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SHORT COMMUNICATION

IN VITRO REGENERATION OF *GARCINIA INDICA* USING LEAF EXPLANTS

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Regeneration of plantlets via adventitious bud differentiation on leaf explants of *Garcinia indica* (Thouars) Choisy, a medicinally important apomictic tropical tree species was done. Leaves were cultured on Murashige and Skoog's medium supplemented with cytokinins (6-Benzylaminopurine, BAP, kinetin and Thidiazuron, TDZ) alone and in combination with auxin (α-Napthaleneacetic acid, NAA) for direct shoot proliferation. Maximum number of shoots were formed in 63.89% explants on MS medium supplemented with 1.0 µM TDZ after 2-4 weeks of culture. Elongation of the induced shoots was achieved on MS basal medium containing 0.2% activated charcoal. Maximum rooting (83.33%) was observed in shoots cultured on half-strength MS medium supplemented with 10 µM IBA. The plantlets were successfully transferred to soil after hardening.

Key words: **Adventitious buds, apomictic seeds, *Garcinia indica*, leaf explants, micropropagation**

Garcinia indica (Thouars) Choisy (Clusiaceae) commonly known as 'kokum' is a fruit tree found in tropical rain forests of Western Ghats of India. It has high economic value as the seeds yield valuable edible fat known in commerce as 'kokum butter' (Wealth of India 1980). The fruit rind is rich in (-) Hydroxycitric acid (HCA), an important biologically active plant metabolite used as an anti-obesity drug which accelerates fat burning and inhibits fatty acid synthesis by inhibition of ATP citrate lyase, an important enzyme in Kreb's cycle (Heymsfield *et al.* 1998). The fruit has an agreeable flavour and a sweetish acid taste. Fresh fruits and dry rind is used in curries to give an acidic flavour and also for preparing cooling syrups during summer months (Wealth of India 1980).

The major constraint in popularizing this species as a potential horticultural crop is the dioecious nature of the tree. Male and female trees can be differentiated only at the flowering stage (7-9 years of age). The seeds are recalcitrant due to their high sensitivity to desiccation and freezing and have shelf life of only four weeks (Malik *et al.* 2005a). Thus it is not possible to raise seedlings throughout the year. Softwood grafting, the other method used for clonal propagation of *G. indica* (Nawala and Karmakar 1997) is seasonal, space requiring and cumbersome. Availability of rootstocks for grafting is also limited.

Most of the studies pertaining to *in vitro* culture of genus *Garcinia* have been conducted in *G. mangostana* using seed and leaf explants (Goh *et al.* 1990, 1994, 1997, Normah *et al.* 1992, 1995, Prakash *et al.* 1997, Te Chato and Lim 2000 and Huang *et al.* 2000). *G. indica*, which has been recognized as an important horticultural and medicinal tree species has gained limited attention. Malik *et al.* (2005b) reported plantlet regeneration from immature seeds and *in vitro* conservation of shoots. Kulkarni and Deodhar (2002) used immature seeds, apical and axillary buds for *in vitro* establishment of *G.*

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indica while Deshpande *et al.* (1999) and Mathew *et al.* (2001) conducted preliminary studies on *in vitro* establishment of kokum apical buds. However, no reports are available regarding *in vitro* multiplication of *Garcinia indica* using leaf explants.

Considering the horticultural and medicinal importance of *G. indica,* attempts were made to develop an efficient and reproducible method for *in vitro* multiplication of *Garcinia indica* using leaf explants.

Fruits of *Garcinia indica* (IC-136682-2) were procured from the orchard of NBPGR regional station Thrissur, Kerala, India and brought to the laboratory at NBPGR, New Delhi. The depulped and decoated seeds were used for *in vitro* experiments. The decoated seeds were germinated on MS (Murashige and Skoog 1962) basal medium after surface sterilization with 0.1% mercuric chloride for 12 min followed by 3-4 washings with sterile distilled water. Leaves collected from these plantlets were used for adventitious bud differentiation experiments. In addition, young leaves collected from mature trees were also used for experiments.

The leaves collected from *in vitro* raised seedlings and mature trees were cultured on media supplemented with various concentrations of cytokinins namely 6-Benzylaminopurine (BAP, 0-50.0 μM), Kinetin (0-50.0 μM) and Thidiazuron (TDZ, 0-12.5 μM) added singly. To study the interactive effect of TDZ and α-Napthaleneacetic acid (NAA), explants were cultured on MS medium supplemented with TDZ (0.1–12.5 μM) and NAA (1.0 and 2.5 μM).

The leaves with induced adventitious buds (2-7 mm) were transferred to MS basal medium supplemented with 0.2% activated charcoal for shoot elongation (2-4 sub cultures). The elongated shoots, 15 to 25 mm long were excised and transferred to either rooting or multiplication medium.

The induction and elongation media were supplemented with 3% sucrose and solidified with 0.8% agar (pH 5.8). Cultures were maintained under 16 h photoperiod of cool white fluorescent light (30 μmol m^{-2} s^{-1}) at 25 ± 2°C temperature.

The shoots (15-25 mm long) were transferred to half-strength MS medium supplemented with 5-25 μM IBA or NAA and 2% sucrose for root initiation. The well formed plantlets, after 4 weeks on rooting medium were washed thoroughly and transferred to wide mouth bottles containing quarter-strength liquid MS medium with 1% sucrose and absorbent cotton as a support for hardening. The hardened plantlets were established in pots containing a mixture of soil, vermiculite and farmyard manure (FYM) in 1:1:1 ratio.

For regeneration experiments, 12 explants per treatment were used and each experiment was repeated twice. Observations were made fortnightly and treatment effects were quantified on the basis of the number of explants showing regeneration and the number and length of regenerates per explant. The data was subjected to Analysis of Variance (ANOVA) and significant differences between means were analyzed by Duncan's Multiple Range Test (DMRT) at 5% probability level.

The leaf explants from *in vitro* raised seedlings as well as mature trees when cultured on MS basal medium showed no change in morphology even after 5 weeks of culture. Comparison of adventitious shoot regeneration response of leaf explants towards different cytokinins and their various concentrations indicated that the young leaves collected from mature trees were not responsive to any of the media combinations tested. However, the leaves obtained from *in vitro* raised seedlings developed multiple shoot primordia within 2-3 weeks on MS media supplemented with TDZ (Table 1). Loss of competence of mature explants has been attributed to progressive specialization of the tissue, which reduces the plasticity and capability of the cells to dedifferentiate (Abdullah *et al.* 1987 and Goh *et al.* 1990). No adventitious buds were formed on explants cultured on MS media supplemented with BAP or kinetin. An average of 2.67 buds developed directly without callus development on upper leaf surface in 63.89 % explants on 1.0 μM TDZ supplemented medium (Figure 1A, B). Green friable callus appeared at the base of all the cultured explants. The leaves cultured on higher TDZ concentrations (2.5 to 12.5 μM) showed 5-6 times growth in size due to swelling followed by appearance of compact, green callus on swollen leaf area and petiole bases. However, low

Table 1. Effect of TDZ on induction of adventitious buds on leaf explants of *Garcinia indica* (accession IC-136682-2) as recorded after 5 weeks of culture. Means with same superscript are not significantly different from each other at a 5% level according to Duncan's Multiple Range Test.

Cytokinin Concentration (µM)	Response (%)	Average bud number	Average bud length (mm)
Control	0.00 [d]	0.00 [c]	0.00 [b]
TDZ			
0.5	44.46 [b]	1.58 [b]	2.67 [a]
1.0	63.89 [a]	2.67 [a]	2.72 [a]
2.5	11.11 [c]	1.13 [b]	2.36 [a]
5.0	2.78 [d]	0.33 [c]	0.85 [b]
12.5	0.00 [d]	0.00 [c]	0.00 [b]
LSD ($P < 0.05$)	6.99	0.48	1.09

or no adventitious buds were formed. The dosage of cytokinin in the culture medium is known to be critical for shoot organogenesis. TDZ has been reported to be the most active non-purine cytokinin-like substance for shoot induction. It facilitates efficient micropropagation of many recalcitrant woody species at lower concentrations (Huettman and Preece 1993). Effects of supra-optimal concentrations of cytokinins as reported here were also reported in *G. mangostana* (Goh *et al.* 1990) and seed explants of *G. indica* (Malik *et al.* 2005b).

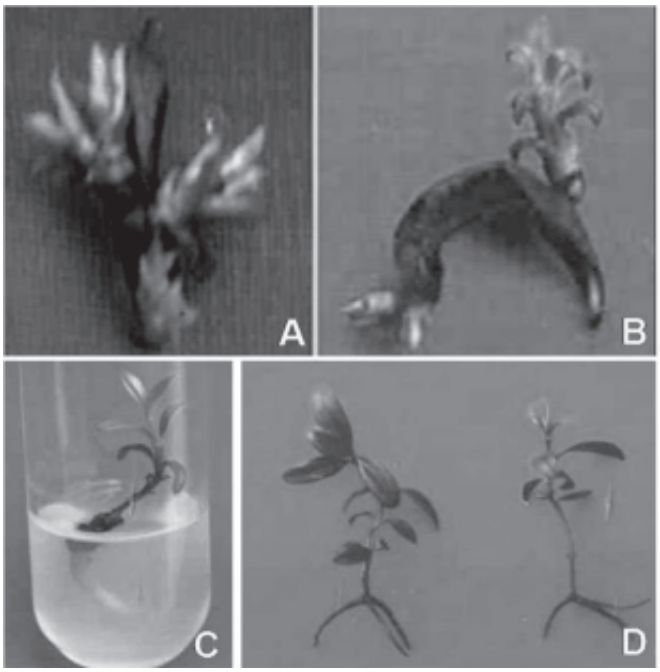

Fig. 1. Plant regeneration of *G. indica* from leaf explant. (A, B) Induction of adventitious buds on leaf lamina and midrib; (C, D) Rooting of *in vitro* raised shoots.

Addition of NAA in combination with TDZ only enhanced callusing. MS medium supplemented with 1.0 µM TDZ produced the optimal response. Incorporation of NAA in the medium encouraged callus formation as was earlier reported in *G. mangostana* (Normah *et al.* 1992 and Huang *et al.* 2000). On the contrary, positive effect of NAA in morphogenetic responses of seeds of *G. indica* was reported by Kulkarni and Deodhar (2002). The variable response of different species to auxin supplemented media may be due to different endogenous levels of auxins. Inhibition of shoot formation may be due to action of auxins accumulated at the basal end of the explants (Marks and Simpson 1994). The small shoot buds (2-7 mm) sub-cultured 2-4 times on MS basal medium supplemented with 0.2% activated charcoal attained height of 15-25 mm. Subculture of small shoot buds on hormone free MS basal medium has been previously observed in many other species including *Pinus roxburghii* (Kalia *et al.* 2007). Exposure of explants to higher TDZ concentrations during induction phase may have led to accumulation of cytokinins, which inhibited shoot bud differentiation and instead enhanced swelling and callusing of explants. Activated charcoal was added to the elongation medium as it is reported to adsorb the metabolites inhibiting morphogenesis thus supporting better growth (Thomas 2008). Direct rooting was achieved in 52.77 - 83.33 % shoots on half strength MS medium supplemented with IBA or NAA (Table 2, Fig. 1C, D). Both the auxins were most effective at the concentration of 10 µM. The roots produced in IBA supplemented media were thin and long compared to short, swollen and stunted roots produced on NAA supplemented media. All the roots were produced without an intervening callus phase on diverse culture media tested. Similar results were reported in *G. mangostana* (Goh *et al.* 1990). In contrast are the results of Kulkarni and Deodhar (2002) on *G. indica* where rooting of adventitious shoots induced on seed

Table 2. Effect of different concentrations of IBA and NAA on induction of rooting in shoots of *G. indica* (accession IC-136682-2) after 4 weeks of transfer on rooting media. Means in a column with same superscript are not significantly different from each other at a 5% level according to Duncan's Multiple Range Test.

Auxin Concentration (µM)	Response (%)	Average root number	Average root length (cm)
Control	19.44 ^{e}	1.00 ^{f}	3.43 ^{de}
IBA			
5	69.44 ^{abc}	1.37 ^{de}	4.63 ^{b}
10	83.33 ^{a}	2.54 ^{a}	5.25 ^{a}
15	75.00 ^{ab}	2.09 ^{b}	5.33 ^{a}
20	72.22 ^{abc}	1.74 ^{c}	4.09 ^{c}
25	69.44 ^{abc}	1.17 ^{ef}	3.10 ^{e}
NAA			
5	52.77 ^{d}	1.17 ^{ef}	2.57 ^{f}
10	77.77 ^{ab}	1.47 ^{cde}	3.74 ^{cd}
15	69.44 ^{abc}	1.55 ^{cd}	3.78 ^{cd}
20	66.66 ^{bc}	1.46 ^{cde}	3.08 ^{e}
25	58.33 ^{cd}	1.16 ^{ef}	2.44 ^{f}
LSD (P <0.05)	13.0	0.29	0.41

explants occurred through an intervening callus phase on NAA supplemented media.

The *in vitro* raised plantlets were delicate and required hardening before field transfer as the non-hardened plantlets did not survive when transferred to the field. In the liquid hardening medium, the root system developed and elongated further. For hardening, medium with reduced mineral salt and sucrose concentration was used so as to force the regenerants to rely on their own photosynthetic apparatus for nutrition (Kozai *et al.* 1988). The rooted plantlets were transferred to pots containing soil, vermiculite and FYM in 1:1:1 ratio. The plantlets showed 90% survival in the pots with normal growth and morphological characteristics.

The present study demonstrates that direct shoot bud regeneration can be achieved from leaves of *Garcinia indica*. Leaves are perennially available and can be used for direct shoot regeneration and micropropagation overcoming the seasonal dependence of other propagation methods like seeds and soft wood cutting. Nodal segments from *in vitro* raised shoots can be employed as propagules for further multiplication obviating the dependence on field material unlike grafting methods. The *in vitro* cultures can give a stable supply of the bioactive secondary product (HCA) as already demonstrated by Kulkarni and Deodhar (2002). The *in vitro* methods can also be employed for conservation of this recalcitrant species as demonstrated by Malik *et al.* (2005b). The meristems derived from *in vitro* cultures can also be cryopreserved to achieve long-term conservation of the species.

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