Discussion
DISCUSSION

Organogenesis de novo in tissue culture has provided a useful system for studying the development of higher plants. Early observations by Skoog and Miller (1957) presented the evidence that shoot organogenesis is regulated by the balance of auxin and cytokinin in the culture medium. In recent years, shoot induction, differentiation and development were intensively studied using various annual plants by genetic, molecular, biochemical and cellular approaches (Burritt and Leung 1996, Lakshmanan et al. 1997). The process of organogenesis from plant tissue cultures has three main phases:

1. Induction of explants competence,
2. determination of cell fate following the exogenous application of the growth regulators, and
3. morphogenesis.

It has been established that shoot meristem initiation and development follows an ordered sequence of changes in tissue organization before they emerge from the explants. Later experiments, determined the sequence of stages in the initiation and developmental pattern of root and shoot organogenesis in cultured leaves of *Nicotiana tabaccum* (Att-field and Evans 1991a and b).

Development of genetic manipulation system to produce transgenic plant greatly depends on the establishment of a reliable and efficient regeneration system via organogenesis and/or embryogenesis. Regardless of the sufficient regeneration levels achieved for some of the medicinal plants with different
explant, the regeneration in many other plants remain difficult and seem to be strongly genotype dependent.

In the present investigation, two medicinally important plants viz. *Vitex negundo* and *Ruta graveolens* have been tried in order to achieve rapid and reproducible in vitro regeneration system. The results obtained during the study have been discussed in the light of existing literature. Several explants can be used to propagate plants in vitro. However, the most simple and realistic method of cloning the plants in vitro is the single node culture that resulted in the production of a number of shoots that can also regenerate roots with ease. Nodal segments containing axillary buds have quiescent or active meristem which has the potential of developing into complete plantlets. In nature, these buds remain dormant for specific period depending on the growth pattern of plant. The mechanism of dominance has also been demonstrated to be under control of various growth regulators, the proportion of these regulators in the culture media can be manipulated to induce the regeneration of each meristem into liable shoots. The growth regulator type, concentration, and the source of explants played an important role during the optimization of culture conditions to regenerate shoot and roots in vitro.

5.1 Direct shoot regeneration

Nodal explants of *Vitex negundo* and *Ruta graveolens* failed to develop shoot buds in growth regulator free medium. In contrast, when the same explants were grown on culture media containing cytokinin, axillary shoots developed precociously, which proliferated to form clusters of secondary and tertiary shoots. Multiple shoot emergence from nodal explants was observed on MS medium containing BA at different concentrations. The role of BA in bud breaking and shoot formation from nodal explants has been reported in many plants such as *Anemopaegma arvense* (Pereira et al. 2003), *Feronia limonia*
(Hiregoudar et al. 2003), *Annona squamosa* (Nagori and Purohit 2004), *Davidsonia* spp. (Nand et al. 2004), *Bupleurum raoi* (Chen et al. 2006), *Artemisia vulgaris* (Sujatha and Kumari 2007), *Sida cordifolia* (Sivanesan and Jeong 2007), *Pongamia pinnata* (Sugla et al. 2007) and *Ulmus parvifolia* (Thakur and Karnosky 2007). BA at the concentration of 5 μM and 10 μM were found optimum for maximum shoot induction in *Vitex negundo* and *Ruta graveolens* respectively. The increase in concentration of BA beyond optimal level had a negative effect and the shoot exhibited a stunted nature with the reduction in number of shoots generated from each explant. These findings are in consonance with the results obtained earlier in *Withania somnifera* (Sen and Sharma 1991), *Kaempferia galanga* (Vincent et al. 1992), *Vitex negundo* (Sahoo and Chand 1998), *Rauvolfia tetraphylla* (Faisal and Anis 2002) and *Pogostemon heyneanus* (Hemborm et al. 2006).

Nodal segments also started growing after 3 weeks of culture on MS medium containing Kn and 2iP with 20-75% regeneration frequency, whereas high (34-87%) regeneration frequency was exhibited by the use of BA. Thus, all cytokinins (BA, Kn and 2iP) were capable of shoot induction in nodal segments and BA was found to be significantly more effective than other cytokinins. Similar observations with different cytokinins have been reported in other plant species such as *Holostemma ada-kodien* (Martin 2002), *Anemopaegma arvense* (Pereira et al. 2003), *Garcinia indica* (Malik et al. 2005), *Morus alba* and *Morus indica* (Chitra and Padmaja 2005) and *Tylophora indica* (Faisal et al. 2007).

The combination of various auxins (IAA, IBA and NAA) with the optimal cytokinin concentration was also studied for their ability to effect the shoot induction and multiplication rate and to optimize the medium composition for maximum plantlet regeneration in both the species. Both regeneration
frequency and average number of shoots produced per explants were enhanced significantly when compared with cytokinin only. All the three auxins tested were capable of inducing more than 70% explants to respond positively but the combination of BA (5.0 μM) with NAA (0.5 μM) was found to be the best combination in *Vitex negundo*. However, the optimum response in case of *Ruta graveolens* was found at BA (10.0 μM) + NAA (2.5 μM). The use of NAA at relatively low concentrations positively enhance shoot induction in the presence of cytokinin at the optimal level is in agreement with the results obtained in *Tylophora indica* (Sharma and Chandel 1992), *Liquidambar stytraciflua* (Kim et al. 1997), *Psoralea corylifolia* (Anis and Faisal 2005), *Aloysia polystachya* (Burdyn et al. 2006), *Dioscorea nipponica* (Chen et al. 2007), *Sida cordifolia* (Sivanesan and Jeong 2007) and *Erigeron breviscapus* (Liu et al. 2008). These result indicated that the growth regulators, BA and NAA could ensure in vitro regeneration and synergism of BA and NAA in proper concentration was extremely favourable for their multiplication. In contrast, deleterious effect of combination of NAA with BA was also reported in *Cunila galioides* (Fracaro and Echeverrigaray 2001), *Artemisia judaica* (Liu et al. 2003) and *Garcinia indica* (Malik et al. 2005). However, in *Holostemma ada-kodien* (Martin 2002) and *Hagenia abyssinica* (Feyissa et al. 2005), the best combination among various auxins (IAA, IBA and NAA) was observed on a medium containing BA and IBA, whereas IAA was most effective auxin with BA in *Chonemorpha grandiflora* (Nishitha et al. 2006). Almost similar response was observed with BA and IBA or NAA combination in case of *Pogostemon heyneanus* (Hemborn et al. 2006).

The promoting effect of Kn and auxin on proliferation of axillary buds was also studied and it was observed that the combination of Kn with NAA produced maximum number of shoots and showed synergistic effect on shoot
multiplication and elongation in both the species. Similar culture response on a medium containing Kn and NAA has been observed in *Chlorophytum arundinaceum*, however, combination of IAA and IBA was evident in shoot morphogenesis of *Teucrium stocksianum* (Bouhouche and Ksiksi 2007). These reported results support the findings that the interaction of auxin and cytokinin (Kn) is necessary for in vitro organogenesis and high concentration of cytokinin and low concentration of auxin appeared to be a prerequisite for differentiation of adventitious buds.

Shoot tip explant of both the species, *Vitex negundo* and *Ruta graveolens* were also tested for the induction of shoot morphogenesis on a media containing various PGRs (BA, Kn, 2iP, IAA, IBA and NAA) singly as well as in various combinations and concentrations. Among all the treatments tested, the best response was observed on a combination of cytokinin (at higher) and an auxin (at lower) concentration. Similar effect of auxin-cytokinin has also been reported in other medicinal plants such as *Costus speciosus* (Chaturvedi et al. 1984), *Picrorhiza kurroa* (Upadhyay et al. 1989), *Holarrhena antidysenterica* (Kulkarni et al. 1992), *Decalepis hamiltonii* (Gridhar et al. 2005), *Vitex negundo* (Rani and Nair 2006) and *Eclipta alba* (Husain and Anis 2006a).

Plant hormones play an important role in many aspects of growth and development including dormancy which may also be hormonally controlled. Cytokinins have been shown to stimulate or accelerate release of buds from dormancy. Different cytokinin generally express different activities in affecting axillary shoot formation in vitro (Preece and Sutter 1991). Besides, the $N^6$ substituted adenine derivatives (BA, Kn and 2iP), several urea derivatives also have cytokinin like activity, e.g. the thidiazolylurea derivative, thidiazuron (Matsubara et al. 1990). TDZ was developed by Schering as a cotton defoliants (Arndt et al. 1976) and its cytokinin activity is similar to that of highly active N-
phenyl-N’-4-pyridylurea derivatives (Mok et al. 1982). In addition, TDZ could be substituted for adenine-type cytokinins in various cell culture systems including both callus cultures and micropropagation of many woody plants. There is growing evidence that TDZ may be involved in increasing the biosynthesis or accumulation of endogenous cytokinin (Murthy et al. 1995).

Therefore, the goal of this segment of current study was to determine the role of TDZ as cytokinin for the formation of morphologically well developed shoot tip and axillary shoots at various concentrations in both the plant species. In *Ruta graveolens*, TDZ showed inhibitory effect and resulted in abnormal shoot development whereas in *Vitex negundo*, the concentration of TDZ was highly significant on multiple shoot induction in both the explant i.e. nodal segment and shoot tips. Number of shoots increased with the increasing level of TDZ upto 1.0 μM, thereafter a slight decrease in number of shoots was observed. Therefore, 1.0 μM TDZ proved to be the optimum concentration for maximum regeneration in *Vitex negundo*. Similar response with the low concentration of TDZ which is most frequently used for micropropagation of various plant species has been reported (Huetteman and Preece 1993, Lu et al. 1993, Khurana et al. 2005, Ahmad et al. 2006a and b, Ahmad and Anis 2007a) and other medicinal plants (Ledbetter and Preece 2004, Faisal et al. 2005b, Faisal et al. 2005c, Ahmad and Anis 2007a and b, Husain et al. 2007, Siddique and Anis 2006a and b). In contrast, TDZ was found to be least effective and yielded low shoot in comparison to other cytokinins in *Vitex negundo* as reported by Sahoo and Chand (1998) and could be because of time and exposure of explant with TDZ.

TDZ has been reported to inhibit shoot elongation on prolonged exposure (Huetteman and Preece 1993). In the present study, continuous or more than 4 weeks exposure to TDZ resulted in abnormal shoot development
with fasciation and distortion of regenerated shoots in *Vitex negundo*, and this problem was overcome by transfer of TDZ exposed explants to a medium lacking TDZ or on a medium containing reduced concentration of BA (1.0 μM) and NAA (0.5 μM) combination for different time period (2, 4, 6 and 8 weeks). Similar response with BA and NAA on TDZ exposed tissues was also evaluated in *Liquidambar styraciflua* (Kim et al. 1997).

Present finding illustrated that TDZ can substitute both auxin and cytokinin requirement reported earlier (Chandramu et al. 2003b, Sharma et al. 2006, Vadawale et al. 2006). There are now more than 500 publications that deals with various effect of TDZ on plant regeneration physiology. One of the reasons is due to its unique mode of action which may involve, at least in part, the modulation of the levels of endogenous growth regulators, polar auxin transport, and accumulation of proline in TDZ-induced tissues (Jones et al. 2007). The precise mode of action of TDZ in shoot induction has remained elusive. The complex nature of biochemical and morphological responses that have been reported for plant tissues exposed to TDZ has provided some indication of the cascade of physiological reactions within the plant tissues but there have been relatively few investigations that have utilized radio labelled TDZ for characterization of the fate of TDZ molecule (Murch and Saxena 2001).

*Vitex* is a woody tree and availability of fresh sprout is season-dependent. Thus, the availability of explants for the clonal propagation becomes a limitation. To circumvent this problem, after four sub-cultures, in vitro proliferated shoots (microshoots) were further used as source of explants. Recurrent production of shoots form microshoots has been reported in number of tree taxa like *Caesalpinia pulcherrima* (Rahman et al. 1993), *Lagerstromia parviflora* (Quraishi et al. 1997), *Paulownia tomentosa* (Rout et al. 2001), *Pistacia vera* (Onay 2000) and *Syzygium cuminii* (Jain and Babbar 2000).
Promotory effect of TDZ was also evident on the axillary shoot proliferation response of micronodes which exhibited higher regeneration response as compared to the nodal explant derived from tree (Data not shown). The micronodes from new regenerated shoots were subcultured after each passage to confirm the recurring nature of the developed protocol. Through repeated subculture of micronodes from microshoots, over 6000 shoots could be produced in a span of 8-10 months from a single nodal explant derived from adult tree.

Besides, cost-culturing, this segment of present work has eliminated the dependence on season for collection of plant material from the tree by developing a protocol for recurrent production of shoots from nodal explants derived from microshoots, thus making it possible to raise any number of clones of the selected tree at any time of the year.

5.2 Indirect shoot organogenesis

Crop improvement by traditional methods (mass selection, inbreeding and hybridization) is labour intensive and time consuming and strongly influenced by environmental conditions. In contrast, in vitro regeneration via callus culture may be a quicker and easier than conventional breeding technique to induce morphological and chemical variations (Gao and Bjork 2000). Regeneration potential of cultured explant evidently depends on various factors such as nutrient medium, explants, temperature, length, age and importantly endogenous level of phytohormones (Reddy and Vaidyanath 1990, Malik and Saxena 1991, Gulati et al. 1996, Arora and Chawla 2005).

Interestingly, it has been observed that in the same plant, different explants showed differential response for callus induction at various levels of growth regulators which may be explained on the basis that different explants
were at different biochemical status at the time of inoculation (Paterson and Everett 1985).

The aim of this segment of work was to establish the composition of plant growth regulators in the culture medium allowing for effective regeneration and rooting of the shoots from the callus induced from various explants (leaf, nodal and internodal) of *Ruta graveolens*. It was observed that shoots were initiated through indirect organogenesis from a callus that was formed in the presence of all the tested PGRs. Callusing started from the cut end of explants and covered the whole surface within 4-6 weeks. Cut end served as an initiation point for callus induction and may be due to the accumulation of auxins at the cut portion, which stimulate cell proliferation in the presence of growth regulators. The observation recorded revealed that the fastest growing callus formation was achieved in nodal segments on MS medium containing 2,4-D and 2,4,5-T at various levels. The findings are in accordance with the results obtained in *Guizotia abyssinica* (Sarvesh et al. 1993), *Tylophora indica* (Faisal and Anis 2003) and *Withania somnifera* (Rani et al. 2003). Addition of lower concentration of BA (2.5 μM) to various concentration of 2,4-D was found to promote callus induction in all the three types of explants. Nodal segments were found to be superior to inter-node or leaf explants for callus induction. The combination of an auxin with cytokinin greatly affected the characteristics of callus and relatively friable and yellow callus was obtained on a medium containing 2,4-D (10 μM) and BA (2.5 μM) which is in accordance with the results obtained in *Guizotia abyssinica* (Sarvesh et al. 1993), *Bacopa monnieri* (Srivastava and Rajani 1999) and *Potentilla potaninii* (He et al. 2006). However, this differs slightly from the results obtained in *Paspalum simplex* (Molinari et al. 2003) and *Hypericum perforatum* (Wojcik and Podstolski 2007) who showed that medium containing 2,4-D and
Kn was most effective for callus growth. Combination of NAA also reported to promote callus induction in *Leucaena leucocephala* (Maity et al. 2005) and *Stylosanthes guianensis* (Mrogniski and Kartha 1981). TDZ too has been found to have positive effect on callus induction either singly or in various combinations with 2,4-D (Chang and Chang 1998, Nyochembeng and Garton 1998, Chen et al. 2000, Lata et al. 2002, Shahzad et al. 2006). However, a combination of TDZ and NAA on callus induction in *Cicimifuga racemosa* (Lata et al. 2002) has also been reported.

The growth pattern of callus as observed during the present investigation has clearly demonstrated that combination of auxin and cytokinin was absolute pre-requisites for callus induction in *Ruta graveolens* and also combination is often crucial for setting off certain physiological processes that could ultimately lead to the callus formation. Callus growth was enhanced when auxin level in the medium kept higher compared to cytokinin (Maity et al. 2005). The improved efficiency in callus induction may be attributed to the role of auxins-cytokinins in DNA synthesis and mitosis (Skoog and Miller 1957). Auxins and cytokinins might have acted synergistically to promote either cell division or cell expansion depending upon other factors within the cell, which react with these hormones.

Incubation of callus on a media containing cytokinin and auxin at different level, resulted in shoot buds differentiation in *Ruta graveolens*. BA alone at slightly higher (7.5 µM) concentration induced the development of greenish nodular callus with shoot buds which later converted into shoots. Such response with BA has been reported in literature (Gharyal and Maheshwari 1990, Maity et al. 2005). Increasing concentration of BA from 7.5 to 10.0 µM resulted in decrease in shoot regeneration ability in *R. graveolens* callus which is in agreement with the reports available on *Coleus forskohlii*.
(Reddy et al. 2001) and Tylophora indica (Faisal et al. 2005c). Stimulation of shoot bud differentiation and proliferation was achieved by supplementing the medium with NAA at various levels with optimum concentration of BA (2.5 μM) and it was observed that the presence of NAA was crucial for regeneration of shoot buds. Maximum morphogenetic response with highest number of shoots was noticed on nodal callus clumps of Ruta graveolens cultured on 10.0 μM BA + 2.5 μM NAA. Clearly, NAA showed the synergistic effect with BA and enhanced shoot bud induction from callus and increased the shoot morphogenetic response. Similar, observations with auxin and cytokinin combination have been recorded in Petasites hybridus (Wldi et al. 1998), Eucalyptus grandis (Liu et al. 1999), Coleus forskohlii (Reddy et al. 2001), Tylophora indica (Faisal and Anis 2003, 2005), Leucocaena leucocephala (Maity et al. 2005), Potentillia potaninii (He et al. 2006) and Hypericum perforatum (Wojcik and Podostolski 2007).

5.3 Rooting in regenerated shoots

During micropropagation, rooting in microshoots is often problematic and losses at this stage have vast economic consequences (De Klerk 2002). At the same time, adventitious root formation is a fascinating scientific subject matter as many genotypes are recalcitrant to root (Hartmann et al. 1990). Thus, research on adventitious root formation is highly important from the practical point of view.

Adventitious root production in isolated micro-cutting of Vitex negundo and Ruta graveolens was achieved in the presence of various auxins (IAA, IBA and NAA) in MS or ½ MS medium. In both species, equimolar concentration (1.0 μM in Vitex negundo and 0.5 μM in Ruta graveolens) of IAA and IBA induced maximum rooting response. Similar results were reported in Garcinia indica (Malik et al. 2005) and Rauvolfia tetraphylla (Faisal et al. 2005b).
Rooting in in vitro regenerated microshoots of *Vitex negundo* also started after 2 weeks of incubation on MS medium supplemented with NAA with (61%) rooting frequency whereas quite high percentage (95%) of shoots rooted well when IBA was used. Thus, from the results obtained in *V. negundo*, it appeared that all auxins (IAA, IBA and NAA) expressed the positive response for in vitro root induction but IBA was found to be most effective. The effectiveness of IBA in rooting has also been reported in various medicinal plants like *Liquidambar styraciflua* (Kim et al. 1997), *Holostemma ada-kodien* (Martin 2002), *Anemopaegma arvense* (Pereira et al. 2003), *Feronia limonia* (Hiregoudar et al. 2003), *Tylophora indica* (Faisal and Anis 2003) and *Pongamia pinnata* (Sugla et al. 2007). However, in *Artemisia vulgaris*, IAA was found to be superior (Sujatha and Kumari 2007).

MS strength also plays an important role in in vitro rooting as observed in *Ruta graveolens* where ½MS was found superior over MS medium, which is in consonance with the earlier reports viz. *Cucumis sativus* (Ahmad and Anis 2005), *Tylophora indica* (Faisal and Anis 2005), *Acacia sinuata* (Shahzad et al. 2006), *Mucuna pruriens* (Faisal et al. 2006c and d) and *Clitoria ternatea* (Shahzad et al. 2007).

Time and exposure to auxin also play a critical role in in vitro rooting. In *Vitex negundo*, 1.0 μM (IBA) proved to be the best concentration for maximum rooting which is in agreement with the results reported in *Vitex negundo* (Sharma et al. 2006). In contrast Chandramu et al. (2003b) and Vadawale et al. (2006) achieved rooting on higher concentration of auxins in the same species. Superiority of combination of two auxins was also reported for the same plant (Sahoo and Chand 1998, Rani and Nair 2006). Short pulse treatment of higher concentration of an auxin prior to transfer to PGR free medium was also found.

In vitro rooting response is perhaps related to endogenous auxin or cytokinin levels. Control over the type of organ produced in culture is governed by the balance of exogenous and endogenous growth regulators. Cells of the same plant can have different endogenous levels of plant growth regulators and therefore it is reasonable to postulate that responses to exogenous or endogenous auxin also vary during in vitro rooting. Further, differences in rooting responses may also provide progress towards improving rooting efficiencies of recalcitrant genotypes (Kim et al. 1998).

In vitro raised micro-cuttings of *Vitex negundo* were rooted ex vitro by shoot pulse treatment of various auxins prior to transfer to soilrite. High rooting efficiency (97%) with 500 μM IBA was observed, which is in accordance with the results obtained in *Annona squamosa* (Nagori and Purohit 2004), *Hydrangea quercifolia* (Ledbetter and Preece 2004) and *Cyamopsis tetragonoloba* (Ahmad and Anis 2007b). However, auxin application did not affect the rooting response in *Porteranthus trifoliantus* (Bruno et al. 2007).

Ex vitro rooting method combines rooting and hardening step, thus reducing the time for clonal multiplication. Rooting in microshoots of *Vitex negundo* with this technique eliminates the additional in vitro rooting steps as tried earlier (Sahoo and Chand 1998, Chandramu et al. 2003b, Vadawale et al. 2006).

Because, the environmental culture condition and auxin treatments were quite different, direct comparison of root development between in vitro and ex vitro rooting studies are difficult. The ex vitro rooting method, however, resulted in favorable root development and shoot growth without producing callus which is in consonance with the results obtained in *Fraxinus*.
pennsylvanica (Kim et al. 1998) and Nyctanthes arbor-tristis (Siddque et al. 2006). Rhizogenesis of micro-cuttings excised from greenhouse stock plants and shoots excised from in vitro grown plant showed similar pattern of PGR influence in Ulmus parvifolia (Thakur and Karnosky 2007) but the number of rooted shoots were higher from the in vitro grown shoot as compared to ex vitro grown.

5.4 Synthetic seed production

Numerous techniques are now available for rapid and large scale commercial multiplication of elite and desirable plant species in vitro. In order to make system economically viable, many researchers encapsulated somatic embryos as well as axillary buds in sodium alginate to prepare synthetic seeds (Bapat and Rao 1988). Na₂-alginate encapsulation technique can be applied in conjunction with micropropagation for in vitro conservation. It can be used for germplasm storage or as a means to reduce the need for transferring and subculturing during off-season periods (West et al. 2006).

The encapsulation of in vitro derived axillary buds has been employed in recent years to develop synthetic seeds in many plant species like Eucalyptus grandis (Watt et al. 2000), Quercus spp. (Tsvetkov and Hausman 2005), Hibiscus moschentos (West et al. 2006), Morus spp. (Kavyashree 2006), Pimpinella pruatjan (Roostika et al. 2006), Chonemorpha grandiflora (Nishitha et al. 2006), Rauwolfia tetraphylla (Faisal et al. 2006a) and Tylophora indica (Faisal and Anis 2007). Successful application of encapsulation of somatic embryos has also been reported in various medicinal plants viz. Santalum album (Bapat and Rao 1988), Arnebia euchroma (Manjkhola et al. 2005) and Rotula aquatica (Chithra et al. 2005). The alginate coat protects the micropropagules and thus has a practical application in germplasm exchanges and conservations. The provision of alginate coat as an artificial endosperm to provide nutrient is essential for
development of encapsulated micropropagules (Danso and Ford-Lloyd 2003). Therefore, the encapsulation experiment in both the plant species i.e. Vitex and Ruta were tested. In Vitex negundo, Kn was given in the medium at various level singly as well as in combination with NAA, however combination of BA with NAA was found suitable for in vitro conversion of encapsulated beads into young shootlets in Ruta graveolens. Axillary buds emerged from capsules after 2 weeks of planting in case of Vitex. Besides supporting shoot growth, the treatments (Kn + NAA) also support the root growth. However, the high shoot growth was accompanied by low root growth. It seems that they did not need in vitro root induction, thus the root induction was enough to be conducted ex vitro prior to the acclimatization. Similar response in Pimpinella pruatjan (Roostika et al. 2006) and Tylophora indica (Faisal and Anis 2007) were also reported. The percent conversion of capsules in case of Vitex negundo was quite high (more than 90%), while, in Ruta graveolens, it was considerably reduced.

The percentage development of plantlets from encapsulated nodal segments decreased as the period of storage increased beyond 4 weeks. Decline in conversion response could be attributed to inhibition of tissue respiration by the alginate matrix, or a loss of moisture due to partial desiccation during storage as reported earlier (Danso and Ford Lloyd 2003, Faisal et al. 2006a, Faisal and Anis 2007). Encapsulated nodal segments of both the species were viable (50%) even after 8 weeks of cold-dark storage. However, only 8% conversion frequency was observed in non-encapsulated nodal segments. The observation with cold stored encapsulated segments of Vitex and Ruta are in accordance with the previous studies (Kinoshita and Saito 1990, Adriani et al. 2000, Tsvetkov and Hausman 2005, Faisal et al. 2006a, Faisal and Anis 2007).
5.5 Acclimatization

During the last three decades, plant micropropagation has developed from a laboratory curiosity to a real industry. However, it’s wide spread use is restricted for several reasons, one of these being that a high percentage of micropropagated plants are lost or damaged during acclimatization i.e. transfer from culture vessel to soil. Due to the special environment in vitro, it is difficult to produce plants which can be acclimatized to the outside environment (Kozai 1991). High humidity of the environment in vitro does not allow synthesis of cuticle and epicuticular wax on the epidermis of leaves of regenerated plants. Consequently, when such plants are transferred to a relatively less humid external environment, they undergo desiccation and death (Selvapandian et al. 1988). Malik et al. (2005) used medium with reduced mineral salts and sucrose concentration for hardening of in vitro raised shoots of *Garcinia indica* as it probably forced the regenerants to rely their own photosynthetic apparatus for nutrition (Kozai et al. 1988). A lengthy and cumbersome acclimatization procedure is required for such cultured plants during which they develop thickening, epicuticular wax and the mechanisms of closure of stomata become operative. In order to solve these problems, I have standardized the acclimatization technique which not only reduces the time of acclimatization but also allowed a high survival rate in both the plant species. To ensure high humidity, the transplanted in vitro raised plantlets of *Vitex negundo* and *Ruta graveolens* were covered with clear polyethylene bags individually and maintained in culture room (25 ± 2°C) conditions initially for 2 weeks (with gradual removal of polybags in order to acclimatize) and transfer to normal laboratory conditions for another 2 weeks, finally under full sun. In *Vitex negundo*, ex vitro rooting was also performed which eliminates the additional in vitro rooting step reported earlier in *Vitex negundo*. Potting
substrate (garden soil, soilrite and vermicompost) also plays an important role in acclimatization of plantlets and the highest survival rate for the micropropagated plants in both the species was achieved in vermicompost. Similar response of potting mixture was also recorded in *Holostemma* ada-Kodidien (Martin 2002), *Tylophora indica* (Faisal et al. 2007), *Sida cordifolia* (Sivanesan and Jeong 2007) and *Erigeron breviscapus* (Liu et al. 2008). Properly hardened plantlets were transferred to field where they grew well without any detectable morphological variations.

**5.6 Physiological study**

Acclimatization is the final step in a successful micropropagation system. During this stage plants have to adapt to the new environment of greenhouse or field. Malfunctioning of the water house-keeping (hyperhydricity) as well as poor photosynthetic apparatus are major constraints for better acclimatization (Preece and Sutter 1991). After transplanting ex vitro, all the abnormalities caused by extreme environmental condition in the cultivation vessel need to be repaired. The plantlets usually need some weeks of acclimation in shade with the gradual lowering of air humidity (Pospisilova et al. 1998). Considering the importance of this step of acclimation, various physiological parameters, like chlorophyll and carotenoids contents, net photosynthetic rate and carbonic anhydrase activity was studied during the acclimatization period in the present study.

The total chlorophyll (a+b) and carotenoids concentration increased significantly with the increase in number of days during acclimatization. A similar observation with chlorophyll content was also reported earlier (Pospisilova et al. 1999). The increase in chlorophyll concentration may be attributed to the induction of chlorophyll synthesis enzymes required for chlorophyll biosynthesis (Jeon et al. 2005).
Net photosynthetic rate ($P_n$) in both the species decreased in the first week after transplantation and increased thereafter. The decline in photosynthetic rate during the first week after the transfer from in vitro to ex vitro condition indicates that climatic conditions create stress in micropropagation plants. Similar results were observed earlier in *Spathiphyllum* (Huylenbroeck and Deberg 1996) and *Rosa hybrida* (Genoud et al. 1999).

Enzymatic activity has also been reported to be associated with the acclimatization of micropropagated plants. Carbonic anhydrase activity increased during the acclimatization indicating that enzyme plays a determinant role in transport and exportation of sugar within the plant (Aragon et al. 2005).

### 5.7 Genetic fidelity

The hardened tissue culture raised clonal progeny may posses deviant phenotypes due to some somaclonal variations (Cullis and Clearly 1986). Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span (Chaterjee and Prakash 1996). The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerants seriously limits the broader utility of micropropagation systems (Rani and Raina 2000). The application of higher concentration of growth regulators and the recurrent subculture for indefinite period hinders maintenance of genetic fidelity in the tissue culture clones (Sahijaram et al. 2003), somaclonal variation at phenotypic, cytological, biochemical and molecular levels were detected among micropropagated plants (Ray et al. 2006). Chemical variability in the medicinal plant propagation is a common problem which leads to inconsistent results in fundamental and applied research (Murch and Saxena 2006). A well characterized production system can provide small and large quantities of genetically and chemically consistent plants throughout the year and thus in
vitro germplasm maintenance and genetic stability assessment systems can be implemented for the preservation of important medicinal plants (Alan et al. 2007). Several techniques such as cytological, isozymes and molecular markers have been employed to detect variation if any or to confirm the genetic stability of micropropagated plants (Gupta and Varshney 1999).

Culture conditions adopted during the present investigation did not seem to affect the genetic integrity as it was confirmed by adopting DNA based ISSR technique. Four primers (UBC-801, UBC-880, UBC-899 and UBC-900) that produce distinct amplification profiles displayed same banding pattern in the 10 randomly selected plants and the DNA sample taken from mother plant. This indicates the clonal or true to type nature of the developed progenies of *Vitex negundo*. ISSR have been proven to be a suitable molecular technique to analyze the clonal integrity of descendents of a single individual as well as to detect the variation that is induced or occurred during in vitro culture (Archak et al. 2003, Venkatchalam et al. 2007). ISSR technique was chosen because it amplifies different regions of the genome allowing better analysis of genetic stability/variation of plantlets as well as the simplicity and cost effectiveness which is in accordance with the result obtained in *Prunus dulcis* (Martin et al. 2004).

In conclusion, we have established successfully a direct in vitro system for *Vitex negundo* and both direct and indirect for *Ruta graveolens*.

The developed direct culture protocol is simple, one step, reproducible and efficient for shoot bud differentiation on medium containing various PGRs. The system is rapid over the other developed methods and can be completed within 12 weeks from initiation of culture to transplantation of regenerants to soil. Since the plantlets were developed directly without intervening callus phase, somaclonal variation is avoided. Additionally, in the present study, the shoots induced, had thick stem and proper expanded leaves which could tolerate the
stress during acclimatization. This appears to be significant achievement over other published reports.

Direct acclimatization (ex vitro rooting) is easy and inexpensive and allows an efficient and fast establishment of plant in soil which is the most important step in micropropagation. Rooting in microshoots with this technique eliminates the additional in vitro rooting step reported earlier.

The regenerated plantlets were similar to their progenitor in relation to morphology and growth characteristics and can be applied for mass propagation, multiplication, improvement and conservation of clones of both *Vitex negundo* and *Ruta graveolens*.

The developed indirect regeneration system in *Ruta graveolens* from callus provides a step towards the development of transformation system of this important medicinal plant.

**Future Work:**

Optimization of various PGRs and recurrent production of plantlets from regenerated as well as mother plant will be included in Future work because there is a need to develop an improved and efficient protocol which should be cost effective and can be utilized for scaling up the same. The success of propagation and multiplication programmes lies in the identification of genetically divergent material and development of genetically superior stock. An understanding of the extent of genetic diversity is critical for the success of breeding programme. Traditional methods using morphological characteristics for establishment of genetic diversity and relationships among the accessions were largely unsuccessful due to strong environmental influence. Hence, selection based on genetic information using molecular markers is essential as it is more reliable and consistent. Therefore, genetic diversity assessment of different genotypes of *Vitex negundo* and *Ruta graveolens* will be included in future scheme. The genetic diversity will be assessed using various PCR based molecular marker techniques like RAPD and ISSR.