DISCUSSION

MICROP Propagation

Shoot induction and multiplication

The present study has demonstrated the successful method or protocol for in vitro proliferation of multiple shoots and complete development of plantlets. For shoot induction and multiplication, the growth regulators have been used both individually and in combinations. By using this method multiple shoots have been induced from two explants viz. axillary bud and apical bud. The results indicate that apical bud is more competent than axillary bud in the process of induction and multiplication of shoots.

The nature of the explant, plant growth regulators, complex extracts (casein hydrolysate, coconut milk, malt extract and yeast extract) and antioxidants markedly influenced in vitro propagation of Gymnema sylvestre (Komalavalli & Rao, 2000). The most commonly used cytokinins are benzyladenine and kinetin. Cell division is regulated by the joint action of auxin and cytokinin, each of which influence different phases of cell cycle. The variations in the regeneration potential among explants are attributable to the differences in their physiological and genetic make up of cells. Auxins affect DNA replication, whereas cytokinin seems to exert some control over the event of mitosis and cytokinesis. Thus auxin and cytokinin levels in cultures need to be carefully balanced and controlled (Vesely et al., 1994). The effective concentrations of growth regulators and supplements requirement for
shoot bud induction were akin to both the explants as also observed by Komalavalli & Rao (1997).

Cytokinins

Bud break and development of shoots from both the explants was a function of cytokinin activity. The induction of multiple shoots in explants varied with cytokinin type and concentrations and was also influenced by explant type. The potential for shoot multiplication in *Cardiospermum halicacabum* appears to be strong in the presence of BA alone in the culture medium. The stimulatory effect of singular supplement of BA on bud burst and multiple shoot formation is similar to that reported in other medicinal plant species including *Ocimum* sp. (Patnaik & Chand 1996, Sahoo et al., 1997). In *Vitex negundo* (Sahoo and Chand, 1998) of the three cytokinins (BA, KN and Thiadiazuran) evaluated, BA at an optimal concentration of 2.0 mg/l was the most effective in inducing bud break and multiple shoot formation on both axillary bud and shoot buds. There was a linear correlation between the increments in BA concentration up to the optimum level (2.0 mg/l) and percentage of shoot developments and number of shoots per explant. No further increase in the frequency and the number of shoots could be observed with the increase in the concentration of cytokinin, indicating that a threshold was possibly achieved. Benzyl adenine above 2.0 mg/l suppressed sprouting of the dormant axillary buds in the nodal explants. Similarly the shoot forming capacity of seeds was influenced by the BA concentration in the medium and multiplication rate of 15-16 fold was achieved on 3.0 mg/l BA (Arya et al., 1999). The differential effects of various concentrations of BA on the stimulation of shoot bud formation from cotyledonary nodes has already been reported for *Glycine max* (Cheng et al., 1980). Arya et al. (1999) concluded that the shoot forming capacity of seeds in vitro was greatly influenced by the BA concentration in the medium. Multiple shoot induction of *Cajanus cajan* cv. Vamban 1 from apical and axillary meristem was obtained on 13.31 µM BA by Franklin et al. (1998). On the whole BA was the most effective growth regulator in all these reports, indicating cytokinin specificity for multiple shoot induction in these tissues. The relative benefit of BA compared to other cytokinins in tissue culture is well documented (Hans & Stephens, 1987). In both
Ocimum species, the percentage of bud break declined with increase in concentration of BA above the optimal level (Patnaik & Chand, 1996).

The efficiency of other cytokinins (2ip, KN) on shoot production was compared with that of BA. The explant response to different cytokinins showed that all other cytokinins are inferior to BA. The number of shoots produced per explant in medium supplemented with BA is significantly higher than with other cytokinins. In general, KN was less effective than BA and 2ip in inducing shoots (Sunnichan et al., 1998). Our observations on the suppression of sprouting at lower concentrations of KN and no response at higher concentrations were in consonance with those of Ahuja et al. (1982) in Ocimum gratissimum and O. viridae and more recently of Patnaik & Chand (1996) in O. americanum and O. sanctum, Sahoo and Chand (1998) in Vitex negundo and Malathy and Pai (1998) in Ixora singaporensis. MS medium containing BA was more effective than KN for inducing proliferation of axillary buds as in previous reports (Krishnan et al., 1995; Kannan & Jasrai, 1996; Sarker et al., 1997; Reddy et al., 1998; Handique & Bora, 1999; Komalavalli & Rao, 1997, 2000). Other than BA, 2ip provided good results over KN on shoot multiplication experiments. The superiority of BA over other cytokinins (KN, 2ip) has been demonstrated in Sapium sebiferum (Siril & Dhar, 1997). In general KN was less effective than BA in inducing shoots (Sunnichan et al., 1998; Sunnichan & Shivanna, 1998). According to Reddy et al. (1998) also KN at different concentrations did not improve the number of proliferating shoots.

There was no improvement in the shoot bud induction frequency, the number of shoots and their quality on BA+ KN containing medium. From these observations and results, it seems reasonable to conclude that independently of the concentrations of BA used, KN suppress the shoot development. The other interpretation is that KN probably counteracted the promotive effect of BA. The synergistic effect of the two cytokinins in the present study is similar to the results obtained in other medicinal plants. Thus the low response of BA+KN on micropropagation was also reported in other medicinal plants, viz Eremostachys superba (Sunnichan and Shivanna, 1998) and Gymnema sylvestre (Reddy et al., 1998). Sunnichan et al. (1998) also reported that there was no enhancement of shoot number on medium containing BA plus KN.
in *Sterculia urens*. Roy *et al.* (1998) also reported the multiple shoot from shoot tip and nodal explants of *Elaeocarpus robustus* on BA and KN media. There was no improvement in the number of shoot and their quality on BA+KN containing medium (Sunnichan & Shivanna, 1998). *Sapindus mukurossi* (Sapindaceae) also yielded high number of shoots in medium containing BA or KN with GA3.

**Synergistic effect of cytokinins and auxins**

A synergistic effect of a range of auxins in combination with BA on promotion of shoot multiplication is well documented for many medicinal plant species. So BA was here tested in combination with auxins, (NAA, IAA and IBA) to enhance the rate of shoot multiplication. BA with IAA proved to be the most effective treatment for promoting shoot multiplication. Sunnichan *et al.* (1998) also reported that multiple shoots could be induced in cultures of shoot tips and nodal segments on MS medium containing BA + IAA. He also mentioned that callusing was induced when IAA concentration was increased beyond optimum level. The potential for shoot multiplication in *C.halicacabum* appeared to be very strong in the presence of cytokinin and IAA than other combinations. Both for apical and axillary meristems, IAA was found to be more effective for bud proliferation than were NAA and IBA. Both IBA and IAA gave more or less equal response both in terms of frequency and number of shoots, except shoot tip on NAA provided slightly less number of shoots. In contrast Reddy *et al.* (1998) obtained maximum number of shoots on BA and NAA media.

Almost all treatments of auxin, except few, produced callus at the explant base with increase in auxin concentration. Among the three auxins (NAA, IAA and IBA), IBA induced the highest frequency of callus initiation on both explants. Bud break and multiple shoots were induced in apical and axillary meristems of *Madhuca longifolia* on BA singly or in combination with NAA, IAA and IBA (Rout & Das, 1993). Various successful combinations have been reported such as BA + IAA for *Adhatoda beddomei* (Sudha and Seen, 1994), *Alpinia galanga* (Anand & Hariharan, 1997) and *Eremostachys superba* (Sunnichan & Shivanna, 1998), BA+NAA for *Ixora singaporensis* (Malathi and Pai, 1998), *Sapiwn sebiferum* (Siril & Dhar, 1997),
Elaeocarpus robustus (Roy et al., 1998) and Ocimum americanum and O. sanctum (Patnaik & Chand, 1996). In Gymnema sylvestre Reddy et al. (1998) proved that nodal explants on BA with NAA combination responded well, BA+IBA for Rheum emodi (Lal & Ahuja, 1989), Gardenia jasmonides (George et al., 1993), Madhuca longifolia (Rout & Das, 1993) and Excoecaria agallocha L., (Rao et al., 1998). Compared to BA alone the addition of auxin significantly improved the responses. Thus BA in combination with NAA and IAA was more effective for inducing axillary bud break, multiple shoot formation and complete plantlet formation (Patnaik & Chand, 1996). The callus formation varied with auxin type and concentration in terms of frequency and the amount of callus produced. Experiments indicated that lower concentrations could modify positively the shoot induction response in axillary bud when combined with cytokinins (BA) as observed in the propagation of Hemidesmus indicus (Patnaik & Debata, 1996), whereas the presence of NAA suppressed shoot formation and callus production in shoot tip explants. Higher concentration of NAA, IAA, and IBA induced callus, occasionally root formation occurred at excised ends and prevented multiple shoot formation and this is supported by Sunnichan & Shivanna (1998). The shoots formed exhibited phenolic exudation and leaf drop, which inhibited the conversion of multiple buds into shoots (Komalavalli & Rao, 2000).

Other supplements

Presence of organic supplements in induction and activation of axillary buds and subsequent proliferation of adventitious buds in many species are well documented (Das gupta et al., 1987). In the present study, the influence of additives was studied after determining the optimum BA level for bud induction and to improve the quality of shoots. There was a great improvement in the number of shoots in 2ip+BA combination only on shoot tip explants. The presence of adenine in the medium is known to promote axillary bud differentiation and development. A higher concentration of adenine is required as compared to other cytokinins such as KN to promote bud differentiation in Ixora singaporensis (Malathy & Pai, 1998). Addition of other organic supplements had no significant promotive effect in the number of shoots in C.halicacabum. Thus the organic supplements were found unsatisfactory.
for mass propagation of this plant. But the leaves were green and healthy and no leaf fall was observed. The ability of Ads and GA₃ in favouring the growth of sprouted axillary buds into healthy green shoots by delaying abscission of petioles is also well documented (Jordan & Balbo, 1985; Demeke & Hughes, 1990). Incorporation of Ads with BA was stimulatory for shoot multiplication from corm bud culture in Gloriosa superba (Sivakumar & Krishnamurthy, 2000). The presence of adenine in the medium is known to promote axillary bud differentiation and development (Malathy & Pai, 1998). A concentration of 146 mg/l⁻¹ (Adenine) was the best for shoot proliferation of Leontochir ovallei (Lu et al., 1995).

Premature leaf drop has been encountered during in vitro shoot multiplication experiments in some other species, where addition of Ads, aminoacids, calcium or iron was essential to overcome the problem (Wang & Hu, 1984; Dhawan & Bhojwani, 1985; Raghava Swamy et al., 1992; Patnaik & Debata, 1996). Among the additives, Ads for shoot bud differentiation, shoot bud conversion and elongation of shoots has been well documented (Chaturvedi, 1975; Reuveni et al., 1990; Shekhawat et al., 1993; Dhar & Upreti, 1999; Pattnaik & Chand, 1996). Complex extracts like coconut water and yeast extract (irrespective of concentrations) were unsatisfactory for mass propagation of this species. In contrast, coconut water and yeast extract have been reported to have promotive effective in other plant species (Jordan & Balbo, 1985; D'Silva & D'Souza, 1992; Gras & Calvo, 1996; Roy et al., 1998). CM, CH and YE did not significantly improve the shoot sprouting frequency, shoot number and shoot length (Komalavalli & Rao, 2000; Kulkarni et al., 1997). Cassells et al. (1999) supplemented MS medium with 0.825 mg/l⁻¹ ammonium nitrate, 150 mg/l⁻¹ sodium dihydrogen phosphate, 1.0 g/l⁻¹ casein acid hydrolysate, 50 mg/l⁻¹ adenine sulphate for micropropagation of Arnica chamissonis. In contrast to our result, it is reported that the addition of CM (10%) and CH (100 mg/l⁻¹) to the medium enhanced the number of shoots (Roy et al., 1998). Like wise the addition of aminoacid also did not improve the number of shoots.
Explants

Among the various explants (leaf, internode, petiole, shoot tip and axillary bud) tested, only those of axillary node and shoot tip explants showed positive morphogenetic response and readily developed multiple shoots, whereas other explants (leaf, petiole and internode segments) produced only callus. The hormonal and additive requirement for shoot bud induction and overall growth were akin to axillary node and shoot tip explants. However, the propagation rate and morphogenetic response significantly varied to a greater extent according to the explant type (Komalavalli & Rao, 2000). Multiple shoots could be induced in cultures of shoot tips as well as nodal segments on MS medium containing BA and IAA (Sunnichan & Shivanna, 1998). Plants of Catalpa ovata were regenerated in vitro from shoot tip and nodal explants as well as from cotyledon derived calli (Lisowska & Wysokinska, 2000).

The percentage of explants forming multiple shoots was higher with shoot tip than axillary bud. Also in our experiments a marked difference was found in the number of multiple shoots obtained from the two explants. However, the effective concentrations of growth regulators and the organic supplements requirements for shoot bud induction were akin to both the explants. Thus it seems that the physiological state of the vine influences the explant behavior in vitro (Shekhawat et al., 1993).

Shoot elongation

The addition of GA₃ to the medium containing BA had no stimulatory effect on the frequency of shoot development and the number of shoots, but it did promote shoot elongation. In almost all concentrations of GA₃, the shoot length doubled or tripled with elongated internodes. The shoot elongation by GA₃ is well documented. Welander et al. (1989) reported that GA 3 stimulated shoot elongation in Populus X Wilsocarpa.
Rooting

All the three auxins induced rooting in all used concentrations. Both IBA and IAA were effective for root induction. There was no difference found in the rooting response and the number of roots between two explants. But the quality and quantity i.e. frequency and the number of roots varied depending upon the concentration and type of auxins. Some of these shoots when transferred individually or in a group to rooting medium showed development of comparatively longer roots (3-5 cm long). Roots were shorter, thin and fibril like nature in NAA medium. However, there were no significant differences between the effects of IAA and IBA concentrations tested. Sunnichan et al. (1998) reported that IBA was effective for root induction in Sterculia urens. Our findings were also in consonance with the report of Sunnichan & Shivanna (1998) that IBA in the concentration range of 0.5-2.0 mg l⁻¹ induced rooting and beyond 2.0 mg l⁻¹ IBA did not support rooting but induced callusing at the cut ends of the shoots. Further it was also described that IBA at 1.5-2.0 mg/l caused 90% of the shoots to develop roots in 12 to 14 days time. Quaraishi & Mishra (1998) obtained rooting response in IAA. IBA was most effective than other three auxins tested for root induction and survival in the field (Komalavalli & Rao, 1997; 2000). Sometimes extensive callusing at the base with thin, delicate root formation was noticed when the medium was supplemented with NAA and IAA respectively (Komalavalli & Rao, 2000). The rooted plantlets appeared to be phenotypically normal. There was no rooting in case of shoots planted on basal medium. In culture where the medium was supplemented with auxin, root primordia emerged from the shoot base 10-20 days after implantation and subsequently developed into roots. In all the cultures supplemented with auxin there was considerable thickening of the shoot base prior to root formation. Depending upon the concentration of auxin in the medium there was simultaneous callusing and rooting at the thickened shoot base. Similar observations have been reported in Hemidesmus indicus (Patnaik & Debata, 1996). Because the callus formed was superficial and highly friable, it could be easily be removed from shoots before transferred to pots. The shoots elongated and rooted directly in vermiculite after a pulse treatment with IBA (25 mg/ml) for 15 min (Das et al., 1999). Thus IBA the was most effective in inducing roots (Sahoo & Chand, 1998).
Callus induction

The NAA, BA combination yielded the highest amount of greenish callus than IAA, BA media from shoot tip explants. Addition of KN was also observed to significantly improve the response of all three auxins permitting efficient callus induction from both explants. The most successful callus induction was recorded in NAA medium containing both BA and KN in both explants. Thus the explants, auxins, BA and their interactions and second order interactions with KN significantly influenced callus yield. In all treatments, shoot tip gave better response compared to axillary bud. Among explant sources leaflet gave 95-100% callus induction, whereas shoot tip gave 77% callus induction (Barna & Wakhlu, 1994). In addition to auxin and BA, the inclusion of KN in the medium improved the callus induction frequency. BA and KN had a promotive effect on callus proliferation only at lower concentrations. MS medium with growth regulators alone did not induce any morphogenic response from either yellow friable or light green compact calli, but promoted further proliferation. The effect of auxin and cytokinin type, and their concentration, and their interactions were significant for callus initiation using various explants.

The growth regulator combinations stimulated callus growth to a lesser extent from shoot tip explants. In Xanthosoma sp. callus production or mass was significantly greater from petioles than shoot tips and adventive regeneration was induced from compact and light green callus (Nyochemberg & Garton, 1998).

Callus texture

Light green compact callus was associated with nodular structures and yellow callus was associated with loosely packed friable regions. Shoot differentiation was achieved from light green compact callus, but not from yellow friable callus (Prakash et al., 1999). But in this study, there is not much correlation between callus nature and regeneration. The regenerated shoot organs developed mostly beneath and within
the callus mass, irrespective of the explant source (Nyochemberg & Garton, 1998). The callus is multilobular in shape with typical green colour and relatively with high chlorophyll content. On the surface of the callus, active growth points are produced that can be subcultured to obtain young shoots (Tiburcio et al., 1991). The callus formation in this study also complies with these reports.

**Shoot differentiation**

Morphogenesis in tissue culture is a prior requirement for much genetic manipulation of plant cells in culture. The most important factors affecting the induction of organogenic callus and plant regeneration through organogenesis include the explant type, media formulations and growth regulators. Van (1981) has stated that "the inherent cellular state" which the explant has brought along into the culture medium after its excision as well as the "past of the mother plant" is as important components (to regeneration) as the nutritional, phytohormonal or environmental factors being applied to the explant. Higher regeneration rate of young tissues has been reported to occur in other species (Baker & Bhatia, 1993; Carneiro et al., 1999). Organogenic or embryogenic callus is generally induced from meristematic tissues such as embryos, basal meristems or shoot tips (Bajaj, 1992). The cell differentiation involves the activation of certain genes and repression of others, which control different basic metabolic and anabolic pathways. Besides growth regulators, several low molecular compounds are also known to be involved in differentiation (Dey et al., 1998).

Even though most cells of a plant contain the identical genetic information, calli obtained from different types of somatic cells vary in their competence to de-and re-differentiate under identical culture conditions (Taras, et al., 1999). Teuter & Reinert (1973) pointed out that the control of morphogenic capacity of plant cells and tissues cultured in vitro is not wholly genetic, but depends at least in part on epigenetic changes, which occur during growth and differentiation in vitro. If this so, then it would appear that Na Citrate and other inorganic and organic substances may play a role in epigenesis of Cardiospermum halicacabum callus favouring shoot regeneration. Thorpe (1994) concluded that a wide variety of biological, physical and
chemical factors affect the specific pattern of organogenesis and its extent. The interactions between growth regulators and other metabolites provide a common mechanism for the regulation of all types of growth including organ formation (Brown & Thorpe, 1986).

Selection of explant

Leaf and internode

Leaf and internode explants from mature plants of *C. halicacabum* were investigated for their morphogenic potential using growth regulators, organic and inorganic supplements. But there was complete absence of regeneration capacity in these explants.

Leaf explants easily produced calli but no shoot formation occurred in *Penstemon serrulatus* and only roots were produced in green callus (Wysokinska, 1993). The regeneration of plants from chickpea callus cultures has been problematic (Barna & Wakhlu, 1994). Unorganized callus developed from leaf, stem, hypocotyls, cotyledon, root, shoot tip and embryo explants of *Capsicum annuum* cv. mathania could not be induced to differentiate shoot buds by using various concentrations and combinations of growth regulators (Agrawal et al., 1988). Callus cultures of the economically important monocots, however, have poor regeneration ability and great differences may exist between genotypes. Such genotypic variation in the morphogenic response is widespread, and can even be observed in genera like *Nicotiana*, widely used as model in tissue culture studies (Purnhauser et al., 1987). There exist variations in the regeneration capacity among genotypes and even within tissues produced by the same genotype (Duncan et al., 1985).

Embryo like structures were obtained from shoot apex derived callus of *Phaseolus vulgaris* cultivars, but no viable plants were recovered (Martin and Sondahl, 1984). Of the *P. vulgaris* cultivars tested, green nodular callus with a competence to regenerate was established from vegetative buds of some genotypes, whereas in others, green nodular callus obtained, but organogenesis did not occur.
This indicates that the genetic factors responsible for regeneration capacity (Willy Dillen et al., 1996) i.e. regeneration was influenced by genotype (Abbas et al., 1997). These traits or genetic factors conferring or controlling regeneration ability are also supported by Reisch & Bingham (1980), Koornneef et al. (1993) and Birhman et al. (1994). However, regeneration of plants from chickpea callus cultures has been problematic. In chickpea, it was observed that shoot differentiation was influenced by the explant source used for initiating callus culture. While the calli initiated from the cotyledon, hypocotyl, epicotyl and apical part of the shoot tip were unable to produce shoots (Barna and Wakhlu, 1994). Leaf explants of *Piper betle* and *Piper nigrum* produced callus but failed to show organogenesis (Bhat et al., 1995).

As there was no regeneration observed in leaf and internode explants, shoot tip and axillary bud were selected for further regeneration studies. Similarly Bhat et al. (1995) highlights the importance of the choice of the proper explant for regeneration.

**Shoot tip**

Between the two explants used in our studies, shoot tip exhibited enormous regeneration ability than axillary bud. Many evidences can support this selection of shoot tip as explant for regeneration studies as follows. An alternative is to use shoot tip as explant for transformation and plant regeneration (Bhaskaran & Smith, 1988; Nahdi & de Wet, 1995). The greatest response of shoot tip is attributable to the differences in their physiological status of various explant sources (Vasil & Thorpe, 1994). The superiority of shoot tip over axillary node has been well documented in the literature. In most of the recalcitrant species, shoot tip is a viable alternative for regeneration via organogenesis. In *Larix decidua* adventitious shoots were produced on callus tissue from shoot tips of 3-month-to 2-year old seedlings (Chalupa, 1983). The study by Idei and Konda (1998) suggested that organogenesis in tissue cultured shoot primordium of *Utricularia praelonga* could be induced by NO₃⁻ in KNO₃.

Abbas *et al.* (1997) reported that the callus was produced on shoot tip of two *Corchorus* species and high frequencies of regenerated shoots were obtained in both species. Aminuddin *et al.* (1993) standardized a protocol for *in vitro* propagation of
*Piper betle* L. from the callus raised from shoot tip explants. Nychemberg & Garton (1998) initiated callus from shoot tip and more than 80% became organized into shoots and confirmed that shoot tip derived callus had greater morphogenic potential. Due to the superior response and friable nature of shoot tip callus, it was utilized to initiate organogenic suspension culture. Previous works on callus initiation in *Xanthosoma* spp using shoot and/or meristem tip has been reported (Hartman, 1974; Licha *et al.*, 1982). Strauss & Arditti (1980) obtained regeneration of *Xanthosoma caracu* plantlets from callus cultures of shoot tip origin. Several reports have mentioned the development of protocorms in shoot tip callus cultures (Abo El-Nil & Zettler, 1976; Nyman *et al.*, 1983; Nguyen & Nguyen, 1987; Sabapathy & Nair, 1995) and directly on agitated axillary, apical and adventitious bud cultures of cocoyam (Acheampong and Henshaw, 1984) media. Willy dillen *et al.* (1996) obtained shoots from nodular callus derived from buds. The best shoot differentiation was obtained from calli derived from shoot tips (Barna and Wakhlu, 1994). Islam *et al.* (1982) reported single and multiple shoot formation from shoot tip derived callus of *C. capsularis*. Rahman *et al.* (1985) showed that callus initiated from apical meristems of *C. capsularis* were able to form adventitious shoots. Ochatt & Azkue (1984) obtained callus proliferation and plant recovery with *Oxalis eosa* shoot tip cultures.

Caulogenic callus cultures were established from vegetative short shoot buds collected from the tree of *Larix X eurolepis*. Shoot formation resulted mostly from adventitious organogenesis on callus tissue, but adventitious organogenesis and axillary shoot development were occasionally observed on neoformed shoots (Laliberter & Lalonde, 1988). A system for rapid plant regeneration through somatic embryogenesis from shoot tip explants of *Sorghum bicolor* was described by Seetharama *et al.* (2000). These observations with shoot tip cultures were similar to the system described by Zhong *et al.* (1992) that differentiation of multiple shoot clumps and somatic embryos from shoot tips.

Further, the shoot induction response of the shoot tip explant was both qualitatively and quantitatively better than that of axillary node explant at all concentrations of sodium citrate tested.
Bohmer et al. (1995) developed a regeneration system for pea protoplast derived callus via organogenesis, by using the lateral shoot buds of cotyledon free embryo axes and induced a high frequency of plant regeneration. Ayabe et al. (1995) used young green leaves, immature etiolated leaves and calli obtained from the in vitro culture of shoot apices as source of protoplasts and regenerated whole garlic plantlets.

**Axillary node**

Even though axillary node yielded less response compared to shoot tip, in some treatments its response is on par with shoot tip. Yam et al. (1990) induced callus from axillary bud of taro (Colocasia esculenta var. esculenta, Araceae) and subsequent plant regeneration. Altvorst van et al. (1995) described that cells within the nodal region of carnation shoots exhibits a high potential for adventitious shoot formation. In axillary bud, stem and leaf explants shoot regeneration originated from the node cells, located at the transition between leaf and stem tissue. Adventitious shoot formation has also been reported for axillary bud explants of carnation (Miller et al., 1991). In *Piper nigrum* and *Piper betle* adventitious shoot bud differentiated from nodal explants. Besides axillary shoots, nodal explants regenerated shoot buds from the proximal end of the internode and also around the nodal ring (Bhat et al., 1995). A procedure for the regeneration of fertile plants by organogenesis from nodal explants of *Glycine max* was described by Wright et al. (1986). Kim et al. (1990) reported that they have obtained efficient regeneration of fertile plants from explants of primary leaf nodes prepared from 7-day seedlings. There have been several reports of shoot regeneration from seedling nodes (Saka et al., 1980) and Wright et al. (1987) investigated the regeneration capacity of explants bearing primary leaf nodes. Embryogenic cell suspension culture was established from nodal callus of *Cymbopogon martini* (Roxb.) Wats. (Patnaik et al., 1997). Nandwani & Ramawat (1991) reported the plant formation from nodal callus cultures of *Prosopis juliflora* (Swartz.) DC. Plants were regenerated through callus culture from nodal segments in patchouli [(*Pogostemon cablin* Benth.) Meena, (1996)]. There are many studies in *Cymbopogon* include the induction of organogenesis, somatic embryogenesis, plant regeneration and somaclonal variation in callus cultures derived from the rhizome,

The greatest capacity of shoot formation (98%) was observed on cotyledonary node derived callus (Cid et al., 1999). The highest shoot regeneration response may have been associated with the origin of the calluses obtained from cotyledonary nodes. This character was also observed on calli of Phaseolus derived from explants containing axillary meristems and cultivated in the presence of TDZ (Zambre et al., 1998). The study by Mendoza (1992) described the shoot regeneration from the callus of immature primary leaves in Vigna radiata. Juvenility of the tissue emerged as the major factor since the response was restricted to the explants from the unexpanded proximal parts of the youngest (juvenile) leaf, as reported earlier (Tanaka & Sakanishi, 1977; Mathews & Rao, 1985; Vij et al., 1994 & Vij & Pathak, 1990).

Medium composition

To optimize the nutrient requirement of tissue culture, it seems that the mineral composition especially of microelements should be examined to achieve maximal regeneration (Sharma & Vij, 1997). Elkonin & Pakhomova (2000) demonstrated that manipulation of the mineral composition of the medium allowed effective control of morphogenesis in sorghum tissue culture. It was believed that cell differentiation was a distinct process that can be induced in the cell cycle by providing suitable regulators (Basu et al., 1989). The second most critical factor is the inorganic salt concentration in the medium (Wright et al., 1986).

1. Plant growth regulators

As observed in this study, the remarkable response of BA over other cytokinins is an obvious one. Regarding concentration, both the explants require higher concentration to effect organogenesis. Both cytokinins (BA and KN) have no role to play in differentiation when growth regulator alone used in the medium. At the same time both induced dramatic regeneration response only in the presence of other organic and inorganic supplements. Various concentrations and combinations of growth
regulators failed to induce differentiation of shoot buds in callus tissue (Agrawal et al., 1988). Auxins, which are essential for callus induction, play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration (Purnhauser et al., 1987). Torimoto and Harrada (1982) found that differentiation of adventitious buds could be induced not only by cytokinin but also by some anticytokinin substances.

A higher concentration of BA (5-10 mg/l) was necessary to elicit good response. BA was the most suitable one between the two cytokinins tested for shoot formation. The percentage of calli forming shoots and the number of shoots formed per callus was optimal in *Cicer arietinum* when 10 mM BA was used (Barna and Wakhlu, 1994). The superior effect of BA over the other cytokinins for shoot induction has been attributed to the abilities of plant tissues to metabolize the natural hormone more readily than other synthetic growth regulators or to the ability of BA to induce endogenous production of zeatin (Zaerr and Papes, 1982). The highest regeneration rates were obtained on 22 mM BA in *Neoregelia cruenta* (Carneiro et al., 1999). The highest frequency of shoot regeneration in sainfoin was achieved from stem segments on a medium containing 20 mM BAP (Murat et al., 1998). Shrivastav & Rajani (1999) found BA to be more suitable than KN as the former resulted in a quicker and better response than the latter. BA was more effective than KN for de novo shoot bud formation. Organogenesis was induced with 30 mg/l BA in *Lycoris aurea* (Huang & Liu, 1989).

2. Sodium citrate

In our study, Sodium citrate plays a major role in regeneration of callus. It has substantial effect in the production of shoot number and quality only when combined with other additional constituents. There was a significant increase in the extent of shoot regeneration with an increase in the concentration of sodium citrate. Incorporation of citric acid (100 mg/l) to the medium prevented phenolic exudation, increased the production of healthy normal shoots and bud differentiation in *Gymnema sylvestre* (Komalvalli & Rao, 2000). The promotive effect of citric acid was supported by Shekhawat et al. (1993). Aminuddin et al. (1993) also fortified MS
medium with antioxidants namely citric acid (150 mg/l), ascorbic acid (100 mg/l) and PVP separately or in combination for shoot initiation from *Piper betle* shoot tip callus. According to Castillo & Jordan (1997) the use of antioxidants helped to prevent browning and favoured organogenesis. He suggested that the morphogenic effect of leaf leading to adventitious shoot was enhanced when one of the following antioxidants like glutathione (15.25 mg/l), cysteine (15 mg/l), aminooxyacetic acid (10 mg/l), glutamine (100 mg/l), casein hydrolysate (200 mg/l), citric acid (250 mg/l) or ascorbic acid (250 mg/l) was used.

Importance of ascorbic acid in plant cells has long been recognized, initially in controlling the redox system of cells and such processes as seed germination, growth, oxidative phosphorylation and photophosphorylation, stimulation of RNA synthesis, bud development and prevention of senescence (Sweet and Guruprasad, 1997). The organic acid can also act as a buffer for cell culture (Dawson *et al*., 1969; Gamborg & Shyluk, 1970; Dougall & Weyranch, 1980; Gleddie *et al*., 1983). Callus cultures of soybean will grow on ammonium as a sole source of nitrogen but only if organic acids are present in the medium (Behrend & Mateles, 1976; Fukunga *et al*., 1978). The effect has been attributed to the metabolism of organic acids such as succinate and Δ-ketoglutarate. Organic acids have been extensively used in the culture of protoplasts (Kao, 1977). According to Sharma & Vij (1997) the most effective treatment for promoting shoot regeneration was that Sodium citrate was present throughout the culture period. Other effects of citrate, observed on morphogenic cultures were a general anti-senescence effect and reduction of adventitious root formation.

A beneficial effect is largely restricted to the acids of the Kreb's cycle. Organic acids and their sodium and potassium salts, stabilize the pH of hydroponic solution (Trelease & Trelease, 1933; George & Sherrington, 1984) or in vitro media (Van Overbeek *et al*., 1941,1942; Arnow *et al*., 1953). Divalent organic acid such as citric, maleic, malic and malonic (depending on species) are found in the xylem sap of the plants, where together with amino acids, they can complex with metal ions and assist their transport (White *et al*., 1981). Gamborg and Shyluk (1970) found that some organic acid could promote ammonium utilization and enhance the metabolism
of ammonia. Murashige & Tucker (1969) showed that citric acid produces the most pronounced growth stimulation. Thus some plants seemed to derive nutritional benefit from the presence of one particular organic acid. Citric acid and tartaric acid can act as a source of chelating agents for some divalent metals (Bobtelsky & Jordon, 1945). Three carrier mediated transport systems seem to be involved with tricarboxylic acid such as citrate, isocitrate and cis-aconitate. Citric acid is an absolute prerequisite for respiration and biological oxidation. The major fate of citrate in plant tissues appears to be metabolism via TCA cycle or via glyoxalate cycle.

There are few reports in the literature on the need of citric acid and ascorbic acid for morphogenesis (Narayanasamy, 1994; Sweet & Guruprasad, 1997; Dhar & Upreti, 1999). The beneficial effect of citric acid may be due to its antioxidant property or due to the increase in morphogenic-specific storage proteins by organic acid (Nichol et al., 1991). Axillary shoots proliferated best on MS medium containing citric acid (104.18M) and PVP (12.5 or 258M) supplemented with 0.44 8M BA in Cleistanthus collinus (Quraishi & Mishra, 1998).

3. Nitrogen salts

Based on our results, it is confirmed that nitrate contributes much to the regeneration of Cardiospermum callus. Both K⁺ and NH₄⁺ salts of nitrogen yielded good response. It was also that there was only little difference in shoot induction response between NH₄NO₃ and KNO₃. Increasing the concentration of NH₄NO₃ or KNO₃ in the medium increased the shoot differentiation potential of light-green compact callus. According to Palmer (1992) 60 µM AgNO₃ significantly enhanced both the percentage of Brassica campestris shoot regeneration and the number of shoots per cotyledon explant and cobalt chloride at 20 and 30 µM increased cotyledon shoot regeneration. Silver nitrate effectively promoted shoot regeneration in wheat (Triticum aestivum) callus cultures derived from immature embryos. Enhancement of shoot regeneration by AgNO₃ was observed in callus cultures of non-regenerating or weakly regenerating mutants of Nicotiana plumbaginifolia (Purnhauser, 1987). Nitrogen generally has both nutritional and regulatory functions in plant development (Mordhorst & Lorz, 1993).
It has also been reported that the inorganic NO$_3^-$: NH$_4^+$ ratio alters cell sensitivity to auxin and regulation of hormone metabolism in rice (Kim et al., 1996). Mathur et al. (1995) observed rapid \textit{in vitro} multiplication of jujube through mature stem explants on modified MS medium containing 3800 mg l$^{-1}$ potassium nitrate and 2475 mg l$^{-1}$ ammonium nitrate. Halperin and Wetherll (1965) reported that some nitrogen sources affect organogenesis in plant culture. The study by Idei and Konda (1998) suggested that organogenesis in tissue cultured shoot primordium of \textit{Utricularia praelonga} could be induced by NO$_3^-$ in KNO$_3$.

4. Amino acids

Isoleucine also has some role to play in callus induction differentiation. Among the different amino acids investigated, isoleucine was found to be the suitable one. According to Basu et al., (1989) addition of optimal concentrations of L-leucine and L- isoleucine enhanced shoot formation significantly. L-methionine, L-threonine and pyruvic acid supported only proliferation but no differentiation. Thus these results suggested that amino acids play a regulatory role in \textit{Brassica} morphogenesis. Thus leucine and isoleucine were shown to support differentiation. Basu et al. (1989) have identified the amino acids as important growth regulators, which involved in tissue differentiation (TD) as a modulator of growth and differentiation, which may be affecting several metabolism and consequently morphogeneisis. Of IAA-amino acid conjugates, the best shoot regeneration of peanut and pigeonpea from leaf callus was obtained on medium with IAA-aspartic acid (Susan & Leela, 1993). Tiburcio et al., (1991) indicated that D, L-alpha-difluoromethylarginine (DFMS) pretreatment could be used to improve the regeneration efficiency from maize callus cultures. Proline also had striking effect on shoot formation, increasing shoot number five to six folds when 1 to 3 g/l was supplied (Kim et al., 1990). Amino acid may induce or inhibit cell proliferation or differentiation. In \textit{Brassica}, leucine and isoleucine were reported to promote differentiation, whereas methionine and threonine activated proliferation (Basu et al., 1989).
5. Adenine and adenine sulphate

Some natural products are known to promote regeneration from calli. Our findings suggested that both adenine and adenine sulphate increased regeneration response. Skoog & Tsui (1951) found that a supplement of adenine in the medium significantly promoted plant regeneration from tobacco callus. Cid et al. (1994) reported that an addition of adenine was effective for the regeneration of plantlets from calli derived from suspension cultures of garlic. Additions of both adenine and coconut milk had dramatic synergistic effect on the regeneration of garlic calli (Ayabe et al., 1995). Our results further revealed that both CH and YE had no much effect on regeneration, in contrast to the stimulatory effect of CH on shoot differentiation in callus culture of Carica papaya (Hosain et al., 1993).

6. Synergistic effect of citrate and nitrogen salts

Citrate (5mM) greatly enhanced the growth of alfalfa (ca. 20 fold) and wheat cells in an ammonium based medium. Alfalfa and wheat cells grown in an L-glutamine-based medium were influenced by citrate. The growth of tobacco cells was slightly enhanced by 5mM citrate (Yukio et al., 1978). In 1970, Gamborg & Shyluk reported that soybean cells grew in a defined medium with ammonium sulfate as the sole source of nitrogen and the TCA cycle acids, citrate, malate, fumarate or succinate were added at concentrations ranging from 2 to 10mM. The beneficial effect of the TCA-cycle acids was explained by assuming that they facilitated ammonium utilization by stimulating ammonium-sensitive reactions directly involving these acids (Gamborg & Shyluk, 1970). Using tobacco cells Behrend and Mateles (1975,1976) found that 10 mM succinate, malate, fumarate, citrate, Á-ketoglutarate, glutamate or pyruvate supported growth on ammonium as the sole nitrogen source, and suggested that the role of the organic acids was to supply Á-ketoglutarate for glutamate and glutamine synthesis. Thus the TCA-cycle acids have a differential effect on growth of cells depending on the concentration used, the nitrogen source supplied, and on the cell species. In general, the results support the conclusions of others that TCA-cycle acids allow plant cells to grow more successfully on ammonium as the sole source of
nitrogen. TCA-cycle acids are required for the efficient assimilation of ammonium-nitrogen (Yukio \textit{et al.}, 1978).

Organic acids can have three roles in plant culture media (George, 1993).

* they may act as chelating agents, improving the availability of some micronutrients.
* they can buffer the medium against pH change.
* they may act as nutrients.

7. Synergistic effect of nitrate and other organic compounds

In addition to different explants, the higher Mg$^{2+}$, K$^+$, Mn$^{2+}$ and Zn$^{2+}$ concentrations as well as the higher concentration of organic compounds in the TE medium possibly play an important role in regeneration of pine (Tang \textit{et al.}, 1998). The addition of casein hydrolysate (500 mg/l) and more potassium phosphate (1.86 mM) to the culture medium enhanced shoot differentiation (Prakash \textit{et al.}, 1999).

The regeneration medium for \textit{C.capsularis} consisted of full strength MS supplemented with 50 mM KNO$_3$, 500 mg/l casamino acids and K12 organics (Saha \textit{et al.}, 1999). Kim \textit{et al.} (1990) recorded that proline and four fold-raised level of MS inorganic micronutrients caused a large increase in shoot number and substantial effects on the efficiency of shoot formation.

Role of nonhormonal chemical treatment on regeneration

The shoot forming ability of calli was enhanced by adding 5mM potassium phosphate to the medium (Barna & Wakhlu, 1994). The results obtained from the regeneration experiments of \textit{Hybanthus enneaspemus} demonstrated that the varying contributions of KH$_2$PO$_4$ concentration in the medium with respect to the differentiation of shoots in different plant species (Prakash \textit{et al.}, 1999). The four fold-raised level of MS inorganic micronutrients caused a large increase in shoot number, thus the effectiveness of mMS medium must in large part due to its higher level of
concentrations (Kim et al., 1990). With CoCl₂, percent regeneration was almost doubled or tripled compared to control and also shoot number several times (Sharma & Vij, 1997). The promotory role of higher doses of CuSO₄·5H₂O has been recorded in several dicot and monocot plants (Sharma & Vij, 1997).

Callus responded to increasing Na₂SO₄ stress by increasing both A and As. This was accompanied by shoot formation and higher regeneration capacity of shoots (Pua et al., 1985a). This finding indicated that osmotic changes might be associated with shoot formation of callus and suggests a regulatory role of Na₂SO₄ on in vitro organogenesis of plants through an altered endogenous phytohormonal and osmotic adjustment. The study also reemphasizes the association of altered water relations with organogenesis (Brown & Thorpe, 1980) and the osmotic adjustment possibly plays a role in enhancing mitochondrial activity in the high energy requiring process (Thorpe, 1983). These results are consistent with the previous findings (Brown et al., 1979; Brown & Thorpe, 1980) by having more negative A and As in shoot forming than non-forming callus. Also with tobacco callus cultures, increase in Na₂SO₄ concentration increased the regeneration of shoots (Pua et al., 1985b).

To our knowledge, the positive effects of timentin on in vitro morphogenesis of cultured tomato tissues has been reported by Ling et al. (1998). For other species the various positive effects of antibiotics like cefatoxime and carbenicillin on shoot regeneration are well documented and reviewed (Billings et al., 1997; Nauerby et al., 1997; Ling et al., 1998). Phenylacetic acid improves bud elongation and in vitro plant regeneration efficiency in Capsicum annum L. (Husain et al., 1999).

Elongation

Most neoformed shoots did not show pronounced elongation of the stem in vitro (Laliberter & Lalonde, 1988). In this study also the elongation of regenerated shoots was very poor.
Regeneration studies on other Sapindaceae members

Litchi is a member of Sapindaceae, has so far proven to be a difficult material for propagation using in vitro culture. Attempt to regenerate plants from explants derived from mature trees have failed to give satisfactory results (Kantharajah et al., 1989). However, the formation of up to 15 adventitious buds was reported when immature litchi embryos were treated with BA (100 mg l⁻¹) for 3 h followed by culture on hormone free medium for 4 weeks (Kantharajah et al., 1992). Multiple shoot induction has been achieved from seeds on 20 mg l⁻¹ BA and rooting in pulse treatment with IBA (25 mg/ml) for 15 min. (Das et al., 1999). Likewise in this study also C. halicacabum requires high concentration of BA.

Shoot morphology

Shoots that developed in the callus cultures showed morphological variations. Most did not show pronounced elongation of the axis. They looked either like young brachyblasts with leaves of fairly uniform size, or possessed a distinct external series of shorter leaves. Occasionally, shoots exhibited a rosette-like growth with their leaves shorter and wider than in the precedent types (Laliberter & Lalonde, 1988)

Conclusion

It was observed that shoot differentiation was influenced by the explant source used for initiating callus culture. From these results it is concluded that the choice of the explant, media components, and conditions during culture are important factors when considering plant regeneration from somatic cells. Mendoza et al. (1992) also concluded from his study that the choice of the variety, the physiological state of the explant, media components and conditions during culture are important criteria when considering plant regeneration from somatic cells. The in vitro morphogenic responses of cultured plant tissues are affected by the different components of the culture media, and it is important to evaluate their effects on plant regeneration (Costa et al., 2000). The differential response could be due to the types of supplements used in the medium.
SOMATIC EMBRYOGENESIS

Reliable plant regeneration systems that culminate in the formation of plants are necessary for the successful utilization of biotechnological methods in the genetic improvement of plant species. Somatic embryogenesis offers special advantages, such as high proliferation rate, singulation and bipolar structure, but in a number of species, conversion to robust seedlings often uneven and problematic (Janick, 1993). Over 70% of the successful cases, MS salts or its derivatives were used for somatic embryogenesis (Evans et al., 1981). Cytokinins, vitamins, organic acids and amino acids were found to have a stimulating role in embryogenesis (Asano et al., 1996).

Of the plant growth regulators, 2,4-D and sometimes other auxins may be required for certain species (Ammirato, 1983). A system for rapid plant regeneration through somatic embryogenesis from shoot tip explants of Sorghum bicolor was described by Seetharama et al. (2000).

Although somatic embryogenesis has been described for more than hundreds of plants species from different families, the number of reports of somatic embryogenesis among the members of the Sapindaceae is still very low. In the present study an attempt has been made to obtain somatic embryogenesis from the two explants of C. halicacabum.

Callus induction

The nature of the growth regulator treatment had a very significant and specific effect on callus induction. Only specific plant growth regulators combinations at particular concentrations were effective in inducing callus on explants. The combination of an auxin with a cytokinin was more favourable for callus induction than only one growth regulator (Kintzios et al., 1998). The application of IAA or IBA as the sole growth regulator was effective in inducing callus from leaf and internode explants. We also used the cytokinins, BA and KN together with auxins for efficient induction of callus. High callus induction rates were observed on IBA+KN and NAA+BA media. Addition of 2,4-D to the culture medium had a negative effect on callus induction. Genotype was the most significant factor influencing explant
embryogenic differentiation in culture. It is interesting that, irrespective of the
picular plant growth regulator combinations, callus induction from mature leaves
took place at various auxin/cytokinin concentration ratios, but somatic embryogenesis
was associated only when the auxin (pCPA- para ChloroPhenoxyAceticAcid) was
supplemented at a concentration 11-fold (53.5sM) that of the cytokinin (KN) in the
induction medium, while other concentrations failed to induce any embryogenic
response at all i.e. rose explants responded to a narrow spectrum of treatments, rather
than demonstrating a continuous pattern of embryogenic differentiation over several
concentrations(Kintzios & Michaelakis, 1999). Thus in callus induction studies by
Prem and Huiling (1999) also, 2,4-D induced poor quality of callus and BA had a
significant effect on callus induction, growth and appearance of callus. In addition,
the type of explant had apparent effect on callus induction, nature of calli and callus
induction rates on explant from both sources were essentially varied. The callus
derived from leaf explant was invariably much greener and granular than the stem
(internode) derived callus (Rout et al., 1999).

Embryo induction

Since the auxin type is the key factor which determines the induction of
organogenesis or embryogenesis, the auxin obviously plays a crucial role in this
experiment. The present observation showed that 2,4-D was found to be essential for
induction of somatic embryos in C.halicacabum. For the induction and development
of somatic embryos, additional cultural manipulation was required such as the use of
low to moderate concentrations of auxins, cytokinins, and/or gibberellic acid in the
medium and also use of abscisic acid or high osmoticum treatments or the physical
treatments such as desiccation (Ammirato, 1983 & Parrot et al., 1992).

Somatic embryogenesis was never observed in the absence of exogenous
hormones. Addition of BA and glutamine slightly enhanced the frequency embryo
production. This indicates that auxin is essential for induction of embryogenesis. BA
and glutamine showed a promotive effect with auxin on embryogenesis.
Explant effect

Some significant differences in the frequency of embryogenesis and the number of somatic embryos were noticed between leaf and internodal explant derived callus. Internode exhibited a high frequency of embryogenesis and more number of somatic embryos than leaf explant. Embryo when transferred to germination medium showed a wide range of developmental changes. Some embryos grew into callus, root or shoot and some failed to develop into plants.

2,4-D

Auxin is the most important factor essentially for the regulation of induction and development of embryogenesis. The induction of somatic embryos was significantly influenced by auxin and cytokinin source, concentration and their interactions. The effectiveness and efficiency of an auxin type appears to be tissue specific on somatic embryo production. Regulation of endogenous auxin plays a crucial role in induction of somatic embryogenesis (Zimmerman, 1993). The data here showed the importance of exogenous auxin in regulating somatic embryogenesis in *C. halicacabum*. Substitution of 2,4-D in the medium with varying concentrations of NAA or IAA did not induce somatic embryos (Inamdar *et al.*, 1990).

It has been demonstrated that 2,4-D has a critical role in induction of somatic embryogenesis in most plant species (Ammirato, 1983). Among the auxin compounds presently used, 2,4-D has been particularly found to be an effective inducer of somatic embryogenesis in more than 50% of plant taxa reported for successful production of somatic embryos (Evans *et al.*, 1981). In the present work also 2,4-D only successfully induced somatic embryogenesis in *Cardiospermum halicacabum*. However, the effect of 2,4-D in induction and/or development of somatic embryos is variously interpreted. In *Corylus avellana*, if 2,4-D did not inhibit the induction of embryo, it did inhibit their further development into plants (Radojevic *et al.*, 1975). In accordance with above statements, the present study also indicates that 2,4-D is very essential in embryo induction, but inhibits its further development into mature embryos in *C. halicacabum*. Like wise in another
Sapindaceae member, *Sapindus trifoliatus*, 5-methyl tryptophan has been used to prevent any extraneous proliferation during the development of embryos as demonstrated by Desai et al. (1986). Thus only 2,4-D was found to be suitable inducer in somatic embryogenesis through callus culture. 2,4-D (2.0mg/l) showed superior response for embryo induction.

Higher concentration of auxin (2,4-D) not only decreased the mean number and frequency, but also delayed the embryogenesis. The decreased response of embryogenesis in the presence of higher concentration of auxin might be due to altered growth regulator levels in the medium that is essential for embryo induction. Ruffoin and Massabo (1996) have also observed a relationship between the number of embryos formed and concentration of 2,4-D in the induction medium in *Lisanthus rumelius*. According to Wakhlu and Sharma (1998), 2,4-D was the most suitable of the four auxins tested for somatic embryogenesis.

**2,4-D plus BA**

The requirement of cytokinin in addition to auxin observed in *C. halicacabum* has also been reported in *Sapindus trifoliatus* (Desai et al., 1986) and *Euphorbia longan* (Litz, 1988). This is in agreement with a previous result reported by Reddy and Reddy (1993) in *Arachis hypogea*. The inclusion of BA with 2,4-D produced higher frequency of somatic embryos. In *Populus deltoides*, MS+2,4-D+BA produced more number of globular shaped embryos (Michler & Bauer, 1991). Similar results have been reported by Evans et al. (1981).

BA combined with 2,4-D yielded maximum embryo production compared to all other combinations examined in the solid medium. Increasing concentration of BA had a significant positive effect up to optimum concentration on promotion of somatic embryogenesis. Optimal embryo forming callus was observed on a medium with 1.0 mg/l 2,4-D plus 1.0 mg/l BA (Ignacimuthu et al., 1999).
Complex extracts

Incorporation of complex extracts, a source of reduced nitrogen, had stimulated embryogenesis in several plant species (Reincrt, 1959; Debeaujon & Branchard, 1993). This is in contrast to the present study, where additives did not significantly improve the embryogenic potential and the development of existing embryos. The role of complex extracts in improving embryo formation is controversial. It has been reported that casein hydrolysate (Preece & Sharon, 1995) and yeast extract (Radojevic, 1980) have a suppression effect on embryo development. However, yeast extract (Chan & Staba, 1964) and casein hydrolysate (Rangaswamy, 1958a,b; Zhang et al., 1987; Button & Botha, 1975) have also been shown to have a promotive effect. Embryogenic tissues were induced on medium having 2,4-D, BA, CH and glutamine in *Picea marina* and *Picea rubens* and embryo development was with ammonium nitrate (Tremblay & Tremblay, 1991). Embryogenic tissues were induced on medium having 2,4-D, BA, CH and glutamine in *Picea marina* and *Picea rubens* and embryo development was with ammonium nitrate (Tremblay & Tremblay, 1991).

Aminoacids

Glutamine as an organic nitrogen source was beneficial to embryogenesis (Das et al., 1995; Higashi et al., 1996; Augustine & D'Souza, 1997; Barrett et al., 1997), since it is the transported form of nitrogen in several species (Tabone et al., 1986). Moreover, it enhances differentiation of predetermined direct embryogenic cells (Kamada & Harada, 1979; Stuart & Strickland, 1984; Muralidharan & Mascarenhas, 1995). This is in agreement with the present study. Whetherell and Dougall (1986) in carrot and Stuart and Strickland (1984) in *Medicago sativa* reported that glutamine with 2,4-D produced maximum number of somatic embryos. In contrast none of the amino acids tested viz. glutamine and alanine appreciably improved the induction of somatic embryogenesis. However, with all the amino acid treatments, the growth of the somatic embryos was much better (Murthy et al., 1996). Media containing 2,4-D and glutamine produced the higher number of somatic embryos. Of all evaluated concentrations, 2,4-D (2.0 mg l\(^{-1}\)) and glutamine (10.0 mg l\(^{-1}\)) combination was found to be suitable for maximum response.
Also some reports showed that amino acids were effective on embryo development (Stuart and Strickland, 1984; Redenbaugh *et al.*, 1991a; Shetty and Mckersie, 1993 and Nichol *et al.*, 1991). The source of nitrogen in the medium also affects *in vitro* embryogenesis in ways, which depend on the plant species used (Chee *et al.*, 1992). Ammonium or amino acids such as proline, alanine, arginine, or glutamine stimulate prolific somatic embryo formation when supplied together with nitrate (Redebaugh *et al.*, 1991b; Stuart *et al.*, 1985).

**Embryo maturation**

A variety of unusual culture conditions and chemicals reportedly enhance embryogenic competence (Carman, 1990). According to Asano *et al.* (1996), in the presence of cytokinin and thiamine, the addition of riboflavin or 3-ketoglutaric acid, a key intermediate in the TCA cycle proved to further enhance embryogenic callus induction from *Zoysia japonica* seeds. The increase of phosphorous content in the media significantly promoted the induction of embryogenic sorghum callus from immature embryos (Elkonin & Pakhomova, 2000).

Organic acids such as citric acid and malic acid that were applied during the last subculture of callus were found to increase the number of embryos formed, the conversion rate of the embryos and the amount of seed-specific storage proteins (Buyukalaca & Mavituna, 1996). Without this pretreatment, it was not possible to obtain mature embryos. The inclusion of 6g/l potassium citrate in the medium had the most pronounced effect on the number of heart shaped embryos in the medium. Such pretreatment may be selection for more embryogenic cells or cause an alteration in cellular metabolism towards differentiation and away from cell proliferation (Redenbaugh *et al.*, 1991b). The pretreatment agents in their study were citric acid and malic acids. Since potassium salts of these acids were used, there would have been an effect of the potassium ions as it is the major and most common cation involved in maintaining the ionic balance of the medium and the osmotic potential of the cytoplasm. The effect of potassium in sweet potato somatic embryogenesis was studied by Chee *et al.* (1992).
The culture pretreatment regime results in decreased callus growth, changes the morphology of the callus and causes an increase in embryo yield after induction and regeneration of the callus. The effective organic acids and their optimum concentrations are K⁺ citrate (20 mM), K⁺ malate (60 mM), K⁺ succinate(< 50 mM). The embryo which formed from callus have more normal morphology than embryo coming from untreated callus cells. The further effect of organic acids pretreatment is to increase the percentage of whole plants (conversion) compared to control treatments. The effect on culture buffering and chelation apparently do not account for the organic acid pretreatment effect. Potassium citrate pretreatment nearly doubled the rate of conversion of embryos to plantlets. It was suggested that only the organic acid treatment contributes to the improvement that leads to greater conversion of somatic embryos to plants. Thus the organic acids are generally useful for the enhancement of alfalfa regeneration (Nichol et al., 1991). Organic acids have been used in a number of reports as a counter ion to NH₄⁺. In these cases the organic acid can also act as a buffer for cell culture (Dawson et al., 1969; Gamborg & Shyluk, 1970; Dougall & Weyranch, 1980; Gleddie et al., 1983). Callus cultures of soybean will grow on ammonium as a sole source of nitrogen but only if organic acids are also present in the medium (Behrend & Mateles, 1976; Fukunga et al., 1978). The effect has been attributed to the metabolism of organic acids such as succinate to α-ketoglutarate. Organic acids have been extensively used in the culture of protoplasts (Kao, 1977). Christianson et al. (1983) used NH₄⁺-citrate in pretreatment regimes with soybean cultures as a counter ion to ammonium and concluded that a change in the reduced nitrogen content of the medium was important in regulating embryogenesis. There are several features of organic acid response in alfalfa cell cultures that make it unusual. Thus Potassium citrate produced good stimulation of embryogenesis. Organic acid do not trigger or induce embryo development as does 2,4-D, but only promotes. First organic acid treatment is effective for a range of di and tri-carboxylic acids. Baruah and Bordoloi (1989) used the vitamin supplements, calcium pantothenate and biotin in the regeneration medium for high frequency somatic embryogenesis and regeneration of plantlets. Emershad & Ramming (1994) added iron citrate as one of the constituents in the embryo induction medium of Vitis vinifera. Somatic embryogenesis occurred in liquid cultures containing 20 mM NH₄NO₃ and 30 mM KCl (Bieniek et al., 1995). Prior to culture, the cotyledon
explants of *Tagetus erecta* were dipped in a filter sterilized solution of sodium citrate (65mM) for 1 min. in order to reduce browning (Bespalhok & Hattori, 1998).

Even in this maturation studies with sodium citrate there was not successful or complete maturation from globular to late cotyledonary stage.

**Embryo germination problems**

Embryo germination (conversion) was not occurred on the 2,4-D/BA media. Therefore, it was not possible to prove any beneficial effect of BA on somatic embryo maturation as has been demonstrated for other species. Thus the induction and maturation of somatic embryos took place only under narrow spectrum of treatments, while other growth regulator combinations and concentrations failed to induce any embryogenic response. Embryo like structures were obtained from shoot apex derived callus of *Phaseolus vulgaris* cultivars, but no viable plants were recovered (Martin and Sondahl, 1984). The friable callus appeared hyperhydric and most cells were unable to differentiate and reorganize into primary meristematic cells and no conversion was observed (Lydia *et al.*, 1999).

The failure and/or low frequency of conversion are often attributed to morphological abnormalities or immaturity of somatic embryos (Ammirato, 1987). Somatic embryos were developed from protoplasts of *C.capsularis* (Saha & Sen, 1992), however plant regeneration could not be achieved as the somatic embryos failed to germinate. Somatic embryos were induced from immature cotyledons and embryonal axes; however, well-developed plants could not be derived from these embryos (Leela & Susan, 1994).

Somatic embryogenesis and subsequent formation of plantlets was obtained from callus cultures derived from leaves of mature soap nut (*Sapindus trifoliatus*). Reduction of 2,4-D concentration during subsequent cultures resulted in formation of embryoids. Unless 5-methyl tryptophan was added and the level of sucrose rose, the embryoids began to recallus and failed to form plantlets. Growth of heart shaped embryos to mature embryos was not achieved in this medium, as residual 2,4-D was
sufficient to induce callus from the heart shaped embryos. In some cases, as in the present the requirements for somatic embryogenesis are for more complex involving various mechanisms. In the present case, somatic embryos never germinated on a medium containing cytokinins or an auxin-free medium even after prolonged subculture, instead they began to recallus. We reasoned that by inhibiting the growth of this callus, it might be possible to trigger the germination of embryos. A tryptophan analogue, 5-methyl tryptophan was found effective not only in preventing recallusing but it also induced elongation of the embryonic radicle in about 40% of the somatic embryos (Desai et al, 1986). In Crataeva nvrvala also embryos developed into plantlets only in the presence of 5-methyl tryptophan (0.5mg/l) (Inamdar et al., 1990).

Generally, the development of embryos is attributed to the biophysical and genetic factors governed by the laws of embryogenesis. Some times in tissue culture (callus tissue) abnormal embryos also develop or the cells at the base develop tannin leading to the browning of callus and ultimate death or dedifferentiation of that tissue (Johri et al., 1996). Somatic embryos formed from leaves of peanut demonstrated a high degree of morphological abnormality (Chengalrayan et al., 1995). Thus it was not possible to prove any beneficial effect of BA on somatic embryo maturation as has been demonstrated for other species (Kintzios et al., 1998). The addition of yeast extract and proline were not beneficial either for somatic embryogenesis efficiency or somatic embryo conversion (Neves et al., 1999).

Somatic embryo germination

No plant was recovered from the somatic embryos obtained in this study. 2,4-D-induced embryos germinated unipolarly, but the plantlets were weak and hyperhydrated and did not survive the subsequent acclimatization process. Embryos, which developed in the presence of sodium citrate germinated unipolarly and developed into hyperhydrated and/or fascinated shoots. Although the fascinated shoots could be maintained in culture, root formation never obtained and plants were not recovered. The addition of different growth regulators (IBA, BA and GA3) and the use of different types of basal media (MS, 1/2MS and B5) did not result in conversion

78
to whole plants. The same result was reported by Bespalhok & Hattori (1998). The hyperhydrated fascinated shoots are similar to those produced in somatic embryos of Sweet Potato (Desamero et al., 1994). The formation of fascinated/hyperhydrated shoots could be caused by an insufficient maturation of the embryos and a chilling or dehydration treatment may be necessary for conversion to whole plants (Raemakers et al., 1995). The failure and/or low frequency embryo conversion is often attributed to morphological abnormalities or immaturity of somatic embryos (Ammirato, 1987). Organic acids such as citric acid and malic acid applied during the last subculture of callus were found to increase the number of embryos formed, the conversion rate of the embryos and the amount of seed specific storage proteins (Redenbaugh et al., 1991b).

Maturation and germination of somatic embryos are two critical steps in eventual recovery of healthy plants (Ramanjini & Prakash, 1998). Generally, in the development of multiple embryos, meristematic primordia developed are attributed to biophysical and genetical factors ungoverned by laws of embryogenesis. Some times in tissue culture (callus tissue) abnormal embryos also develop or the cells at the base of primordial develop tannins leading to the browning of callus and ultimate death and dedifferentiation of that tissue (Johri et al., 1996).

Basal MS medium supplemented with BA and activated charcoal failed to germinate the matured somatic embryo from both solid and liquid culture. The lack of germination of embryos in liquid medium was also observed by Chang (1995). Thus the cultures lack conversion into normal emblings and showed developmental anomalies. The reason for this may lie in the following conditions: -

1. Plant growth regulators and other factors studied were inhibitory at the given concentrations for plantlet formation which occur because of utilization by the tissue for its growth during this prolonged period of incubation.
2. Due to lack of laticifers in somatic embryo (Wilson, 1976; Dunbar et al., 1986; Kerry et al., 1986; Wilson & Dunbar, 1987).
3. Due to blocking of somatic embryos after the formation of cotyledons or
4. Due to lack of somatic embryos with a well structured and functional shoot apex and radical meristem (Chalupa, 1995a,b).

There are several reports of well-formed embryos that failed to produce plantlets (Wilson & Mahlberg, 1977; Radojevic, 1980; Ammirato, 1987; Narayanasamy, 1994). Unfortunately, for many reports, convincing documents on the formation of well-developed true embryos capable of growing into complete plant are lacking. Therefore, we must be concerned not only with those species that have yielded somatic embryos in culture but also the variables and conditions that have regulated their initiation and development. Interestingly, members of the Sapindaceae family have not readily produced somatic embryos from cultured somatic tissues. Thus the embryonic calli remain calcitrant to the interacting influence of plant growth regulators and additives in embryo germination but exhibited unlimited capacity of embryogenic callus proliferation. Though, approaches outlined here had given negative result, the various parameters studied related to embryogenic process serve at least to provide a basis for reconsideration of regulatory systems. All plant tissues have the characteristic cellular totipotency. Hence more works that directly explores tissue interaction should clarify understanding of in vitro tissue competency and perhaps shed light on the reasons for the failure of some kinds of explants or species to undergo any organ formation. Therefore, further studies like dessication or cold treatment (Chalpua, 1995b), thiadiazuran (Huetteman & Preece, 1993) etc., are needed to identify the stimulatory factor that induces somatic embryo germination in C. halicacabum. 

In many studies morphologically abnormal embryos like callused embryos or malformed embryos have been frequently observed (Choi et al., 1997). Therefore, the rate of plant conversion from somatic embryos and their successful soil transfer was low compared to zygotic embryos; these are the main barriers for commercial application of mass propagation (Green and Philips, 1975).
Somatic embryogenesis in liquid medium

The frequency of somatic embryo formation in liquid culture was much higher than in solid culture. Induction of somatic embryogenesis in suspension culture formed large clumps of globular and latter stages of embryos and the embryos underwent proliferation and development in the same medium. The number and the frequency of somatic embryo were dependent on 2,4-D concentrations in the induction medium. It has been demonstrated that 2,4-D has a critical role in induction of somatic embryogenesis in most plant species (Halperin & Whetherell, 1965; Ammirato, 1983). Between the two treatments - 2,4-D alone and 2,4-D+BA, the former one showed maximum percentage of embryogenic response. 2,4-D induced the maximum percentage of response in leaf and internodal culture in 28 days. In the present study, frequency of somatic embryogenesis was found to be the result of interaction between explant type and concentration of auxin and cytokinin used. Similar observation was also reported by Michler and Baur (1991) in Populus. The frequency of somatic embryo formation in liquid culture was much higher than in solid culture in Eleutherococcus senticosus (Choi et al., 1999).

The formation of globular stage embryos was observed within 28 days of time. The developmental stages such as heart, torpedo and early cotyledonary shaped embryos were observed in the first subculture in the same medium. Ammirato (1987) has documented the requirement of exogenous auxins for development of somatic embryos in tissue culture.

From these results, we summarize that unbalanced endogenous hormone distribution by exogenous hormone application may result in the abnormalities of somatic embryos. In many studies, somatic embryos have been reported to be morphologically different compared to zygotic embryos. Abnormal morphology such as poor cotyledon development, callused embryos or malformed embryos has been frequently observed (Pence et al., 1981; Choi et al., 1997). Therefore, the rate of plant conversion from somatic embryos and their successful soil transfer was low compared to zygotic embryos; these are the main barriers for commercial application of mass propagation (Green and Philips, 1975). Somatic embryos of several plant
species, even though they are genetically identical to the donor plant and are morphologically similar to zygotic embryos, can undergo abnormal development such as small and poorly developed cotyledons, low vigour, lack of desiccation tolerance and low germinability (Pence et al., 1981; Parrot et al., 1992; Krochko et al., 1992).

PHYTOCHEMICAL STUDIES

Plant tissue culture are potentially valuable for studying the biosynthesis of secondary metabolites and may also eventually provide an efficient means of producing commercially important plant products (Butcher, 1992). The production of secondary metabolites in plants is often restricted to specific tissues and organs during particular developmental stages. The morphological differentiation of plant cells into specialized cell types and tissues is accompanied by chemical differentiation that establishes the metabolic pathway leading to secondary product formation. The phytohormone, auxin has been proposed to act as a regulator of the switch between cell growth and secondary product formation (Goddijn et al., 1992; Pasquali et al., 1992). Hormone composition is often a critical factor in secondary product accumulation. The type and concentration of auxin and cytokinin usually in combination have also been shown to affect cell division, cell enlargement and root or shoot differentiation.

Differentiation and tissue organization are not only the factors that influence the synthesis of secondary metabolites, but environmental factors also can have marked influence on the production of certain secondary metabolites (Butcher, 1992). Genetic, morphologic, and biochemical factors do affect plant tissue cultures and their ability to produce a significant amount of a product. The expression of these factors is undoubtedly interrelated. A large number of callus and suspension cultures usually harvested after the completion of rapid growth phase, have been examined for the presence of secondary products characteristic of the whole plant or some organs of the plant. The production of a range of chemicals by using tissue culture is remarkable (Dougall, 1980).
In general, callus tissues retain the characteristics of the plant from which they are derived in their biosynthetic and regenerative potentiality. *Vinca, Atropa* and others produced compounds of pharmaceutical interest in their tissue culture as from intact plants (Yeoman, 1986). The potential for synthesis of chemicals by plants is enormous, ranging from highly pure compounds of pharmaceutical value to complex food materials. Growing interest in the practical aspects of plant tissue culture led to the exploration of cell culture system for the biosynthesis of a variety of natural products, a venture that has blossomed into a new technology with significant impact on plant biochemistry and industry. The production of a range of chemicals by using plant tissue culture is remarkable (Yeoman, 1987).

The results obtained by Indra et al. (1998) are consistent with the fact presented by Flores (1987) that the plant cell cultures produce a spectrum of compounds different from those produced by intact plant. Thus there are many uses of cell and organ culture in the production of biological chemicals.

**Callus**

It was recognized that different tissue explants taken from different parts of a plant within and between genotype having varying growth regulator requirements for callus growth. The morphogenesis and effect of various growth regulators on callus induction using different explants in *Lablab purpureus* var. *lignosus* (Thiruvengadam & Jayabalan, 2000). Callus cultures may be derived from a wide variety of plant organs for specific cell types. The general growth characteristics of a callus involve a complex relationship among the plant material used to initiate the callus, the composition of the medium and the environmental conditions during the incubation period. Mohamed & Jayabalan (1996) developed a protocol for callus induction in *Macrotyloma uniflorum*.

The *in vivo* plant material and callus derived from *C.halicacabum* was undergone preliminary phytochemical screening. The results from the phytochemical studies showed many important findings like some compounds are present in both material sources. But some compounds are exclusively present in any one of the two-
studied materials. Thus the synthesis of secondary metabolites varies between *in vivo* and *in vitro* sources. Under *in vivo* condition the production of secondary compounds depends on the environmental conditions, where the plant survives. But *in vitro* condition the production is influenced by the chemical composition of the medium especially plant growth regulators.

Biomass and secondary metabolite accumulation were assayed on a wide range of auxins, cytokinins and their combinations using *Ecballium elaterium* callus tissue (Attard & Spiteri, 2001).

In leaf, alkaloids were mostly absent, but in callus only alkaloids were observed, this might be due to the action of growth regulators and *in vitro* environmental conditions. Alkaloid production has been observed in cotyledonary leaf derived callus tissues, and also in *in vitro* differentiated shoots and roots of *Hyasyamus muticus* (Basu & Chand, 1998).

1. Only three compounds namely volatile oils, tannins and steroids were present ubiquitously in all samples i.e. in all solvent extracts of leaf and callus.
2. Fatty acids are also present in all except one sample i.e. callus benzene extract.
3. Flavanoids were present in all leaf samples except ethyl acetate extract. On the other hand only methanol extract of callus showed positive for flavonoids.
4. Saponin was present in all extracts of callus, but absent in acetone and ethanol extract of leaf.
5. All callus extract showed positive for alkaloids except acetone extract. In the case of leaf, alkaloids present only in benzene and acetone extract.
6. Phenolics were absent in all extracts of callus but present only in ethanol and methanol extract of leaf.