2.5 DISCUSSION

When shoot tip, leaf and stem explants of *Heliotropium indicum* L. were inoculated on MS basal medium, production of phenols caused necrosis and callus turned brown and eventually died. Many plants, especially medicinal and aromatic plants are naturally rich in polyphenolic compounds. When explants are excised and placed on the *in vitro* culture medium, these phenolic compounds are released from cut surface of the explants and oxidize to form phytotoxic products. As a result of this event, the media and explants turn brown and the explants are unable to grow further and eventually die (Bhojwani 1996 & Poudyal 2008). It was true with the present study on *Heliotropium indicum* L.

The browning problem was a result of phenolic secretion which inhibits micropropagation in the present study on *H. indicum* L. The addition of activated charcoal and Poly vinyl pyrolidone (PVP) was used to overcome the browning problem. It may remove contaminants from agar (Kohlenbach and Wernicke 1978) and secondary products secreted by the cultured tissues (Wang and Haung, 1976, Fridborg et al., 1978) or possibly regulate the supply of certain endogenous growth regulators (Reinert and Bajaj, 1977) and in the present study activated charcoal showed prevention of phenol production in the culture and enhanced *in vitro* flowering.
Browning of the surface of plant tissues accompanied by darkening of culture medium due to the oxidation of phenolic compounds resulted in the formation of quinines which are highly reactive and toxic to plant tissue (Chawla H.S. 2002). This phenomenon was undesirable because it affected the growth and establishment of explants, which in turn, affected the results in obtaining callus in the present study on *H. indicum* L. Similar observations were recorded for *Psoralea corylifolia* (Pandey et al., 2013).

As medium supplemented with activated charcoal it has been reported that it stimulates embryogenesis (Kohlenbach and Wernicke, 1978) but in the present study no such phenomenon observed. Activated charcoal is often used in plant tissue culture for the adsorption of inhibitory substances in the culture medium, drastically decreasing the phenolic oxidation or brown exudate accumulation (Thomas T.D, 2008). In the present study to avoid browning activated charcoal and PVP were used.

Activated charcoal has been frequently added into the culture medium for plant tissue with success (Van Winkle et al., 2003). Activated charcoal not only increased the percentage response of rooting but also reduced the production of callus from the explants (Agarwal V. et al., 2002).

Callus during every subculture in the present on *H. indicum* study showed browning adversely affecting the growth. Adding charcoal and PVP to media could
solve this problem. Activated charcoal and PVP proved best for regeneration in *Boswellia serrata* (Purohit *et al.*, 1995).

The data indicated that the highest mean plant height (6.47 cm) was shown with activated charcoal, while the lowest height (3.60 cm) was shown without charcoal in *Ruscus hypoglossum* (Dahab *et al.*, 2005). Similar observation were recorded in the present study on *H. indicum* L.

Addition of 100 mg/l ascorbic acid to MS medium was found as one of the best methods to control browning of explants of *Pyrus bretschneideri* (Poudyal *et al.*, 2008). Morpholine ethane sulfonic acid (*MES*) could be used to inhibit browning problem as a pH effectors (Mansuroğlu, S. *et al.*, 2001).

*In vitro* seed germination was achieved successfully on both full and half strength MS medium. Freshly harvested seeds were showing 100% viability. Seed germination is a response characterized by three parameters percentage, rate, and uniformity (Hartmann *et al.*, 2002). Germination percentage is the number of seeds from a population that germinate. Germination rate is the "speed or velocity" of germination and can be expressed as the time it takes for a defined percentage of seed to germinate. Germination uniformity is a measure of the time it takes for all of the seeds to germinate. (Randall 2008). In the present study on *H. indicum* L. showed germination of seed different with different ages and only freshly harvested seeds showed 100% germination.
Discussion

The source of explant is an important factor in the course of shoot induction. Morphogenetic capacity of explants originated from different organs or tissues of different organs are generally different. In the present both in vivo and in vitro explants were used.

Plant regeneration through in vitro propagation has been reported in some members of the family Boraginaceae including Arnebia euchroma (Manjkhola et al., 2005), Cordea verbenacea (Lameira and Pinto, 2006), Heliotropium indicum (Datta et al., 2003, Senthil Kumar and Rao 2006), Tournefortia paniculata (Bertoluci et al., 2001), Rotula aquatica (Martin, 2003; Chitra et al., 2005) and Trichodesma indicum (Verma et al., 2008).

Datta et al., (2003) reported callus induction from nodal and hypocotyl explants on MS medium augmented with NAA, BA, aspergine and glutamine to induce callus from hypocotyls and organogenesis in Heliotropium indicum L. and Senthil kumar (2006) reported shoot generation from apical and axillary buds explants of Heliotropium indicum L. on MS medium supplemented with KN (1.0 mg/l), BA (0.5 mg/l) and IAA (0.05 mg/l).

Slight and moderate callus were recorded with different colors like light green, white, light brown and brown from both leaf and stem explants of H. indicum L. Explant culture in vitro may be involved in organogenesis and develop shoots or roots depending on the morphogenic potentiality of the cells. There are three distinct stages during organogenesis, namely dedifferentiation,
induction of organogenesis pathway and development of organs (Klerk 2005). The present study showed that the explant response to induce callus depends on different concentrations and combinations and nature of plant growth regulators and type of explant used.

We tried single hormones for stem and leaf explants of *H. indicum* L. When explants cultured on the medium containing different concentrations of 2,4-D alone had no effect on callus induction and shoot induction. 2,4-D proved very effective callus inducing auxin in *Ionidium suffruticosum* (Sonappanavar, 2009), *Viola serpens* Wall. (Vishwakarma *et al*., 2013). NAA alone in the MS medium induces low callus in the present study on *H. indicum* L. In case of *Solanum trolobatum* L. (Alagumanian 2004) and in *Viola serpens* Wall had no effect on induction of callus and shoot. IBA alone was in the medium showed that the formation of direct shoots from shoot apex explant and leaf explants responded for low callus induction in the present study on *H. indicum* L. IBA played a significant role in shoot elongation of *Solanum hainanense* (Loc *et al*., 2011).

When explants treated with BAP alone for *Heliotropium indicum* L shows shoot induction from shoot apex and stem explants and low callusability was observed in leaf explants. Where as in *Stevia rebaudiana* (Mehta *et al*., 2012) BAP proved to be a better choice than KN and maximum shoots obtained on its 2.0 mg/l concentrations. In previous reports on *Cerpegea bulbosa* and *C. jainii*
revealed that the BAP alone can induce axillary shoot multiplication from nodal segments (Patil, 1998).

Kinetin in combination with NAA or IBA induced good number of shoots in the present study on *H. indicum* L. Similarly many results showed that the cytokinin (KN) induced multiple shoot formation and shoot length (Sajina *et al*., 1997; Luo *et al*., 2009). However, Gomes *et al*., (2010) found that KN was more effective in promoting shoot growth of *Arbutus unedo* L. than other cytokinins. Kn was also found superior to BA in case of *Phyllanthus amarus* (Bhattacharya R *et al*., 2001) in the induction of multiple shoots.

The concentration of individual auxin and cytokinin or in combinations will determined the efficiency of callusability and organogenesis (Kohlenbach, 1977). This was supported by Unander (1991) found that the stem explant was ideal explants for callus induction in *P. amarus*. Similar results were observed in the present study on *H. indicum* L.

Among the explants used stem explant have recorded the best response in the present study on *Heliotropium indicum* L. The similar report by Unander (1991) found that the stem explant was ideal explants for callus induction in *P. amarus*. Combination of auxin and cytokinin favored shoot bud differentiation in many plants (Sudha *et al*., 2005; Rathore *et al*., 2005). The ideal concentrations of cytokinins differ from species to species and need to be estimated accurately to achieve the effective rate of multiplication (Gomes *et al*., 2010).
NAA and IBA were found less effective when they were used alone, but in combination with cytokinins they were more effective in callus induction from stem and leaf explant of *Heliotropium indicum L.* of present study.

In the present study combinations of BAP and NAA in different concentrations were not responded well with explants in the *H. indicum* L. to induce callus or to produce shoots but in *Cucumis sativus* L. (A Vasudevan *et al.*, 2001) proved to be very good combinations for subcultures of callus to induce multiple shoots. All combinations of BAP and NAA induced higher degree of callus in *Berberis tinctoria* Lesch (S.Paulsamy, 2004).

An alternative approach to increase the number of shoots has been reported for *Zingiber officinale* and *Curcuma longa* on MS medium containing BAP with NAA to multiply shoots (Haque *et al.*, 1999; Rahman *et al.*, 2004).

The nodal segments of *Cordia verbenacea* proliferated into shoots on MS medium containing KN and NAA (Lameira and Pinto, 2006), and that of *Rotula aquatica* on MS medium with BA and IBA (Martin, 2003); and *Tournefortia paniculata* on WPM fortified with BA (Bertoluci *et al.*, 2001). Similarly in the present study on *H. indicum* L. KN +NAA proved to be good combination for multiple shoot induction via callus culture.

In the present study on *H. indicum* L. NAA in combination with BAP and KN produced callus from leaf explants. Similarly, at higher concentrations of NAA+ KN (2.0+2.0 mg/l) and NAA+BAP (2.0+2.0 mg/l) the matured leaves
induced green callus in *Solanum trilobatum* (Satish Kumar *et al.*, 2011). Lal and Ahuja (1996) reported proliferated calli of *Picrorhiza kurroa* on MS medium containing 4.0 mg/l NAA and 1.0 mg/l KN after 2 weeks culture.

KN 3.0 mg/l + NAA 1.5 mg/l responded well with leaf derived callus sub cultured and produced shoots in the present study on *H. indicum* L. Similarly, combinations of KN with NAA showed highest shoots from callus of the *Solanum tuberosum* L. (Shirin *et al.*, 2007). KN with NAA in MS medium also stimulated proliferation and elongation of shoots in *Allium sativa* L. (Suh S. 1986).

Highest shoot length was observed on MS medium supplemented with 0.5 mg/l Kn and 0.5 mg/l NAA in *Sida cordifolia* L (Pattar *et al.*, 2012).

The young stem derived callus is viable, whereas the callus derived from the leaf could not be maintained beyond a second sub cultures in the present study on *H. indicum* L. Similar observations were also found in *Tylophora indica* (Rao and Narayanaswamy, 1972); *Ceropegia jainii*, *C. bulbosa* var. *bulbosa* and *C. bulbosa* var. *lushii* (Patil, 1998). Indirect shoot regeneration through callus phase obtained from leaf explants was reported for *Justicia gendarussa* (Agastian 2006).

It was observed that in the present study on *H. indicum* L. leaf and stem explants do not have the equal potential to regenerate roots. This also indicates that they are differing in their endogenous content of chemicals. However, frequent rhizogenesis was noticed from the callus cultures of both explants. Good
response for adventitious roots from the leaf callus with BAP 2.0 mg/l + NAA 2.0 mg/l and stem callus with BAP 2 mg/l + NAA 3 mg/l. Profuse rhizogenesis was also observed on MS medium with NAA + BAP in *Ocimum sanctum* (Shazad et al., 2000). The medium containing NAA along with BAP was found to produce good amount of callus with rhizoids like structures in *Ceropegia pusilla* (R. Kondamudi et al., 2010). The concentration of NAA and the rooting ability were directly proportional to one another.

Therefore, it was indicated that the cytokinins also have stimulating effects on root formation. Only root initiation was observed in earlier efforts as in *Glycine max* (Evans et al., 1976). After few weeks of growth, root turns brown and flaccid. The loss of root differentiation ability is one of the drawbacks of maintaining normal root culture for long term as observed in *Duboisia* species (Endo et al., 1985) which was also true with the present study on *H. indicum* L.

However, after taking into consideration of supplemented exogenous cytokinin, the overall ratio between auxin and cytokinin favoured the redifferentiation of callus cells into root cells (Chawla, 2002). During redifferentiation, the callus cells were no longer prepared for cell division, and rather ready to turn into root cells leading towards organogenesis (Mohr et al., 1995).
Root and shoot initiation, and the process of differentiation from unorganised callus tissue are closely regulated by the relative concentrations of auxins and cytokinin in the medium (Ammirato, 1983; Rout and Das, 1997).

During the callus formation, some of the calli developed into excessive roots. The root forming calli have been reported in other plant species such as from root explants of wheat (Chauhan and Singh 1995). Lower concentrations of NAA without BAP gave maximum number of roots in *Hybanthes ennerspermis* (Velayutham et al., 2012), *Datura metal* (Muthukumar et al., 2004) and in *Jasticea gendarussa* (Agastian et al., 2006).

In *Fortunella crassifolia*, maximum rooting is achieved on MS medium supplemented with NAA, Kn and 0.5 g/l AC (Yang et al., 2006). Joshi et al., (2007) reported that on application of AC in plant tissue culture includes *Swertia chirayita* wherein 0.5g/l activated charcoal had been employed along with NAA for rooting. Jeyachandran.R et al., (2013) reported that in *Micrococca mercurialis* (L.) Benth. half strength MS medium with IBA and activated charcoal induced high frequency of roots.

The micro shoots of different species of family Boraginaceae showed variation in nutritional and hormonal requirements during rooting. Martin (2003) reported *ex vitro* rooting in *R. aquatica*, whereas *in vitro* rooting was found successful on hormone-free MS medium in *C. verbenacea* (Lameira and Pinto, 2006,) and on hormonefree WPM in *T. paniculata* (Bertoluci et al., 2001).
Addition of activated charcoal has been reported to establish dark environment (Pan and Staden, 1998) suitable for root initiation, but the micro shoots of *T. indicum* formed callus at the base and subsequently dried when placed on MS medium containing IBA and activated charcoal. The micro shoots of *T. indicum* best rooted on half-MS medium supplemented with IBA (Verma *et al*., 2008).

Our results shows that IBA and NAA could be applied to initiate root formation but IBA was the most appropriate one for *Heliotropium indicum* L.

Shoot tip explants were found to be an excellent explants source to induce direct organogenesis than nodal explants in *Heliotropium indicum* L. The shoot tips are better than nodal segment for multiple shoot production because of the higher cytokinin to auxin ratio present in the shoot tip.

Similarly the shoot tip was found to be the superior explants for micropropagation in many number of plants, for example *Cannabis sativa* (Ren Wang *et al*., 2009); *Boehmeria nivea* (L) Gaud (Sut *et al*., 2004), *Ocimum sanctum* (Girija *et al*., 2006), *Alternanthera sessils* (Wesely *et al*., 2011), *Lippia nodiflora* (Evelyne Priya, S and Ravindhran, R., 2011), *Cicer arietinum* (Islam *et al*., 1995), *Stevia rebaudiana* Bert., (Das *et al*., 2011) and in *Solanum nigrum* L. (Kavitha *et al*., 2012). In *Bacopa chamaedryoides* (Haque *et al*., 2013) shoot tip proved to be better responding explant than nodal explant, inoculated on MS basal medium containing BAP and IAA.
Direct organogenesis from leaf was observed in the present study on *Heliotropium indicum* L. when MS media supplemented with KN and NAA. Whereas in those containing several concentrations of kinetin did not produce any shoots at all and became necrotic in *Digitalis lamarckii* Ivan. (Verma et al., 2011). The interaction between cytokinins (BA and TDZ) with NAA concentrations for the number of shoots per leaf explant and leaf regeneration frequency was significant in *Primula heterochroma* (Hamidoghli et al., 2011).

In the present study on *H. Indicum* L. when compared with leaf and stem explants, it was clear that stem explants were more productive for shoot formation than leaf explants. Similar observations were recorded for *Verbena officinalis*. (Turker et al., 2010)

This situation makes organ cultures a favored option. Shoot cultures have been considered appropriate when the target secondary metabolites are produced in aerial parts of the plant (Saito and Mizukami, 2002). The best shoot induction was observed for the *Aloe barbadensis* on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA. (Baksha, R, 2005).

Studies of Fuller and Fuller (1995) on the micropropagation of *Brassica* spp. showed that the highest number of shoots (88.3%) obtained in medium containing 2 mg/l IBA + 4 mg/l KIN from seedling derived explant.

*In vitro* flowering of *Heliotropium indicum* L. of present study was observed when shoots inoculated onto rooting medium after 20-25 days of
inoculation. The highest in vitro flowering response were 85% in IBA+BAP [Table 2 (2.0 mg/l + 1.0 mg/l)]. Similar observation were reported in Vitex trifolia (Nagaveni et al., 2013) when transferred to rooting medium with IBA at concentration of 2.46-14.76 µM and IAA at concentration of 2.85-17.13 µM initiated flowering along with rooting.

In vitro flowering was observed in Ceropegia pusilla (Kalimuthu et al., 2013) on ½ MS medium containing BAP (4.44µM) + IBA (2.46µM) after 32 days of culture transferred to the rooting medium. In vitro flowering and fruiting was observed in Micrococco mercurialis (L.) Benth when shoots were (Jeyachandran et al., 2013) transferred to rooting media containing IBA and charcoal. Similarly in the present study on Heliotropium indicum L. was also flowering observed in the rooting media contains charcoal.

In the present study, it is clearly observed that auxins and cytokinins are essential for flower induction. Cytokinins alone induced flowering in Bambusa arundinacea (Joshi & Nadgauda 1997) and Plumbago indica (Nitsch & Nitsch 1967). In vitro flowering ability can be increased by increasing IBA concentration when applied to the plantlets as reported by Thulaseedharan and Vaidyanathan (1990) in Vicoa indica. The essentiality of auxins for flower induction and development has been reported in few plants like Torenia (Tanimoto and Harada 1981), Vigna radiata (Avenido and Haulea 1990), Vigna mungo (Ignacimuthu et
In the present investigation, IBA induced maximum number of reproductive buds. Flowering ability was increased with the increase in IBA concentration applied to the regenerated shoots which were grown on MS medium supplemented with low concentrations of BAP and KN. The exogenous hormonal supply might have been added up to the endogenous contents, raising the hormonal level required for triggering the flowering.

Stephen and Jayabalan (1998) opined that flowering was considered as a complex process regulated by both external and internal factors and its induction under \textit{in vitro} culture is extensively rare. While Zimmerman \textit{et al.}, (1985) were of opinion that the interaction of carbohydrate and other nutritional factors with endogenous growth regulators can influence some biological parameters which are altered when plant changes from juvenile to mature phase. High frequency of flowering was observed when the medium was supplemented with 3\% (w/v) sucrose; this result coincides with earlier reports in \textit{Lycopersicon esculentum} (Rastogi and Sawhney 1987), potato (Al-Wareh \textit{et al.}, 1989), \textit{Vigna mungo} (Ignacimuthu \textit{et al.}, 1997), \textit{Pisum sativum} (Franklin \textit{et al.}, 2000), and \textit{Gentiana trifolia} (Zang and Leung 2002). According to the floral nutrient diversion hypothesis, C/N ratios increase in buds during flower induction (Sachs 1977).
Discussion

The supply of exogenous plant growth hormone in the medium was essential for flower bud induction. It is known that the plant growth hormone requirement of plants for in vitro flowering varies. The exogenous auxin plays in an important role in floral induction as reported earlier in *Lycopersicon esculentum* (Mill.), *Solanum nigrum* (L.) (Sheeja and Mandal, 2003, Jabeen et al., 2005).

An in vitro flowering mechanism is considered to be a convenient tool to study specific aspects of flowering and whole mechanisms of the reproductive process such as floral initiation, floral organ development and floral senescence (Murthy et al., 2012).

The flowering plantlets described in the present study may have a practical value in wide hybridization and in vitro fertilization studies, especially in studying the mechanism of fertilization of *H. indicum* L. under experimental condition because in vitro flowering of regenerated plantlets can be induced at any time of the year. It was reported here the simple and efficient protocol for plant regeneration and in vitro flowering in *Heliotropium indicum* L.