50 ± 0.05c
KIN + IBA	4.64 + 2.46	90.0 ± 10.0	2.70 ± 0.18ef	2.60 ± 0.07c	93.3 ± 6.67	2.70 ± 0.13cde	2.13 ± 0.07b
	4.64 + 4.92	63.3 ± 3.33	2.40 ± 0.14de	2.03 ± 0.07b	66.6 ± 3.33	2.90 ± 0.22de	2.11 ± 0.08b

Table 1*B*. Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma longa* (after 6 weeks of culture). Means ± SE, n = 10. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [μM]	MS medium response [%]	shoot number [explant^{-1}]	shoot length [cm]	MSR medium response [%]	shoot number [explant^{-1}]	shoot length [cm]
BA	4.43	0	-	-	56.67 ± 3.33	1.10 ± 0.09a	1.57 ± 0.04b
	13.31	46.67 ± 8.82	1.20 ± 0.20a	1.37 ± 0.12abc	90.00 ± 5.77	2.71 ± 0.11e	2.02 ± 0.11cd
	22.19	40.00 ± 5.77	1.57 ± 0.03abc	1.62 ± 0.02bcd	66.67 ± 3.33	2.00 ± 0.15cd	1.57 ± 0.18c
KIN	2.32	13.33 ± 3.33	1.00 ± 0.00a	1.08 ± 0.06a	56.67 ± 6.67	1.29 ± 0.06a,b	1.49 ± 0.20a
	4.64	46.67± 3.33	1.85 ± 0.08bc	1.89 ± 0.05de	86.67 ± 8.82	2.63 ± 0.26e	2.22 ± 0.06de
	9.29	50.00 ± 5.77	1.08 ± 0.08a	1.27 ± 0.07ab	60.00 ± 5.77	1.78 ± 0.04bc	1.82 ± 0.24bc
BA + NAA	13.31 + 2.68	80.00 ± 10.0	4.27 ± 0.30f	4.25 ± 0.07h	100.00 ± 0.00	8.17 ± 0.17h	4.54 ± 0.13i
	13.31 + 5.37	60.00 ± 5.77	1.71 ± 0.27bc	1.68 ± 0.11c,d	60.00 ± 5.77	2.57 ± 0.14e	2.91 ± 0.08g
KIN + NAA	4.64 + 2.68	70.00 ± 5.77	2.25 ± 0.39cd	2.24 ± 0.02ef	66.67 ± 3.33	3.17 ± 0.12f	3.10 ± 0.05g
	4.64 + 5.37	56.67 ± 3.33	1.37 ± 0.19ab	1.37 ± 0.01abc	63.33 ± 3.33	1.74 ± 0.30bc	2.53 ± 0.01f
BA + IBA	13.31 + 2.46	73.33 ± 3.33	3.61 ± 0.22e	3.58 ± 0.12g	100.00 ± 0.00	7.03 ± 0.15g	3.96 ± 0.10h
	13.31 + 4.92	53.33 ± 3.33	1.87 ± 0.13bc	1.93 ± 0.04de	56.67 ± 3.33	2.42 ± 0.21de	2.42 ± 0.04ef
KIN + IBA	4.64 + 2.46	56.67 ± 3.33	2.49 ± 0.16d	2.50 ± 0.07f	80.00 ± 0.00	3.33 ± 0.11f	3.72 ± 0.06h
	4.64 + 4.92	53.33 ± 3.33	1.81 ± 0.12bc	1.82 ± 0.04d	60.00 ± 5.77	1.58 ± 0.21bc	1.76 ± 0.13bc

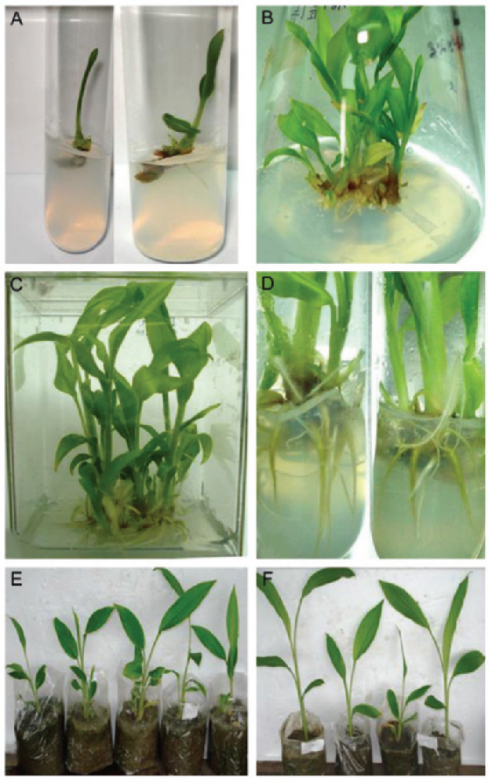

Fig. 1. Plant regeneration in *Curcuma* species: *A* - shoot induction from axillary bud in MSR medium supplemented with BA and kinetin after 2-week culture period in *C. amada* and *C. longa*. *B* - multiple shoots formed from axillary bud of *C. amada* in MSR medium (13.31 μM BA + 2.46 μM IBA) after 8 weeks, *C* - simultaneous rooting and shooting in *C. longa* in MSR medium (13.31 μM BA + 2.68 μM NAA) after 8 weeks, *D* - rooting of shoots in ½ MS + NAA (2.68 μM) after 4 weeks, *E, F* - 2-month-old hardened plants of *C. amada* (*E*) and *C. longa* (*F*).

multiplication, while 0.6 % agar produced the highest shoot length. Shoots produced on medium with 0.8 % agar were long, slender, elongated with some shoots showing signs of drying and browning (data not shown). Carbon source in culture medium had also a significant effect on the growth of *Curcuma* species. MSR medium containing maltose was giving best result as far as shoot induction and multiplication was concerned in both the *Curcum*a species studied when compared to MS medium containing sucrose. Further, a significant increase in shoot length, and number and length of roots were observed when the concentration of sugar was 2 % suggesting that low concentration of sugar could be best for *in vitro* multiplication of *Curcuma* species.

Rooting was observed in all combination of BA with IBA and NAA in both *C. amada* and *C. longa*. This indicated the inherent root inducing tendency of explants of rhizome origin rather than the influence of hormones applied exogenously. Such response has been reported earlier in *C. haritha* (Bejoy *et al.* 2006). 83.3 % of well developed shoots (5 - 6 cm) when excised and transferred to ½ MS medium supplemented with 2.68 μM NAA produced well developed roots (Table 2, Fig 1*D*).

Plantlets developed *in vitro* could be hardened in the greenhouse for two months suggesting the suitability of the *in vitro* protocol (Fig. 1*E*,*F*). Hardened plants of *C. amada* and *C. longa* recorded about 80 and 100 % survival and were reared to maturity.

To confirm the genetic stability of regenerated plants maintained in culture for a period of 2 months, RAPD analysis was carried out. Out of the 10 different RAPD primers tested, 9 and 7 primers produced clear and scorable bands in *C. amada* and *C. longa*, respectively. Nine selected RAPD primers utilized in this study for *C. amada* gave rise to a total of 46 scorable bands, ranging from 300 - 1 800 bp in size. The number of bands

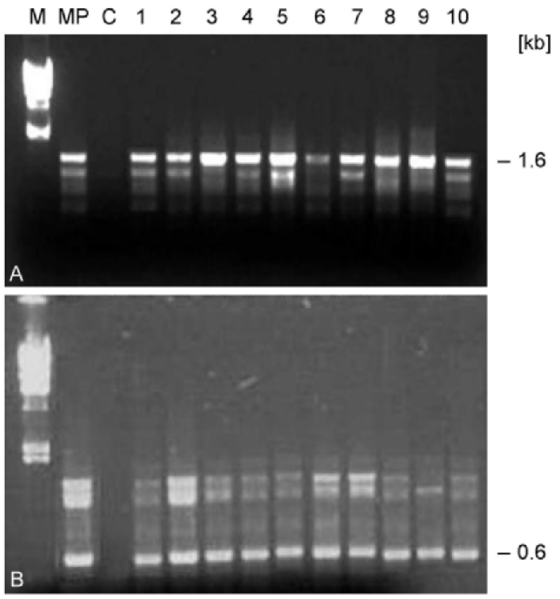

Fig. 2. RAPD profiles of micropropagated plants of *Curcuma* species using the decamer primers: *A* - *C. amada* (OPO-04), *B* - *C. longa* (OPA-03). M - DNA marker, *lane* MP - DNA from the mother plant, *lane* C - water control, *lanes* 1 - 10, DNA from micropropagated plants.

for each primer varied from 1 - 8, with an average of 5.1 bands per primer. Similarly, the 7 selected RAPD primers utilized in this study for *C. longa* gave rise to a total of 36 scorable bands, ranging from 500 - 2 000 bp in size and the number of bands for each primer varied from 1 - 5, with an average of 5 bands per primer. Each primer generated a unique set of amplification products that were monomorphic across all the micropropagated plants. Number of monomorphic bands was highest, six in case of primer OPA-03 for *C. amada* and was lowest in case of the primer OPA-10 in *C. longa*. Overall, no changes in the amplified fragments were detected among all micropropagated plantlets with reference to donor plant, which confirmed the genetic stability of these plantlets derived *in vitro*. Fig. 2 shows representative examples of amplified banding patterns produced by RAPD primers.

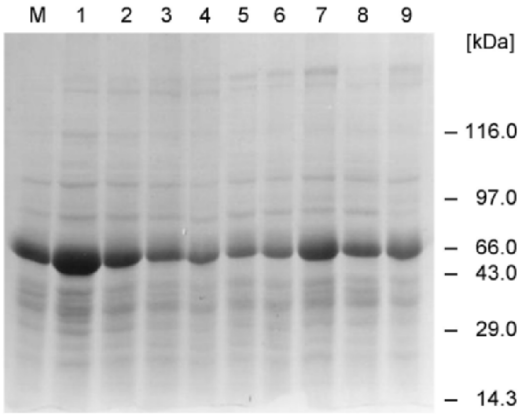

Fig. 3. Polypeptide profiles from leaves of micropropagated plants of *C. amada* using SDS-PAGE analysis. *Lane* M - proteins from the mother plant, *lanes* 1 to 9 - proteins from micropropagated plants, molecular mass marker (14 - 116 kDa) *on the right*.

In SDS-PAGE analysis, 12 unique bands were observed and the protein patterns of both micropropagated plants and the mother plant exhibited relatively high degree of identity (Fig. 3). The difference among polypeptides is in the relative intensity of the stained bands rather than in their numbers. It was observed that the low molecular mass proteins produced high intensity bands while the high molecular mass proteins were present in lower concentrations.

Table 2. Effect of auxin treatments on *in vitro* rooting in shoots of *C. amada* and *C. longa* cultured in half-strength MS medium (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [μM]	*C. amada* response [%]	root number [shoot^{-1}]	root length [cm]	*C. longa* response [%]	root number [shoot^{-1}]	root length [cm]
NAA	2.68	76.6 ± 3.33	5.2 ± 0.18e	4.4 ± 0.12d	83.3 ± 3.33	7.1 ± 0.24d	5.2 ± 0.12e
	5.37	56.6 ± 6.67	1.6 ± 0.12ab	1.7 ± 0.11a	66.6 ± 6.67	3.0 ± 0.13b	3.3 ± 0.09d
IAA	2.85	60.0 ± 5.78	3.8 ± 0.15d	3.1 ± 0.09c	70.0 ± 5.78	3.8 ± 0.20c	2.5 ± 0.10b
	5.70	50.0 ± 5.78	1.2 ± 0.12a	2.1 ± 0.06b	46.6 ± 3.33	2.0 ± 0.15a	1.8 ± 0.12a
IBA	2.46	73.3 ± 3.33	3.0 ± 0.15c	3.0 ± 0.10c	56.6 ± 6.67	3.5 ± 0.15bc	3.0 ± 0.14c
	4.92	56.6 ± 8.29	1.8 ± 0.13b	2.2 ± 0.08b	43.3 ± 3.33	2.1 ± 0.14a	1.5 ± 0.10a

Discussion

In rhizomatous plants, contamination is a major problem during initiation and further successful establishment of aseptic cultures (Balachandran *et al.* 1990, Borthakur *et al.* 1999). It was observed that for *Curcuma* species, percentage response and number of explants showing contamination was highly dependent on the season during

which the material was collected. Spring (March - May), when plants are actively growing, was found to be the most favorable season for initiation of culture; 69 % of cultures developed adventitious shoots and rate of contamination was also less. *In vitro* seasonal effect on bud growth has been reported in rhizomatous species such as *Curcuma zedoaria* (Stanly and Keng 2007) and *Curguligo orchioides* (Wala and Jasrai 2003).

In the present investigation, multiplication was found to occur by development of axillary buds which is ideal for maintaining genetic stability. However, the rate of bud multiplication was significantly different according to the various concentrations of growth regulators and combination of BA with lower concentration of auxin (NAA or IBA) was found to be ideal for shoot multiplication. However, persistence of explants in culture media containing higher concentration of cytokinins suppressed shoot elongation in contrast to other researchers who used rather high concentrations of plant growth regulators for the multiple shoot formation for some of the *Zingiberaceae* species (Chan and Thong 2004, Bharalee *et al.* 2005). Loc *et al.* (2005) reported that MS medium supplemented with 20 % (v/v) coconut water, 3 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA induced the formation of 6 shoots per explants in *C. zedoaria*. Bharalee *et al.* (2005) found that MS medium supplemented with 4 mg dm^{-3} BA and 1.5 mg dm^{-3} NAA was the best medium for shoot multiplication of *C. caesia* (3.5 shoots per explants) and MS with 1 mg dm^{-3} BA + 0.5 mg dm^{-3} NAA for *C. zedoaria* (4.5 shoots per explants). Balachandaran *et al.* (1990) reported that *C. domestica* could produce 3.4 shoots per explants, *C. caesia* produced 2.8 shoots per explants using MS medium supplemented with 3 mg dm^{-3} BAP. Nayak (2000) reported MS medium supplemented with 5 mg dm^{-3} BAP to be effective for shoot multiplication in *C. aromatica* producing an average of 3.3 shoots per explants whereas Mohanty *et al.* (2008) found 3 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA to be optimum medium for multiple shoot induction *via* indirect regeneration in the same species. Our results indicated that 13.39 μM BA and 2.68 μM NAA in the MSR medium was sufficient for the induction of multiple shoots from the buds and shoots of *C. amada* and *C. longa.*

Sucrose is widely used as a standard carbon source for plant tissue culture, and different concentrations and different osmotic environments have been used. However, a significant increase in shoot length and number was observed when maltose was added to the medium suggesting that non reducing sugars such as maltose could be best carbon source for *in vitro* multiplication of *Curcuma* species. Sugars (sucrose or maltose) at concentration 2 % were found to be most suitable for shoot multiplication. At concentration above 3 % *Curcuma* plantlets were etiolated and died. Similar observations were recorded in ginger species (Barthakur and Bordoloi 1992). However higher concentration of sugar source has been found to be ideal for *in vitro* microrhizome production in *Zingiber officinale* (Zheng *et al.* 2008).

RAPD based genetic assessment of genetic stability of *in vitro* grown micropropagated plants has been reported in many other plant species (Rout and Das 2002, Hussain *et al.* 2008, Tyagi *et al*. 2010). The source of the explants and mode of regeneration (somatic embryogenesis/ organogenesis/axillary bud multiplication) are known to play a major role in determining the presence or absence of variation (Salvi *et al.* 2001). We have used rhizomatous buds as explants for micropropagation of *C. longa* and *C. amada* because it lowers the risk of genetic instability. The results concur with earlier reports (Suri *et al.* 1999) that the micropropagation through explants containing organized meristem is generally more resistant to genetic changes that might occur indirect regeneration (Salvi *et al.* 2002).

The results obtained from our study suggest that *in vitro* regeneration and multiplication of *C. longa* and *C. amada* from rhizome with axillary buds could be used for rapid clonal propagation of these two valuable medicinal plants with the least possibility of genetic variations. These can also be used as a source of disease-free planting material for the farmers.

References

Balachandran, S.M., Bhat, S.R., Chandel, K.P.S.: *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). - Plant Cell Rep. **8**: 521-524, 1990.

Bejoy, M.M., Anish, N.P.: Factors affecting the *in vitro* multiplication of the endemic zingiber *Curcuma haritha* Mangaly and Sabu. - Asian J. Plant Sci. **5**: 847-853, 2006.

Bharalee, R., Das, A., Kalita, M.C.: *In vitro* clonal propagation of *Curcuma caesia* Roxb and *Curcuma zedoaria* Rosc from rhizome bud explants. - Plant Biochem. Biotechnol. **14**: 61-63, 2005.

Barthakur, M.P., Bordoloi, D.N.: Micropropagation of *C. amada*. - J. Spices Arom. Crops **1**: 154-159, 1992.

Borthakur, M., Hazarika, J., Singh, R.S.: A protocol for micropropagation of *Alpinia galanga*. - Plant Cell Tissue Organ Cult. **55**: 231-233, 1999.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chan, L., Thong, W.H.: *In vitro* propagation of Zingiberaceae species with medicinal properties. - J. Plant Biotechnol. **6**: 181-188, 2004.

Cloutier, S., Landry, B.S.: Molecular markers applied to plant tissue culture. - *In Vitro* Cell Dev. Biol. Plant. **30**: 32-39, 1994.

Ferreira, J.J., Alvarez, E., Fueyo, M.A., Roca, A., Giraaldez, R.: Determination of outcrossing rate *Phaseolus vulgaris* L. using seed protein marker. - Euphytica **113**: 259-263, 2000.

Hussain, Z., Tyagi, R.K., Sharma, R., Agarwal, A.: Genetic

diversity in *in vitro* conserved germplasm of *Curcuma longa* as revealed by RAPD markers. - Biol. Plant. **52**: 627-633, 2008.

Isabel, N., Tremblay, L., Michaud, M., Tremblay, F.M., Bousquet, J.: RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis in ginger. - Plant Cell Tissue Organ Cult. **32**: 289-292, 1993.

Laemmli, U.K.: Cleavage of structural proteins assembly of the bacteriophage T4. - Nature **22**: 680-685, 1970.

Loc, N.H., Duc, D.T., Kwon, T.H., Yang, M.S.: Micropropagation of zedoary (*Curcuma zedoaria* Roscoe) – a valuable medicinal plant. - Plant Cell Tissue Organ Cult. **81**: 119-122, 2005.

McCouch, S.: Miniscale DNA Extraction Suitable for PCR from Rice Leaf. - Genome Mapping Laboratory, PBGB, Manila 1992.

Mohanty, S., Panda, M.K., Subudhi, E., Nayak, S.: Plant regeneration from callus culture of *Curcuma aromatica* and *in vitro* detection of somaclonal variation through cytophotometric analysis. - Biol. Plant. **52**: 783-786, 2008.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nayak, S.: *In vitro* multiplication and microrhizome production in *Curcuma aromatica* Salisb. - Plant Growth Regul. **32**: 42-47, 2000.

Panda, M.K., Mohanty, S., Subudhi, E., Acharya, L., Nayak, S.: Assessment of genetic stability of micropropagated plants of *Curcuma longa* L. by cytophotometry and RAPD analyses. - Int. J. Intregrative Biol. **1**: 189-194, 2007.

Prakash, S., Seshadri, S., Elangomathavan, R., Kathiravan, K., Ignacimuthu, S.: Efficient regeneration of *Curcuma amada* Roxb plantlets from rhizome and leaf sheath explants. - Plant Cell Tissue Organ Cult. **78**: 159-165, 2004.

Rout, G.R., Das, G.: An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers. - Biol. Plant. **45**: 27-32, 2002.

Salvi, N.D., George, L., Susan, E.: Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. - Plant Cell Tissue Organ Cult. **66**: 113-119, 2001.

Salvi, N.D., George, L., Susan, E.: Micropropagation and field evaluation of micro propagated plants of turmeric. - Plant Cell Tissue Organ Cult. **68**: 143-151, 2002.

Sit, A.K., Tiwari, R.S.: Micropropagation of turmeric (*Curcuma longa* L). - Recreational Horticult. **4**: 145-148, 1997.

Stanly, C., Keng, C.L.: Micropropagation of *Curcuma zedoaria* Roscoe and *Zingiber zerumbet* Smith. - Biotechnology **6**: 555-560, 2007.

Suri, S.S., Jain, S., Ramawat, K.G.: Plantlet regeneration and bulbil formation *in vitro* from leaf and stem explants of *Curculigo orchioides*, an endangered medicinal plant. - Scientia Hort. **79**: 127-134, 1999.

Tyagi, P., Khanduja, S., Kothari, S.L.: *In vitro* culture of *Capparis decidua* and assessment of clonal fidelity of the regenerated plants. - Biol. Plant. **54**: 126-130, 2010.

Wala, B.B., Jasrai, Y.T.: Micropropagation of an endangered medicinal plant: *Curculigo orchioides* Gaertn. - Plant Tissue Cult. **13**: 13-19, 2003.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V.: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. - Nucl. Acids Res. **18**: 6531-6535, 1990.

Zheng, Y., Liu, Y., Ma, M., Xu, K.: Increasing *in vitro* microrhizome production of ginger (*Zingiber officinale* Roscoe). - Acta. Physiol. Plant. **30**: 513-519, 2008.