Original Research Article
Optimization of IBA for efficient survival of micropropagated *Garcinia indica* Choisy shoots using In-vitro and Ex-vitro rooting techniques

ABSTRACT

Aims: The study focuses on optimization of concentration and time of rooting hormone exposure of IBA for efficient survival of tissue culture raised *Garcinia indica* Choisy plantlets for in-vitro and ex-vitro rooting techniques

Study design: The subcultured microshoots of *Garcinia indica* were subjected to in-vitro and ex-vitro rooting trials by treating them with IBA of varying concentration and time, to standardize these particular parameters required by this auxin to induce rooting.

Place and Duration of Study: Rooting trials were carried out in Plant tissue culture-Biochemical Sciences Division of CSIR- National Chemical Laboratory, Pune 411008 between June 2018 and April 2019.

Methodology: Regularly subcultured five to six years old shoots from female trees of *Garcinia indica* were used for the study. Various concentrations of IBA in correlation with time were used for in-vitro and ex-vitro root induction. The rooted plantlets were then transferred to polyhouse for acclimatization and will further be planted in open field locations in June 2019.

Results: Induction of rooting was observed within thirty days of treatment with IBA. It was observed that 500ppm of IBA gave 30% rooting for in-vitro rooting trials whereas 2000ppm of IBA induced 80% rooting for shoots given ex-vitro rooting treatments. An interesting phenomenon that was observed for 70% of the shoots which failed to induce rooting by in-vitro treatment was that they survived with 100% rooting success under ex-vitro rooting conditions. The hardened plantlets were successfully acclimatized in the polyhouse with survival rate of 90% and were further transferred to polythene bags with rooting mixtures of sand: soil: farmyard manure. These plantlets have been healthy for the last 6 to 9 months and will be transported for field trials in June 2019.

Conclusion: Ex-vitro rooting technique was found to be more effective than in-vitro rooting. Thus, by optimizing the rooting hormone parameters, female plants of *Garcinia indica* can be successfully grown using tissue culture technology and can be propagated in large numbers to increase the female plant number in plantations.

Keywords: Garcinia indica, IBA, optimization, in-vitro, ex-vitro, hardening.

1. Introduction

Garcinia indica: A fruit bearing tree species commonly known as 'kokam' or 'Indian butter tree', is one of the native underexploited tree species for research and development found in

the Western Ghats of Maharashtra, Costal Karnataka and Goa [1]. Rind and seeds of the fruits are used in preparations of beverages and kokam butter respectively. Kokam butter is used as an essential ingredient in many beauty and cosmetic products owing to its soothing and smoothing properties. It is a saturated fat which is solid at room temperature and liquid when heated [2]. The fruit rind of kokam is rich in Garcinol, Anthocyanins and (–) Hydroxycitric acid (-HCA), which is an important biologically active plant metabolite used as an antiobesity drug [3],[4],[5],[6]. Being a tree species, it is cross-pollinated and Polygamodioecious in its natural population i.e. 50% would be female and 50% would be male trees which can be differentiated only after flowering and fruiting stage. In nature it takes seven to eight years for the tree to start bearing flowers [7]. The seeds are recalcitrant and are quick to lose viability. Therefore, it is difficult to raise seedlings throughout the year. However, greater variability among the genotypes which differ in production and quality, dioecious nature of plants, dominance of tropism in vegetative phase and harvesting of fruits at the onset of rainy season are a few of the obstacles faced in commercial cultivation of kokum.

Protocols of micropropagation using seed explants have been developed for this recalcitrant tree species [8],[9],[10]. The ultimate success of micropropagation depends on the ability of successful rooting of in-vitro shoots. As, in-vitro grown shoots are adapted to growing in 80% relative humidity and in specialized nutrient media components supplied with sugar and other growth regulators, direct transfer of in-vitro grown shoots to ex-vitro conditions is a critical step. The present paper describes the protocol for optimizing the rooting parameters to ensure the efficient survival of *G.indica* by in-vitro and ex-vitro rooting techniques. The in-vitro grown shoots which were used for these rooting trials were subcultured shoots of the last five to six years developed from female tree shoot buds collected during 2014-15 between January-May. Shoots were regularly sub-cultured in multiplication media standardized previously [11]. This medicinal plant is listed as endangered species of Southern India [12],[13]. Due to increasing importance for traditional medicine in recent years, reintroduction of *Garcinia indica* in locations where it is endangered along with its in-situ conservation is very essential for human race. Thus, using biotechnological tools for ensuring the survival of rare species is necessary. Various protocols for in-vitro propagation of threatened species is standardized and can be used for conserving plants facing the threat of extinction [14]. Rapid in-vitro multiplication and conservation of *Garcinia indica* offer the only safe and cost effective method for long term conservation of such species [15]. Root explants of *Garcinia indica* could also be used effectively used for direct shoot regeneration and large scale multiplication [16].

Since *G.indica* is an economically important tree and endangered in southern part of India, for in-vitro conservation by micropropagation to become a reality in future, in-vitro and ex-vitro rooting methods have been standardized using various concentrations of IBA during present study.

2. Material And Methods

2.1 Place of study

The research work was carried out at Plant Tissue Culture-Biochemical Science Division in CSIR-National Chemical Laboratory, Pune.

2.2 In-vitro shoots for rooting

Regularly subcultured microshoots of five to six years old *Garcinia indica* Choisy cultures incubated at 25±1℃ and 16/8 photoperiod were used to carry out rooting experiments (Fig 1a).

2.3 Subculturing

Shoots were regularly subcultured in autoclaved jam bottles of dimensions 5.5cm x 12.5cm with autoclavable polyvinyl caps containing multiplication WPM media (Woody Plant Medium,

[13] *Lloyd* and McCown 1982) supplemented with growth hormones like BAP 2 mg/L, KIN 1mg/L, IBA 1 mg/L along with 3% sucrose (w/v) and 0.4% phytagel (w/v). All growth regulators were incorporated into medium before autoclaving. In order to avoid contamination, 50mg/L of antifungal agent Bavistine (BAIF, India) was added. Medium was autoclaved at 120℃ for 20 mins at 105 kPa. Sterile 100mg/L of antibacterial agent Cefotaxim (Alkem, India) were added to the autoclaved medium as a maintenance dose.

2.4 In-vitro Rooting

For in-vitro root induction, in-vitro grown shoots of length 4-5 cm long from which lower leaves were removed were used and a slanting cut was given to the base of each shoot. Varying concentrations of filter sterilized IBA of 300, 400, 500, 700, 800, 2000 mg/L with varying time periods of 24, 48 and 72 hours were used for root induction. IBA was filter sterilized to eliminate contamination and maintain aseptic conditions. The shoots were then dipped into 5 ml of each WPM basal liquid rooting medium in test tubes of 2.5cm x 15cm. Each tube was fortified with these concentrations of filter sterilized IBA and incubated for 24, 48 and 72 hours in dark at 25±1^{o}C. The treated shoots were shifted to *G.indica* rooting media containing WPM basal medium, 1.5% Sucrose, 0.4% Phytagel devoid of growth hormones in test tubes and incubated in culture room at 25±1^{0}C and 24 hrs light conditions. The shoots were regularly observed for rooting.

2.5 Ex-vitro Rooting

In-vitro grown, elongated shoots of *G. indica* were tested for ex-vitro rooting response. The shoots were washed carefully with water to remove traces of stuck agar and treated with 0.1% bavistin for 10min. Slanting cuts were given at 1.5 cm from the basal portion of the shoots. They were dipped in aqueous solution of IBA of different concentrations ranging from 400, 1000, 2000, 3000 and 4000 mg/L for 5, 15, 20, 25 and 30 mins. These were then planted in plastic cups containing pre sterilized fine sand and sealed with another cup on top using cling wrap/paraffin to minimize the loss of moisture and maintain high humidity during rooting and hardening. Cups were maintained at 25±1ºC in 24 hrs light with watering after every third day by dipping the base of the cups (in which holes were made previously), using autoclaved water.

2.6 Hardening

In-vitro rooted plantlets having atleast two roots of 2 to 4cm in length were washed carefully with water to remove traces of agar and then transferred to plastic cups containing sterilized sand in 1:1 ratio; covered properly to prevent desiccation. Cups were maintained at 25±1ºC in 24 hrs light of 2000 lux photoperiod. After 1 month the plastic covers were removed and the plantlets were maintained in moist sand for more than 30 days. After 2 months plants were shifted to plastic pots and kept in the polyhouse at 27±1ºC. Water was given to the plants every morning and evening by a sprayer and also the pots were dipped in water as done for ex-vitro shoots. After 4 months the plants were shifted into the polythene bags and were ready for field trials.

The same procedure was carried for ex-vitro rooted plantlets but the cups were maintain for more than 2 months as both rooting and hardening took place simultaneously and the data was recorded every 15th day for each concentration of IBA. Minimum of 30 plants were used for the standardization experiments and repeated thrice; and the data was analyzed by Standard deviation for both in-vitro and ex-vitro rooting experiments.

3. RESULT AND DISCUSSION

3.1 In-vitro rooting

In-vitro root induction was noticed between 20-30 days at 300-400 mg/L of IBA treated shoots. Callus formation was not seen in these shoots at any of the IBA concentrations. Only 15-20% of shoots demonstrated rooting at these said concentrations of IBA. However, shoots

which were treated with 500 mg/L of IBA for 48 hours, root induction was observed within 15-20 days directly from the shoot base (Fig 2a). Out of 24, 48 and 72 hours incubation with IBA, shoots treated for 48 hours were healthier and showed rooting. Hence, 48hours incubation time was seen to be optimum for the present study (Fig 2a). Maximum of 30% rooting was observed for shoots supplemented with 500 mg/L IBA concentration (Fig 2b). Shoots supplemented with more than 700 mg/L of IBA concentration dried and failed to induce roots, may be due to excess concentration of IBA for shoots.

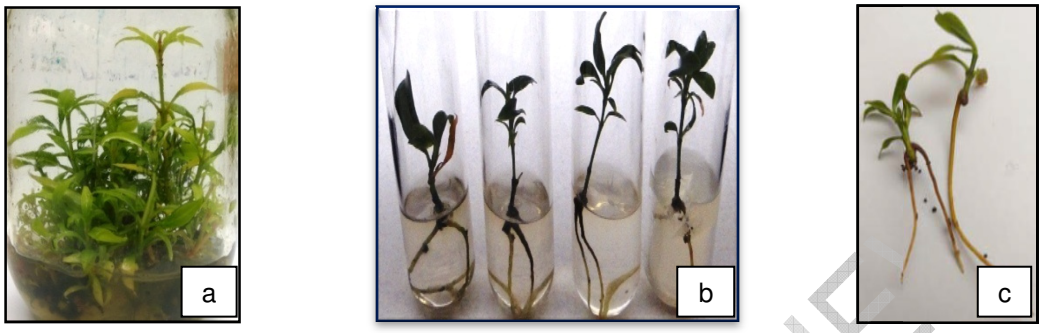


Figure 1: a. 5-6 years old elongated in-vitro shoots of *Garcinia indica* regularly subcultured on Multiplication media b. In-vitro rooting on medium supplemented with WPM + 1.5% Sucrose + 0.4% Phytagel with 500 mg/L IBA dip for 48hrs. c. Adventitious root system after in vitro root induction.

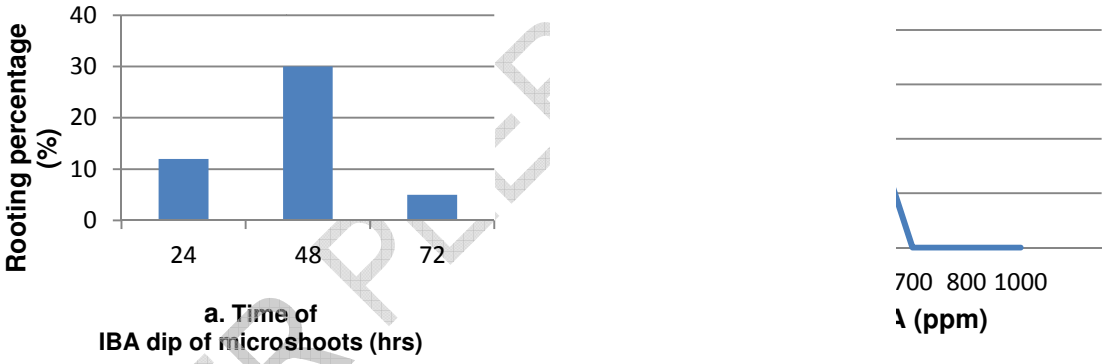


Figure 2a: Effect of time parameter on rooting percentage. Dipping the shoots for 48 hours in IBA solution gave maximum rooting of 30%. b. Effect of different IBA concentrations on in-vitro rooting of shoots, 500ppm was found to be most effective

3.2 Ex-vitro rooting

For ex-vitro rooting, 80% of the shoots which were treated with dipping in 2000 mg/L of IBA for 15 minutes were rooted within 30 days (Fig 3a). However, there was a decline in rooting success with the further increase in IBA concentration. We have tried IBA dip from varying time range of 5 mins to 30 mins for all concentrations of IBA and found that dipping of shoots for 15 mins was an optimum time to induce rooting in 80% of the total shoots (data not shown). Simultaneous rooting and hardening of shoots is the highlight of ex-vitro rooting technique. The acclimatization of these ex-vitro rooted shoots was seen by change of leaf color to darker shade, elongation of the leaves, sturdiness of shoots and base of the shoots became swollen before roots emerged (Fig 3b). Elongated ex-vitro roots sometimes push the shoots above sand and could be observed easily by eyes (Fig 3c). No callus was seen at the root shoot junction and a clear root-shoot connection was observed (Fig 3d). Once the leaves of the shoots became steady in culture room under sterile conditions, they were shifted for further acclimatization in the polyhouse in plastic cups (Fig 4a). After 2 months of hardening in humidity controlled normal polyhouse the plantlets were shifted to bigger pots (Fig 4b) and they were transferred to low cost polyhouse (data will be discussed in next paper) for further

acclimatization. After 8 months the length of these roots ranged from 20 to 34cm and these acclimatized plants in pots were shifted to black polythene bags (Fig 4c). It has been planned to transport these plantlets for the field trials at their native locations once the monsoon set-in in June 2019 in India.

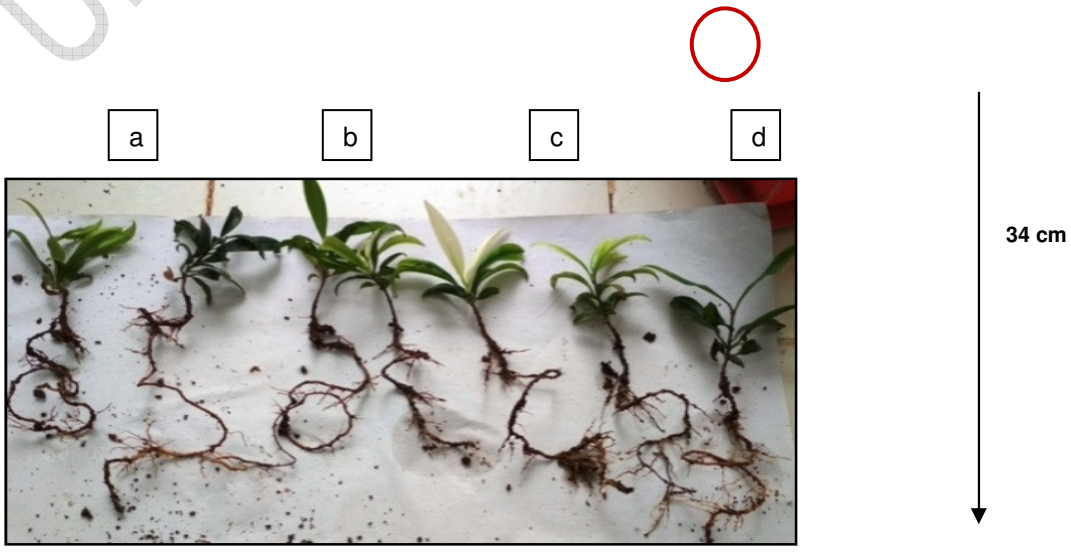
An interesting observation made during this present study was how the shoots which were given in-vitro rooting treatment initially showed a greater success in rooting after ex-vitro rooting treatment. Around 70% of the unrooted in-vitro grown healthy shoots whose leaves were turning dark green in color but had not shown any signs of root induction even after 30 days, were given ex-vitro rooting treatment with 2000 mg/L IBA. On observation after 15 days, rooting had been induced and on observation after 90 days demonstrated 100% rooting. These plants are much more healthy in appearance as compared to both in-vitro and ex-vitro rooted shoots (Fig 3f).

Table 1 shows the percent rooting at different IBA concentrations and average root and shoot length at different IBA concentrations during present study.

IBA Conc. (mg/L)	No. of Shoots inoculated	Rooting % of shoots after 30 days (%±SD)	Average root length (cm) after 60 days (cm±SD)	Average root length (cm) after 80 days (cm±SD)	Average shoot length after 60 days(cm) (cm±SD)
400	40	24±0.57	2.5±0.63	5.5±0.50	3.5±0.50
1000	40	50±2.30	3.5±0.41	5.3±0.43	3.5±0.40
2000	**40**	**80±4.61**	**3.7±0.25**	**5.4±0.52**	**5.0±0.50**
3000	40	65±2.30	3.1±0.35	5.5±0.50	3.6±0.28
4000	40	60±3.45	3.4±0.28	5.2±0.32	3.2±0.25

Table 1: Effect of varying IBA conc. on ex-vitro shoots for rooting.

For 40 shoots that were inoculated, it was seen that 2000mg/L of IBA induced rooting in 80% of the shoots; as seen after 30 days. The average root length was 3.7cm and 5.4cm, as seen after 60, 80 days respectively. The average shoot length after 60 days was seen to be 5cm.



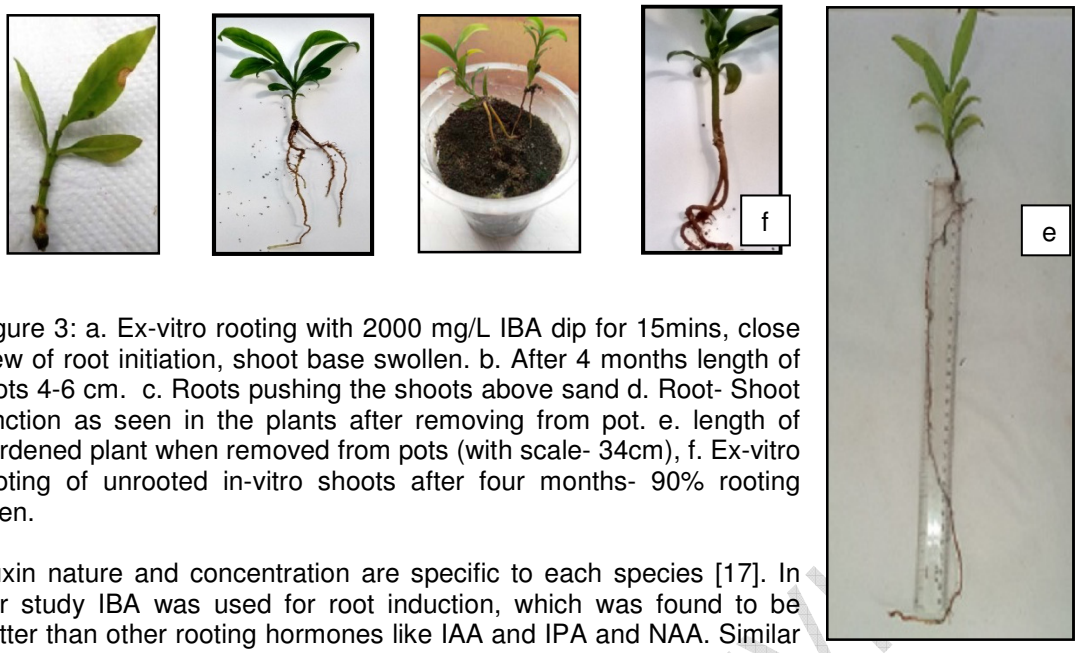


Figure 3: a. Ex-vitro rooting with 2000 mg/L IBA dip for 15mins, close view of root initiation, shoot base swollen. b. After 4 months length of roots 4-6 cm. c. Roots pushing the shoots above sand d. Root- Shoot junction as seen in the plants after removing from pot. e. length of hardened plant when removed from pots (with scale- 34cm), f. Ex-vitro rooting of unrooted in-vitro shoots after four months- 90% rooting seen.

Auxin nature and concentration are specific to each species [17]. In our study IBA was used for root induction, which was found to be better than other rooting hormones like IAA and IPA and NAA. Similar results were also observed by Deodhar et al,(2014) for *G.indica* Choisy, where they reported IBA as the optimum hormone for root induction [11]. Panwar et al, (2018) for *Tinospora cordifolia*, Jaime et al, (2018) for Smoke tree propagation has also shown such results [18],[19]. Carbohydrate concentration plays a major role in acclimatization as plantlets are transferred from heterotrophic to autotrophic conditions. Therefore, in order to increase the survival percentage it is necessary to transfer the plants to field gradually through various hardening stages with high light intensity and low humidity conditions [20]. In-vitro rooting and hardening of plantlets from immature seed culture of *G.indica* have been developed but the survival rate of acclimatized plantlets when transferred to greenhouse has not been mentioned [21]. As compared to many other tree species studied in this laboratory in the last 40 years, hardening of *G.indica* seems multi-step and laborious as compared to other tree species.. Only survival in soil is not enough unless field trials are performed for next 3-6 years till fruiting occurs in the tissue culture raised female plants. Our main objective is to supply the tissue culture raised plants of this laboratory in various kokam growing regions in Konkan with the help of foresters and Konkan Agriculture University for field trials.

When rooting was induced by IBA in *G.indica* in in-vitro and ex-vitro rooting trials, during the present study adventitious roots system was initially observed. However, when the plantlets were shifted to pots from cups, natural tap root system had developed in the plants with good root hair. Plantlets developed through this method have roots without any callus at the base of micro-cuttings, just like the natural root system and has higher rooting rates, root length and survival rate; as compared to in-vitro developed plantlets. This technique is economical in terms of labor and time saving. Deodhar et al, (2014) discussed about 60% plantlets survival in greenhouse after their transfer to soil [11]. There are several reports on in-vitro and ex-vitro rooting and ex-vitro survival of medicinal plants [22], [23] but field trials of the survived plants have not been mentioned by them

Figure 4: a. shows shoots planted in cups after rooting treatment. b. Survived, rooted plants transferred from cups to pots to enhance root growth. c. successfully rooted plants transferred to black polythene bags and maintained in Low-cost polyhouse, ready to be supplied for field trials in natural locations around Konkan region of Maharashtra.

4. CONCLUSIONS

This study demonstrates an efficient rooting method for propagation of female trees of *G.indica*- a tree of great economic and medicinal importance. Using the method reported in this study 90% survival of acclimatized plantlets is achieved. Development of well-formed and functional root system is one of the essential steps for water and mineral uptake and the plants to get acclimatized in the soil, which has been achieved during present study. Such plants when transported for field trials at natural locations will give a good survival rate.

CONSENT (WHERE EVER APPLICABLE)

No consent was required for publication of this manuscript.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

No animal studies have been carried out in during the course of this study.

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