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BRIEF COMMUNICATION

In vitro plant regeneration and genotype conservation of eight wild species of *Curcuma*

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Abstract

In vitro protocols for plantlet regeneration and medium-term genotype conservation of eight wild species of *Curcuma* have been optimized. Both the phenomena were genotype-dependent and influenced significantly by type and concentration of cytokinins used. In general, benzyladenine (BA) was found superior to other cytokinins tested for plantlet regeneration and γ,γ-dimethylallylaminopurine (2iP) for conservation. Number of shoots per culture ranged from 1.3 to 7.2 and conservation period from 264 to 379 d. In 30-d-old cultures, highest frequency of shoot regeneration could be obtained in *C. malabarica* (7.2 shoots per culture) on MS + 11.4 μM zeatin. *Curcuma* sp. (unidentified wild species) could be conserved for maximum period (379 d) on MS + 24.6 μM 2iP followed by *C. aromatica* (363 d) on MS + 22.8 μM zeatin. The tissue culture-raised plantlets were morphologically similar to their parents. The *in vitro*-conserved plants multiplied rapidly in tissue cultures and produced normal rhizomes upon transfer to soil in net house.

Additional key words: germplasm, genetic resources, micropropagation, tissue culture.

The genus *Curcuma* (family *Zingiberaceae*) comprises more than 80 species of rhizomatous perennial herbs and has a wide-spread occurrence in the tropics of Asia and extends to Africa and Australia (Purseglove *et al*. 1981). *In vitro* techniques could be used for conservation of *Curcuma* germplasm, as proven in case of potato, cassava and yams germplasm (Ashmore 1997). Tissue culture has been applied as a potent method for multiplication and conservation of various vegetatively propagated crop species in our laboratory also (Mandal *et al*. 2000, Yusuf *et al*. 2001). Direct regeneration (without callus) of functional plantlet in tissue culture is a pre-requisite for any successful *in vitro* conservation programme as the regeneration through callus is known to induce somaclonal variations in *Curcuma longa* (Salvi *et al*. 2001). Nadgauda *et al*. (1978) and Balachandran *et al*. (1990) reported the *in vitro* multiplication in *C. longa* using rhizome bud as explants, and micropropagation of *C. longa* through callus cultures from leaf (Salvi *et al*. 2001). However, reports on micropropagation of wild species are not available, except for *C. aeruginosa*, *C. caesia* (Balachandran *et al*. 1990) and *C. aromatica* (Nayak 2000). No systematic work is reported so far about the conservation of wild species of *Curcuma* using *in vitro* techniques. The present studies were carried out to optimize the *in vitro* protocols for direct plantlet regeneration and effective conservation of wild species of *Curcuma.*

Rhizomes buds were collected from sprouted rhizomes of eight wild species of *Curcuma*, namely, *C. aeruginosa* Roxb. (TCR 2237), *C. aromatica* Salisb. (IC 88850), *C. brog* Val. (IC 29881), *C. caesia* Roxb.

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Abbreviations: BA - N^6-benzyladenine; MS - Murashige and Skoog (1962); NAA - α-naphthalene acetic acid, 2iP - γ,γ-dimethyl-allylaminopurine.
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(IC 311735), *C. malabarica* Velayudhan *et al*. (IC 88846), *C. raktakanta* Mangaly and Sabu (IC 88862), *C. soloensis* Vel. (IC 136971), and *C.* sp. (TCR 220, unidentified wild species) from the plants grown in a net house of National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. The buds (2 - 3 cm) were excised and thoroughly washed under running tap water to remove adhered soil particles. Outer scales of the buds were removed and were immersed in water containing 2 or 3 drops of *Tween-20* for 20 min and washed 7 or 8 times with distilled water and used as explants. Surface sterilization of the buds was done using 1 g dm^{-3} mercuric chloride and 2 or 3 drops of *Tween-20* for 10 - 15 min and subsequently thoroughly washed 7 or 8 times with sterilized distilled water. The sterilized buds were cultured onto Murashige and Skoog (1962) medium (MS) + 11.1 μM N^6 benzyladenine (BA) to obtain the shoots *in vitro* that were further used to study plantlet regeneration and conservation.

Shoots were excised from the 2-week-old cultures raised on MS + 11.1 μM BA and the leaves were removed. The basal shoot portion containing the shoot tip (swollen base) encircled with the whorls of leaves was cut to 1 - 2 cm above from the base and cultured on MS media supplemented with various cytokinins. Two concentrations (2.5 mg dm^{-3} and 5 mg dm^{-3}) of four cytokinins *i.e.* 11.1 μM and 22.2 μM of BA, 11.6 μM and 23.2 μM of kinetin, 11.4 μM and 22.8 μM of zeatin and 12.3 μM and 24.6 μM of γ,γ-dimethylallylaminopurine (2iP) were tested. The pH of all the media was adjusted to 5.8 with 0.1 M NaOH prior to addition of the agar-agar (7.5 g dm^{-3}) and approximately 20 cm^{-3} of the medium was dispensed into culture tubes (25 × 150 mm, *Borosil*, Mumbai, India). Each culture tube received one explant. The medium was autoclaved at 121 °C temperature and 1.06 kg cm^{-2} pressure for 20 min. Cultures were incubated at 25 ± 2 °C and an irradiance of 40 μmol m^{-2} s^{-1} provide by cool white flourescent lamps (*Philips*, Mumbai, India) at a 16-h photoperiod. All the chemicals used were of analytical grade (*Hi-media*, Mumbai, India and *Sigma*, St Louis, USA).

Some regenerated shoots were subcultured when the leaves showed chlorosis but the basal shoot portion containing shoot tip was green. The period from the initiation of cultures to next subculture was considered as conservation period for a given culture. Remaining plantlets were hardened for 2 weeks in plastic pots (diameter 15 cm) containing a mixture of horticultural grade pelrite : Irish peat moss : vermiculite (1:1:1) (*Kelpelrite*, Bangalore, India) moistened with ¼ MS major salt solution. The plantlets were kept at 25 ± 2 °C covered for 1 - 2 weeks to maintain high humidity (75 - 85 %) and subsequently exposed to *ex vitro* conditions. The 3-week-old hardened plantlets were transferred to soil in earthen pots (diameter 30 cm) and allowed to grow in net house.

Each treatment comprised 18 cultures and repeated 3 times. Thus, data were recorded at a periodic interval of 30 d and presented as mean value of 54 cultures for number of shoots per culture and conservation period. The experiments were conducted in completely randomized block design. Data were subjected to analysis of variance and Duncan's Multiple Range Test (DMRT, Gomez and Gomez 1984) to test the significance of differences.

Rhizome buds started sprouting within a period of 6 - 8 d. Two or three shoot buds were initiated from a single rhizome bud explant, cultured on MS + 11.1 μM BA. Initially, 40 - 45 % healthy cultures were established; remaining contaminated cultures were re-sterilized twice and re-cultured onto fresh media to obtain *ca*. 55 % healthy cultures. In 2 weeks, 1 - 2 cm long shoots were obtained on the same medium, which were used for further regeneration and conservation studies.

Table 1. Shoot regeneration in wild species of *Curcuma*; number of shoots per culture on various cytokinin-supplemented MS media. Data recorded after 30 d from culture. Values with different letters in the same column are significantly different at 1 % level using DMRT. *F* values are highly significant.

Species	BA [μM]		Kinetin [μM]		Zeatin [μM]		2iP [μM]	
	11.1	22.2	11.6	23.2	11.4	22.8	12.3	24.6
C. aeruginosa	3.0^{g}	3.0^{g}	2.3^{g}	2.5^{g}	3.2^{fg}	3.3^{d}	2.3^{gh}	2.6^{d}
C. aromatica	3.7^{cd}	5.4^{cd}	4.1^{a}	3.6^{c}	4.4^{c}	2.6^{ef}	2.6^{ef}	3.1^{b}
C. brog	3.8^{cd}	5.2^{cd}	3.3^{bc}	3.4^{de}	3.8^{ed}	4.8^{b}	2.8^{ef}	4.1^{a}
C. caesia	3.5^{e}	3.6^{fg}	2.6^{ef}	2.5^{f}	1.3^{h}	1.5^{g}	2.2^{gh}	2.2^{e}
C. malabarica	5.8^{ab}	5.7^{ab}	2.7^{ef}	4.0^{b}	7.2^{a}	4.3^{c}	5.0^{a}	4.1^{a}
C. raktakanta	3.8^{cd}	3.7^{fg}	2.9^{d}	3.3^{ed}	3.1^{fg}	2.5^{ef}	3.6^{bc}	1.7^{f}
C. soloensis	3.3^{f}	4.0^{c}	3.1^{cb}	5.0^{a}	5.0^{b}	3.3^{d}	3.5^{bc}	2.8^{c}
C. sp.	5.9^{ab}	5.6^{ab}	2.7^{ef}	2.5^{f}	3.9^{ed}	5.7^{a}	3.1^{d}	4.1^{a}
F	20.19	20.23	21.83	19.85	22.81	20.8	18.85	21.8
SE	0.12	0.15	0.08	0.11	0.13	0.10	0.09	0.03
Variance [%]	3.52	6.12	4.56	5.52	4.98	5.05	4.02	3.58

Table 2. Effects of four cytokinins (at two concentrations) on *in vitro* conservation (number of days) of wild species of *Curcuma.*

Species	BA [μM]		Kinetin [μM]		Zeatin [μM]		2iP [μM]	
	11.1	22.2	11.6	23.2	11.4	22.8	12.3	24.6
C. aeruginosa	311	281	289	306	312	279	324	296
C. aromatica	307	286	331	314	342	363	299	294
C. brog	280	280	273	270	267	269	271	264
C. caesia	287	292	295	323	318	274	305	301
C. malabarica	280	290	290	285	296	284	291	277
C. raktakanta	288	286	294	298	316	286	291	318
C. soloensis	296	304	272	292	290	291	311	304
C. sp.	304	280	300	366	305	308	323	379

All the cytokinins at both concentrations used, induced the shoot buds from the basal swollen portion in 100 % explants of all the species tested. However, the quantitative response for shoot bud formation was different in various species (Table 1). In 3-week-old cultures, the shoot buds developed into small shoots (1 - 2 cm) along with formation of new shoot buds on swollen base. No further increment was observed in number of shoots after 4 - 5 weeks from culture. Shoot regeneration followed the distinct pattern for each species. A significant difference was observed with regard to shoot regeneration in tested species and cytokinins. Considering all the species together, the mean number of shoots per culture ranged from 1.3 to 7.2 (Table 1). *C. malabarica* produced the highest number of shoots (7.2 shoots per culture) on MS + 11.4 μM zeatin. In *C. aeruginosa*, *C. aromatica*, *C. brog*, *C. caesia*, *C. raktakanta* and *C.* sp. maximum shoot regeneration potential was expressed on MS + 11.1 μM BA. Superiority of BA for shoot multiplication of *C. longa* (Balachandran *et al.* 1990, Tyagi *et al.* 1998) and *C. aromatica* (Nayak 2000) has been well documented in literature. Efficacy of 2iP for shoot regeneration in *C. longa* is reported by Salvi *et al.* (2002) but in our studies none of wild species produced higher number of shoots per culture on medium containing 2iP than that on other cytokinin-supplemented media. No definite pattern was observed for the length of shoot of the cultures raised on various cytokinin-supplemented media. However, highest shoot length (5.6 cm) was recorded in *C. brog* on MS + 11.6 μM kinetin closely followed by *C. aromatica* (4.8 cm) on MS + 11.1 μM BA.

Table 3. Optimum cytokinin concentration in MS media for shoot regeneration (maximum number of shoots per culture) and conservation (maximum period in d) for wild species of *Curcuma.* Data for shoot regeneration were recorded after 30 d from culture.

Species	Shoot regeneration	Conservation
C. aeruginosa	11.4 μM or 22.8 μM zeatin (3.3)	12.3 μM 2iP (324)
C. aromatica	22.2 μM BA (5.4)	22.8 μM zeatin (363)
C. brog	22.2 μM BA (5.2)	11.1 or 22.2 μM BA (273)
C. caesia	11.1 μM or 22.2 μM BA (3.6)	23.2 μM kinetin (323)
C. malabarica	11.4 μM zeatin (7.2)	11.1μM or 22.2 μM BA (296)
C. raktakanta	11.1 μM BA (3.8)	24.6 μM 2iP (316)
C. soloensis	11.4 μM zeatin or 23.2 μM Kn (4.3)	12.3 μM 2iP (311)
C. sp.	11.1 μM BA (5.9)	24.6 μM 2iP (379)

Rooting has not been a constraint to develop the shoots into plantlets. Development of roots was observed in all 4-week-old cultures of *Curcuma* species on all the media tested. No attempt was made to record the data on precise number of roots per shoot, as origin of roots from individual shoot was indistinguishable in culture form. However, 2 - 4 thick roots per shoot were observed in the plants transferred to soil. As high as 96 - 100 % of the hardened plants of each species survived transplantation to the soil which is in agreement of the observations of Salvi *et al.* (2002). The tissue culture-raised plants resembled to their parents phenotypically. The genetic stability of the regenerants was studied using 8 isozymes and RAPDs analyses, and no variation was detected in *in vitro*-conserved plants. The detail results of studies on genetic stability will be reported in a separate paper.

Each of the tested media supported the growth of all the cultures for a minimum of 270 d. Amongst the eight wild species of *Curcuma* under study, *C.* sp. was adjudged the best storer; it could be conserved up to 379 d on MS + 24.6 μM 2iP followed by *C. aromatica* for 363 d on MS + 22.8 μM zeatin (Table 2). The data on shoot regeneration and conservation period revealed that the optimum medium for shoot regeneration and conservation is not necessarily the same. In none of the species except *C. brog*, did the same cytokinin or its concentration favour both shoot regeneration and conservation (Table 3). Kinetin and 2iP were not suitable to yield the highest number of shoots per culture in any species, but kinetin was found optimum for conservation of shoot cultures of *C. caesia* and 2iP for *C. aeruginosa*,

C. raktakanta, *C. soloensis* and *C.* sp. (Tables 2, 3). Chandel and Pandey (1992) reported the efficacy of 2iP in conserving the shoot cultures of various *Allium* species for 14 - 23 months.

The simple and reproducible protocols for plantlet regeneration and conservation have been optimized for eight wild species of *Curcuma*. Following these protocols, *ca.* 20 cultures of each species are conserved for about 3 years in the *In Vitro* Genebank at NBPGR, New Delhi. *In vitro* conservation of wild species of *Curcuma* for extended periods under conditions as described above may allow cost-efficient plant regeneration and medium-term conservation. Plants that have been conserved for 2 years, rapidly multiplied in tissue culture have been transferred to soil in earthen pots kept in net house. These grew as normal plants showing morphological similarity to their mother plants, and produced normal rhizomes. Future efforts are focused to develop the protocol for shoot tip cryopreservation for long-term conservation.

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