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National Academy of Agricultural Science (NAAS)
Rating : 3. 03

In vitro propagation of *Curcuma karnatakensis* –An Endemic taxon

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Abstract: *Curcuma karnatakensis belongs to the family Zingiberaceae, is endemic to Western Ghats of Karnataka region and is totally underexploited scientifically. In vitro propagation was initiated for the first time in this taxon using tuber as source of explants. Segments of tubers were inoculated on Phillips and Collins medium supplemented with various growth regulators. Callus was obtained on a medium containing a single hormone. Combination of two cytokinins has induced the growth of bud into a single shoot. Multiple shoots were obtained from the culture on a medium supplemented with combination of auxins and cytokinins. The rate of induction of multiple shoots was enhanced when yeast extract / CM was added to auxins and cytokinins combinations. Maximum of 8 to 9 shoot buds were induced on L2+NAA (32.22µM) +2, 4-D (27.12µM) +Kin (37.12µM) +2-ip (39.36µM). Thus obtained shoots were sub cultured to ½ L2 medium supplemented with IBA for root induction. Regenerated plantlets are then sequentially acclimatized by transferring them to vermiculate, then to soil, sand, and manure mixed in the ratio of 1:1:1. Nearly 50 percent of survival is recorded.*

Keywords*: Conservation, Curcuma karnatakensis, endemic species, In vitro multiplication.*

INTRODUCTION

The genus *Curcuma* is well known for its various medicinal properties. Several species of *Curcuma* are used in indigenous systems of medicine including Chinese medicine since ages. The active principle Curcumine was reported to have very high antioxidant activity and anti-cancer preventive effects[1-4].

Curcuma karnatakensis Amalraj, Velayudhan and Muralidharan is endemic to Karnataka state, India and has been reported from only two places- Hirehalli and Dharwad.Since it is endemic with rare occurrence, conservation of this taxon is of top priority. Plant tissue culture is emerging as an alternate strategy for conservation of rare and threatened taxa through micropropogation and establishment of *in vitro* gene banks. As this taxon is scientifically unexplored, the present investigation is an attempt to study the feasibility of utilizing *in vitro* culture for mass multiplication through micropropogation.

MATERIAL AND METHODS

Source of explants

Healthy plants are collected from Dharwad during flowering season from June to November since plants can be located only during this season. They are maintained in the polyhouse, department of Botany, Bangalore University, Bangalore. Tubers of about 4×2cm were excised and washed thoroughly under running tap water for 30 min. They were cut into 4 segments of each 1×2 cm –apical, middle 2 segments and basal and used as explants.

Surface sterilization

Tubers were surface- sterilized sequentially in Bevastin- a fungicide for 30 min followed by Tween

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20 for 30 min, mercuric chloride for 4 min and streptomycin- an antibiotic for 2 min. Explants were thoroughly washed with sterile double distilled water after each treatment.

Media and culture condition

Surface sterilized explants were inoculated on to Murashige and Skoog's[5] (MS) and Phillips and Collins[6] (L2) media supplemented with various growth regulators. Sucrose (3%) was used as carbon source and bacteriological Agar agar (0.8%) as gelling agent. The P^H of the medium was adjusted to 5.6. The cultures were incubated under white fluorescent lights of intensity of 25µmol $m^{-2}s^{-1}$at 25 ± 2^0c. The cultures are maintained under 16:8 h light and dark period regime.

Rooting and acclimatization

The excised shoots from the culture were subcultures to ½ L2supplemented with IAA and IBA at various concentrations for root induction. Thus obtained plantlets were transferred to plastic cups filled with vermiculate fed were with ½ L2 liquid medium. After four weeks in culture room, the plantlets were transferred to pots containing soil, sand and manure in 1:1:1 ratio.

Histology

Segments of responding tubers were fixed in FAA(Formalin: Acetic Acid: Alcohol) for 24 h. Customary paraffin technique was followed. Microtome sections of about 15-18 m thickness were taken and stained with Haidenhains haematoxylin and counter stained with orange-G/Eosin. Photomicrograph of sections was taken with Nikon binocular microscope using Canon camera.

Data analysis

All the experiments conducted were repeated thrice in five replicates .The mean was calculated and represented as mean± SE. The data was subjected to one way ANOVA for analysis. Significant 'F' ratios between group mean were further subjected to DMRT using SPSS version 1.5. Probability values <0.05 were considered as significant [7].

RESULTS

Initially both MS and L2media were used to raise the cultures. However, L2 medium was found more suitable than MS medium in supporting the growth of the cultures. Hence, the data was accordingly recorded from the cultures grown on L2medium (Table 1). The medium was supplemented with various auxins -2, 4-D, IAA, IBA, NAA and cytokinins –Kin, BAP, TDZ, 2-ip either alone or in combinations. Presence of single hormone has induced only whitish friable callus from the cut surface(Fig .1a).However combinations of two Cytokinins such as TDZ (2.27µM) +Kin(27.84µM) /2-ip(29.52µM) had promoted the growth of the single shoot from the

Table 1
Effect of Growth Regulators on multiple shoot regeneration from the cultures of *Curcuma karnatakensis*

Sl. No.	*L2+Growth Regulators(µM)*	*Mean±SE*
1.	L2+Kin(37.212)	1.6 ± 0.46^{i}
2.	L2+Kin (37.212) +CM (10%)	2.0 ± 0.77^{h}
3.	L2+Kin(37.212)+Yeast(0.5g)	3.0 ± 0.77^{e}
4.	L2+2-ip (29.52) +TDZ(2.27)	4.0 ± 0.77^{cd}
5.	L2+NAA(21.48)+Kin(18.56)	3.0 ± 0.70^{ef}
6.	L2+NAA(21.48)+TDZ(4.54)	3.0 ± 0.55^{g}
7.	L2+NAA(32.22)+2,4D(27.24)+Kin(37.12)+2-ip(39.36)	5.0 ± 0.76^{ab}
8.	L2+ +Kin (37.12) \2-iP (39.36) + TDZ (0.45)+ yeast extract(0.25 - 0.5 gm)\ CM(10%)	5.58 ± 0.77^{a}
9.	L2+2-ip(39.36)+TDZ(0.45)+GA_3(5.76)	4.0 ± 0.44^{c}

Data represented by Mean ± SE of the independent experiments. Mean followed by the different letter within columns are significantly different ($p<0.05$) using Duncan's multiple range test.

explants after two weeks of culture (Fig. 1b). Induction of multiple shoots in the cultures was dependent on the type and combination of growth regulators in the medium. Presence of NAA (21.48µM) along with TDZ (4.54µM) had induced friable white callus from the explants without any shoot growth .However, NAA (21.48µM) along with Kin (18.56µM) had promoted the growth of a single shoot and multiple shoots with TDZ (4.54 µM)(Fig.1c). Multiple shoots were initiated from the explants on L2 +NAA (32.22µM) +2, 4-D (27.24µM) +Kin (37.12µM) +2-ip (39.36µM). When yeast extract (0.25 to 0.5 gm) /CM (10%) was added to L2+Kin (37.12µM) +2-ip (39.36µM) +TDZ (0.45µM) multiple shoots were initiated from the basal region within one week of inoculation (Figs.1d&e).

Thus obtained multiple shoots were subcultured to L2 +2-ip (39.36µM) +GA_3 (5.76µM) for shoot elongation. When the shoots were grown up to 6-8cms with two pairs of leaves, they were subcultured to rooting medium (half strength) containing IAA/IBA at various concentrations along with GA_3 (5.76µM). Induction of roots from the basal part of shoots was achieved on IBA (19.68µM) +GA_3 (5.76µM). Then the plantlets were sequentially hardened by transferring them on to small plastic cups containing vermiculate fed with 1/2 L2 liquid medium and then to pots containing sand, soil and manure in 1:1:1 ratio (Fig.1f).

The microtome sections of the cultures have shown the origin of multiple shoots from the

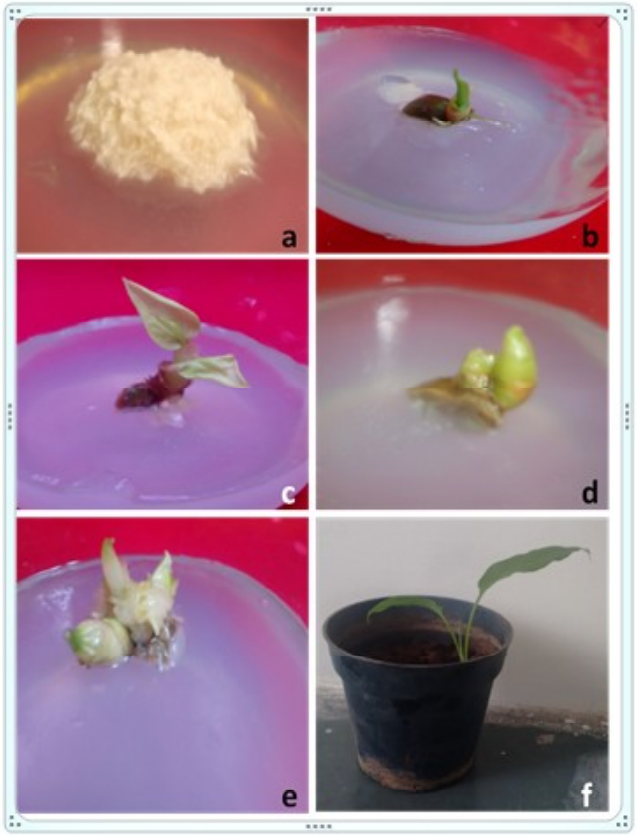

Figure 1: Micropropagation of *Curcuma karnatakensis*.

1a. Formation of Whitish friable callus from the cut surface of rhizome on L2+Kin(37.212 µM); 1b. Single shoot growth on L2+2-ip (29.52µM) +TDZ (2.27 µM); 1c. Sprouting of leaves from the single shoot on NAA(21.48µM) +Kin (18.56 µM); 1d. Multiple shoot formation on L2+NAA(21.48µM) +TDZ (4.54 µM); 1e. Multiple shoot formation on L2+ +Kin (37.12 µM) +2-iP (39.36 µM)+TDZ(0.45µM)+ CM(10%); 1f. Hardening of tissue cultured plant on Vermiculate.

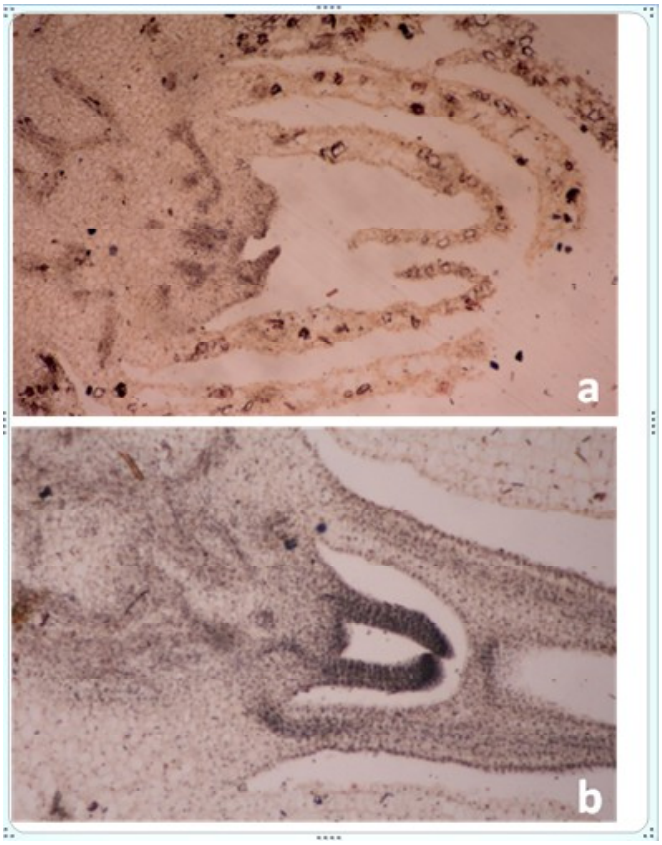

Figure 2: Histological Studies

2 a & b. Sections of cultures showing the direct origin of shoot buds.

explants directly without formation of callus (Fig.2a, b) confirming the direct organogenesis.

DISCUSSION

Amalraj et al. [8] have reported this taxon from Karnataka for the first time in 1999 near Hirehalli. Later Kotresha et al. [9] in 2008 have located this species in Dharwad. As in other Zingiberaceae members, it has a rootstock of about 5×2cm that bears 10 to 12 stipitate, fusiform tubers of about 4×2cm in size. Tuber segments of about 1×2 cm were inoculated onto L2medium supplemented with various growth regulators. MS medium was used to raise the culture of several species of *Curcuma* [9-13]. Woody plant medium was used by Zuraida et al. [14] to raise the cultures of *C.caesia*. However, L2 medium was found to be superior in the expression of morphogenetic potential of the explants in the present studies

L2 medium was formulated by Phillips and Collins[6]in 1979 to initiate the callus and suspension cultures of Red clover and a few legumes .Since than it has been known as leguminous medium (L2). It differs from MS medium in not having nicotinic acid, which is found to be inhibitory for callus induction and growth in legumes. Further, L2 medium has less quantities of ammonium salts than MS. Explants inoculated onto basal medium have not responded. They have dried after 2-3 weeks in the culture .Addition of either auxin or cytokinin alone had induced only callus without any morphogenetic responses. Where as in the rhizome cultures of *C.angustifolia*[10], BAP at lower concentrations had induced multiple shoots. Combinations of two cytokinins had promoted the growth of the bud into a single shoot in the present study. However Gomathy et al.[13], have recorded proliferation of multiple shoots when MS was supplemented with BAP and Kin .The combination of auxin with cytokinins was found to be more favorable for induction of multiple shoots in various taxa[15-20]. Critical balance between auxin and cytokinins is essential to induce the shoot buds from the culture. Skoog and Miller [21] and Coenen and Lomax[22] were also of the opinion that cytokinins and auxin act synergistically to control various physiological and developmental responses. BAP along with NAA had promoted the growth of multiple shoots from the rhizome cultures of *C.aeroginosa*[23], *C.longa*[11, 24] and *C.caesia*[12],whereas Gomathy et al.[13]have obtained best result in shoot proliferation on BAP combined with IAA. In the present study, combination of two auxins and two cytokinins were found to be optimal for the induction of multiple shoots. When yeast extract or/and CM added to these combinations, the numbers of multiple shoots were induced at a faster rate than on the medium without yeast extract and CM. The percentage of amino acids in yeast extract is very high [25]. It is normally enhance growth in the media containing relatively low concentration of nitrogen [26]. L2 medium is relatively contain less concentration of nitrogen compounds than MS medium. Coconut milk was first used in tissue culture by Van Over beek et al. [27] who found that its addition to the culture medium was necessary for the development of young embryos of *Datura stramonium*. Since then CM has been used as an additive in the culture medium [28]. It is classified as undefined supplement whose composition can be vary considerably [29]. The coconut milk has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis[29].

The regenerated shoots in the present study were rooted on 1/2 L2 medium supplemented with IBA. Root induction is a crucial step in micropropagation, which is controlled by the growth of roots in both *in vivo* and *in vitro* conditions [30]. Role of IBA as an effective root inducing hormone is well established in several taxa [16, 31-33] .This has been attributed to its slow movement and degradation of IBA facilitates its localization near the site of its application [34]. Half strength MS supplemented with IBA was found to be suitable for induction of roots in *C.longa*[11, 13] and *C.*caseia[12] .However in *C.aeroginosa* [23] and *C.angustifolia* [10] roots were initiated on shoot multiplication media itself. The rooted plantlets were then transferred to plastic cups filled with vermiculate fed with 1/2 L2 liquid medium. Vermiculate is a versatile mineral and known for its thermal suitability and inertness. It has the capacity to hold high moisture content and provides more air space for the plant roots. Hence, it is one of the best substrate used for the

establishment of tissue-culturedplantlets.[35-37]. Finally, tissue cultured plantlets were transferred for pots containing soil, sand and manure in 1:1:1 ratio .It can be concluded from the above data, that the mass multiplication is possible by *in vitro* cultures that can be exploited further for conservation of these endemic taxon.

ACKNOWLEDGEMENT

Thanks are due to UGC, New Delhi for granting BSR-Faculty fellowship for one of the author (DHT). The present work is carried out under this fellowship.

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