*Asian Journal of Conservation Biology*, July 2020. Vol. 9 No. 1, pp. 151-155 AJCB: FP0133
ISSN 2278-7666 ©TCRP Foundation 2020

# Conservation of *Curcuma caesia* Roxb.- A critically endangered species *via in vitro* plant regeneration from organogenic callus

**Indrani Sarma*, A.C. Deka**

*The Energy and Resources Institute, North Eastern Regional Centre, Guwahati- 781036, India*

(Received: September 20, 2019; Revised: January 25, 2020 ; Accepted: March 12 , 2020)

## ABSTRACT

A novel protocol for callus mediated shoot regeneration and somatic embryogenesis was established for *Curcuma caesia* Roxb, a critically endangered species of North east India. Vigorous callus growth was observed in MS medium containing higher concentration of 5.0 $mgl^{-1}$ 2, 4-D from pseudostem explants. More than 70% of the pseudostem explants of this species responded for callus induction within 22 days of culture. After 4 subcultures these callus showed embryogenic response in MS medium containing 2.0 $mgl^{-1}$ BAP with 0.05 mg $l^{-1}$ NAA. Shoots were successfully regenerated on MS medium with a concentrations of 0.25 $mgl^{-1}$ Kn and 0.05 $mgl^{-1}$ $GA_3$ within 25 days of transfer. Single shoots transferred into MS basal medium free of plant growth regulator rooted well with (80%) survivality under field condition.

**Key words:** *Curcuma caesia*, callus induction, somatic embryogenesis, plant regeneration

## INTRODUCTION

*Curcuma caesia* Roxb. of Zingiberaceae Family, is a perennial, erect rhizomatous herb with large leaves and bluish-black rhizome. It is commonly known as black turmeric or kali haldi, native to North-East and Central India. Fresh rhizomes are aromatic with intense camphoraceous odour contains camphor, ar-turmerone, (Z)-ocimene, ar-curcumene, 1, 8-cineole, borneol, bornyl acetate and curcumene as the major constituents. The rhizomes of this plant have a high economic importance owing to its putative medicinal properties. Traditionally, the rhizomes of *Curcuma caesia* Roxb. is used in the treatment of leucoderma, asthma, tumours, piles, bronchitis, rheumatic pains etc (Kagyung *et al*., 2010). This herb also gives protection against Alzheimer's disease and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease (Behar *et al*., 2013). Due to high medicinal value, black turmeric has been exploited indiscriminately from their natural habitat which make it under the category of critically endangered species (Behar *et al*., 2014; Kumar *et al*., 1998). Thus, efforts should be made to conserve and work for the betterment of this plant species.Vegetative modes of propagation and absence of seed setting in *C. caesia* prevented the production of new and improved cultivars through conventional plant breeding.

An alternative method for creating improved genotypes through selection of somaclonal variation has been reported in many species by several workers (Nayak *et al*., 2003; Mohanty *et al*.,2008). Callus induction and plantlets regeneration from different parts of other species of *Curcuma* has been reported earlier by other workers (Mohanty *et al*., 2008). However, very least report is available for callus culture of *Curcuma caesia*. Therefore, attempt has been made in the present work for plantlet regeneration from callus culture of *C. caesia* and *in vitro* somatic embryogenesis.

## MATERIALS AND METHODS

Healthy rhizomes of *Curcuma caesia* Roxb. were collected and grown in the experimental garden of Department of Botany, Gauhati University, Guwahati. Dormant rhizome axillary buds were excised from the clean rhizome, which were used as the source of explants. Freshly collected rhizomes were cleaned with running tap water and immature buds were excised with sharp blade and washed with detergent (Tween-20, 0.1% v/v) for 15 min and subsequently rinsed with clean water. Explants were then surface sterilized in disinfectant (0.1% $HgCl_2$ to which two – three drops of Tween-20 were added) for 10 minutes. Under sterile conditions, $HgCl_2$ solution was decanted and the explants were rinsed five-six times with sterile distilled water. Sterilized explants were trimmed aseptically with a sharp and pre sterilized surgical blade. Trimmed rhizome axillary bud (3.0-4.0 mm size) served as the primary explant and initially cultured on shoot induction medium to obtain contamination free cultures. Murashige and Skoog (1962) MS basal media supplemented with 3% sucrose and 2.2 $gl^{-1}$ Gelrite modified with various concentrations of N6-Benzyl-aminopurine (BAP 0.05 to 3.0 $mgl^{-1}$) in combination with Kinetin (Kn; 0.5-2.0 $mgl^{-1}$), Indole-3-acetic acid (IAA; 0.5-1.0 $mgl^{-1}$), ά-naphthaleneacetic acid (NAA; 0.5-1.0 $mgl^{-1}$) and 2, 4-D (0.5-4.0 $mgl^{-1}$) were used for establishment of the culture. Laboratory reagent-grade sucrose was replaced by locally available commercial sugar (market sugar) as carbon source for reducing the cost of the media.

The pH of the media was adjusted to 5.8 prior to the addition of the gelling agent. The media was autoclaved at 121° C with 15 lbs pressure for 15 minutes. Excised buds were inoculated into culture media under laminar air flow. Cultures were incubated at 25±2°C under 16 hours of photoperiod from cool white fluorescent tube giving 12.5µmol $m^{-2}s^{-1}$ at culture level. Most

*Corresponding Author's E-mail: indranisarma@gmail.com

of the cultures sprouted within 7-12 days of inoculation and the emerged shoots (1.0-2.0 cm) were sub cultured in the same media for further multiplication. A total of 20 explants were used for each of the treatments and the number of shoots per explant was recorded after four weeks of inoculation. Each set of experiment was repeated thrice.

### *Callus induction and shoots regeneration*

***Callus induction****:* Leaf sheath, pseudostem and root segment derived from *in vitro* grown 4 weeks old seedlings were inoculated in freshly prepared media for callus induction. MS basal media supplemented with various concentrations of N6-Benzyl-aminopurine (BAP 0.05 to 3.0 $mgl^{-1}$) in combination with Kinetin (Kn; 0.5- 2.0 $mgl^{-1}$), Indole-3-acetic acid (IAA; 0.5-1.0 $mgl^{-1}$), ά-naphthaleneacetic acid (NAA; 0.5-1.0 $mgl^{-1}$) and 2, 4-D (0.5-4.0 $mgl^{-1}$) sucrose and 2.2 $gl^{-1}$ Gelrite were used as callus inducing media. Cultures of all treatments were divided in to two groups. The first group incubated and maintained under 16-h photoperiod. The second group maintained in dark. All cultures incubated at 26 ± 1° C for 8 weeks. After callus development the cultures were transferred in to new media for somatic embryogenesis and shoot regeneration.

### *Embryogenic test*

Test of callus cells for the presence of embryos was carried out by acetocarmine stain (Gupta and Durzan, 1987). Acetic acid solution (45%) was prepared by mixing 45 ml of glacial acetic acid with 55 ml of sterile distilled water heated this solution and stir with glass rod. Continue boiling until the dye dissolves and then cool to room temperature. Filter the solution and stored in refrigerator.

Calli extracted from cultures responded for somatic embryogenesis are macerated gently to break down to small pieces. Small amount of cells are taken on a clear slide and treated with 1-2 drops of acetocarmine reagent on cells and stir well with a needle. Warm the slide for 1-2 minutes gently over the flame then washed the sample by destaining solution on warm water for 2-3 times and then examined under microscope. The resulted stained crimson red cells are embryogenic while unstained cells are non embryogenic.

## RESULT AND DISCUSSION

### *Callus induction and shoots regeneration*

Shoot multiplication of *Curcuma caesia* was found to be best in MS medium supplemented with BAP (1.0 $mgl^{-1}$) and 2, 4-D (0.25 $mgl^{-1}$) within 8 weeks in (23.80±0.51shoots/explant). Out of the three explants (pseudostem, leaf sheath and root segment) studied, pseudostem showed vigorous callus growth in MS medium containing higher concentration of 2, 4-D (5.0 $mgl^{-1}$) (Plate-d ). More than 70% of the pseudostem explants of this species responded for callus induction within 22 days of culture. It was followed by the MS media supplemented with 2.0 mg/l 2,4-D and MS with 1.0 $mgl^{-1}$ BAP, 4.0 $mgl^{-1}$ 2, 4-D within 25-30 days of culture(Plate-a ; Plate-b ).The Calli thus generated from this combination was found to be highly friable, transparent white in colour, loose in nature with vigorous growth. During subcultures, secondary proliferated calli were subsequently produced from initially induced calli on the same medium. Similar results also reported by Roopadarsini (2010) on *in vitro* callus induction with the supplementation of 3.0 $mgl^{-1}$ 2, 4-D in other *Curcuma* species. Callus induction was observed under dark treatment only while in

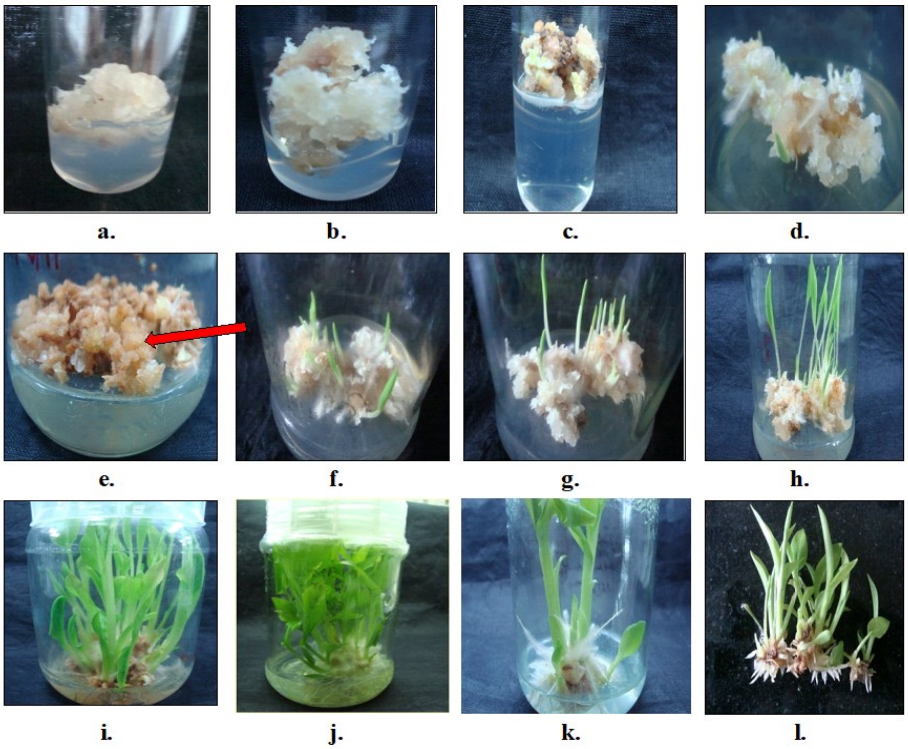


**Plate 1.** *Growth of callus from pseudostem explants of Curcuma caesia (a) Callus initiation after 2 weeks in 2.0 mg/l 2,4 -D (b) Callus growth after 4 weeks MS + 1.0mg/l BAP+4.0 mg/l 2, 4-D (c) Callus growth after 6 weeks (d) Callus growth after 8 weeks in MS + 5.0 mg/l 2,4-D (e) Embryonic development (f)pre shoot development (g) Development of plantlet from the somatic embryos of Curcuma caesia in MS 2.0 mg/l+ 0.05 mg/l NAA medium. (h) Plantlet initiation from somatic embryos after 4 weeks of culture (i)Rhizogenesis and platlet developement after 8 weeks of culture (j) Plantlet development and growth after 10 weeks of culture,(k)Rooting in media without growth hormone(l)Plantlets with roots.*

**Table 1.** Effect of MS basal media on callus induction and growth from various explants (leaf sheath, root, pseudostem) of *Curcuma caesia*

| Explant | Media | Percent of explant for callus induction | Days required for callus induction | Callus Growth | Response |
|---|---|---|---|---|---|
| Leaf sheath | MS | 0 | 0 | 0 | No response |
| | MS + 1.0 mg/l 2,4D | 22 | 25 | + | Callus induced with poor growth |
| | MS + 3.0 mg/l 2,4D | 40 | 22 | + + + | Highly friable callus with profuse growth |
| | MS+ 5.0 mg/l 2,4-D | 56 | 20 | + +++ | White friable callus induced |
| | MS + 8.0 mg/l 2,4D | 30 | 22 | ++ | Callus induced, compact in nature |
| | MS + 1.0 mg/l BAP+ 2.0 mg/l 2,4-D | 50 | 24 | ++++ | Callus induced friable in nature |
| | ½ MS+ 1.0 mg/l 2,4-D+ 0.5 mg/l NAA | 28 | 26 | + | Compact callus induced with poor growth |
| | ½ MS+ 0.5 mg/l 2,4-D+ NAA 2.0 mg/l | 20 | 27 | + | Little callus induced, compact in nature |
| Root | MS+ 1.0 mg/l 2,4-D | 0 | 0 | - | No response |
| | MS+ 2.0 mg/l NAA | 10 | 38 | ++ | Callus induced with moderate growth |
| | MS+ 1.0 mg/l 2,4-D+ 0.5 mg/l NAA | 0 | 0 | - | No response |
| | MS+1.0 mg/l 2,4-D+ 1.0 mg/l NAA | 20 | 33 | + | Callus induced, hard in nature |
| | ½ MS+ 0.5 mg/l 2, 4-D+ 2.0 mg/l NAA | 23 | 30 | ++ | Callus induced, Compact in nature |
| | ½ MS+ 2.0 mg/l NAA | 0 | 0 | - | No response |
| Pseudo-stem | MS+ 0.05 mg/l BAP+ 1.0 mg/l 2, 4-D | 32 | 28 | + | Callus induced with moderate growth |
| | MS+ 1.0mg/l BAP+ 4.0 mg/l 2,4-D | 47 | 25 | +++++ | Friable callus induced with high growth |
| | MS+ 0.5 mg/l BAP+ 1.5 mg/l 2,4-D | 45 | 27 | ++ | Callus induced |
| | MS+ 0.5 mg/l 2,4-D+ 2.0 mg/l NAA | 12 | 35 | + | Compact Callus induced |
| | MS+ 1.0 mg/l 2,4-D | 35 | 30 | +++ | Whitish Friable Callus induced, with moderate growth |
| | MS+2.0 mg/l 2,4-D | 50 | 30 | ++++ | Friable Callus induced |
| | MS+ 5.0 mg/l 2,4-D | 70 | 22 | + + + ++ | Highly friable callus induced, transparent white in colour |
| | ½ MS+ 0.5 mg/l 2, 4-D+ 2.0 mg/l NAA | 12 | 30 | + | Hard Callus with slow growth |

photoperiod 16 hrs light, explants showed no response. After 4 subcultures these callus showed embryogenic response in the same medium. Microscopic observation of these calli showing embryogenic response revealed intense cell multiplication and divisions towards the periclinal globular structures of somatic embryos. Moderate response for callus induction from pseudostem explants was also recorded in MS media containing 0.05 $mgl^{-1}$ BAP, 1.0 $mgl^{-1}$ 2, 4-D and 1.0 $mgl^{-1}$ 2, 4-D within 28 and 30 days respectively. The growth of the induced calli was also vigorous with transparent white in colour. Compact callus with poor growth was observed from pseudostem explant of *Curcuma caesia* in half strength of MS media along with 0.5 $mgl^{-1}$ 2, 4- D+ 2.0 $mgl^{-1}$ NAA within 30 days of culture. Complete absence of light was a requirement for best callus induction. Similar results for preference of callus induction and growth in darkness was also reported by various workers in different species of zingiberaceae (Taha *et al*., 2013; Mello *et al*., 2001). Callus induction of other *Curcuma* species was also reported earlier on MS medium with 10 mg/l BA and 1 mg/l 2, 4-D or the medium with 10 $mgl^{-1}$ BA and 15 mg $l^{-1}$ NAA (Mukhri and Yamaguchi, 1986). However, no callus formation was recorded on MS media without growth regulators (Table 1, Plate 1).

Callus induction of *Curcuma caesia* was also observed from leaf sheath explant in half strength MS medium fortified with 5.0 $mgl^{-1}$ 2, 4-D within 20 days of culture. Tuan *et al*. (2011) reported higher callus induction in MS medium supplemented with 1.0 $mgl^{-1}$ 2, 4-D, 1.0 $mgl^{-1}$ BA and 2% sucrose, from leaf-base explants of *Curcuma zedoaria* (Table 1, Plate 1).

Among the explants studied the root explants of *Curcuma caesia* showed poor response for callus formation. Callus induction from root explants was observed in the medium containing ½ strength MS basal medium with 2.0 $mgl^{-1}$ NAA and 0.5 $mgl^{-1}$ 2, 4-D within 30 days of culture. In contrast to the present findings Miachir *et al*.(2004) reported callus induction from root segments of *Curcuma zedoaria* on MS medium supplemented with 1.0 $mgl^{-1}$ NAA in dark.

### *Somatic embryogenesis and Plantlet Regeneration*

Highly proliferated callus tissue of *Curcuma caesia* initiated in MS medium were aseptically transferred to the organogenesis inducing medium fortified with various auxins and cytokinins singly or in combinations. Embryonic callus were proliferated from the explants of *Curcuma caesia* after 30 days of transfer on MS medium

**Table 2.** Effect of different phytohormones on embryogenic development of *Curcuma caesia*

| Media | Embryogenic response% | Days required for embryogenic development | Remarks |
|---|---|---|---|
| MS | 0 | 0 | No embryogenic response |
| MS+ 1.0 mg/l BAP | 4 | 30 | Embryogenic callus developed. |
| MS+ 1.0 mg/l 2,4-D | 0 | 0 | No response |
| MS+ 5.0 mg/l 2,4-D | 0 | 0 | No embyogenic callus |
| MS+ 3.0 mg/l 2, 4-D+ 1.0 mg/l NAA | 10 | 47 | Pre embryogenic callus |
| MS+0.5 mg/l BAP+ 0.05 mg/l 2, 4-D | 11 | 28 | Formation of globular embryo like structure |
| MS+ 1.0 mg/l+ 0.05 mg/l 2, 4-D | 6 | 41 | Plantlet initiated from callus |
| MS+ 1.5 mg/l BAP+0.25 mg/l 2, 4- D | 8 | 34 | Embryogenic friable callus |
| MS+ 1.5 mg/l BAP+ 2.0 mg/l 2, 4 –D | 10 | 30 | Embryogenic callus developed. |
| MS+ 1.0 mg/l BAP+ 0.05 mg/l NAA | 4 | 28 | Pre embryogenic response |
| MS+ 2.0 mg/l BAP+ 0.05 mg/l NAA | 20 | 30 | Formation of globular embryos and shoots |
| MS + 0.25 mg/l Kn + 0.05 mg/l GA3 | 16 | 25 | Formation of greenish friable callus with pre shoot |
| MS+ 0.5 mg/l BAP+ 0.25 mg/l GA3 | 10 | 30 | Development of globular embryos |
| MS+ 1.0 mg/l BAP+ 0.25 mg/l GA3 | 5 | 33 | Development of embryo like structure |
| MS+ 2.0 mg/l 2,4-D | 0 | 0 | No response |
| MS+ 2.0 mg/l BAP+0.05 mg/l 2, 4- D | 9 | 40 | Maturation of somatic emryos and shoot initiation |
| MS+ 0.5 mg/l BAP+ 0.05 mg/l GA3 | 6 | 31 | Development of globular embryos |
| MS+ 0.75 mg/l BAP+ 0.05 mg/l 2, 4- D | 8 | 38 | Maturation of somatic emryos and shoot initiation |
| MS+ 1.0 mg/l BAP+ 0.05 mg/l Kn | 5 | 42 | Embryogenic development |
| MS+ 1.0 mg/l BAP+ 0.05 mg/l GA3 | 0 | 0 | No response |

supplemented with 2.0 $mgl^{-1}$ BAP with 0.05 $mgl^{-1}$ NAA. Malamug *et al*. (1991) was also able to regenerate multiple shoots from callus derived shoots of ginger using BAP and NAA in modified MS medium. The embryogenic calli were routinely maintained on the same induction medium. Numerous somatic embryos of varied shapes were developed on the surface of the embryo genic callus. In this medium globular embryo developed with numerous shoots of *Curcuma caesia* within 30 days of transfer. Greenish friable callus with preshoots were developed while transferring these embryos to freshly prepared media fortified with 0.25 $mgl^{-1}$ Kn and 0.05 $mgl^{-1}$ $GA_3$ within 25 days of transfer(Table 2) (Plate-e).There is strong evidence that the combination of BAP and NAA enhances shoot regeneration from organogenic calluses of *Alstroemeria* (Gonzales-Benito and Alderson, 1990) and *Dianthus chinensis* (Jethwani and Kothari, 1996). The effect of combining these two growth regulators was also described for micro propagation of *Curcuma amada* (Barthakur and Bordoloi, 1992) and other members of the Zingiberaceae family (Babu *et al.,* 1992; Reghunath and Priyadarshan, 1993; Dogra *et al.,* 1994; Hung, 1995; Illg and Faria, 1995). Addition of $GA_3$ in the culture media promoted germination of somatic embryos of *Curcuma caesia* as reported earlier in *Curcuma* species by Prakash *et al*. (2001). Mature somatic embryos germinated readily and developed into normal plantlets after 4 weeks on half strength MS basal medium. Well rooted plantlets were successfully acclimatized in soil at high rate of survivality (80 %).

## CONCLUSION

It may be concluded from this study that the protocol developed will be useful for rapid *in vitro* propagation of *Curcuma caesia* through somatic embryogenesis. Mass multiplication within a short period of time may provide a viable approach through callus culture and somatic embryogenesis for germplasm conservation of this species.

## REFERENCES

Babu, K.N. Samsudeen, K. Ravindran, P.N.1992. Direct regeneration of plantlets from immature inflorescences of ginger (*Zingiber officinale* Rosc.) by tissue culture. J. Spices Aromat. Crops. 1: 43–48

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

Behar, N, Tiwari, K.L. and Jadhav, S.K. 2013. Comparative phytochemical screening of bioactive compounds in *Curcuma caesia* Roxb. and *Curcuma longa*. Research Journal of Medicinal Plant. 7(2): 113 – 118.

Behar N, Tiwari, K. L. and Jadhav, S. K. 2014. A Review on Non-Conventional Turmeric: *Curcuma caesia* Roxb. Current Trends in Biotechnology and Pharmacy. 8 (1): 91-101

Borthakur M and Bordoloi D.N.1992. Micropagation of *Curcuma amada* Roxb. J. Spice and Aromatic Crops. 1: 154–156

Dogra, S. P.; Korla, B. N. and Sharma, P. P.1994. *In vitro* clonal propagation of ginger (*Zingiber officinales* Rosc.). Hort. J., 7: 45-50

Gonzalez-Benito, E. and Alderson, P. G. 1990. Regeneration from *Alstroemeria* callus. Acta Hortic. 280: 135-138

Hung, J. H. 1995. *In vitro* propagation and preservation of ginger germplasm resources. Scientia Agricultura Sinica. 28: 24-30

Illg, R.D. and Faria, R.T. 1995. Micropropagation of *Alpinia purpurata* from inflorescence buds. Plant Cell Tiss. Org. Cult. 40: 183-185

Jethwani, V and Kothari, S. L. 1996. Phenylacetic acid induced organogenesis in cultured leaf segments of *Dianthus chinensis*. Plant Cell Rep., 15: 869-872

Kagyung R, Gajurel P.R, Rethy P and Sing B. 2010. Ethnomedicinal plant used for gastrointestinal diseases by Adi tribe of Dehang-Debang biosphere reserve in Arunachal Pradesh. Journal of Traditional Knowledge, 9(3):496-501.

Kumar, S., Singh, J., Shah, N.C., Ranjan, V. 1998. Indian Medicinal Plants Facing Genetic Erosion. CIMAP: Lucknow. P- 219.

Malamung, J.J.F., Inden, H. and Asahira, T. 1991. Plantlet regeneration and propagation from ginger callus. Scientia. Hort. 48: 89-97

Mello, M.O. 2001. Histological Analysis of the Callogenesis and Organogenesis from Root Segments of *Curcuma zedoaria* Roscoe. Brazilian Archives of Biology and Technology. An Int Jr., 44(2):197–203.

Mohanty, S., Panda, M.K., Subudhi, E. and Nayak, S. 2008. Plant Regeneration from Callus Culture of *Curcuma Aromatica* and *In Vitro* Detection of Somaclonal Variation through Cytophotometric Analysis. *Biologia Plantarum*, **52**: 783-786.

Miachir, J.I., Romani, V.L.M., Amaral, A.F.C., Mello, M.O., Crocomo, O.J., Melo, M. 2004. Micropropagation and Callogenesis of *Curcuma zedoaria* Roscoe. Sci. Agric. (Piracicaba, Braz.), 61(4): 427 -432.

Mukhri, Z. and Yamaguchi, H. 1986. *In vitro* plant Multiplication from Rhizomes of Turmeric (*Curcuma domestica* val.) and Temoe Lawak (*C. xanthoriza* Roxb.). Plant Tissue Culture Letters. 3(1): 28-30

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

Prakash, E., Khan, S.V, Meru, E. and Rao, K.R. 2001. Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. and Subr., an endangered medicinal plant of Tirumala hills. Current Science. 81(9):153-155

Prakash, S., R. Elangomathavan, S. Seshadri, K. Kathiravan and S. Ignacimuthu. 2004. Efficient regeneration of *Curcuma amada* Roxb. plantlets from rhizome and leaf sheath

explants. *Plant Cell, Tiss. Cul*. 78: 159-165

Roopadarshini, V. 2010. High Frequency Shoot Multiplication and Callus Regeneration of Turmeric. International Journal of and Biochemistry. 6(5): 723–733

Reghunath, B.R. and Priyadarshan, P.M. 1993. Somaclonal variation in cardamom (*Elettaria cardamomum* Maton) derived from axenic culture of juvenile shoot primordia. Acta Hortic., 330: 235-242

Tuan, V.C.; Hoang, V. D.; Loc, N. H. 2011. Cell suspension culture of Zedoary (*Curcuma zedoaria* Roscoe). VNU Journal of Science. Natural Sciences and Technology. 27: 64-70