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In vitro Clonal Propagation of *Cardiospermum halicacabum L.* Through Nodal Segment Cultures

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A simple and efficient protocol for in vitro cloning of mature plants of *Cardiospermum halicacabum* using nodal shoot segments has been successfully developed. 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. Three to four shoots were initiated per axillary 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 (Kn). Repeated transfer of the initial explants for up to five passages on MS medium with 0.5 mgl-1 BAP and Kn + 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, Cloning, Nodal Shoot Segments, *Cardiospermum Halicacabum*.

INTRODUCTION:

Cardiospermum halicacabum L., known as the Balloon plant or Love in a puff, which is a climbing plant widely distributed in tropical and subtropical Africa and Asia. The genus Cardiospermum (family Sapindaceae) represents more than 30 recognized species throughout the world. [1] 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. [2] *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.

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The whole plant is applied to reduce swellings and hardened tumors. [3] There is a claim that roots are used by some local tribes to treat rheumatoid arthritis in Asian and African communities. [4,5] Scientific studies have provided an explanation for some of the medicinal properties of balloon vine. *In vitro* antifilarial activity of extracts of *C. halicacabum* against *Brugia pahangi* has mild but definite direct macrofilaricidal action. [6] *C. halicacabum* also showed antipyretic activity against yeast-induced pyrexia in rats [7] and cure for gastric ulcers in rats [8]. The whole plant has been used as anti-inflammatory, [9, 10] antipyretic, [11] analgesic, antiparasitic, [12] as well as an effective non toxic antifertility herb. [13]

Traditionally this plant is highly useful in ayurveda, shiddha, homeophathic and unani Indian Systems of medicines to treat rheumatoid arthritis, [14] gastrointestinal diseases, [15] respiratory diseases, [16] urogental diseases, inflammatory diseases [17,18] etc. in India and China. [19] Deepan [20] studied the phytochemical screening and *In-vitro* anti-microbial activity leaves of *C. halicacabum*.

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 micropropagation protocol to restore the genome.

Materials and Methods

1. Collection and sterilization of explants and induction of shoots

The mature plants of *Cardiospermum halicacabum* (Baloon vine) were selected from East Coast region of South India, (Pondicherry). Mother plants were also grown in greenhouse at 28ºC under a 14/10 hrs (light/dark) photoperiod. The experiments were performed using nodal 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 [21]. 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). The test tubes were sealed with cotton plugs and incubated in a growth chamber at 26 ± 2ºC 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).

2. 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.

3. 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} along with 200 mgl^{-1} activated

charcoal. These individual inoculated shoots were then placed under diffused light.

4. Hardening of plantlets in green house

The *in vitro* rooted shoots were inoculated on soilrite 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 plantlets were transferred to black polybags 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 explant collection, explants size and plant quality are some of the factors which play important role in the initiation and multiplication of culture. [22,23] The explants harvesting during the months of January-February found to be the most suitable for the initiation of cultures of *C. halicacabum*.

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 (Fig. 1A,B & C). 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 (Table 1). 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. [24,25,26,27,28] 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.

Table 1. Effect of different concentrations of cytokinins (BAP and Kin) + 0.5 mgl^{-1} IAA on bud breaking from nodal explants of *C. halicacabum*.

Sl. No.	BAP (mgl^{-1})	Kn (mgl^{-1})	Shoot length (cm) (Mean ± SD)
1	0.0	0.00	0.0 ± 0.00
2	0.5	-	1.0 ± 0.23
3	1.0	-	1.2 ± 0.31
4	1.5	-	2.2 ± 0.77
5	2.0	-	2.5 ± 0.29
6	2.5	-	2.5 ± 0.35
7	3.0	-	2.2 ± 0.62
8	3.5	-	1.8 ± 0.82
9	4.0	-	1.2 ± 0.67
10	-	0.5	0.6 ± 0.56
11	-	1.0	1.1 ± 0.89
12	-	1.5	1.5 ± 0.27
13	-	2.0	2.0 ± 0.50
14	-	2.5	1.9 ± 0.72
15	-	3.0	1.3 ± 0.74
16	-	3.5	1.0 ± 0.98
17	-	4.00	0.7 ± 0.47

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 (Table 2). Once the existing meristem is activated and freed from the developmental constraints of the mother explants, they are conditioned. [29] The maximum number of shoots multiplied when the medium was supplemented with IAA. IAA at very low concentration (0.5 mgl^{-1}) was found the best during this study. 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 [30] where addition of either of the auxins significantly reduced the number of shoots.

Table 2. Effect of Cytokinins (BAP and Kin) with 0.5 mgl^{-1} IAA on multiplication of shoots of *C. halicacabum*.

Sl. No.	BAP (mgl^{-1})	Kin (mgl^{-1})	No. of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)
1	0.00	0.00		
2	0.5	-	7.1 ± 0.83	9.7 ± 0.66
3	1.0	-	5.8 ± 0.28	8.9 ± 0.34
4	1.5	-	4.6 ± 0.32	7.7 ± 0.16
5	2.0	-	3.4 ± 0.77	6.8 ± 0.35
6	2.5	-	2.1 ± 0.62	5.9 ± 0.63
7	3.0	-	1.0 ± 0.51	5.2 ± 0.33
8	-	0.5	5.4 ± 0.32	8.8 ± 0.81
9	-	1.0	5.0 ± 0.91	7.8 ± 0.29
10	-	1.5	4.3 ± 0.35	6.4 ± 0.36
11	-	2.0	3.8 ± 0.66	5.2 ± 0.57
12	-	2.5	2.9 ± 0.42	4.6 ± 0.71
13	-	3.0	1.4 ± 0.26	2.9 ± 0.84

The rate of shoot multiplication was 7.7 ± 0.27 shoots per inoculum or culture bottle (Fig. 1D & E). 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^{-1}BAP 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*, [29] *Capsicum annuum* L., [26] *Cassia angustifolia* [27] and *Ocimum basilicum*. [10]

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. [31,32] 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.

Fig. 1.

Fig. 1A, B & C: Different stages of induction of shoots from nodal meristem on MS medium.
Fig. 1D & E: Multiple shoot formation in cultures.
Fig. 1F & G: *In vitro* rooted shoots on IBA containing medium.
Fig. 1H: Hardened plant in pot.

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. 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 (Table 3), (Fig. F & G). This supports the findings of [33] where IBA was effective but showed contrast with studies made by [34,31] where IAA proved to be a better root inducer compared to other auxins.

Table 3. Effects of auxin amendments (IBA and IAA) of half-strength MS medium + 200 mgl^{-1} activated charcoal on root induction from *in vitro* raised shoots of *C. halicacabum*.

Sl. No.	IBA (mgl^{-1})	IAA (mgl^{-1})	Response (%)	No. of roots (Mean ± SD)	Root length (cm) Mean ± SD)
1	0.00	0.00	00		
2	0.5	-	69	1.5± 0.43	1.3 ± 0.71
3	1.0	-	78	2.3 ± 0.77	2.6 ± 0.87
4	1.5	-	93	2.4 ± 0.19	3.3 ± 0.21
5	2.0	-	100	3.3 ± 0.27	3.6 ± 0.13
6	2.5	-	92	3.3 ± 0.65	2.6 ± 0.81
7	3.0	-	47	3.1 ± 0.12	2.2 ± 0.77
8	4.0	-	58	2.7 ± 0.36	2.1 ± 0.89
9	-	0.5	71	1.7 ± 0.87	1.6 ± 0.82
10	-	1.0	86	2.6 ± 0.28	2.3 ± 0.65
11	-	1.5	83	2.8 ± 0.33	2.6 ± 0.19
12	-	2.0	48	3.1 ± 0.64	2.9 ± 0.82
13	-	2.5	64	3.0 ± 0.37	2.6 ± 0.26
14	-	3.0	72	2.9 ± 0.93	2.1 ± 0.11
15	-	4.0	81	2.7 ± 0.87	1.5 ± 0.49

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. [35]

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 (Fig. H). When the plants are transferred to pots/soil/polybags there should be minimum disturbance to the root system. 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 field transferred.

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