3. MATERIALS AND METHODS

3.1. Explant source

Different healthy young explants [(Axillary bud (AB), Shoot tip (ST) and Inter node (IN), Leaf (L)] were collected from three year old plants of *Tinospora cordifolia* established in the field of Department of Plant Science, Bharathidasan University campus, Tiruchirappalli, India (Plate1a). Cotyledonary node (CN), cotyledon (C), Young axillary bud (YAB) and young shoot tip (YST) explants were collected from fifteen days old seedlings grown in vitro (Plate1b).

3.2. Sterilization

Sterilization procedure for the explants varied depending upon their sensitivity to sterilizing chemicals. The collected explants were washed under running tap water for 2-5 minutes and then washed with teepol solution for 30 seconds. The explants were then surface sterilized with 70% alcohol for 30 seconds followed by treatment with 0.1% HgCl$_2$ for 2-4 minutes. The explants were then cut into pieces (1.0-1.5 cm long) before being cultured on the nutrient medium.

3.3. Culture medium

The various media tested in the present study were Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), woody plant medium (WPM) (Lloyd and Mc Cown, 1981) and modified MS medium (mMS -MS+B$_5$ vitamins). The culture media consisted of the mineral salts and organic nutrients, 3% sucrose, Myoinositol (100 mg W/V), 0.8% agar (Hi media) and different growth regulators at different concentrations (Cytokinins and Auxins). The pH of the medium was adjusted to 5.7 before adding molten agar. The culture tubes were then sterilized by autoclaving at 104 KPa and
121° C for 20 minutes. The molten agar medium was then dispensed into culture tubes and plugged tightly with non-absorbent cotton.

3.4. Culture conditions

After the inoculation of the explants, the culture tubes were maintained in a culture room under controlled conditions of 25 ± 2°C for 16h photoperiod by cool white fluorescent light (35μEM^-2 S^-1) with 55-66% relative humidity. Each treatment had 10 replicates and was repeated thrice.

3.5. Acclimatization

Six-week-old well-rooted plantlets were removed from the culture tubes and washed free of agar. The plantlets were then transferred to plastic containers (5 cm diameter) containing red soil and vermiculite (1:3) and maintained at 25±2° C, 16h day length (35-50 m EM^-2 s^-1) and at 75-80% relative humidity. The potted plants were irrigated with half-strength MS basal medium devoid of sucrose and myo-inositol, every four days for a period of two weeks. The plants were then transplanted to earthenware pots (10 cm diameter) containing natural soil and kept under shade for 2 weeks. The potted plants were then moved to the garden.

3.6. Micropropagation

3.6.1. Explant collection and sterilization

Young and mature axillary buds (YAB and MAB), young and mature shoot tips (YST and MST) and cotyledonary nodes (CN) were the different explants used for micropropagation. The explants after collection were washed thoroughly under running tap water for 5 minutes, followed by rinsing with teepol for 30 seconds. The explants were then surface sterilized with 70% alcohol for 30 seconds followed by treatment with 0.1% HgCl2 for 4 minutes. After each treatment the explants were washed thoroughly with sterilized distilled water for 3 minutes. The explants were then cut into pieces (1.0 cm) and inoculated vertically on the culture medium containing various growth regulators and the cultures were maintained under optimal growth conditions.
3.6.2. Culture media

3.6.2.1. Shoot initiation
Different media (MS, mMS and WPM) fortified with cytokinins Benzyl adenine (BA) (0.1 - 4.0 mg/l) and Kinetin (KN) (0.1- 4.0 mg/l) alone or in combination were tested along with auxins, Indole acetic acid and Naphthalene acetic acid (IAA/ NAA 0.1 - 1.0 mg/l) for shoot initiation. The effect of gibberellic acid (GA3) and adenine sulphate (AdS) on shoot induction, growth and development were tested. Various antioxidants (citrate, polyvinylpyrrolidone, ascorbic acid and charcoal) were also tested for their effective reduction of phenolics from explants.

3.6.2.2. Shoot bud induction and multiplication
After 2 weeks of shoot initiation the initiated shoots were transferred to MS media fortified with optimal concentrations of BA (2.0 mg/l)+KN (0.75 mg/l)+auxins (IAA/NAA 0.1-1.0 mg/l) +GA3 (0.1-1.0 mg/l) +0.8% sucrose and ascorbic acid (100 mg/l). Subculturing was done on the same medium at periodic intervals of 2 weeks.

3.6.2.3. Shoot elongation
Proliferated shoots (2-4 cm) were cultured on MS medium containing MS basal salts along with GA3 (0.1-2.0 mg/l).

3.6.2.4. Rooting of shoots
Healthy and elongated shoots (6-7 cm length) formed in vitro were excised and transferred to half-strength MS basal medium supplemented with IBA (0.1-1.0 mg/l) or NAA (0.1-1.0 mg/l).

3.6.3. Acclimatization and hardening
Acclimatization and transfer of plantlets to soil and treatments given were the same as described above.

3.6.4. Statistical analysis
At the end of the period of 45 days, the shoot formation experiments were evaluated by determining the percentage of explant response, shoot bud induction, number of shoots/culture, shoot length and root initiation. All the experiments were repeated thrice.
with 10 replicates for each treatment. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation wherever possible, the effects of different treatments were quantified on the basis of percent cultures showing the response/culture. The experimental design was random and factorial with cytokinins and auxins as independent variables. The data pertaining to frequencies of shoot proliferation, shoot elongation and rooting was carried out using Duncan's New Multiple Range Test (DMRT). Significance was determined at 5% level (Gomez and Gomez, 1976).

3.7. Organogenesis

3.7.1. Explant source and sterilization
Healthy young and mature leaves (YL and ML) and internodal (IN) explants were collected from 3-year-old field grown plants and cotyledons were collected from in vitro seedlings. The explants were washed under running tap water for 2-3 minutes and then surface sterilized with 70% alcohol for 30 seconds followed by treatment with 0.1% HgCl₂ for 2 minutes. Thorough washing with sterilized distilled water was done after each treatment. The explants were cut into pieces (1.0-1.5 cm) and slightly wounded on the abaxial side and placed horizontally on the culture medium. The cultures were maintained under optimal culture conditions.

3.7.2. Indirect organogenesis
3.7.2.1. Callus induction
The explants were cultured on mMS medium supplemented with different concentrations and combinations of (0.1-4.0 mg/l) IAA, 2, 4-D, NAA and IBA along with 3% sucrose (W/V), 0.8% agar (W/V) for callus initiation and proliferation. Different concentrations of antioxidants/adsorbents-PVP, ascorbic acid, citric acid and charcoal in the range of (10-150 mg/l) were also tested in effective reduction of browning of tissues. The callus was subcultured at 10 day interval. Callus initiation was observed from 15-20 days of inoculation.

3.7.2.2. Shoot regeneration and multiplication
The proliferated yellow nodular calli were cultured on shoot bud regeneration medium containing various concentrations of BA (0.1-4.0 mg/l), KN (0.1-2.0 mg/l), IAA (0.1-
1.0 mg/l) and NAA (0.1-1.0 mg/l). After shoot bud induction, the cultures were subcultured on optimum concentrations of BA (3.0 mg/l) + KN (0.75 mg/l) + IAA (0.75 mg/l) + GA₃ (0.05 mg/l) and BA (3.0 mg/l) + KN (1.0 mg/l) + NAA (0.75 mg/l) + GA₃ (0.05 mg/l) at periodic interval of 10 days.

3.7.3. Direct organogenesis

3.7.3.1. Shoot bud induction and shoot multiplication
Leaf and cotyledon explants were cultured on MS medium containing high cytokinin (BA and KN) and low auxin ratio (IAA and NAA). After direct shoot bud induction the cultures were transferred to shoot multiplication medium as that of indirect organogenesis.

3.7.3.2. Shoot elongation, rooting and acclimatization
Regenerated shoot buds (0.5-2.0 cm) were transferred to MS medium containing GA₃ (1.0 mg/l). Shoot elongation was observed from 10-15 days. The elongated shoots were then transferred to rooting media (1/2 MS+IBA) and acclimatization was done as described above.

3.7.4. Statistical analysis
All the experiments were repeated thrice with 10 replicates for each treatment. The data pertaining to frequencies of callus induction, shoot bud differentiation, shoot multiplication, root initiation and number of shoots/culture were subjected to analysis of variance and mean separation was carried out using Duncan’s New Multiple Range Test (DMRT). Significance was determined at 5% level.

3.8. Somatic embryogenesis

3.8.1. Somatic embryogenesis on solid medium

3.8.1.1. Callus induction and proliferation
Leaf explants collected from 3-year-old field grown plants were used as the source for somatic embryogenesis. Few incisions were made perpendicular on the midrib region of the foliar blade and the abaxial side was placed horizontally on MS medium containing various concentrations of 2,4-D (0.1-3.0 mg/l). After callus initiation they were subcultured twice on same medium for callus proliferation at 2 week interval.
3.8.1.2. Somatic embryo induction and maturation

Proliferated whitish yellow friable calli were transferred to induction medium containing various concentrations of 2,4-D (0.5-3.0 mg/l) in combination with BA (0.1-2.0 mg/l). At the end of 20-25 days, the globular embryos formed were transferred to MS medium fortified with different concentrations of BA (0.1-3.0 mg/l) + GA₃ (0.05-1.0 mg/l) + glutamine (1-10 mg/l) for further development and maturation. The number of different stages of embryos was counted after 4 week under stereomicroscope.

3.8.1.3. Germination of somatic embryos

The mature somatic embryos were then transferred to germination medium containing only BA (0.05 - 1.0 mg/l) and medium containing BA (0.5 - 1.0 mg/l) + GA₃ (0.05 - 1.0 mg/l) + glutamine (1-10 mg/l).

3.8.2. Somatic embryogenesis in liquid medium

3.8.2.1. Callus induction and proliferation

Callus induction and proliferation were carried out in the same manner as for solid medium.

3.8.2.2. Somatic embryo induction

Two week old whitish yellow friable calli (500 mg were aseptically transferred to 150 ml Erlenmeyer flask containing 50ml of MS basal medium with 3% (W/V) sucrose, various concentrations of 2, 4-D (0.1-3.0 mg/l) and BA (0.1-3.0 mg/l). The flasks were agitated on rotary shaker with 100 rpm under light conditions of 8 EM² s⁻¹ at 25 ± 2 °C. The cultures were subcultured at regular intervals of 10 days (500 mg fresh mass). The globular embryos formed in the suspension were observed under a microscope during the culture period.

3.8.2.3. Somatic embryo maturation and germination

The globular embryos formed were transferred to MS basal medium fortified with BA (0.5 - 3.0 mg/l) + GA₃ (0.05 - 1.0 mg/l) + glutamine (10-50 mg/l). Subculturing was done at two week interval. Different stages of embryos heart, torpedo and cotyledonary stages developed after a certain period. For germination of somatic embryos they were
transferred to germination medium as described for solid medium. The number of somatic embryos at different stages i.e. globular, heart, and torpedo were recorded after 30 days.

3.8.3. Statistical analysis
Data were collected after six weeks of culture. There were 10 replicates for each experiment and the experiments were repeated thrice. The experimental design was completely randomized and factorial with growth regulators as independent variables. The data pertaining to frequencies of somatic embryo induction and maturation were subjected to statistical analysis. Analysis of variance and mean separation was carried out using Duncan's New Multiple Range Test (DMRT). Significance was determined at 5% level.

3.9. Determination of Berberine

3.9.1. Explant source and sterilization
*Tinospora cordifolia* plants growing in experimental field were used as the donor plants for the initiation of cultures. Stem segments (internode) excised from the plants were washed several times in running tap water, rinsed with few drops of teepol solution and surface sterilized with 70% ethanol for 30 seconds and 0.1% HgCl₂ for 4 minutes. Segments were then washed thoroughly with sterile water to remove HgCl₂ before culturing them on the nutrient medium.

3.9.2. Initiation of callus cultures
Sterilized stem explants were cut into pieces of 5mm, wounded and placed on sterilized MS medium containing different concentrations of IAA (0.5 - 3.0 mg/l), NAA (0.5 - 3.0 mg/l) and 2,4-D (0.5 - 3.0 mg/l). The effect of cytokinins BA and KN were tested in combination with IAA (0.5 mg/l) (regenerative callus induction). The pH of the medium was adjusted to 5.7. Agar (0.8%) was used as the gelling agent. The medium was autoclaved at 15 psi and 121 °C for 15 minutes. The cultures were maintained under controlled conditions of light, temperature and humidity as mentioned earlier. The maximum biomass production period was tested at regular intervals of 15, 25, 35 and 45 days period and their fresh weight (FW) and dry weight (DW) were calculated. Subculturing was done at 15 days interval on the same medium with the same
Materials and Methods

concentration of growth regulators. Dry weight (DW) of callus was determined by
drying the callus at 60 °C in an oven to a constant weight. Results were expressed as
percentage dry weight (g/l). Effect of light and temperature on biomass production of
callus at 35 days was studied by maintaining the cultures at different temperatures
(25 °C and 30 °C) and under white fluorescent light (control) and UV light (treated).
Assuming there is correlation between maximum biomass production and berberine
accumulation the callus with higher biomass was analyzed quantitatively for berberine
accumulation in all samples. A minimum of three replicates were used for all the
growth treatments and the experiments were repeated thrice. The data were expressed
as mean performance of all the replicates.

3.9.3. Extraction of Berberine

3.9.3.1. Thin layer chromatography
The field grown plants of *Tinospora cordifolia* (stems) were sun dried and defatted
with petroleum ether and the residue was subjected to chloroform and methanol
extraction for 72 h (soxhlet). The chloroform and methanol extracts were evaporated
and subsequently taken up for the isolation and identification of major alkaloids. One
gram of dried callus from each sample was used for the quantification of berberine.
Thin layer chromatogram was performed using silica gel G with MeOH: CHCl₃ (20:80)
as solvent system. The alkaloids were identified by their fluorescent colours by
spraying Dragendorff’s reagent and comparing the Rf values. The alkaloid profile of
callus and plant material was compared and taken for HPLC analysis for further
quantification.

3.9.3.2. High Performance Liquid Chromatography
HPLC analysis of various methanol extracts of field grown plants and callus cultures
were carried with reverse phase silica gel HPLC (SHIMADZU, JAPAN) using
analytical –Shim-Pack CLC-ODS –C18 W column, methanol : acetonitrile (60 : 40) as
the solvent system with a flow rate of 1ml/min and the detector set at 254 nanometer.
The berberine alkaloid present in callus and plant material was identified by
comparison of their retention times with the authentic berberine sample received from
Sigma Chemicals Co., Bangalore, India.