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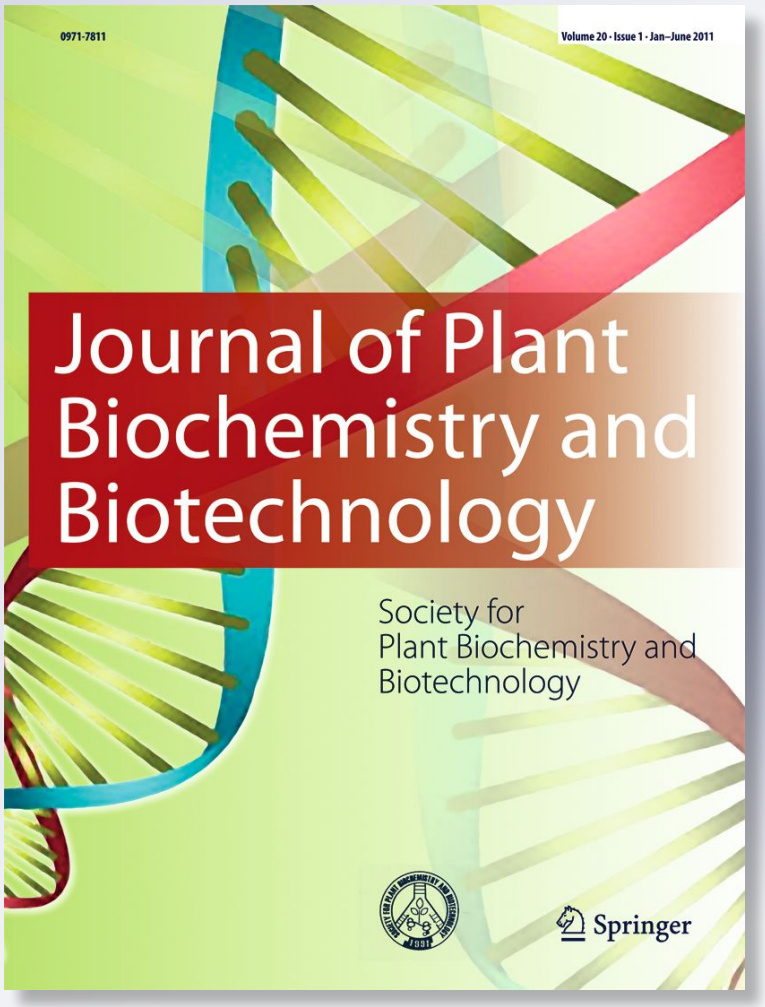

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SHORT COMMUNICATION

In vitro morphogenetic studies on three apomictic species of *Garcinia*

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Abstract Comparison of morphogenetic potential of three important Indian species of *Garcinia*—*G. indica*, *G. cambogia* and *G. xanthochymus* has been reported. Apomictic seeds of *G. indica* were found to be morphogenetically most potential followed by *G. cambogia*. The explants of *G. xanthochymus* were highly recalcitrant towards in vitro conditions and failed to induce adventitious buds on any of the media tested. High frequency direct shoot bud differentiation was induced in aseptic seed cultures of *G. indica* and *G. cambogia* on MS medium supplemented with cytokinins (BAP, kinetin or TDZ). Amongst the three cytokinins tested, TDZ (0.1–0.5 μM) was most effective for adventitious bud differentiation in both *G. indica* and *G. cambogia,* however, the proliferating buds failed to elongate. Substantial number of buds induced on BAP supplemented media elongated into shoots after subculture on elongation medium. Addition of NAA along with cytokinins in the induction medium enhanced callusing without improvement in bud induction response. The induced adventitious buds were elongated on MS basal medium containing 0.2% activated charcoal. Direct rooting was achieved in both *G. indica* and *G. cambogia* on auxin supplemented media with best response at 10 μM IBA concentration in both the species. The in vitro raised plantlets showed 90% survival in the field when transferred after hardening and acclimatization.

Keywords *Garcinia indica* · *G. cambogia* · *G. gummi-gutta* · *G. xanthochymus* · Micropropagation · Adventitious bud differentiation · Apomictic seeds

Abbreviations

BAP	6- Benzylaminopurine
IBA	Indole-3-butyric acid
MS	Murashige and Skoog's medium (1962)
NAA	α-Napthalene acetic acid
TDZ	Thidiazuron

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Genus *Garcinia* L. belonging to family Clusiaceae (syn. Guttiferae) is mostly distributed in Western and Eastern Ghats in India. Out of 30 species of *Garcinia* producing edible fruits, three important Indian species are Kokum -*G. indica* (Thouars) Choisy, Malabar tamarind—*G. cambogia* (Gaertn.) Desr. (syn. *G. gummi-gutta* (L.) Robs) and Mysore gamboge—*G. xanthochymus* Hook.f.ex T. Anderson. These three species grow in Western Ghats of India as wild and semi-domesticated species. Fruits are valued for their nutritional and medicinal properties (Wealth of India 1980). The fruit rind of *G. indica* and *G. cambogia* is rich in (−) Hydroxycitric acid (HCA), an important biologically active plant metabolite used as an anti-obesity drug (see Malik et al. 2005a). *G. indica* and *G. cambogia* are facultative agamosperms while *G. xanthochymus* in obligate agamosperm (Malik et al. 2005b). None of the three *Garcinia* species

have well defined endosperm/cotyledons and embryonic axis indicating that it is not a true seed.

Garcinia indica, G. cambogia and *G. xanthochymus* are underexploited, dioecious tropical fruit tree species with promising economic value. Differentiation between male and female trees is known only at the flowering stage (approximately after 7–9 years). The tree growth is slow; and the conventional vegetative propagation methods of softwood grafting and rooting of cuttings are not very successful (see Malik et al. 2005a). Moreover, these methods are season dependent, space requiring and cumbersome in nature thus finding limited application. Another bottleneck is the limited availability of rootstocks for grafting. Thus, propagation is usually dependent on seeds, which are highly recalcitrant in behavior and cause difficulties in producing planting stock material throughout the year (Malik et al. 2005c).

Most of the studies pertaining to in vitro culture of genus *Garcinia* have been conducted in *G. mangostana* using seed (Teo 1992; Normah et al. 1992, 1995; Huang et al. 2000) and leaf explants (Goh et al. 1990, 1994, 1997; Prakash et al. 1997; Te-Chato and Lim 1999; 2000). Recently, attention has been given to micropropagation of *G. indica* using immature seeds, young leaves, apical and axillary buds for in vitro establishment (Deshpande et al. 1999; Mathew et al. 2001; Kulkarni and Deodhar 2002; Malik et al. 2005a). However, no reports are available regarding in vitro multiplication of *G. cambogia* and *G. xanthochymus*. Thus, the present investigation was taken up to develop an efficient and reproducible method for micropropagation of *G. indica, G. cambogia* and *G. xanthochymus* using seeds and to compare their morphogenetic potential under in vitro conditions.

Mature fruits of *G. indica* (IC 136685–1) and *G. cambogia* (IC 244081–2) were collected from NBPGR regional station, Thrissur, Kerala while those of *G. xanthochymus* (IC 136680–1) were collected from Western Ghats of India. The depulped and decoated seeds were surface sterilized under aseptic conditions in laminar air flow cabinet with 0.1% mercuric chloride for 12 min followed by 3–4 washings with sterile distilled water.

Whole seeds and seed segments were used for in vitro establishment of *G. indica, G. cambogia* and *G. xanthochymus* on media supplemented with different concentrations of plant growth regulators. The apomictic seeds lacking differentiation were segmented into 4 pieces and these segments were cultured on MS medium supplemented with various concentrations of BAP, TDZ or Kinetin for adventitious bud differentiation. Seed segments were also cultured on MS medium supplemented with lower concentrations (0.1, 0.2 or 0.5 μM) of TDZ. To study the interactive effect of BAP and NAA, explants were cultured on MS medium supplemented with BAP (5–50 μM) and NAA (1.0 or 2.5 μM).

The seed segments with induced adventitious buds, after 5 weeks of culture on plant growth regulator supplemented medium, were further segmented into smaller parts and transferred to MS basal medium supplemented with 0.2% activated charcoal for shoot elongation. The well developed shoots (2–3 cm long) were excised and 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 liquid quarter-strength MS medium containing 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.

The induction and elongation media were supplemented with 3% sucrose and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving it at 15 psi and 121°C for 20 min. Cultures were maintained under 16 h photoperiod of cool white fluorescent light (30 μmol m^{-2} s^{-1}) at 25±2°C.

For all the regeneration experiments, 12 explants of each accession were used per treatment 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 adventitious buds induced in each explant. All the four segments of a single seed showed similar response; hence the data of all the segments was merged for statistical analysis. 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 morphogenetic potential of explants derived from mature trees holds great commercial value as it can be applied directly for cultivar improvement. However, in many tropical tree species in vitro establishment and multiplication using mature vegetative tissues is difficult. Seeds or seed segments have been used in species producing apomictic seeds (Huang et al. 2000; Kulkarni and Deodhar 2002; Malik et al. 2005a) for in vitro establishment as they resemble the parent plant in genetic makeup. Thus, in the three agamospermous tropical tree species of *Garcinia* viz. *G. indica, G. cambogia* and *G. xanthochymus*, apomictic seeds and seed segments, which can represent the mature trees, were the ideal explants for in vitro establishment and multiplication. The apomictic whole seeds of *Garcinia indica*, *G. cambogia* and *G. xanthochymus* (Fig. 1a–c) cultured on MS basal medium developed a shoot along with 1–3 roots from one end while only a root emerged from the other end of the seed after 2–3 weeks of culture. Apomictic seed segments (one seed sliced into four pieces) cultured on MS basal medium also developed a shoot along with roots from one end and only

roots from the other end as was recorded in whole seeds in all the three species. However, the middle segments produced only single shoots in *G. indica* and *G. cambogia*.

In the present study, wide variations in morphogenetic potential of apomictic seeds of the three *Garcinia* species were observed. Seed segments of *G. indica* were morphogenetically most potential producing a maximum of 99.75 and 54.31 buds respectively on TDZ and BAP supplemented MS media. *G. cambogia* producing and average of 66.97 and 28.89 buds respectively per seed segment had intermediate potential while the seed segments of *G. xanthochymus* which failed to produce adventitious buds on any of the media tested had least morphogenetic potential among the three species. However, major differences in morphogenic response were not observed within the segments of a single apomictic seed in *G. indica* and *G. cambogia.* Interdependence of morphogenetic potential and juvenility of explant has been reported earlier. Goh et al. (1990) reported that physiological and ontogenic state of explant affect the shoot organogenic capacity. Loss of competence of explants has been attributed to progressive specialization of the tissue, which reduces plasticity and capability of the cells to dedifferentiate.

Addition of cytokinins in the culture medium led to development of multiple shoot primordia within 2–3 weeks of culture (Fig. 1d–e), which gradually developed, into shoots in the next 2 weeks. Analysis of Variance revealed that the percent response as well as the number of adventitious buds differentiating in the species were significantly ($P<0.0001$) affected by the exogenous treatment of cytokinins. Amongst the three cytokinins tested, lower concentrations of TDZ (0.1–0.5 μM) were most effective for adventitious bud differentiation in both *G. indica* and *G. cambogia.* An average of 99.75 and 66.97 buds developed in 74.08 and 67.59% explants respectively at 0.5 μM TDZ concentration in *G. indica* and *G. cambogia* (Table 1). However, the shoot buds induced on TDZ supplemented media showed a poor bud to shoot conversion frequency (only 5–10%) due to vitrification of shoots during elongation phase. On the other hand, higher TDZ concentration of 1.0–12.5 μM led to development of callus on the whole explant surface with only 11.0 and 6.19 shoot buds developing per seed segment in *G. indica* and *G. cambogia* respectively.

Apomictic seed segments of *G. indica* produced an average of 23.98 to 54.31 buds in 87.04 to 96.30% explants on BAP (5–50 μM) supplemented MS media while those of *G. cambogia* produced 20.28 to 28.89 buds in 50.92 to 89.81% explants (Table 1). Average bud length was higher in media supplemented with 5 and 12.5 μM BAP in both the species. Further increase in BAP concentration led to clustering of shoots which were stunted and failed to elongate and their leaves did not expand even after transfer to hormone-free MS medium. Addition of kinetin at equimolar concentrations of optimal BAP levels induced fewer but longer shoot buds compared to BAP. Thus, BAP when added singly in the medium was the most effective plant growth regulator for adventitious bud differentiation indicating the cytokinin specificity of *G. indica* and *G. cambogia* seeds for multiple shoot regeneration. Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue (Zaerr and Mapes 1982). The promotive effect of BAP in inducing multiple shoots has been previously reported in *G. mangostana* (Goh et al. 1990) and *G. indica* (Malik et al. 2005a) also.

The level of BAP used in the medium significantly influenced the multiple shoot formation capacity of explants. Maximum number of shoots per explant was produced with 50 μM and 25 μM BAP concentrations in *G. indica* and *G. cambogia* respectively. However, shoot buds developing on lower BAP concentrations of 5 and 12.5 μM showed better elongation during elongation phase compared to those produced on higher BAP concentrations. Increase in BAP concentration beyond the optimal levels was found to exhibit inhibitory effect. Similar effect of BAP at supra-optimal concentrations was also reported in *G. mangostana* and *G. indica* where decrease in shoot length and aggregation of shoot buds was observed (Goh et al. 1990; Malik et al. 2005a).

Incorporation of NAA in the medium did not improve the shoot bud formation rather encouraged callus formation. Decrease in percent response as well as average bud number was registered when NAA was incorporated in the medium. However, shoot length was not significantly affected by NAA addition. Similar effect of NAA was earlier reported in *G. mangostana* (Normah et al. 1992; Huang et al. 2000) and *G. indica* (Malik et al. 2005a). Teo (1992) also reported callusing of explants with increasing concentration of NAA (from 0–2 ppm) in *G. mangostana*. On the contrary, Kulkarni and Deodhar (2002) reported a positive effect of NAA in morphogenetic responses of seeds of *G. indica*. The variable response of different genotypes and species to auxin supplemented media may be due to different endogenous levels of auxins. The inhibition of shoot formation may be due to action of auxins accumulated at the basal end of the explants.

The plant growth regulators are rarely specific in their ultimate influence on growth and development, and the responses of cells, tissues and organs in vitro can vary with cultural conditions, the type of explant and the genotype: this is because of the considerable variability existing among genera, species and even cultivars in the type and amount of hormones required for induction of morphogen-

Fig. 1 Seeds of **a** *Garcinia indica*, **b** *G. cambogia* and **c** *G. xanthochymus*. Induction of adventitious shoot buds in **d** *Garcinia indica* and **e** *G. cambogia;* **f** Development of shoot and root from respective ends of *G. xanthochymus*. **g**–**i** Rooting, hardening and field transfer of in vitro raised plantlets of *Garcinia indica*

esis. A maximum of 99.75 and 66.97 shoot buds were induced on each seed segment of *G. indica* and *G. cambogia* respectively in the present study. This number is significantly higher than the average number of shoot buds per seed as reported in *G. indica* (16.7, Kulkarni and Deodhar 2002) and *G. mangostana* (7.0, Normah et al. 1992).

Subculture of small shoot buds on hormone free MS basal medium was found essential for elongation. Segmentation of the explant into smaller pieces with 8–10 buds each enhanced the rate of bud elongation. Addition of 0.2% activated charcoal to the basal medium supported a better shoot elongation rate. Charcoal has been reported to absorb the metabolites inhibiting morphogenesis thus supporting better growth. The induction medium had a profound effect on rate of elongation of buds. The shoot buds developed on TDZ supplemented medium showed a poor percentage of bud to shoot conversion with only 5–10% buds elongating into healthy shoots and rest shoots vitrifying during the elongation phase. The shoot buds induced on BAP supplemented media showed better bud to shoot conversion frequency of 95–100%. Shoot elongation was more in explants initially cultured on lower concentrations of BAP (5–12.5 μM). The shoots elongated to a length of 2–3 cm after 2 subcultures on basal medium. However, the buds developed on higher concentrations of BAP (25–50 μM) required 4–5 subcultures for reaching a length of 2–3 cm. Similar observations were reported in *Pinus roxburghii* (Kalia et al. 2007). Continued exposure

Table 1 Effect of cytokinins on induction of adventitious buds in seed segments of *G. indica* (accession IC-136685-1), *G. cambogia* (IC-244081-2) and *G. xanthochymus* (IC 136680–1)[i] as recorded after 5 weeks of culture

Cytokinin	Concentra-tion	*G. indica*			*G. cambogia*		
		Response	Average bud number	Average bud length	Response	Average bud number	Average bud length
	(μM)	(%)		(mm)	(%)		(mm)
Control	0.0	0.00 ^{f}	0.00 ^{i}	0.00 ^{f}	0.00 ^{e}	0.00 ^{h}	0.00 ^{d}
BAP	5.0	87.04 ^{ab}	23.89 ^{g}	3.32 ^{b}	87.04 ^{a}	20.28 ^{f}	3.57 ^{a}
	12.5	94.45 ^{a}	37.39 ^{f}	2.92 ^{bc}	89.81 ^{a}	23.53 ^{ef}	3.42 ^{a}
	25.0	92.59 ^{a}	48.25 ^{de}	2.22 ^{d}	75.93 ^{b}	28.89 ^{d}	2.50 ^{b}
	37.5	94.44 ^{a}	45.22 ^{ef}	1.43 ^{e}	69.45 ^{b}	25.92 ^{de}	1.51 ^{c}
	50.0	96.30 ^{a}	54.31 ^{d}	1.03 ^{e}	50.92 ^{c}	24.89 ^{def}	1.14 ^{c}
Kinetin	12.5	63.89 ^{d}	16.50 ^{gh}	4.00 ^{a}	54.63 ^{c}	11.00 ^{g}	3.83 ^{a}
TDZ	0.1	77.78 ^{bc}	72.17 ^{c}	2.82 ^{bc}	75.93 ^{b}	44.67 ^{c}	2.65 ^{b}
	0.2	69.45 ^{cd}	85.75 ^{b}	2.89 ^{bc}	70.37 ^{b}	59.78 ^{b}	2.71 ^{b}
	0.5	74.08 ^{cd}	99.75 ^{a}	2.42 ^{cd}	67.59 ^{b}	66.97 ^{a}	2.25 ^{b}
	12.5	36.11 ^{e}	11.00 ^{h}	1.43 ^{e}	25.93 ^{d}	6.19 ^{g}	1.38 ^{c}
LSD	(P <0.05)	10.24	7.92	0.52	9.52	4.83	0.48

[i] Explants of *G. xanthochymus* were highly recalcitrant towards in vitro conditions and no adventitious bud differentiation could be achieved on any of the culture media tested

Means with same superscript are not significantly different from each other at a 5% level according to Duncan's Multiple Range Test

of explants to high BAP concentrations during induction phase seems to cause accumulation of cytokinins, which inhibit further shoot growth. The elongated shoots were excised after every subculture and the explant was cultured again on fresh basal medium for elongation of the remaining smaller shoot buds.

Occasionally, rooting was observed during the elongation phase in some shoots in *G. indica* and *G. cambogia*. Auxins had a significant (P<0.0001) influence on root forming capacity of in vitro raised shoots (Table 2). Direct rooting was achieved in 66.67 to 91.66% and 63.88 to 80.55% shoots in *G. indica* and *G. cambogia* respectively

Table 2 Effect of different concentrations of IBA and NAA on induction of rooting in shoots of *G. indica* (accession IC-136685-1) and *G. cambogia* (IC-244081-2) after 4 weeks of transfer on rooting media

Auxin	Concentra-tion	*G. indica*			*G. cambogia*		
		Response	Average root number	Average root length	Response	Average root number	Average root length
	(μM)	(%)		(cm)	(%)		(cm)
Control	0	13.88 ^{f}	1.00 ^{f}	3.50 ^{e}	8.33 ^{f}	1.00 ^{f}	3.41 ^{bc}
IBA	5	72.22 ^{bcd}	1.28 ^{ef}	3.14 ^{f}	63.88 ^{bc}	1.08 ^{ef}	3.01 ^{bcd}
	10	91.66 ^{a}	2.47 ^{a}	5.88 ^{a}	80.55 ^{a}	2.28 ^{a}	5.50 ^{a}
	15	83.33 ^{ab}	2.10 ^{b}	5.16 ^{b}	75.00 ^{ab}	2.06 ^{ab}	3.82 ^{a}
	20	74.99 ^{bc}	1.86 ^{bc}	3.81 ^{cd}	66.67 ^{bc}	1.87 ^{bc}	3.57 ^{bc}
	25	66.67 ^{cde}	1.21 ^{ef}	2.97 ^{f}	63.89 ^{bc}	1.30 ^{ef}	2.61 ^{def}
NAA	5	52.77 ^{e}	1.15 ^{ef}	2.65 ^{g}	44.44 ^{e}	1.20 ^{ef}	2.40 ^{ef}
	10	77.77 ^{abc}	1.42 ^{de}	4.02 ^{c}	72.22 ^{ab}	1.44 ^{de}	3.56 ^{b}
	15	69.44 ^{bcd}	1.60 ^{cd}	3.76 ^{d}	58.33 ^{cd}	1.68 ^{cd}	3.33 ^{bc}
	20	66.66 ^{cde}	1.40 ^{de}	3.12 ^{f}	50.00 ^{de}	1.32 ^{ef}	2.91 ^{cde}
	25	58.33 ^{de}	1.18 ^{ef}	2.48 ^{g}	50.00 ^{de}	1.16 ^{ef}	2.26 ^{f}
LSD	(P <0.05)	14.53	0.27	0.23	10.98	0.37	0.55

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

on half strength MS medium supplemented with IBA. The explants cultured on NAA supplemented media developed roots in lesser number of shoots i. e. 52.77 to 77.77% and 44.44 to 72.22% in *G. indica* and *G. cambogia,* respectively. Both the auxins were most effective at 10 μM concentration. IBA proved better for root formation as it induced long and thin roots in higher number of explants compared to thick and stunted roots produced on NAA supplemented media in both *G. indica* and *G. cambogia*. Goh et al. (1990) reported similar results in *G. mangostana*. Roots were induced directly from the shoot base without intervening callus phase. However, Kulkarni and Deodhar (2002) reported rooting of shoots in *G. indica* through an intervening callus phase on NAA supplemented media.

The in vitro raised plantlets were delicate and required hardening before field transfer. The root system showed further development in the liquid hardening medium and elongated to 8–12 cm. The rooted plantlets were finally transferred to pots containing soil, vermiculite and FYM in 1:1:1 ratio. The plantlets resumed growth and showed 90% survival in the pots. The regenerated plantlets showed normal growth and morphological characteristics when compared with seedlings. During hardening, use of medium with reduced mineral salt and sucrose concentration was used which forces the regenerants to rely on their own photosynthetic apparatus for nutrition. In the present study, use of this strategy resulted in better survival of plantlets when transferred to ex vitro conditions.

Comparison of in vitro morphogenetic potential of three horticulturally important *Garcinia* species using agamospermous (apomictic) seeds showed that the seeds of *G. indica* and *G. cambogia* were morphogenetically potential while *G. xanthochymus,* an obligate agamospermous species, was highly recalcitrant towards in vitro conditions. Lower concentrations of BAP and TDZ were found effective for induction of adventitious buds, however, buds induced on BAP showed better elongation into shoots compared to those induced on TDZ. The seeds being produced apomictically in the species ensures true-to-type or clonal multiplication of the selected mother trees. Commercial exploitation of developed protocol for multiplication of these commercially important horticultural species is possible, as the axillary buds from in vitro raised shoots can be employed as propagules for further multiplication. Development of medium-term conservation strategies using in vitro techniques for these highly recalcitrant seed species will ensure their existence in future since no long-term conservation methods are available for these recalcitrant seeded species. In vitro conservation of *G. indica* has been standardized (Malik et al. 2005a) while the same is in process for the other two species. Further, the apical meristems derived from in vitro cultured plantlets can be employed for long-term conservation of the species using cryopreservation techniques, once a protocol is standardized.

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