DISCUSSION

5.1. Adult Explants

Sterilization of Explants

The most crucial step during the development of protocols of plant regeneration in woody species like the present investigation is the method of explant sterilization for reduced contamination. All the explants to be used in the investigation (shoot tips, nodal segments, internodes and leaves) were washed very intensively with running tap water for 25 minutes. Detergent laboline (0.1% v/v) and Tween-20 were used for washing the explants followed by pretreatment with bavistin (0.5% w/v) for 30 min. each. Use of bavistin as pretreatment has also been reported in *Litchi chinensis* (Dass *et al.*, 1999) and *Nyctanthes arbor-tristis* (Reeth and Shivamurthy, 2005).

After this treatment, nodal segments were surface sterilized by HgCl$_2$ (0.1% w/v) for 3 min. A similar sterilization protocol result has also been reported in *Crocus corms* (Homes *et al.*, 1987), *Plumbago rosea* L. (Harikrishnan and Hariharan, 1996), papaya (Saraswati *et al.*, 2005) and *Vitex negundo* L. (Sharma *et al.*, 2006). A similar exposure has been reported in *Elaeagnus* shoot sterilization by application of 0.1% HgCl$_2$ (Economou and Spanoudaki, 1988) and sterilization of seed of *Echinacea* sp. (Lata *et al.*, 2006). Although HgCl$_2$ has high environmental hazard but its effectiveness for surface sterilization of nodal segments was inevitable as the explants were collected from an old shrub. Similarly, shoot tip explants were disinfested with NaOCl (1.0% v/v) for 10 minutes. This efficient method of explant sterilization for reduced contamination in nodal segments and shoot tip explants has also been shared in *Viburnum tinus* (Nobre *et al.*, 2000), bamboos (Thakur and Sood, 2006) and Kentucky bluegrass (Hu *et al.*, 2006). Unlike the present investigation, 20-30 minutes in 0.5% NaOCl resulted in explant necrosis in *Berberis thunbergii atropurpurea* (Uno and Preece, 1987). Internodal segments, leaves and hypocotyl explants were disinfested by NaOCl (1% v/v) for 5 min. Bactericidal action of hypochlorite solution is due to both hypochlorous acid (HOCl) and OCl$^-$ ion

There was a significant effect of season on the contamination of explants. In winter the infection rate was low regardless of the length of time
of surface sterilization due to the less fungal interference like that found in *Berberis thunbergii atropurpurea* by Uno and Preece (1987).

**Control of Browning**

Establishment of *in vitro* cultures of several plant species, especially woody plants, is usually hindered by the fatal browning of the explants and culture medium. Browning is generally considered to result from the oxidation of phenolic compounds released from the cut end of the explants, by polyphenol oxidases, peroxidases or air. The oxidized products, quinones are known to be highly reactive and inhibit enzyme activity leading to death of explants (Bhat and Chandel, 1991). Since the plant material (*Berberis lycium*) is rich in phenols, it may result in browning of cultures and media.

To overcome the browning, basal end of branch was cut and frequently sub cultured (3-4 days interval) on the fresh medium for a cumulative period of two weeks. A similar method was successfully employed in eliminating phenolic compounds produced in *Berberis trifoliata* (Mackay *et al.*, 1996); *Pisonia alba* (Jagadishchandra *et al.*, 1999) and *Rotula aquatica* (Sebastian *et al.*, 2002).

Best result in overcoming and neutralization of phenols with respect to explant establishment were also obtained when explants were soaked in 0.1 mg/L citric acid for 5 to 10 min. In this way about 90% of the cultures were able to produce shoots. Similar observations were also reported in *Nyctanthes arbor-tristis* (Reth and Shivamurthy, 2005). Addition of activated charcoal did not show desired results as it is known to adsorb other compounds from the medium such as growth regulators, as reported in *Miscanthus xigiformis* ‘Honda Giganteus’ by Peterson *et al.* (1999), Ebert and Taylor (1990) and Buter *et al.* (1993). However, Wang and Haung (1976) reported beneficial effects of activated charcoal in plant tissue culture. Explants taken from the basal portion of the branch during the months of November - April resulted in minimum phenols, while as maximum release of phenols was observed during May-October. This seems to be obvious as more phenols are produced when the plants resume new growth (Reeth and Shivamurthy, 2005).
Use of Fungicides in the Culture Media

Release of excessive phenols in the medium together with die-back at the tip of the shoots led to the conclusion that the infection was present in the internal tissues of the original explants. Similar observations were reported in *Oenothera hookeri* leaf tip cultures (Bove and Garnior, 1998; Garcia *et al.*, 2003). Further, the endogenous microorganisms which exuded from the cut surface of the explants and then grew throughout the medium, limited the success of *in vitro* studies of *Berberis lycium*.

To remove the infection in the internal tissues of the original explant, fungicides such as nystatin, fluconazole, griseofulvin, bavistin and amphotericin-B were used in the medium. Amphotericin-B with 2.5μg/ml concentration was found to have effectively reduced the infection. Use of amphotericin-B has also been tested in *Eucalyptus grandis*, maize callus and barrenwort (Matar, 1996), *Nicotiana tabacum, N. plumbaginifolia*, callus cultures of *N. tabacum* and tobacco seedlings (Shields *et al.*, 1984). Bavistin also controlled the infection but started toxicity in explants in the third week of culture. Use of bavistin in the media has also been reported in *Litchi chinensis* (Das *et al.*, 1999) and *Bacopa monnieri* (Tiwari and Tiwari, 2006). Reduced infection due to bavistin has also been documented by Saraswathi *et al.* (2005) in papaya and other plant texa (Skirvin *et al.*, 1999; Drew and Smith, 1986).

Micropropagation

Multiple Shoot Induction

The outcome of the present study related to multiple shoot induction indicates that apical dominance proved to be very strong in *Berberis lycium* microshoot proliferation. When the results of all treatments in the cytokinin (BAP, Kn, TDZ) experiments were considered, significantly more axillary shoots were formed on tipless shoot parts than on shoot tips. Basal media lacking these phytohormones failed to produce shoots.

Similarly, media with cytokinins only produced shoots with short internodes which turned into rosette leaves at the tip while with addition of GA₃ this tip produced shoots showing that GA₃ fortified with cytokinins had synergistic effect. Use of GA₃ with BAP has also been observed in *Berberis*
thunbergii (Karhu and Hakala, 1991), Ocimum basilicum (Sahoo et al., 1997) and Rosa hybrida (Barna and Wakhlu, 1995). However, these results along with that of B. thunbergii and O. basilicum are contrary to that of Arora and Bhojwani (1989) in Saussurea lappa where BAP+GA₃ combination enhanced multiple shoot formation. Addition of GA₃ helps in the tracheidal and xylem fiber formation as observed in Citrus limon callus (Khan, 1996). Concentration of GA₃ had not significantly effected on shoot number, though 1mg/L GA₃ elongated the shoots which were delayed in lower concentrations while as higher concentration of GA₃ hampered shoot formation or produced abnormally elongated shoots. GA₃ below 0.5mg/L failed to produce shoots in conjunction with cytokinins.

Of all the concentrations of cytokinins (Kn, TDZ, BAP) tested, BAP at1 mg/L concentration induced the formation of higher number of shoots per explant. The promotive effect of BAP for inducing multiple shoots has been previously reported in several Berberis species (Karhu and Hakala, 1991; Uno and Preece, 1987; Arena et al., 2000). Superiority of BAP over other cytokinins for shoot induction may be attributed to the abilities of plant tissue to metabolize the natural hormone readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue and thus work through natural hormone system (Zaerr and Mapes, 1982). An increase in BAP level (>2 mg/L) was inhibitory to the formation of shoots or axillary bud growth. These results are in corroboration with that obtained in Berberis thunbergii (Karhu and Hakala, 1991), B. thunbergii atropurpurea (Uno and Preece, 1987) and B. buxifolia (Arena et al., 2000). Shoots produced on the TDZ treatments had a poor appearance (thin and long) with some discoloration of the basal portion of the shoots as has also been reported for Berberis trifoliata (Mackay et al., 1996). The shoot number was also increased by inoculating 2 explants in one culture vessel which developed at least 2 shoots in one tube. This way the purpose of tissue culture could also be fulfilled.

There was no significant influence of auxins (IAA, IBA and NAA) on the shoot induction. However, the shoot formation response of Berberis lycium was better on a medium that contained a combination of BAP and NAA. Formation of shoots on the medium fortified with auxin (NAA) along with
cytokinin has also been demonstrated in B. thunbergii (Karhu and Hakala, 1991). Higher concentration of auxins in the medium did not improve the response of shoot formation but instead encouraged formation of callus at the base of the explants as seen in earlier reports of Karhu and Hakala (1991) in Berberis thunbergii, Islam et al. (1993) in Morus laevigata and Monier and Ochatt (1995) in Cotonea genotypes. This inhibition of shoot formation may be attributed to the action of accumulated auxins at the base end of the explants (Marks and Simpson, 1994).

Among the three basal media tested (MS, B5 and WPM) for the shoot induction, MS basal medium was most effective for the present study as also previously observed in Berberis thunbergii (Karha and Hakala, 1991), Morus australis (Pattnaik et al., 1996) and Stericulium urens (Purohit and Dave, 1996). Oka and Ohyama (1986) have reported that both dilution and strengthening of the original MS salt concentration decreased the growth of buds in Morus alba. They further observed that addition of ammonium nitrate and potassium nitrate in basal medium was better for growth of buds than the addition of potassium nitrate alone.

Nodal explants gave better shoot regeneration response than shoot tip explants in our study. The differential response of nodal and shoot tip explants has been attributed to the difference between the physiological states of the buds on different regions of a stem as also observed by Vieitez et al. (1985) as well as strong apical dominance exerted by the shoot tips even in the presence of BAP (Lakshmanan et al., 1997). Superiority of nodal explants in terms of the number of shoots formed per explant has also been reported in several Berberis species including B. thunbergii (Karhu and Hakala, 1991). The differential response of explants attributed to various levels of endogenous plant growth regulators of explants from different parts has also been stated by Komlavalli and Rao (2000) in Gymnema sylvestre.

A significant effect of explanting season on shoot regeneration was observed in this study. Nodal explants collected during April to December showed better shoot forming response than the explants taken during December to March as denoted previously in Sapium sebiferum (Siril and Dhar, 1997), Morus australis (Pattnaik et al., 1996), Psidium guajava (Amin and Jaiswal, 1988) and Liquidambur styraciflua (Sutter and Barker, 1985).
Better shoot forming ability of nodal explants collected during April to September may be attributed to the most active growth phase of the plant material. In *B. trifoliata* summer is the explanting season (Mackay *et al.*, 1996). The axillary shoot number did not increase with increase in subculture period from 4 to 7 weeks.

**Multiple Shoot Proliferation**

Nodal explants excised from *in vitro* raised shoots produced on MS medium containing 1mg/L BAP in conjunction with 1mg/L GA₃, were used for shoot proliferation.

Best response in terms of number of shoots per explant (4.8±1.54) and the length of shoots (2.7±0.63 cm) was obtained on a medium containing 1mg/L BAP + 2mg/L GA₃. Similar response in growth and shoot proliferation was reported in *Berberis thunbergii* (Karhu and Hakala; 1991). On the contrary, high BAP level (2mg/L) and low level of GA₃ (0.5 mg/L) suppressed the proliferation of shoots. This suppression might be due to accumulation of high cytokinin levels in them which inhibits further shoot development. Multiplication rate was not so high due to very strong apical dominance in micro shoots in *Berberis lycium* like that reported in *B. thunbergii* (Karhu and Hakala, 1991) and the decapitation of shoots was, therefore, necessary to achieve shoot multiplication. Overall micropropagation rates of *Berberis lycium* were lower than those reported for *B. trifoliata* (Mackay *et al.*, 1996) and *Ulmus pumila* (Corchete *et al.*, 1993).

In the present investigation, sucrose was most favourable carbon source for shoot formation. It is in accordance with *Berberis thunbergii* (Karhu and Hakala, 1991), *B. trifoliata* (Mackay *et al.*, 1996) and *B. thunbergii atropurpurea* (Uno and Preece 1987). Sucrose has also been reported to be favourable for micropropagation of shoots in *Morus laevigata* (Islam *et al.*, 1993) while as Oka and Ohyama (1982) have shown that fructose is superior over sucrose for multiplication of shoots in *Morus alba*.

The effect of agar level was significant for shoot proliferation in *Berberis lycium* species as investigated in the present study. Best shooting response was obtained in agar level varying between 0.6-0.8/%. Media that lacked agar or contained low agar level (0.4%) resulted in the production of
vitrified shoots. A suppression of shooting response was observed with high agar levels (1-1.2%) as has also been reported in *Morus alba* (Oka and Ohyama, 1978). 0.8% agar levels proved to be more suitable. This result is similar to that found in *B. trifoliata* and *Berberis thunbergii* (Mackay *et al.*, 1996; Karhu and Hakala, 1991).

Multiple shoots produced *in vitro* were proliferated further as nodal explants by repeated sub culturing at 4 week intervals. The number of shoots per explant increased upon five passages of subculture and thereafter the rate of shoot multiplication declined. A similar effect of sub culturing on multiplication of shoots has been reported in *Berberis trifoliata* (Mackay *et al.*, 1996) and *Morus australis* (Pattnaik *et al.*, 1996).

### Rooting and Plant Establishment in Field

The most Herculean task of plant regeneration in the present study like other woody species was the induction of roots on the newly formed microshoots. Rooting of nodal explants excised from *in vitro* raised shoots was low in the presence of auxins. Similar results were observed in *Berberis thunbergii atropurpurea* (Uno and Preece, 1987), *B. buxifolia* (Arena *et al.*, 2003), *Podophyllum hexandrum* (Arunugam and Bhojwani, 1989), *Prunus mume* (Harada and Murai, 1996), *Tinospora cordifolia* (Kumar *et al.*, 2003) and peach (Hammerschlag *et al.*, 1987). Low levels of rooting of microshoots by auxin treatment can be attributed to presence of some anatomical barriers or inhibitors as has been found in the past with rooting in stem cuttings by auxin application (Spiegel, 1955; Fadl and Hartmann, 1967; Joshi *et al.*, 1992) and absence of some rooting cofactors (Hess, 1959, 1965, 1968; Challenger *et al.*, 1965) impeding root differentiation. This recalcitrance to form roots may also be due to the treatments used to produce shoots *in vitro* i.e. addition of GA₃. This fact is supported by the observations of Mohan *et al.* (1995) on *Moringa pterygosperma*.

In plants which produce berberine such as *Berberis lycium*, growth regulators such as auxins promote the synthesis of the secondary metabolites (Jayakumaran *et al.*, 1992). Production and release of berberine into the culture medium can affect the morphogenetic response (Uno and Preece 1987; Jayakumaran *et al.*, 1992; Arena and Martinez, 2001; Arena *et al.*, 1982).
especially in rhizogenesis (Arena et al., 2000). The excreted berberine accumulated in the medium, turns yellow in colour which could be associated with the increase of peroxidase activity and the decrease of polyamine concentrations during the root expression stage. Though little is known about the primary mechanism of action of polyamines but their positively charged amino groups cause them to combine with negatively charged phosphate groups in DNA and RNA in the nucleus and ribosomes. Polyamines tend to mimic some of the effects of the auxins and it is presumed that polyamine synthesis precedes adventitious root formation (Tiburcio et al., 1989). Therefore, instead of stimulating the rooting, the decrease in endogenous level of polyamine concentration in the present investigation, which is not enhanced even by addition of auxins, formation of root primordia becomes difficult.

However, IBA could induce 40% rooting in the explants. IAA and IBA have also been reported to be potent auxins for inducing root formation in *Berberis thunbergii* (Karhu and Hakala, 1991) and IBA and NAA in *Berberis trifoliata* (Mackay, 1996) and mulberry species (Jain et al., 1996; Patnaik et al., 1996; Singh and Wakhlu, 1999). Reduced rooting rates in *B. lycium* can also be attributed to endophytic infection as noticed in strawberry runner explants by Tanprasert and Reed (1998).

The root formation response was improved with supplementation of BAP in the medium. Increased rooting ability following cytokinin application together with auxin has also been achieved in *Berberis thunbergii* (Karhu and Hakala 1990, 1991), *Prosopis* sp. (Yao et al., 1989) and *Petroselinum crispum* (Vandemoortele et al., 1996).

Sucrose was most effective carbon source for root formation among the different carbon sources. Superiority of sucrose over glucose for inducing root formation on axillary shoots has been demonstrated in *Cordyline fruticosa* and *Beaucarnea recurvita* (Samyn, 1995, 1997).

Rooting response of the presently worked species of *Berberis* was increased by addition of phloroglucinol (10-15 mg/L) in the medium as also reported in *Prunus cerasifera* (Hammerschlg, 1982). Keeping explants in dark for two weeks drastically declined root formation as also been previously reported in *Liquidambar styraciflua* (Sutter and Barker, 1985), *Chaenomeles*
*japonica* (Margaret and Boe, 1982) and *Prunus persica* (Damiano *et al.*, 1991). Increase in rooting percentage by dark treatment has been demonstrated in *B. thunbergii* (Karhu and Hakala, 1991).

The effect of potting mixture on establishment of micropropagated plants was observed to be crucial. Vermiculite and sand (1:1) irrigated with $\frac{1}{2}$ MS salt solution resulted in the production of a maximum percentage of successful hardened plants. Variation in survival rate during *ex vitro* establishment of plants has been reported in *Gareinia mangostana* (Normah *et al.*, 1995). De Fossard (1987) reports that crucial factor in the acclimatization of *in vitro* raised plants is the initial step of inducing the formation of fully functional roots in a potting mixture while ensuring that the delicate shoot system is protected against desiccation. In *Berberis buxifolia* stress has been laid to know more about the improvement in the quality of *in vitro* root formation and survival rate during acclimatization (Druart *et al.*, 1982; Gaspar *et al.*, 1991; Kevers *et al.*, 1997; Arena *et al.*, 2000).

The micropropagated plants were similar to mature adult plants in morphological characters. Variation in some parameters such as leaf weight and stomatal frequency was noticed in micropropagated plants in the first year of transplantation. These variations did not persist in the plants after 2nd year of transplantation and the plants appeared morphologically similar to the adult mother plant. Zaman *et al.* (1992) reports that in spite of slight morphological variations, the nutritional value of micropropagated plants compares strictly with those of cutting raised plants in various *Morus* genotypes. The number of plants produced/ explant after 12 weeks of culture was found to be 94.

**Callogenesis**

Callus was formed from leaf, internodal segments and hypocotyl explants on auxin rich medium. Among the different auxins, 2, 4-D was effective for callus initiation. The favourable effect of 2, 4- D over other auxins and its enhancement by incorporating Kn for callus initiation has been previously reported in several *Berberis* species (Breuling *et al.*, 1985; Deliu *et al.*, 1994). It is possible that auxin level increases in the explants during culture on an auxin rich medium and pre treatment of these explants with Kn compensâtes differences in endogenous cytokinin/auxin ratio.
Optimal callus initiation was achieved on MS basal medium although explant types varied significantly in their capacities to initiate callus induction. Hypocotyl explants showed maximum capacity to form callus while as leaf explants did not produce much callus. Variation in callus forming ability of different explant types has been reported in *Beta vulgaris* (Krens and Jamar, 1989) and *Morus alba* (Kathiravan *et al.*, 1995). Similarly, the growth of the callus was good on MS basal medium as previously observed in *Berberis parvifolia* (Deliu *et al.*, 1994) and *B. wilsoniae* (Breuling *et al.*, 1985). A profound effect of explant type on callus growth was also observed in the present work with hypocotyl explants showing maximum growth rate.

The callus morphology was influenced by the composition of the medium. Friable callus was formed in the presence of 2, 4-D alone while gelatinous callus was obtained on 2, 4-D and Kn supplemented medium. Compact callus was produced in the presence of a combination of NAA and BAP. These results agree with the findings of Deliu *et al.* (1994) who also reported that callus morphology in *Berberis parvifolia* was dependant on the medium composition. Hunault *et al.* (1989) have demonstrated that the presence of a cytokinin in the medium tends to increase compactness of callus tissue in *Foeniculum vulgare* which corroborates with our observation.

**Organogenesis**

Organogenesis from calli of *Berberis lycium* failed and no shoot formation was observed. This was thought to be genotypically controlled as has been earlier reported in *Actinida chinensis* (Gui, 1979), *Liquidambar styraciflua* (Brand and Lineberger, 1992) and various mulberry varieties (Singh and Wakhlu, 1999). Even the use of GA3 did not modify the organogenetic response of which according Khan (1996) is the best xylogenic hormone to mimic auxin + Kn together. Yellow colour of the medium suggested that the callus tissue produced some alkaloids that might have shown self inhibiting effects leading to the blackening and death of the calli.

Analysis of the yellow colour pigment in the cultures of *Berberis lycium* revealed the presence of berberine. It was one of the main findings in the present investigation. Similar results have been observed in *Berberis parvifolia* (Deliu *et al.* 1994) and *Thalictrum minus* (Ikuta and Tokawa, 1988).
Numerous yellow crystals of the alkaloid released into the medium also kept increasing after first week of subculture which we feel hampered uptake of nutrients leading death of the calli.

Blackening of callus may be due to conversion of berberine into jatrorrhizine and subsequently into quinones. The explanation of berberine being precursor of jatrorrhizine through an unknown mechanism has been given by Beecher and Kelleher (1983) and Ruffer et al. (1985). Further, the production and release of berberine into the culture medium can affect the morphogenetic response as reported in B. buxifolia (Arena et al., 2003). As excreted berberine is accumulated, the medium turns yellow in colour, which could be associated with the increase of peroxidase activity and the decrease of polyamine concentrations in the callus. Decrease of polyamines also leads to the inhibition of callus growth and regeneration from callus via organogenesis.

Results of the present investigation show that alkaloid accumulation depends on the combination, type and level of auxins and cytokinins in the nutrient medium. Optimal condition for growth and synthesis of secondary metabolites were found on MS medium with 3% sucrose and NAA and Kn. This result does not agree with the findings of Deliu et al. (1994) on Berberis parvifolia in which secondary metabolite got enhanced by replacing Kn with BAP.

5.2. Seedling Explants

Like adult plant material, the method of seed sterilization for contamination free cultures was done after depulping the berries by rubbing on cloth. Extracted seeds, somewhat triangular, tough but permeable to water were washed with distilled water using liquid detergent laboline followed by pretreatment with bavistin. Seeds were finally surface disinfected for 2 minutes in HgCl₂ and rinsed 5-6 times in sterile distilled water.

Sterilized seeds were inoculated on basal medium with 2% agar and then transferred to 0.8% agar supplemented medium. In contrast, 4% agar was used for germination of seeds of Berberis parvifolia (Deliu, et al. 1994). The seedlings produced a long tap root, hypocotyl and two green cotyledons with free terminal lobes. However, the plumule failed to elongate and
produced no shoots even after 102 days. This may be due to the fact that in nature, the seedling takes 6-7 years to reach the mature stage and hence the shoot or the meristem between the cotyledons would be elongating gradually or by biotic association which was not possible in the culture vial in a short span of time.

Using seedling explants, an improved regeneration protocol was developed for *Berberis lycium*. To our knowledge there are no reports on *in vitro* shoot production of this plant using various nutrient media on cotyledonary node explants. In this study, we have compared multiple shoot production using various nutrient media and growth regulators and have reported the best treatment for the mass production of this plant.

**Micropropagation**

**Multiple Shoot Induction**

Cotyledonary nodes (CN) (1cm long) of 30 days-old seedling resulted in more axillary shoots than adult plant material on all treatments of cytokinin experiments. The mean proliferation count was 3.4 on the cotyledonary node explants. Medium lacking cytokinins did not regenerate shoots as has been observed in cotyledonary explants of *Swainsona salsula* (Yang *et al.*, 2001; Jusaitis, 1997). Like adult material, there is synergistic effect of GA₃ with cytokinins. Incorporation of GA₃ facilitated elongation and proliferation of shoots. A similar phenomenon was obtained in *Bauhinia vahlii* (Bhat and Dhar, 2000) and *Trapa japonica* (Hoque and Arima, 2003). The juvenility of the seedling explants probably contributed to the high proliferation rate than adult material and agrees with rapid multiplication of jojoba seedlings by *in vitro* culture (Roussos, *et al.*, 1999) and *Podophyllum hexandrum* (Arumugam and Bhojwani; 1989).

Higher concentration of GA₃ hampered shoot formation as in adult plant material. Superiority of BAP over other hormones for sprouting and inducing proliferation of axillary buds of CN has also been reported in *Gymnema sylvestra* (Komalavalli and Rao, 2000).

Cytokinins TDZ, Kn, BAP produced reduced shoot number and shoot length when supplemented with NAA, IBA or IAA. Inclusion of NAA in combination with cytokinin produced callusing. A similar result has been
observed in *Gymnema sylvestre* seedling explant (Komalavalli and Rao, 2000). When the original axillary node of seedling explant (C.N) was cultured (after removal of the multiple shoots to leave a stump), the number of shoots increased for the next 2-3 subcultures and reduced thereafter. Hence, from a single node explant it was possible to obtain a significant number of new shoots within a period of 3 months.

**Multiple Shoot Proliferation**

Nodal explants excised from shoots grown on a medium containing BAP level (0.8 mg/L) required transfer to a medium containing 0.6 mg/L BAP for further growth and shoot proliferation. Higher BAP levels during induction and proliferation stages appeared to have caused accumulation of higher cytokinin levels in them which inhibited further shoot development.

As observed in *Bauhinia vahlii* cotyledonary node explants (Bhatt and Dhar, 2000), the favourable carbon source for shoot formation from cotyledonary node explants of *Berberis lycium* with better shoot length, fresh and dry weight of shoots was sucrose (30mg/L). Best shoot response was obtained in agar level varying between 0.6-0.8 percent. Media lacking agar or containing low agar level (0.4%) resulted in the production of vitrified shoots. Higher level of agar (1-1.2%) suppressed shoot formation. Proliferation of shoots was increased by repeated subculturing at 4 weeks intervals. The number increased upon five passages of subculture and thereafter shoot multiplication rate declined. In short, the agar level and subculture used for proliferation pattern closely corresponds to that obtained in adult material.

**Rooting and Plant Establishment**

A good system of plant regeneration *in vitro* has been achieved from cotyledonary node explant. Excised axillary node derived *in vitro* shoot (1.9 cm long) were rooted only upon transfer to full strength MS medium containing auxin, whereas no rooting was noted in hormone free MS medium. MS medium supplemented with auxins at different concentrations showed varied effect on rooting. In most of the cases two roots emerged from the node nearest to the transected end of the stem within 30 days. Of the three auxins tested, IBA (1mg/L) was most effective for root induction and survival
in the field with minimum callus formation. Extensive callusing at the base without root formation and thin delicate root with intervention of callus were noticed when the medium was supplemented with NAA. A similar result is found in *Gymnema sylvestra* (Komalavalli and Rao, 2000) and in *Swainsona salsula* (Yang *et al*., 2001). The root formation response was improved with supplementation of BAP in the medium unlike *Cypripedium flavum* (Yan *et al*., 2006) where media without BAP produced more roots than that supplemented with BAP.

Among different carbon sources for rooting, sucrose was most effective. Rooting response of the *invitro* raised shoots was enhanced by addition of phloroglucinol (10-15 mg/L) in the medium containing IBA. Rooted plantlets were transferred to the phytatrails filled with sterilized sand and vermiculite (1:1) up to 5 cm topped with ½ MS salt solution resulted in the production of a maximum percentage of successfully hardened plants. Normal growth of potted plants was visible 10-15 days after transfer to field conditions. The transplantation success was 58%. The regenerated plants did not show detectable variation in morphological growth characteristics when compared with the field grown plants. Variation in some parameters such as leaf weight and stomatal frequency was noticed in micropropagated plants in the first year of transplantation. These variations did not persist in the plants after 2nd year of transplantation and the plants appeared morphologically similar to the adult plants. These results are substantiated by cotyledonary node explant derived plantlets in *Swainsona salsula* (Yang *et al*., 2001). The number of plants produced / explant after 12 weeks of culture was found to be 256.

**Callogenesis**

Hypocotyl, roots and cotyledon explants of germinated seedling were used for callus initiation on auxin rich medium. Among the different auxins, 2,4-D was effective for callus initiation. It was enhanced by incorporating kinetin in the medium. Among the different explant types, hypocotyl showed maximum capacity of callus formation. Callus of all types of explants failed to differentiate shoots on any of the media tested.
Organogenesis

As observed in calli derived from hypocotyl, intermodal segments and leaves from adult plant material, organogenesis did not occur in calli raised from hypocotyl, roots and cotyledon explants of germinated seedling under all conditions tested. This corresponds to the findings of Jordan et al. (2001) in Sophora toromiro seedling explants. This could be caused by blackening and ultimately the death of cultures due to self inhibiting effects of berberine in the callus cultures. This is in sharp contrast to the Nandina tissue cultures where successful morphogenesis was witnessed in the presence of berberine in the medium (Gould and Murashige, 1985).

Interestingly, the synthesis and release of the active principle berberine in cultured callus provide an opportunity for bypassing the extraction from plant and hence the conservation of this species which is on the verge of extinction. The growth regulators (type and level in the medium) have a link with the metabolism of protoberberine and remarkably stimulate berberine production. To improve the quantity and loss incurred during leaching in medium the development of an immobilized continuous culture system will be advantageous. Further, to improve the quantity there is a need of manipulating the hormones and the basic salt solution along with the study of physiological process behind the release of berberine.