MATERIALS AND METHODS

Explant source

Healthy young twigs (10-15 cm) were collected from 3-5 months old plants of *Cardiospermum halicacabum* L. growing in the field of the Bharathidasan university campus, Tiruchirapalli. After trimming off the larger leaves, the shoot material was cut into pieces (2-3 cm), each containing one single node. These were washed in running tap water for 30 min.

Sterilization

*Axillary node*

2-3 cm long nodal segments were washed in an agitated solution of liquid detergent (Teepol) for 15 min. The washed explants were surface sterilized by immersion in a solution of commercial bleach (10% Sodium hypochlorite) followed by 2-3 min in a solution of mercuric chloride (0.1% w/v). The buds were finally rinsed 4-5 times with sterile distilled water and cut into pieces (1.0-1.5 cm long) before being implanted into the culture medium.

*Shoot tip*

1-2 cm long shoot tips were collected separately and disinfected using the same procedures as described for nodal explants with exception of 5% v/v sodium hypochlorite solution. Shoot tip ca. 5mm in length, free from visible buds but
consisting of apical meristems and protective sheath of 1-4 young leaves and primordial, were excised from sterilized pieces.

*Leaf and internode*

Leaf and internode were isolated from the aerial parts and were sterilized using the method followed for shoot tip.

*Culture medium*

The basal medium consisted of the mineral salts and organic nutrients of Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), 3% sucrose, myo-inositol (100 mg w/v) and 0.8% agar (Bacteriological grade). All the chemicals were analytical grade (Sigma, Sd fine and Himedia). After, all of the supplements have been added, the pH of the medium was adjusted to 5.8 before adding molten agar. About 10-15 ml of molten medium was dispensed into boiling tubes (Borosil, India) and the culture tubes were capped with cotton plug. The culture media were sterilized by autoclaving at 1.06 kgcm⁻¹ and 121°C for 20 min. The explants were implanted vertically onto culture medium (15ml) in culture tubes (25x150mm) and sub cultured every 4 weeks unless otherwise stated.

*Culture conditions*

The cultures were maintained in a culture room at 25±2°C under a 16-h photoperiod with a light intensity of 35μS⋅m⁻²⋅S⁻¹ from Philips cool white fluorescent tubes with 55-60% relative humidity. Each treatment had 10-25 replicates and was repeated thrice.

**MICROPROPAGATION**

Depending on the experiment, the basal medium was variously supplemented with factorial combinations of different growth regulators such as Benzyl adenine (BA), Kinetin (KN) and 2-isopenteny1 adenine (2ip) with the range of 1.0 to 2.0 mgl⁻¹, and
Indole butyric acid (IBA), Indole acetic acid (IAA), Naphthyl acetic acid (NAA) at different concentrations (0.01-1.0 mg l⁻¹). The effect of amino acids and other organic supplements on shoot induction, growth and development were also determined.

**Multiplication of shoots**

After 4 weeks of culture initiation, primary shoots formed *in vitro* were isolated and sectioned into one shoot tip with swollen base after removing the leaves. The shoot segments each containing the shoot tip were subcultured on MS medium fortified with same growth regulator concentrations and combinations depending upon the experiment for further multiplication. Also a range of plant growth regulator types and concentrations and other additives were tested for multiple shoot initiation with axillary and apical explants. Subsequent cultures were in the same medium at a periodic interval of 4 weeks. The same procedure was used for the *in vitro* raised shoots form axillary buds.

**Shoot elongation**

For shoot elongation, regenerated shoot buds (2-3cm) were cultured on MS medium containing BA (2.0 mg l⁻¹) + GA₃ (0.1-2.0 mg l⁻¹).

**Rooting of shoots**

For rooting, shoots (4-5 cm in length) obtained from the proliferated buds of the two types of explants were harvested and individually transferred to MS medium supplemented with 0.5-2.0 mg l⁻¹ IAA, IBA and NAA individually. The cultures were grown for 30d before data were collected.

**Acclimatization and transfer of plantlets to soil**

Four to six weeks old regenerants with well-developed roots were removed from the culture tubes and washed free of agar. The plants were transferred to plastic containers (5 cm diameter) containing a mixture of vermiculite and red soil (3:1) and
maintained inside a culture room set at 26±1°C, 16h day length (35 - 40 μS m⁻² S⁻¹) and 75-80 % relative humidity was maintained by covering the plant with polythene bags. The potted plants were irrigated every 4d with half strength MS basal salt solution devoid of sucrose and inositol for a period of 5 weeks.

Statistical analysis

At the end of the initial 4-week culture period and another 4-week multiplication cycle, the following variables were determined: 1) percentage of explant response, 2) number of shoots longer than 8mm, and 3) the length of tallest shoots produced by each explant. Rooting experiments were evaluated by determining percentage of rooting and root number using ten shoots per treatment.

Minimum of 10-25 replicates were taken for each treatment and all the experiments repeated thrice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. Whenever possible, the effect of different treatments were quantified on the basis of percent cultures showing the response per culture. The experimental design was random and factorial with cytokinin and auxin as independent variables. The data pertaining to frequencies of shoot proliferation and multiplication, shoot elongation and rooting were subjected to mean ± SE.

ORGANOGENESIS

Callus induction

The sterilized explants (axillary node and shoot tip) were cultured on MS medium added with different concentrations and combinations of auxins [IAA, NAA (1.0-5.0 mg/l) ] and cytokinins [BA, KN (0.5 mg/l) ].
Shoot regeneration and multiplication

Calli derived from both the explants were cultured on shoot regeneration medium. The different constituents include plant growth regulators, inorganic, organic and natural products such as BA, KN, Sodium Citrate, Citric acid, Ammonium nitrate (NH₄NO₃), Potassium nitrate (KNO₃), Isoleucine, Adenine, Adenine sulfate and other amino acids in various concentrations and combinations based upon the earlier reports. The plant regeneration from leaf and internode derived callus could not be obtained in this plant. Therefore, an attempt was made to induce callus tissue from shoot tip explants and regenerate them into plantlets. The effects of various treatments on morphogenesis from C. halicacabum were investigated.

Elongation, rooting and hardening

For elongation, rooting of shoots and hardening of the plantlets, treatments were given as described in micropropagation procedure.

SOMATIC EMBRYOGENESIS

Solid medium
Callus induction and proliferation

Sliced sections of leaflet and internode (5-10mm) were placed onto MS solid medium incorporated with various concentrations of different auxins (2,4-dichlorophenoxy acetic acid-2,4-D, NAA, IAA and IBA) individually or in combination with cytokinins (BA and KN) for inducing callus. Few incisions were made in each foliole perpendicularly to the mid-vein using a sharp razor blade, and the folioles were oriented so that the abaxial side was in contact with the medium for induction of embryogenic -competent callus. Twenty-one days old callus was subcultured on to fresh medium with same growth regulator combinations for further proliferation. Callus was subcultured twice with 3 weeks interval.
**Induction of somatic embryos**

Somatic embryos were obtained in a two-step procedure. In the first step, three week old friable callus and yellow white in colour was cultured on induction medium amended with various concentrations of 2,4-D (0.0-5.0 mg l⁻¹) alone or combined with BA (0.1-2.0 mg l⁻¹) for initiation of embryogenic competent callus. From these investigated combinations, best one was selected for further proliferation of somatic embryos. Ten callus pieces per treatment (each 300-400 mg FW) with five replicates were used. The embryogenic calli were subcultured at 4 weeks intervals on a 2,4-D supplemented medium for proliferation. For investigating the role of amino acids and other organic supplements on induction of somatic embryos, friable calli were transferred to their respective medium. In the second step, the same medium was used but with 2,4-D and sodium citrate to allow somatic embryo development.

**Maturation of somatic embryos**

To obtain mature embryos, the calli bearing globular embryos were transferred to maturation medium fortified with BA (1.0 mg l⁻¹) plus sodium citrate (25-125 mg l⁻¹). The number of different stages of embryos was counted after 4 weeks under stereomicroscope.

**Liquid or suspension culture**

**Callus induction and proliferation**

As described in above section.

**Somatic embryo induction**

Suspension cultures were initiated by transferring 3 weeks old callus (500 mg) to 25 ml of induction medium in 100ml Erleynemeyer flask containing 2,4-D in various concentrations (0.0-5.0 mg l⁻¹). The flasks were agitated on a rotary shaker with 100 rpm under light condition of 80 μ M S at 25±2° C. Embryogenic suspension culture
(500mg fresh mass) were subcultured with every three weeks interval for two months, after which cultures were discarded.

After induction of somatic embryos, the number of embryos with different stages were counted on different days (5-20) of incubation. Samples of suspension cultures were taken at random and the number of somatic embryos were counted under microscope. Counting was made from 10 replicates. The frequencies of somatic embryo production and total number of embryos in each culture were calculated.

Various concentrations of amino acids (Methionine and Glutamine) were tested for induction and enhancement of somatic embryogenesis with 2,4-D.

**Data collection**

Data were collected after 6-8 weeks of culture. There were 10 replicates per treatment and all experiments repeated thrice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The fresh weight of callus was taken by gently removing the tissue from medium. Each embryogenic tissue was then dispersed in 15mm petridish filled with 10ml of sterile water and embryos were counted under a dissecting microscope. The numbers of somatic embryos at different developmental stages (globular, heart, torpedo shaped) were recorded after 30 days. Data on somatic embryos forming either roots or shoots were also collected.

**Statistical analysis**

The experimental design was random and factorial with growth regulators as independent variables. The data pertaining to frequencies of somatic embryo induction and maturation were subjected to statistical analysis. Data from the growth regulator experiments were analyzed as a factorial arrangement and followed by multiple comparison procedure and the mean differences were tested with Duncan’s Multiple Range Test (Ê=0.05)(Gomez & Gomez, 1976).
PHYTOCHEMICAL STUDIES

Collection and storage of plant material

The preliminary phytochemical screening tests has been attempted in *C. halicacabum* plant parts and callus material to find out the presence or absence of certain bioactive compounds. The method of extraction and the respective test procedures were given below. For each experiment triplicates were maintained. Phytochemical screening of plant was carried out by using the following methods (Amarasingham *et al.*, 1964); Das & Bhattacharjee (1970); Gibbs (1974); Santaram (1983) and Harborne (1998).

The plant parts were collected from 3-5 months old matured plants and washed with water and then chopped into small fragments. Then materials were air dried under shade conditions for 4-5 days and the drying operation was carried out under controlled conditions to avoid chemical changes. The dried samples were powdered roughly with hands. The powdered samples were stored in polythene containers at room temperature. The callus material (friable and white) obtained from various explants were collected and dried in hot air oven at 60°C for 48 hrs. Then the dried material was powdered using mortar and pestle and the powdered samples were stored in polythene containers at room temperature.

Extract preparation

The organic constituents from dried plant tissue and callus material were obtained by continuously extracting the powdered material in Soxhlet apparatus with a range of organic solvents, starting from non-polar solvents petroleum ether, benzene, chloroform (to separate lipids and triterpenoids) to polar solvents acetone, ethyl acetate, ethanol and methanol (for more polar compounds). The extracts were concentrated to one third of their original volume and used for testing the chemical constituents.

The coarse powder of leaf and callus was extracted separately with 1000 ml of petroleum ether at the temperature 60-80°C by continuous hot percolation using
soxhlet apparatus until the extraction was completed (24 hrs). After completion of extraction, the extract was filtered and concentrated to dryness under pressure. A yellowish dark green residue was obtained. The marc left after petroleum extraction was dried and extracted further with other solvents like benzene, chloroform, ethyl acetate, ethanol and methanol in this sequence (non polar to polar solvents). But the extraction was done with each solvent at their respective boiling points viz. benzene (79-81°C), chloroform (59-61°C), ethyl acetate (60-70°C), acetone (55-56°C), butanol (60-80°C), ethanol (35-40°C) and methanol (35-40°C). After each extraction, marc was dried completely and reused. Likewise after every extraction the residue was collected separately and dried. The petroleum ether and benzene extracts were dark-green and paste like. Next the chloroform and acetone and ethyl acetate residues were light green, brownish in colour and finally ethanol and methanol residues look light brown and whitish powder.

Screening tests

All the above extracts were used for testing alkaloids, volatile oils, fatty acids, emodins, flavonoids, steroids/triterpenoids, anthracene glycosides, tannins, phenolics and saponins. The methods of preliminary phytochemical analysis based on the methods of Brindha et al. (1977) and Kokate (1995) is given in Table-3.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Experiment</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Test solution+minimum amount of chloroform+3drops of con.H₂SO₄ (Liebermann Burchard Test)</td>
<td>Purple colour changing to blue or green</td>
<td>Presence of steroids</td>
</tr>
<tr>
<td>2.</td>
<td>Test solution+piece of Tin+3drops of thionylchloride</td>
<td>Violet or purple colour</td>
<td>Presence of triterpenoids</td>
</tr>
<tr>
<td>3.</td>
<td>Test solution + shaken with 2N HCl, aqueous layer formed decanted and to which one or two drops of Meyer's reagent is added</td>
<td>White turbidity or precipitate</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>4.</td>
<td>Alcoholic solution on drop of Ferric Chloride</td>
<td>Intense colour</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>5.</td>
<td>Test solution +water, shaken well (Froth test)</td>
<td>Foaming lather</td>
<td>Presence of saponins</td>
</tr>
<tr>
<td>6.</td>
<td>Water soluble portion of the extract treated with basic lead acetate solution</td>
<td>White precipitate</td>
<td>Presence of Tannins</td>
</tr>
<tr>
<td>7.</td>
<td>Test solution +Magnesium powder and treated with con.HCl, cooling the test tube under running water (Shinoda test)</td>
<td>Orange colour</td>
<td>Presence of flavanoids</td>
</tr>
<tr>
<td>8.</td>
<td>Evaporation of the extract on a porcelin crucible</td>
<td>Aromatic smell of the residue</td>
<td>Presence of volatile oils</td>
</tr>
<tr>
<td>9.</td>
<td>Evaporation of the extract on a filter paper</td>
<td>A translucent spot on the paper</td>
<td>Presence of fatty acids</td>
</tr>
</tbody>
</table>