

**PLANT REGENERATION IN *CARDIOSPERMUM HALICACABUM* L.
THROUGH NODAL SEGMENTS, CALLUS ORGANOGENESIS AND TRUE-
TWO-TYPE CONFORMITY OF PLANT BY HISTOLOGICAL STUDIES**

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ABSTRACT

Background: *Cardiospermum helicacabum* L. known as the balloon plant or love in puff, belongs to the family Sapindaceae, is a long lived, scrambling vine. *C.halicacabum* leaves contain some pharmaceutically important compounds like saponin, stigmasterol.

Methods: In the present investigation an attempt has been made to study rapid micro propagation from nodal explant of *C.halicacabum* and to check the anatomical features in the *invitro* and *exvitro* level. The nodal segments were cultured on MS medium supplemented with various plant growth regulators. The main reason for the micro propagation of this species is its high medicinal value in ayurveda, siddha, homeopathic and unani.

Results: Highest direct regeneration percentage is shown in MS+BAP (0.3 mg/l) and induction of friable the callus from the cut end of nodal explants showed in the hormonal combination of MS+BAP+2,4-D at 0.3+0.2/L produced green, compact callus. The minimum period for callus induction from nodal plant was 11 days. Anatomical studies in the *exvitro* and *invitro* studies revealed that the presence of 5-8 vascular bundles in stem, and pith is present in the centre. In leaves, the non-glandular and glandular trichomes are present in both cases. Also the contamination frequency was checked and showed 85% in the combination of MS+2,4D (0.03mg/l).

Conclusion: The propagation method in *Cardiospermum halicacabum* is highly useful in the conservation and mass clonal propagation. It will definitely help in the horticultural and ornamental improvement of this medicinal plant and can lead to the analysis of secondary metabolites and bioactivity of *C.halicacabum*.

Keywords: *Cardiospermum halicacabum*, Tissue culture, Sapindaceae, organogenesis, Callus

INTRODUCTION

Cardiospermum halicacabum L. is a climbing plant widely distributed in tropical and subtropical Africa and Asia. The genus *Cardiospermum* represents more than 30 recognized species throughout the world. Leaves of *C. halicacabum* contain largely tannins, saponins and traces of alkaloids [1]. Many scientists studied the various effects of *C. halicacabum* like antipyretic activity, antibacterial activity, antihyperglycaemic effects, *invitro* antifilarial activity, antidiarrhoeal activity, antioxidant activity, larvicidal and ovicidal efficacy, antiparasitic activity, anxiolytic property, silver nanoparticle development and its activity, diuretic activity etc. [2-6]. The seed oil of *C. halicacabum* have 11-eicosenoic acid as the major fatty acid in its glycerides [7]. The phytochemical screening and in-vitro antimicrobial activity in leaves of *C.halicacabum* [8]. The pharmacological potential of *C.halicacabum* collected from Aligarh, India using morphological, phytochemical, physiochemical and spectroscopic studies. Aqueous plant extracts showed the presence

of alkaloid sugars, amino acid, proteins, saponin and tannins [9].

Waako *et al.*, 2005 studied *C. halicacabum* and *Momordica foetida* and in their study it was revealed that the water extract of *C. halicacabum* was toxic to mice, none surviving beyond day 4 of oral administration, with no evidence of protection against *Plasmodium berghei* malaria [10]. The anti-inflammatory activity of *Cassia occidentalis* leaf powder and an ethanol extract of *C. halicacabum* [11]. Ethnolic extract of *C. halicacabum* showed better antioxidant activity than that of aqueous extract [12]. The rapid micro propagation of *C. halicacabum* through plant regeneration from leaf and nodal explants showed adventitious shoots (28 per callus) formed at 8 μ M Kin and 0.5 μ M IAA [13]. An efficient protocol for *C. halicacabum* by using different parts of the plants, i.e cotyledon, hypocotyl, cotyledonary node, leaf, internode and node had the potential to produce calli on MS medium supplemented with BAP and NAA [14].

Also, developed a rapid and efficient protocol for micropropagation of *C. halicacabum* via axillary bud multiplication [15-17].

MATERIALS AND METHODS

Selection of explant

C. halicacabum L. (Sapindaceae), is a valuable medicinal climber was selected for the *in vitro* regeneration procedure. It is an important medicinal herb in ayurvedic, siddha, unani, homeopathic systems of medicines and the tender and younger shoots are used as vegetable. The major chemical components of the whole plant and seed are saponin and cyanogenic glyceride respectively. The plant is also used for filariasis and diarrhoea in cow and oxen. The herb is also used as fodder. Nodal explants were cut out from 8 weeks old *in vitro* cultures of *C. halicacabum* build up from 4 years old adult plant collected from Mallaka Village, Thrissur, Kerala. The healthy shoots were collected and the plant parts were separated into leaves, stems and nodes for surface sterilization. Throughout the process, the explants were sterilized separately.

Preparation of MS Basal Medium

The Murashige and Skoog (1962) medium were variously supplemented with growth regulators for callus induction and shoot regeneration [18]. It was supplemented

with 3% sucrose and 0.8% agar was used during the investigation. The PH of the medium was adjusted to 5.8 with 1N NaOH or HCl prior to autoclaving. The MS basal medium (1L) was composed of 4.4 g MS powder (including all the macronutrients, micronutrients, FeNaEDTA and vitamins) or PGR (if needed). Sucrose at 30 g (w/v) was added as the carbon source and then dissolved completely with distilled water. The pH of the medium was adjusted to 5.7 ± 0.1 by using NaOH or HCl with a pH meter (METTLER TOLEDO, 320pH Meter). Agar Technical at 8 g (w/v) was added to solidify the medium. The medium was then autoclaved at 121°C and 15 psi for 21 minutes. After autoclaving, the autoclaved medium was let cooled before dispensing into the sterile culture tubes in the laminar flow hood. MS medium without any supplementation of PGR was used as the control. The combinations of PGR with MS medium are given below in **Figure 2**.

Surface Sterilization

All apparatus such as forceps, scalpel, petri dishes, conical flasks etc. need to be cleaned and sterilized before being utilized. These culture apparatus were wrapped in aluminium foil and autoclaved for 30 minutes. Forceps and scalpel were dipped in 99.9% alcohol, sterile distilled water, dried

and dipped into hot bead sterilizer to ensure the sterility of culture apparatus. Forceps and scalpel need to be cooled by dipping them into sterile distilled water before being used to excise plant tissues. All the cultures were labeled and incubated in the culture room under a photoperiod of 16 hours light and 8 hours darkness at $25 \pm 1^\circ\text{C}$ with a light intensity of 1000 lux provided by cool white fluorescent tubes. This was suitable to allow cultures to respond and grow.

After trimming off the larger leaves, the shoot material was cut into pieces (2 - 3cm). These were immersed for surface sterilization by using the HgCl_2 solution and the explants were first washed thoroughly under running tap water for 2 hours and washed with Dettol for 3 minutes and 6 minutes, respectively. After 2 hours, the explants were transferred into the laminar flow chamber (MDH, MICROFLOW) to be surface-sterilized. The explants were then rinsed with 70% ethanol for 1 minute and rinsed 3 times with sterile distilled water for 2 minutes. Finally, the explants were rinsed with sterile distilled water for 5 times. The leaf explants were then cut into squares with approximate size of 0.5 cm x 0.5 cm, stem

and nodal explants were cut into approximately 1-2 cm in length.

Inoculation, direct regeneration and Callus induction from the nodal explants

The uncontaminated leaf and stem explants were transferred into MS medium supplemented with different concentrations and combination of MS + BAP (0.5 to 0.3mg/L), MS + 2,4-D (0.5 to 0.3mg/L) MS + BAP + 2,4-D (0.5 to 0.3mg/L). All the cultures were labeled and incubated in the culture room under a photoperiod of 16 hours light and 8 hours darkness at $25 \pm 1^\circ\text{C}$ with a light intensity of 1000 lux provided by cool white fluorescent tubes. This was suitable to allow cultures to respond and grow. After 4 weeks of culture, plants directly regenerated and the callus was also formed. Also calculated contamination frequency and the Percentage of shoot formation.

Contamination Frequency

Data collected for contamination frequency were the type and morphology of the contaminant over 2 weeks of culture. The data calculated were the total percentage of contamination and the percentage of contamination for each contaminant with the following formula:

$$\text{Percentage of contamination for /each contamination\%} = \frac{\text{Number of contaminated explants} \times 100\%}{\text{Total number of explants cultured}}$$

Plant Regeneration

For shoot multiplication, data collected were the number of shoots formed from the nodal part and shoot morphology. The data

calculated were the percentage of shoot formation and average number of shoots formed over 4 weeks of culture. The formulas were shown as below [19]:

$$\text{Percentage of shoot formation (\%)} = \frac{\text{Number of explants that formed shoot} \times 100}{\text{Total number of explants cultured}}$$

RESULTS AND DISCUSSION

Shoot regeneration

Nodal segments are cultured on Basic medium without growth regulators growth did not show any regeneration responds. MS + BAP 0.3(mg/L) showed 82% of regeneration after 8 days of inoculation. Maximum responds was observed in the MS medium supplemented with MS + BAP + 2, 4-D followed by MS+BAP.

Effect of BAP

MS medium supplemented with various concentrations of plant growth regulators, when MS medium was supplemented with BAP only, shoot formation occurred within 2 weeks of cultured. The maximum number of the shoots and highest shoot regeneration frequency was achieved at 0.3 μ M BAP. Reduction in the number of shoots was observed at lower level of BAP that is 0.1 μ M BAP. Effect of regeneration percentage is shown in **Figure 1 (C&D)**.

Effect of 2, 4-D

When MS medium supplemented with 2, 4-D the regeneration frequency of explant is very low and the contamination rate is very high (**Table 2**). The plantlets thus obtained were successfully hardened and transferred to greenhouse.

Callus induction: callus induction frequency in responds to different combinations of BAP and 2, 4 - D in MS medium is presented in **Figure 1(E)**. Callus were developed in all hormonal combination but the callus frequency high in the hormonal combination of BAP + 2, 4 - D in 0.3 μ M and 0.2 μ M per 100 ml.

Anatomical comparison of ex vitro and in vitro cultured leaves and stem

There is no anatomical difference between ex vitro and in vitro cultured leaves. Result shows that the species has non-glandular and glandular trichomes and transverse section of midrib showed that, the presence of protruding structure on the right and left of adaxial side and U- shaped on abaxial side. Cuticle on the epidermal of leaf

surface often showed the presence of striae in the epiphyll part and usually radiating from the stomata. The result obtained in the study can be used to differentiate the anatomy. The midrib region possesses thick, distinct epidermal layers of fairly large squarish, thick walled cells. Lower part of midrib consists of fairly thick walled, angular cells. The vascular stand is fairly prominent. It is a triangular and comprises a cluster of wide circular thin walled xylem elements. The mesophyll differentiated into palisade and two or three layers of lobed loosely arranged spongy parenchyma cells. In stem, there is the presence of 5-8 vascular bundles are present, and pith is present in the centre (Figure 3).

Histological features of callus

In *C. halicacabum*, callus is developed from nodal segments and it is from the basal cut end. Here, specific auxin to cytokinin ratio in plant tissue culture medium give rise to an unorganized growing and dividing mass of callus cells. These were pale yellow in colour and irregular in shape. The calli were soft, friable, organogenic and greenish.

The success of in vitro techniques largely depends on the availability of efficient and robust tissue culture protocols. In addition the selection of plant growth

regulators and their proper combination is necessary to get high percentage of callus induction and direct regeneration. In the present study addition of MS+BAP in the hormonal concentration of 0.1 to 0.3mg/L and MS+ 2,4-D in the same concentrations shouldn't produce any callus regeneration, but BAP and 2,4-D to MS medium significantly improved callus induction in this plant (MS + BAP + 2,4-D in 0.3 + 0.2 mg/L) (Figure 1). Here we produce the callus from *C. halicacabum*, the production of callus would be advantageous for genetic engineering and fast development of new varieties. This study is mainly conducted for the mass propagation of *C. halicacabum* from nasal and leaf segments. The system described for the mass propagation of *C. halicacabum* by using callus from leaf and nodal segments. In this study the addition of BAP in optimum concentration to MS medium significantly improved direct regeneration of this plant and addition of hormonal combination (BAP + 2, 4-D) into the MS medium also improved the callus production. Also we noticed that, medium containing no or higher concentration of plant growth regulators produced lower quality of callus. Early study reveals that that the concentration of BAP is highly significant for callus production. Here in this

study, higher concentration of 2, 4-D is necessary for callus induction from leaf and nodal segments.

Contamination Percentage

Contamination frequency can be calculated by given equation. It is calculated by using the available data of number of

contaminated tubes and total number of tubes. Here the results showed that the highest contamination range was in the combination of MS+2,4D (0.03) is 85% and the lowest level is in MS+BAP (0.03) is 25% only (**Figure 4**).

$$\text{Percentage of shoot frequency} = \frac{\text{Number of contaminated tube} \times 100}{\text{Total number of tubes}}$$



Figure 1: A) Plant Habit B) Fruit of *C. halicacabum* C&D) Direct regeneration of *C.halicacabum* from axillary bud by Effect of BAP E) Effect of hormonal combination of BAP and 2,4-D shows induction of friable callus from the cut end of nodal explants

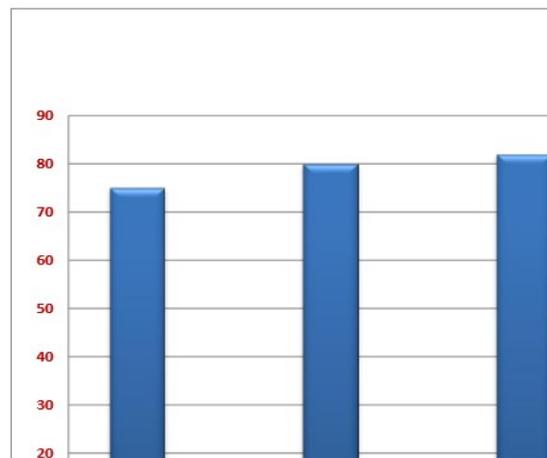


Figure 2: Effect of hormonal combination in direct regeneration of *C. halicacabum*

Table 1: Effect of BAP and 2, 4-D in MS medium on callus induction frequency from nodal segments of *C. halicacabum* plantlet

MS medium + PGR	Concentration (mg/L)	Callus %	Days for callus induction	growth of after 2 weeks	Color and Texture
MS+BAP+2,4-D	0.3+0.1/L	66	16	+	Green,compact
MS+BAP+2,4-D	0.3+0.2/L	87	14	+	Green,compact
MS+BAP+2,4-D	0.3+0.3/L	70	11	+	Green,compact

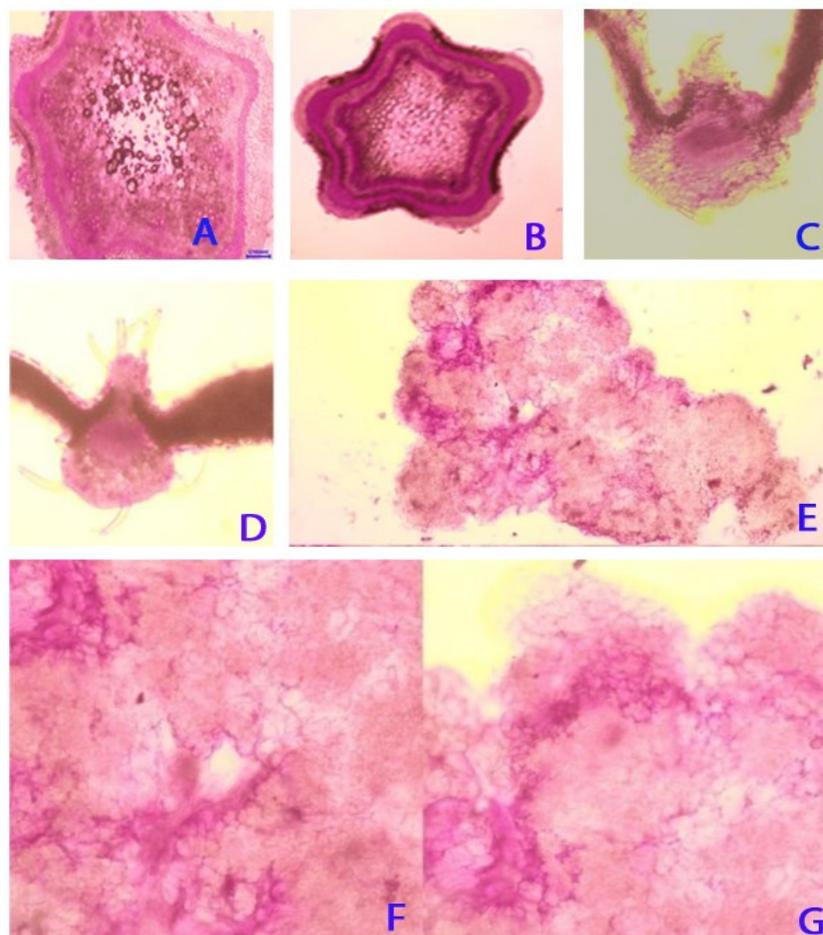


Figure 3: Anatomical studies of *C. halicacabum* A) ex vitro Stem B) invitro stem C) ex vitro leaf D) invitro leaf E-G) Callus

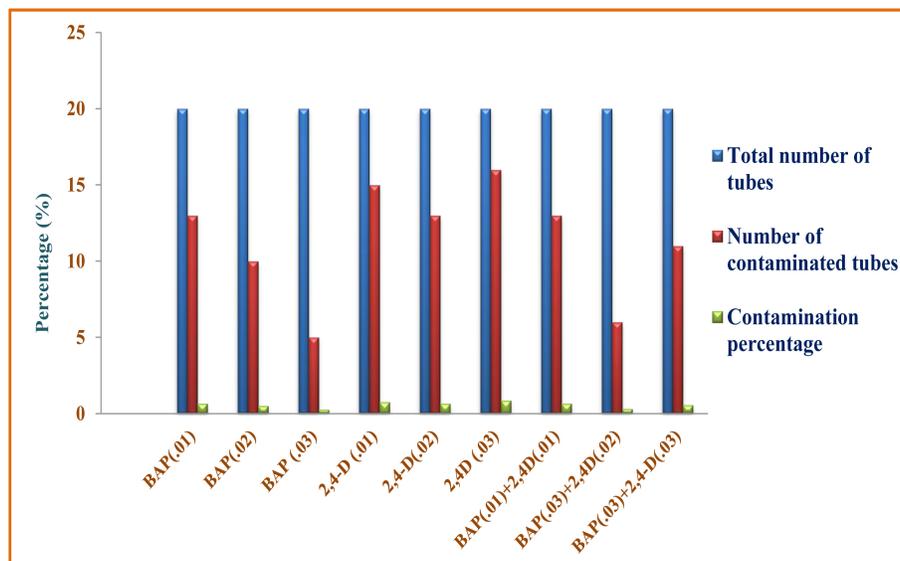


Figure 4: Contamination frequency invitro studies in *C.halicacabum*

CONCLUSION

Anatomical studies of micropropagated plants of *C.halicacabum* was achieved for the first time using the callus and nodal segments. So, this work reveals the importance of the micropropagation of this valuable medicinal plant in a commercial production level. Traditional approaches to plant regeneration from callus by manipulating the relative ratio of plant growth regulators have been successfully used in this study. Also the propagation method is highly useful in the conservation and mass clonal propagation as well as genetic transformation, ecological and physiological studies for horticultural and ornamental improvement of this medicinal plant. In addition the protocol can be used for the analysis of secondary metabolites and bioactivity of *C.halicacabum*.

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Conflict of interest

The authors declare that they do not have any conflicts of interest in this research.

Abbreviations

MS – Murashige and Skoog, BAP – 6-Benzylaminopurine, NAA – 1-

Naphthaleneacetic acid, IAA - Indole-3-acetic acid, 2,4 - D -2,4-Dichlorophenoxy acetic acid, Kn -Kinetin, TDZ - Thidiazuron, ECH- Ethnolic extract of *C. halicacabum*.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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