Materials & Methods
3. Materials and Methods

3.1. *Hildegardia populifolia*

3.1.1. Plant material collection

3.1.1.1. Source of plant material
Twigs and dry follicles of *Hildegardia populifolia* were collected in the month of November during 2004 from Kalrayan hills (300-600 m), Eastern Ghats of Tamil Nadu, India and cuttings were maintained in the garden of Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India (Plate 1).

3.1.1.2. Cuttings
The twigs collected in different thickness were cut in a slant position and the tip of the twigs was dipped in wax. The basal part was treated with IBA (10mg/100ml) for 2 hours and then, the pretreated twigs were planted in the polythene bags with sand and soil mixture.

3.1.1.3. Seed germination
The infertile seeds were discarded by water floatation method and the healthy seeds were selected for further treatment. To raise the seedling, the three types of treatments were adopted. In the first treatment, seeds were directly sown in the pots containing sand and soil and irrigated frequently.

In the second treatment, the seeds were treated with Conc. H$_2$SO$_4$ for different time duration (5, 15, 30, 45, 60 and 90 min.) and the seeds were scrubbed over rough surface to soften the hard outer coat and washed thoroughly in running tap water. Then, the seeds were sown in the soil and sand mixture followed the proper irrigation.
Hildegardia populifolia - Habit

a. & b. Natural habitat c. A twig with pods
In the third treatment, seeds were treated with Conc. H₂SO₄ to soften the outer hard seed coat for different time duration (5, 15, 30, 45, 60 and 90 min.). The acid treated seeds were scrubbed over rough surface and the seeds were washed thoroughly in running tap water. The seeds were soaked in sterile distilled water and placed in a rotary shaker for the uniform imbibitions of water by the softened seeds for 2 days to improve the breakage of seed coat. On the third day, the acid treated seeds were again washed thoroughly in sterile distilled water after 1-2 drops of liquid detergent (Teepol) for 5 minutes. The washed seeds were disinfected by rinsing in 70% ethyl alcohol for 5 minutes and followed by aqueous solution of 0.1% (W/V) mercuric chloride (HgCl₂) for 10 minutes. Then, the seeds were rinsed 4-5 times with sterile distilled water. Sterilized seeds were aseptically inoculated in both MS medium supplemented with GA₃ (1.0 mg/l) and cotton soaked with sterile water. The inoculated seeds were incubated under optimal culture condition.

The in vitro raised seedlings were transferred to earthen pots containing soil and sand (1:1) and maintained in the garden. The explants were collected from two months old in vitro derived seedling and cuttings.

3.1.2. Explant sterilization
Explants such as axillary bud, apical bud, internodes, leaf and petiole of 1-2 cm length from healthy plants were initially washed with few drops of liquid detergent (Teepol) for 5 min followed by rinsing in running tap water. The washed explants were disinfected by rinsing in running tap water. The washed explants were disinfected by rinsing in 70% ethanol for 3 min and with aqueous solution of 0.1 % (W/V) mercuric chloride for 5 min. Finally the explants were rinsed 4-5 times with sterile distilled water and cut into pieces each 0.5-1.0 cm long. Nodal segments containing the nodal region with axillary buds were inoculated upright with the end of the node inserted a few mm into the culture medium. Apical bud of 0.5 cm length was inoculated on culture medium.
Internode and petiole were also cut into a few mm size and inoculated on culture medium.

3.1.3. Culture medium

Various types of basal media such as MS medium (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) medium, mMS medium (MS + organic supplements of B5 medium) and woody plant medium (Lloyd and McCown, 1981) consisted of the mineral salts, organic nutrients, 3% sucrose, meso-inositol and 0.8% agar (Agar Type I, Himedia) were used. Various plant growth regulators were tried such as cytokinins [BA (6- Benzyl adenine), TDZ (Thidiazuron) and KN (6-Furfuryl amino purine)], auxins [NAA (α – Naphthalene acetic acid), IBA (Indole-3-Butyric acid), 2,4-D( 2,4 - Dichlorophenoxo acetic acid), IAA( Indole-3- acetic acid) or Picloram)] and Gibberellic acid (GA3) alone or in combination at various concentrations based on the experiments. The pH of the medium was adjusted to 5.7 before gelling with agar. Aliquots of the medium (10-15ml) were dispensed into 150mm x 25mm rimless glass culture tubes plugged with non absorbent cotton plugs and autoclaved at 1.06 Kg/Cm² and 121°C for 15 mins.

3.1.4. Culture conditions

The cultures were incubated in culture room maintained at 25± 2°C, under a 16 h photoperiod with a light intensity of 35μE m⁻²S⁻¹ from Philips cool white fluorescent tubes with 55- 60% relative humidity. For each treatment 25 replicates were used and each experiment was repeated thrice.

3.1.5. Micropropagation

3.1.5.1. Shoot proliferation

The nodal segments (axillary bud) and shoot tips (apical bud) were cultured on MS medium containing of BA, KN and TDZ (0.5-5.0mg/l) individually or in combinations for initiation. For further proliferation the shoots were subcultured on MS medium supplemented with BA (1.0 mg/l) and combination with KN (0.05-2.0 mg/l).
3.1.5.2. Shoot multiplication and elongation

For further multiplication and elongation, the shoots were sub cultured on MS medium supplemented with BA (1.0 mg/l), KN (0.5 mg/l) and GA₃ (0.1-3.0 mg/l).

For further increase in shoot quality, the additives like adenine, adenine sulphate, glutamine and sodium citrate (10-50 mg/l) were added to the medium containing BA (1.0 mg/l), KN (0.5 mg/l) and GA₃ (1.0 mg/l).

3.1.5.3. Rooting

Elongated shoots from the clump of shoots were excised and subcultured on different strength MS medium containing IBA, NAA (0.1-3.0 mg/l) with different concentration of sucrose (1-3%) and without sucrose.

3.1.6. Organogenesis

3.1.6.1. Callus induction

The sterilized explants (internode, petiole and leaf) were cultured on MS medium supplemented with different combinations and concentrations of auxins [IAA, IBA, NAA and 2, 4-D (0.1-3.0 mg/l)] for callus induction.

3.1.6.2. Callus proliferation

Callus was transferred to MS medium containing cytokinins [BA and KN (0.5-3.0 mg/l)] individually and in combination with auxins [IAA and IBA (0.01-0.5 mg/l)] for proliferation. For further enhancement, the callus was transferred to BA (2.0 mg/l), IAA (0.1mg/l) and additives such as adenine, adenine sulphate, glutamine and sodium citrate (10-50 mg/l).

3.1.6.3. Shoot regeneration and multiplication

Green compact nodular callus was cultured on shoot regeneration medium containing BA (2.0 mg/l), IAA (0.1 mg/l), glutamine (25 mg/l) and varying concentration of TDZ (0.1 mg/l-3.0 mg/l). Callus was sub-cultured on MS
medium supplemented with BA (2.0 mg/l), IAA (0.1 mg/l), glutamine (25 mg/l) and various concentrations of antioxidants (activated charcoal, PVP and ascorbic acid) (5-25 mg/l) to increase the number of regenerants.

3.1.6.4. Rooting
The regenerated multiple shoots were excised and sub-cultured on MS medium containing IAA, IBA and NAA (0.1-3.0 mg/l) for root induction.

3.1.6.5. Acclimatization and transfer of plantlets to soil
Four weeks old in vitro raised plantlets with well developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transferred to plastic containers and earthen pots containing mixture of sand: soil (1:2) maintained inside a culture room at 25 ± 2°C and 35μS m⁻² S⁻¹ light intensity provided by cool white fluorescent tubes and 70-80% relative humidity was maintained by covering the plant with polythene bags. Plantlets were watered with 1/4 – strength MS basal solution devoid of sucrose and meso-inositol at 3 days intervals for a period of 3 weeks. The acclimatization plantlets were then transferred to pots containing soil and kept under shade for another 8 weeks before transferring to the field.

3.1.7. Somatic embryogenesis
3.1.7.1. Somatic embryogenesis in solid medium
3.1.7.1.1. Callus induction and proliferation
The sterilized petiole and internodal explants were transferred to MS medium supplemented with 2, 4-D and NAA (0.5-4.0 mg/l) for callus induction. Fifteen days old friable callus was sub-cultured onto fresh medium with same growth regulator for further proliferation.

3.1.7.1.2. Induction of somatic embryos
Two week old brown, shiny, friable callus (i.e.) proembryogenic mass was transferred to MS medium containing various concentrations of 2, 4-D and
picloram (0.1-3.0 mg/l) alone or in combination with cytokinins [BA (0.05-1.0 mg/l)] for initiation of somatic embryo callus.

3.1.7.1.3. Enhancement of embryo induction
The somatic embryo callus and proembryogenic mass were transferred to medium containing 2, 4-D (1.0 mg/l), BA (0.5 mg/l) and glutamine (10-50 mg/l) for the increase in somatic embryo formation.

3.1.7.1.4. Maturation of somatic embryos
The callus containing globular embryos were transferred to varying strengths of MS medium supplemented with varying concentrations of sucrose (1-4%) for maturation.

3.1.7.1.5. Somatic embryo germination
For the germination, the matured embryos were transferred to medium containing ABA (0.01-0.5 mg/l).

3.1.7.2. Somatic embryogenesis in liquid medium
3.1.7.2.1. Callus induction and proliferation
As described in above section.

3.1.7.2.2. Somatic embryo induction
Two weeks old friable callus was transferred to the suspension cultures (i.e.) 100 ml of induction medium in 500 ml Erlenmeyer flask containing 2,4-D and picloram in various concentrations (0.1-3.0 mg/l) and glutamine (25 mg/l). The flasks were agitated on a rotary shaker with 100 rpm under light condition. Embryogenic suspension cultures were sub cultured with every two weeks intervals for one month after which cultures were discarded. The number of embryos was counted and stages identified under stereo microscope and photographed.
3.1.7.2.3. Maturation of somatic embryos

The embryos were transferred to MS liquid medium supplemented with BA and GA$_3$ (0.1-3.0 mg/l) for the maturation. The number of somatic embryos at different developmental stages (globular, heart and torpedo shaped) was recorded in different days (10, 20 and 30). Data on somatic embryos forming either roots or shoots were also collected.

3.2. *Simarouba glauca*

3.2.1. Plant material collection

Seeds were procured from the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India. Seeds were treated with Conc. H$_2$SO$_4$ for one hour. The acid treated seeds were scrubbed over rough surface to soften the hard seed coat and the seeds were washed thoroughly in running tap water. The seeds were soaked in sterile distilled water and placed in a rotary shaker for the uniform imbibitions of water by the softened seeds for 3 days to improve the breakage of seed coat. On the fourth day, the seeds were again washed thoroughly in sterile distilled water and the seeds were sown in the plastic bags containing soil and sand mixture followed the proper irrigation. The raised seedlings were transferred to earthen pots containing soil and sand (1:1) and maintained in the garden. The explants were collected from two month old seedlings (Plate 2).

3.2.2. Explant sterilization

Explants such as cotyledonary node, axillary bud, apical bud, internodes and cotyledon of 1-2 cm length from healthy plants were initially washed with few drops of liquid detergent (Teepol) for 5 min followed by rinsing in running tap water. The washed explants were disinfected by rinsing in running tap water. The washed explants were disinfected by rinsing in 70% ethanol for 1 min and with aqueous solution of 0.1 % (W/V) mercuric chloride for 3 min. Finally the
Simarouba glauca - Explant source

a. Tree  
b. Closeup view of twig  
c. Two month old *in vivo* germinated seedlings in field condition
explants were rinsed 4-5 times with sterile distilled water and cut into pieces each 0.5-1.0 cm long.

3.2.3. Micropropagation

3.2.3.1. Shoot bud induction
The nodal segments (axillary bud) and shoot tips (apical bud) and cotyledonary nodes were cultured on MS medium containing of BA and KN (0.5 - 4.0 mg/l) individually or in combinations for shoot bud initiation.

3.2.3.2. Shoot multiplication
For further multiplication, these shoots were cultured in the medium supplemented with BA (3.0 mg/l), KN (0.5 mg/l) and different concentrations of auxin, IAA, NAA and IBA (0.05-1.0 mg/l).

3.2.3.3. Shoot proliferation
For further enhancement in shoot multiplication, the additives like adenine sulphate, glutamine and sodium citrate (5-20 mg/l) were added to the medium containing BA (3.0 mg/l), KN (0.5 mg/l) and NAA (0.5 mg/l).

For the enhancement of shoots, the multiplied shoots were transferred to the medium containing BA (3.0 mg/l), KN (0.5 mg/l), NAA (0.5 mg/l) and glutamine (10 mg/l) with the antioxidants such as ascorbic acid and Poly vinyl pyroolidone (5-25 mg/l).

3.2.3.4. Shoot elongation
The microshoots were transferred to MS medium supplemented with GA3 (0.5-5.0 mg/l) individually or in combination with cytokinin for shoot elongation.
3.2.3.5. Rooting
The microshoots were excised and subcultured on MS medium containing IAA, IBA, NAA (0.5-4.0 mg/l) individually and in combination with KN (0.1-2.0 mg/l) for root induction.

3.2.4. Organogenesis
3.2.4.1. Callus induction
The sterilized explants (cotyledon and internode) were cultured on MS medium supplemented with different concentrations of auxins [IAA, IBA and NAA (0.5-4.0 mg/l)].

3.2.4.2. Shoot regeneration
The callus derived from all explants were cultured on shoot regeneration medium containing BA, KN (0.05-4.0 mg/l) and GA3 (0.1-2.0 mg/l) individually and in combination for shoot regeneration.

Callus was sub-cultured on MS medium supplemented with BA (3.0 mg/l), KN (0.5 mg/l), GA3 (1.0 mg/l) and various concentrations of sodium citrate, glutamine (5-20 mg/l) and coconut water (5-20%) to enhance the shoot quality.

3.2.4.3. Rooting
The regenerated multiple micro shoots were excised and sub-cultured on MS medium containing IAA, IBA and NAA (0.1-3.0 mg/l) for root induction.

3.2.4.4. Acclimatization and transfer of plantlets to soil
Four weeks old in vitro raised plantlets with well developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transformed to plastic containers and earthen pots containing mixture of sand: soil (1:2) maintained inside a culture room at 25 ± 2°C and 35μE m⁻²S⁻¹ light intensity provided by cool white fluorescent tubes and 70-
80% relative humidity was maintained by covering the plant with polythene bags. Plantlets were watered with 1/4 - strength MS basal solution devoid of sucrose and meso-inositol at 3 days intervals for a period of 3 weeks. The acclimatized plantlets were then transferred to earthen pots containing soil and kept under shade for another 8 weeks before transferring to the field.

3.2.5. Somatic embryogenesis

3.2.5.1. Somatic embryogenesis in solid medium

3.2.5.1.1. Callus induction and proliferation
The cotyledon and internode explants were sterilized and transferred to MS medium supplemented with 2, 4-D and NAA (0.1-3.0 mg/l) for callus induction. Fifteen days old green compact callus was subcultured onto fresh medium with same growth regulator for further proliferation.

3.2.5.1.2. Induction of somatic embryos
Two week old green, compact callus (i.e.) proembryogenic mass was transferred to MS medium containing NAA (1.0 mg/l) with various concentrations of BA (0.1-3.0 mg/l) for initiation of somatic embryo callus.

3.2.5.1.3. Maturation of somatic embryos
The callus containing white coloured globular embryos were transferred to MS medium supplemented BA (2.0 mg/l), NAA (0.5 mg/l), with varying concentrations of additives adenine sulphate, glutamine and sodium citrate (5-20mg/l) for maturation.

3.2.5.1.4. Somatic embryo germination
For the germination the matured embryos were transferred to MS medium containing BA (1.0 mg/l), ascorbic acid (10 mg/l) with varying concentrations of TDZ (0.05-1.0 mg/l).
3.2.5.2. Somatic embryogenesis in liquid medium

3.2.5.2.1. Callus induction and proliferation
As described in above section.

3.2.5.2.2. Somatic embryo induction
Two weeks old compact callus were transferred to the suspension cultures (i.e.) 100 ml of induction medium in 500ml Erlenmeyer flask containing NAA and TDZ in various concentrations (0.05-3.0 mg/l) individually and in combination. The flasks were agitated on a rotary shaker with 100 rpm under light condition. Embryogenic suspension cultures were sub cultured with every two weeks intervals for one month after which cultures were discarded. The number of embryos was counted and stages identified under stereo microscope and photographed.

3.3. Statistical analysis
A minimum of 25 replicates were taken for each treatment and all the experiments were repeated thrice. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The experimental design was random and factorial. The data pertaining to shoot proliferation frequencies and shoot number, shoot elongation and rooting were subjected to mean and mean separation analysis by using Duncan’s Multiple Range Test (DMRT) (Gomez and Gomez, 1976).