J. Crop Sci. Biotech. 2016 (JUNE) 19 (2) : 157~165
DOI NO. 10.1007/s12892-015-0119-4

**RESEARCH ARTICLE**

# Direct Shoot Organogenesis from Rhizomes of Medicinal Zingiber *Alpinia calcarata* Rosc. and Evaluation of Genetic Stability by RAPD and ISSR Markers

**Sudipta Shekhar Das Bhowmik[1,2], Adreeja Basu[3], Lingaraj Sahoo[1*]**

[1]*Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati – 781039, India*
[2]*Center for Tropical Crops and Biocommodities, Queensland University of Technology, Gardens Point Campus QLD 4000, Brisbane, Australia*
[3]*Center for Energy, Indian Institute of Technology Guwahati, Guwahati – 781039, India*

Received : November 6. 2015 / Accepted: January 14. 2016


## Abstract

A simple and efficient protocol for direct *in vitro* shoot multiplication and plant regeneration was established for an important aromatic medicinal plant, *Alpinia calcarata*. Preinduction of rhizome segments in medium containing 8.8 µM 6-benzylamino purine (BAP) rescued the buds from dormancy in 60% of the cultures. An average of 6.2 shoots were produced from rhizomatous bud explants on Murashige and Skoog (MS) medium supplemented with 5 µM BAP, 10 µM kinetin, and 2.5 µM α-Naphthalene acetic acid (NAA). The mother cultures retained their morphogenetic potential upto four subcultures and a maximum of 1.77-fold increase in shoot multiplication was recorded after the 3rd subculture. Rooting was simultaneously induced during subculture on shoot multiplication medium eliminating an additional step for rooting induction. Rooted plantlets were successfully acclimatized in pots in the greenhouse and subsequently established in the experimental garden without any visible symptoms of wilting and necrosis. The genetic fidelity of regenerated plants was evaluated by adapting to two PCR-based DNA marker techniques, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) which detected no variability in the *in vitro* multiplied plantlets of *A. calcarata.* This efficient method of clonal multiplication may be useful for commercial scale multiplication, and *in situ* and *ex situ* conservation of elite germplasm of *A. calcarata*.

**Key words :** *Alpinia calcarata*, genetic uniformity, micropropagation, rhizome

## Introduction

*Alpinia calcarata* Rosc. is a commercially important aromatic medicinal plant, native to India, China, and Sri Lanka (Mangaly and Sabu 1992). The rhizomes of this perennial herb are used extensively in traditional systems of medicine as an aphrodisiac and in the treatment of bronchitis, cough, respiratory ailments, diabetes, asthma, and arthritis in Southeast Asia (Arambewela et al. 1995; Ramanayake 1994). Recently, the rhizomes have been reported to have antimicrobial (Robinson et al. 2009), antinociceptive (Arambewela et al. 2004),and anti-inflammatory activities(Arawwawala et al. 2012). Apart from these bioactivities, the rhizome exhibits insecticidal activity (Dutta et al. 1985). In India, the dried rhizomes form a major ingredient of several Ayurvedic drug formulations such as Rasnadhi Kazhayam, Rasnadhi Choornam, Rashnadhi Thailam, and Ashawagandharishtam (Sabu 2006). The aromatic compounds, 1,8-cineole and β-pinene were reported as the major constituents in leaf, flower, and rhizome oil, and α-fenchyl acetate from the root oil of the plant (Koul et al. 2005). In addition, the leaves and roots are also a good source of protocatechinic acid, quercetin, 4-O-methyl-syringic acid, vanillic acid, methyl cinnamate, and numerous terpenes and diterpenes constituents (Merh 1986). Unfortunately, the ruthless overexploitation and collection of the rhizome from its natural habi-

**Lingaraj Sahoo**( ✉ )
Email : ls@iitg.ernet.in

tat has led to the depletion of wild populations of *A. calcarata* at an alarming rate. Overutilization of the *A. calcarata* rhizomes by deracination risks the extinction of this species in the future. In India, there is an annual demand of 1.7 tons of dried rhizome of *A. calcarata* (Sasidharan and Maraleedhara 2000).The plant is conventionally propagated by rhizome cuttings which are insufficient to meet the demand of commercial scale production. Furthermore, the cultivation through rhizome cuttings is not sustainable as they are the raw material for drug preparation. Moreover, the long maturity period of the rhizomes (36-46 months) for obtaining suitable propagules for propagation is the major limitation of rhizome based cultivation throughout the year in *A. calcarata.* Improvement of *A. calcarata* by conventional breeding is impeded by its rare flowering and lack of seed set. Consequently, an effective approach is needed for rapid and mass propagation of *A. calcarata* to ensure the sustainable utilization of this species.

*In vitro* propagation methods have proven to be powerful tools for commercial scale propagation of medicinal plants which are difficult to propagate through conventional means, and conservation of elite and rare plant species that are threatened or on the verge of extinction (Murch et al. 2000). Micropropagation through direct regeneration from resident meristems without an intervening callus stage is preferred since extensive callus formation and long-term callus culture can lead to somaclonal variations (Evans and Bravo 1986). Further, *in vitro* propagation through axillary bud culture is an easy and economic way to obtain a large number of consistently uniform and true-to-type plants (Hu and Wang 1983). However, the tissue culture regenerants are often susceptible to genetic alteration due to culture-induced stress and mode of regeneration (Cecchini et al. 1992; Kaushal and Kamlesh 2003). Therefore, ensuring the genetic stability of *in vitro*-regenerated plants is an essential requisite for large-scale multiplication. Despite the numerous advantages of the *in vitro* propagation, genetic instability is often observed in micropropagated plants, and therefore, it is necessary to establish a system that produces genetically stable and identical plants. *In vitro* clonal multiplication of *A. calcarata* through rhizome buds has been reported (Sudha et al. 2012), however the regeneration is accompanied with formation of meristemoids and callus. Moreover, the absence of any evidences of genetic integrity in the *in vitro*-grown regenerants is the major bottleneck in adapting the protocol in commercial scale propagation of *A. calcarata.*

Several strategies have been described to assess the genetic stability of *in vitro*-derived plants such as karyological analysis and isozyme markers, however they have their own limitations (Isabel et al. 1993). PCR-based techniques such as Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) are the simple, reliable, and cost effective techniques that do not require any prior information for primer designing and appears to be useful for the analysis of genetic fidelity of *in vitro* propagated plants (Agnihotri et al. 2009; Jokipii et al. 2004; Mandl et al. 2007). The DNA polymorphism-based profiling facilitates direct and reliable measurements to detect culture-induced variation at the DNA level (Cloutier and Landry 1994). In contrast to restriction length polymorphism (RFLP) no radioactive probes are required for RAPD and ISSR, and thus they are found to be suitable for the assessment of *in vitro*-raised and -conserved plant materials (Lakshmanan et al. 2007). Moreover, these markers are not developmentally regulated and can be used for rapid identification with a minimum starting material. These PCR-based techniques have been extensively used for assessment of clonal fidelity in *in vitro* plants in many recent studies (Leva and Petruccelli 2012; Mohanty et al. 2011; Nayak et al. 2011; Parida et al. 2011; Rout et al. 2009).

Considering the importance of an efficient *in vitro* propagation method for generation of consistently uniform and true-to-type plantlets in medicinally important *A. calcarata,* we established an efficient and high frequency multiple, direct shoot, regeneration system from mature rhizome buds of *A. calcarata*. To validate the genetic stability of *in vitro*-raised plants, we performed RAPD and ISSR based analysis for genetic purity assessment.

## Materials and Methods

### Plant material and culture initiation

Mature rhizomes of *A. calcarata* were collected from Jagiroad, Assam, India during the rainy season and placed in soil:compost (1:1) to allow sprouting of buds. The rhizome segments (~2.0 x 1.5 cm) with axillay buds embedded in scales were excised, washed with liquid detergent for 10 min, followed by rinsing with sterile distilled water and then surface sterilized with 0.1% (m/v) mercuric chloride for 5 min followed by rinsing five times with sterile distilled water. The rhizome segments were inoculated in culture tubes (Borosil, Mumbai, India) containing Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium fortified with 30g $L^{-1}$ sucrose and 0.8% agar (HiMedia, Mumbai, India). The phytohormones, BAP and kinetin (Kin) were added (2.3 - 23.2 µM) to the basal medium for efficient priming of the rhizome segments. The pH of the media was adjusted to 5.8 prior to adding agar and autoclaved at 121ºC for 20 min. The cultures were maintained at 25 ± 2ºC, with 16 h photoperiod with irradiance of 40.5 µmole $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

### Multiple shoot induction and regeneration of plantlets

The axillary buds (~ 2.5 x 2.5 mm) produced from rhizome segments, cultured for 4 weeks on MS medium supplemented with BAP and Kin, were used as explants. The explants were cultured on MS medium containing BAP and Kin at different concentrations (5.0-20 µM) either singly or in combinations for shoot multiplication. The synergistic effect of auxin on

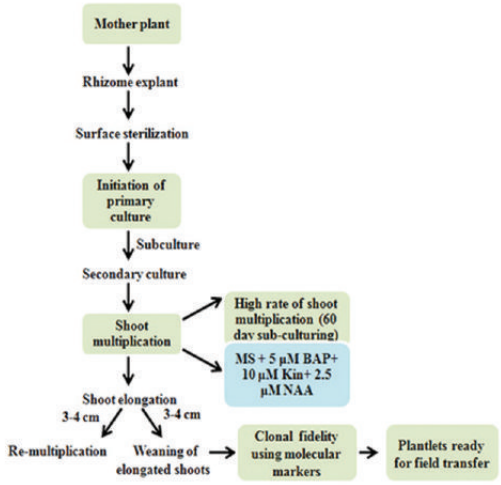


**Fig. 1.** Flow diagram of steps involved in rapid multiplication of shoot and root from rhizome of *Alpiniacalcarata*

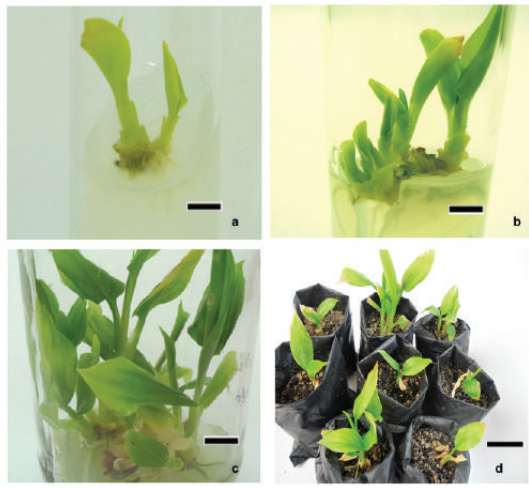


**Fig. 2.** Multiple shoot induction and plant regeneration from rhizome buds explants of *A. calcarata Rosc.* (a) Initiation of aseptic primary culture from swelling bases of rhizome explants within4 weeks (Bar 15 mm). (b) Multiple shoot bud regeneration from secondary rhizome explants within 8 weeks(Bar 15 mm). (c) Shoot elongation and root formation in culture vesselwithin 8 weeks (Bar 15 mm). (d) Four-week-old weaned plantlets in soil and compost mixture under greenhouse (Bar 15 mm).

shoot multiplication was tested by adding 2.5 µM of NAA to the shoot multiplication medium (Table 1). The percentage of explants forming multiple shoot buds, mean number of shoots per explant, mean shoot length,and mean number of roots per explant was recorded after 6 weeks of culture.

## Plant acclimatization and transfer to field

The plantlets with well-developed roots were removed from the medium, washed thoroughly under tap water, and transferred to pots containing soil and compost (1:1). The pots were covered with polyethylene bags and sprinkled with water at 2-day intervals for the first 2 weeks to maintain a relative humidity of 85-90%. The plantlets were then shifted to the greenhouse and acclimatized under 70% relative humidity with a 16 h photoperiod at 30 ± 2 ºC. The acclimatized plantlets were eventually transferred to garden soil in the experimental garden.

## Statistical analysis

Each treatment contained 10 replicates and the experiments were repeated thrice. All experiments were conducted in a completely randomized manner. The data were analyzed by Analysis of variance (ANOVA) at the 5% significant level and means compared using Fischer's LSD test (PC version Origin 7.0. NORTHAMPTON, MA, USA).

## Evaluation of genetic stability of regenerated plants

The clonal fidelity of *in vitro*-raised plants was tested using RAPD and ISSR markers. For this purpose, 6-month-old hardened plants were chosen randomly from the population and compared with the mother plant. Total genomic DNA of the mother plant and *in vitro*-raised plantlets was extracted from 100 mg of young leaf tissue using DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instruction. Quality and quantity of DNA preparations were checked by both spectrophotometrically and by running on 1% agarose gels containing 0.5 µg $mL^{-1}$ of ethidium bromide.

A total of 20 RAPD (Operon Technologies, USA) and 10 ISSR primers (UBC primer set No. 9, University of British Columbia, Canada) were used for initial screening. The RAPD and ISSR analyses were performed according to Williams and Zeitkiewicz, respectively (Williams et al. 1990; Zietkiewicz et al. 1994). The PCR amplifications were carried out in a total volume of 25 µL containing template DNA (100 ng for RAPD and 50 ng for ISSR, respectively), 10× PCR buffer containing 1.5 mM $MgCl_2$ (Bangalore Genei, Bangalore, India), 100 µM of dNTP mix (Bangalore Genei), 1 U of Taq polymerase (Bangalore Genei), and 1 µM of primer. PCR amplifications were carried out in a thermal cycler (Applied Biosystems 2720, USA). The five RAPD and five ISSR primers showing discrete and consistent polymorphic bands were then selected (Table 3) for analyzing the clonal fidelity of *in vitro*-regenerated plants.

For RAPD, PCR amplification was programmed for initial denaturation at 94ºC for 3 min, followed by 40 cycles of 1 min denaturation at 94ºC, 1 min annealing at 34ºC, and 2 min extension at 72ºC, with a final extension at 72ºC for 10 min. The same PCR amplification conditions were maintained for ISSR analysis, but the annealing temperature was set based on the ISSR primers (Table 3). Amplified products were resolved by electrophoresis on 1.4% agarose gels in tris-acetate EDTA (TAE) buffer stained with 0.5 µg $ml^{-1}$ of ethidium bromide and gel images were taken by using the gel documentation system gel documentation system (Bio-Rad Laboratories, USA).

## Data scoring and analysis

Consistent, well-resolved fragments in the size range of 100

**Table 1.** Effect of different concentrations and combinations of plant growth regulators on shoot regeneration and root formation from rhizomatous bud explants of *A. calcarataon* MS medium after 8 weeks of culture.

| Growth regulators (µM) BAP | kin | NAA | Regeneration response (%) | Mean number of shoots/explants | Mean shoot length (cm) | Mean number of roots/explants |
|---|---|---|---|---|---|---|
| - | - | - | 76.6 ± 3.3$^{a}$ | 0.86 ± 0.2$^{a}$ | 1.2 ± 0.46$^{abc}$ | 0.0 ± 0.0$^{a}$ |
| 5.0 | - | - | 83.3 ± 8.8$^{a}$ | 1.6 ± 0.2$^{a}$ | 1.9 ± 0.98$^{abc}$ | 0.0 ± 0.0$^{a}$ |
| 10.0 | - | - | 93.3 ± 3.3$^{a}$ | 2.2 ± 0.34$^{abde}$ | 2.5 ± 0.8$^{abcd}$ | 0.66 ± 0.33$^{a}$ |
| 15.0 | - | - | 96.6 ± 3.3$^{a}$ | 2.5 ± 0.29$^{acde}$ | 1.5 ± 0.99$^{abc}$ | 1.33 ± 0.33$^{a}$ |
| 20.0 | - | - | 76.6 ± 3.3$^{a}$ | 1.1 ± 0.24$^{abc}$ | 3.5 ± 0.40$^{ad}$ | 1.66 ± 0.33$^{ab}$ |
| - | 5.0 | - | 80.0 ± 0.0$^{a}$ | 1.9 ± 0.7$^{a}$ | 2.5 ± 0.86$^{abcd}$ | 1.0 ± 0.0$^{a}$ |
| - | 10.0 | - | 86.6 ± 8.8$^{a}$ | 2.9 ± 0.4$^{acdef}$ | 3.4 ± 1.4$^{ad}$ | 1.3 ± 0.33$^{a}$ |
| - | 15.0 | - | 96.6 ± 3.3$^{a}$ | 3.2 ± 0.4$^{def}$ | 2.3 ± 0.54$^{abcd}$ | 2.0 ± 0.57$^{abc}$ |
| - | 20.0 | - | 83.3 ± 3.3$^{a}$ | 1.7 ± 0.46$^{ad}$ | 2.1 ± 0.30$^{abc}$ | 2.0 ± 0.0$^{abc}$ |
| 5.0 | 5.0 | - | 96.6 ± 3.3$^{a}$ | 3.3 ± 0.63$^{def}$ | 4.4 ± 0.74$^{ad}$ | 1.6 ± 0.33$^{ab}$ |
| 10.0 | 10.0 | - | 90.0 ± 3.3$^{a}$ | 3.8 ± 0.11$^{defg}$ | 4.0 ± 0.37$^{ad}$ | 2.0 ± 0.33$^{abc}$ |
| 15.0 | 15.0 | - | 100 ± 3.3$^{a}$ | 3.6 ± 0.76$^{defg}$ | 4.2 ± 0.32$^{ad}$ | 2.6 ± 0.33$^{abcd}$ |
| 20.0 | 20.0 | - | 76.6 ± 3.3$^{a}$ | 2.3 ± 0.43$^{abc}$ | 2.1 ± 0.63$^{abc}$ | 2.6 ± 0.33$^{abcd}$ |
| 5.0 | 2.5 | 2.5 | 96.6 ± 3.3$^{a}$ | 3.6 ± 0.24$^{defg}$ | 5.8 ± 1.2$^{ef}$ | 3.3 ± 0.33$^{abcd}$ |
| 10.0 | 5.0 | 2.5 | 100 ± 0.0$^{a}$ | 4.5 ± 0.43$^{efg}$ | 7.5 ± 0.81$^{efgh}$ | 4.6 ± 0.66$^{cde}$ |
| 15.0 | 10.0 | 2.5 | 93.3 ± 3.3$^{a}$ | 3.7 ± 0.482$^{defg}$ | 8.1 ± 1.99$^{efgh}$ | 7.0 ± 0.57$^{efg}$ |
| 20.0 | 15.0 | 2.5 | 86.6 ± 3.3$^{a}$ | 3.4 ± 0.52$^{defg}$ | 3.6 ± 0.24$^{a}$ | 5.3 ± 0.33$^{cdef}$ |
| 2.5 | 5.0 | 2.5 | 96.6 ± 3.3$^{a}$ | 4.9 ± 0.93$^{efg}$ | 3.3 ± 1.7$^{ad}$ | 6.3 ± 0.33$^{cdef}$ |
| 5.0 | 10.0 | 2.5 | 100 ± 0.0$^{a}$ | 6.2 ± 0.5$^{gh}$ | 4.4 ± 1.1$^{ad}$ | 10.0 ± 1.15$^{fgh}$ |
| 10.0 | 15.0 | 2.5 | 93.3 ± 3.3$^{a}$ | 5.0 ± 0.59$^{efgh}$ | 5.4 ± 1.9$^{ef}$ | 9.0 ± 0.57$^{fgh}$ |
| 15.0 | 20.0 | 2.5 | 83.3 ± 3.3$^{a}$ | 4.6 ± 0.58$^{efg}$ | 4.9 ± 1.0$^{ef}$ | 6.6 ± 0.33$^{efg}$ |

Values represent means ± se

Each treatment comprised of 20 explants in three replicates

Means followed by same letter within columns are not significantly different ($P \leq 0.05$) according to Fischer LSDtest

bp to 2.5 kb were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel and a binary data matrix was constructed. The amplification fragments were analyzed as alleles assuming the presence of Hardy-Weinberg equilibrium and segregation of all loci in a dominant Mendelian fashion. The binary datasets were analyzed using SIMQUAL (similarity for qualitative data) module of NTSYS-pc for computation of Jaccard's coefficient of similarity ($J_{ij}=a/(a+b+c)$, where $J_{ij}$ is the measure of genetic similarity between regenerants $i$ and $j$, $a$ is the number of monomorphic bands present in both regenerants, $b$ is the number of bands present only in $i$ while $c$ is the number of bands present only in $j$). These similarity coefficients were used to generate dendrograms using Unweighted Pair Group Methods with Arithmetic averages (UPGMA) employing the Sequential Agglomerative Hierarchic Non-overlapping (SAHN) programs from NTSys PC version 2.02 k software (Rohlf 1998).

# Results

## *In vitro* sprouting and culture initiation

Rescuing the cultures from repetitive in borne contamination was critical and only 60% of cultures survived as germ-free. MS medium without cytokinins (control) did not stimulate any morphogenic response and the axillary buds embedded in scales of rhizome segments remained dormant until 3 weeks of culture. However, supplementation of BAP at different concentrations facilitated stimulation of bud formation from cultured rhizome segments. The addition of 8.8 µM of BAP to MS medium induced initiation of shoot buds from swelling bases of 60% of cultures (data not shown) within a month of culture (Fig. 2a). An average of 1.5 aseptic shoot buds were obtained when the rhizome segments were subcultured on the same induction medium (data not shown). Supplementation of kinetin at various concentrations failed to induce initiation of the shoot buds from the cultured rhizome segments even after 2 months of culture.

## Multiple shoot induction and plant regeneration

Shoot bud regeneration potential was significantly enhanced when explants were derived from the rhizome segments and primed on 8.8 µM of BAP (Table 1) (Fig. 1). Initial swelling and greening of vegetative buds followed by initiation of new shoot buds resulted within 20 d of culture (Fig. 2b) of explants on shoot multiplication medium containing BAP and Kin at different concentrations and combinations with varying percentage of response (Table 1) (Fig. 2). The highest shoot bud forming response (100%) was noticed on medium containing both BAP and Kin and the lowest response (76%) was recorded in basal medium devoid of any phytohormone (Table 1). The explants cultured on medium fortified with a combination of BAP and Kin induced a significantly higher number of shoots per explant than medium containing either of them (Fig. 2). A maximum of average 6.2 shoots per explants with 100% shoot bud initiation efficiency were obtained within 8 weeks of culture on MS medium supplemented with 5 µM BAP, 10 µM Kin and 2.5 µM NAA (Figs. 2b, c) (Table 1). The shoot multiplica-

**Table 2.** Evaluation of morphogenetic potential of shoot culture of *A. calcarata* for four subculture passages on shoot multiplication medium*.

| Subculture passage | Mean number of shoots | Fold increase in mean no of shoots length (cm) | Mean shoot |
|---|---|---|---|
| First | 6.2 ± 0.5[a] | 1.0 | 4.4 ± 1.1[a] |
| Second | 8.3 ± 0.3[a] | 1.33 | 5.0 ± 0.28[a] |
| Third | 11.0 ± 1.0[b] | 1.77 | 5.4 ± 0.32[a] |
| Fourth | 7.0 ± 0.6[a] | 1.12 | 4.3 ± 0.23[a] |

*Shoot cultures were subcultured on MS medium containing 5 µM BAP, 10 µM kinetin, and 2.5 µM NAA
Values represent means ± SE
Means followed by the same letter within columns are not significantly different ($P \leq 0.05$)
Fischer by the LSD test

tion rate was significantly less in explants cultured on medium containing either BAP or kinetin, in the absence of NAA. A combination of a higher dose of BAP, Kin and NAA in the medium resulted in reduction in shoot multiplication efficiency (Table 1). The shoots buds obtained from vegetative bud cultures differentiated into shoots after 20 d of culture (Fig. 2b) which elongated within 60 d of culture on the same medium in all the treatments with varying degrees of shoot length (Table 1; Figs. 1, 2c).

The multiple shoots thus obtained were subcultured on the same medium for 3-4 passages to obtain a clump of healthy, elongated, and robust shoots with roots (Fig. 2c). This technology essentially eliminated a step of *in vitro* rooting thereby reducing the overall cost of micropropagation. The highest mean number of roots per culture (10.0 ± 1.15 i.e., 10.0 X 6.2) was observed on shoot multiplication medium that contained BAP (5 µM) and Kin (10 µM) and NAA (2.5 µM), and the lowest (0.66 - 1.0) on medium containing BAP (10 µM) and Kin (5 µM), respectively (Table 1).

On repeated subcultures, upto four passages on the shoot multiplication medium, the mother cultures demonstrated retention of their morphogenetic potential and the rate of shoot multiplication depended on the number of subcultures, and a maximum of 1.77-fold increase in shoot multiplication was recorded after the third subculture (Table 2). However, no significant change in mean shoot length was observed during subculture passages (Table 2). No further increase in shoot multiplication was observed after subsequent subculture (data not shown).

## Weaning and field transfer

More than 90% *in vitro*-raised plantlets of *A. calcarata* were hardened and acclimatized in soil and compost mixture within 4 weeks in the greenhouse (Fig. 2d). The plants were healthy and without any visible symptoms of wilting and necrosis. All the plantlets transferred to natural soil in experimental garden showed vigorous growth and development.

## Assessment of genetic fidelity

RAPD and ISSR analysis was employed to analyze the genetic fidelity of seven randomly selected regenerants. A total of 20 RAPD primers were tested for initial screening but only five primers produced clear and reproducible bands. The five primers produced 21 distinct and scorable bands, with an average of 4.2 bands per primer. The number of scorable bands for each RAPD primer varied from seven (OPA-07) to two (OPK-16) (Table 3). Each primer generated a unique set of amplification products ranging in size from 3250 bp (OPA-07) to 900 bp (OPA-07) (Fig. 3a). No polymorphism was detected during the RAPD analysis of the seven *in vitro*-regenerated plants and the mother plant in all the primers except OPS-09 which showed polymorphism at a single locus. The primer (OPS-09) yielded a total of five bands out of which four bands were monomorphic in all the regenerants producing an average of 95% monomorphism pattern across the *in vitro*-raised plants tested. Five resolvable ISSR primers produced a total of 27 scorable bands with an average of 5.4 per primer. Among the five primers, UBC-810 produced a maximum of seven bands with size in the range of 3530 - 947 bp (Table 3) (Fig. 3b). The seven *in vitro*-raised plants tested using all the ISSR primers, produced 100% monomorphic banding pattern in *in vitro*-raised plants as well as in the mother plant. A total of 48 scorable bands were produced by RAPD and ISSR primers with an average of 4.8 per primer. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the *in vitro*-raised plants derived from different explants was 0.979, indicating 98% similarity (Table 4). The matrix generated dendrogram clearly indicated that all the *in vitro*-raised plants of *A. calcarata* had close similarities with the stock mother plant (Fig. 4).

**Table 3.** Description of RAPD and ISSR primers used for fingerprinting analysis of micropropagated plants and the source plant of *A. calcarata.*

| Sl. No. | Primer code | Primer's sequence (5'-3') | Total number of bands amplified | *Alpiniacalcarata* No. of monomorphic bands | Percentage monomorphism |
|---|---|---|---|---|---|
| RAPD primers | | | | | |
| 1 | OPA-07 | GAAACGGGTG | 7 | 7 | 100 |
| 2 | OPG-02 | GGCACTGAGG | 4 | 4 | 100 |
| 3 | OPK-07 | AGCGAGCAAG | 3 | 3 | 100 |
| 4 | OPK-16 | GAGCGTCGAA | 2 | 2 | 100 |
| 5 | OPS-09 | TCCTGGTCCC | 5 | 4 | 80 |
| Total | | | 21 | 20 | 95.23 |
| ISSR primers | | | | | |
| 1 | I1 | (AG)9 | 4 | 4 | 100 |
| 2 | UBC-807 | (AG)8T | 6 | 6 | 100 |
| 3 | UBC-810 | (CA)8T | 7 | 7 | 100 |
| 4 | UBC-815 | (CT)8G | 5 | 5 | 100 |
| 5 | UBC-899 | CATGGTGTTG GTCATTGTTCCA | 5 | 5 | 100 |
| Total | | | 27 | 27 | 100 |

**Table 4.** Similarity coefficients among mother plant and micropropagated plants of *A. calcarata* based on RAPD and ISSR markers.

| | *In vitro* plantlets | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mother | M | AC1 | AC2 | AC3 | AC4 | AC5 | AC6 | AC7 |
| M | 1.0 | | | | | | | |
| AC1 | 1.0 | 1.0 | | | | | | |
| AC2 | 0.979 | 0.979 | 1.0 | | | | | |
| AC3 | 1.0 | 1.0 | 0.979 | 1.0 | | | | |
| AC4 | 1.0 | 1.0 | 0.979 | 1.0 | 1.0 | | | |
| AC5 | 1.0 | 1.0 | 0.979 | 1.0 | 1.0 | 1.0 | | |
| AC6 | 1.0 | 1.0 | 0.979 | 1.0 | 1.0 | 1.0 | 1.0 | |
| AC7 | 1.0 | 1.0 | 0.979 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

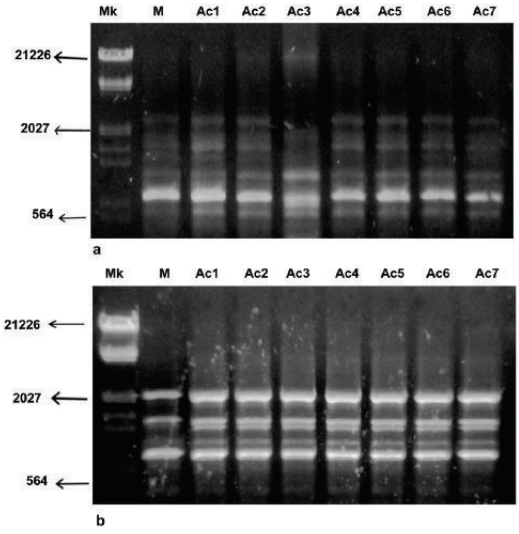


**Fig. 3.** RAPD profiles of micropropagated plants of *Alpinia calcarata*. (a) RAPD (OPA07)and (b) ISSR (UBC-810) banding pattern in micropropagated plants (6-months) and field grown mother plant of *A. calcarata* (lane 1-mother plant, lanes 2 to 8- micropropagated plants, M - ladder).

## Discussion

The root oil and oleoresin of *A. calcarata* in particular have immense medicinal value. India, along with Thailand and Indonesia, is the major supplier of *A. calcarata*. Micropropagation is one of the key approaches for large-scale multiplication of true-to-type plantlets of economically important medicinal plants including Zingiberaceae species. *In vitro* regeneration of several medicinally important galangal species has been reported (Jinu and Aravinthan 2008; Kochuthressia et al. 2012; Rao et al. 2011). However, there is no convincing report on micropropagation of *A. calcarata.*

In general, contamination is a major problem in initiating aseptic cultures in rhizomatous plant species (Rout et al. 2001). In Zingiber species, the contamination rate in *in vitro* cultures has been primarily attributed to presence of outer leaf sheaths in rhizomes and the time of their collection (Stanly and Keng 2007). We recorded a maximum frequency of contamination-free cultures (60%) after treatment with 0.1% mercuric chloride for 5 min and further exposure was lethal to the tissue. The low rate of contamination in the present system might be due to the appropriate steps for surface sterilization. During the dry season, the rhizomes of Zingiber species are known to remain dormant. Breaking of bud dormancy on rhizomes of many Zingiber species is very important for overcoming the problem of eye bud emergence and to produce the plant year round (Thohirah et al. 2010). The axillary buds of many Zingiber species have been reported to remain dormant. We found supplementation of BAP at different concentrations facilitated stimulation of bud formation in cultured rhizome segments. Use of BAP in culture has been reported to break the dormancy of axillary buds in many medicinal Zingiber species including *Costuspictus* (Punyarani and Sharma 2012) and *Curcuma cordata* (Thohirah et al. 2010). In the present investigation, shoot multiplication was found to occur from rhizomatous bud explants in a medium containing two cytokinins, BAP and Kin, either in the presence or absence of NAA. Explants cultured on medium containing either BAP or Kinwere ineffective for multiple shoot bud induction. The synergistic effect of two cytokinins, BAP and kinetin has been found to be enhancing the shoot multiplication. However, the rate of shoot bud multiplication varied based on the dose and the combination of phytohormones used. The combination of two cytokinins (BAP and Kin) in the culture medium has been reported to be ideal for shoot multiplication in *Zingibermoran* and *Z. zerumbet* (Das et al. 2013), *Kaempferia galangal* (Kochuthressia et al. 2012), *Alpinia galangal* (Parida et al. 2011), *Alpiniazerumbet* (Jinu and Aravinthan 2008), and *Bosenbergia pulcherrima* (Anish et al. 2008). Our results indicated that a combination of BAP (5 µM), Kin (10 µM) and NAA (2.5 µM) in the MS medium was sufficient for the induction of maximum multiple shoots from the rhizomatous bud explants in *A. calcarata*. The combination of two cytokinins along with auxin has been reported to increase the rate of shoot multiplication by several fold in *Zingiber officinale* (Palai et al. 1997). The addition of NAA to shoot multiplication medium has been reported to induce optimal multiple shoot induction in *Z. petioletum* (Prathanturarug et al. 2004), in genus *Curcuma* (Bharalee et al. 2005), and *B. rotunda* (Yusuf et al. 2011). However, contradictory results have been reported in a few galanga species such as *A. galangal* with high efficiency shoot multiplication in medium containing a single cytokinin (Rao et al. 2011). The rhizomatous bud cultures of *A. calcarata* were rooted, during subculture, in shoot multiplication stage in medium supplemented with NAA in conjunction with BAP and Kin. Hence, additional treatment was not required for root induction. The simultaneous production of shoots and roots were also reported in other Zingiberaceae species (Balachandran et al. 1990; Chan and Thong 2004; Kochuthressia et al. 2010; Punyarani and Sharma 2012). We observed induction of rooting in rhizomatous bud cultures of *A. calcarata* due to the synergistic effect of low concentrations of NAA with BAP and Kin. The synergistic effect of NAA at a low concentration along with BAP has been found to be highly effective for enhanced shoot bud proliferation and simultaneous rooting in rare zingibers like

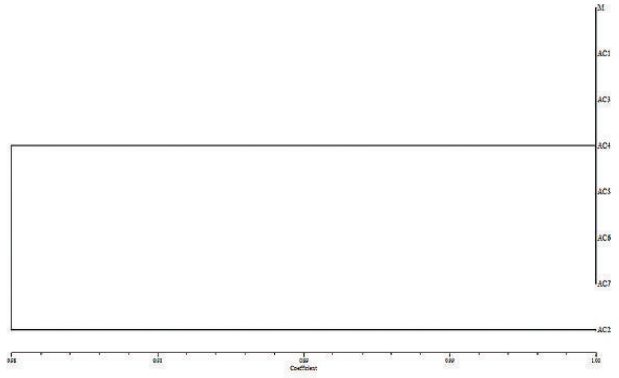

**Fig. 4.** Dendrogram showing the relationship of micropropagated plantlets of *A. calcarata* with their mother stock.

*Zingiber petiolatum*, *Mantisia spathulata*, *Mantisia wengeri*, and *Kaempferia galangal* (Bhowmik et al. 2009; Prathanturarug et al. 2004; Shirin et al. 2000).

Maintenance of genetic stability is one of the most important criteria in the micropropagation of valuable medicinal plants. A major problem often encountered with the plantlets generated by *in vitro* multiplication of parent stock is the induction of somaclonal variation, arising as a direct consequence of *in vitro* culture of plant cells, tissues, or organs. The source of explants, method of regeneration (Goto et al. 1998), use of sub- and supra-optimal levels of plant growth substances, especially synthetic ones (Martins et al. 2004), and long-term culture may contribute to somaclonal or epigenetic variations in micropropagated plants (Das et al. 2010; Kishor and Devi 2009). Therefore, it is necessary to verify thegenetic purity of the micropropagated plants by DNA marker-based analysis in order to ensure clonally uniform plant materials for commercial scale propagation. Evaluation of genetic stability by using a combination of two types of markers that amplify different regions of the genome has been proved to be precise and useful (Lattoo et al. 2006; Martin et al. 2006; Ray et al. 2006; Venkatachalam et al. 2007). Hence, in the present study, the clonal fidelity of the *in vitro*-raised plant materials was confirmed by adopting two PCR-based DNA marker techniques, RAPD and ISSR. No variability was detected among the *in vitro*-multiplied plantlets of *A. calcarata*. In our study, we obtained plantlets from the sprouting of dormant buds situated in the axils of the bracts. These findings support the fact that a meristem-based micropropagation system is much more stable genetically than those in which regeneration occurs via the callus phase.

## Conclusion

This is the first time through the present study that we describe an efficient protocol for micropropagation of *A. calcarata* and demonstrate the absence of genetic variation in *in vitro*-raised plantlets by two PCR-based DNA marker techniques, RAPD and ISSR that targeted the non repetitive and repetitive (microsatellite) regions of genomic DNA, respectively. This efficient method of clonal multiplication can be successfully employed for commercial scale multiplication without much risk of genetic instability and *in situ* and *ex situ* conservation of elite germplasm of *A. calcarata*.

## Acknowledgements

Post Doctoral Fellowship to SSDB by Department of Biotechnology, Government of India (R&D/07/BT/P/(RA)/SSB/01/2010) is gratefully acknowledged.

Conflict of Interest: The authors declare that they have no conflict of interest.

## Reference

Agnihotri RK, Mishra J, Nandi SK. 2009. Improved *in vitro* shoot multiplication and rooting of *D. hamiltonii* NeesetArn. Ex Munro: production of genetically uniform plants and field evaluation. Acta Physiol. Plant. 31: 961-967

Anish NP, Dan M, Bejoy M. 2008. Conservation using *in vitro* progenies of threatened ginger-*Boesenbergia pul cherrima* (Wall.) Kuntze. Int. J. Bot. 4(1): 93-98

Arambewela LSR, Arawwawala LDAM, Ratnasooriya WD. 2004. Antinociceptive activities of aqueous and ethanolic extracts of *Alpiniacalcarata* rhizomes in rats. J. Ethnopharmacol. 95: 311-116

Arambewela LSR, Basnayake CS, Serasinghe P, Tissera MS, Dias S, Weerasekara DR. 1995. Traditional treatment in Sri Lanka for chronic Arthritis. NARESA Printing Unit, Colombo, Sri Lanka

Arawwawala LDAM, Arambewela LSR, Ratnasooriya WD. 2012. *Alpinia calcarata* Roscoe: A Potent Anti-inflammatory Agent. J. Ethnopharmacol. 139 (3): 889-892

Balachandran SM, Bhat SR, Chandel KPS. 1990. *In vitro* clonal multiplication of turmeric (Curcuma spp.) and ginger (*Zingiber officinale* Rosc.). Plant Cell Rep. 8: 521-524

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

Bhowmik SS, Kumaria S, Rao SR, Tandon P. 2009. High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata* Schult. And *Mantisia wengeri* Fischer and analysis of genetic uniformity using RAPD markers. Ind. J. Exp. Biol. 47: 140-14

Cecchini E, Natah L, Cavallini A, Durante M. 1992. DNA variation in regenerated plants of pea. Theor. Appl. Genet. 84: 874-878

Chan LK, Thong WH. 2004. *In vitro* propagation of

Zingiberaceae species with medicinal properties. J. Plant Biotechnol. 6: 181-188

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

Das A, Kesari V, Rangan L. 2010. Plant regeneration in *Curcuma* species and assessment of genetic stability of regenerated plants. Biol. Plant. 54: 423-429

Das A, Kesari V, Rangan L. 2013. Micropropagation and cytogenetic assessment of *Zingiber* species of Northeast India. 3 Biotech. 3(6): 471- 479

Dutta TR, Ahmed R, Abbas SR, Rao MKV. 1985. Plants used by Andaman Aborigines in gathering rock honey. Ecol. Bot. 39(2): 130-138

Evans DA, Bravo JE. 1986. Phenotypic and genotypic stability of tissue cultured plants. In RH Zimmerman, RJ Griesbach, FA Hammerschlag, RH Lawson, Ed., Current Plant Science and Biotechnology in Agriculture: Tissue Culture as a Plant Production System for Horticultural Plants, Martinus Nijhoff Pub. Dordrecht, pp 73-94

Goto S, Thakur RC, Ishi K. 1998. Determination of genetic stability in long term micropropagated shoots in *Pinus thunbergii* Parl, using RAPD markers. Plant Cell Rep. 18: 193-197

Hu CY, Wang PJ. 1983. Meristem. shoot tip and bud culture. In DA Evans, WR Sharp, PV Ammirato, Y Yamada, Ed., Handbook of Plant Cell Culture, Macmillan Pub. New York, pp 177-227

Isabel N, Tremblay L, Michaud M, Tremblay FM, Bousquet J. 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. Theor. Appl. Genet. 86: 81-87

Jinu J, Aravinthan KM. 2008. Micropropagation of *Alpinia zerumbet* Vaigata through *in vitro*rhizome bud culture. Ind. J. Bot. Res. 4(2): 197-200

Jokipii S, Ryynanenb L, Kallioc PT, Aronenb T, Haggman H. 2004. A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. x *Populus tremuloides* Michx. Plant Sci. 166: 799-806

Kaushal B, Kamlesh K. 2003. Random amplified polymorphic DNA (RAPDs) markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. Euphytica 132: 41-46

Kishor R, Devi HS. 2009. Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb.f × *Vanda stangeana* Reichb.f) using thidiazuron and analysis of their genetic stability. Plant Cell Tiss. Org. Cult. 97: 121-129

Kochuthressia KP, Britto SJ, Jaseentha MO. 2012. *In vitro*-multiplication of *Kaempferia galanga* L. an endangered species. Int. Res. J. Biotech. 3(2): 27-031

Kochuthressia KP, Britto SJ, Joelri L, Raj M, Jaseentha MO, Senthilkumar SR. 2010. Efficient regeneration of *Alpinia purpurata* (Vieill.) K. Schum. plantlets from rhizome bud explants. Int. Res. J. Plant Sci. 1(2): 43-47

Koul A, Gangar SC, Sandhir V. 2005. Pitfalls journey from traditional to modern medicine. Nat. Prod. Rad. 4: 6-13

Lakshmanan V, Venkataramareddy SR, Neelwarne B. 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electron. J. Biotech. 10: 1-8

Lattoo SK, Bamotra S, Sapru DR, Khan S, Dhar AK. 2006. Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker-an endangered medicinal herb. Plant Cell Rep. 25: 499-506

Leva AR, Petruccelli R. 2012. Monitoring of cultivar identity in micropropagated olive plants using RAPD and ISSR markers. Biol Plant. 56(2): 373-376

Mandal AB, Thomas VA, Elanchezhian R. 2007. RAPD pattern of *Costus speciosus* Koen ex. Retz., an important medicinal plant from the Andaman and Nicobar Islands. Curr. Sci. 93: 369-373

Mangaly JK, Sabu M. 1992. A taxonomic revision of South Indian *Alpinia* Roxb. (Zingiberaceae). Rheedea. 2: 38 - 51

Martin KP, Pachathundikandi S, Zhang CL, Slater A, Madassery J. 2006. RAPD analysis of a variant of banana (Musa sp.) cv. grandenaine and its propagation via shoot tip culture. In Vitro Cell. Dev. Biol. Plant. 42: 188-192

Martins M, Sarmento D, Olivera MM. 2004. Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Rep. 23(7): 492-496

Merh PS, Daniel M, Sabnis SD. 1986. Chemistry and taxonomy of some members of the Zingiberaceae. Curr. Sci. 55: 835-839

Mohanty S, Panda MK, Sahoo S, Nayak S. 2011. Micropropagation of *Zingiber rubens* and assessment of genetic stability through RAPD and ISSR markers. Biol. Plant. 55(1): 16-20

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

Murch SJ, Krishna RS, Saxena PK. 2000. Phytopharmaceuticals: mass production, standard, and conservation. Sci. Rev. Alt. Med. 4: 39-43

Nayak S, Kaur T, Mohanty S, Ghosh G, Choudhury R, Acharya L, Subudhi E. 2011. *In vitro* and *ex vitro* evaluation of long-term micropropagated turmeric as analyzed through cytophotometry, phytoconstituents, biochemical and molecular markers. Plant Growth Reg. 64: 91-98

Palai SK, Rout GR, Das P. 1997. Micropropagation of ginger *Zingiber officinale* Rosc-interaction of growth regulators and culture conditions. In S Edison, KV Ramana, B Sasikumar, BK Nirmal, SJ Eapen, Ed., Biotechnology of Spices, Medicinal and Aromatic Plants,India,Indian Society for Spices, Kerala. pp 20-24

Parida R, Mohanty S, Nayak S. 2011. Evaluation of genetic fidelity of *in vitro* propagated greater galangal (*Alpinia galanga*) using DNA based markers. Int. J. Plant Animal Environ. Sci. 3: 123-133

Prathanturarug S, Angsumalee D, Pongsiri N, Suwacharangoon S, Jenjittikul T. 2004. *In vitro* propagation of *Zingiber petiolatum* (Holttum) I. Theilade, A rare

Zingiberaceous plant from Thailand. In Vitro Cell. Dev. Biol. Plant. 40: 317-321

Punyarani K, Sharma JG. 2012. Micropropagation and microrhizome induction in *Costus pictus* D. Don using *in vitro* and *ex vitro* nodal segments as explant. Not. Sci. Biol. 4(2): 72-78

Ramanayake LOV. 1994. Publication of Department of Ayurveda. Colombo, Sri Lanka, 68-71.

Rao K, Chodisetti B, Gandi S, Mangamoori LN, Giri A. 2011. Direct and indirect organogenesis of *Alpinia galanga* and the phytochemical analysis. Appl. Biochem. Biotech. DOI 10.1007/s12010-011-9353-5

Ray T, Dutta I, Saha P, Das S, Roy SC. 2006. Genetic stability of three economically important micropropagated banana (Musaspp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant Cell Tiss. Org. Cult. 85: 11-21

Robinson PJ, Balakrishnan V, Raj JS, Britto SJ. 2009. Antimicrobial activity of *Alpinia calcarata* Rosc. and characterization of a new $\alpha\beta$ unsaturated carbonyl compound. Adv. In. Biol. Res. 3 (6): 185-187

Rohlf FJ. 1998. NTSYSpc Numerical Taxonomy and Multivariate Analysis System, Version 2.02, Exeter Software, Setauket

Rout GR, Palai SK, Samantaray S, Das P. 2001. Effect of growth regulator and culture conditions on shoot multiplication and rhizome formation in ginger (*Zingiber officinale* Rosc.). In Vitro Cell. Dev. Biol. Plant. 37(6): 814-819

Rout GR, Senapati SK, Aparajita S, Palai SK. 2009. Studies on genetic identification and genetic fidelity of cultivated banana using ISSR markers. Plant Omics J. 2(6): 250-258

Sabu M. 2006. Zingiberaceae and Costaceae of South India. Indian Association of Angiosperm Taxonomists, Calicut, p 52

Sasidharan N, Muraleedhara PK. 2000. Survey on the commercial exploitation and consumption of medicinal plants by the drug industry in Northern Kerala. Res. Rep. No. 193, Kerala Forest Research Institute, Thrissur, Kerala

Shirin F, Kumar S, Mishra Y. 2000. *In vitro* plantlet production system for *Kaempferia galanga*, a rare Indian medicinal herb. Plant Cell Tiss. Org. Cult. 63: 193-197

Stanly C, Keng CL. 2007. Micropropagation of *Curcuma zedoaria* Roscoe and Zingiberzerumbet Smith. Biotech. 6(4): 555-560

Sudha CG, George M, Rameshkumar KB, Nair GM. 2012. Improved clonal propagation of *Alpinia calcarata* Rosc., a commercially important medicinal plant and evaluation of chemical fidelity through comparison of volatile compounds. Am. J. Plant Sci. 3: 930-940

Thohirah LA, Flora CLS, Kamalakshi N. 2010. Breaking bud dormancy and different shade levels for production of pot and cut *Cucurma alismatifolia*. Am. J. Agr. Biol. Sci. 5(3): 385-388

Venkatachalam L, Sreedhar RV, Bhagyalakshmi N. 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Elect. J. Biotech. 15: 106-113

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: 6531-6535

Yusuf NA, SuffianAnnuar MM, Khalid N. 2011. Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. Afr. J. Biotech. 10(7): 1194-1199

Zietkiewicz E, Rafalski A, Labuda D. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics. 20: 176-183