BIOLOGIA PLANTARUM 55 (3): 554-558, 2011

BRIEF COMMUNICATION

High frequency plant regeneration from the mature seeds of *Garcinia indica*

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Abstract

A high frequency plant regeneration system was developed for the production of high yielding elite clones of *Garcinia indica via* direct organogenesis. A maximum number of 86.2 shoot buds per explant were induced from the mature seed segments cultured on Woody plant medium (WPM) supplemented with 2.0 mg dm^{-3} N^6-benzyladenine and 1 mg dm^{-3} indole-3-acetic acid. Rooting was achieved on half-strength WPM medium supplemented with 3 mg dm^{-3} indole-3-butyric acid. Rooted plantlets were acclimatized and transferred to greenhouse for further growth. The highest survival rate of 95 % was recorded using a mixture of garden soil and sand. Histological studies clearly revealed multiple shoot formation from mature seed segments.

Additional key words: direct organogenesis, kokum elite clones, mass multiplication, multiple shoots.

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Garcinia indica Choisy, an endemic plant to Western Ghats of India, belongs to the family *Clusiaceae* and is popularly known as kokum (Shetty and Kaveriappa 2001). The fruit rind contains bioactive compounds of medicinal importance such as hydroxycitric acid (HCA) and garcinol (Jena *et al*. 2002, Jayaprakasha and Sakariah 2002).

Though *in vitro* shoot regeneration of kokum using apical buds (Mathew *et al*. 2001), immature seeds, young leaves, apical and axillary buds (Kulkarni and Deodhar 2002, Thengane *et al*. 2006), *in vitro* root explants (Deodhar *et al*. 2008) and matured seeds (Malik *et al*. 2005) were reported earlier, the average number of shoots formed ranged from 2 to 58 per explant. In the present study we have developed and standardized an efficient protocol for high frequency plant regeneration from the mature seed segments of *Garcinia indica* for the production of elite clones.

Ripened fruits were collected from the trees with high yielding, large fruit size and high acid taste of *Garcinia indica* Choisy during April - June from the Western Ghats of Goa, India. The fruits were washed thoroughly with clean tap water, followed by quick dip in 70 % ethanol. Seeds were separated and then seed coat was removed. Further steps were carried out under sterile condition in a laminar air-flow chamber. The mature seeds were washed with sterile distilled water, followed by rinsing with 70 % (v/v) ethanol for 2 min. The seeds were then surface sterilized with 0.1 % mercuric chloride for 1 min and rinsed with sterile distilled water 3 - 4 times. Each seed was cut into three pieces (endosperm) and used as explants. The seed segments were cultured on Woody plant medium (WPM; Lloyd and McCown 1980) supplemented with N^6-benzyladenine (BA; 1.0, 2.0, 3.0, 4.0, 4.5 and 5.0 mg dm^{-3}) or in combinations with indole-3-acetic acid (IAA; 1.0 mg dm^{-3}), α-naphthalene acetic acid (NAA; 1.0 mg dm^{-3}) and indole-3-butyric acid (IBA; 1.0 mg dm^{-3}). The medium was fortified with 3 % sucrose and 0.75 % agar. The pH was adjusted to 5.7 ± 0.2 with 0.1 M HCl and 0.1 M NaOH. IAA was filter sterilized and added to autoclaved media. For shoot elongation, multiple shoot buds were cultured on WPM

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Received 8 November 2009, *accepted* 31 March 2010.

Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthalene acetic acid; PGR - plant growth regulator; WPM - woody plant medium.

Acknowledgements: Authors acknowledge the financial support provided by the Council of Scientific and Industrial Research (CSIR) (No.38(1168)/07/EMR-II), New Delhi, India and the University Grants Commission (UGC), New Delhi, India, under Special Assistance Programme (SAP) to carry out the above research work.

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supplemented with 1.0 mg dm^{-3} BA and 0.1 % activated charcoal. Induction of roots were standardized using half-strength WPM medium containing 1.5 % sucrose and different concentration of IBA and NAA (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg dm^{-3}). The cultures were incubated at 25 ± 2 ºC, a relative humidity of 50 - 60 % and 16-h photoperiod (50 µmol m^{-2} s^{-1}; cool white fluorescent tubes). For acclimatization, healthy plantlets were transferred to a mixture of equal volume of garden soil and sand. Plants were grown under high relative humidity in a growth room for two weeks and then they were moved to a greenhouse. The data were analyzed by using *WASP-2.0*. Ten replications were maintained for each treatment and each experiment was repeated three times. Observations were made every week. For histological studies, thin transverse sections of 8 - 10 µm were obtained using cryo-microtome (*Leica CM1800*, *Leica Instruments*, Nussloch, Germany) at the temperature of -6 ± 1 °C and stained with either 1 % safranin or 0.1 % toluidine blue for 5 min. The sections were mounted in dilute glycerin (5 %) and photographed using *Nikon E800* microscope attached with *Nikon Coolpix4500* digital camera (*Nikon*, Tokyo, Japan).

Seed segments showed prominent swelling after one week of culture in WPM supplemented with various plant growth regulators (Fig. 1*A*, Table 1). Induction of multiple shoot primordia was noticed on all over the surface of explants after 2 - 3 weeks (Fig. 1*B*). After one month of culture the shoot primordia differentiated into shoot buds. Malik *et al*. (2005) observed similar response of swelling of explants along with shoot bud primordia in MS medium supplemented with plant growth regulators. Direct somatic embryogenesis using immature seeds revealed the initiation of small protuberances after 2 - 3 weeks of culture (Thengane *et al*. 2006).

The frequency of shoot buds induction ranged from 20 to 100 % in the media supplemented with BA alone. Maximum number of shoot buds (27 per explant) was observed in 4.5 mg dm^{-3} of BA. Increasing the concentration of BA in the media greatly increased the percentage of shoot bud induction (Table 1). Similar results were also reported in *Garcinia indica* (Malik *et al*. 2005), *Phaseolus vulgaris* (Dang and Wei 2009) and *Citrus volkameriana* (Tavano *et al*. 2009). Maximum shoot bud length of 5.6 mm was observed in presence of 1 mg dm^{-3} BA. Similarly, maximum length of shoot buds with lower concentration of BA was reported by Malik *et al*. (2005). An increase in the concentration of BA beyond the optimum (4.5 mg dm^{-3}) did not affect shoot bud induction significantly, but the buds appeared to be developmentally suppressed and did not grow further. Similar responses of reduction in number of shoots were observed with high concentrations of BA in *Garcinia indica* (Malik *et al*. 2005), *Garcinia mangostana* (Goh *et al*. 1990) and *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu 2009). Stunted growth of shoot buds was also reported in *Garcinia mangostana* in presence of higher concentration of BA (Goh *et al*. 1990).

Table 1. Influence of plant growth regulators [mg dm^{-3}] on induction of multiple shoot buds in *Garcinia indica*. Regeneration frequency [%], shoot number [$explant^{-1}$] and shoot length [mm] were measured. Means ± SE, n = 10; means followed by the same letter were not significantly different at $P < 0.05$. No buds were induced in basal medium.

BA	IAA	NAA	IBA	Regen.	Shoot number	Shoot length
1.0				20	06.0 ± 0.81^{s}	5.6 ± 0.51^{a}
2.0				80	10.0 ± 0.66^{r}	5.1 ± 0.73^{a}
3.0				100	17.0 ± 0.87^{l}	4.4 ± 0.51^{b}
4.0				100	21.0 ± 0.78^{k}	3.5 ± 0.52^{de}
4.5				100	27.0 ± 0.87^{i}	3.6 ± 0.51^{cd}
5.0				100	16.0 ± 0.66^{m}	2.1 ± 0.56^{i}
1.0	1.0			40	13.0 ± 0.67^{n}	3.0 ± 0.66^{efg}
2.0	1.0			100	86.2 ± 0.78^{a}	2.1 ± 0.59^{i}
3.0	1.0			100	47.2 ± 0.63^{c}	2.4 ± 0.69^{hi}
4.0	1.0			100	27.2 ± 0.63^{i}	3.2 ± 0.63^{def}
4.5	1.0			100	22.2 ± 0.56^{j}	3.2 ± 0.63^{def}
1.0		1.0		50	11.0 ± 0.81^{q}	4.1 ± 0.56^{bc}
2.0		1.0		100	35.0 ± 0.66^{g}	3.2 ± 0.63^{def}
3.0		1.0		100	38.5 ± 0.52^{e}	3.1 ± 0.87^{def}
4.0		1.0		100	42.5 ± 0.52^{d}	2.9 ± 0.87^{fgh}
4.5		1.0		100	61.2 ± 0.78^{b}	2.1 ± 0.56^{i}
1.0			1.0	80	12.5 ± 0.52^{op}	3.4 ± 0.51^{def}
2.0			1.0	100	30.0 ± 0.81^{h}	2.5 ± 0.52^{ghi}
3.0			1.0	100	35.7 ± 0.48^{f}	2.2 ± 0.42^{i}
4.0			1.0	100	38.5 ± 0.70^{e}	3.4 ± 0.51^{def}
4.5			1.0	100	42.7 ± 0.48^{d}	3.2 ± 0.42^{def}

BA in combination with auxins was found to be effective for shoot bud induction; however, it took one week more for the induction of shoot buds in comparison with BA alone. Among the BA and IAA combinations, 2 mg dm^{-3} BA with 1 mg dm^{-3} IAA produced highest number of shoots (86.2 per explant) with 100 % regeneration (Fig. 1*E*). Parimalan *et al*. (2009) reported that BA and IAA combinations produced high number of shoots in *Bixa orellana*. BA (4.5 mg dm^{-3}) in conjugation with NAA (1.0 mg dm^{-3}) also produced 61.2 shoot buds per explant (Fig. 1*C*,*D*). Dai *et al*. (2009) observed similar response in *Brassica oleracea*. The synergistic effect of BA and NAA on shoot formation using seed explants has been reported in *Garcinia mangostana* (Sirchl *et al*. 2008). BA and NAA combination produced 20 - 40 % callus along with shoot bud formation similarly as Malik *et al*. (2005) reported in *Garcinia indica*. BA in combination with IBA was less effective in shoot bud induction when compared to BA + IAA and BA + NAA (Table 1).

Induced shoot buds were cultured on WPM supplemented with 1.0 mg dm^{-3} BA and 0.1 %

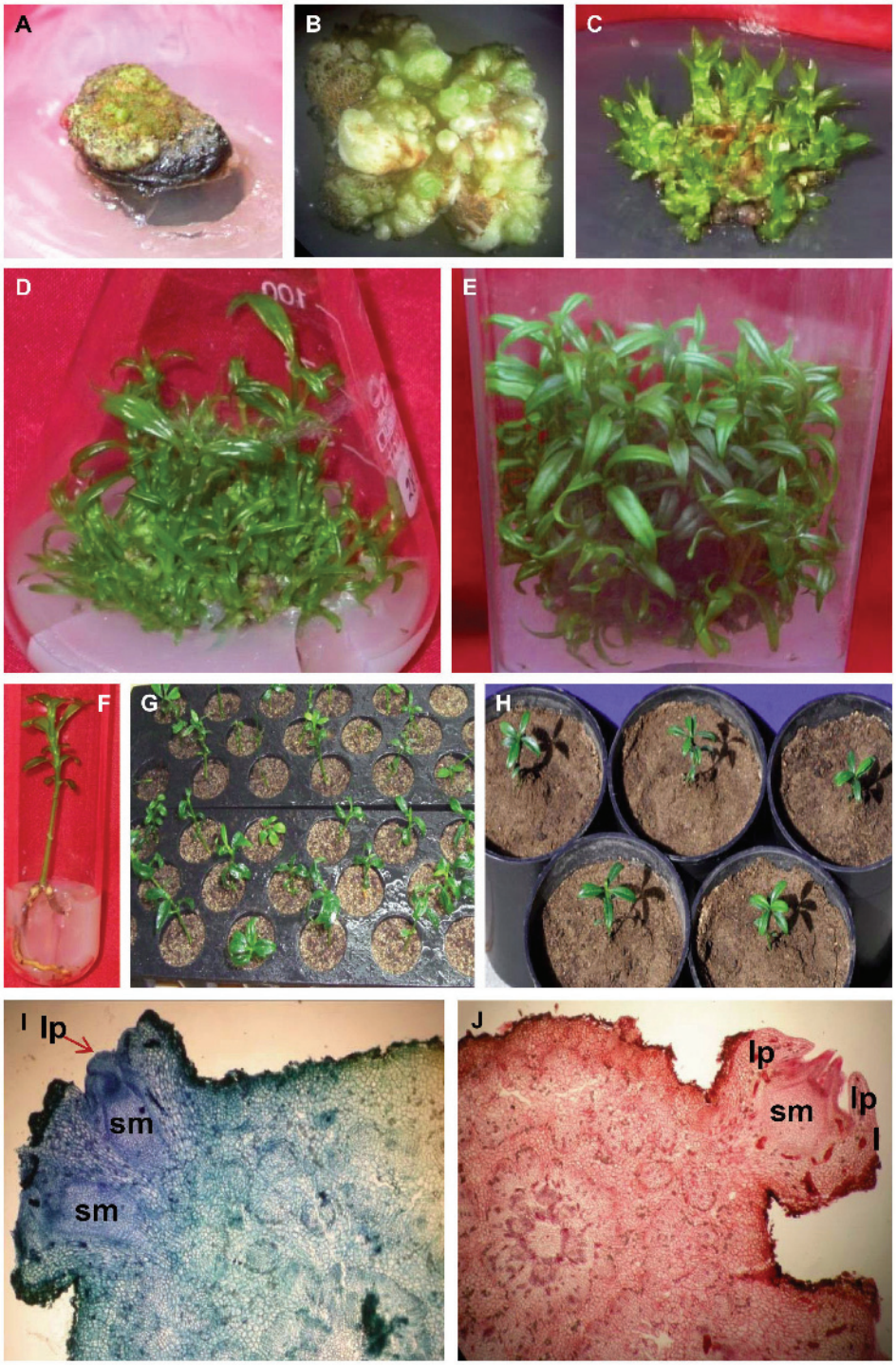

Fig. 1. Plant regeneration from mature seed segments of *Garcinia indica*: *A* - swelling of explants after one week of culture; *B* - initiation of shoot buds after 2 weeks of culture on WPM supplemented with 2 mg dm^{-3} BA and 1 mg dm^{-3} IAA; *C* - early stages of multiple shoot formation on WPM supplemented with 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 40 d of culture; *D* - multiple shoots developed on WPM 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 60 d of culture; *E* - multiple shoots developed on WPM 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 90 d of culture; *F* - rooting in half-strength WPM supplemented with 3 mg dm^{-3} IBA; *G* - hardened plants transferred to seed trays; *H* - after 15 d of growth in seed tray plantlets transplanted into large plastic pots for further growth in a greenhouse; *I* - transverse section of seed segment after two weeks of culture stained with toluidine blue showing early stage of development of shoot meristem (sm) and leaf primordium (lp) (× 20); *J* - transverse section of seed segment after 3 weeks of culture stained with safranin showing well developed shoot meristem and leaf primordium (× 20).

Table 2. Effect of IBA and NAA on rooting of *Garcinia indica*. Means ± SE, $n = 10$; means followed by the same letter were not significantly different at $P < 0.05$. No rooting in basal medium.

IBA [mg dm^{-3}]	NAA [mg dm^{-3}]	Frequency [%]	Number [shoot^{-1}]	Length [cm]
0.5		30	1.00 ± 0.06^{d}	0.60 ± 0.04^{e}
1.0		70	1.28 ± 0.42^{cd}	3.14 ± 0.31^{b}
2.0		70	1.42 ± 0.51^{bc}	3.57 ± 0.52^{a}
3.0		100	2.80 ± 0.42^{a}	3.65 ± 0.51^{a}
4.0		100	1.70 ± 0.48^{b}	1.75 ± 0.48^{d}
5.0		80	1.50 ± 0.52^{bc}	2.58 ± 0.52^{c}
	0.5	60	2.00 ± 0.66^{d}	1.20 ± 0.12^{d}
	1.0	60	2.00 ± 0.47^{d}	1.81 ± 0.11^{a}
	2.0	70	2.10 ± 0.56^{d}	1.83 ± 0.09^{a}
	3.0	80	3.80 ± 1.13^{b}	1.21 ± 0.15^{d}
	4.0	80	5.00 ± 0.47^{a}	1.33 ± 0.08^{c}
	5.0	90	2.90 ± 0.73^{c}	1.60 ± 0.09^{b}

activated charcoal for better shoot growth and elongation. Two weeks later, the elongated shoots of 3 - 5 cm were cut at basal region and placed on rooting media. The smaller shoots were again cultured on elongation medium. Kalia *et al*. (2007) and Li *et al*. (2009) reported that removal of the elongated shoots at regular intervals was necessary to allow the elongation of smaller shoot buds.

Rooting was achieved in all the combinations of IBA and NAA within a period of 2 - 3 weeks (Table 2). IBA induced the highest frequency of rooting (100 %) at 3 and 4 mg dm^{-3} when compared to NAA. However, highest number of roots (5 per explant) was observed at 4 mg dm^{-3} NAA after 15 d. Among the IBA and NAA concentrations, maximum length of the roots (3.65 cm) was recorded in 3 mg dm^{-3} IBA (Fig. 1*F*). Even though the number of roots produced by NAA was higher, the roots were stunted, swollen, greenish white and did not support the acclimatization process as compared to thick white roots produced by IBA. Shoots cultured on media without IBA and NAA did not develop any roots. Similar responses of root induction by IBA and NAA were reported in *Garcinia indica* (Malik *et al*. 2005, Thengane *et al*. 2006, Deodhar *et al*. 2008). In contrast to the above, half-strength WPM with 2.5 - 245 μM IBA did not induce any rooting in *Garcinia indica* (Chabukswar *et al*. 2006). It was reported that IBA promotes better root induction in plants such as *Azadirachta indica* (Srivastava *et al*. 2009), *Acanthophyllum sordidum* (Meratan *et al*. 2009), *Bixa orellana* (Parimalan *et al*. 2009) and *Plantago algarbiensis* (Goncalves *et al*. 2009).

For hardening, the rooted plantlets were transferred to culture tubes containing quarter-strength liquid WPM without sucrose for 10 - 12 d. The plantlets with healthy roots that were transplanted into a mixture of equal volume of garden soil and sand recorded 95 % survival rate (Fig. 1*G*). Different potting mixtures such as soil, *Vermiculite* and farm yard manure (Malik *et al*. 2005), sand and soil (Thengane *et al*. 2006) and cocopeat (Deodhar *et al*. 2008) were used for acclimatization of regenerated plantlets of *Garcinia indica*. After two weeks growth, hardened plants were transplanted into large pots and moved to a greenhouse (Fig. 1*H*).

Histological studies were carried out to understand the origin and development of shoot buds. Transverse section of seed segments after one week of culture revealed the presence of numerous meristematic zones. After two to three weeks of culture, the meristematic zones developed into shoot and leaf primordium (Fig. 1*I,J*).

In the present study we have developed and standardized an efficient protocol for high frequency plant regeneration from mature seed segments of *Garcinia indica*. This protocol will be useful for the production of elite clones for large scale cultivation and genetic manipulation to enhance the bioactive compounds in future.

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