2.3 MATERIALS AND METHODS

_Heliotropium indicum_ L., an herbaceous plant belongs to the family Boraginaceae was selected as the experimental material. It is commonly known as Indian heliotrope. Authenticated plants of _Heliotropium indicum_ L., were collected from Hubli, Dharwad district of Karnataka. The following criteria favored its choice.

Plant is readily available and a literature revealed that the plant has not been previously worked out for tissue culture of all parts of plant.

Source & choice of plant material

_H. indicum_ L., plants are grown & maintained in the garden Department of Botany. P.C. Jabin Science College, Hubli, Karnataka and also in the department of Botany KUD were used as the source of explants. Excised shoot tips, stem segments, tender leaves, old leaves and seeds were used as explants.

Glasswares & instruments

Glassware Borosil grade consisting of beakers, conical flasks, Petri dishes, standard flasks, pipettes, measuring cylinder etc were used. Explants were culture in wide neck Erlenmeyer’s conical flask (50ml, 100ml, 150ml, and 200ml), culture tubes (150mm long & 25mm diameter).

All the glass wares in use were regularly cleaned to ensure no contamination. Glasswares were thoroughly washed in running water using ‘Laboline’ detergent & finally rinsed in distilled water. All cleaned glass wares
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were placed in hot air oven for 3 hours at 100c to make them dry. Once used culture vessels were first autoclave & washed following the conventional procedure. Plugs for the tubes & plants were made out of nonabsorbent surgical cotton wrapped in muslin cloth.

Accessories used like scalpels, forceps, spatula, needles, holder were made of stainless steel & were sterilized every time before use. The equipment & instruments used in lab included Laminar Air Flow cabinet, hot air oven, refrigerator, distillation unit, electronic mono pan balance Digital electronic pH meter, Autoclave, Hotplate, microscope, Rotary microtome, steel racks, magnetic stirrer etc.

The LAF hood surface was wiped clean with paper towel soaked in 70% ethanol and sterilized by germicidal ultra violet light for at least 30 min prior to use. All the surgical instruments like scalpel, forceps and scissors were sterilized by dipping in 100% ethyl alcohol and flaming prior to use.

Culture room

The explants were incubated in a culture room where the temperature was maintained at 25-26°C, humidity at 85% and photoperiod of 16h light and 8h dark.

Preparation of culture media

The media formulation described as Murashige and Skoog (1962) referred as MS medium was selected as the optimal culture medium. Stock solutions of generally 10 times major elements, 1000 times minor elements, 100 times organic
constituents were prepared. These stock solutions were stored in a freeze chest at -4°C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than month.

MS (Murashige and Skoog 1962) inorganic salts, organic supplements and vitamins were used as basal media for seed germination, callus induction, callus multiplication, shoot and root induction. The formulation and composition of MS medium is given in appendix.

The reagents used were of Analytical Reagent Grade. Each salt was dissolved separately one after one to avoid precipitation. All the constituents except agar were mixed and then the pH of the solution was adjusted to 5.8 by using 1N NaOH. Later, agar was added and the medium was heated to boil so as to homogenize agar.

After the preparation of the medium definite aliquots of the medium were then added depending upon the capacity of the culture vessel. Generally 25 ml, 50 ml, 100 ml of the medium was distributed into the test tubes, 100 ml and 250 ml flasks respectively. After plugging the glassware with cotton plugs, media were steam-sterilized at 121°C for 15 - 20 minutes. After autoclaving, tubes were placed in slanting stands to prepare the slants. These were then left to cool and solidify.

**Growth regulators**
Auxins and cytokinins were the two major phytohormones used in different concentration and combination in various media for induction of callogenesis, caulogenensis and rhizogenesis.

**Auxins:** Powder of Auxins were dissolved in 1N NaOH and made up the volume with sterilized distilled water and then used or stored in freezer as stock for further use.

The three Auxins used in the present study were,

1. 2,4-Dichlorophenoxy acetic acid
2. Indole-3-butyric acid
3. Naphthalene acetic acid

**Cytokinins:** The cytokinins were dissolved in 1N NaOH and then used or stored as stock for further use. The two cytokinins used were

1. 6-Benzyl amino purine (BAP) and
2. Kinetin (KN)

Absorbents like Activated Charcoal and Polyvinyl pyrrolidone were used directly in the media for controlling browning.

**Inoculations**

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a bactericidal U. V. tube. The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula,
forceps, scalpels, blade etc.), Spirit lamp, tube containing absolute alcohol etc were also cleaned with alcohol.

The fresh material to be inoculated was kept in a Petri dish covered with a piece of black paper in order to protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber and the chamber was then sterilized with U.V. rays continuously on for one hour.

In the process of sterilization living materials should not lose their biological activity, but only bacterial or fungal contaminants should be eliminated. The commonly used sterilants are bleach, ethanol, sodium hypochlorite, mercuric chloride. The type of sterilant used, concentration and time depends on the nature of explant and species (Razdan, 1993).

The plant tissues or explants collected from the wild or the green house are usually contaminated with microorganisms and other contaminants. These microorganisms such as bacteria or virus must be removed during the preparation of aseptic explants otherwise they would kill the explants either due to their overgrowth or due to the release of toxic substances into the medium.

The potential sources of contamination in the cultures are the plant tissues, instruments, culture medium, environment of the transfer area, technicians and incubation room (Dodd’s and Roberts, 1995).

In fact, the sterilization treatment may vary from season to season as the microbial populations are dependent on seasons (George and Sherrington, 1984).
The mother plants were given 0.1% Carbendazim spray at weekly intervals. The explants like stems, leaves and shoot apices were taken from the plants growing under the in vivo conditions. The stem pieces, leaves, shoot apices were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface.

The explants were then washed with liquid detergent (Laboline) for another 15 minutes and then washed properly to remove the detergent. The explants were treated with 0.1% (w/v) Carbendazim for 5 minute to remove fungal contamination and then washed properly to remove the fungicide. Sterilizing agents used for obtaining aseptic tissues are sodium hypochlorite for one minute then explants were washed distilled water to remove sterilant for different periods with different explants.

Then 70% alcohol for 30 seconds and were followed by sterile distilled water. The explants taken from field borne plants were treated with 0.1% mercuric chloride Hg Cl₂ solution for 1-2 minutes respectively depending upon the explants. Then the explants were washed thoroughly with sterile distilled water for five times with one minute interval each to remove sterilants. Last two steps were carried out under laminar air flow.

Seeds of *Heliotropium indicum* L. were used to raise the seedlings under sterile conditions. Like explants, seeds were washed under running tap water, then liquid detergents and finally under Carbendazim to remove dust particles,
microbes and fungi. Under the laminar air flow seeds were surface sterilized with 0.1% HgCl₂ for 7 - 8 minutes.

Constant shaking was done during this period to get thorough sterilization. Rinsing with sterile distilled water 4 - 5 times was necessary for the removal of sterilant from the seeds. These were then inoculated on Basal MS medium for germination. Various parts like root, stem, leaves and shoot apices were excised from 4 weeks old seedlings and transferred separately to different experimental media.

Hands and arms which were to be used inside the inoculation chamber were scrubbed with alcohol before inoculation. The rims of the test tubes and the sides of the plugs were flame sterilized. Instruments (like forceps, scalpels, spatula etc.) were all sterilized by dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. The explants were then planted on variously augmented MS medium.

**Cultural conditions**

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 2°C. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). The intensity of illumination was 3500 lux at the level of cultures and a 16 hour light regime was followed by 08 hour darkness.
A minimum of 10 culture tubes were raised for each combination and all experiments were performed 3 times. Analysis of variance (ANOVA) and mean separations were carried out using Duncan’s multiple range test to assess the statistical significance. $P \leq 0.05$ was considered as statistically significant, using statistical software SPSS ver. 17.

Data Collection

The day of initial callus formation, the morphology and colour of the callus were recorded. At the end the observation period, percentage of the explants forming callus as well as the degree of callus formation was measured.