2.4 RESULTS

SEED GERMINATION (PLATES: 27-28)

Seeds of *Heliotropium indicum* L. were used for germination test. The seeds were placed in full strength MS medium without any plant growth regulator. When a fresh seeds were inoculated, there was remarkable increase in the percentage of germination, whereas percentage was go on decreasing for stored seeds and only 10 % germination was noticed for one month old seeds and it was zero for 6 month old seeds. In another test, where ½ strength MS medium was used devoid of PGR showed percentage of seed germination was comparatively very low (Table 7, Plate 27). Therefore the present studies revealed that full strength MS medium was better choice for *in vitro* seed germination. After two weeks *in vitro* plants were raised. *In vitro* grown seedlings were later used as sterilized explant source.

CALLUS INDUCTION/ CALLOGENESIS (PLATES: 29-31)

A literature survey indicated that the *in vitro* protocol for leaf and stem callus culture of *Heliotropium indicum* L. was not yet standardized. In view of its medicinal importance and the lack of tissue culture protocols, the present
study reports the prime protocol for regeneration from leaf and stem callus culture of *Heliotropium indicum* L. Callus initiation, callus growth, shoots and root initiation data were recorded to evaluate indirect organogenesis.

**MORPHOGENETIC EFFECTS OF INDIVIDUAL HORMONES ON EXPLANTS**

The manipulation of plant growth regulators was essential to optimize the induction of callus. After 4 weeks of observation, all the plant growth regulators tested on shoot apex, leaf and stem explants showed poor callus formation (Table 8, 9 & 10). However, the degree of the callus induced from the leaf and stem explants varied from each plant growth regulator. The leaf and stem explants were cultured on MS medium containing different concentrations of BAP, KN, 2,4-D, IBA and NAA alone and in combinations. However for the leaf explants the rate of callus formation was very poor. These results indicate that the callus induction from leaf segments was relatively difficult. There was no callus formation in the absence of growth regulator.

Whenever explants were inoculated on medium there were many difficulties i.e. cultures turn brown due to secretion of phenolic compounds.

The production of chemicals that deter or kill pests and pathogens represents one mean of self-protection. Plant phenolics are secondary metabolites
involved in the defence mechanisms of plants against fungal pathogens and insect herbivores. Plants respond to diverse environmental enemies with a bewildering array of responses, which use constitutive and induced phenolic substances. (Lattanzio et al., 2006)

Callus browning is a typical feature of callus cultures of *Heliotropium indicum* L. Brown callus results in decreased regenerative ability, poor growth and even death. Callus induction and growth in *in vitro* culture of *Heliotropium indicum* L. meet a lot of difficulties, connected especially with rapid aging of culture. Browning of explants and progressive tissue necrosis is the result of intensified oxidation of phenolics compounds.

In order to eliminate unfavorable changes in *in vitro* culture of *Heliotropium indicum* L. media were supplemented with various antioxidants and absorbents.

Absorbents were added directly to the basal medium, these media were also useful in browning limitation. The lowest browning was obtained in callus induced on media supplemented with PVP 10 (10mg/l) and activated Charcoal. (10mg/l).
MORPHOGENETIC EFFECTS OF DIFFERENT CONCENTRATIONS AND COMBINATIONS OF HORMONES ON EXPLANTS

In order to study the effects of various concentrations and combinations of different auxins and cytokinins on callus induction from the leaf and stem explants, the auxins tested were 2,4-Dichlorophenoxyacetic acid (2,4-D), NAA and Indole-butyric acid (IBA) and cytokinins tested were Kinetin and 6,benzylaminopurine (BAP).

Callus formation was observed within three to four weeks on leaf and stem explants cultured on MS medium supplemented with varying concentrations of plant growth regulators. Callusing was initiated after 7-10 days of inoculation. The callus induction always preceded by swelling of the explant.

Results were recorded for callus by different parameters like colour, degree of callusing and percentage of explants responded for callusing. Among the three auxins tested with, NAA and IBA were found to be more effective in callus induction than 2,4-D.

To maximize the callus proliferation, a medium supplemented with different concentrations and combinations of hormone, BAP (1.5 to 5.0 mg/l) + 2,4-D (1.5 to 2.5 mg/l), BAP (1.5 to 5.0 mg/l) + IBA (1.5 to 2.5 mg/l) BAP (1.5 to 5.0 mg/l) + NAA (1.5 to 2.5 mg/l), KN (1.5 to 5.0 mg/l) + 2,4-D (1.5 to 2.5 mg/l) would likely be effective.
mg/l), KN (1.5 to 5.0 mg/l) + IBA (1.5 to 2.5 mg/l) and KN (1.5 to 5.0 mg/l) + NAA (1.5 to 2.5 mg/l) were tried (Table 11 and 12).

For leaf explants BAP + IBA, BAP + NAA, KN + IBA and KN + NAA combinations responded and produced calli. KN (4.0 mg/l) + NAA (2.0 mg/l) proved as good combinations for callus proliferation from leaf explants after two weeks (Table 11, Plate 29, Fig F &G) and callus after three and four weeks of inoculation (Plate 31, Fig B&D) Simultaneously both KN (4.5 mg/l) + NAA (2.5 mg/l) and BAP (4.5 mg/l) + IBA (2.5 mg/l) were proved to second good combinations to induce callus from leaf explants.

KN (4.0 mg/l) + NAA (2.0 mg/l) (Table 12) was proved as good combinations for callus proliferation from stem explants. Later in the same NAA+KN medium microshoots were observed. (Plate 29). For stem explants BAP (4.5 mg/l) + NAA (2.5 mg/l) (Table 12, Plate 31, Fig A) produces whitish callus. When the leaf and stem explants treated with MS medium supplemented with high concentration auxin and low concentration of cytokinins callus was induced without PVP. Brown, light brown, white and light green coloured callus were recorded. There was no good callus production.

Other combinations like 2.0 mg/l KN and 2.0 mg/l IBA, 2.0 mg/l BAP and 2.0 mg/l IBA, 2 mg/l KN and 1.0 mg/l IBA were also well responded.

The callus obtained from leaf and stem explants were subcultured. Callus was divided into small pieces and cultured onto MS medium supplemented
with various concentrations and combinations of auxins and cytokinins with BAP + NAA, BAP + IBA, and KN + NAA and KN + IBA.

The subcultured calli enhanced shoot regeneration potential after two weeks. Among these combinations 3.0 mg/l KN with 1.5 mg/l IBA (Table 13, Plate, 32, Fig A) and 2.5±0.3 microshoots (Plate32 Fig B) were produced when MS medium was supplemented with 3.0 mg/l KN and 1.5 mg/l NAA showed an organogenic response and produced shoot buds from callus derived from leaf explant and with other combinations only callusing was noted with very few shoot buds. BAP was poor cytokinin compared with KN to establish microshoots and callusing was noticed. Also it proves that stem derived callus had more potential to produce shoots than leaf callus. (Table 14)

Whereas in 1.5 mg/l KN and 1.5 mg/l NAA with optimum number 11.4±0.4 shoots (Plate 32, Fig E) were induced on the medium and 1.5 mg/l KN and 1.5 mg/l IBA induced 10.9±0.4 (Plate32, Fig F) shoots was second highest number of shoots for stem derived callus was subcultured.

Based on these results KN was proved to be good cytokinin for sub culturing of stem derived callus. The response from leaf explants was slower and callus induction was less than that from stem explants.

Synthetic auxin 2,4-D is known to induce good-quality callus in a number of medicinal plants. Our results showed that 2,4-D had no effect on callus induction and shoot initiation of *H. indicum* L.
INDUCTION OF ROOTS FROM IN VITRO SHOOTS (PLATE : 33)

Isolated shoots were transferred to MS medium supplemented with various concentrations of IBA with BAP and KN for rooting of in vitro shoots with PVP. 2.5 mg/l IBA + 0.5 mg/l BAP produced callogenic base with maximum number roots (Table15, Plate 33). Much elongated and good number of roots was produced on medium supplemented with 2.0 mg/l IBA and 1.0 mg/l with charcoal (Table18, Plate 33). Other combinations of IBA and BAP were also a good hormonal combination when compared with other hormonal combinations of NAA with BAP and KN.

IN VITRO RHIZOGENESIS FROM CALLUS (PLATES: 35-36)

BAP 2 mg/l + IBA 2.0 mg/l and BAP 2.0 mg/l+ NAA 2.0 mg/l were effective for induction rhizogenic callus from leaf explants of Heliotropium indicum L. (Table 16) Better induction of rhizogenic callus was observed on MS medium supplemented with BAP 2 mg/l +NAA 3.0 mg/l, BAP 2 mg/l +NAA 2.0 mg/l, BAP 2 mg/l +IBA 3.0 mg/l and KN 2.0 mg/l + IBA 3.0 mg/l for stem explant.(Table 16)
Adventitious roots from the leaf callus with BAP 2.0 mg/l + NAA 2.0 mg/l, KN 2.0 mg/l + IBA 3.0 mg/l and BAP 2.0 mg/l + IBA 2.0 mg/l. For Stem callus with BAP 2 mg/l + NAA 3 mg/l, BAP 2 mg/l + NAA 2.0 mg/l and BAP 2.0 mg/l and IBA 3.0 mg/l (Table 16, Plate 34& 35) proved to be good combination to produce rhizogenic callus with maximum adventitious roots.

Other combinations like 2.0 mg/l BAP and 3.0 mg/l IBA, 2.0 mg/l KN and 3.0 mg/l NAA and 2.0 mg/l BAP and 2.0 mg/l IBA were also produced rhizogenic callus. Leaf explant with 2.0 mg/l BAP and 2.0 mg/l NAA proved to be very good rhizogenic callus.

**DIRECT ORGANOGENESIS FROM DIFFERENT EXPLANTS**

*(PLATES: 37-39)*

In the present study, BAP and Kinetin were two cytokinins tested on shoot tip, stem and leaf explants of *Heliotropium indicum* L. Shoot initiation shoot growth, and root initiation data were recorded to evaluate direct organogenesis.

BAP alone treated with different concentrations (0.5 mg/l to 10 mg/l) on different explants. For shoot tip explant when treated with 2 mg/l shows caulogenesis (Table 8). Whereas for stem explant 1 mg/l and 2 mg/l shows
caulogenesis and for leaf explant there was no response when BAP was treated alone.

When KN treated with different concentrations (0.5 mg/l to 10 mg/l) on different explants of *H. indicum* L. 2 mg/l KN was responded for caulogenesis (Table 8) in shoot tip explant and stem or leaf explants do not show any response with any of these concentrations of KN (Table 9 &10).

2,4-D, IBA and NAA were three auxins tested on shoot tip, stem and leaf explants of *H. indicum* L. 2,4-D when treated with different concentration (0.5 mg/l to 10.0 mg/l) no morphogenic response were recorded. For auxin IBA 5.0 mg/l and 2 mg/l shows direct rhizogenesis in shoot tip where as leaf and stem explants were not responded. For NAA alone in the medium, no direct organogenesis was recorded.

Cytokinins concentration combined with auxin was more effective than cytokinins used alone on the regeneration of *Heliotropium indicum* L. The results showed that the combination of BAP with NAA and IBA, the combination of KN with NAA and IBA gave better cell division and regeneration than the BAP and KN used alone for shoot tip, stem and leaf explants.

BAP 2.5 mg/l + IBA 1 mg/l gave direct multiple shoots from shoot tip explant and second highest multiple shoots from shoot tip explants when supplemented with KN 3.0 mg/l +NAA 1.0 mg/l (Table 17, Plate 39 ).
There were direct shoots from shoot tip and stem explants from the MS media containing charcoal and hormones BAP with IBA and NAA with different concentrations shows direct shoot formation. Shoot tips and stem explants cultured on basal medium containing charcoal typically develop into single seedling like shoot with strong apical dominance (Plate 40). On the contrary when the shoots of the same explant material are grown on culture medium containing PVP axillary shoots develop clusters shoots (Plate 39).

For stem explants MS media with PVP and different combinations and concentrations of cytokinins (BAP & KN) with auxins (2,4-D, IBA & NAA) were tested. KN 1.0 mg/l +NAA 1.5 mg/l & BAP 0.5 mg/l + IBA 1.5 mg/l shows direct caulogenesis. There was no regeneration from the media containing cytokinins or auxins alone for leaf explants.

Although statistical data (table 11), indicates that there was direct shoot regeneration from combinations KN 1 mg/l+NAA 1.5 mg/l (Plate 37, Fig A, C and D) from leaf explants and 0.5 mg KN/l +1 mg IBA/l and BAP 1.0 mg/l and IBA 1.5 mg/l gave direct root formation from leaf explant but other combinations showed callus formation.
IN VITRO FLOWERING FROM SHOOT APICES (PLATES: 41-43)

When shoot tip explant treated with MS medium supplemented with IBA 2.0 mg/l + BAP 1.0 mg/l, IBA 1.5 mg/l + BAP 1.0 mg/l with 10 mg/l charcoal show highest in vitro flowering. The in vitro inflorescence almost has normal in vivo grown inflorescence characters (Table 18, Plate 41, 42).

In vitro flowers were developed from stem explants inoculated on MS medium supplemented with 3.5 mg/l BAP+2.0 mg/l IBA and 10 mg/l PVP after two weeks (Table 12). In vitro flowers were induced from stem explants inoculated on MS medium supplemented with 4.0 mg/l KN+ 2.0 mg/l IBA and 10mg/l PVP after two weeks (Table 12). In vitro grown shoot on MS medium supplemented with IBA 2 mg/l and BAP 0.25 mg/l with PVP. (Table 15, Plate 43).

Very interestingly, an inflorescence developed on PVP supplemented media does not show normal floral morphology and they were unusual and green in colour.