



Clonal propagation of female plants of *Garcinia indica* Choiss: a tree species of high medicinal value

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ABSTRACT

Fruits of *Garcinia indica*, a medicinally important tree species from Western Ghats of India is a rich source of medicinally important (-) Hydroxycitric acid that shows anticholesterol and antiobesity activities. Since this species is polygamodioecious, sex of the plant can be identified only after flowering that takes approximately 7-8 yrs. From commercial and horticultural point of view, plantation of female trees is much preferred. In this study, an efficient protocol for propagation of female plants was developed using *in vitro* techniques. For establishment of sterile cultures, fungal contamination and browning of explants were the major hurdles. Season of plant material collection and culture vessels used for *in vitro* sprouting were also observed significant factors in establishment of sterile cultures. The use of WPM basal fortified with 2% sucrose and 8.87 μ M BAP resulted maximum sterile and healthy buds. TDZ in the range 0.22 – 4.54 μ M along with BAP induced multiple shoots. Highest 63% rooting was obtained on WPM medium with 4.90 μ M IBA whereas, maximum 60% plantlets survived in green house after their transfer to soil.

Abbreviations: BAP: 6-Benzyl amino purine, TDZ : Thidiazuron ((N-phenyl-N¹-1,2,3-thidiazol-5-ylurea), IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, IPA: Indole-3-propionic acid, NAA: α -Naphthalene acetic acid, SDW: Sterile Distilled Water, ANOVA: Analysis of Variance, WPM: Woody Plant Medium.

1. INTRODUCTION

Garcinia indica Choiss. (Family Clusiaceae) commonly known as 'Kokum Butter Tree' is an underexploited fruit tree species found in tropical rain forest of Western Ghats of India. Fruits of *Garcinia* have many remarkable medicinal properties, the most important of which is antiobesity effect. Fruit rind of *G. indica* contains hydroxycitric acid (-) (HCA) and garcinic acid. (-) HCA being the strong inhibitor of citrate cleavage enzyme is widely used in formulations of antiobesity drug worldwide [1-4]. Beside antiobesity properties, fruits also possesses anthelmintic, cardio tonic and anti-tumour properties and is useful in the treatment of piles and dysentery. Kokum butter, a preparation from fruits is an emollient and is useful in burns, scalds, cosmetics as well as extender in confectionary items [5]. Fruits also contain several active compounds that exhibit biological activities against bacteria [6], diabetes [7] and HIV-1 protease [8].

Being a tree species, it is slow growing, cross pollinated and polygamodioecious in nature. The differentiation between male and female plants is possible only after flowering stage. Mostly it takes 7-8 years for the plants to bear flower. In horticultural practices, propagation of *G. indica* is generally done by seeds and soft wood grafting [9]. The seeds are recalcitrant due to high sensitivity to desiccation and freezing with shelf life of only 4 weeks [10]. Thus, it becomes difficult to raise seedlings throughout the year. Moreover, in an orchard established with seedlings, 50% plants are generally male. As reported by various workers, grafting is another method that can be used for clonal propagation of *G. indica* [11,12]. The successful clonal propagation of *G. indica* is season dependent, space requiring and moreover grafted plants show change in the canopy with reduced fruit yield, thus has limited applications. Availability of root stock is another limitation for large scale successful grafting.

This medicinally rich plant species is listed as endangered species of Southern India [13], which makes it necessary to conserve this tree species. Conservation of genetic diversity is presently being undertaken on limited scale by maintaining trees *in*

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situ which is associated with various problems. *In vitro* conservation and cryopreservation offer the only safe and cost effective option for long term conservation of genetic resources of recalcitrant species such as *G. indica* [14].

Most of the studies on *in vitro* propagation of genus *Garcinia* have been conducted in *G. mangostana* using variety of explants like seed [15], leaves [16] and via nodular callus formation by Te-Chato and Lim [17]. Repetitive somatic embryogenesis from seed explants in *G. indica* was obtained by Thengane and coworkers [18], while *de novo* shoot regeneration from root cultures was reported by Deodhar coworkers [19]. A method for regeneration of plantlets via adventitious bud differentiation from mature seeds of *G. indica* has been developed by Malik *et al.* [20]. Kulkarni and Deodhar (2002) used immature seeds and young leaves for *in vitro* production of *G. indica* plants [21] while Deshpande *et al.* (1999) and Mathew *et al.* (2001) conducted preliminary studies on *in vitro* establishment of kokum apical buds [22,23]. However, no reports are available on *in vitro* multiplication of female plants of *G. indica*.

In the present study, few female plants with high fruit bearing and HCA content were identified and collected from the different locations in Maharashtra region of the Western Ghats of India and used for clonal propagation. With the aim of clonal multiplication and its conservation, *in vitro* propagation method for these identified elite plants was standardized using different hormones, antimicrobial agents, additive and *in vitro* as well as *ex vitro* rooting.

MATERIAL AND METHODS

Plant material

Apical and axillary buds of *G. indica* were collected from identified elite female trees in different seasons of the year. Buds were first kept in running water for 1 hour and then washed with labolene (Qualigens, India) as a surfactant followed by washing with 10% Savlon (v/v) (Johnson and Johnson Ltd, India) as an antiseptic. This was followed by treatments first with 0.1% (w/v) polyvinyl pyrrolidone (Sigma, USA) for 30 min and later by 1% (w/v) Bavistin (BASF, India) for 60 min. After each treatment, buds were washed thoroughly with sterile distilled water (SDW). All further operations were carried out in laminar air flow (LAF) chamber under sterile conditions. In LAF, buds were first rinsed with 70% ethanol (v/v); washed with SDW for 5-6 times. After treatment with 70% ethanol, buds were treated with 1% HgCl₂ (w/v) (Qualigens, India) for 7 min. and again washed thoroughly 5-6 times with SDW.

Media preparation

These surface sterilized buds were then inoculated on Woody Plant Medium (WPM) [24] supplemented with 6-benzylamino purine (BAP; 8.87 µM) (Sigma, USA). Medium was fortified with 2% sucrose (Qualigens, India) and solidified with 0.75% agar (Qualigens, India) after adjusting the pH to 5.8-6. All growth regulators were incorporated into the medium before autoclaving. The media were sterilized by autoclaving at 120°C

for 20 min at 105 kPa. The inoculated cultures were incubated at 25 ± 1°C at 16/8 hrs photoperiod with 35µE⁻²m⁻²s⁻¹ illumination provided by cool white fluorescent tubes.

Contamination due to fungus was a major hurdle in establishment of sterile cultures. Therefore initially standardization of protocol for obtaining sterile cultures was the first step. To avoid microbial contamination, anti-fungal agent Bavistin alone in concentration range 65 – 85 mg/L or in combination with antibiotic Cefotaxim (300 and 500 mg/L) (Alkem Laboratories, India) were tested. The effect of additive such as Plant Preservative Mixture (PPM) as antifungal and antibacterial agents on microbial contamination was also tested.

To study the effect of culture vessel was studied by using both, cotton plugged culture tubes and plastic capped jam bottles containing 20 and 80 ml medium respectively. For each medium formulation 20 tubes and 15 bottles were used. Single bud was cultured per vessel and each experiment was repeated thrice. The established sterile cultures were tested for *in vitro* response on WPM basal medium supplemented with different combinations and concentrations of plant hormones 6-Benzyl Amino Purine (BAP), Kinetin, Indol-3-acetic acid (IAA) and indol-3-butyric acid (IBA).

In vitro Multiplication

The effect of these hormone on sprouting of buds was studied by supplementing WPM with BAP in the range 4.44-8.87 µM, kinetin in the range 2.32-4.65 µM, IAA 5.71 µM and IBA 4.90 µM either singly or in combination. All the media were fortified with Bavistin (75 mg/L) and Cefotaxim (400 mg/L) along with 2% sucrose and 0.75% agar. After 6 weeks, sprouted sterile buds were shifted to WPM containing BAP (8.87 µM) with Thidiazuron (TDZ) in the range 2.27-4.54 µM for multiplication without any antifungal and antibacterial agent. Explants were subcultured once in two weeks to fresh respective medium.

Elongation and induction of roots

Multiplied shoots were separated and inoculated individually on WPM with 0.88 µM BAP for elongation. For *in vitro* root induction, shoots with 4.4-5 cm height were transferred to half strength WPM macro salts liquid medium fortified with IBA (2.45-4.90 µM) and 1% sucrose for 48 hrs, and then shoots were shifted to half strength plain WPM with 1% sucrose and solidified with 0.2% phytagel (Sigma, USA). After induction of roots in 6 weeks, plantlets were hardened in pots containing a mixture of sterilized soil and sand (1:1) and maintained in controlled growth chamber conditions (25 ± 2 °C, 16-h photoperiod, 80 % relative humidity and irradiance of 50 µmol m⁻² s⁻¹).

Ex vitro rooting

In vitro raised shoots of *G. indica* were also tested for *ex vitro* rooting response. The shoots were treated with aqueous solution containing IAA (342 and 684 µM) and IBA (295 and 588 µM) for 2 hrs, planted in plastic pots containing sand and coco

peat (1:1) and covered with polythene bags to minimize loss of moisture and maintain high humidity. Pots were maintained at $25 \pm 1^\circ\text{C}$ in 24 hrs light photoperiod with watering after every 7 days. The established plants were transferred to greenhouse.

Statistical Analysis

For statistical analysis, analysis of variance (ANOVA) was done by completely randomized block design (CRBD) using Agrobase 99 software for the experiments and the angular transformation values derived as per Snedecor and Cochran [25].

RESULTS AND DISCUSSIONS

G. indica being woody tree species harbors high microflora on the surfaces as well as endophytes. This was quite evident by the high percentage of microbial contamination during establishment of sterile cultures. Endogenous and/or surface contamination was the main factors limiting culture establishment. Ten to 70% of inoculated explants were observed contaminated was after fourth day of culture initiation. Beside contamination, another limiting factor in culture establishment was browning of explants. Like most of the different species of genus *Garcinia*, *G. indica* is also rich in phenolic compounds which can be attributed to the browning of *in vitro* cultures. When inoculated on WPM, latex like secretions were observed that resulted the browning of the explants and adjacent medium. The explants which did not contaminate turned brown and subsequently did not survive. Thus, the initial responses observed were tissue browning and contamination along with bud growth and shoot elongation. Similar limitations due to microbial contamination and browning in micropropagation have been observed by Diaz-Sala *et al.* [26], Yu and Reed [27] and Nas *et al.* [28].

For the establishment of sterile cultures and sprouting, different parameters like effects of different antifungal and antibiotic agents, season of explant collection and type of culture vessel used were studied.

Effect of antimicrobial agents

To avoid the contamination, antifungal agent Bavistin and antibacterial agents Cefotaxim were tested. These antimicrobial agents were chosen based on some initial experiments done. WPM containing $8.87 \mu\text{M}$ BAP with Bavistin 75 mg/L and Cefotaxim 400 mg/L resulted maximum 86% sterile cultures (Table 1).

The supplementation of media with Plant Preservative Mixture (PPM) reduced the sterility to 52.5-62.5% and moreover these explants turned brown after sprouting. Thus in comparison to MG/L, supplementation of media with Bavistin and Cefotaxim was found more effective for establishment of sterile cultures. Also sterile buds on Bavistin + Cefotaxim containing media were healthy (Table 1). Previously, Bavistin in the culture medium was reported to reduce fungal contamination in Litchi [29]. Likewise, Likewise, Cefotaxime was found to be effective in controlling bacterial growth in plant tissue cultures of Chrysanthemum [30], *Lycopersicon* [31] Cucumber [32] etc. Therefore, in this present

study, this antibiotic was used to raise microbe free culture. Niedz (1998) reported that PPM at 0.2% could prevent post culture contamination in plant culture vessels [33]. Babaoglu and Yorgancilar (2000) reported similar observations in the regeneration of salad burnet [34] while PPM was used to establish axenic cultures in wych elm [35]. However, in our study, 75 mg/L Bavistin and 400 mg/L Cefotaxim were found most optimum for establishment of sterile *G. indica* cultures.

Table 1: Effect of antifungal antibiotic and additive agents on control of contamination in *G. indica*.

Medium	Antimicrobial Agents		Additive Plant preservative Mixture (ml)	% Sterility	% Contamination
	Bavistin (mg/L)	Cefotaxim (mg/L)			
WPM + BAP(8.87 μM)	75	0	0	42.5	57.5
	75	300	0	30	70
	75	400	0	68	14
	75	500	0	40	60
	0	0	1	52.5	47.5
	0	0	2	62.5	37.5

Basal media WPM + 2% sucrose + 0.7% agar

Effect of seasonal variation

The season in which plant material was collected for raising *in vitro* cultures showed varied response in terms of contamination and bud break. With optimized sterilization procedure described above, the cultures initiated during the months of January – May showed highest bud break and less contamination than those raised in June – November (Table 2). Since the later period coincides with rainy season in India, 100% explants raised during this season were contaminated. On the contrary, January- May is the pre-fruiting season of the plant which had good response in terms of percent sterility of the cultures. Similar seasonal effect on culture establishment was reported in other tree species such as apple [36], papaya [37], sweet gum [38] and guava [39].

Table 2 - Effect of collection season on establishment of sterile cultures of *G. indica*

Month of collection of explant	Phynology of Plant	WPM+ Bavistin (75mg/L)		WPM+ Bavistin (75 mg/L) + Cefotaxim (400 mg/L)	
		% Sterile cultures	% Contamination	% Sterile cultures	% Contamination
January	Flowering	42.5	57.5	86	14
April	Fruiting	46	55	34	63
May	Fruiting	31	57.5	37	61
September	Vegetative	17.5	72	13	96
November	Vegetative	8	92	4	96

Data scored after 6-8 days

Effect of culture vessels

In the present study, the effect of culture vessel on *in vitro* response was also investigated. High response in tubes than in bottles on WPM media containing $8.87 \mu\text{M}$ BAP was observed. In bottles, the leaves were comparatively small, only partially opened and leaf fall was seen after 4-5 days of incubation. On the

contrary, the leaves were fully opened and green in tubes. This observed response in tubes might be due to proper exchange of gases and the moisture creating a drier microenvironment for the tissue. On the contrary, the moisture in bottles is trapped and the microenvironment of the culture remains saturated with moisture. Similar influence of culture vessels on growth and development of culture was previously emphasized in the cultures of woody species by McClelland and Smith [40].

Effect of Auxines and cytokinins

Nodal explants when cultured on WPM with different concentrations of BAP, Kinetin, IBA and IAA alone or in combination, initiated the axillary bud initiation. Shoot sprouting was initiated after 1-2 weeks. Percent response varied with the type of growth regulator used and its concentration. Among the various auxins and cytokinins tested, BAP alone (8.87 μ M) induced maximum number of explants to initiate shoots (Fig 1b) with 82% response (Table 3). Similar effect of BAP on axillary bud proliferation was observed by previously Martin [41] and Hiregoudar *et al.* [42]. BAP at higher concentrations not only reduced the number of shoots but also resulted stunted growth of shoots. These results indicated that increase in BAP concentrations above certain level does not support enhancement of % response. Shoots were incubated on these particular media for 45 days with shifting on fresh media after every 15 days. Addition of kinetin in media could not significantly increase the % response while addition of 4.60 μ M kinetin reduced the % response. Along with BAP and kinetin, addition of IBA and IAA on bud proliferation was also tested. Like kinetin, IBA and IAA also had no significant effect.

Table 3 Effect of different hormones on % response in *G. indica*.

BAP (μ M)	Kinetin (μ M)	IBA (μ M)	IAA (μ M)	% response
4.44	-	-	-	79
8.87	-	-	-	82
13.32	-	-	-	68
17.76	-	-	-	62
22.20	-	-	-	55
4.44	2.32	-	-	72
8.87	2.32	-	-	75
13.32	2.32	-	-	71
17.76	2.32	-	-	68
22.20	2.32	-	-	65
4.44	4.64	-	-	52
8.87	4.64	-	-	48
13.32	4.64	-	-	41
17.76	4.64	-	-	32
22.20	4.64	-	-	31
4.44	2.32	4.90	-	21
8.87	2.32	4.90	-	25
13.32	2.32	4.90	-	28
4.44	2.32	-	2.85	31
8.87	2.32	-	2.85	35
13.32	2.32	-	2.85	37

Effect of TDZ on multiplication/ multiple shoot induction

TDZ, is a phenylurea derivative known to exert cytokinin like action. It was reported that exposure of explants to an optimum TDZ supplemented medium followed by the withdrawal of growth regulators effectively triggers shoot multiplication in many plant species such as *Psoralea corylifolia* [43], *Tamarindus indica* [44], *Saintpaulia ionantha* [45], *Medicago truncatula* Gaertn. cv. Jemalong and *Medicago truncatula* ssp. Narbonensis [46] and *Hibiscus cannabinus* [47] etc. On the contrary, cultures continuously grown on TDZ containing media resulted in fasciated and distorted shoots. Deleterious effect of continued incubation in TDZ was reported on growth and multiplication of *Cicer arietinum* [48] and *Rauwolfia tetraphylla* [49]. In the view of these reports, *G. indica* tissue was also exposed to WPM media containing TDZ in the range 2.27-4.45 μ M with 8.87 μ M BAP for 45 days with subculture on fresh media after every 15 days. Initiation of multiple shoots was observed after 20 days on media supplemented with TDZ in the range 0.22- 4.54 μ M along with BAP 8.87 μ M. Significantly higher multiplication rate was noted with the combined treatment of 8.87 μ M BAP and 4.54 μ M TDZ (significant at 1% level). With these concentrations of TDZ and BAP, 3-4 shoots with average shoot length of 5.92 cm were induced after third subculture (Table 4) (Fig 1 c and d).

Table 4: Effect of TDZ on no. of multiple shoot induction and elongation of shoots in nodal explants of *G. indica*

Hormones		Number of subculture					
BAP (μ M)	TDZ (μ M)	S1		S2		S3	
		No. of shoots	Length of shoots (\pm SE)	No. of shoots	Length of shoots (\pm SE)	No. of shoots	Length of shoots (\pm SE)
8.87	0.22	1.11	1.1 \pm 0.06	1.5	1.3 \pm 0.8	2.22	1.5 \pm 0.5
8.87	0.91	1.63	0.9 \pm 0.5	2.11	1.2 \pm 0.7	2.44	1.4 \pm 0.8
8.87	1.82	1.78	0.8 \pm 1.2	2.64	1.2 \pm 0.5	2.85**	1.3 \pm 0.1
8.87	2.72		1.4 \pm 0.8	3.62*	2.3 \pm 1.1	3.75*	2.4 \pm 1.1
		2.75**					
8.87	3.63	2.66	1.3 \pm 0.01	3.33*	2.6 \pm 0.9	3.33*	2.8 \pm 0.7
8.87	4.54	2.75**	1.4 \pm 0.5	3.87*	2.9 \pm 0.9	3.87*	3.4 \pm 0.9

Basal media WPM + 2% sucrose + 0.7% agar Values are Mean \pm SE of three experiments. ** * Significant at 5% and 1% level respectively

The shoots were maintained by sub-culturing on the same media after 15 days. At every stage of subculture on same media, shoot multiplication was observed. These shoots were observed to elongate further with repeated subculture on respective media. After 40-45 days, these multiple shoots were separated and transferred to fresh media every 15 days interval. This frequent transfer was noted to be essential to prevent defoliation and to maintain culture vigor for sustained shoot growth. Transfer of original explant directly to media supplemented with TDZ in the range 0.22- 4.54 μ M along with BAP 8.87 μ M did not induce any

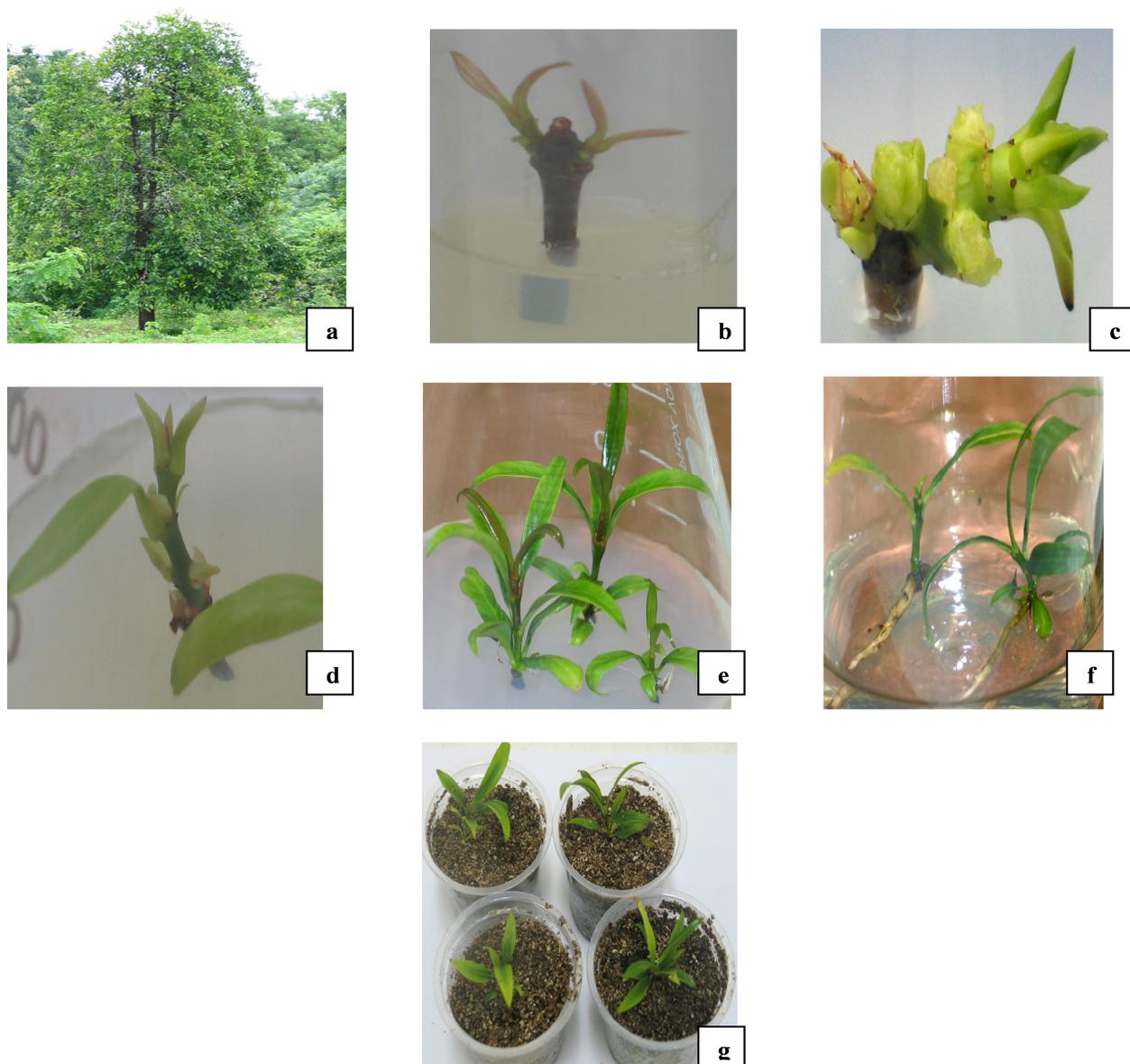


Fig. 1 a. Habitat of *G. indica*. **Fig. 1 b.** Sprouting of axillary bud on WPM containing 8.87 μM BAP with 75 mg/L Bavistin and 300 mg/L Cefotaxim. **Fig. 1 c and d.** Multiple shoots induction on WPM media with 8.87 μM BAP and 4.54 μM TDZ. **Fig. 1 e.** Elongation of multiple shoots on 0.87 μM BAP. **Fig. 1 f.** Rooting of shoots on WPM media with 4.90 μM IBA. **Figure 1 g.** Hardened plants.

additional multiple shoots. New shoots harvested at each subculture were inoculated individually on same media to obtain further multiplication.

Although the phenomenon of fasciated shoots was previously reported to be associated with the use of TDZ [50]. The regenerated plants produced in this study exhibited neither fasciated shoots nor any other abnormal characters. Thus for micropropagation of *G. indica*, the use of TDZ was found best without any adverse effect.

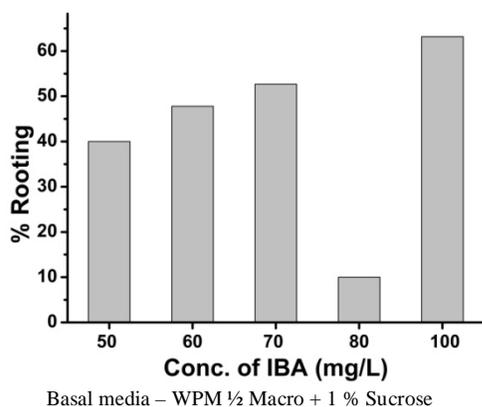
Induction of *in vitro* roots

Shoots when maintained on multiplication media for longer period remained very small and required further elongation step

for rooting induction. Therefore WPM with a low BAP (0.89 μM) was used for shoot elongation. It was observed that reduced concentration of BAP stimulated shoot elongation. Similar observations wherein reduced BAP concentration resulted shoot elongation were made by Chalupa (1987) in *Tila cordat* [51]. However higher concentration of BAP was reported to be inhibitory for shoot elongation [52,53]. It was found that shoots elongated to maximum 4.4-5.0 cm length within 15–20 days after their transfer to BAP containing medium (Fig 1e). To check their rooting competence, these shoots were then transferred to WPM containing half strength macro stock, IBA in the range and 2.45-4.90 μM) and 1% sucrose without agar for 48 hrs. After 48 hr., they were shifted to WPM media containing half strength macro

salts with 1% sucrose and 0.2% phytigel without any hormones. Root induction was observed within 20-25 days from the base of shoots at nodal portion (Fig 2). Maximum 63.2% shoots were observed to induce rooting on WPM supplemented with 4.90 μM IBA with an average 3.4 cm length of shoots. Rooting % was very less (10%) on WPM containing 3.94 μM IBA while with 3.44 μM IBA, 52.7% rooting was observed (Fig 1f).

The well developed plantlets were later shifted to potting mixture of sterilized sand and soil (1:1) for acclimatization. Initially these pots were covered with polythene bags for 15 days which then was gradually removed. Acclimatized plants were then transferred to green house, where 70% survival was achieved.



Basal media – WPM ½ Macro + 1 % Sucrose
Fig. 2: Effect of different IBA concentrations on *in vitro* rooting in shoots of *G. indica*

Ex vitro rooting

In this study, experiments were also performed to induce the roots to *in vitro* grown shoots *ex vitro*. For *ex vitro* rooting, shoots were dipped in IAA (3.42 and 6.84 μM) and IBA solutions (2.95 and 5.88 μM) for 2 hrs and then shifted to potting mixture containing sterilized sand and cocopeat (1:1), and covered with polythene bags to maintain moisture. Forty percent shoots induced *ex vitro* rooting to IAA (3.42 μM) treated shoots within 50-60 days (Table 5). Such *ex vitro* induced rooted shoots in pots were maintained in hardening room with controlled temperature (25°C) and continuous light. Plants were watered after every 8 days and were then shifted to greenhouse where 60% plantlets were observed survived and healthy (Fig 1g).

These results demonstrated the feasibility of the application of tissue culture technique for raising female and elite trees of *G. indica* - a tree of great economic importance. Generally woody plants are known to be recalcitrant to *in vitro* regeneration and multiplication. In the view of this fact, the rate of multiplication in *G. indica* was also low since the explants were derived from mature woody trees. Unlike for other horticultural plants, the use of tissue culture techniques for the propagation of tree species like *G. Indica* was beset with very special problems. Some of these include the physiological state of the material (Juvenile/ mature phases), general recalcitrant response of the explants vis a vis medium, inadequate rooting of the regenerated shoots and the associated problems of poor transfer ratio of

established plants into soil. Nevertheless, micropropagation using tissue culture is the best and widely used method for clonal propagation of selected clones. Previously, many plant species of medicinal importance such as *Baliospermum montanum* [54,55], *Spilanthes acmella* [56], *Curculigo orchioides* [57], *Azadirachta indica* [58] etc. were successfully clonally propagated.

Table 5: Effect of different auxin concentrations on *ex vitro* rooting

Auxin concentration	% rooting
IBA (2.95 μM)	10
IBA (5.88 μM)	10
IAA (3.42 μM)	40
IAA (6.84 μM)	20

CONCLUSION

This study reporting the clonal method of propagation of *G. indica*, a economically important horticultural tree species with high medicinal importance, offers a great potential for mass multiplication of female plants for large scale plantation. Along with this, the elite female plants having maximum % HCA can also be propagated by this method. Using the method reported in this study, 4-5 plants from single apical bud can be produced within 130-135 days through *in vitro* rooting and in 165-170 days through *ex vitro* rooting.

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