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In vitro Regeneration and Estimation of Curcumin Content in Four Species of *Curcuma*

Sumana ROY and Sarmistha Sen RAYCHAUDHURI*

Plant Tissue Culture and Molecular Biology Laboratory Department of Biophysics, Molecular Biology and Genetics University of Calcutta 92, Acharya Prafulla Chandra Ray Road, Kolkata 700009, India
**Correspomding author* E-mail address: src@cubmb.ernet.in

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Abstract

Four species of *Curcuma, C. longa, C. amada, C. aromatica* and *C. zedoaria* were collected from different parts of West Bengal, India and propagated in our experimental garden. *In vitro* regeneration of *C. longa* and *C. aromatica* was carried out from nodal explants and that of *C. zedoaria* from rhizome explants. Shoots were successfully regenerated from both nodes and rhizomes in *C. amada*. Plants regenerated *in vitro* produced rhizomes when planted in pots containing sterile soil. Curcumin contents in rhizomes of these plants were determined by spectrophotometric analysis. All accessions of *C. longa* uniformly showed a high curcumin content, while *C. amada* and *C. zedoaria* did not. A novel accession of *C. aromatica* (accession number v_{10}) was found to contain curcumin even higher than that of *C. longa*, suggesting it to be useful as an alternative source of curcumin.

Key words: *Curcuma longa, C. amada, C. aromatica, C. zedoaria*, regeneration, curcumin.

Abbreviations

MS, Murashige and Skoog medium; NAA, α-naphthaleneacetic acid; Kin, 6-furfurylaminopurine; BA, benzyladenine.

The genus *Curcuma* belongs to the family Zingeberaceae and includes species *C. longa, C. amada, C. aromatica* and *C. zedoaria*, which are of high medicinal importance. *C. longa*, commonly known as turmeric, has been used as aromatic ingredient for cooking. Curcumin extracted from *C. longa* rhizome is an anti-inflammatory agent (Ammon *et al.,* 1993) and has anti-carcinogenic properties (Piper *et al.,* 1998). *C. aromatica*, a closely related species of *C. longa*, also contains curcumin. Powdered rhizome of this species is of high caloric value and used as a substitute for baby food (Moral and Suhrawardy, 1999). Rhizomes of *C. amada* have special aroma like mango and commonly known as mango ginger. It is used to make chutney and pickles and is a popular condiment in eastern India (Middleditch, 1753). Rhizomes of *C. zedoaria* are eaten as vegetable (Middleditch, 1753).

The present investigation was carried out to propagate these economically important plants *in vitro* and to assess curcumin content of the rhizome by spectrophotometer. Rhizomes of 12 accessions of the above 4 different *Curcuma* species were collected from different areas of West Bengal and cultivated in the experimental garden in the University College of Science, Calcutta. The phenotypic characteristics of 12 accessions of the genus *Curcuma* are compared (**Table 1**). Plant height varied from 38 to 69 cm and leaf size also varied (12 to 19 cm). Color of the rhizome was found to be bright yellow in *C. longa*, orange yellow in *C. aromatica*, lemon yellow in *C. amada* and white in *C. zedoaria*. Curcumin content was determined from rhizomes of four *Curcuma* species by solvent extraction and spectrophotometric method (ASTA method, 1997). A 100-mg of dried rhizomes was taken in an extraction flask (100 ml flat bottom, with TS 24 / 40 ground joint) and 30 ml of 95% alcohol was added and refluxed for 3h. The refluxed residue was cooled and taken on a filter paper, washed with 100 ml of 95% alcohol. A 20-ml of the filtrate was taken and diluted to 250 ml with 95% alcohol. The absorbance of the diluted sample and that of the standard curcumin solution was measured at 425 nm by a spectrophotometer (Hitachi U 2001). The standard calibration curve with 25 mg of stock curcumin dissolved in 100 ml 95% alcohol showed a linear relationship between absorbance at 425 nm and curcumin concentration (data not shown). Up to the concentration of 13×10^{-4} g l^{-1}, absorbancy of

Table 1 Morphological properties and curcumin content in 12 accessions of 4 different species of *Curcuma*

Species	Accession Number	Collection Site	Height of Plant (cm) (mean ± sd)	Size of leaf (cm) (mean ± sd)	Curcumin (mg/100 mg rhizome)	Color of rhizome
C. longa	v_1	Thekua	67.4 ± 3.50	19.2 ± 1.48	4.6	Yellow
C. longa	v_2	N. 24 Pgs	67.0 ± 3.39	18.8 ± 1.48	4.5	Yellow
C. longa	v_3	Malda	67.0 ± 4.30	18.0 ± 1.22	4.4	Yellow
C. longa	v_4	Bongaon	65.6 ± 4.03	18.4 ± 2.1	3.8	Yellow
C. longa	v_5	Geyonkhali	65.4 ± 4.15	18.6 ± 3.20	3.0	Yellow
C. longa	v_6	Patna	66.0 ± 4.74	18.0 ± 2.54	4.4	Yellow
C. amada	v_7	Thekua	66.4 ± 4.16	17.4 ± 2.30	Absent	Lemon yellow
C. amada	v_8	Malda	69.2 ± 3.11	17.6 ± 1.94	Absent	Lemon yellow
C. amada	v_9	Geyonkhali	69.4 ± 2.88	19.2 ± 1.30	Absent	Lemon yellow
C. aromatica	v_{10}	Malda	38.8 ± 2.86	11.8 ± 1.30	5.0	Orange yellow
C. aromatica	v_{11}	Bongaon	46.0 ± 1.58	12.4 ± 1.14	0.03	Orange yellow
C. Zedoaria	v_{12}	Thekua	32.4 ± 4.27	21.0 ± 3.39	Absent	White

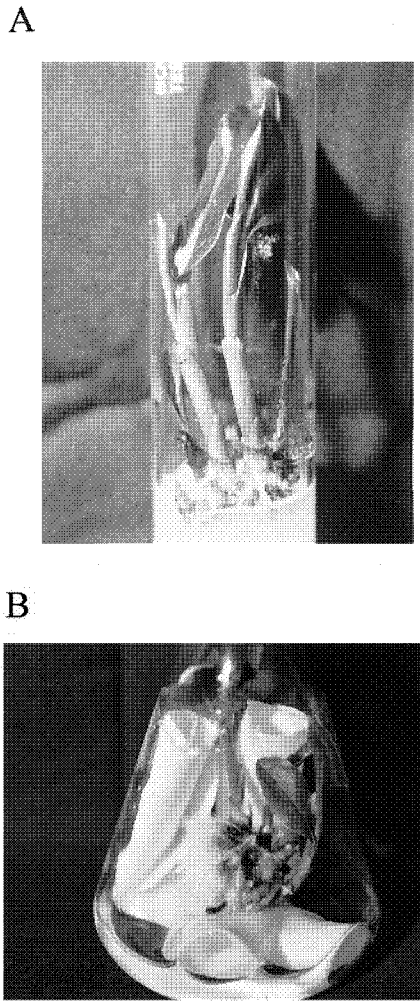

Fig. 1 Regeneration of *C. longa*. *C. longa* in MS media from nodal explant (A) and in liquid media (B).

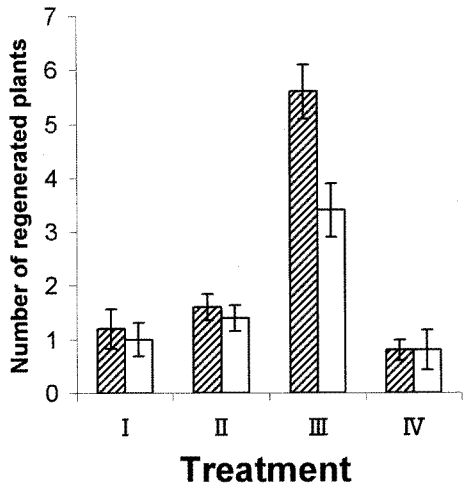

Fig. 2 Number of shoots regenerated from rhizome explant. Number of shoots are shown in *C. amada* (shaded bar) and *C. zedoaria* (open bar) (mean ± SE) at different treatment. The concentration and ratio of NAA (mgl^{-1}) and kinetin (mgl^{-1}) were 4/3 (I), 4/4 (II), 4/5 (III) and 5/3 (IV), respectively.

curcumin solution follow Beer's law. Curcumin concentration of the samples was estimated using the above standard calibration curve (**Table 1**).

Young nodes (2-3 cm) and fresh rhizomes (2-3 cm) were used as explants. Explants were sterilized with 0.1% $HgCl_2$ solution for 25 to 27 min with vigorous shaking and then washed in sterile distilled water five to six times in a laminar air flow bench and inoculated in MS media (Murashige and Skoog, 1962) with different concentrations of auxins and cytokinins. The pH of the media was adjusted to 5.6-5.8, solidified with 0.9% agar and autoclaved for 20 min at 14.062 x 10^3 Kg/m^2 (20 Ibs/Sq inch) pressure. Slices of young rhizomes and nodes were kept horizontally in culture tubes containing 20 ml

Table 2 Comparison of shoot regeneration of *C. longa, C. amada* and *C. aromatica* from nodes in MS-media containing NAA and kinetin

Concentration of NAA/Kin (mg l^{-1})	No. of shoots in		
	C. longa	C. amada	C. aromatica
4/5	$6.3^a \pm 0.83$	$4.0^a \pm 0.44$	$4.2^a \pm 0.19$
4/4	$1.4^b \pm 0.24$	$1.6^b \pm 0.24$	$1.6^b \pm 0.24$
4/3	$1.2^b \pm 0.37$	$1.2^b \pm 0.19$	$1.2^b \pm 0.37$
5/3	$0.8^b \pm 0.37$	$0.8^b \pm 0.19$	$1.6^b \pm 0.24$

Duncan's multiple range test was performed to compare the mean number of shoot tips regenerated from nodes and rhizomes with different treatments of plant growth regulators (Gomez and Gomez, 1984). When every possible pair of treatment means is compared to identify pairs of treatments that are significantly different, one can apply DMRT. The procedure is explained as follows: Step I : All treatment means are ranked in decreasing order. Step II : Difference between ith and jth treatment means computed. StepIII: Standard error of mean difference sd is calculated. StepIV: The shortest significant ranges for (t-1) values which is $R_p = (r_p)$ (sd) $\sqrt{2}, p=2,3...t$ is calculated (p=2 means two mean values with consecutive rankings and p=t for the highest and lowest means)

r_p = tabular values of significant studentized ranges at p at 5% level of significance ($P \geq 0.5$).

Step V: All treatment means that do not differ significantly from each other are identified and grouped together.

The difference between largest treatment mean and largest R_p value is determined in all treatment values which are less than the computed difference are declared as significantly different from the largest treatment mean. Next the range for the remaining treatment means were computed and compared the range with R_p value at R_p = m, where m = the number of treatments in the group.

Alphabets (a, b) were used for groupings not significantly different. When treatments are not arranged according to ranks, same groups are given common alphabets (which may be overlapping for treatment results).

media (stab). The cultures were maintained at 22-25℃ and at 55 to 60% relative humidity under Philips fluorescent day light tubes emitting 32 x 10^8 moles $s^{-1}m^{-2}$ for 16/8 h light/dark period. Different concentrations of NAA (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg l^{-1}) and kinetin (Kin) (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg l^{-1}) were added to MS media for plant regeneration from young nodes (**Fig. 1A**) and rhizomes. The same hormone concentrations were maintained for two subcultures each of 30 days duration. Plantlets multiplied in solid media and were transferred aseptically to liquid MS medium with the same hormone combination for root initiation on filter paper bridge (**Fig. 1B**). After two weeks, they were transferred to sterilized soil and maintained in a culture room with occasional exposure to the external environment and finally transferred to pots containing garden soil. Each experiment was repeated three times.

Among the combinations tested, only 4 combinations were found to give reproducible results and the best result was obtained with 4 mg l^{-1} NAA and 5 mg l^{-1} Kin in *C. longa* (5-7 shoots/node) and *C. aromatica* (4-5 shoots/node) using nodes as explants (**Table 2**). Rhizome explants of *C. longa*, and *C. aromatica* produced only small buds whereas *C. amada* produced maximum3-4 shoots/node (**Table 2**) and 5-6 shoots/rhizome (**Fig. 2**) in MS media with 4 mg l^{-1} NAA and 5 mg l^{-1} Kin. In *C. zedoaria*, maximum (3-5) shoots were regenerated (**Fig. 2**) from rhizome and microrhizome induction was also observed in the best combination.

Mrudul et al. (2001) observed *in vitro* microrhizome production in *C. longa* from sprouted buds of small rhizome portions. According to these authors, BA had an inhibitory effect on microrhizome production. On the other hand, Meenakshi *et al.* (2001) found little success in *in vitro* shoot production in *C. longa*, while direct regeneration of shoots from immature inflorescence of *C. longa* was observed (Neeta *et al.*, 2000). Microrhizome was obtained *in vitro* from shoot meristem of *C. zedoaria* in MS media with BA (Mello *et al.*, 2000). Nayak (2000) reported shoot multiplication and plant regeneration from sprouted buds of *C. aromatica*. In the present investigation, node explant of *C. longa* and *C. aromatica* regenerated to produce shoots, and shoot regeneration was most successful from rhizome explant in *C. zedoaria*. In *C. amada* both nodes and rhizomes produced shoots *in vitro*. In the present investigation, the range of curcumin content in *C. longa* varied from 3.0-4.4%. The other two species (*C. amada* and *C. zedoaria*) do not contain curcumin but have a range of other compounds with medicinal value (Middleditch, 1753). It was found that a variety of *C. aromatica*

with accession number v_{10} with short in height and small leaves, is a novel plant with very high curcumin content (5.0%). This may be propagated as an alternative source of anti-carcinogenic chemical curcumin.

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