Micropropagation offers means of rapid and mass multiplication of the existing stock of germplasm for biomass production and conservation of rare and threatened species (Bonga and Durzan 1987, Gupta et al., 1993, Husain and Anis 2004, Anis et al., 2005)

Leguminous plants have greater application from economic and ecological point of view. It is necessary to apply tissue culture technique for their rapid multiplication. During recent years, a number of woody legumes have been successfully propagated *in vitro* using juvenile as well as mature plant parts (Trigiano et al., 1992).

### 5.1. Direct shoot regeneration

Explants of *B. tomentosa* failed to develop shoot buds in growth regulator free medium. Various cytokinins (BA, K, and 2-iP) were tested to facilitate bud initiation. In the present study, BA was proved to be most effective than other cytokinins. Maximum shoot bud induction from CN, aseptic nodal as well as field grown nodal explants was obtained on MS medium supplemented with BA at a concentration of 5.0 μM, 7.5 μM and 5.0 μM respectively. The superiority of BA over other cytokinins in tissue culture has been well documented in fabaceous plants viz. *Acacia mearnsii* (Beck et al., 1998), *Albizia chinensis* (Sinha et al., 2000), *Ceratonia siliqua* (Romano et al., 2002), *Cassia angustifolia* (Agrawal et al., 2003), *Sesbania drummondii*
(Cheepala et al., 2004), *Sesbania rostrata* (Jha et al., 2004), *Pterocarpus marsupium* (Chand and Singh 2004, Anis et al., 2005).

Increase in concentration of BA beyond optimal level had a negative effect and exhibited reduction in regeneration frequencies and shoots number from each explant. Reduction in shoot number at concentration higher than optimal level has also been reported in *Albizia chinensis* (Sinha et al., 2000), *Pterocarpus marsupium* (Anis et al., 2005).

The combination of auxins (IAA and NAA) with optimal concentrations of cytokinin was also studied for their ability to effect the shoot induction and multiplication rate and to optimize the medium composition for maximum plantlet regeneration. IAA and NAA tested were capable of inducing more than 75% explants to respond positively and the combination of 5.0μM BA with 0.5μM NAA was found to be the best combination in CN and field grown nodal explants. However in case of aseptic nodal 7.5μM BA along with 0.5μM NAA gave the maximum result. The synergistic effect of BA along with NAA has been demonstrated in Strasberry (Bhatt and Dhar, 2000), *Terminalia arjuna* (Pandey 2006). The results corroborate with the earlier findings of several workers, who reported the addition of low level of auxin with cytokinin promoted shoot induction and proliferation as in *Wrightia tinctoria* (Purohit and Kukda, 1994), *Acacia catechu* (Kaur et al., 1998), Strawberry (Bhatt and Dhar, 2000), *Psoralea corylifolia* (Anis and Faisal 2005), *Terminalia arjuna* (Pandey 2006).
However, in *Holstemma ada-Kodien* (Martin 2002), *Cassia angustifolia* (Siddique and Anis, 2007), the best combination among various auxins and cytokinins were observed on a medium containing BA + IAA and TDZ + IAA respectively.

In addition to other cytokinins, a considerable effect of TDZ was observed on *Bauhinia tomentosa*. TDZ could be substituted for adenine-type cytokinins in various cell culture systems including both callus cultures and micropropagation of many woody plants. Its mode of action may be attributed to its ability to induce cytokinin accumulation (Victor et al., 1999) or to enhance the accumulation and translocation of auxin (Murch and Saxena, 2001). In the present study, cotyledonal node (CN) explant cultured on MS medium supplemented with different concentrations of TDZ and maximum shoot regeneration frequency (62%) was achieved at 0.8μM TDZ. Similar results have also been reported for axillary shoot proliferation in several woody plant species (Huetteman and Preece 1993, Lu et al., 1993, Upreti and Dhar 1996, Khurana et al., 2005, Ahmad et al., 2006 a and b, Siddique and Anis 2006, Ahmad and Anis, 2007).

The regeneration frequencies and number of shoots declined with an increase or decrease in TDZ concentration beyond the optimal level. The continuity of TDZ to CN explants resulted in the formation of fasciated or distorted shoot buds. The serious effect of continued presence of TDZ has also been reported on the growth and multiplication of *Cassia angustifolia* (Siddique and Anis, 2007a), *Rhododendron* (Preece and Imel, 1991), *Dalbergia sissoo* (Pradhan et al., 1998a).
This problem was overcome by transferring the TDZ exposed cultures to TDZ lacking MS medium. Subculturing had a significant effect on shoot multiplication. The highest rate of shoot per cotyledonary node explant was observed up to fourth culture passage and beyond which a gradual decline was noticed. These results corroborate to the previous reports for *Pterocarpus santalinus* (Arockia Samy et al., 2000), *Syzygium cumini* (Jain and Babbar, 2000). In contrast, TDZ was found to be least effective and yielded low shoot in comparison to other cytokinin in *Bauhinia tomentosa*. Low effect of TDZ has also been reported on *Vitex negundo* (Sahoo and Chand, 1998).

The multiplication and establishment of microshoots were further improved by the supplementation of Ads to the aforesaid optimal combination. Adenine in the form of adenine sulphate can stimulate cell growth and greatly enhanced shoot formation (Murashige 1974). It provides a good source of nitrogen to the cell and can generally be taken up more rapidly than inorganic nitrogen (Thom et al., 1981). Ads reported to exhibit synergistic effect with other cytokinins and the strategy of using Ads as an adjuvant has been adopted effectively for many woody plant species including *Tectona grandis* (Devi et al., 1994), *Bauhinia vahlii* (Dhar and Upreti, 1999), *Jatropha curcus* (Rajore and Batra, 2005).

*In vitro* regeneration from field grown explant has been observed to be difficult due to several inherent problems such as severe microbial contamination, seasonal morphogenetic variation, having slow growth capacity and browning of medium (Purohit and Kukda, 1994, Quraishi and Mishra, 1998, Agrawal et al., 2002, Kumar et
Browning was one of the major obstacle in the establishment of culture of *Bauhinia tomentosa*. This problem was circumvented by using some adjuvants polyvinylpyrrolidone (PVP), casein hydrolysate (CH) and ascorbic acid (AA). Presoaking of nodal explants in antioxidant solution has been suggested to control browning in guava (Amin and Jaiswal 1987), *Prosopis cineraria* (Shekhwat et al., 1993).

The effect of other treatment like distilled water (Cress well et al., 1982) and PVP (Amin and Jaiswal, 1988) have proved to be effective but in present study, minimum reduction in browning was observed, using the above treatment (PVP, CH and AA).

Our work clearly shows that seasonal changes greatly influenced the *in vitro* response. Plant material collected during March to June gave the maximum response. The percentage establishment declined gradually during the subsequent month. March to June is considered to be the active growth phase to establish culture. The better response of actively growing shoot buds over dormant buds have been reported by (Siril and Dhar, 1997, Husain and Anis, 2004, Kumar et al.,2005).Thus, seasonal changes seem to be one of the major critical factor in the establishment of culture. Such findings have earlier been reported by Tisserat (1985), Prasad and Chaturvedi (1988) and Dhar and Upreti (1999).
5.2. Rooting in regenerated shoots

Rooting of *in vitro* regenerated shoots and transplantation of the plantlets to the field is the most important, crucial and essential step, but difficult task in tissue culture of Woody trees. In the present study, optimization of rooting response of tissue culture raised microshoots of *B. tomentosa* was achieved using different growth regulators at varying concentration. Adventitious rooting has been considered and manipulated as a single phase process in which auxin is reported to play major role (Bellamine et al., 1998). In case of *B. tomentosa*, auxins (IAA, IBA and NAA) exhibited differential response for rhizogenesis and IBA (1.0 μM) was found better as compared to IAA and NAA in stimulating adventitious root formation. IBA has been observed to induce strong rooting response and has been extensively used to promote rooting in a wide range of plant and woody species (Thakur et al., 1998, Siddique and Anis 2007a and b).

Rooting of the shoots can be promoted by low concentrations of auxins (Mott, 1981, Amerson and Mott, 1982). The higher concentration of auxins caused callus induction at the base of shoots with roots. The callus formation with roots reduces the survival rate of *in vitro* raised plantlets (Nemeth, 1986).

In another experiment, the microshoots were rooted by a two step culture procedure; involving a strategy of giving pulse treatment of an auxin (IBA) at different concentration (100μM, 200μM and 300μM) for 24 h, followed by transfer of such treated shoots to hormone free ½ MS medium. The two step culture procedure
has been used in many tree species such as *Pterocarpus marsupium* (Anis et al., 2005), *Prosopis cineraria* (Shekhwat et al., 1993), *Ceratonia siliqua* (Romano et al., 2002), *Rauvolfia tetraphylla* (Faisal and Anis 2002).

The percentage of rooting was markedly enhanced by augmenting the medium with a phenolic compound, chlorogenic acid at different concentration along with IBA. A maximum frequency 70% of root formation and the highest number (9.6 ± 0.50) of roots with maximum root length (2.3 ± 0.35) was achieved on MS medium with IBA (2.5μM) and chlorogenic acid (5.0μM). The promotive effect of phenolic compound has been identified in several woody plant species including *Pterocarpus marsupium* (Husain et al., 2007), *Malus pumila* (James, 1983, Zanol et al., 1998).

5.3. Acclimatization

Hardening and acclimatization are crucial steps in achieving success in any micropropagation programme. Micropropagated tree species when transferred from *in vitro* to *ex vitro* conditions of green house or field show low survival or reduced growth rate due to sudden changes in environment (Eliasson et al., 1994, Pospisilova et al., 1999). The special conditions during *in vitro* culture resulted in the formation of plantlets of abnormal morphology, anatomy and physiology. When *in vitro* raised plantlets are transferred to a relatively less humid external environment they undergo desiccation and death (Selvapandiyam et al., 1988). So they need a period of acclimatization to overcome these abnormalities. In the present study, the rooted plantlets of *B. tomentosa* were successfully hardened off inside the growth room in
selected planting substrate. The plantlets were covered with transparent polythene bags to maintain high humidity and also to prevent desiccation with the control temperature, photoperiod and irradiance. These were then transferred from soilrite to garden soil after 4 weeks.

5.4. Synthetic seed

Somatic embryos formed in vitro are coated (encapsulated) in a gel containing nutrients and other additives are designated as synthetic or artificial seeds (Demarly, 1986, Redenbaugh K et. al.,1984). 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 species (Tsvetkov and Hausman, 2005), Morus species (Kavyashree, 2006), Tylophora indica (Faisal and Anis, 2007).

In the present investigation, the potential of alginate encapsulated nodal explants of Bauhinia tomentosa for propagation as well as effect of cold storage on conversion frequency and number of shoots were evaluated. An optimal concentration exchange between Na\(^+\) and Ca\(^{2+}\) producing firm, clear, isodiametric, beads was achieved using a 3% of sodium alginate upon complexion with 100 mM calcium chloride.

Combination of BA with NAA was found suitable for in vitro conversion of encapsulated beads into young shootlets in Bauhinia tomentosa. Conversion into complete plantlets was achieved after 8 weeks of culture on the same medium. This is

The percentage development of plantlets from encapsulated nodal segments decreased as the period of storage increased beyond 4 weeks. The decline in the conversion response observed in a synthetic seed stored for a period of 5-6 weeks may be due to inhibited respiration of plant tissue by alginate or to a loss of moisture due to partial desiccation during storage.