Direct organogenesis from leaf explants of *Garcinia indica* Choisy: An important medicinal plant

Devendra K Chauhan*, A K Thakur, A Dass, J M Lima and S K Malik

1Tissue Culture and Cryopreservation Unit, National Bureau of Plant Genetic Resources (NBGPR), New Delhi 110 012, India
2Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan 173 230, India
3Biotechnology Laboratory, Allahabad Agriculture Institute-Deemed University, Allahabad 211 007, India

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A high frequency plant regeneration protocol through direct organogenesis has been developed for *Garcinia indica* Choisy using leaf explants. The leaf explants were procured from the *in vitro* developed shoots raised from the nodal explants comprising of axillary buds. The leaf explants were cultured on MS medium supplemented with different concentrations of BAP. The highest number of shoots per explant was obtained on the medium supplemented with 22.2 µM BAP, while the lowest number of shoots was recorded on the medium supplemented with 0.45 µM BAP. The highest percentage of rooted plants was obtained on MS basal medium (MS +0.45 µM BAP) supplemented with 0.81-1.07 µM NAA, while maximum root length was recorded on the medium supplemented with 0.05 µM NAA. MS medium supplemented with 0.45 µM BAP and 0.81 µM NAA was recorded optimal for rooting of the shoots. The plantlets were hardened and successfully acclimatized to the open field conditions.

Keywords: *Garcinia indica*, leaf explants, organogenesis, regeneration

Introduction

*Garcinia indica* Choisy belongs to the family *Guciaceae*. It is endemic to western Ghats of Maharashtra, Goa, Karnataka, Kerala and Tamil Nadu provinces of India. It is a dioecious, cross-pollinated and heterozygous tree. Its center of origin is southern Maharashtra and Goa. Its fruits are valued for its nutritional and medicinal properties. The dried fruits are used as an excellent substitute of tamarind and lime in the preparation of *sambar* and non-vegetarian curries, especially for fish, for imparting a special flavour and aroma. It is used in preparing syrup during summer days in Goa and Karnataka. Its active ingredient (-)-hydroxyl citric acid (HCA) is pharmaceutically very important because it possesses anti-obesity and anti-cholesterol properties. It accelerates fat burning and inhibits fatty acid synthesis. Seeds of this plant also produce valuable fat known as Kokum butter, which is edible and suitable for ointment in medicinal preparations for fissures of lips and heel.

Due to the severe deforestation and habitat destruction, *G. indica* is included in the list of endangered species of medicinal plants of our country. Differentiation between male and female trees can only be made 7-8 years after planting at the flowering stage. Dioecious nature of this species is also a major constraint in producing good quality planting material. The tree is slow growing and propagation is generally done either by softwood grafting or by seeds. Seeds of the plant are recalcitrant in nature due to high sensitivity to desiccation and freezing. The shelf life of seeds is very short ranging from 2-4 wk. Therefore, it is difficult to raise the plants throughout the year. Plantlet generation by grafting is season dependent, which is further limited by root stock availability. The possibility of improvement in *G. indica* by conventional breeding methods is limited because of its complex genetic makeup. The demand of *Garcinia* products, its dioecious nature of plants and short supplies of quality planting material are the constraints in its commercial cultivation. *In vitro* plantlet development from different explants and direct shoot bud formation from leaf explants of seedling and mature trees have been reported in *G. mangostana*. However, very limited regeneration and conservation work was carried out in *G. indica* using leaf explant.
In the present work, we are reporting the development of a high frequency plant regeneration protocol in *G. indica* via direct organogenesis using leaf explants.

**Materials and Methods**

Seed material of *G. indica* was obtained from National Bureau of Plant Genetic Resources (NBPGR), Regional Station Trissure, Kerala, India. Seeds were initially used to raise the *in vitro* plants for deriving nodal sections for further propagation *in vitro*. The seeds were washed with 1% solution of Tween-20 for 10 min and placed under running tap water for 1 h. Surface sterilization of seeds was done with 0.1% of HgCl$_2$ (w/v) for 10 min, followed by 4 rinses with sterilized doubled distilled water to remove the traces of HgCl$_2$.

**Medium and Culture Conditions**

Murashige & Skoog’s (MS) medium$^{15}$ supplemented with 3.0% sucrose (w/v) was used for shoot bud differentiation and multiplication. The pH of the medium was adjusted to 5.75 using 0.1 N NaOH or 0.1 N HCl and 0.75% agar was added before autoclaving. Routinely, 30 mL of the molten medium was dispensed into culture tubes (25 × 150 mm$^2$) plugged with plastic caps and autoclaved at 121°C and 1.05 kPa pressure for 20 min. All the cultures were incubated in a culture room maintained at 25±2°C temperature and 65 to 75% relative humidity with 8 h photoperiod with an intensity of 55 µmol m$^{-2}$ s$^{-1}$ attained with PAR lamps OSRAM® Lumilux Plus and Plus Eco-Cool white.

**Shoot Regeneration**

Hormone free MS medium was used for culture of seeds in test tubes to raise seedlings. After 25-30 d, seedlings attained the height of 4-6 cm with 3 to 4 nodes. A preliminary experiment was conducted with MS medium supplemented with different hormone combinations to find better responding composition for axillary bud culture. On the basis of culture response, MS medium supplemented with 0.45 µM BAP was selected for final experimentation. The nodal explants (0.5 to 1.0 cm) comprising of axillary buds from *in vitro* raised seedlings were sub-cultured on MS medium containing 0.45 µM BAP. Young and tender leaves were harvested after 21 d of sub-culturing, cut into small pieces and inoculated on MS medium supplemented with various concentrations of BAP (Table 1) for culture initiation and multiplication. All the cultures were transferred to fresh medium after 3 wk duration. The cultures were evaluated for average number of shoots per explants and percentage of regenerated healthy shoots after 4 wk of culture.

**Root Regeneration**

For root induction, 25-d-old shoots were sub-cultured on MS medium supplemented with 0.45 µM BAP and various concentrations of NAA (Table 2). MS medium containing 0.45 µM BAP was used to maintain the cultures. Excised shoots with 3-4 nodes were placed in culture tubes containing 25 mL medium with 0.75% of agar. For each treatment, 24 culture tubes were inoculated. Roots appeared within 7-8 d of inoculation. The data on per cent root regeneration and root length was recorded after 21 d of sub-culture.

### Table 1—Effect of various concentrations of BAP (in MS medium) on regeneration of shoots from leaf explants of *G. indica*

<table>
<thead>
<tr>
<th>No.</th>
<th>BAP conc. (µM)</th>
<th>No. of shoots/explant</th>
<th>% regenerated healthy shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.45</td>
<td>0.75 ± 0.433* (0-1)</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>2.22</td>
<td>1.42 ± 0.640 (0-2)</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>4.44</td>
<td>1.83 ± 0.553 (1-3)</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>8.89</td>
<td>3.42± 1.115 (1-5)</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>13.32</td>
<td>6.75 ± 2.454 (2-8)</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>16.67</td>
<td>10.83 ± 2.967 (3-14)</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>22.20</td>
<td>15.75 ± 4.850 (5-30)</td>
<td>93</td>
</tr>
</tbody>
</table>

*5% level of significance; 60 tubes/treatment

### Table 2—Effect of different NAA concentrations (in MS medium+0.45 µM) on rooting of *in vitro* regenerated shoots of *G. indica*

<table>
<thead>
<tr>
<th>No.</th>
<th>NAA conc. (µM)</th>
<th>% rooted plants</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.0</td>
<td>0.00± 0.00</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.6</td>
<td>2.10± 0.107*</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>9.0</td>
<td>1.80± 0.097</td>
</tr>
<tr>
<td>4</td>
<td>0.27</td>
<td>47.1</td>
<td>1.60± 0.082</td>
</tr>
<tr>
<td>5</td>
<td>0.54</td>
<td>71.6</td>
<td>1.50± 0.081</td>
</tr>
<tr>
<td>6</td>
<td>0.81</td>
<td>100</td>
<td>1.40± 0.071</td>
</tr>
<tr>
<td>7</td>
<td>1.07</td>
<td>100</td>
<td>0.80± 0.043</td>
</tr>
</tbody>
</table>

*5% level of significance; 24 tubes/treatment
Hardening and Acclimatization

After proper root development when plantlets attained a height of 4-5 cm, they were taken out from the culture tubes gently, washed under gentle stream of running tap water to remove the adhering medium. These were transferred to plastic pots containing autoclaved peat moss and then covered with plastic wrapper to prevent humidity losses. Plantlets were maintained at 25±2°C temperature and 60-70% relative humidity under bright sunlight. Plantlets were frequently watered and gradually exposed to the natural environment.

Statistical Analysis

All experiments were set up in completely randomized design and repeated thrice. About 60 tubes were used per treatment for shoot regeneration from the leaf explants. Data pertaining to mean percentage of cultures showing shoot induction response in terms of average number of shoots per explant, per cent explants regeneration, per cent root regeneration and root length were recorded and analyzed. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences (P< 0.05) among the treatment means.

Results and Discussion

Leaf explants of *G. indica* were inoculated on MS solid medium supplemented with varying levels of BAP. Explants started to swell after 2-3 d of culture and shoot initiation started after 1 wk of culture from the mid rib of leaf explants (Fig. 1A). Simultaneously, light-green coloured adventitious shoot buds appeared from the cut margins of leaf explants (Figs 1B & C). Multiple shoot induction was recorded on MS medium supplemented with 4.44 to 22.2 µM BAP (Table 1). Number of shoots per explant increased with respect to the increasing concentration of BAP. Significantly more adventitious shoots were observed on leaf explants exposed to 22.2 µM of BAP with an average of 15.75 shoots per explant and 93% frequency of explants regeneration as compared to other concentrations of BAP. Minimum average number of shoots per explant was recorded on MS medium containing 0.45 µM BAP; while not a single explant could respond to shoot bud induction in hormone free MS medium. BAP is considered as one of the most useful cytokinins for achieving the multiplication and microprogation of the plants.

Shoot regeneration in *G. indica* was also successfully achieved from seed explants on MS medium supplemented with BAP (12.5-50 µM)\(^{11}\). Plant regeneration in *G. indica* via somatic embryogenesis and repeated somatic embryogenesis route using seed as explants material has also been reported\(^{12,13}\). *De novo* shoot regeneration by culturing roots of the plantlets of *G. indica* for a long time on ½ strength MS medium supplemented with BAP (0.44-2.22 µM ) has been well elucidated\(^{14}\). The seeds of *G. indica* remain viable only for a short period of time, i.e., for about a month. So establishment of shoots under *in vitro* and regeneration and multiplication of complete plants using leaf explants procured from the *in vitro* developed shoot material is a very wise strategy to get the regular availability of the plant material throughout the year.

In our study, percentage of rooted plants increased with the increasing concentration of NAA, but root length decreased on MS medium containing 0.81 µM NAA and onwards (Table 2). MS medium supplemented with 0.45 µM BAP and 0.81 µM NAA

![Fig. 1 (A-D)—Direct organogenesis from leaf explants of *G. indica*: A. Direct organogenesis from mid rib of leaf explant cultured on shoot regeneration medium; B & C. High frequency shoot regeneration from leaf explants on MS medium supplemented with 22.2 µM BAP; & D. Rooting in *in vitro* developed shoots on MS medium supplemented with 0.45 µM BAP and 0.81-1.07 µM NAA](image-url)
were found to be the optimal medium for rooting, where 100% of the regenerated shoots developed roots (Fig. 1D, Table 2). The minimum percentage of rooted plants was observed on MS medium supplemented with 0.05 μM NAA, while maximum root length was observed in this medium. Auxin free MS medium did not respond for rooting. Earlier, root induction in G. indica cultured shoots was reported on ½ strength MS medium supplemented with 10 μM IBA\textsuperscript{11}. In another report, rooting in \textit{in vitro} developed shoots of \textit{G. indica} was obtained by pulse treatment of 9800 μM IBA dip for 30 seconds\textsuperscript{18}. Nearly 100% rooting (\textit{in vitro}) was obtained on ½ MS medium with 2% sucrose and 0.6% agar. A protocol for restoring the rooting competence in a mature plant of \textit{G. indica} through serial shoot tip grafting \textit{in vitro} has also been developed\textsuperscript{19}. The root regeneration in \textit{G. indica} shoots could take place on WPM medium supplemented with NAA (2.69-10.74 μM) and IBA (4.90 μM) within 20-25 d\textsuperscript{14}.

Hardening is also a crucial step prior to transplantation of plantlets to the soil. The \textit{in vitro} plantlets live in 100% relative humidity and also depend on the media composition. Plantlets were, therefore, allowed to grow on rooting media for about 1 month after root initiation. During this phase, the nutrients in the culture gradually depleted and plants became sturdy and easy to aclimatize in the mist house\textsuperscript{20}. The hardened plantlets were then transferred to the pots containing autoclaved peat-moss. During the weaning process, the plantlets were kept covered with plastic covers for 15 d to maintain 100% relative humidity. After 2 wk, perforations were made in the plastic cover to gradually lower the humidity. In the mean time, the plantlets developed an efficient root system, built up new and photosynthetic active leaves and were ready to be transferred to the field.

In the present study, production of multiple shoots from leaf explants of \textit{G. indica} was highly reproducible whenever the experiments were repeated. The regenerated plantlets showed normal morphological and growth characteristics as there was no intervening callus phase and also the time taken for complete regeneration of plantlets under \textit{in vitro} conditions was only 45-50 d. The present protocol offers a solution to the large scale production of \textit{G. indica} plants throughout the year to abridge the gap of demand and supply. This protocol will also provide a platform for genetic transformation of this species for the introgression of various desirable traits.

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**References**