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Indian Journal of Natural Products and Resources
Vol. 3(1), March 2012, pp. 65-72

In vitro and *in vivo* adventitious bud differentiation from mature seeds of three *Garcinia* spp.

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Received 13 December 2010; Accepted 17 September 2011

An efficient method of propagation of three endemic species of *Garcinia* Linn., *viz. G. indica* Choisy, *G. tinctoria* Dunn. and *G. gummi-gutta* (Linn.) N. Robs. was developed to produce plantlets with high level of shoot multiplications and root formations since the traditional methods of propagation of *Garcinia* has some limitations. Propagation studies conducted in present investigation has shown that MS medium supplemented with 6-benzyl amino purine (BAP, 2.5 mg/l) gave best response and induced multiple shoot initiation. Root initiation took place in MS medium with or without α-Naphthalene Acetic Acid (NAA) but root elongation was faster in MS medium supplemented with 2 mg/l of NAA. Variation in *in vitro* responses were observed in all these three species. *In vivo* seed germination studies were conducted to understand the seed germination pattern in these species. This technique can be used for multiplication of elite genotypes and conservation of *Garcinia* species.

Keywords: *Garcinia indica, Garcinia tinctoria, Garcinia gummigutta,* Propagation; *In vivo, In vitro,* Seed germination, Adventitious bud.

IPC code; Int. cl. (2011.01)—A61K 36/00, A23L 1/221.

Garcinia spp., belonging to family Clusiaceae, are potential underexploited tree spice and medicinal plants. It is currently gaining much medicinal and culinary importance. Irrespective of its importance, many species of *Garcinia* are threatened due to habitat destruction. It was reported that the population density of many species is dangerously low and is reduced to one or two trees in a given location making survival of these species very difficult[1]. This is further aggravated by the fact that seeds fail to produce seedlings due to various physiological and environmental factors making natural multiplication and maintenance of these species impossible. Some *Garcinia* spp. are included in the list of endangered species of medicinal plants of southern India[1].

Among the *Garcinia* species, *G. indica* Choisy, *G. tinctoria* Dunn. and *G. gummi-gutta* (Linn.) N. Robs. are endemic to Western Ghats. The tree growth is slow and propagation is usually done by seeds and softwood grafting[2]. The seeds are recalcitrant due to high sensitivity to desiccation and freezing with shelf-life of only 4 weeks[3]. Hence, it is not possible to raise seedlings throughout the year. Softwood grafting, another method used for clonal propagation of *G. indica*[4, 5] is season dependent and is cumbersome in nature thus finding limited application. Another bottleneck is the limited availability of rootstocks for grafting. Improvement of tropical fruit trees by conventional breeding methods in general is limited due to complex genetic systems and extended juvenility. Hence there is an urgent need to find alternate and efficient methods of propagation for multiplying these species. The present study is to develop a simple and efficient method for high frequency plant regeneration from the seeds of these three species. Two approaches, viz. micropropagation and *in vivo* germination of seed fragments were attempted.

Most of the studies pertaining to *in vitro* culture of genus *Garcinia* spp. have been conducted in *G. mangostana* Linn. using seed and leaf explants[6-9]. Limited attention has been given to *G. indica*, *G. tinctoria and G. gummi-gutta* which have recently been recognized as important horticultural and medicinal tree species. Kulkarni and Deodhar[10] used immature seeds, young leaves, apical and axillary buds for *in vitro* establishment of *G. indica*, while Deshpande *et al*[11] and Mathew *et al*[12] conducted preliminary studies on *in vitro* establishment of kokum apical buds. However, no reports are available

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regarding *in vitro* multiplication and conservation of *G. tinctoria* and *G.gummi-gutta*.

Joshi *et al*[13] studied that seeds of *G. gummi-gutta* follow 'garcinia-type' of germination in which the primary root and shoot emerge from the opposite ends of the seed when the seeds were allowed to germinate in sterile moist germination paper. *In vivo* seed germination studies were not reported in *G. tinctoria* and *G. indica*. Therefore, present investigation was taken up to study the *In vitro* and *in vivo* adventitious bud differentiation from mature seeds.

Materials and Methods

In vitro propagation

Fruits of three species *G. indica, G. tinctoria* and *G. gummi-gutta* were collected from the orchard of Indian Institute of Spices Research, Calicut, Kerala. Fruits were washed under running tap water and then wiped with alcohol. The fruits were cut open in laminar air flow hood and the seeds were removed. The seed coat was removed carefully with the help of scalpel. The seeds were then treated with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes and washed three or four times in sterile distilled water before placing them on sterilized filter paper to dry.

The seeds were cultured in two ways: as whole seeds and seeds cut vertically into two fragments. The whole seeds and cut segments were cultured on MS medium (Murashige and Skoog)[14] supplemented with various concentrations of BAP (0, 1, 2, 2.5, 3, 4 mg/l). The media were supplemented with 30 g/l sucrose and solidified with 8g/l agar 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. After the shoot developed, the cultures were transferred to media containing BAP (1mg-4mg/l) and NAA (1 mg and 2 mg/l). The cultures were first kept in dark for 72 h and incubated at 22°C. They were given a photoperiod of 16 h with a light intensity of 2000 lux. Subculturing was done in every 21 days. The data were subjected to statistical analysis to obtain the least square difference.

In vivo seed germination studies

Fruits of *G. indica, G tinctoria* and *G.gummi-gutta* were cut open and the seeds were removed. The seed coat was removed and the seeds were washed with alcohol followed by washing with sterile water for three or four times. The seeds were placed on sterile filter paper to dry. The seeds were then kept in sterile moist germination paper at room temperature (25 ± 2°C).

Results and Discussion

Micropropagation of Garcinia

Garcinia seeds developed apomictically are said to be homogenous. *Garcinia* is usually propagated through seeds. The size of the seed and age of parent plant have effect on the seedling vigor. Conventional vegetative methods have not been successful as the cuttings do not root and the grafted buds are killed by the fermentation of the yellow resinous latex released when the cortex is cut opened to receive the bud[15]. Seeds are produced at particular season. *Garcinia* seeds do not germinate in nature easily, slow growth, loss of early seed viability and difficulties in rooting of stem cuttings has led to search for alternative means for propagation of *Garcinia* spp. Basra[16] reported that the endosperm of some species is responsible for seed dormancy. In case of *G. gummi-gutta* the seed dormancy of 7-8 months was reported by Rajendran *et al*[17]. In view of the above, research work was undertaken for developing methods of propagation of *Garcinia indica*, *G. tinctoria* and *G. gummi-gutta*.

The seeds of the three *Garcinia* species were initially cultured in MS medium containing cytokinin BAP concentrations of 0, 1, 2, 2.5, 3 and 4 mg/l. After the shoots developed to a height of about 8 cm, the cultures were transferred to MS medium containing cytokinin BAP (1 to 4mg/l) and auxin NAA (1 and 2 mg/l). The cultures were subcultured every 21 days. A perusal of Table 1 would reveal that there were statistical difference among species as well as among the treatments besides a significant interaction. The Figure 1 would indicate that among the species studied, *G. indica* showed very fast response in culture and *G. gummi-gutta* was slow to respond. Among the BAP levels tested, the response was significant at 2.5 mg/l.

In *G. indica*, whole seeds gave rise to rooted plantlet within 2 months (Plate 1). The proximal end of the seed that was cut into two segments gave rise to shoots within a period of six to ten days (Plate 2c) but the distal end took a longer time for shoot initiation. After shoot initiation, when the shoot had reached 8 cm height they were subcultured in a medium containing NAA of concentration 1 and 2 mg/l. Primary root develops from the distal end and multiple

Table 1—Effect of BAP on induction of shoot, root and leaves in three species of *Garcinia*

S. No.	Species	Conc. of BAP (mg/l)	Time of shoot initiation (days)	Time of primary root initiation (days)	Time of adventitious root initiation (days)	Time of leaf initiation (days)
1.	*G. indica*	0	11	12	28	35
		1	9	10	18	27
		2	7	8	16	23
		2.5	6	6	16	20
		3	7	6	18	21
		4	7	6	17	23
2.	*G. tinctoria*	0	25	26	35	45
		1	15	16	20	32
		2	15	16	23	30
		2.5	13	15	25	35
		3	13	14	26	35
		4	15	17	25	39
3.	*G. gummi-gutta*	0	28	30	55	48
		1	23	23	48	45
		2	21	22	50	49
		2.5	21	20	45	42
		3	24	28	55	52
		4	23	23	49	47
Lsd (0.05)			3.145	4.210	4.402	7.375

shoot proliferation was observed in the distal end after 2 months when the medium was supplemented with BAP (Plate 2e). Shoot proliferation was observed in the middle segment also but that took a longer time (after 2 months). Hence, it was possible to get shoots and root from all the segments of the seed. *G. indica* cultures exhibited multiple shoot proliferation when segments were inoculated in a medium containing BAP concentration of 1 to 4 mg/l and maximum shoot proliferation was observed at BAP concentration of 2.5 mg/l (Plate 2g). Malik *et al*[18] reported that BAP was found to be more potent compared to other cytokinins used. Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue[19]. The shoot buds were formed either from the peripheral cells or the cells beneath. So, the meristematic activity of the cells was intensified and uniform throughout the seed segment. Similar results were reported by Bhojwani and Razdan[20] in many species of Angiosperms. These shoot buds were differentiated or originated from the peripheral cell of the endosperm and possessed distinct shoot apices and well differentiated vasculature[20, 21].

Murthy and Patil[22] conducted *in vitro* regeneration studies in *G. tinctoria.* They observed that seed explants developed an average of 11.1 multiple shoots on the medium supplemented with 3mg/l BAP. In the present study, multiple shoots were not observed in *G. tinctoria* but shoot initiation took place earlier in the medium supplemented with 2.5 mg/l of BAP (Plate 3a). Shoot initiation took place within 13 days. Adventitious root formed within 23 days. Leaves developed after 30 days. In the medium without BAP, shoot and root initiation was noticed but later than 45 days (Table 1). Whole seeds gave rise to plantlet faster than the seed fragments (Plate 3 a, b and c). Shoot and root initiation in seed fragments took the same time as in whole seed, but for shoot elongation it took a longer time. After shoot initiation, when the shoot had reached 8 cm height they were subcultured to medium containing NAA of concentration 1 and 2 mg/l. In the present study only the whole decoated seed of *G. tinctoria* has given rise to a healthy plantlet and transferred to sterile soil after 3 months (Plate 3c). Though the seed fragment initiated shoot and root but fully formed plantlet was not noticed within five months.

G. gummi-gutta seeds take more time to germinate than the other two species. The shoot bud differentiation from inoculated seed and seed segments was observed 21 days after inoculation onto MS medium supplemented with 2.5 mg/l of BAP (Plate 4b and e). After shoot initiation, when the shoot had reached 8 cm height they were subcultured to medium containing NAA of concentration 1 and 2

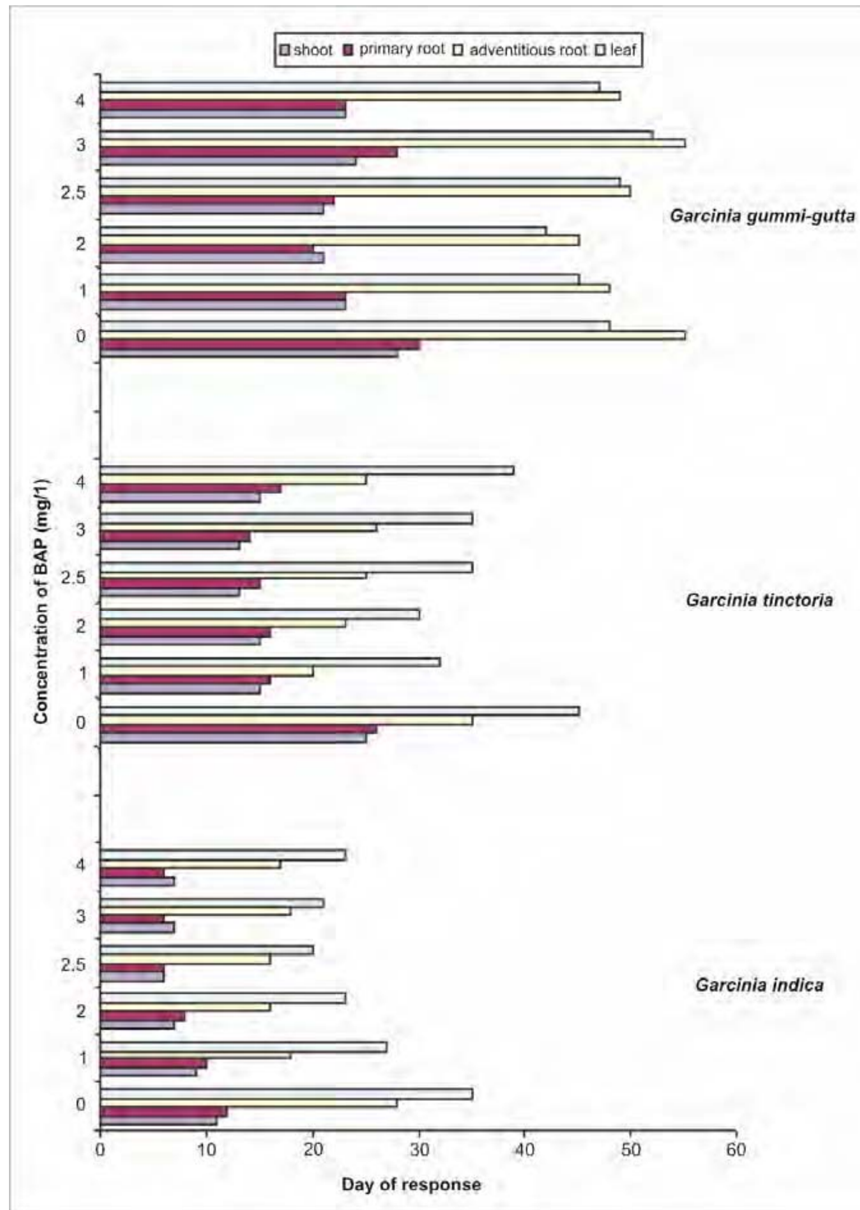

Figure 1—Effect of BAP concentration on initiation of shoot, root and leaves in three species of *Garcinia*

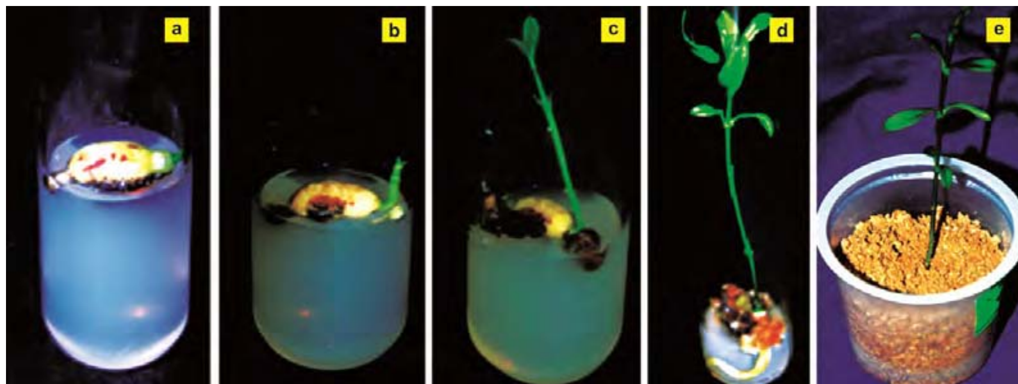

Plate 1—*In vitro* plantlet regeneration from whole seed of *G. indica* (a) after 2 weeks (b) after one month (c) after 2 months (d) after 3½ months (e) plantlets transferred to soil.

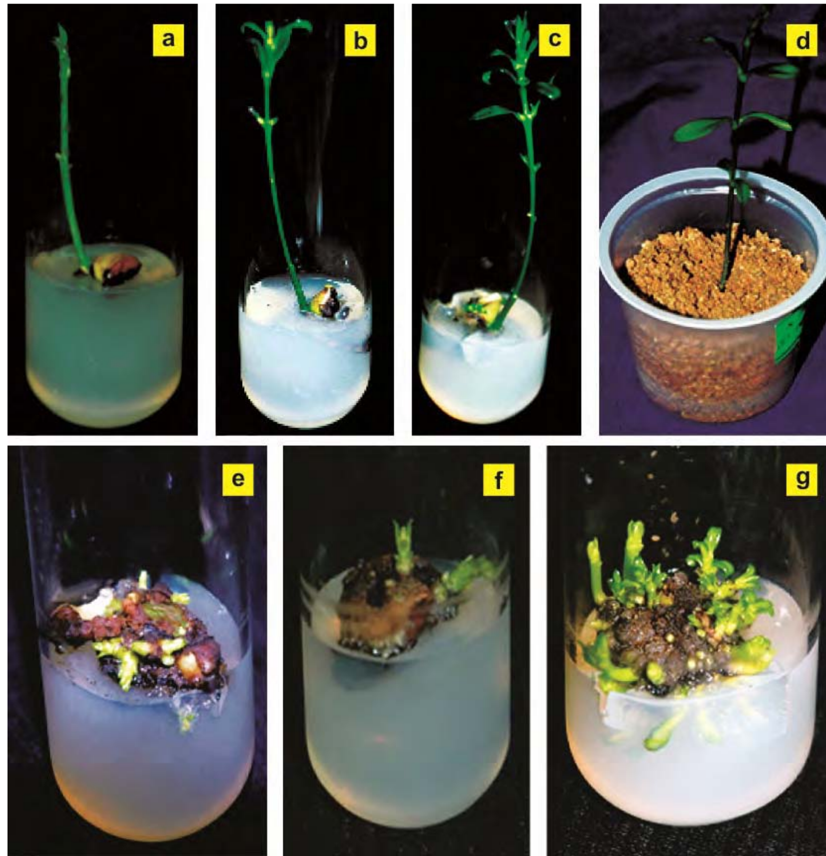

Plate 2—*In vitro* plantlet regeneration from seed segments of *G. indica* (a) shoot initiation in proximal end after one month (b) after 2 months (c) after 3½ months (d) plantlet transferred to soil (e) shoot proliferation from the distal end after 2 months (f) shoot proliferation from middle piece (g) multiple shoot proliferation from seed segment of *G. indica* when medium supplemented with 2.5 mg/l of BAP.

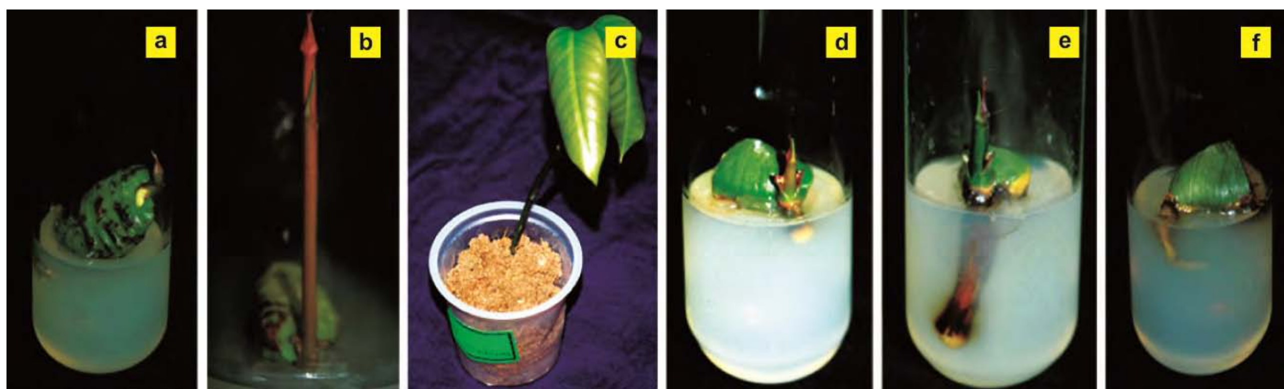

Plate 3—*In vitro* plantlet regeneration from seed and seed segments of *G. tinctoria* (a) shoot initiation from whole seed after 3 weeks of culture (b) after 1 ½ months (c) after 3 months (d) shoot initiation from the seed cut into two fragments proximal end after 1 month (e) proximal end after 2 months (f) primary root developed from the distal end after 2 months.

mg/l. The time taken for shoot elongation is faster than *G. tinctoria*. Leaves developed after 45 days. After 2 months the rooted plantlets were transferred to soil (Plate 4c). Multiple shoot proliferation was observed in *G. gummi-gutta*. The results of the present study are almost similar with the findings of Rajendran *et al*[23].

It has been observed in the present study that BAP concentration of 2.5 mg/l is the best for shoot initiation in two species, *G. indica* and *G. gummi-*

gutta and 2 mg/l in *G. tinctoria.* Root initiation took place in MS medium with or without NAA but root elongation was faster in MS medium supplemented with 2 mg of NAA per litre.

The highest number of multiple shoots obtained in this medium was twenty in *G. indica,* followed by eight in *G. gummi-gutta*. *G. tinctoria* did not respond to the above medium and gave only one shoot. This indicates species differences in *in vitro* response; *G. indica* being the most responsive and *G. tinctoria* the least responsive. The multiplication rate obtained was 1:20 in *G. indica* and 1:9 in *G. gummi-gutta* compared to 1:1 ratio when seeds were used and 1:15 with earlier reported *in vitro* protocol indicating the efficiency of the micropropagation. The cultured plants were hardened and planted out with over 95% success.

In vivo seed germination studies

Regeneration of *Garcinia* spp. takes place by the formation of primary root at the distal end followed by the appearance of shoot at the proximal end. Adventitious roots were formed at the proximal end beneath the shoot before leaf initiation. This type of seed germination was reported as *Garcinia* type of seed germination[13].

Joshi *et al*[13] have demonstrated the production of supernumerary plants from seed fractions of *G. gummi-gutta*. Germination in *G. gummi-gutta* begins with the emergence of the primary root (PR) at the distal end of the seed followed by the appearance of a shoot from the proximal end (‘proximal’ end of the seed refers to the end towards the peduncle, whereas the other end is the ‘distal’ end). Subsequently, prior to leaf differentiation, an adventitious root (AR) originates from the base of the shoot. This pattern has already been described as ‘garcinia-type’ of seed germination. The PR along with the seed disintegrates over a six-month period and eventually the adventitious root takes over as the main root system of the plant.

The embryo, which fills up the seed, is an elongated hypocotyl with vasculature connecting the two poles. The absence of differentiated embryo, endosperm or embryonic axis indicated that the so called "seed" is not a true seed. Germination characteristics of "seed" showed the clear cut presence of polarity in *Garcinia* species. Regeneration of multiple seedlings from whole seed and seed pieces further indicated the apomictic nature of seed[18].

According to Joshi *et al*[13] primary root arises from the distal end and shoots from the proximal end. In the present study in *G. indica*, the primary roots developed at the distal end of the seed and the shoot developed at the proximal end after fifteen days (Table 2). Adventitious roots developed at the proximal end beneath the shoot after 3 weeks (Plate 5a). From whole seed one to two adventitious roots developed and one shoot from the proximal end, thus a single seedling is obtained from one whole seed while from the seed that was cut into three fragments, three seedlings were obtained. Two shoots arose from the proximal end and one shoot from the middle piece (Plate 5f, a). Adventitious roots formed at the proximal end just below the shoot and primary roots from the distal end of segment (Plate 5f).

The primary roots and the shoot developed after 20 days in *G. tinctoria* (Table 2). Adventitious roots develop faster in *G. tinctoria* (Plate 6 a) than in *G. indica* but the overall plant development is slow in

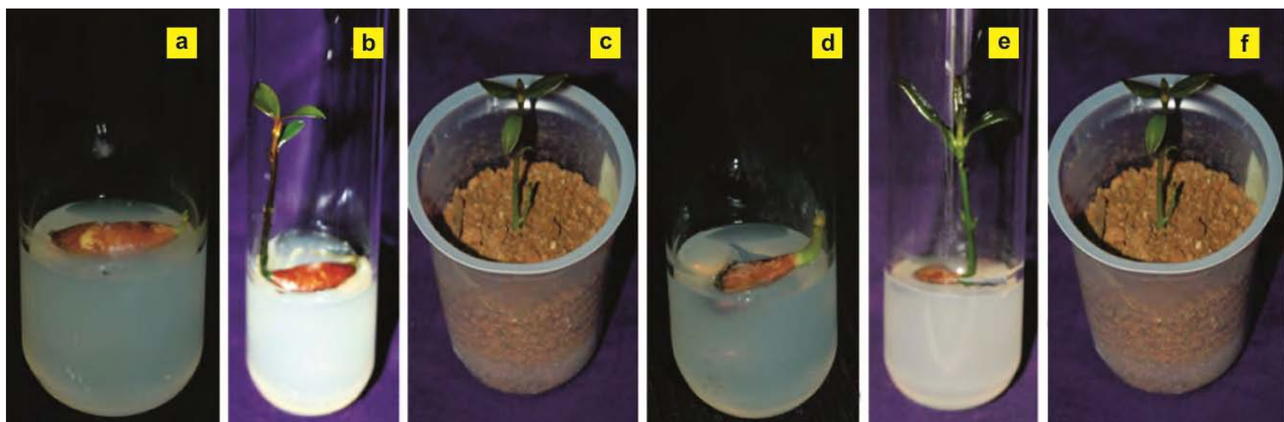

Plate 4—*In vitro* plantlet regeneration from seed and seed segments of *G. gummi-gutta* (a) shoot initiation from whole seed after one month (b) after 2 months (c) after 2½ months (d) Shoot initiation from half seed after one month (e) after 2 months (f)after 2½ months.

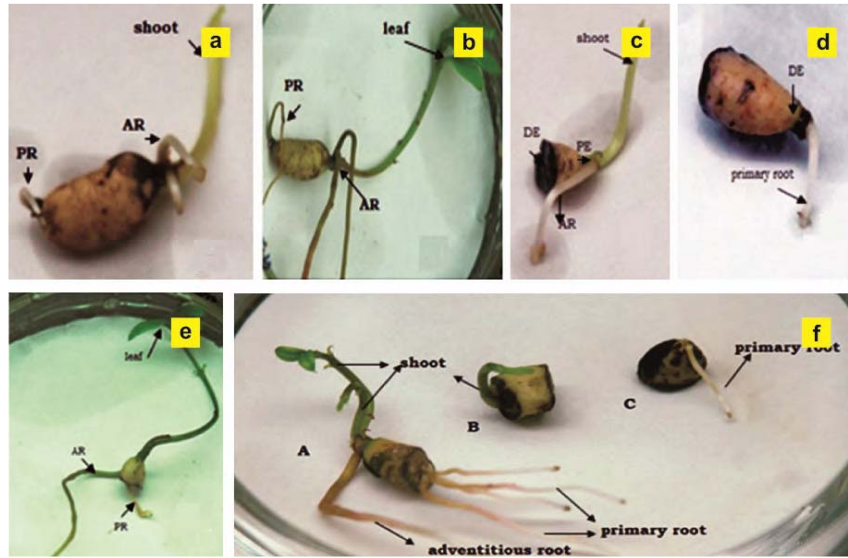

Plate 5—*In vivo* seed germination of *G. indica* on germination paper (a) whole seed after 1 month (b) whole seed after 2 months (c) proximal end (PE) after 1 month (d) distal end (DE) after 1 month (e) proximal end after 2 months (f) seed cut into three fragments A, B and C and Fragment A is the proximal end, Fragment B is the middle fragment and Fragment C is the distal end. The figure shows their growth after 2 months. AR- adventitious root, PR-primary root.

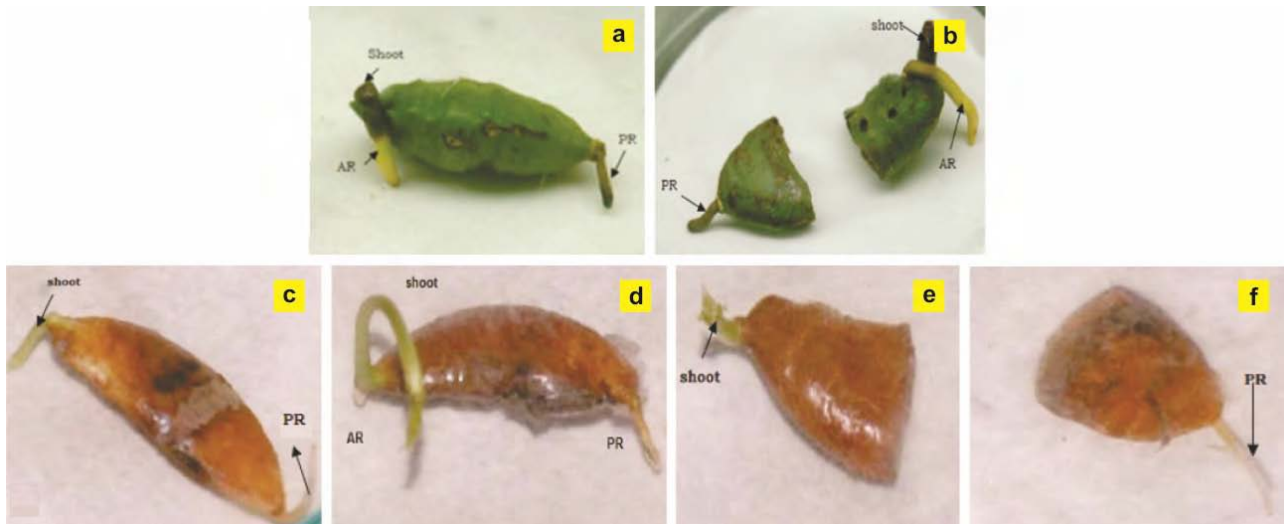

Plate 6—*In vivo* seed germination of *G. tinctoria* and *G. gummi-gutta* (a) germination of the whole seed of *G. tinctoria* (after 1 month) (b) seed which is cut into two fragments (after 1 month) (c) seed germination of *G. gummi-gutta* (whole seed after 2 months) (d) whole seed after 2½ months (e) proximal end after 2½ months (f) distal end after 2½ months. AR is the adventitious root and PR is the primary root.

Table 2—*In vivo* seed germination in three species of *Garcinia*

S. No.	Sample	Time of shoot initiation (in days)	Time of primary root initiation (in days)	Time of adventitious roots generation (in days)	Time of leaf generation (in days)
1	*G. indica*	15	15	27	45
2	*G. tinctoria*	20	20	23	60
3	*G. gummi-gutta*	32	32	50	90

G. tinctoria. Initial shoot development is fast but further development takes a long time.

Decoated seeds of *G. gummi-gutta* took a longer time to germinate compared to the other two species. Primary roots and shoot developed after 32 days. Adventitious roots developed after 50 days (Plate 6d).

Conclusion

Efficient methods for multiplication were developed for three species of *Garcinia,* viz. *G. indica.*

G. gummi-gutta and *G. tinctoria* through low cost multiplication using seed fragments. Studies on *in vivo* seed germination and *in vitro* propagation using whole seeds as well as fragments of seeds resulted in 100 % germination. However species wise differences were noticed in germination pattern and plant development. This technology can be used for multiplication of elite genotypes and conservation of *Garcinia* species.

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