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RESEARCH ARTICLE

INVESTIGATIONS ON THE EFFECTS OF PLANT GROWTH REGULATORS ON *IN VITRO* PROPAGATION OF *Garcinia cambogia* L.

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ABSTRACT

Methods were developed for cloning and large scale plantlet production of *Garcinia cambogia* L. from germplasm selected from East coast region of India. These explants were dressed and surface sterilized with help of 0.1 % $HgCl_2$. Multiple shoots were induced by proliferation of axillary buds/meristems on Murashige and Skoog's (MS) medium incorporated with 2.0 mgl^{-1} 6-Benzylaminopurine (BAP). Incorporation of higher amount of auxins in the culture medium caused callusing from the explants. The shoots of *G. cambogia* were further multiplied on MS medium supplemented with 1.0 mgl^{-1} Indole-3 acetic acid (IAA) + 0.5 mgl^{-1} each of BAP and Kinetin (Kn). On this medium healthy and sturdy shoots were produced. The *in vitro* generated shoots were rooted on half-strength MS medium containing 2.5 mgl^{-1} Indole-3 butyric acid (IBA). By this method 78% shoots were rooted. The *in vitro* rooted plantlets could be hardened and acclimatized in green house.

INTRODUCTION

The number of medicinal and aromatic plants, which constitute the viable component of human health care at one time or another, is above eighty thousands (Kumar *et al*., 2003). They occur most preponderantly in tropical and subtropical countries. *Garcinia* is a large genus (family Clusiaceae) of polygamous trees or shrubs, distributed in the tropical Asia, Africa and Polynesia and is a rich source of bioactive molecules including xanthones, flavonoids, benzophenones, lactones and phenolic acids (Patil, 2005). The genus comprises of 200 old world tropical species of which 20 species are found in India. As a part of traditional materia and medica of India, *Garcinia indica, Garcinia mangosteena, Garcinia cambogia* and *Garcinia morella* are documented in Ayurvedic medicines which are generally known as 'Red Mango or Kokam'. It has antifungal properties (Selvi *et al*., 2003) and prevents acute ulceration in rats when orally administered (Yamaguchi, 2000). The nutraceutical studies on *Garcinia* indicated enormous medicinal properties. Hence, it is termed as an antithelmentic and cardiotonic. It is also useful in curing piles dysentery, tumor pains and cardiac problems. Para hydroxyl citric acid, present in *Garcinia* fruits has inhibiting effect, on lipogenesis (fat production) (Mathew *et al*, 2001). Apart from this, it is widely used as fish preservatives and good source of acid for coagulating rubber. *Garcinia cambogia* extracts are commonly added to weight loss supplements containing other ingredients, such as chromium picolinate and L-carnitine, and in appetite-suppressor products including snack bars, drinks, and chewing gums.

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In these products, the calcium salt of (-)-hydroxycitric acid is usually used (Woodward, 2002). As *Garcinia* product reduces fat, cools body, purifies blood, fights cholesterol, wine and liquors made from *G. cambogia* will attract health conscious people (Veerkar *et al*., 2001).The fruits are the rich source of hydroxycitric acid (HCA), an important biologically active plant metabolite used as an antiobesity and anticholesterol drug.

A simple and efficient method has been developed for rapid regeneration of plantlets via adventitious bud differentiation from mature seeds of *G. indica* by Malik *et al*., (2005). Deodhar *et al*., (2005) worked on repetitive somatic embryogenesis and plant regeneration in *G. indica* from immature seeds. Thengane *et al*., (2006a,b) excised immature fruits and cultured on Lloyd and McCown (1980), woody plants medium with different combinations of auxins and cytokinins. Sirchl *et al*., (2008) developed a micropropagation system for mangosteen using leaf section and mature seeds. Deodhar *et al*., (2008) reported *de novo* shoot regeneration from root cultures of *G. indica*. Malik *et al*., 2010) regenerated plantlets via adventitious bud differentiation on leaf explants of *G. indica*. Rostika *et al*., (2008) also developed micropropagation protocol for Mangosteen (*G. mangostana*).

There is no literary evidence available in the tissue culture and micropropagation of *G. cambogia* L. in India as well as at international level. According to Rostika *et al*., 2008) conventional propagation of *Garcinia* is still facing some problems, such as limited fruiting season and seed number, and the slow growth of seedling. *In vitro* culture is an alternative technique to solve the problems. A study was

carried out to obtain a suitable technique for *in vitro* propagation of *G. cambogia* that enable to produce plantlets with high level of shoot multiplications and root formations.

MATERIALS AND METHODS

Field surveys were conducted during rainy season of 2010-2011. Plants were identified in and around Puducherry and Tamil Nadu. The plants establish in the Govt. Horticultural Farms were pruned and used as sources of explants. Fresh shoot sprouts from the source plants were harvested during November-March. Different types of explanting materials namely, axillary/terminal shoots, shoot apices/nodal shoot segments were harvested from selected plants. 2 - 3 cm long shoots each with 1-2 nodes were used as explants. The explants were cleansed, dressed and washed with soap water. Explants collected were thoroughly washed with water containing 0.1% Bavistin for 4-5 minutes. These were surface sterilized with 0.1% $HgCl_2$ for 4-5 minutes and washed 6-8 times with autoclaved water under Laminar Air Flow Cabinet (Technico Systems, Chennai) and inoculated aseptically onto MS basal medium (Murashige and Skoog, 1962). The pH of the media was adjusted to 5.8±2 with 0.5N NaOH or 0.1N HCl before autoclaving. The medium was poured in the culture vessels. Finally the medium was steam sterilized by autoclaving at 15 psi pressure at 121°C for 15 minutes.

The explants were inoculated vertically on MS medium for culture initiation. Different concentration and combination of cytokinins (BAP and Kn ranging from 1.0 to 5.0 mgl^{-1}) and auxins (IAA and IBA ranging from 0.1 to 2.0 mgl^{-1}) were incorporated in the medium to induce bud breaking. These cultures were incubated at 28±2°C in the dark for 2-3 days. Subsequently these were kept under diffused light (22 µ $molm^{-2}$ s^{-1} Photosynthetic Photon Flux Density, PPFD) for 8-10 days. The light was provided by fluorescent tubes and incandescent bulbs. The *in vitro* regenerated shoots were multiplied by repeated transfer of mother explants on fresh medium and subculturing of *in vitro* produced shoots on fresh medium. The shoots were sub-cultured in the culture flasks. For multiplication of cultures, MS medium supplemented with various concentration and combination of cytokinins (BAP and Kn; 0.1 to 3.0 mgl^{-1} and IAA 1.0 mgl^{-1}) were used. The cultures were incubated at 28±2°C temperature, 60-70% RH and 30-35 µmol $m^{-2}s^{-1}$ PPFD for 12 h/d. For the rooting of *in vitro* produced shoots, the shoots were isolated of appropriate size and these were rooted on half and one- fourth strengths of MS medium containing auxins (ranging 0.5 mgl^{-1} to 5.0 mgl^{-1} IBA and IAA). The *in vitro* rooted plantlets were washed with autoclaved distilled water to remove adhered nutrient agar and then transferred to soil mixture (sand, soilrite, organic manure and black soil in 1:1:1:1 ratio) filled in plastic bottles moistened with one-fourth strength of MS basal salts. After 12-15 some days the caps were loosened and finally removed. Plants hardened in bottles were transferred to polybags containing sand, soilrite, organic manure and black soil in 1:1:1:1 ratio or in different ratio. The hardened plantlets were finally transferred to the pots.

Observation and Data Analysis

The cultures were regularly subcultured on fresh medium after 4-5 weeks interval. The observations were taken after every five days of inoculation. The experiments were repeated thrice with ten replicates per treatment. The rate of multiplication represents number of shoots produce per explant on a specific medium after number of days of its inoculation as mentioned in the results. The data were subjected to statistical analysis.

RESULTS

Explants collected in the month of February were found to be most suitable for culture initiation. Shoot buds appeared within 15 - 20 days from the nodes of the explants. Explants collected from pruned plants respond earlier (within 15 days) than the explants collected from old branches. Percentage of response varied from 23% to 91% on MS medium. MS medium supplemented with BAP was found to be more effective than Kn. 2.0 mgl^{-1} of BAP proved to be the most effective for induction of multiple shoots (Table 1). On this medium 3-4 shoots were induced from each node of the explant (Fig. a-d). The shoots produced were fast growing and lanky. If the concentrations of BAP were increased (above 2.0 mgl^{-1}) healthy but dwarf shoots were produced. The incorporation of auxins, IAA or IBA (more than 1.0 mgl^{-1}), in the medium caused callus formation from the explants and the number of shoots were less (Table 2**)**. On MS medium with BAP + Kn at higher concentrations, healthier shoots were produced but the numbers of shoots were less. Only 2-3 shoots were regenerated from each node on this combination. Amongst various cytokinins and auxin tested for shoot multiplication, MS medium containing 1.0 mgl^{-1} IAA, 0.5 mgl^{-1} each of BAP and Kn proved to be the best for shoot multiplication. The effect of different cytokinins, their concentrations and combination on shoot multiplication is shown in Table 3. In a single culture flask about 4-5 shoots (Fig. e) regenerated on this media combination. Sub-culturing was found to be essential after a period of 25-30 days; otherwise necrosis and tip burning of the shoots were observed. The individual shoots produced in culture were excised and transferred on root induction medium. Half strength MS medium containing 2.5 mgl^{-1} IBA was found to be most suitable medium for induction of strong and viable root from isolated shoots within 2 weeks (Fig. f). On this combination 78% shoots were rooted (Table 4**)**. *In vitro* rooted plantlets were transferred to soil mixture filled in plastic bottles moistened with one-fourth strength of MS basal salts. These were kept in the green house. After 30 days these plantlets were transferred to polybags (Fig. g).

DISCUSSION

Demand for *Garcinia* in the world market is growing due to increasing consumption as Healthy Supplement and due to supportive research on *Garcinia* as a weight controlling herb. *Garcinia cambogia* L. controls fat metabolism, decreases appetite and reducing weight at the same time prevent gastric ulceration. Micropropagation protocols based on the tendency of excised stem tip, nodes or lateral buds to form single or multiple axillary shoots have been utilized to clone a number of plant species (Al-Bahrany and Al-Khayri, 2003; Shekhawat, 2007). The source of the explant cultured is important in determining the regenerative potential (Narayanaswamy, 2002). In the present investigation, nodal segments of *G. cambogia* were used to initiate the cultures. Nodal segments induced multiple shoots in many plants like

Table 1: Effects of cytokinin (BAP) on induction of shoots from explants of *G. cambogia* on MS medium

BAP Concentrations (mgl^{-1})	% of Response	Number of Shoots / Explant ± SD
Control (0.0)	16	0.0 ± 0.00
0.5	25	1.2 ± 0.62
1.0	50	1.7 ± 0.33
1.5	77	2.9 ± 0.57
2.0	91	3.6 ± 0.26
2.5	83	3.0 ± 0.27
3.0	75	2.7 ± 0.63
3.5	70	2.1 ± 0.63
4.0	61	2.0 ± 0.19
4.5	49	1.8 ± 0.45
5.0	23	1.1 ± 0.20

Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with ten replicates per treatment.

Table 2: Effects of auxin (IAA) on shoot induction from axillary buds on MS Medium + BAP 2.0 mgl^{-1}.

Auxin (IAA) (mgl^{-1})	Number of shoots / explant ± SD
0.1	1.0 ± 0.47
0.2	1.4 ± 0.56
0.5	1.8 ± 0.36
0.6	2.0 ± 0.78
0.8	2.5 ± 0.23
1.0	3.3 ± 0.12
1.5	3.0 ± 0.68
2.0	2.4 ± 0.23

Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with ten replicates per treatment.

Table 3: Effects of cytokinins (BAP + Kinetin) concentrations on multiplication of shoots from *in vitro* regenerated shoots on MS Medium containing 1.0 mgl^{-1} IAA.

BAP + Kinetin Concentrations (mgl^{-1})	Shoot Numbers ± SD	Shoot Length (cm) ± SD
0.1	2.3 ± 0.36	2.1 ± 0.56
0.5	3.2 ± 0.28	2.6 ± 0.37
1.0	3.0 ± 0.76	2.8 ± 0.81
1.5	2.4 ± 0.84	2.7 ± 0.43
2.0	2.0 ± 0.38	2.3 ± 0.29
2.5	1.8 ± 0.23	1.9 ± 0.38
3.0	1.4 ± 0.11	1.5 ± 0.18

Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with ten replicates per treatment.

Table 4: Effects of auxin (IBA) on *in vitro* regeneration of roots from *in vitro* generated shoots on half strength MS medium

IBA Concentrations (mgl^{-1})	% of Response	Number of Roots ± SD	Length of Roots (cm) ± SD
0.5	28	1.4± 0.48	1.3 ± 0.67
1.0	41	2.1 ± 0.37	1.6 ± 0.48
1.5	59	2.4 ± 0.81	1.9 ± 0.28
2.0	67	3.2 ± 0.72	2.1 ± 0.18
2.5	78	3.8 ± 0.65	2.6 ± 0.84
3.0	63	3.1 ± 0.91	2.6 ± 0.87
4.0	57	2.8 ± 0.27	2.1 ± 0.28
5.0	36	1.9 ± 0.57	1.6 ± 0.11

Note: The observations were taken after every twenty days of inoculation. The experiments were repeated trice with ten replicates per treatment.

Fig. a, b, c & d. Different stages of induction of shoots from nodal meristems of *G. cambogia*.
e. Cultures in multiplication stage.
f. Induction of roots in *in vitro* produced shoots.
g. *In vitro* regenerated plantlet in hardening condition.

Ceropegia candelabrum (Beena *et al*., 2003), *Echinopus spinossimum* (Murch *et al*., 2003) etc. The physiological age of the explant is another factor, which exercises an influence on organ formation. Nodes are better and more convenient explant type than shoot tips for micropropagation. The use of nodes in preference of shoot tips is reported in other species such as *Adhatoda beddomei* and *Aegle marmelos* (Ajithkumar and Seeni, 1998). Observations in the present study indicated that suitable combinations of auxins and cytokinins were important for shoot induction from nodal segment explants. Nodal segments in *G. cambogia* showed higher rate of multiple shoot induction when BAP alone was used. When cytokinin enriched media were supported with higher concentrations of auxins for nodal cultures caulogenesis was recorded, with a reduced number of shoots. Similar types of results were observed by Shekhawat *et al*., 2011. No multiple shoot induction was observed in nodal explants when cultured in media without cytokinin. Instead the preexisting axillary bud in nodal explants alone gave rise to a single shoot. This was also observed in *Avicennia marina* (Al- Bahrany and Al-khayri, 2003). The explants without growth regulators initiated shoot growth but failed to multiply. However, the multiplication rate and shoot length was significantly higher in cultures supplemented with plant growth regulators.

In present experiments, multiplication was found to occur by the development of adventitious or axillary buds, which is ideal for maintaining genetic stability. However, the rate of bud multiplication was significantly different according to the various concentrations and combinations of cytokinins supplemented. Cytokinin level produced a significant response upon the number of shoots formed per explant. Hu and Wang, (1983) reported that in many plant species, cytokinins play an important role in overcoming the influence of apical dominance and in enhancing branching of lateral buds. Nodal segments of *G. cambogia* produced maximum number of shoots on BAP (2.0 mgl^{-1}). When cytokinins (BAP, Kn) alone were used, BAP produced higher number of shoots than Kn. BAP induced multiple shoots at low concentration in *Adenia hondala* and *Baliospermum montanum.* Increase in the concentration of BAP suppressed bud break and decreased the

number of shoots and retarded the elongation of shoots. Dantu and Bhojwani (1987) and De Bryan and Ferreira (1992) commented about the beneficial effect of BAP over other cytokinins (Kn and 2iP) for shoot multiplication. In this study, BAP only at low concentration was found to promote shoot development. BAP is the most effective cytokinin for adventitious shoot regeneration from explants of Asiatic genotype (Bacchetta *et al*., 2003).

The rooting and acclimatization of shoots represent a difficult and critical stage in the tissue culture process. Efficient rooting of *in vitro* regenerated plantlets and subsequent field establishment is the last, crucial stage of rapid clonal propagation. In the present investigation, rooting was not induced in chorus with shoot formation during culture initiation and multiplication in the cytokinin regime. Auxins have been widely used for *in vitro* and *ex vitro* rooting of plants. Purohit *et al*., 2002, and Shekhawat and Dixit, 2007 reported that auxins are essential for root growth and development. In most of the plant systems, IBA was used for efficient rooting (Loreti *et al*., 1988; Roy *et al*., 1991). Preference of IBA for rooting over NAA was reported in *Actinida delicosa* by Kumar *et al*., (2001). IBA induced high frequency of roots in *Leptadenia reticulata* (Hariharan *et al*., 2002), *Lobelia* sp. (Jabeen *et al*., 2002), and *Trapa* (Hoque and Arima, 2003). In *G. cambogia* also IBA (2.5 mgl^{-1}) induced more number of roots as compared IAA. The protocol developed is efficient and reproducible. It can be used for large-scale amplification of selected clones.

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