

In Vitro Regeneration and Hydroxycitric Acid Production in Tissue Cultures of *Garcinia indica* Chois.

Manik D Kulkarni and Manjushri A Deodhar*

Kelkar Education Trust's V G Vaze College of Arts, Science and Commerce, Mithagar Road, Mulund (E),
Mumbai 400 081, India

Received 8 November 2001; accepted 3 April 2002

Garcinia indica is a rich source of hydroxycitric acid (HCA), an important biological active plant metabolite used as an anti-obesity drug. The female plants are the major source of main isomer, (-) - hydroxycitric acid, a powerful inhibitor of citrate cleavage enzyme. *G. indica* being a polygamodioecious sp, differentiation between male and female plants is detected only at the flowering stage (approx. 7 yrs). Tissue culture methods, offer scope for large scale propagation of female plants. An efficient protocol for multiple shoots and callus has been standardized to produce HCA. Multiple shoots were obtained from immature seed explants on MS basal medium supplemented with NAA (2.69 μM), BAP (8.9 μM) and KN (0.93 μM). Elongation of shoots was achieved on half MS medium supplemented with NAA (0.54 μM), BAP (0.44 μM) and KN (0.93 μM). The shoots developed roots when they were treated with 4900 μM IBA for 30 seconds and cultured on half MS basal medium. Multiple shoots and callus were obtained from explants derived from *in vitro* developed plantlets. The regenerated plantlets were successfully transferred to soil in pots. Multiple shoots and the callus cultures produced HCA.

Keywords: *Garcinia indica*, organogenesis, micropropagation, hydroxycitric acid

Garcinia indica Chois. (Family-Guttiferae; Clusiaceae) occurs in tropical rain forests of western Ghats of India. The natural flora shows wide variation in the fruit yield and quality. Fruits of *G. indica* obtained from coastal regions of Maharashtra, are a rich source of hydroxycitric acid (HCA), an important biologically active plant metabolite used as an anti-obesity drug. HCA inhibits the conversion of carbohydrates to fat through an enzyme, ATP citrate lyase (Watson *et al*, 1969). Of the four isomers of HCA, only (-)-HCA is a powerful inhibitor of citrate cleavage enzyme (Watson *et al*, 1969). The major

source for (-)-HCA are fruit rinds of *G. indica* and *G. cambogia* (Lewis & Neelakantan, 1965).

G. indica (Kokam) rinds are traditionally used in curry preparation. Since it is a polygamodioecious species (Anonymous, 1956), the differentiation between male and female plants is detected only at the flowering stage (approx. 7 yrs). Tissue culture methods, would therefore, offer scope for clonal propagation of elite trees of *G. indica* and for large scale production of female plants. Plant organ and cell culture methods could also present an alternative method for production of HCA. The present paper describes methods for regeneration of plants from *in vitro* cultures and extraction of HCA from the cultured tissues.

The fruits, collected from Konkan Krishi Vidyapeeth Dapoli, Maharashtra, India, were processed for extraction of HCA. The immature green fruits were washed with water containing 2-3 drops of Tween 80 and treated with 70% alcohol for 1 min for surface sterilization. The seeds were removed aseptically from the fruit and cut into 2 segments. Explants such as tender leaves, axillary and apical leaf buds, nodal segments, and petiole were surface sterilized with 70% alcohol for 1 min followed by 2% sodium hypochlorite for 5-7 min and washed with sterile distilled water and cultured on MS medium (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and various combinations of growth regulators, such as 2,4-D (9 μM) + KN (0.93 μM); NAA (10.74 μM) + KN (0.93 μM); NAA (2.69 μM) + BAP (22.2 μM); NAA (5.37 μM) + BAP (8.9 μM) + KN (0.93 μM); IBA (9.8 μM) + KN (0.93 μM); IBA (9.8 μM) + BAP (0.89 μM); IAA (11.42 μM) + BAP (0.89 μM).

The cultures were incubated at $25 \pm 20^\circ\text{C}$ under illumination $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ with 16 hrs photoperiod. The explants were sub-cultured at 30-day intervals. The frequency of explants producing shoots was scored after every 15 days by Analysis of Variance (ANOVA). There were at least 15 or 20 replicates for each experiment. Shoot elongation was evaluated by incubating the shoots on half MS medium supplemented with NAA (0.54 μM), BAP (0-0.89 μM) and KN (0-1.86 μM). Explant materials such as leaves, petiole, and nodal segments from *in vitro*

*Author for correspondence:

Tel: 91-22-5900392/93 ; Fax: 91-22-5647334

E-mail : kelkar_src@vsnl.net

developed plantlets were also cultured on MS medium consisting of NAA (5.37-21.48 μM) and BAP (2.2-13.2 μM).

For rooting, the elongated shoots were initially treated with different concentrations of NAA (1342.5, 2685, 5370 & 10740 μM), IAA (1427.5 & 5710 μM) or IBA (1225, 2450, 4900 & 9800 μM) solutions for 30 seconds and cultured on half strength MS basal medium. Rooted plantlets were subsequently hardened in pots containing soil, sand and cocopeat in the ratio 1:2:1. For callus initiation the various explants like immature seeds, *in vitro* developed leaf, root and nodal explants were cultured on MS basal medium supplemented with NAA (5.37 - 21.48 μM), and BAP (2.2, 4.4 & 13.2 μM).

(-) - HCA was isolated by the method described by Singh *et al* (1995). Five hundred mg of *in vitro* shoots and callus were autoclaved in 15 ml of water at 1.05 kg cm^{-3} for 20 min and filtered. To the hot filtrate, about 2 gm of calcium chloride and ammonia (NH_4OH) were added, which precipitated as its calcium salt. The calcium salt was filtered, washed with water, and dried at 37°C. Detection of HCA was carried out on Varian HPLC model 9010/9050 using reverse phase C18 μ Bondapak column (25 cm $\times$ 4.6 mm). The mobile phase was 0.05 M: KH_2PO_4 , pH 2.5, flow rate, 0.7/ml per min; temperature, 25°C, and detection carried out at, 210 nm. The identity of HCA in the cultures was confirmed through co-chromatography on HPLC and comparison of IR spectrum with standard HCA marketed by Cipex Specialities Pvt Ltd, Mumbai.

Different explants tested, immature seed explants responded in MS + NAA (5.37 μM) + BAP (8.9 μM) + KN (0.93 μM) medium giving multiple shoot-buds in

3 to 4 weeks. At lower concentrations of BAP (2.2 μM), most of the seed explants showed only bulging. As the BAP concentration increased (4.4 or 8.9 μM), these bulges developed plumule like structures, which shriveled after two to three weeks and subsequently, shoot-buds developed from the entire surface of seed explants (Fig. 1a). Increase in concentration BAP (2.2-8.9 μM), gave a gradient increase in the development of number of shoots from the explants. Maximum number of shoots developed from the seed explant in medium containing BAP 8.9 μM . Twenty replicates were used and the number of shoots were scored every 15 days up to 3 months with regular subcultures every 30 days. The average number of shoots per explant obtained after 3 months in culture is summarized in Table 1. The data were statistically examined by ANOVA.

Varying the NAA concentration in the medium caused distinct differences in morphogenetic responses of the seed explants. NAA concentrations (1-5.37 μM) induced shoot regeneration. NAA at 2.69 μM gave maximum number of shoots, however, concentrations higher than 5.37 μM caused callus induction with sporadic root initiation. MS + NAA (2.69 μM) + BAP (8.9 μM) + KN (0.93 μM) was therefore, selected as the optimum medium for shoot proliferation from immature seeds.

Axillary and apical buds from *in vitro* plantlets responded in medium having composition MS + NAA 2.69 μM + BAP 8.9 μM + KN 0.93 μM and developed multiple shoots (Fig. 1b). *In vitro* roots also developed multiple shoots in MS medium incorporated with NAA 5.37 μM + BAP 4.4 μM + KN 0.93 μM (Fig. 1c).

Table 1—Effect of various concentrations of NAA and BAP on the formation of multiple shoots from immature seed explants.

NAA (μM)	Average number of shoots per explant $\pm$ SE			
	BAP (μM)			
	2.2	4.4	8.9	13.2
1.07	0 ^{ap*}	0.85 $\pm$ 0.58 ^{ap}	14.9 $\pm$ 3.89 ^{bq}	8.1 $\pm$ 3.12 ^{aq}
2.69	0.7 $\pm$ 0.43 ^{ap}	2.75 $\pm$ 1.16 ^{ap}	16.7 $\pm$ 4.22 ^{bq}	0.65 $\pm$ 0.64 ^{bp}
5.37	0.05 $\pm$ 0.04 ^{ap}	1.7 $\pm$ 0.76 ^{aq}	4.25 $\pm$ 2.52 ^{aq}	7.55 $\pm$ 2.64 ^{bq}
10.74	0 ^{ap}	1.95 $\pm$ 0.76 ^{aq}	0 ^{ap}	2.8 $\pm$ 0.97 ^{aq}

Means followed by the same letter are not significantly different at $p=0.05$ according to ANOVA test ($n=20$). The letters a and b indicate significance in columns and the letters p and q indicate significance in rows. For the above combinations KN=0.93 μM was kept constant.

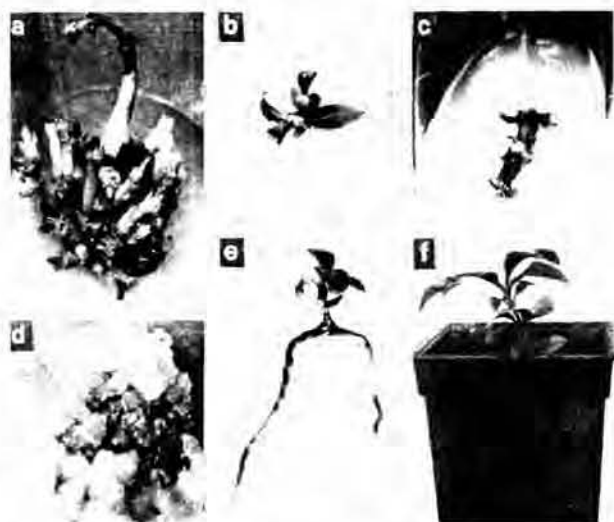


Fig. 1—Morphogenetic responses of explants obtained from immature seeds and *in vitro* developed plantlets of *G. indica*.

a - Multiple shoots from immature seed on medium containing MS+NAA (2.69 μ M)+BAP (8.9 μ M)+KN (0.93 μ M).

b - Shoot bud initiation from nodes of *in vitro* regenerated plants with MS+NAA (2.69 μ M)+BAP (17.6 μ M)+KN (0.93 μ M).

c - Shoot bud initiation from root of *in vitro* regenerated plants with MS + NAA (5.37 μ M) + BAP (4.4 μ M) + KN (0.93 μ M).

d - Callus formation from *in vitro* root with MS + NAA (16.11 μ M) + BAP (2.2 μ M) + KN (0.93 μ M).

e - Direct rooting from regenerated shoots on basal MS $\frac{1}{2}$ medium with IBA dip at 4900 μ M.

f - Hardened plant in pot containing sand, soil and cocopeat.

Elongation of *in vitro* shoots was evaluated by keeping NAA concentration constant at 0.54 μ M. Among the various concentrations of BAP (0-0.89 μ M) and KN (0-1.86 μ M) tested, half MS medium containing NAA (0.54 μ M) + BAP (0.44 μ M) + KN (0.93 μ M) was most effective.

Seventy per cent rooting of the elongated shoots was achieved in half MS medium, supplemented with 10.74 μ M NAA. However, the root initiation occurred through the intervention of unorganized callus. As a result most of the plantlets failed to survive in soil. When the elongated shoots were dipped in different concentrations of NAA (1342.5, 2685, 5370 & 10740 μ M) or IBA (1225, 2450, 4900 & 9800 μ M) for 30 seconds and cultured on half MS basal medium, 70% rooting was achieved with 4900 μ M of IBA dip in 25 days. IAA did not induce rooting in the *in vitro* plants. The resulting plantlets were devoid of callus at the root shoot junction (Fig. 1e) and survived well in pots.

The rooted plantlets were transferred to the pots containing soil, sand and cocopeat in the ratio 1:2:1. Such mixture gave 60% survival and the plantlets

showed growth in the apical region after about 40 days (Fig. 1f).

For immature seed explants, NAA 5.37 μ M + BAP 13.2 μ M + KN 0.93 μ M was most conducive for callus initiation. *In vitro* leaf explants showed bulging at the petiole end, which subsequently developed unorganized proliferation to give friable callus in medium containing NAA 16.11 μ M + BAP 2.2 μ M + KN 0.93 μ M. Callus initiation also occurred from root explants in the same medium. The callus initiated from all the three explants grew well in subsequent transfers to fresh media containing MS + NAA 10.74 μ M + BAP 13.2 μ M + KN 0.93 μ M (Fig. 1d) supplemented with (w/v) sucrose, 3% (w/v) agar 0.8% at pH of the medium at 5.7.

Successful regeneration of plantlets from immature seed explants indicated that a large number of plants could be obtained by *in vitro* culture. The data on shoot-bud initiation from *in vitro* developed leaf segments and roots indicated that vegetative tissues could also be employed to regenerate plants. Since the plant is polygamodioecious, it may be of interest to raise female progeny through this method. The observation of shoot bud regeneration from *in vitro* nodal explants was maximum at NAA (2.69 μ M) + BAP (17.6 μ M). This medium is comparable with the observations made in *G. mangostana* (Goh *et al.*, 1990), where the optimum level of BAP conducive for shoot bud development was 22.2 μ M.

In vitro raised shoots and callus gave the characteristic sour taste like the parent plant leaves and fruits. Extracts from cultures of multiple shoots growing in half MS + NAA (0.54 μ M) + BAP (0.44 μ M) + KN (0.93 μ M) and from callus cells, growing in MS + NAA (10.74 μ M) + BAP (13.2 μ M) + KN (0.93 μ M) gave calcium salts in the form of thick precipitate. HPLC analyses of the precipitates from the fruits and from callus cultures showed the presence of HCA as a major peak at retention 5.1, with smaller amounts of citric acid at 7.1, respectively (Fig. 2). However, the precipitate obtained from multiple shoots showed a single peak corresponding to HCA. Citric acid was not present in the shoot cultures. There are quantitative differences in the calcium salt of HCA obtained in the fruit, callus and multiple shoots (Table 2).

Threo (-) isomer of HCA present in *Garcinia* species is a stereo specific anti-obesity drug which is difficult to produce by conventional methods. The synthesis of HCA in *in vitro* shoots and callus

Table 2—HCA synthesis in fruits and *in vitro* cultures of *G. indica* estimated by HPLC analysis

Material	Total HCA as Ca salt dry weight (%)	Relative area for HCA (%)	Relative area for citric acid (%)
Fruit	22	60.18	5.07
<i>In vitro</i> shoots	3.0	89.22	0
Callus	3.0	84.88	0.43

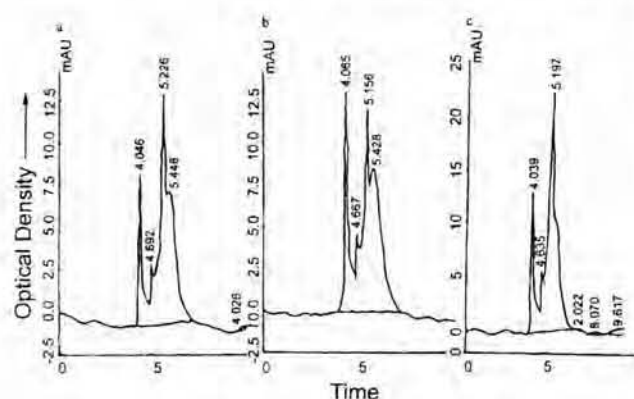


Fig. 2—HPLC profiles of calcium salt of HCA.
a - HPLC of standard HCA extracted from fruits.
b - HPLC of HCA extracted from callus.
c - HPLC of HCA extracted from *in vitro* leaves.

indicated that these cultures could be an alternative source for HCA production. *G. indica*, *G. cambogia* and *G. mangostana* are economically important tree species, both for their fruits and bioactive ingredients. HCA is an exciting natural product with different biological activities and least toxicity. It is, therefore, important to explore possibilities of producing this molecule for large scale HCA through other

biotechnological avenues such as callus and shoot and root cultures *in vitro*. Further research for germplasm conservation and shoot multiplication of these species is essential.

Acknowledgment

Authors thank Mr G D Kelkar, Chairman, Kelkar Education Trust's Scientific Research Centre; Dr M R Heble, Scientific Advisor, SRC and Dr M R Kurup, Principal of KET's V G Vaze College for encouragement. They also thank Dr G D Joshi, Konkan Krishi Vidyapeeth, Dapoli, for providing plant material, Cipex Specialities Pvt Ltd (Mumbai), for authentic sample of hydroxycitric acid and Dr Inamdar from Ashkot Analytical Services, Mumbai, for HPLC method.

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