DISCUSSION

Micropropagation from axillary buds, callus culture from in vitro harvested leaf discs, isozyme analysis of callus derived plants, somatic embryogenesis, protoplast culture, electrophoretic analysis of callus proteins and effect of seasonal variation on shoot initiation from axillary buds were studied in Terminalia arjuna, Madhuca longifolia and Mentha piperita. The results were discussed below:

I. MICROPROPAGATION

In the present study axillary buds were used as explants for micropropagation because, axillary buds have proven to be useful for multiplying mature selected angiosperm trees, such as teak (Gupta et al., 1980), Eucalyptus (Gupta et al., 1981) and aspen (Ahuja, 1984).

1. SHOOT INITIATION

1.1. Basal Media

The success of tissue culture mostly depends on the type of basal medium used. In the present study MS medium supplemented with BAP and KN was found...
to be suitable than the SH and WPM for shoot initiation in *T. arjuna*, *M. longifolia* and *M. piperita*. It was suggested that a suitable starting point for initiation of the callus from a dicot tissue explant would be the MS basal medium (Dodds and Lorin, 1985).

From the table-4 it is obvious that in *T. arjuna* and *M. longifolia* the response for shoot initiation was in the order of MS > WPM > SH and in *M. piperita* MS > SH.

1.2. Growth regulators and shoot initiation

1.2.1. BAP and KN

Supplementing MS medium with BAP (1 or 2 mg/l) alone was found not effective for shoot initiation from axillary buds of *T. arjuna* and *M. longifolia*. In *T. arjuna*, MS medium supplemented with BAP (1 mg/l) showed neither shoot initiation nor death of explant even after 45 days of culture. But the addition of KN (0.01 mg/l) to the above said medium showed 63.1% of shoot initiation after 18 days (Table 1).

In *M. longifolia* 9.3% of shoot initiation in MS + BAP 1 mg/l and 46.3% in MS + BAP (1 mg/l) + KN (0.1 mg/l) was observed (Table 2). Hence, the addition
of BAP and KN to the MS medium was found to be suitable for effective shoot initiation. Similar results were observed in *Dalbergia latifolia* (Swamy et al., 1992). In *Pterocarpus santalinus*, B5 medium supplemented with two cytokinins (BAP and KN) was found to be suitable for single shoot growth from shoot tips (Sita et al., 1992).

In *M. piperita* BAP (2 mg/l) alone was found to be suitable for shoot initiation from axillary buds. Addition of BAP (2 mg/l) to MS medium showed 82.3% of shoot initiation, but the addition of KN (0.01 mg/l) to the same medium showed 68.5% of shoot initiation (Table 3).

1.2.2. GA₃

Shoot initiation period was reduced in MS medium supplemented with lower concentrations of GA₃ in *T. arjuna, M. longifolia* and *M. piperita*.

In *T. arjuna* shoot initiation period was reduced from 18 days to 16 days by the addition of GA₃ 0.01 mg/l to MS + BAP 1 mg/l + KN 0.01 mg/l. Addition of GA₃ 0.05 mg/l to the above medium reduced the number of days to 10, but it also reduced the percentage of shoot initiation (Table 5).
In *M. longifolia*, shoot initiation period was reduced from 14 days to 12 days by the addition of GA$_3$ 0.05 mg/l to MS + BAP 1 mg/l + KN 0.1 mg/l. Addition of GA$_3$ 0.1 mg/l to the above medium reduced the number of days to 8, but it also reduced the percentage of shoot initiation (Table 6).

In *M. piperita* shoot initiation period was reduced from 16 days to 14 by the addition of GA$_3$ 0.05 mg/l to MS + BAP 2 mg/l. No further reduction in number of days was observed by increasing the concentration of GA$_3$ (upto 1 mg/l) in the above medium (Table 7).

In *Carica papaya* also the addition of GA$_3$ to modified MS medium showed shoot initiation within a short period (Mondal et al., 1990).

1.3. **Anti-oxidant and adsorbents on browning of explants**

Phenolic exudation and browning of explants especially during culture initiation (Thorpe and Patel 1984; Dan-Hua and Meredith, 1986) are common in tissue culture. Browning of cultures due to the formation of various phenol derivatives leads the inhibition of morphogenic response. Recommended various approaches to reduce this problem include pre-treatment of explants with anti-oxidants and incorporation of antioxidants into the culture medium (Dalal et al., 1991).
In the present study activated charcoal (AC) combined with ascorbic acid (AA) was found to be suitable for controlling the browning of explants. AC will adsorb many organic and inorganic molecules from the culture medium, and this substance has been used in a variety of tissue culture systems. It may remove contaminants from agar and secondary products secreted by the cultured tissues or possibly regulate the supply of certain endogenous growth regulators (Wang and Huang, 1976 and Fridborg et al., 1978).

_Cocculus pendulus_ tissues grown in medium containing different concentrations of AC and PVP showed less phenolic effect and maximum protein content (Bhardwaj and Ramawat, 1993).

2. SHOOT MULTIPLICATION

2.1. BAP and KN

MS medium supplemented with BAP was found to be suitable for multiple shoot formation in _T. arjuna_ and _M. longifolia_. However, addition of KN to MS medium along with BAP increased number of shoots per explant.
In *T. arjuna*, 2.6 shoots per explant was developed in MS medium supplemented with BAP 2 mg/l, addition of KN 1 mg/l to the above medium showed the formation of 4.6 shoots per explant (Table 9).

In *M. longifolia* 1.6 shoots per explant were observed in MS medium supplemented with BAP 2 mg/l and addition of KN 1 mg/l showed 2.1 shoots per explant. Further improvement in number of shoots per explant was observed in MS + BAP + KN + NAA treatment (Table 9). Similar results were observed in *Pterocarpus santalinus*, wherein the addition of BAP and KN was found to be suitable for multiple shoot formation (Sita et al., 1992).

In *M. piperita* 8.6 shoots per explant was observed in MS medium supplemented with BAP 2 mg/l. Whereas, the addition of KN (1 mg/l) reduced the number of shoots per explant to 2.5 (Table 9). Hence, KN was found to be ineffective for multiple shoot formation as it was also observed in *Brassica* (George and Rao, 1980) and *Carthamus* (George and Rao, 1982). In sesame KN was found totally ineffective for the induction of multiple buds and only a single shoot was developed from explant (George et al., 1987).

In the present study, supplementing the medium for shoot initiation and shoot multiplication with BAP was found to be essential. But increased concentration of
BAP (above 2 mg/l) reduced the response in all the three plants. In Populus hybrids, high BAP concentration resulted in stunted growth and Zeatin was found superior to BAP in stimulating axillary shoot growth (Coleman and Ernst, 1990). Supra-optimal levels of BAP resulted in stunted shoots has been reported for other woody genera (Brand and Lineberger, 1986 and Sutter and Barker, 1985).

2.2. NAA

In T. arjuna number of shoots per explant was reduced from 4.6 to 3.3 by the addition of NAA (0.1 mg/l) to MS + BAP (2 mg/l) + KN (1 mg/l). The addition of NAA resulted in development of callus at the base of explant (Table 9) as reported in Pterocarpus santalinus (Sita et al., 1992).

In M. longifolia, supplementing MS medium with NAA (0.1 mg/l) along with BAP (2 mg/l) and KN (1 mg/l) showed the maximum number of shoots (3.3) per explant. Deletion of NAA from the medium reduced the number of shoots per explant to as low as 2.1 (Table 9). The positive effect of NAA on apical and axillary meristems derived from 10 day old seedlings of M. longifolia was reported (Rout and Das, 1993).
In *Carica papaya* the addition of NAA to shoot multiplication medium was found to be essential (Mondal *et al.*, 1990). In *Populus X Wilsoecarpa* also the addition of NAA with BAP showed the best multiplication (Welander *et al.*, 1989).

In *M. piperita*, the addition of NAA (0.1 mg/l) to MS + BAP (2 mg/l) + KN (1 mg/l) reduced the number of shoots per explant from 2.5 to 2.1. Moreover, callus development at the cut end of explant was also observed (Table 9).

3. **SHOOT ELONGATION**

3.1. **Basal medium and BAP**

MS medium having half strength macronutrients was found to be better than the full strength MS medium for shoot elongation in *T. arjuna, M. longifolia* and *M. piperita* (Table 10).

In *T. arjuna*, 2.3 cm (maximum) of shoot elongation was observed in MS (half strength macronutrients) supplemented with BAP 0.01 mg/l. In MS medium (full strength macronutrients) supplemented with BAP 0.01 mg/l, 0.8 cm of shoot elongation was observed.
In *M. longifolia*, 2.1 cm (maximum) of shoot elongation was observed in MS (half strength macronutrients) + BAP 0.01 mg/l and 0.9 cm in MS (full strength macronutrients) + BAP 0.01 mg/l.

In *M. piperita*, shoot elongation was 2.1 cm in MS (half strength macronutrients) + BAP 0.01 mg/l and 1.1 cm in MS (full strength macronutrients) + BAP 0.01 mg/l.

In the present study, half strength macronutrients -MS medium was effective for shoot elongation than the full strength macronutrients-MS medium. Shoot buds (0.5-1 cm) of *Dalbergia latifolia* subcultured to MS medium with reduced major elements or low salt Woody Plant Medium showed a maximum of shoots with 3-4 cm length (Swamy et al., 1992).

3.2. GA₃

In *T. arjuna* and *M. longifolia*, addition of GA₃ (0.1 mg/l) has not improved shoot elongation. Similar observation was reported in a hybrid popular (Agarwal and Gupta, 1991). The failure of GA₃ for differentiation and elongation of shoots in internodal/nodal explants may be due to its inhibitory effect (Douglas, 1989). Whereas, Welander et al., (1989) reported that GA₃ stimulated shoot elongation in *Populus x Wilsocarpa*. 

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In *M. piperita*, maximum shoot elongation (4.9 cm) was achieved with the addition of GA$_3$ (0.1 mg/l) to MS (half strength macro nutrients) + BAP (0.01 mg/l). In MS (full strength macronutrients) + BAP (0.01 mg/l) + GA$_3$ (0.1 mg/l), 1.3 cm of shoot elongation was observed. Increasing the concentration of GA$_3$ to 1 mg/l showed less shoot elongation.
II. CALLUS CULTURE

The effect of auxins (2,4-D, NAA & IAA), cytokinins (BAP & KN) and gibberellins (GA3) supplemented to MS medium on callus culture from leaf discs of *T. arjuna*, *M. longifolia* and *M. piperita* were studied. The shoots differentiated from callus were analysed for catalase isozyme pattern.

1. CALLUS INITIATION AND CULTURE

Callus was initiated from leaf discs. The initiated calli were subcultured on to the same medium for further development. Shoots were differentiated after subcultures.

1.1. Callus initiation

1.1.1. 2,4-D and NAA

2,4-D was found to be the suitable auxin for callus initiation and development from leaf discs of *T. arjuna* and *M. longifolia* than NAA. Callus development response was improved with increase of the concentration of 2,4-D (Table 11 & 12).
NAA was found to be the suitable auxin for initiation and development of callus from leaf discs of *M. piperita*. The response of leaf discs to 2,4-D was lower than the response to NAA (Table 13). Similar results were reported in *Mentha* by Eck and Kitto (1990). Regeneration from *M. piperita* callus (initiated from mature embryos) occurred on basal medium containing BAP and NAA (Eck and Kitto, 1990). Budding from callus cultures of *Mentha arvensis* var. *piperascens* was observed on medium supplemented with NAA and KN (Ono, 1982).

1.1.2. BAP and KN

Supplementing the callus induction medium with BAP as the sole cytokinin source resulted in low level of response in all three species. Whereas, the combination of BAP and KN proved better for callus induction and culture. However, it was reported that BAP, with an auxin, induced callus in hybrids of *Populus alba* x *P. grandidentata* (Son and Hall, 1990), *Mentha* (Eck and Kitto, 1990), peanut and pigeon pea (Eapen and George, 1993).
1.2. Shoot differentiation

1.2.1. 2,4-D, IAA and NAA

In *T. arjuna*, shoot differentiation from callus was observed on MS medium supplemented with 2,4-D, IAA and BAP. Whereas, the deletion of 2,4-D from medium lead to a failure or showed a slight decrease in percentage of shoot differentiation (Table 14).

In *M. longifolia*, the removal of 2,4-D from the best shoot differentiation medium (MS + BAP + IAA) increased the percentage of shoot differentiation (Table 15).

IAA was found to be the effective auxin for shoot differentiation from calli of *T. arjuna, M. longifolia* and *M. piperita*. At lower concentration (0.01 mg/l), it induced shoot differentiation with BAP in all three plants. Higher levels of IAA (above 0.01 mg/l) reduced the shoot differentiation. The beneficial effect of IAA in shoot differentiation was supported by earlier works. The presence of IAA and BAP in the medium showed shoot and root development from tomato hypocotyl.
cultures (Magnus et al., 1992). The maximum shoot regeneration was induced in peanut and pigeon pea leaf discs in medium containing IAA or IAA-amino acid conjugate in combination with BAP (Eapen and George, 1993).

The maximum percentage of shoot differentiation in T. arjuna was in MS + NAA + BAP. NAA was totally ineffective in shoot differentiation of M. longifolia. In M. piperita low percentage (4.3%) of shoot differentiation was observed in NAA supplemented medium (Tables 14, 15 & 16). Earlier studies in M. piperita revealed that NAA and BAP showed shoot differentiation in callus developed from mature embryos (Eck and Kitto, 1990). But, in the present study leaf discs were used for callus culture. Leaf discs of M. piperita, cultured on basal medium supplemented with BAP and NAA, showed no shoot regeneration, but the leaf discs of M. citrata regenerated in the same medium (Eck and Kitto, 1992). Hence, NAA may not be effective for shoot regeneration from leaf or leaf derived callus of M. piperita. The beneficial effect of NAA on callus embryogenesis in Kaempferia galanga was also reported (Vincent et al., 1992).

1.2.2. BAP

Shoot regeneration from callus of T. arjuna, M. longifolia and M. piperita was not observed on MS medium supplemented with BAP alone (Tables 14, 15 & 16).
Whereas, in *M. citrata* and *M. piperita* shoots were regenerated from leaf disc explants on medium containing BAP and coconut water (Eck and Kitto, 1992).

1.2.3. GA₃

GA₃ (0.01 mg/l) combined with BAP and IAA showed (13.3%) shoot differentiation in *M. piperita*. But, deletion of GA₃ in the medium increased the percentage of shoot differentiation (22.3%) (Table 16). In *T. arjuna* and *M. longifolia* GA₃ inhibited the differentiation of shoots. Hence, GA₃ had no promotary effect in the concentration of 0.01 mg/l combined with BAP and IAA/ NAA. In contrast, Kumar et al. (1983) have used GA₃ combined with BAP and NAA in Blayde’s medium for regeneration from leaf callus of pigeon pea.

2. ISOZYME ANALYSIS

Biochemical traits, such as isozymes, provide a tool to study the extent of somaclonal variation in a manner analogous to their use in elucidating genetic variation in natural populations (Adams and Joly, 1980 and Bhaskaran et al., 1987).
In the present study catalase isozyme variations were observed among *T. arjuna* and *M. piperita* plants, regenerated from callus cultures (Plate III), but no visible morphological variation was observed.

In *T. arjuna* the variation was observed in the Rf value of slow moving band. In the parental pattern the Rf value was 0.64, but in the variant the Rf value was 0.65. The Rf value of fast moving band (0.76) was similar in parental and variant pattern.

In *M. longifolia* no variation was observed. The Rf value of the band was 0.31.

In *M. piperita* the parental band was a fast moving band (Rf 0.49) when compared to its variant (0.46).

Several tissue-specific isoforms of catalase subunits were reported in castor bean. The endosperms and cotyledons have 54 and 56-kDa sub-units and the hypocotyls have 56 kDa sub-units (Ota et al., 1992).

Heinz and Mee (1971) found that 30-80% of the regenerants of sugarcane showed variation in peroxidase, amylase and trans-aminase isozymes. No direct correlation between isozyme variations and cytological or morphological characteristics was observed.
III. ROOTING

The shoots excised from multiple shoots of axillary buds and callus culture of leaf explant of *T. arjuna*, *M. longifolia* and *M. piperita* were subjected to rooting by using different concentrations of basal medium, cytokinin and auxins (Tables 17, 18 & 19).

1. Basal medium

Half strength MS medium was found to be suitable for rooting of *T. arjuna*, *M. longifolia* and *M. piperita* than the full strength MS medium. In *Dalbergia latifolia*, shoots showed 80% of root initiation in half strength MS medium, supplemented with IBA (Swamy et al., 1992). In *Carica papaya* half strength modified MS medium (Mondal et al., 1990) and in *Quercus acutissima* also half strength MS medium (Moon et al., 1987) were found to be suitable for rooting.
2. Growth regulators

2.1. NAA, IAA and IBA

NAA, IAA and IBA were used for rooting with BAP in half strength MS medium. Among the three auxins, IBA was found to be better than the other two auxins. However, the combination of two auxins showed maximum rooting.

In *T. arjuna*, NAA combined with IBA showed maximum rooting percentage (33.6%) and substitution of NAA with IAA reduced the rooting percentage (30.4%).

In *M. longifolia*, maximum rooting was observed on NAA, IAA and IBA combination (18.5%) or NAA and IBA combination (17.4%). Substituting the NAA with IAA (half MS + BAP + IAA +IBA) reduced the percentage of rooting to 14.3%.

In *M. piperita* maximum rooting (62.4%) was observed on half strength MS medium supplemented with IBA. Addition of IAA (1 or 2 mg/l) reduced the percentage of rooting.

Rout and Das (1993) observed that IBA (1 mg/l) alone induced rooting, in *M. longifolia* shoots developed from seedling explants, and at IBA 1.5 mg/l
reduced rooting and initiated the basal callus formation. But in the present study, IBA (2 mg/l) with NAA (1 mg/l) and with or without IAA (1 mg/l) showed the maximum rooting percentage. The differential behaviour may be due to the explant selection as reported by Rout and Das (1993). Whereas, Sita et al. (1992) reported that in *Pterocarpus santalinus* IAA showed better rooting than NAA and IBA. D’Silva and D’Souza (1992) reported the optimum rooting on MS + IAA + IBA in Cashew.

In *T. arjuna* and *M. longifolia* single or double thick roots were developed on IBA and NAA supplemented medium (Plte IV - A & B) as reported in *Pterocarpus santalinus* (Sita et al., 1992).

Supplementing the rooting medium with NAA (2 mg/l) showed the callus formation at the basal end of plantlets. *M. piperita* showed callus formation even at NAA 1.0 mg/l. Similar results were observed in Cashew on rooting medium supplemented with NAA (D’Silva and D’Souza, 1992), and in *M. longifolia* on rooting medium supplemented with IBA (1.5 mg/l) (Rout and Das, 1993).
2.2. BAP

BAP (0.001 mg/l) supplemented to half strength MS medium with auxin was used for rooting of *T. arjuna*, *M. longifolia* and *M. piperita*. However, the deletion of BAP from the rooting medium was not showing much difference in rooting percentage. The plantlets of *T. arjuna* and *M. longifolia* grown on BAP deleted medium showed poorly developed leaves, whereas, in *M. piperita*, the leaf development was normal.
IV. HARDENING AND SOIL TRANSFER

*T. arjuna*, *M. longifolia* and *M. piperita* plants grown and rooted in *in vitro* were transferred to culture tubes having 10-15 ml of distilled water for hardening. After 15 days, the plants were transferred to plastic containers filled with growth medium [sand, red soil and vermiculite (1:1:2)]. The plants were kept under high humid condition. After 20 days, the plants were transferred to room temperature and after 30 days to the nursery condition. During acclimatization, 1/10 MS basal liquid medium (without sucrose) was added to the growth medium.

Acclimatization and survival in field condition was high in *M. longifolia* (58%) than the *T. arjuna* (21%) and *M. piperita* (56%). Since *M. longifolia* is a deciduous, drought tolerant plant, it has thick leaves. So, they can acclimatize easily by tolerating the *ex vitro* conditions during soil transfer. The leaves of *T. arjuna* showed wilting symptoms within 1-2 days after soil transfer. Similarly, *M. piperita* leaves were also showed wilting symptoms within a short period after soil transfer. Eventhough the plant survival after soil transfer were low it can be improved with the help of mist chamber and glass house facilities.
V. SOMATIC EMBRYOGENESIS

Direct somatic embryo formation was observed on leaf discs on solid medium. Somatic embryos were obtained from suspension culture of callus induced from leaf discs.

In the present study MS medium was used for somatic embryogenesis. In 70% of instances somatic embryogenesis was mostly achieved on MS medium and on a slightly modified MS medium (Reinert, 1958). One of the characteristics of this medium is its relatively high concentration of nitrate, potassium and ammonium ions in comparison to other nutrient media (Dodds and Lorin, 1985). Moreover it had proved that MS medium was effective for the growth of a variety of dicot and monocot plants (Dixon, 1987).

1. DIRECT SOMATIC EMBRYO FORMATION

1.1. Glutamine and direct somatic embryo and embryogenic callus formation

L-glutamine promoted direct embryogenesis but inhibited embryogenic callus formation in T. arjuna and M. piperita. Similar results were also observed in Populus spp. (Michler and Bauer, 1991). No direct somatic embryos were formed on leaf discs of M. longifolia even with the addition of glutamine. But it inhibited
the amount of embryogenic callus formation (Table 20). Nitrogen provided by MS salts was probably at low optimal level for stimulating embryogenic callus formation, and glutamine may have had a more specialized role to induce differentiation of pre-determined direct embryogenic cells as hypothesised by Kamada and Harada (1979).

Glutamine at 5 mM added to White's basal medium which contained 3.2 mM inorganic nitrogen proved effective for embryogenesis, while higher concentrations of KNO₃ (40 mM) were necessary to achieve the same results. However, embryos were never formed with nitrate as a sole source of nitrogen at any concentration within the physiological range but was necessary for the development of advanced stages (Reinert and Bajaj, 1992).

In Colocasia esculenta var. antiquorum, addition of glutamine was found to improve the in vitro germination of zygotic embryos (Nyman et al., 1987).

2. SUSPENSION CULTURE

2.1. Globular-shaped somatic embryo formation

2.1.1. 2,4-D and NAA

The importance of auxins in somatic embryogenesis is well documented
Among the auxin compounds presently used, 2,4-D has been particularly found to be an effective inducer of somatic embryos in more than 50% of plant taxa reported for successful somatic embryogenesis (Evans et al., 1981).

In the present study, globular-shaped somatic embryo formation was not observed in the 2,4-D deleted medium (MS + BAP + KN) in T. arjuna and M. longifolia (Tables 21 & 22). However, the effect of 2,4-D in induction and/or development of somatic embryos is variously interpreted.

Inhibition of somatic embryo induction by 2,4-D was reported in Liriodendron tulipifera (Merkle and Sommer, 1986). In Corylus avellana, if 2,4-D did not inhibit the induction of embryo, it inhibits their further development into plantlets (Radojevic et al., 1975). However, the present study indicated that 2,4-D is highly essential in somatic embryo induction and its further development into mature embryos in T. arjuna and M. longifolia as reported in Crataeva nurvala (Inamdar et al., 1990).

In M. piperita, NAA was found to be the better auxin than 2,4-D for somatic embryogenesis (Table 23) as observed in callus culture. No somatic embryo formation was observed in NAA deleted medium (MS + KN + BAP).
2.1.2. BAP and KN

BAP alone supplemented to MS medium with an auxin showed low number of globular-shaped somatic embryos, and the addition of KN increased the number. In T. arjuna 16 somatic embryos/ml were observed in MS medium supplemented with BAP (0.1 mg/l) + 2,4-D (3 mg/l), addition of KN (2 mg/l) to the above medium showed 362 somatic embryos/ml. In M. longifolia, 5 somatic embryos/ml was observed in MS medium supplemented with BAP (0.1 mg/l) + 2,4-D (3 mg/l), addition of KN (2 mg/l) to the above medium showed 174 somatic embryos/ml. In M. piperita, 46 somatic embryos were observed in MS medium supplemented with BAP (0.1 mg/l) + NAA (3 mg/l), addition of KN (2 mg/l) to the above medium showed 232 somatic embryos/ml (Tables 21, 22 & 23).

Whereas in Populus deltoides BAP alone (MS + 2,4-D + BAP) produced more number of globular-shaped embryos (Michler and Bauer, 1991). The effect of BAP and KN were variously interpreted. Halperin (1966) reported that, KN is effective in maintaining the embryo-forming potential in solid cultures of Carrot for a long period. In Asparagus, KN allows differentiation of embryos at concentrations above $5 \times 10^3$M (Wilmar and Hallendoorn, 1968). In Apium, KN at a concentration of 0.1 mg/l promoted embryogenesis (Halperin and Jensén, 1967).
2.1.3. Ascorbic acid

Ascorbic acid (AA) in the suspension culture medium promoted the formation of more somatic embryos. In *Oldenlandia umbellata* addition of AA with BAP promoted the formation of more somatic embryos than in BAP alone (Rao and Bahadur, 1990).

2.2. Maturation of globular-shaped somatic embryos

The globular-shaped somatic embryos of *T. arjuna*, *M. longifolia* and *M. piperita* were transferred to liquid MS medium with 1.5% sucrose and low level of auxin (2,4-D / NAA). Three percent sucrose inhibited the maturation of these three plants. In *Zea mays* 6% of sucrose was found to be favourable for embryo maturation. However, higher percentage of sucrose (9%) did not improve the maturation efficiency (Emons et al., 1993).

In the maturation medium low concentration of auxin (2,4-D/NAA) was found to be necessary in the present study. But in *Populus* spp., removal of 2,4-D from the culture medium favoured the maturation of globular-shaped somatic embryos (Michler and Bauer, 1991).
2.3. Germination of somatic embryos

Germination of *T. arjuna* and *M. longifolia* somatic embryos were not satisfactory. The germination was not complete. This may be due to the growth condition provided at early or later stages of development as reported in *Populus* spp., where most germinated (in liquid medium) somatic embryos, that were regenerated solely in liquid media, were vitrified and did not survive the transition *ex vitro* (Michler and Bauer, 1991).

Solid MS medium was used for germination of mature somatic embryos as reported in *Populus* spp. (Michler and Bauer, 1991).

MS medium supplemented with IAA, BAP and ABA was found to be favourable for germination of somatic embryos of *T. arjuna*, *M. longifolia* and *M. piperita* than using single or any two growth regulators. Culturing embryos on medium supplemented with IAA, BAP and ABA for a week period and transfer to growth regulator free medium induced germination in all the three plants. In *Populus* spp. embryos proceed to germinate after one week pulse treatment of IAA on solid media (Michler and Bauer, 1991).
Inclusion of ABA was found to be favourable for germination. ABA has been implicated to be involved in controlling many events during zygotic embryogenesis including morphogenesis (Quatrano, 1987). Abnormal morphology of somatic embryos has been remedied by exogenous application of ABA to the culture media (Ammirato, 1974). ABA could be a limiting factor needed to regulate cell division, cell size, and differentiation and thereby reduce overall embryo size and attain normal developmental morphology (Michler and Bauer, 1991).
VI. PROTOPLAST CULTURE

1. PROTOPLAST ISOLATION AND CULTURE

1.1. Protoplast isolation

Protoplasts were isolated from leaves (obtained from micropropagated plants) of T. arjuna, M. longifolia and M. piperita and cultured up to callus level.

In the present study macerozyme and cellulase were used for protoplast isolation. These enzymes were found to be suitable for protoplast isolation from hypocotyls of Sesamum indicum (Dhingra and Batra, 1990), leaves of peppermint (Sato et al., 1993) and leaf mesophyll of hybrid poplar (Park and Son, 1992).

In the present study plasmolysis of leaves in 9% mannitol increased protoplast yield in all the three plants. Mannitol is considered to be relatively inert metabolically and infuses slowly into the protoplasts (Eriksson, 1985).

1.2. Protoplast culture

Protoplasts were cultured in liquid K3 medium. One important advantage of liquid culture is that it allows a gradual change of the osmolarity of the culture medium and in this way promotes rapid cell regeneration.
Sorbitol and mannitol were added to protoplast culture medium because, the wall pressure must be replaced by osmotic pressure in the culture media. The stability, viability and future growth of protoplasts are closely related to the maintenance of proper osmotic conditions during isolation and subsequent culture (Eriksson, 1985).

The protoplasts were initially cultured in K3 medium supplemented with an auxin (2,4-D/NAA concentration higher than cytokinin) and a cytokinin (BAP). After microcolony formation the concentrations of growth regulators were increased. In general, protoplast culturing starts with a relatively high (1-3 mg/l) concentration of NAA or 2,4-D along with a lower (0.1-1.0 mg/l) of BAP or Zeatin. When protoplast division has started, it is often recommended to change the exogenous hormone supply (Eriksson, 1985).

The protoplasts of *T. arjuna*, *M. longifolia* and *M. piperita* were cultured in dark condition. Generally, high light intensity inhibits protoplast growth when applied from the beginning of the culture. The inhibition by high light intensity is not clearly understood but might be related to the rapid bleaching of the chloroplasts. There are reports of better protoplast growth when the cultures are kept in continuous dark (Santos et al., 1980 and Arcioni et al., 1982). In contrast, it has been shown that for legume species light was necessary for initiating protoplast division (Oelck et al., 1982).
Morphological differences between embryogenic and non-embryogenic callus phenotypes are well known and provide a basis for selection of cultures with a high efficiency for plant regeneration (Nabors et al., 1983). Several biochemical changes occur during the process of callus development and shoot differentiation. The use of soluble protein profiles to identify embryogenic tissue is gaining acceptance as in maize (Everett et al., 1985). In the present study protoplast-callus protein profiles are compared with the protein profiles of embryogenic leaf-callus protein profiles in *T. arjuna*, *M. longifolia* and *M. piperita*.

Protein profiles of protoplast-calli of *T. arjuna*, *M. longifolia* and *M. piperita* closely resembled to the protein profile of their corresponding leaf derived embryogenic callus. So, analysis of soluble proteins of embryogenic and non-embryogenic calli showed distinct differences in rice (Chen and Luthe, 1987). Polypeptides in the range of 35-45 kDa were observed in protoplast and leaf callus of these three plants. In rice, 40-45 kDa polypeptides were reported to be more abundant in embryogenic calli and in embryo extracts (Chen and Luthe, 1987).
In *M. longifolia* two polypeptides with molecular weight of less than 29 kDa were observed. Presence of low molecular weight bands (below 29 kDa) are reported to be dense in non-embryogenic callus of sandalwood (Mhatre *et al.*, 1991). In contrast Yasuda *et al.* (1980) reported that synthesis of low molecular weight proteins (16,000 to 20,000 daltons) may be associated with early stages of adventitious bud formation in Douglas fir cotyledons cultured under *in vitro* condition.
VII. SEASONAL VARIATION

Seasons influence the various stages of life activities such as growth, metabolism, reproduction etc. The mineral nutrient status of the plant also differs depending upon the season as reported in golden delicious apple in which leaf nitrogen content declined gradually with the active growth period from May to end of August and then rose slightly during September; Potassium content decreased continuously from May to early August (Verma and Singh, 1990). In Guava maximum extension growth was observed in spring season (Dwivedi et al., 1991). So, the explants collected at a particular season responded differently under in vitro condition as observed in Lilium speciosum (Robb, 1957), in which bulb scales regenerated bulbils freely when taken from plants during spring and autumn periods of growth, but not if taken during summer or winter.

In the present study July to September showed better shoot initiation in all three plant species with slight variations. Likewise, during May-June low or no shoot initiation was observed. These results show similarities with the results reported in Eucalyptus tereticornis (Das and Mitra, 1990).