Direct somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss

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Direct somatic embryogenesis without an intervening callus phase was induced from immature seeds of *Garcinia indica* Choiss. Woody plant medium supplemented with 6-benzyl amino purine (BAP) (4.44–22.19 μm) alone or in combination with α-naphthaleneacetic acid (2.69 μm) produces somatic embryos within a period of 2–3 weeks with 80% frequency. Embryo induction was observed all over the explant surface. Origin of the embryo was confirmed histologically from sub-epidermal layer of the seed. Maturation of these embryos was achieved after 12 weeks of culture on a medium containing BAP (16.08 μm) in combination with indole-3-acetic acid (2.85–5.71 μm) and/or kinetin (4.65 μm). About 75% of the regenerated somatic embryos germinated into complete plantlets. The plantlets were acclimatized successfully with 92% survival in greenhouse.

Keywords: *Garcinia indica*, Guttifereae, seeds, somatic embryos.

GARCINIA indica Choiss., popularly known as 'kokam butter tree' belongs to the family Guttifereae and is distributed in and along the Western Ghats of India. This habitat is one among the 25 globally identified biodiversity hotspots. *Garcinia* is a polygamodioeceous[1], slow-growing and moderate-sized slender tree with drooping branches[2]. The plants show wide variation with respect to fruit yield, which is approximately 10–15 kg annually. The fruit has remarkable medicinal properties. It is an anthelmintic, cardio-tonic and is useful in piles, dysentery, tumours and heart complaints. Kokum butter is an emollient, and useful in burns, scalds and skin care preparations. The fruit rind contains two acids, viz. garcinic acid and hydroxycitric acid (HCA). HCA is a powerful inhibitor of the citrate cleavage enzyme[3]. It inhibits lipidogenesis by the inhibition of fatty acid synthesis and is a well-known anti-obesity drug. The rind also contains garcinol, a yellow pigment and anthocyanin, a dark red pigment. Seeds of *G. indica* remain viable for a short period of time, i.e. about one month. Thus, *in vitro* propagation may offer an answer to the large-scale production of plants throughout the year.

Somatic embryogenesis offers alternative and efficient means for plant multiplication and regeneration. Conventionally, enhanced proliferation of meristem, followed by rooting in a two-stage process is practised for higher multiplication of plants. For genetic improvement of a crop, embryogenic cultures are a pre-requisite. Moreover, direct somatic embryogenesis offers several advantages in crop improvement[4], such as cost-effectiveness and large-scale propagation using a bioreactor.

In the present work, we describe the induction of direct somatic embryogenesis in *G. indica* leading to plantlet regeneration and transfer to soil.

Immature fruits (Figure 1 *a* and *b*) were collected along the Western Ghats of India, especially from the Konkan region, Maharashtra during March. The fruits were washed with labolein (Qualigens, India) as surfactant (0.1% v/v), followed by washing with 10% (v/v) Savlon (Johnson and Johnson Ltd) as an antiseptic. This was followed by treatment with 1% (w/v) Bavistin (BASF, India) as antifungal agent for 30 min. After each treatment the fruits were washed thoroughly 3–4 times with single-distilled water. All the further operations were carried out under sterile conditions in a laminar airflow chamber. The fruits were dissected so as to recover the seeds, which were first rinsed with 70% (v/v) ethanol, washed 3–4 times with sterile distilled water (SDW) followed by treatment with 0.05% (w/v) $HgCl_2$ (Qualigens, India) for 10 min and washed thoroughly 4–5 times with SDW.

The surface-sterilized seeds were inoculated on Woody Plant Medium[5] (WPM) supplemented with 6-benzyl amino purine (BAP) (2.22–22.19 μm) alone or in combination with α-naphthaleneacetic acid (NAA) (2.69 μm). The media were supplemented with 2% sucrose (w/v) (Qualigens, India) and solidified with 0.85% agar (Qualigens, India) after adjusting the pH to 5.7–5.8. All the growth regulators were incorporated into the media before autoclaving. The media were autoclaved at 120°C for 20 min at 15 psi. The cultures were incubated at 25 ± 1°C at 16/8 h photoperiod with 35 $\mu E^{-2} m^{-2} s^{-1}$ illumination provided by white cool fluorescent tubes. The frequency of explants inducing the embryos was scored after 30 days based on 20 replications for each experiment, which was repeated three times. Explants showing embryogenesis were transferred to WPM medium supplemented with BAP (16.08 μm) in combination with indole-3-acetic acid (IAA) (2.85–5.71 μm) and kinetin (4.65 μm) for maturation. As development of the root pole was poor, embryos with well-developed shoots were shifted to the rooting medium with half-strength WPM supplemented with NAA (5.37–10.74 μm) in combination with indole-3-butyric acid (IBA; 4.90 μm) or indole-3-propionic acid (IPA; 5.29 μm) and solidified using 0.25% phytagel (Himedia, India). The germinated embryos were subsequently shifted to half-strength MS medium[6] supplemented with 0.89 μm BAP and solidified with 0.25% phytagel for elongation. The well-developed plantlets were later hardened in pots containing a mixture of soil and sand (1 : 1).

For histological studies, the explants were fixed in glacial acetic acid and ethanol (1 : 3) and later stored at 4°C in 70%

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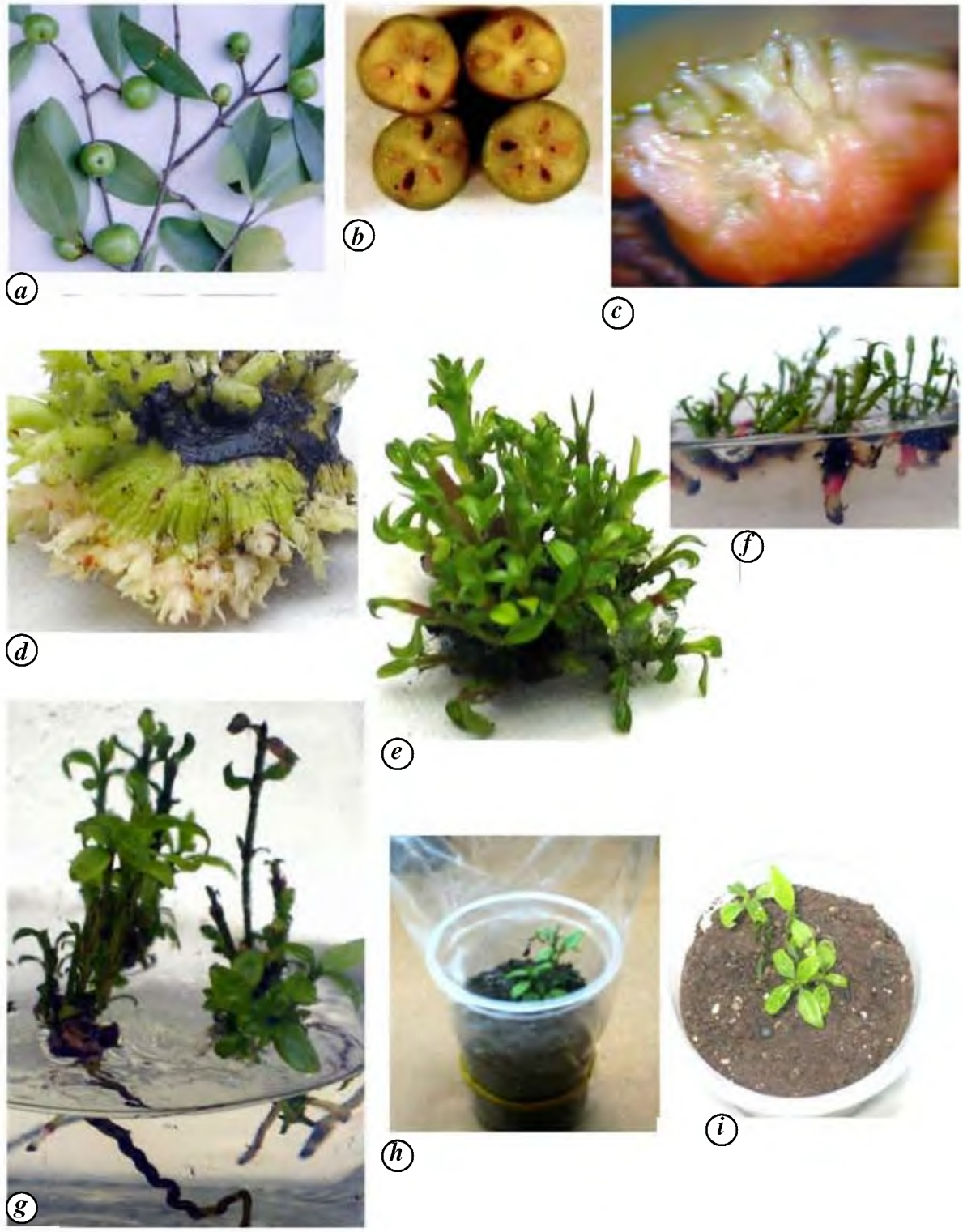

Figure 1. Plant regeneration via direct somatic embryogenesis from immature seeds of *Garcinia indica* Choiss. ***a***, Immature fruits of *G. indica*. ***b***, Transverse section of fruits showing position of seeds. ***c***, Embryogenic tissue induced from seed explant. ***d***, Precocious germination of somatic embryos with increase in embryogenic mass. ***e***, Germinated embryos with well-developed shoot pole. ***f***, Rooting of embryos. ***g***, Elongation of plantlets. ***h***, Acclimatization of plantlets. ***i***, Plantlets transferred to greenhouse.

ethanol. For dehydration, the tissue was passed through different grades of water : ethanol : tertiary butyl alcohol (TBA)[7] and embedded in paraffin wax (melting point 59–60°C). Serial sections were cut at 10 μm and stained with Hematoxylene (1% w/v)-Eosin (1% w/v) and mounted in DPX [2-chloro-*N*-(4-methoxy-6-methyl-1,3,5-triazin-2-yl

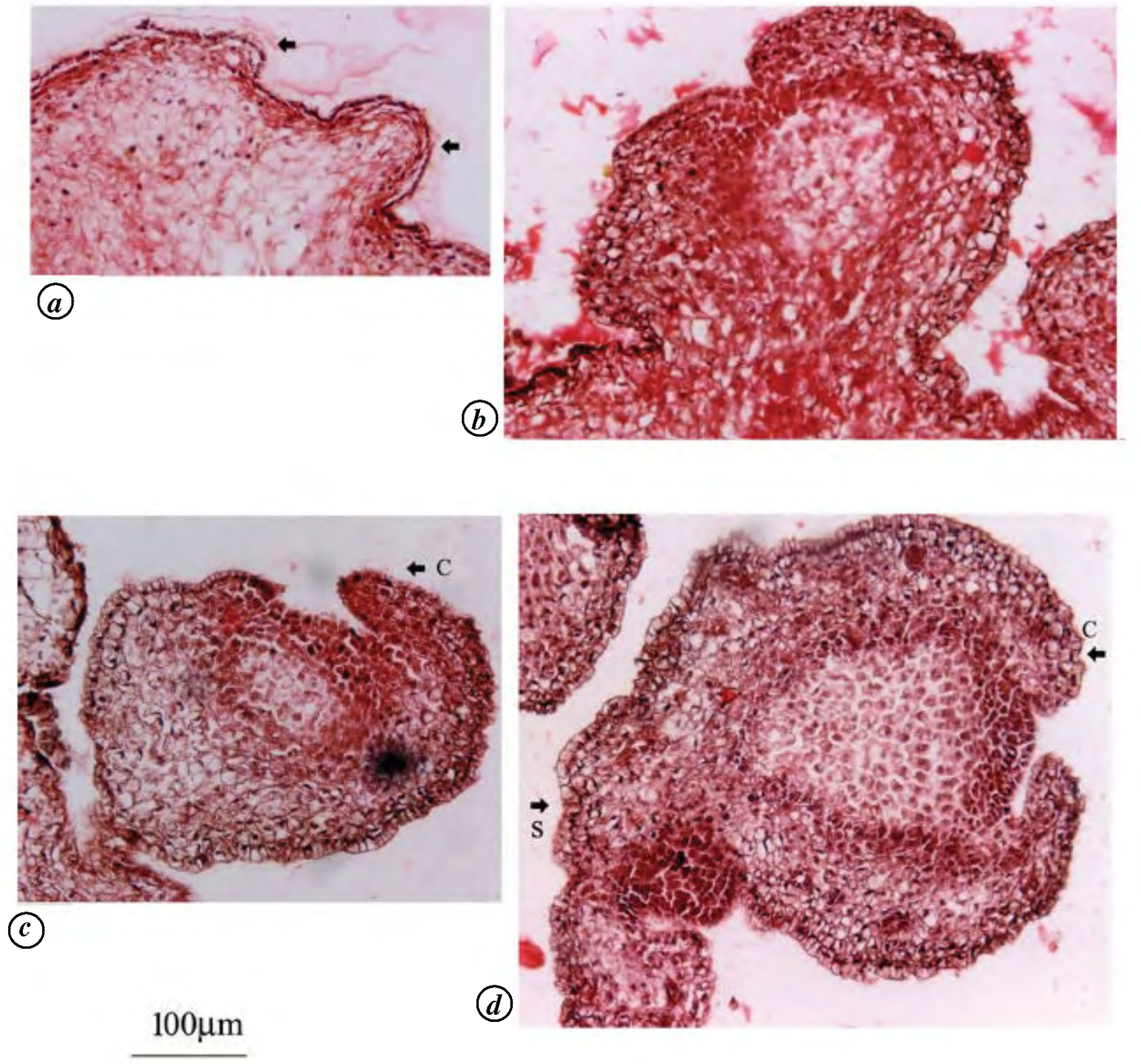

Figure 2. Histology of somatic embryogenesis of *G. indica*. Longitudinal sections through various stages of embryo development. ***a***, Induction of somatic embyrogenesis – sub-epidermal layer. ***b***, Heart-shaped embryo attached to the explant. ***c***, Cotyledonary stage, embryo detaching from explant. ***d***, Mature somatic embryo with suspensor. C, Cotyledon; S, Suspensor.

amino carbonyl) benzene sulfonamide] (Qualigens, India) and observed microscopically.

Analysis of variance was done by completely randomized block design using Agrobase 99 software and angular transformation values were derived[8].

The immature seeds were incubated on a series of media for embryo induction. The seeds showed swelling in one week and initiation of small protuberances was observed after 2–3 weeks of incubation (Figure 1 *c*). Embryogenic clumps were visible all over the surface of the seed within a period of 5 weeks. An increase in BAP from 2.22 to 22.19 µm showed increase in the frequency of somatic embryo induction up to 80% (Table 1). The higher concentration of BAP (22.19 µm) also showed the highest number of somatic embryos per seed explant, i.e. 48.53. Combination of BAP (2.22–22.19 µm) and NAA (2.69 µm) was also found responsive for embryo induction, with low frequency, which ranged from 8 to 28%. However, low concentration of BAP (2.22 µm) with NAA (2.69 µm) produced 24.83 somatic embryos per explant. ANOVA for average number of embryos per explant showed one treatment significant at 5% level, while five treatments were significant at 1% level. Similarly, ANOVA for mean frequency showed seven treatments significant at 1% level.

To confirm the origin of embryo induction, histological studies were conducted. Initiation of direct somatic embryo was observed from the sub-epidermal layer of seed

Table 1. Influence of different growth hormones on somatic embryo induction

BAP (μm/l)	NAA (μm/l)	Mean frequency of somatic embryo induction (%)	Avg. no. of somatic embryos per explant
2.22	–	43.33**	2.70
4.44	–	73.33**	7.33**
16.08	–	66.66**	3.67*
22.19	–	80.00**	48.53**
2.22	2.69	28.00**	24.83**
4.44	2.69	08.00	7.63**
16.08	2.69	12.00**	20.27**
22.19	2.69	16.00**	1.80

Data was scored after 6 weeks of culture.
Basal medium: WPM + sucrose 2% + Agar 0.85%.
For frequency: LSD (0.05) = S.E.D. X '*t*' at 90 degrees of freedom (5%) = 1.511.
LSD (0.01) = S.E.D. X '*t*' at 90 degrees of freedom (1%) = 2.097.
For avg. no. of embryos: LSD (0.05) = S.E.D. X '*t*' at 90 degrees of freedom (5%) = 1.581.
LSD (0.01) = S.E.D. X *t* at 90 degrees of freedom (1%) = 2.194.
*Significant at 5% level; ** Significant at 1 % level.

Table 2. Effect of different growth hormones on embryo development and maturation

BAP (μm/l)	IAA (μm/l)	Kinetin (μm/l)	Mean frequency of embryo maturation (%)	No. of mature embryos per explant mean (± SE)
16.08	5.71	–	80	20.66 ± 1.78
16.08	2.85	4.65	60	19.30 ± 1.35
16.08	5.71	4.65	90	21.87 ± 2.50

Data were scored after 12 weeks of culture.
Basal medium: WPM + sucrose 2% + agar 0.85%.
SE, Standard error.

explant without an intervening callus phase. Differentiated meristematic pockets were seen at the periphery of explants that further developed into embryos (Figure 2 *a*). Development of heart-shaped embryos with induction of suspensor (Figure 2 *b*), followed by well-differentiated cotyledonary stage embryos, which was attached to the explant were also observed (Figure 2 *c*). These embryos later got detached from the mother tissue and well-developed germinating embryos with shoot–root pole along with the suspensor were seen (Figure 2 *d*).

Prolonged incubation of the cultures for 8–10 weeks on any of the induction media did not ensure maturation of embryos. However, an increase in the embryogenic mass was observed (Figure 1 *d*). The embryogenic masses were, therefore, shifted to the maturation medium, viz. WPM with BAP (16.08 μm), IAA (2.85–5.71 μm) and/or kinetin (4.65 μm). Upon transfer to these media, maturation of embryos and growth of the shoot pole were prominently observed (Figure 1 *e*). Maturation frequency varied from 60 to 90% after 12 weeks of incubation, where maximum number of mature embryos was 21.87 per explant on the medium containing BAP (16.08 μm), IAA (5.71 μm) and kinetin (4.65 μm; Table 2). Figure 3 shows the effect of incubation period on the number of mature embryos. A gradual increase in the number of mature embryos was evident from the 12th week onwards. In 24 weeks, 43.80% embryos showed maturation with well-developed shoots. However, as development of the root pole was poor, the germinating embryos in clusters with well-developed shoot pole of size 1.5–2.0 cm were transferred to the rooting media (Table 3), which comprised half-strength WPM supplemented with NAA (5.37–10.74 μm) and IBA (4.90 μm) or IPA (5.29 μm). Development of root pole was observed in all media combinations in 20–25 days. The best hormonal combination was NAA (10.74 μm) with IBA (4.90 μm), where root development was 76.03% with 15.33 roots per explant. A combination of NAA (5.37 μm) and IPA (5.29 μm) also gave similar results with 69.76% rooting. However, roots induced on NAA and IBA combination showed faster growth than those with incorporation of IPA in the media (Figure 1 *f*). The well-developed plantlets were transferred to half-strength MS medium supplemented with 0.89 μm BAP for further elongation (Figure 1 *g*). Plantlets of size about 5–6 cm were washed in water and then shifted to sterilized potting mixture of sand and soil (1 : 1) for acclimatization (Figure 1 *h*) and transferred to greenhouse conditions (Figure 1 *i*).

Direct somatic embryogenesis proceeds from the cells that are already determined for embryogenic development. In the presence of growth regulators and culture conditions, such cells undergo cell division and express embryogenesis[9]. Induction of direct somatic embryos from

Table 3. Frequency of root induction on varying auxin combinations

NAA (μm/l)	IBA (μm/l)	IPA (μm/l)	Mean frequency of rooting (%)	No. of roots per explant mean (± SE)
5.37	4.90	–	22.44	8.25 ± 1.83
10.74	4.90	–	76.03	15.33 ± 1.75
5.37	–	5.29	69.76	16.36 ± 1.00

Data were scored after 20 days of culture.
Basal medium: Half-strength WPM salts + sucrose 2% + Phytagel 0.25%.
SE, Standard error.

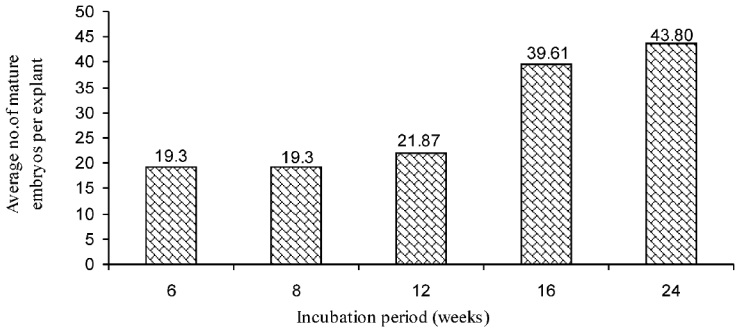

Figure 3. Effect of incubation period on the number of mature embryos. Increase in the average number of mature somatic embryos per explant on WPM + BAP (16.08 μm) + IAA (5.71 μm) + kinetin (4.65 μm).

different explants, especially in woody perennials is reported from many plant species[10–13]. There are several reports on the propagation of *Garcinia mangostana* using different explants. Direct shoot-bud formation from leaf explants of *G. mangostana*[14,15] and shoot proliferation from seed explants[16] have been reported. *In vitro* multiple shoot induction on MS media with BAP + NAA + kinetin was also worked out in *G. indica*[17]. In the present work, we report a complete cycle of plant regeneration via direct somatic embryogenesis, which requires about 28–30 weeks. Using this protocol, from a single seed about 40–50 plants can be produced within this period.

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