Introduction

Plants are the most important resource gifted by nature. Man has exploited them since the time of evolution for their own benefits. Thus, the important phytodiversity, especially pharmacological as well as medicinally important plants have become extinct, or many of them are on the verge of extinction, threatened etc. This situation has been created by man himself and pharmacological companies for therapeutic purposes to treat the various ailments of human being. The entire history reveals that ancient people have been aware of the fact about the vegetative propagation of plants. Unfortunately, only a small number of plants have the ability to propagate through vegetative means while most of them cannot be regenerated through vegetative propagation due to binding of conventional vegetative propagation methods. Thus, micro propagation has came into existence as an alternative means for conservation of various endangered, threatened and endemic, medicinal plants, or for those which are not being propagated through vegetative means.

Therefore, for the genetic improvement of food crops to continue at a pace sufficient to meet the need of the 8.3 billion people projected to be on this planet at the end of the quarter century, micropropagation has been considered as a significant tool for this critical job.

Tissue culture is now commercially the most viable and efficient biotechnological tool particularly for the developing world. The one singular area, where tissue culture as a technology has found its widest practical

application is in the realm of micropropagation. Micropropagation can simply be described as an *in vitro* method of producing clonal offsprings identical to the stock (Aitken-Christie and Connett, 1992). The so called theory of totipotency, which states that the cells are autonomic and, in principle, capable of regenerating to give a complete new plant, has been in fact the foundation of micropropagation (Pierik, 1987).

Micropropagation offers the advantages in quality, quantity and economics over conventional vegetative propagation for many species, providing an efficient method for the production of a large number of uniform plantlets in a short span of time (Altman and Colwell, 1997; Celiktas *et al.,* 2010). Furthermore, they also facilitate the rapid propagation of valuable genotypes, production of disease free plants, non-seasonal production throughout the year and the germplasm conservation (Gurel, 2009; Khan *et al.,* 2010).

Micropropagation of plants produces elite clones and play key role in the development of high quality plantation, foliage, horticulture and economically important plant species. As conventional propagation techniques are time consuming and labour intensive, thus making the availability of a large number of plants for plantation or for afforestation is a difficult and challenging task (Ray *et al.,* 2005). These problems could be overcome by using micropropagation technique and propagules can be produced at a faster rate and in an economical manner. Besides, being means for the mass multiplication of existing stocks of germplasm for biomass energy production, it is also amenable for the conservation of elite and rare

species, that are threatened with the danger of extinction. Micropropagation focuses on the effects of medium components (sugar, minerals, salts, phytochromes, vitamins, amino acids, etc.) on the dedifferentiation, differentiation, rooting and further growth of plantlets under aseptic conditions (Nguyen and Kozai, 1998).

Micropropagation basically relies on the use and manipulation of medium components in *in vitro* conditions. Micropropagation begins with the selection of plant material to be tissue cultured. Once the plant material is chosen for the cultures, the collection of explant begins and their further processing, such as sterilization, inoculation, dedifferentiation, redifferentiation and organogenesis etc. is taken in to account.

During the last forty two years, micropropagation and other *in vitro* techniques have been widely used and have become commercial technique for horticultural and agricultural crops for their mass scale propagation (George and Sherrington, 1984; Dodds, 1991; George, 1993; Das *et al.,* 1996; Kambaska and Santilata 2009; Girijashankar, 2011; Arumugam *et al.,* 2011; Rani *et al.,* 2012).

Some recent reports on micropropagation of rare/endangered medicinal plants include- zygotic embryo culture in *Hyphorbe lagenicaulis* (Sarasan *et al.,* 2002), leaf culture and petiole culture in *Thapsia garganica* (Makunga *et al.,* 2003), epicotyl culture in *Saussurea obvlilata* (Dhar and Joshi, 2003), axillary bud culture in *Rotula aquatica* (Martin, 2003), inflorescence axis with floral bud in *Chlorophytum borivilianum* (Debnath *et*

al., 2007); *Gerbera jamesonii* (Chakrabarty and Datta, 2008), nodal segment culture in *Synsepalum dulcificum* (Ogunsola and Llori, 2008), Micropropagation has been also carried out in *Ceropegia bulbosa* (Goyal and Bhadauria, 2006), *Adhatoda vasica* (Abhyankar and Reddy, 2007), *Zingiber officinale* (Mohanty *et al.*,, 2008), *Crataeva nurvala* (Babbar *et al.,* 2009). *Jatropha curcas* (Rajore and Batra, 2007); *Ceratonia siliqua* L. (Naghmouchi *et al.,* 2008). *Rauvolfia serpentina*. L. (Singh *et al.,* 2009); *Phyllanthus amarus* (Sen *et al.,* 2009) *Azadirachta indica* (Shekhawat *et al.,* 2009); *Senecio macrophyllus* (Trejgell *et al.,* 2010); *Thevetia peruviana* (Zibbu and Batra, 2010; Siwach *et al.,* 2011) and *Boerhavia diffusa* (Saini *et al.,* 2011); *Murraya Koenigii* (Rani *et al.,* 2012). Further, direct regeneration of plants has been done in many plants species *viz., Scoparia dulcis* (Majumder *et al.,* 2011); *Acacia auriculiformis* (Girijashankar, 2011); *Withania somnifera* (Arumugam and Gopinath, 2011); *Cicer arietinum* L. (Khan *et al.*, 2010), *Clitoria ternatea* L., (Anand *et al.*, 2011) , *Agave vera-cruz* Miller. (Dafedar *et al.,* 2012), *Sarcostemma acidum* (Roxb.) (Rathore *et al.*, 2013) and Aerva lanata (L.) (Varutharaju *et al.*, 2014).

Advantages of micropropagation

- Micropropagation can be used as an alternative to conventional method of vegetative propagation, with the objective of enhancing the rate of multiplication.

- Through *in vitro* clonal propagation, large number of plants can be raised from a small even microscopic piece of plant tissue within a short span of time.

- It provides reliable and economical method of maintaining pathogen free plants in a state that can allow rapid multiplication and also allows international exchange of germplasm.

- Plant multiplication can continue throughout the year irrespective of seasons.

The stages of micropropagation in plants have been outlined as-

- Multiple shoot proliferation through axillary bud sprouting.
- Apical bud proliferation through apical shoot meristem culture.
- Callus induction and its differentiation.
- Induction of rooting in regenerated shoots.
- Hardening and pot transfer of regenerated plants.

Material and methods

During the present research work, micropropagation experiments were conducted in *Tinospora cordifolai* through proliferation of nodal segments. Sterilization of explants was performed *via* the normal procedure as described in chapter-3. All the surface sterilized nodal segments were

inoculated on Woody Plant Medium, supplemented with various hormonal concentrations and combinations.

All the chemicals used were of high purity grade. The cultures were inoculated at 26±2°C under 16/8 hour photoperiod and 55±5% relative humidity. Fifteen replicates were taken for each treatment and experiments were repeated thrice for confirmation. Periodic observations were recorded and the results were subjected to statistical analysis.

Observation and results

Establishment of protocol for multiple shoot proliferation was difficult because after initial culture period 50-60% explants showed high amount of contamination. Though, the morphogenetic responses in inoculated explants were observed on all media combinations, the frequency of morphogenetic responses and number of shoots regenerated varied, depending upon the type and concentration of different hormones added to the medium. During the present set of experiments, multiplication of shoots was obtained from explants bearing nodal segments. In *Tinospora cordifolia* 9-10 shoots were produced, when nodal segments were inoculated on Woody Plant medium supplemented with BAP (2.5 mg/l.).

Evaluation of different factors on multiple shoot proliferation through nodal segments was also observed.

Size, orientation and physiological state of explants

Explants of various sizes (1.0-4.0 cm) were cultured on Woody Plant medium supplemented with auxin/cytokinin. It was observed that 1.0 cm long explants showed better survival and regeneration capacity as compared to much longer or smaller explants. With this orientation of nodal/apical segments at the time of inoculation also plays an important role in survival and regeneration capability of nodal explants. It was found that vertically placed nodal segment gave better results, regarding their survivability and number of multiple shoots, than the oblique or horizontal orientation.

Growth medium

Nutrient salts together with plant growth regulators play an important role in the optimization of plant responses *in vitro*. *In vitro* tissue response mainly depends on the basal medium on which explants are inoculated. The choice of a particular medium depends on the species of plant, tissue or organ to be cultured and the purpose of the experiment. The success of the application of *in vitro* methods is largely due to a better understanding of the nutritional requirements of cultured cells and tissues. Results revealed that only Woody Plant media was most suited for the present experiments (Table-4.1).

Carbohydrate source

Experiments were performed for testing effect of different carbohydrates sources on shoot multiplication and plant regeneration. For

that different carbohydrates *viz*. sucrose, glucose, fructose, mannitol and maltose were used in Woody Plant medium at various concentrations (1-6%). However, among all the carbohydrates tried sucrose at 3.0% (w/v) was the most significant medium constituent for survival, multiplication and growth. While other carbohydrates exhibited poor responses, due to this their use in Woody Plant medium was omitted from our experiments

(Table-4.2).

Light

All the three characteristics of light, *viz*. intensity, period and quality were observed. During the present experimentation, the range of light intensities was set between 1000-5000 lux. However, light intensity of 3000 lux was found to be the best responsive for the growth of cultures

(Table-4. 3).

Phytohormones

In addition to the nutrients, it is generally necessary to add one or more growth substances, to support good growth of tissues and organs. However, the requirement for these substances varies considerably with the tissues and it is believed that it depends on their endogenous levels. During the present studies various cytokinin and auxin were used.

A. CALLUS DEVELOPMENT AND ITS DIFFERENTIATION

Callus is a mass of unorganized cells produced from any type of explant *in vitro*, when the macro and micro nutrients along with growth promoting hormones are supplied to the excised explants in the form of medium. Callus is a homogeneous unorganised mass of parenchymatous cells, trachery elements or sieve elements, which has the potentiality to produce normal roots, embryoids and in turn development of complete plantlets. Under the stimulus of endogenous growth substances or hormones added to the medium the metabolism of cells which is in quiescent state, is triggered resulting in division, growth and development. For plant cells to develop into a callus it is essential that the nutrient medium contains plant hormones, i.e. auxin, cytokinin and gibberellins. Concentrations of these hormones were tested for different tissue explants (leaf, internode and stem node) taken from the plant.

Callus growth can be monitored by fresh weight and dry weight measurements but dry weight measurements could be more accurate. Callus cultures can be maintained for indefinite period by repeated sub culturing of tissues on fresh medium at regular intervals after having observed the growth index. These cultures under appropriate conditions undergo organogenesis leading to complete plantlet formation after going through various stages like shoot development and rooting etc. It is known as caulogenesis, which is helpful in the quick multiplication of explants depending on a number of factors like source of explant, type of explant, size of explant, hormonal combination, plant genotype etc. Media used in

either of the pathways was controlled by the balance of cytokinin and auxin, low auxin with high cytokinin concentration promotes the shoot formation, while high ratio of auxin induces root formation. Quality, quantity and high pH of medium also plays an effective role in the induction of organogenesis (Chidananda and Massimo, 2008).

Material and methods

During the present course of experimentation explants *viz*. leaves and internodes were tried for callus induction. *In vivo* explants were taken from 2-3 months old plant and sterilized by using the regular sterilization procedures as described in Chaper-3. After sterilization of explants they were inoculated on MS medium supplemented with various concentrations of auxins and in combination with cytokinins.

Callus cultures were maintained through subculturing at a regular interval. Evaluation of growth, fresh and dry weight of callus was determined to study the kinetics of callus development. The growth index (GI) was determined as:

$$G.I. = \frac{\text{Final biomass} - \text{Initial biomass}}{\text{Initial biomass}}$$

Observation and results

(a) **Selection of suitable explant for callus induction**

Three different explants i.e. leaf segment, internodal, nodal segments were employed in callus induction studies. Explants were inoculated on MS medium supplemented with different auxins (IAA, IBA, NAA and 2,4-D) in the range of 0.1-4.0 mg/l. Out of the used explants, callus developed from internodal segments was yellow, brown, hard and brittle, which later turned into dark brown colour (Plate-4.1; Fig. A). Whereas, callus developed from nodal segment was green but callus was brittle and growth was too slow (Plate-4.1; Fig. B). However, leaf explants used showed the best responses for callus induction and its further development. It was brown, compact, homogenous and showed rapid growth pattern (Plate-4.1; Fig. C). Leaf margins curled after 15 days of inoculation and turned into callus after 35 days of inoculation, which was subcultured again and again to get stock callus on M.S. medium fortified with 2,4-D (0.5 mg/l) (Table 4.4).

(b) **Effect of various auxins on callus induction**

For the development of callus from leaves, different auxin *viz*. IAA, IBA, NAA and 2,4-D were tried at various concentrations (0.1-4.0 mg/l) to evaluate their effects on callus induction. It was observed that callus induction could take place at all the concentrations of auxins tried. However, IAA could initiate callusing only at the leaf margins and further growth of this callus was not observed. Further, on NAA moderate callusing was observed, the callus was pale yellow which later turned brown (Table 4.4).

Furthermore, IBA did not produce any positive responses regarding the callus induction. Further, 2,4-D at all the concentrations was found to be beneficial for callus induction, proliferation and further growth of the callus concerned (Plate-4.2, Fig A-D). Furthermore, the optimum callusing was found on MS medium supplemented with 2,4-D (0.5 mg/l), which gave brown, moderate growing rate, homogenous maximum amount of callus and after repeated subculturing of this callus, it served as 'stock callus' (Plate 4.3-Fig. A-C).

Effect of 2,4-D in combination with cytokinin on callus development

Experiments were set up to enhance callus growth by incorporation of cytokinin with optimized 2,4-D. Callus obtained on 2,4-D (0.5 mg/l) was subcultured on medium containing BAP/Kn (0.1-4.0 mg/l) and TDZ (0.1-4.0 mg/l). No significant responses were observed in the callusing. Furthermore, it has also been observed that none of the cytokinins was beneficial to induce shooting from the callus.

B. MULTIPLE SHOOT REGENERATION *VIA* NODAL SEGMENTS

Effect of cytokinins

During the present research work, experiments were conducted to study the effect of various concentrations of cytokinin alone or in combination (BAP/Kn) on the pre-prepared explants for shoot initiation from nodal stem segments of *Tinospora cordifolia* on Woody Plant medium.

a. Effect of 6-benzyl amino purine (BAP)

During the experiment, 0.1-5.0 mg/l concentrations of BAP were tried on T*inospora cordifolia.* The results proved that BAP (0.6 mg/l) elicited 2-3 initiated shoots per explant after 20-25 days of inoculation (Table 4.5) (Plate 4.4; Fig.A-B).

b. Effect of Kn

In order to study the effect of kinetin on multiple shoot formation, MS-medium containing Kn (0.1.-5.0 mg/l) alone was used. Lower concentrations of Kn proved to be effective for shoot initiation. However, 3-4 shoots were produced from nodal segment in case of *Tinospora cordifolia,* when nodal segments cultured on medium containing Kn (0.6 mg/l) (Table 4.5) (Plate 4.4; Fig. C-D).

c. Effect of BAP for shoot proliferation

During the present research work**,** W.P.M**.** containing Kn (0.5.-8.0 mg/l) alone was used In *Tinospora cordifolia* BAP (2.5 mg/l) alone proved to be beneficial for multiple shoot proliferation, 8-9 shoots were reported. (Table-4.6) (Plate- 4.5 A-D).

d. Effect of TDZ for shoot proliferation

During the present research work**,** W.P.M**.** containing TDZ (0.5.-8.0 mg/l) alone was used In *Tinospora cordifolia* TDZ(3.0 mg/l) alone proved to be beneficial for multiple shoot proliferation (Table-4.6) (Plate- 4.6; A-C).

e. Effect of BAP combination with TDZ for shoot proliferation

To study the combined effect of cytokinin on *in vitro* shoot regeneration Woody plant medium was supplemented with BAP (2.5 mg/l) with pre evaluated optimum concentrations of TDZ (3.0 mg/l). However, only 3-4 shoots were produced with this combination. Hence, this combination was not fruitful for growth and multiplication. (Table-4.6).

Effect of cytokinin in combination with auxin on shoot proliferation:

To evaluate the effects of cytokinin in combination with auxin on *in vitro* shoot proliferation, various auxin *viz*. IAA, 2,4-D, NAA and IBA at 0.5- 6.0 mg/l were added in Woody Plant medium with pre-evaluated optimum dose of cytokinin (BAP at 2.5 mg/l). However, out of various auxins used neither of them was beneficial for proliferation, elongation and growth of the shoots. However, with the addition of IAA bud break was observed but growth was sort of arrested, with the addition of 2,4-D approximately 1-2 shoots were produced at all the concentrations tried with low amount of callusing at the base of the nodal segments. Furthermore, addition of NAA at all the concentrations used was also insignificant for the

production of multiple shoots, only bud break was observed with this. IBA at any of the concentrations used did not produce any significant results concerning to multiplication.

C. INDUCTION OF ROOTING IN THE *IN VITRO* REGENERATED SHOOTS

Rooting is a crucial step in the development of complete plantlets through micropropagation. It is a complex process, which is affected by multiple factors including phytohormones, phenolic compounds, nutritional status and genetic characteristics, as well as associated stress responses (Bennett, 2003). Auxins appear to play a central role in the determination of rooting capacity of a specific tissue (Neto *et al.,* 2000). However, IBA was superior in terms of producing the rooting during the present studies. Similarly, through incorporation of IBA, rooting has been developed in several woody plant species at the cut ends of *in vitro* developed shoots (Drew *et al.*, 1993; Saxena *et al.*, 2000).

Material and methods

In vitro regenerated shoots produced from nodal segments were carefully separated and transferred to rooting medium. Rooting media consisted of full, ½ strength of MS salts. Media were prepared in routine manner and supplemented with various auxins (IAA, NAA, 2,4-D and IBA) at their different concentrations (0.5-6.0 mg/l). The cultures were maintained at 26±2ºC temperature and 55±5% relative humidity under 16/8 hour photoperiod. Observations were recorded periodically.

Observation and results

The success of *in vitro* regeneration relies on the rooting percentage and survival of the plantlets in the field condition. During the present studies rooting of regenerated shoots did not occur on shoot induction medium even after culturing and sub-culturing for a long period. Therefore, the *in vitro* regenerated shoots were sub-cultured on to rooting medium consisting of MS and on ½ MS with auxins (IAA, IBA, NAA and 2,4-D), respectively.

Incorporation of IAA, 2,4-D and NAA with ½ MS medium, at any concentration (0.2-6.0 mg/l) did not evoke rooting responses.. Furthermore, IBA at 0.2-6.0 mg/l was found to be fruitful in respect to the induction and development of roots. Best rooting response was observed on ½ MS medium fortified with IBA at 0.6 mg/l. Roots were healthy, branched, fast growing and whitish-brown in colour. Rooting percentage (86-90%), number of roots (2-3) and root length (6-7 cm) were observed on IBA at 0.6 mg/l (Table 4.7-Plate 4.7-Fig. A-B).

D. HARDENING AND POT TRANSFER OF *IN VITRO* REGENERATED PLANTLETS

After the development of roots, regenerated plantlets were removed from glass vessels and the traces of adhering agar to the roots were thoroughly washed by sterile distilled water. The plantlets with well developed roots were transferred to earthen pots containing vermicompost and autoclaved soil (1:3). The earthen pots containing plantlets were incubated in the growth chamber for about 2 weeks, where controlled

environmental conditions like light, humidity and temperature were provided. High humidity (80±5%) was provided by keeping in the plant growth chamber then in the pot. After 2 weeks, the beakers were gradually removed each day for a few hours and potted plantlets were watered regularly. Plantlets were gradually exposed from partial light to sun light (Plate 4.8; Fig. A-C). Later on, the plantlets were transferred to bigger pots with garden soil and finally to field condition, where they showed 85-90 % survivability.

Disscusion

Biotechnology has several ramifications, which may broadly be differentiated into the aspects of biomedicine and agriculture which include horticulture and forestry as well. Plant tissue culture providing a wide range of *in vitro* techniques has developed over the years into a technology without parallel techniques. Amongst different techniques micropropagation of plants through tissue culture has emerged as an invaluable aid for the production of elite clones and has been playing a key role in the development of high quality economically important plant species in agriculture, forestry, horticulture and medicinal plant industries for the past decades (Rani and Raina, 2000; Sunandakumari *et al.,* 2004; Sujatha and Ranjitha Kumari, 2007; Durkovic, 2009).

The successful micropropagation protocol depends on a number of factors. The type, age, nature, physiological state of the explant, seasonal variations and environmental conditions play a crucial role in the

establishment of cultures and their subsequent development into plant regeneration. Sterilization methods, media and culture conditions also affect the micropropagation. A few problems often encountered during the process are:

(1) Browning/blackening of the culture medium and explants: may be due to the oozing of secondary products from the used plant inoculums, hence the medium gradually turns dark brown to black, respectively. The oxidation product of phenols can be phytotoxic and causes necrosis and eventually death of the explants. This problem is especially common in woody legumes (Pierik, 1987).

(2) Hyperhydricity or vitrification: morphological, physiological and metabolic derangement frequently affecting herbaceous and woody plants during their *in vitro* culture. Hyperhydrated shoots generally show poor growth, become necrotic and finally die. The plants showing hyperhydration have low survival rate during acclimatization (Gasper, 1991).

(3) Contamination: it is one of the most serious problems in commercial micropropagation as it can cause a disastrous situation, if it occurs at an advanced stage of the production schedule. It is therefore extremely important to maintain standards of hygiene in and around the sterile area.

The plant family Menispermaceae consists of about 70 genera and 450 species that are found in tropical lowland regions. They are generally climbing or twining, rarely shrubs. Leaves are alternate or lobed, flowers

small cymose, seeds usually hooked or reniform. This family is rich source of alkaloid and terpenes. Tinospora is one of the important genera of the family, consisting of about 15 species. Some medicinally important species includes *T. Cordifolia, T. Malabarica, T. Tementosa, T. Crispa, T. Uliginosa, etc.*

Present investigation was undertaken to study the micropropagation system in medicinally important plant *Tinospora cordifolia*. It is a very important plant in Indian and in Chinese system of medicine. Further, it is exploited from its natural habitat on account of its uses in therapeutics. However, the lack of proper cultivation practices, destruction of their habitat and ruthless exploitation of this plant has led to a rapid decline in wild population of this plant. Keeping in mind the immense importance of this plant and to fulfil the needs for medicinal purposes micropropagation of this plant has been taken up for multiplication of *Tinospora cordifolia*.. Moreover, very little work has been done on this plant concerning tissue culture. Therefore, there is need to develop an efficient protocol for the *in vitro* multiplication of this plant. Present studies were carried out to develop an efficient regeneration system for this plant through nodal segment culture and also to extract secondary metabolites from the calli.

Callus formation and its differentiation

Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins). The specific concentrations of plant regulators needed to

induce callus formation, varies from species to species and even depends on the source of explant (Jain and Datta, 1992; Charriere *et al.,* 1999). Furthermore, genotype is also one of the most important factors for callus induction (Punia and Bohorova, 1992; Sarrafi *et al.,* 1996). Callus cultures could be initiated from a variety of explants such as leaves, cotyledons, stems, shoot tip, petiole, anthers, seeds, internodes etc. (Molina, 2004).

During the present studies callus was obtained from *in vivo* leaf segments, which showed best results at 2,4-D at 0.5 mg/l concentration. Callus is healthy, brown and moderate growing with homogenous fragile cells

In consonance to this, *Thevetia peruviana* on MS medium supplemented with 2,4-D and Kn (Zibbu and Batra, 2010), *Hibiscus cannabinus* on MS medium supplemented with 2,4-D and NAA, Kinetin, BAP, and TDZ (Mahmood *et al.,* 2014), *Podophyllum hexandrum* Royle. also on MS medium supplemented with 2,4-D (Rajesh *et al.,* 2014).

In contrast of above study few plants showed results on other plant growth regulators they are- *Piper nigrumon* on MS medium supplemented with BA and TDZ (Ahmad *et al.*, 2013) *Gerbera jamesonii* Bolus on MS medium fortified with BA and Kinetin (Minerva *et al.,* 2013), *Gentiana kurroo* Royle on MS medium fortified with NAA and BA (Sharma *et al.*, 2014), *Prunus persica* on MS medium supplemented with IBA and BA (Pérez *et al.*, 2014).

Multiple shoot formation through nodal segments

The plant hormones, auxin and cytokinin are thought to have a major role in controlling the bud initiation as well as shoot proliferation process (Napoli *et al.*, 1999). It has been evaluated that auxins have an inhibitory effects on the growth of nodal segment, whereas cytokinin promotes *in vitro* shooting.

During the present research endeavour, various concentrations of BAP, Kn and TDZ were tried to obtain multiplication through nodal segment. Results indicated that among all the cytokinins tried BAP alone was the most suited hormone for proliferation of nodal segment. After 3-4 weeks BAP at 2.5 mg/l produced maximum number of shoots. However, increased concentrations of BAP didn't evoke any positive responses. However, the combination of auxin with cytokinin showed poor responses and didn't prove to be beneficial for multiplication.

Similar observation have been reported for *Gentiana kurroo* Royle (Sharma *et al.*, 2014), *Aerva lanata* (L.) (Varutharaju *et al.*, 2014), *Simmondsia chinensis* (Muhammad Azhar Bashir *et al.*, 2013), *Jatropha Curcas* (LINN.) (Narayan Pandhure *et al.,* 2012), *Vitis vinifera* (L.) (Ashrafuzzaman *et al.,*2012), *Bacopa monneiri* (L.) (Chandra Gurnani *et al.,* 2012).

However in contrast to this, Ananthi *et al.,* (2011) (*Rorippa indica*) (L.), Ray and Bhattacharya (2008) (*Eclipta alba*) (L.) reported best results on kinetin, whilw, Mohanty *et al.,* (2013) (Hedychium coronarium) showed

best results on BA along with NAA, Raomai *et al.*, (2013) (*Homalomena aromatica* Schott) reported best results on BAP, Kaul *et al.*, (2013) (*Ajuga bracteosa*) showed best results on MS medium, supplemented with BA along with IAA.

Induction of rooting in the regenerated shoots

After successful multiplication, the next step of micropropagation is rhizogenesis. The process of root formation is essential step of micropropagation. Several workers pointed out that the endogenous and exogeneous auxins play a key role in root induction (Neto *et al.*, 2001). During the present studies out of all the auxins tried IBA proved to be optimum for the induction of roots. About 6-7 cm long root, shoot tips with two to three leaves, harvested from the elongation medium, were used for rooting. Rooting percentage was lower in full strength MS medium than in half strength MS medium.In half strength MS medium supplemented with 0.6 mg/l IBA and 3% sucrose, profuse root formation was observed after 25 days of inoculation .

It was also supported by the studies carried out by various scientists in different plants such as *Tridax procumbens* (Sahoo and Chand, 1998), *Jatropha curcas* (Rajore *et al.,* 2002), *Leptadenia reticulata* (Hariharan *et al.,* 2002), *Lobelia nicorianfolia* (Jabeen *et al.,* 2002), Trapa (Hoque and Arima, 2003), *Sesbania drumondii* (Cheepala *et al.,* 2004), *Mucuna pruriens* (Faisal and Anis, 2006), Wheat (Rahman *et al.,* 2008), *Larrea divaricata* (Palacio *et al.,* 2009), *Cannabis sativa* (Lata *et al.,* 2010), *Cedrela odorata* (Millan-Orozco *et al.,* 2011), Gentiana kurroo Royle (Sharma *et al.,* 2014), Aerva lanata (L.) (Varutharaju *et al.*, 2014).

In contrast to our study, other auxin such as IAA has also been reported optimum for the induction of roots in many plant species viz. *Charybdis numidica* (*Kongbangkerd et al.,* 2005), *Penthorum chinense* (Yang and Peng, 2009), *Andrographis neesiana* (Karuppusamy and Kalimuthu *et al.,* 2010), *Euphorbia pulcherrima* (Castellanos *et al.,* 2010), Moreover, Dhabhai *et al.,* (2010) in *Acacia nilotica* and Escutia *et al.,* (2010) in *Tigridia pavouia* , *Homalomena aromatica* Schott.(Raomai *et al.,* 2013) found NAA to be the best for *in vitro* rooting..

Hardening and acclimatization

Plantlets developed *in vitro* with well developed shoots and roots were removed from rooting medium and washed thoroughly but delicately to remove adhering agar. The plantlets were then transferred to pots containing coco peat only under cool, white, florescent light 2000-3000 lux, 16/8 hour photoperiod and high humidity (80%) in plant growth chamber (Shivam instruments, New Delhi). The potted plants watered every day with few drop of half strength MS salt solution twice a week for about 2 weeks. These plants kept in plant growth chamber for about three weeks for their hardening. After 3 weeks, gradually these plants were exposed partially to sunlight for their acclimatization in natural conditions. Later on, to see the effect of potting mixture (Coco peat) on enhancement and further root development. They were taken out from thermacol cups and exposed to view. Then these plantlets were transferred to large earthen pots containing a mixture of autoclaved garden soil and coco peat (3:1) and exposed to the natural conditions. During the experiment it was observed that 85% plantlets survived in *Tinospora cordifolia*.