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In vitro clonal multiplication of C*ardiospermum halicacabum* L.

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A simple and efficient protocol for in vitro multiplication of mature plants of *Cardiospermum halicacabum* using nodal and shoot segments has been successfully developed. The stem of *C. halicacabum* being soft and delicate is very sensitive to physical handling and sterilization. In case of *C. halicacabum* extra care must be taken while selecting the explant and surface sterilizing it. Three to four shoots were initiated per auxiliary meristems on Murashige and Skoog (MS) medium supplemented with 2.0 mgl/1 BAP and 0.5 mgl/1 IAA within two weeks, while less numbers of shoots produced on MS medium augmented with Kinetin (KIN). Repeated transfer of the initial explants for up to five passages on MS medium with 0.5 mgl/1 BAP and KIN + 0.5 mgl/1 IAA yielded maximum numbers of shoots. Healthy and elongated shoots were rooted on 1/2 MS medium + 2.0 mgl/1 Indole-3 butyric acid (IBA). The plantlets thus obtained were successfully hardened in green house and transferred to the field.

Keyword: *In Vitro*, Clonal, Nodal and Shoot Segments, *Cardiospermum Halicacabum*

Cardiospermum halicacabum L., known as the Balloon plant or Love in a puff, which is a climbing plant widely distributed in tropical and subtropical region of Africa and Asia. The genus *Cardiospermum* (family Sapindaceae) (Neuwinger, 2000) represents more than 30 recognized species throughout the world. In rural south India, this plant has been harvested and sold in urban and local market as green vegetable providing a source of revenue for low-income families. In the global market, balloon vine has been utilized as several products, 'Love in a Puff', 'Balloon Vine' and 'Heart-seed'.

Various forms of products like gel, cream, shampoo, spray etc. of *C. halicacabum* are available in the market. These products are useful for dry itchy skin and scalp. These products are supported by the various claims concerning with medicinal properties of balloon vine. *Cardiospermum* is an active ingredient in creams and lotions for dermatitis, eczema, and psoriasis. Since it is not a steroid, it is often used as an alternative to cortisone creams. The whole plant is applied to reduce swellings and hardened tumors (Ganesan *et al.,* 2007). Here is a claim that roots are used by some local tribes to treat rheumatoid arthritis in Asian and African communities. Scientific studies have provided an explanation for some of the medicinal properties of balloon vine. *In vitro*

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antifilarial activity of extracts of *C. halicacabum* has mild but definite direct macrofilaricidal action. *C. halicacabum* also showed antipyretic activity against yeast-induced pyrexia in rats and cure for gastric ulcers in rats. The whole plant has been used as anti-inflammatory, antipyretic, analgesic, antiparasitic, as well as an effective non toxic Antifertility herb. Traditionally this plant is highly useful in ayurveda, homeophathic and unani Indian Systems of medicines to treat rheumatoid arthritis, gastrointestinal diseases, respiratory diseases, urogental diseases, inflammatory diseases etc. The phytochemical screening and *in-vitro* anti-microbial activity leaves of *C. halicacabum* (Deepan *et al.,* 2012). Therefore, the uses of this plant are enormous, each and every part of this plant is useful and people are using this plant in day to day life as medicine or as vegetable all over the world. Due to higher demand and unavailability of farming practices, the plant population is decreasing. It is urgent need to develop a cost effective and rapid micro propagation protocol to restore the plant.

Materials and Methods

Collection and sterilization of explants and induction of shoots

The mature plants of *Cardiospermum halicacabum* (Baloon vine) were selected from Nammakal District of Tamil Nadu. The experiments were performed using nodal and shoot segments as explants. The fresh sprouts from mature plants were initially cleaned with 0.5 % (w/v) solution of detergent, for 15 min. These were thoroughly washed with autoclaved sterile distilled water. These explants were then treated with 0.2 % (w/v) solution of systemic fungicide, Bavistin. Explants were surface sterilized with aqueous solution of 0.1 % (w/v) $HgCl_2$. These were then washed with autoclaved water for several times before inoculation. Each explant was inoculated in a 50 ml test tube containing 10 ml (MS) medium (Sredni *et al*., 1992). The surface sterilized juvenile and young explants were aseptically placed on basal MS medium gelled with 0.8% agar, 3% sucrose and various concentrations (0.5 to 4.0 mgl-1) of BAP, Kn and auxins (IAA and IBA) (Uede *et al*., 2002). The test tubes were sealed with cotton plugs and incubated in a growth chamber at 26 ± 2^{o}C under a 14/10 hrs (light/dark) photoperiod with light supplied by white fluorescent lighting at an intensity of 46 µmol m-2 s-1 PPFD (photosynthetic photon flux density).

Multiplication of shoots

The cultures were multiplied by (i) Repeated transfer of mother explants along with regenerated shoots on fresh medium, and (ii) Subculture of *in vitro* produced shoots, were cut into nodal shoot segments with 1-2 nodes and cultured on fresh media. Different concentrations (0.50 to 3.0 mgl-1) of BAP and Kn were used separately and in combinations. BAP (0.5 mgl-1) in combination with varying concentrations of Kn (0.50 to 3.0 mgl-1) and 0.5 mgl-1 IAA was also incorporated in the MS medium in order to optimize culture conditions for shoot amplification.

Rooting of *in vitro* produced shoots

Healthy and strong *in vitro* regenerated shoots were harvested and washed with autoclaved water to remove adhered nutrient medium. These shoots were individually transferred on to root induction media. The media evaluated for this purpose was full and half-strength MS semisolid medium containing IAA and IBA in concentration range of 0.5 to 4.0 mgl-1. These individual inoculated shoots were then placed under diffused light.

Hardening of plantlets in green house

The *in vitro* rooted shoots were inoculated on soil rite containing bottles moistened with

MS macro salts dissolved in distilled water. These bottles were then put in green house for acclimatization. After 7 to 8 weeks the plant lets were transferred to black poly bags and were placed near fan section of green house for gradual acclimatization and hardening. Hardened plants were then transferred to earthen pots containing mixture of garden soil, organic manure, sand, and vermicompost in 3:1:1:1 ratio and were placed in nursery.

Results and Discussions

During present investigation the studies were therefore conducted to establish cultures from selected plants of *C. halicacabum*. Plants were first established in the garden for better management of the source plants, so as to facilitate harvesting desired type(s) of explanting materials at appropriate time. The stem of *C. halicacabum* being soft and delicate is very sensitive to physical handling and surface sterilization. In case of *C. halicacabum* extra care must be taken while selecting the explant and surface sterilizing it. The explant age (physiological status), the season of explants collection, explants size and plant quality are some of the factors which play important role in the initiation and multiplication of culture. The explants harvesting during the months of February- March found to be the most suitable for the initiation of cultures of *C. halicacabum* (Smith, 2000). Maximum shoots were induced on MS medium +2.0 mgl-1 BAP + 0.5 mgl-1 IAA while on MS + Kn medium less number of shoots were regenerated from the nodal explants. Nodal segments cultured on growth regulator free MS medium did not exhibit any regeneration response. However, when MS medium was supplemented with different cytokinins singly, multiple shoot formation occurred within 4 weeks of culture. The maximum number of shoots and the highest shoot regeneration frequency was achieved at 2.0 mgl-1 BAP. Reduction in the number of shoots and shoot length was observed at higher levels of BAP as well as Kn. The ability of cytokinins in multiple shoot bud differentiation at lower concentrations has been reported by several workers. The addition of IAA in low concentration with cytokinins (BAP as well as Kn) has increased the number of shoots from the explants as compared to the effect of IBA (Philip *et al*., 2006)

Shoot multiplication

The shoots could be better multiplied on MS medium + 0.5 mgl-1 BAP and Kn + 0.5 mgl-1 IAA by sub-culturing. Thus the cytokinin requirements were low for shoot multiplication than those required for initiation of shoot cultures from the nodal explants. Once the existing meristem is activated and freed from the developmental constraints of the mother explants, they are conditioned. The maximum number of shoots multiplied when the medium was supplemented with BAP. BAP at very low concentration (0.5 mgl-1) was found the best during this study (Boonmars *et al*., 2005).

The shoots multiply rapidly in culture and these elongate enormously. Some of these acquire length up to 9-10 cm. Inclusion of IAA together with BAP is showing good response in shoot multiplication. Our finds are contrary to the observation of the findings of where addition of either of the auxins significantly reduced the number of shoots (Sheeba and Asha, 2006). The rapid elongation of shoots creates difficulties in their handling. The lanky shoots could not be rooted with easy. On half strength MS medium + 0.5 mgl-1BAP and Kn + 0.5 mgl-1 IAA healthy, thick and sturdier shoots differentiated but the number of shoots were less than full strength MS medium. Similarly, BAP and Kn alone at the same concentration produced lesser number of shoots. The rate of shoot multiplication is known to differ from

species to species during the subculture passages. This enhanced shoot multiplication by subsequent cultures substantiates the earlier reports on *Momordica dioica*, *Capsicum annuum* L., *Cassia angustifolia* and *Ocimum basilicum* (Zayova *et al.*, 2008). After standardization of the conditions for shoot proliferation, the effects of pH was also tested because many important aspects of the structure and activity of biological macromolecules are determined by pH of the medium which directly affects the nutrient uptake and shoot proliferation. The optimum pH for shoot proliferation and elongation was found to be 5.8, while higher level of acidic medium severely inhibits the shoot multiplication rate.

Table 1: Effect of BAP on Multiple Shoot Induction

S. No	Concentration of BAP(mg/l)	Total no of explants	No of explants responded	Presence of multiple shoot	Percentage of response (%)	M±SD
1.	0.01	30	21g	+	55g	3.56±2.1f
2.	0.02	30	22f	+	60f	4.23±1.9d
3.	0.03	30	20h	+	45g	5.2±2.6bc
4.	0.04	30	27c	++	85b	4.1±3.4de
5.	0.05	30	26d	++	80c	3.9±1.5e
6.	0.06	30	28b	++	90b	2.96±2.3h
7.	0.07	30	23	++	65e	2.8±1.6hi
8.	0.08	30	26de	++	80cd	3.3±2.3g
9.	0.10	30	28bc	++	90ab	4.6±3.1c
10.	0.15	30	28bc	++	90ab	5.9±2.5a
11.	0.20	30	29a	+++	95a	5.3±1.4b
12.	0.50	30	28b	++	90ab	4.6±2.6cd
13.	1.0	30	27cd	++	85bc	2.5±1.9i

+++ - more number of shoots; ++ - moderate number of shoots; + - less number of shoots

Means and standard errors (± SE) are pre-sented for each column. Means sharing at least one letter are no significantly different at the p ≤ 0.05 level (Duncan's multiple range test)

Table 2: **Effect of BAP and IAA on shoot regeneration**

S. No	BAP (mg/l)	IAA (mg/l)	Total no of explants	Total no of explants responded	M±SD	Percentage of induction (%)
1.	0.2	0.05	30	25b	5.4±2.8d	75b
2.	0.2	0.1	30	28ab	6.1±3.2bc	90ab
3.	0.2	0.2	30	29ab	6.5±4.1a	95ab
4.	0.2	0.4	30	30a	6.2±1.9b	100a
5.	0.2	0.6	30	28ab	5.9±2.3c	90ab

Means and standard errors (± SE) are pre-sented for each column. Means sharing at least one letter are no significantly different at the p ≤ 0.05 level (Duncan's multi-ple range test)

Table 3: Effect of KIN and IAA in the multiple shoot induction

S.No	BAP (mg/l)	NAA (mg/l)	Total no of explants	Total no of explants responded	M±SD	Percentage of regeneration (%)
1.	0.2	0.2	30	25b	4.1±3.2a	75b
2.	0.5	0.4	30	24ab	2.4±4.5c	70ab
3.	1.0	0.6	30	27a	3.6±1.2b	85a
4.	1.2	0.8	30	23ab	3.2±1.5bc	65ab
5.	1.4	1.0	30	22ab	1.9±1.8d	60ab

Means and standard errors (± SE) are pre-sented for each column. Means sharing at least one letter are no significantly different at the p ≤ 0.05 level (Duncan's multi-ple range test

Table 4: Effect of IBA on root induction

S. No	IBA (mg/l)	Total no of explants	Total no of explants responded	M±SD	Percentage of response (%)
1.	0.2	30	19cd	2.5±2.9de	45cd
2.	0.4	30	25a	3.1±2.8b	75a
3.	0.6	30	21c	2.94±2.0c	55c
4.	0.8	30	23ab	3.56±2.9a	65b
5.	1.0	30	20cd	2.6±2.5d	50cd

Table 5: Effect of BAP, IBA, Kin for the Multiple Shoot, Leaf and Root Induction

S.No	BAP (mg/l)	KIN (mg/l)	IBA (mg/l)	Total no of explants used	Total no of explants responded	Percentage of response (%)
1.	0.2	0.2	0.04	30	27ab	85ab
2.	0.2	0.4	0.04	30	23b	65b
3.	0.2	0.6	0.04	30	28a	90a
4.	0.2	0.8	0.04	30	26ab	80ab
5.	0.2	1.0	0.04	30	25ab	75ab

Rooting of *in vitro* regenerated shoots

Well developed, healthy and sturdy shoots (6-7 cm long) were excised, washed with sterilized water and transferred to different strength of MS medium for rooting. Among these, 1/2 strength MS medium was found to be superior as compared to full strength MS medium. Strength of MS medium appeared to be an important factor in influencing the rooting efficiency. Sometimes, the endogenous level of auxins present in the tissue is sufficient to induce roots in hormone free MS medium (Murashige and Skoog., 1962). However, at times a very low concentration of exogenous auxin is required for better rhizogenesis. But in present study auxin requirement was essential to induce root from the cut part of shoots. The best

rooting response was obtained in 1/2 MS medium augmented with 2.0 mgl/1 IBA and 200 mgl/1 activated charcoal. This supports the findings of where IBA was effective but showed contrast with studies made by where IAA proved to be a better root inducer compared to other auxins. All the shoots could be rooted on IBA containing medium. The root induction was earlier (within 7 days) on IBA supplemented medium while on IAA it took 2-3 weeks and less number of shoots rooted. The superiority of IBA as effective rooting auxin is very well known (Chung *et al*., 1998).

Hardening and acclimatization of plantlets in green house

The hardening of micropropagated plants of *C. halicacabum* has been the most difficult part. As the plants are very delicate and soft, these take time in hardening under green house conditions. Under very high humid conditions, these plants attract fungal contamination and under low humidity conditions and extended exposure they dry up rapidly. The micropropagated plants of *C. halicacabum* require special treatments for hardening /acclimatization. While the plants are hardened, these should get physical support and they also require habitat soil to for survival of the plants. After one month, the micropropagated plants were planted in earthen pots containing garden soil and vermicompost (1:1) and maintained in a greenhouse. Some of the plants could be transferred to the field. (Bonga and Von Aderkas, 1992, Koroch *et al*., 1997).

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