Chapter - 4

Discussion

The somatic embryogenesis in conifers is restricted to explants like mature and immature zygotic embryos, cotyledons from germinating seeds, secondary needles, female gametophytes etc. But till date there is no report of induction of somatic embryos in any conifer using explants from mature trees. In *Pinus kesiya* it was possible to induce somatic embryogenesis from mature zygotic embryos, secondary needles from seedlings grown in a glasshouse and apical dome sections from mature trees.

Dissecting immature zygotic embryos from seeds throughout the growing season to provide sufficient embryogenic material for experimentation is very labour-intensive and the material is available for only a short duration annually. The induction of somatic embryogenesis from mature embryos dissected from stored seed can extend the period during which embryogenic material can be
generated from just a few months to more than a decade (Gupta and Durzan, 1986a; Tautorus et al., 1990). Since mature seeds contain embryos of similar developmental stage which are available throughout the year, the variability of response observed with immature embryos of differing maturity is eliminated, thus permitting detailed studies of factors influencing induction. Mature seeds of *P. kesiya* collected during late January to late February produced better embryogenic cultures (Table 3). This is the period when seeds dehisced. The seeds collected during March gave poor response, as the embryos in these seeds were not healthy.

In the present study, a poor embryogenic response was recorded in case of zygotic embryos dissected from dry seeds. The stratification of seeds for 24 h at 4°C prior to dissection of embryos exhibited a pronounced promotive effect on the formation of embryogenic cultures. Increase in the stratification period and use of 1 week old germinating embryos (cultured at 25°C) resulted in poor embryogenic response. The effect of seed imbibition period on induction of somatic embryogenesis was investigated by various workers. Tremblay (1990) reported better frequency of induction of embryogenic cultures following a 4 h imbibition of *P. glauca* seeds. Other workers like
Hakman et al. (1985) in *P. abies*; Hakman and Fowke (1987a) in black and white spruce; Finer et al. (1989) in *P. strobus*; Attree et al. (1990b) in black and white spruce; Nagmani et al. (1993) in *P. palustris*; Lelu et al. (1994) and Bonga et al. (1995) in *L. decidua* reported that, pre-cotyledonary to pre-germinating embryos were suitable for initiation of embryogenic culture. In *P. strobus* (Finer et al., 1989) reported that the best stage of embryo development for embryogenic culture initiation was prior to cotyledon development of the zygotic embryos. Cotyledonary stage embryos yielded embryogenic cultures with a frequency of ~0.1%. The decline in embryogenic response of germinating embryos of *P. kesiya* could be due to the fact that, biochemical and molecular events may have set in which lead to germination.

Secondary needles of *P. kesiya* from 5-6 weeks old seedlings collected during April to May produced better embryogenic cultures than collected during the other parts of the year. A heavy rainfall and moderate temperature are experienced which are ideal during April to May, which are ideal for seed germination and formation of healthy needles in the natural conditions. From June onwards the secondary needles become harder and show poor initiation of callus, which is
mostly non-embryogenic, and hard in texture. There are several reports available in literature on the formation of embryogenic cultures using secondary needles. In *P. abies* embryogenic callus was established from explants excised from 7 day old seedlings (Krogstrup, 1986; Lelu *et al.*, 1987). Embryogenic callus was also initiated from 12-30 day old seedlings of *P. glauca* and *P. mariana* germinated from 4 and 10 year stored seeds (Attree *et al.*, 1990b). Ruau et al. (1992) described induction of somatic embryogenesis of *P. abies* using needles of 14 months old somatic seedlings growing in a greenhouse. Wescott (1992) could induce embryogenic callus from buds and needles of seven year old trees of *P. abies*.

The sections of apical dome of *P. kesiya* collected during May to July (before flushing of needles) formed better embryogenic cultures. During this period pine forests rejuvenate and cambial activity is maximum. The apical domes are also highly meristematic. The thinner the apical dome sections (0.2-0.5 mm thick) better was the embryogenic response. Priming of apical dome sections at 4°C for 72 h on ½DCR medium containing 0.4% activated charcoal resulted in optimum embryogenic cultures (Figure 4 and 5). A higher temperature of 25°C resulted in browning of cultures. The low temperature
treatment of explants in the medium containing activated charcoal may have helped in adsorption of phenolic compounds and other inhibitors. The promotary effect of activated charcoal on the initiation of polyembryogenesis has been documented. Webb and Flinn (1991) added 1.0% activated charcoal in the initiation medium which triggered embryogenic callus formation in *P. strobus*. To induce polyembryogenesis in *P. menziesii*, Gupta *et al.* (1995b) used activated charcoal in the medium for absorbing endogenous ABA along with other plant growth regulators and other inhibitory metabolic by-products.

In the present study, the browning of the medium was maximum in case of apical dome sections followed by secondary needles and zygotic embryos. The browning of the medium could be prevented by incorporating different anti-oxidants (200 mg/l PVP for zygotic embryos and secondary needles and 200 mg/l PVP + 100 mg/l citric acid for apical dome sections). Gupta (1980) reported that in case of shoot tip cultures of apple and teak agitating the plant materials for an hour or so in an liquid medium containing PVP, β-mercaptoethanol, dithiothreitol, glutathione (0.5-2%) helped in preventing the oxidation of phenolic compounds. Narayanaswamy (1994) discussed that culture
medium fortified with anti-oxidants like ascorbic acid and citric acid (500-2000 mg/l) can curtail the effects of phenolic oxidation.

The effect of different media and inorganic and organic nitrogen sources on somatic embryogenesis in conifers has been extensively studied (Chirstie and Butler, 1994; Bonga et al., 1995; Barrett et al., 1997; Norgaard, 1997). Amongst the different media tried, mMS medium for zygotic embryos, MS medium for secondary needles and ½DCR medium for apical dome sections were found suitable for initiation of embryogenic cultures in *P. kesiya*. In the present study, pH levels of 5.5-5.75 were found to be suitable for culture of all the explants. For initiation of embryogenic cultures of *P. palustris*, the pH of the medium was adjusted to 6.0 (Nagmani et al., 1993). The zygotic embryos showed better embryogenic response in MS medium when KNO₃ level was increased to 4460 mg/l and NH₄NO₃ was decreased to 550 mg/l whereas secondary needles responded well on MS full strength inorganic salts and organic nutrients (Table 8 and 9 respectively). The apical dome sections produced better embryogenic cultures on half strengths of all inorganic salts and full organic nutrients of DCR medium. All the three explants of *P. kesiya* also required higher concentrations of CH (1000 mg/l) and L-glutamine.
(500 mg/l) besides inorganic nitrogen sources for initiation of embryogenic cultures. Gupta and Durzan (1986b) used MS medium with modified levels of NH₄NO₃ (550 mg/l), KNO₃ (4676 mg/l). A half strength of modified MS medium supplemented with CH (500 mg/l), myo-inositol (1000 mg/l), L-glutamine (450 mg/l) and sucrose (3.0%) was used for somatic polyembryogenesis of *P. abies*. For induction of embryogenic callus formation in *P. strobus*, in MSG medium (Wann et al., 1987a; Becwar et al., 1988) NH₄NO₃ was completely replaced by 1450 mg/l glutamine, KNO₃ was reduced from 1900 to 100 mg/l and KCl was added at 745 mg/l. Furthermore 1.0% activated charcoal was used. Finer et al. (1989) used DCR medium containing 50 mg/l glutamine to induce embryogenesis in *P. strobus*. For mature zygotic embryos comparisons of full strength LP and half strength Litvay media gave similar induction frequencies when tested for both *P. glauca* (Tremblay, 1990) and *P. mariana* (Tautorus et al., 1990). In these cases CH was included in most of the media tested. In a comparative study Hristoforoglu et al. (1995) reported, an increase of number of *P. abies* embryogenic lines by three times in a medium containing glutamine and asparagine than the one containing NH₄NO₃ and KNO₃. Embryogenic lines proliferated faster on medium
containing glutamine and CH than medium lacking them. Norgaard (1997) reported better embryogenic culture formation of *A. nordmanniana* on ½BLG medium (Verhagen and Wann, 1989) when NH₄NO₃ was removed completely, reduced KNO₃ (50 mg/l) and KCl (372.5 mg/l) added along with L-glutamine (750 mg/l) and L-asparagine (50 mg/l). Barrett *et al.* (1997) investigated the effects of glutamine-based dipeptides, glutamine and CH as well as deletion of organic nitrogen on induction of somatic embryogenesis in *P. glauca*. They reported that the removal of organic nitrogen sources was promotive for embryogenesis. Li *et al.* (1998) reported that BM₁ medium (Gupta and Pullman, 1991) was superior over other media tried for initiation of embryogenic cultures from immature zygotic embryos of *P. taeda*. It was further reported that BM₁ medium enhanced extrusion and proliferation frequency compared to DCR₁ basal medium, while LP basal salts were ineffective for initiation of culture. Kim *et al.* (1999) used LM (Litvay *et al.*, 1985), LP and MS media for induction of somatic embryogenesis from immature zygotic embryos of *L. leptolepis* and recorded 60.0%, 67.0% and 59.0% embryogenic tissue formation respectively. Though LP medium was slightly better at the initial stage, with time LM medium proved to be
more effective in that species since embryogenic tissue on LP medium failed to proliferate.

In general, glutamine is beneficial for the induction of conifer somatic embryogenesis. Negative effects have only rarely been reported (Barrett et al., 1997). Von Arnold (1987) found that the rate of initiation of *P. abies* embryogenic cultures was higher in NH₄NO₃-containing medium without glutamine. Incorporation of myo-inositol and CH both at 1000 mg/l in the medium resulted in better embryogenic cultures of all the explants of *P. kesiya* compared to use of either of these compounds singly.

Generally for initiation of somatic embryogenesis in conifers, lower concentration of organic carbon sources were more effective (Von Arnold and Hakman, 1986; Von Arnold, 1987; Becwar et al., 1988; Nagmani et al., 1993; Bonga et al., 1995; Li et al., 1998). In the present study, for initiation of embryogenic cultures, lower concentration of sucrose (2.0%) was found to be very effective for zygotic embryos and secondary needles, except apical dome sections where no significant difference in embryogenic response was recorded between media containing 2.0% and 3.0% sucrose. Becwar et al. (1988) reported that low sucrose concentration (1.0%) produced more
ESMs. Using maltose, glucose and sucrose as organic carbon source for initiation of embryogenic cultures of *P. palustris* from zygotic embryos and female gametophytes the better embryogenic cultures could be initiated on medium containing 3.0% sucrose. Kim *et al.* (1999) reported that 2.0% sucrose in the medium resulted in better initiation of embryogenic cultures of *I. leptolepis*.

Usually both an auxin and a cytokinin are necessary for induction of embryogenic cultures in conifers (Attree and Fowke, 1991). 2,4-D generally has been the preferred auxin for the initiation of ESMs of most conifer species (Gupta *et al.*, 1991; Tatorous *et al.*, 1991). NAA has also been successfully used in some cases. Verhagen and Wann (1989) found that 2,4-D and NAA were equally effective in promoting induction of somatic embryogenesis from mature embryos of Norway spruce. Von Arnold (1987) obtained slightly higher frequencies with 2,4-D (at 20 μM) compared to NAA at the same concentration, but NAA was more effective at lower concentrations (5 μM). Jain *et al.* (1988) found that NAA was more effective than either IAA or 2,4,5-T, but did not compare 2,4-D in the same experiment. For *P. kesiya* though 2,4-D and NAA were the preferred auxins like other conifers (Verhagen and Wann, 1989; Gupta *et al.*, 1991;
Tautorus et al, 1991; Gupta and Grob, 1995; Gupta et al., 1995a, 1995b; Norgaard, 1997; Li et al., 1998; Kim et al., 1999), NAA singly was found to be more effective than 2,4-D for all the explants. 2,4-D and NAA at 5.0 mg/l each were highly effective in initiation of embryogenic cultures for zygotic embryos and apical dome sections while for secondary needles 3.0 mg/l each of 2,4-D and NAA were required for optimum response. Combined effect of 2,4-D and NAA was beneficial compared to use of either of them alone. It was observed that incorporation of BAP in auxin rich medium was stimulatory for initiation of embryogenic cultures. The present investigation is in agreement with the observations of other reports on conifers (Cornu and Geoffrion, 1990; Gupta et al., 1991; Nagmani et al., 1993; Bonga et al., 1995; Guevin and Kirby, 1997). Li et al. (1998) reported that a combination of 3.0 mg/l 2,4-D and 0.5 mg/l BA was found better than higher concentrations for initiation of embryogenic cultures in P. taeda. In P. kesiya, the media containing cytokinins (BAP and kinetin) were found to be inhibitory for embryogenic culture formation except apical dome sections where BAP and kinetin (each at 3.0 mg/l in combination) could produce little reddish brown callus which subsequently degenerated. However, BAP
in the medium along with auxins accelerated embryogenic culture formation but at higher concentrations resulted in hard non-embryogenic cultures. ESM culture from A. nordmanniana were best initiated with cytokinins (BA and kinetin) alone, with auxin found to be inhibitory (Norgaard and Krogstrup, 1991).

In the present study, light was found to be inhibitory for initiation of embryogenic cultures, which produced more green and non-embryogenic cultures. Dark was preferred but light at 20 lux formed moderate embryogenic cultures except apical dome sections, where completely hard and green callus resulted. In general, induction of embryogenic cultures in conifers has mostly been done in the dark (Gupta and Durzan, 1986a; Von Arnold, 1987; Gupta and Grob, 1995) except in case of Norway spruce where initiation of embryogenic culture was equally well under light and dark (Verhagen and Wann, 1989). Von Arnold (1987) reported that in Norway spruce, culture under light of 20 h photoperiod was inhibitory for induction of embryogenic cultures. Further, lower induction frequencies were reported in the light compared to the dark, but no significant differences existed.
Cultures on semisolid medium from all the explants after few subcultures ceased proliferation and in some cases started browning on auxin rich medium (Table 22). It was necessary to transfer the cultures on basal media containing reduced levels of growth regulators before culture proliferation ceased. The gradual removal of growth regulators resulted in the formation of PEMs and proembryos. Higher auxin concentration interfered with the development of polarity. When auxins were withdrawn gradually, the polarity developed followed by the formation of proembryos. It was observed that complete removal of growth regulators resulted in degeneration of cultures. Durzan and Gupta (1988) maintained the ESMs in the medium containing lower concentrations of plant growth regulators (1-2 mg/l 2,4-D and 0.1 mg/l kinetin and BA). Nagmani et al. (1993) reduced the 2,4-D levels gradually to 0.5 mg/l for maintenance of long leaf pine cultures and transferred them from dark to diffuse light conditions. Gupta et al. (1995a) reported that it was necessary to reduce the growth regulator levels in the maintenance medium compared to initiation medium for *P. abies*.

In the present study, the culture raised from apical dome sections on semisolid medium were not very soft and translucent until
they were cold treated at 4°C for 24 h before transferring in liquid medium. After cold treatment softer embryogenic cultures resulted.

For initiation of suspension cultures of *P. kesiya*, lower inoculum size (150-200 mg/15 ml medium) was found to be suitable. By decreasing the inoculum (<150 mg/15 ml medium) a poor culture growth was recorded which may be due to inadequate culture density. Higher inoculum at the initial stage slowed the proliferation of cultures which may be due to over crowding of cells. Finer *et al.* (1989) reported that suspension cultures proliferated well with a low culture density in *P. strobus* while, Krogstrup (1990) reported that culture density was crucial and determined the quality of early stage embryos in suspension cultures in *P. sitchensis*.

In the present study, it was necessary to separate embryogenic cultures from non-embryogenic parts grown on semisolid medium. It was necessary to free single cells from mother tissues in suspension cultures failing which degeneration of cultures resulted. The ESMs formation resulted from elongated single cells through cleavage in the suspension medium with reduced growth regulators. They started accumulation in the medium free of growth regulators but containing higher sucrose concentrations. The cultures required low agitation for
proembryonal head formation because at higher speed the embryonal suspensor cells maintained a distance which was not enough for accumulation of cells.

Abscisic acid has been used for cotyledonary embryo development in many plant species (Skriver and Mundy, 1990) including conifers (Gupta and Durzan, 1987). In the present study, the proembryos converted into cotyledonary embryos on respective semisolid basal media containing higher concentrations of sucrose in conjunction with ABA (Table 23). It was observed that neither sucrose nor ABA singly could promote maturation of somatic embryos. A concentration of 4.0% sucrose and 4.0 mg/l ABA was found to be effective for somatic embryo maturation. It is believed that the higher osmoticum helped in the development of embryos while ABA caused desiccation stress resulting in maturation of somatic embryos. Finer et al. (1989) found that elevated sucrose levels (6-12%) were beneficial for somatic embryo maturation and differentiation in P. strobus. Tremblay and Tremblay (1995) reported that sucrose (4-6%) in the medium can serve as an osmotic agent and as a carbon and energy source. Black spruce somatic embryo maturation. Further, the maturation medium containing mannitol could not promote somatic
embryo maturation. Norgaard (1997) reported that maltose (3.4-4.3%) used singly was better organic carbon source than sucrose (3.0%) for maturation of somatic embryos and germination in *A. nordmanniana*. The combination of both maltose and sucrose was more effective than either of them used singly. Carrier *et al.* (1997) studied the effect of exogenous sucrose on maturation and germination of somatic embryo of interior spruce and reported that embryos placed on the medium with added sucrose developed roots and epicotyls and increased their fresh mass by about 13 fold by consuming 25% of the available sucrose in the medium. This exogenously supplied sucrose promoted the formation of linolenic acid, which participated in the maturation of embryos. Durzan and Gupta (1987) discussed that ABA inhibits cleavage polyembryony and allows embryo singulation and further development in Douglas fir. Roberts (1991) reported that a higher concentration of mannitol (6.0%) as an organic carbon source promoted the formation of globular embryos in callus culture of spruce, but few of them could convert into cotyledonary embryos. Like mannitol, ABA also promotes formation of globular embryos. But combination of mannitol and ABA enhanced the effect of ABA on production of globular embryos. Higher concentration of mannitol was inhibitory.
Although mannitol promoted formation of globular embryos, it inhibited the maturation of the embryos. Webster et al. (1990) reported that 40 μM ABA was optimum for the production of mature embryos. In *P. abies*, the embryo development occurred following the removal of auxins and cytokinins and addition of ABA (Gupta et al., 1995a).

Vagner et al. (1998) reported that with increase in exogenous ABA levels (5-40 μM), an increase in somatic embryo maturation of *P. abies* was recorded both in semisolid and liquid medium. In the absence of the exogenous ABA, embryo yield was negligible. Embryogenic cultures on auxin rich medium produce ethylene which act as inhibitor for somatic embryo development (Biddington et al., 1993). They reported that ethylene inhibited induction of embryogenic cultures and development of somatic embryos. Accumulation of ethylene in embryogenic cultures in conifers was reported by Noland et al. (1986) in loblolly pine, Wann et al. (1987a) in *P. abies* and Kumar et al. (1989) in *P. glauca* cultures. It was observed that more ethylene was produced in non-embryogenic than in embryogenic cultures. Ethylene interfered with the development of polarity. The media supplemented with ethylene inhibitors like ABA inhibited ethylene formation and promoted somatic embryo maturation. Kong
and Yeung (1994) studied the influence of ethylene on somatic embryo maturation of white spruce by incorporating various ethylene inhibitors in the medium. They reported that the addition of ABA in the development and maturation medium caused decrease in ethylene production. In the absence of any ethylene inhibitor, the cotyledonary embryo formation was very poor. ABA decreased ethylene formation in the initial stage of cultures but the production of ethylene increased during later. It was further reported that polarity developed with decrease in ethylene production.

In the present study, the somatic embryos (developed from zygotic embryos and secondary needles) became distinct and elongated on basal media without any growth regulators, CH and L-glutamine but containing activated charcoal (0.2% and 0.4% respectively). Activated charcoal adsorbed all the endogenous growth regulators and growth inhibitors and stimulated better singulation and elongation of embryos. Becwar et al. (1989) transferred the Norway spruce cultures with ESMs on hormone-free medium containing activated charcoal for 1 week. It was thought that the charcoal adsorbed cytokinins and auxins, which caused the cultures to cease cleavage polyembryony. Pullman and Gupta (1991) could produce
cotyledonary embryos of *P. abies* on medium containing ABA (50 mg/l) and activated charcoal (1.25 g/l). Further, the combined ABA and charcoal treatment improved embryo quality. Gupta *et al.* (1993) reported that a combination of increased osmolarity with ABA and activated charcoal produced good quality cotyledonary embryos. In *P. menziesii* the embryo development and maturation was promoted by amending the medium with activated charcoal (1.25 g/l) and ABA (30 mg/l) (Gupta *et al.*, 1995a).

In the present study, somatic embryos germinated in the light (1900 lux) at 12 h photoperiod and 40% germination was recorded. The embryos of *P. abies* germinated best after 7 days in the dark followed by continuous light and 80-90% germination was recorded (Gupta *et al.*, 1995b).

A very low specific activity of peroxidase and protein content were recorded in all the explants of *P. kesiya* before culture. With growth on respective initiation media, the peroxidase activity and protein content increased gradually and were maximum at day 10 of the first subculture. This was the period when embryogenic cultures started forming PEMs. The peroxidase activity and protein content declined subsequently during second subculture. Increase in
peroxidase activity and protein content was correlated with the initiation of somatic embryogenesis. Once the process initiated, the peroxidase activity and protein content decreased. The peroxidase activity and protein content could be used as useful markers to identify the onset of embryogenesis (Jain et al., 1990). Zhou et al. (1992) studied the role of peroxidase on induction of somatic embryogenesis in *L. sativa*. They reported the peroxidase activity increased prior to visual manifestation of embryoids and also synthesized specific isoperoxidases. Once this process started, peroxidase activity decreased. This phenomenon was not observed in the non-embryogenic cultures.

Pitel et al. (1992) studied the activities of 10 different enzymes and protein contents during maturation of somatic embryos of *L. eurolepis* hybrid. Further, there was no significant difference in protein contents and patterns in non-embryogenic and embryogenic cell lines on maintenance medium. On maturation medium, all the 10 enzyme activities increased up to 15 days of culture after which gradually decreased. In cultures grown on maturation medium, the protein content started increasing after day 9 and reached the peak day 15 followed by gradual decrease in the quantity and number of bands. Donga and Dunstan (1994) reported that proteins rapidly
accumulated to the highest value (1109 mg/l) at day 9 at the rate of
94.7 mg/l/d during early stages of initiation of embryogenesis of P.
glauc. The intracellular protein content decreased after day 9 of
culture.

In the present study, the initiation, development and maturation
of P. kesiya somatic embryos have been achieved. This opens up a
great potential for large-scale propagation of this species which is
facing rapid denudation. Further work on somatic embryo
development in liquid medium and increase in conversion frequency
of somatic embryos developed on semisolid medium need to be
worked out in future.