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BRIEF COMMUNICATION

Plant regeneration from callus culture of *Curcuma aromatica* and *in vitro* detection of somaclonal variation through cytophotometric analysis

S. MOHANTY, M.K. PANDA, E. SUBUDHI and S. NAYAK*

Centre of Biotechnology, Siksha 'O' Anusandhan University, Bhubaneswar, 751030, Orissa, India

Abstract

Callus cultures initiated from shoot base explants of *Curcuma aromatica* Salisb. were maintained on Murashige and Skoog (MS) media supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid alone or with 0.5 mg dm^{-3} kinetin. Plantlets were regenerated from 60 and 180-d-old callus on MS media supplemented with 3 mg dm^{-3} benzyladenine and 0.5 mg dm^{-3} α-naphthalene acetic acid. Approximately 8 - 10 plantlets were produced after 30 - 40 d of culture per 50 mg of callus inoculated. Out of 113 regenerants analyzed 85 plants were exclusively diploid and 28 were predominantly diploid revealing presence of polyploid nuclei. Frequency of polyploid cells were more in regenerants obtained from 180-d-old callus then from 6-d-old callus which might be attributed to the ageing of callus.

Additional key words: DNA content, tissue culture, wild turmeric.

Curcuma aromatica Salisb., commonly known as wild turmeric, is an under exploited medicinal plant of family *Zingiberaceae*. Vegetative modes of propagation and absence of seed setting in *C. aromatica* has prevented the production of new and improved cultivars through conventional plant breeding. An alternate method for creating improved genotypes through selection of somaclonal variation has been reported in many species by several workers (Larkins and Scowcroft 1981, Mathur *et al.* 1988, Al-Zahim *et al.* 1999, Nayak *et al.* 2003). *In vitro* detection of somaclonal variation through cytophotometric analysis and flow cytometric analysis has been reported previously in other species (Nayak and Sen 1993, 1997, Sliwinska and Thien 2007). Limited information is available on *C. aromatica* callus initiation, microrhizome induction and regeneration through shoot multiplication (Yasuda *et al.* 1988, Nayak 2000, Tyagi *et al.* 2004). Therefore attempt has been taken in the present work for plant regeneration from callus culture of *C. aromatica* and *in vitro* detection of somaclonal variation through cytophotometric analysis.

Shoot base explants of about 0.7 cm excised from freshly sprouted shoots of rhizomes of *C. aromatica* were washed thoroughly with tap water and were cleaned with *Extran*, a liquid detergent solution, and surface sterilized in 0.1 % (m/v) $HgCl_2$ solution for 10 - 12 min. It was then rinsed 3 - 4 times with sterile distilled water, prior to inoculation on MS medium containing various types of plant growth regulators. Murashige and Skoogs (1962; MS) basal media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 - 5 mg dm^{-3}) and α-naphthalene acetic acid (NAA; 0.1 -3 mg dm^{-3}), benzyladenine (BA; 0.5 - 3 mg dm^{-3}) and kinetin (KIN; 0.5 - 3 mg dm^{-3}) singly and in various combinations were used for callus induction. One explant was inoculated per tube and 15 replications were maintained for each set. All media were supplemented with 30 g dm^{-3} of sucrose, 0.8 % (m/v) of agar and pH was adjusted to 5.7 before autoclaving at 121° C and 1.05 kg cm^{-2} for 20 min. Cultures were kept under white fluorescent tubes providing irradiance of 50 μmol m^{-2} s^{-1} for 16-h photoperiod. Temperature of culture room was maintained at 25 ± 2 ºC. Calli from different hormonal sets were subcultured at 30 - 35 d interval. Growth rate of callus was determined on fresh mass basis and calli showing more than three fold increase in fresh mass after 30 d were

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Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; KIN - kinetin; MS medium - Murashige and Skoog (1962) medium; NAA - α-naphthalene acetic acid.

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* Corresponding author; e-mail: sanghamitran@yahoo.com

selected and maintained further for plant regeneration.

Shoot buds were regenerated from callus inoculated on MS media containing various combinations of BA (2 - 5 mg dm^{-3}), NAA (0.1 - 1 mg dm^{-3}), KIN (0.1 - 1 mg dm^{-3}) and indole-3-acetic acid (IAA; 0.1 - 1 mg dm^{-3}) after 60 and 180 d. Cultures were grown under environmental conditions mentioned above. After 45 d, data on average number of shoot buds per culture and length of shoots were taken from each replica. Regenerated shoots (at least 3 - 4 cm) were then separated and each one was then placed on full and half strength MS media with varying concentration of BA (1 - 5 mg dm^{-3}) fresh media for rooting and rapid multiplication. 15 replicates for each treatment were used for data recording.

In vitro grown plants of *C. aromatica,* regenerated from 60- and 180-d-old callus were subjected to cytophotometric analysis to determine the ploidy of regenerated plants. Root tips were fixed over night in 1:3 propionic acid:ethanol. This treatment was followed by hydrolysis in 1 M HCl of 60 °C for 12 min, washed in distilled water and stained in Schiff's reagent for 2 h at 14 °C and squashed with 45 % (v/v) acetic acid. The DNA content of nuclei was measured with Leitz Wetzler micro-spectrophotometer using the single wavelength (550 nm) method (Sharma and Sharma 1980). *In situ* DNA content was obtained on the basis of values of absorbance using Van't Hof's (1965) 4C nuclear DNA value of *Allium cepa* (67.1 pg) as standard. A minimum of 100 dividing cells per plant were scored for estimation of 4C nuclear DNA content.

In vitro grown plants of *C. aromatica* with well developed root and shoot system were transferred to pot and kept in green house for acclimatization. After one month, these plantlets were transferred to field. *In vitro* grown plants showing polyploid nuclei were labeled and maintained separately in field for further analysis.

Callus was induced from shoot base explants of normal field grown plants of *C. aromatica* on MS medium supplemented with different concentration of auxins alone or in combination with cytokinins. Swelling of shoot base was observed after 15 - 20 d of culture followed by initiation of callus in the subsequent 20 - 30 d on MS media in all treatments (Table 1). All the media tried have shown callus initiation but MC_2 medium containing 2 mg dm^{-3} 2,4-D showed optimum response with callus induction in 82.16 % explants, followed by MC_6 medium containing 2 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} KIN showing response in 80.2 % explants (Table 1). The positive role of 2,4-D in callus induction has been reported in *Curcuma longa* (Yasuda *et al*. 1988, Salvi *et al*. 2001, Shirgurkar *et al.* 2006). Faster proliferation of calli occurred when they were sub-cultured to fresh medium after 30 d of culture in callus induction medium. Of all the media, which were effective in callus induction in *C. aromatica*, MC_2 and MC_6 showing more than three fold proliferation rate was selected for maintenance of calli for subsequent experiment of plant regeneration. Calli were maintained for more than 6 months in MC_2 and MC_6 media. Calli grown in these media were light yellow and friable.

Table 1. Effect of different growth regulators on induction and proliferation of callus of *C. aromatica* on MS medium after 30 d. Means ± SE, *n* = 15, *P* < 0.01.

Medium	Growth regulators [mg dm^{-3}]	Callus induction [%]	Fresh mass [mg]
MC_1	2,4-D (1.0)	70.90 ± 1.31	43.3 ± 0.8
MC_2	2,4-D (2.0)	82.16 ± 1.93	66.6 ± 0.8
MC_3	2,4-D (5.0)	47.63 ± 0.97	42.3 ± 1.2
MC_4	NAA (1.0)	16.00 ± 0.64	22.3 ± 0.8
MC_5	NAA (3.0)	10.70 ± 0.89	20.6 ± 1.2
MC_6	2,4-D (2.0) + KN (0.5)	80.20 ± 1.25	64.0 ± 1.1
MC_7	2,4-D (2.0) + KN (1.0)	36.73 ± 0.89	42.3 ± 1.2
MC_8	2,4-D (2.0) + BA (0.5)	62.76 ± 1.21	20.0 ± 0.5
MC_9	2,4-D (2.0) + BA (1.0)	47.03 ± 0.66	19.0 ± 0.5
MC_{10}	2,4-D (2.0) + NAA (0.5)	66.70 ± 0.78	39.3 ± 0.6
MC_{11}	2,4-D (2.0) + NAA (1.0)	43.43 ± 0.42	46.6 ± 0.3
F value		498.7	341.7

Table 2. Effect of different growth regulators on plantlet regeneration from callus of *C. aromatica* after 30 d. Means ± SE, *n* = 15, *P* < 0.01.

Growth regulators [mg dm^{-3}]	Regeneration [%]	Number of shoots [$callus^{-1}$]
BA (3.0)	67.13 ± 0.66	7.13 ± 0.18
BA (5.0)	45.63 ± 0.84	5.73 ± 0.34
KN (3.0)	36.73 ± 0.65	2.23 ± 0.29
BA (3.0) + NAA (0.1)	77.96 ± 1.05	8.70 ± 0.26
BA (3.0) + NAA (0.5)	80.53 ± 0.83	10.13 ± 0.56
BA (5.0) + NAA (0.5)	55.53 ± 0.84	5.70 ± 0.37
BA (3.0) + KN (0.1)	51.50 ± 0.37	2.80 ± 0.17
BA (3.0) + KN (0.5)	54.60 ± 0.80	4.03 ± 0.08
BA (3.0) + IAA (0.5)	20.60 ± 0.83	3.06 ± 0.20
KN (3.0) + NAA (0.5)	32.56 ± 0.83	3.10 ± 0.26
F value	595.2	77.39

Shoot buds were induced from callus of 60-d and 180-d-old culture from MC_2 and MC_6 media when transferred to MS basal media supplemented with cytokinins and auxins of varying concentration. MS media containing different concentration of growth hormones showed different response towards regeneration (Table 2). MS basal medium containing 3 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA showed optimum response, inducing an average of 10.13 shoot buds per culture within 30 - 40 d. Multiplication rate remained unchanged in subsequent subculture. BA at higher concentration has inhibitory effect on shoot regeneration (reduced number of shoots and % of response; Table 2). Significant role of BA in shoot induction from callus of *C. aromatica* was in close agreement to other species of *Zingiberaceae* including that of *Curcuma* spp. (Balachandran *et al.*

1990, Rout and Das 2002, Tyagi *et al*. 2007). Addition of low concentrations of NAA (0.1 - 0.5 mg dm^{-3}) to media containing BA (3 mg dm^{-3}) stimulated shoot regeneration (Table 2), but NAA at higher concentrations inhibited shoot formation and only roots were formed (data not shown). Regenerated shoots attained a length of 3 - 4 cm within 30 d of culture with simultaneous formation of slender roots. These plantlets developed better root system upon transfer to MS media containing only BA (3 mg dm^{-3}). Length of shoots varied from 4 to 5 cm within 30 d. Use of BA in development of healthy root system in *C. aromatica* is in agreement with earlier reports on species of *Curcuma* (Nayak 2000, 2002)

The ploidy status of callus regenerated plants of *C. aromatica* was determined by *in situ* estimation of nuclear DNA content. Out of 113 *in vitro* grown regenerants analyzed 85 plants was exclusively diploid revealing unimodal distribution of a peak corresponding to 4C value (7.65 pg) as in control parent from field. The range of mean DNA content in root tip of these plants varied from 7.61 - 7.75 pg which was almost similar to the range obtained in the root tip of source plant which was 7.63 - 7.76 pg. Other 28 plants were predominantly diploid revealing polymodal distribution of DNA content, showing peaks at approximately 4C (7.65 pg), 8C (15.3 pg), 16C (30 pg) thereby confirming the presence of diploid, tetraploid and octaploid cells. The mean DNA content in roots of these plants varied from 7.65 - 10.25 pg. Result of cytophotometric analysis revealed, that aging of callus was more effective in induction of somaclonal variation in *C. aromatica* culture then the plant growth regulators used as evidenced by the range of frequency of polyploid cells observed in regenerated plants. It was observed that the frequency of polyploid cells in regenerants obtained from both MC_1 and MC_6 callus line was always higher (16 - 35 %) in plant regenerated from 180-d-old callus than the plants derived from 60-d-old callus with 1 - 6 % of polyploid cells. The higher frequency of polyploid cells could be attributed to aging of callus as has been reported in other species (Cassells and Morrish 1987, Nayak and Sen 1997).

Differential frequency of polyploid cells was also induced by different plant growth regulators in media. In *C. aromatica* plantlets developed from 60-d-old callus on MC_1 medium containing 2,4-D (2 mg dm^{-3}) showed a maximum polyploid cells (6 %), whereas those on MC_6 (2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} KIN) only 2 % polyploid cells. Similarly in regenerants derived from 180-d-old culture, plantlets on MC_1 showed 35 % polyploid cell whereas those on MC_6 up to 20 %. Thus 2,4-D used singly could induced more polyploid cell than when combined with KIN. The role of 2,4-D in induction of genetic instability *in vitro* has been reported in many species (Kar and Sen 1985, Nayak and Sen 1993).

Callus derived *in vitro* grown plantlets of *C. aromatica* were transferred to pots containing sterilized soil. Regenerated plants which were detected *in vitro as* predominantly diploid in nature containing variable amount of polyploid cells were labeled and maintained separately. All the plants were then kept in greenhouse for acclimatization. After one month these were transferred to normal field and grown to maturity. About 95 % plants survive when transferred to field. Presently micropropagated plants are under evaluation in the field condition for various qualitative attributes for selecting improved genotype of *C. aromatica*. Thus the procedure outline above has got enough significance in enhancing the spectrum of variation through callus culture for selecting plants with new genetic make up with improved qualitative traits in *C. aromatica.*

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