Chapter IV

Discussion

Somatic embryogenesis in conifers was first documented from immature zygotic embryos of *Picea abies* (Hakman *et al.*, 1985; Chalupa, 1985). Since then there have been successful regeneration of conifers via somatic embryogenesis.

Polyembryogenesis being a common and natural phenomenon in conifers, most conifers undergo one of the two types of polyembryony, either simple or cleavage. In simple polyembryony, as in *Picea* and *Pinus* (Singh, 1978) proembryo is a result of fertilization of more than one egg per ovule by gametes from separate pollen grains, and therefore every proembryo is genetically different. One proembryo usually dominates and continues development while the others abort. When proembryos are cultured *in vitro* they mature into distinctive stages S1, S2 and S3. First the continued cell divisions in all planes in the head region produce club shaped S1 embryos (Fig.19). As the shoot apex starts to protrude, the head of a somatic embryo becomes cone shaped, at this intermediate stage these embryos are denoted as S2 embryos (Zhang *et al.*, 1999). With development of cotyledons, the embryos transit from early stage cotyledonary embryos to mature cotyledonary S3
embryos. These stages from I to III were obtained in the present study carried out in *Pinus kesiya* (Fig. 19).

In conifers and *Pinus*, in particular, factors limiting commercialisation include low culture survival, culture decline causing low or no embryo production and inability of somatic embryos to fully mature, resulting in low germination rates. Tautorus *et al.* (1991) indicated that 50% of the *Picea mariana* suspension cultures tested were discarded due to browning.

Gymnosperms in general show suspensor cleavage polyembryony. While many embryos develop simultaneously in an ovule by proliferation of suspensor cells it is usually the leading proembryos with suspensors which join to form the embryonal head, and which mature into early stage cotyledonary embryos.

Female gametophyte was collected 4-8 weeks after fertilization when the immature embryos were 1.0-1.11 mm in size. This stage was found to be optimum for formation of embryogenic cultures in *P. kesiya*. The developmental stage of immature zygotic embryos has proven to be a critical factor in the initiation of somatic embryogenesis in *P. sylvestris* and *P. pinaster*. The responding stage was different for both species. While initiation of embryonal masses from advanced stage of zygotic embryos have been reported in *P. strobes* (Finer *et al.*, 1989; Klimaszewska and Smith, 1997) in *P. taeda* (Becwar *et al.*, 1990) in *P. pinaster* (Bercetche and Paques, 1995) and in *P. koraiensis* from mature zygotic embryos (Bozhkov *et al.*, 1997). In pines, early
stages of development was found to respond well in *P. taeda* (Gupta and Durzan, 1987) and in *P. caribaeae* (Laine and David, 1990).

For all these studies, it was observed that in pine somatic embryogenesis, the precise determination of the development stage is important for optimum response.

Stratification of seeds before culture, have been found to yield better embryogenic response. In *P. kesiya*, stratification of mature zygotic embryos at 4°C for 12 hrs resulted in better proliferation of embryogenic callus (Table 5). Schneider and Gifford (1994) and Stous and Gifford (1997) working on loblolly pine seeds observed that stratification of seeds helped to mobilize the primary storage reserves (lipids and proteins) that are contained in the lipid bodies and protein bodies respectively. Gori (1979), and Krasowski and Owens (1993) reported that thought these reserves are present in both the embryo and female gametophyte (Ching, 1966; Sasaki and Kozlowski, 1969; Kovae and Kregar, 1989; Gifford, 1998) the majority are stored in the latter. During stratification, these storage proteins get mobilized and degraded to amino acids (Durzan and Chalupa, 1968; Salmia, 1981; Lammer and Gifford, 1987; King and Gifford, 1997) and transported to the growing tissue.

Cultures in liquid medium have the added advantage of having the ability to proliferate proembryonal suspensor masses, which can be maintained for a long duration in culture. Proembryonal masses also existed in callus raised on semisolid medium; however, it was found that callus
cultures raised from mature zygotic embryos could not be maintained for long at least as observed during the present study period in *P. kesiya*. The callus raised from immature female gametophyte when eventually transferred to liquid medium, showed rapid proliferation of PEMs, and at a later stage formation of embryonal suspensor masses and eventually to embryonal heads (Fig 17).

Somatic polyembryogenesis has been described by many workers and termed the embryogenic tissues as ESMs due to their high degree of organization. The ESMs are reported to be white, transluscent in appearance when cultured on semisolid medium appear to glisten due to production of mucilage. The mucilage anatomically consists of a variable mixture of elongated cells, early stage embryos which have embryonal head and a suspensor system (Gupta and Durzan, 1987). Nonembryogenic callus appears opaque, and may not have an anatomical organization.

A possible explanation lies in the degenerating nucellar tissue present in immature female gametophyte that stimulates adjacent cells to divide and form proembryonal suspensor masses. The absence of this tissue in mature zygotic embryo cultures may possibly be the reason, why callus raised from it show lower percentage conversion to somatic embryos.

In the present study, mature zygotic embryos used as explants formed somatic embryos via embryonal suspensor mass formation following stratification for 12 hrs at 4°C (Table 5). However, conversion to somatic
emblings was relatively less than when proliferated in cell suspensions. Immature female gametophyte raised in semisolid medium and later transferred to liquid medium formed embryogenic suspension cultures on the onset and suspension cultures showed ESM formation and embryonal heads formation eventually. Nonembryogenic suspension cultures proliferate as nonembryogenic cultures form the start if the cells lack the potential to form suspensor heads (Fig. 17 and Fig. 18).

The present study was carried out to observe the physiological and biochemical changes that were taking place in the culture right from its initiation to conversion into embryogenic masses. Cultures were grown in various media composition and culture conditions were studied in the light of to compare the different stages beginning with callus initiation till the formation of proembryonal suspensor masses and early stage somatic embryos.

The growth medium supplying the correct combination of salts is of great significance in any tissue culture medium, even more so in cultures turning embryogenic. In the present study BM medium supplemented with two synthetic auxins (2,4-D and NAA) and a cytokinin (BAP) was found most suitable for embryogenic response in culture (Tables 6, 7 and 8). While MS basal medium showed initial proliferation a low response towards proliferation and conversion to embryogenic cultures was observed (Table 8). It seems that Ca(NO₃)₂ plays a significant role in profuse callusing and
embryogenicity. The BM medium contained CaNO₃ whereas it is absent in the MS medium. The presence of Ca²⁺ MS and BM media can be correlated with high proliferation of embryogenic callus (Table 7). Of all the media studied there was presence of Mg(NO₃)₂ in BM medium only, that supported higher percentage of embryogenic response (Table 8). Besides, the increased concentration of CaNO₃ in the BM medium could be related to the high percentage response in BM medium compared to other media using zygotic embryos as explant. Nitrates may also be a deciding factor in the conversion of stage I to stage II callus with conversion to early cotyledonary embryos.

Under *in vitro* conditions, the medium pH have significant influence on the potentiality of the cultures to convert to embryogenic stage. In the study carried out in *P.kesiya*, the medium pH made a significant change in the type of response of cultures to form stage III embryogenic tissue. pH 5.8 was found optimum in the present studies (Tables 9, 10 and 11). There was a low embryogenic response at lower and higher pH which is in conformity with the report of Minocha (1987) where lower pH of cell walls (about 5.5-5.8) kept the carboxyl group of the auxin less dissociated in plasma membrane than in the cytosol (where the pH is higher around 7.0). And because membranes are more permeable to noncharged solutes, undissociated auxins are believed to move from the wall into the cytosol where higher pH causes growth and differentiation of cells. Although there are many reports showing that cells in culture can significantly alter the pH of the external medium through
differential uptake of nutrients and/or through H+ fluxes. This observation may be substantiated again with auxin mode of action and the fact that the polar transport of auxins is regulated by the pH of the cells (Minocha 1987; Smith and Krikorian, 1990). At pH 4.2, less nitrogen and phosphate were available from the MS medium than at pH 5.7. NH4+ was consumed preferentially to NO3⁻ in all culture stages (Taber et al., 1998).

The incorporation of plant growth regulators into tissue culture medium has, historically, been of special significance. Growth hormones are the natural compounds occurring in the plant systems, which in small amounts promote, inhibit or otherwise modify the physiological activities within the system. They are endogenously produced and their regulation is controlled by the physiological needs of the plant. Auxins are known to be principal agents to mediate transition from somatic to embryogenic cells (Smith and Krikorian, 1990). Auxins promote cell elongation by inducing wall acidification. In soyabean, hypocotyl sections elongation in response to a low pH only occurs for an hour or two. After this relatively short period, sections still respond to auxins for a day or two, but they respond no longer to H+ ie., pH. Sustained growth therefore requires auxin-induced production of cell wall precursors to promote transcription of enzymes that enhance cell wall synthesis (Vanderhoef, 1980). But sustained growths of cells require much more than cell wall synthesis. It requires synthesis of plasma membrane (lipids & proteins), because that membrane grows in contact with the wall. At
the same time it requires that the turgor pressure in the cells do not decrease too much, even though the wall is loosened, because if the turgor drops excessively as the cell expands, insufficient pressure will be exerted against the walls preventing sustained growth.

It is therefore probable that this enhanced formation of plasma membrane and maintenance of turgor pressure was being kept up in the presence of two synthetic auxins, NAA and 2,4-D in *P. kesiya* (Table 26) while cultures maintained only in IAA a phytohormone, did not convert embryogenic. Turgor pressure within the cells is maintained also by enhanced solute uptake from the growth medium, and it is here that the importance of a proper concentration of mineral salts in the growth medium for auxin induced growth becomes necessary.

Application of external growth regulators is believed to effect cell polarity by interfering with the pH gradients and electrical fields around the cell (Dijak *et al.*, 1986; Smith and Krikorian, 1990). Therefore, a proper combination of auxins and cytokinins only may result in the development of somatic embryogenesis from the cultured cells, and this varies from species to species, ranging over different genus and taxa. In *P. kesiya*, the best combination was found to be 22.6 μM 2,4-D, 26.8 μM NAA and 11.1 μM BAP.

Most studies on carbohydrate utilization *in vitro* have indicated that sucrose is the best carbon source for optimal growth. However, depending on the genotype, too, explants selectively take up sucrose or maltose, i.e.,
respond differentially to carbohydrate sources (Tremblay and Tremblay, 1991). Low percentage of sucrose (1-2%) resulted in more ESMs formation (von Arnold, 1987; Becwar et al., 1988a, b). In the present study in P. kesiya, callus from mature zygotic embryos showed better response in maltose supplemented medium (Table 17) compared to sucrose (Table 14). Maltose is reported to be broken down more slowly than sucrose, providing a metabolizable carbon source over a longer period of culture (Orshinsky et al., 1990). In general, sucrose in a medium can serve as an osmotic agent and also as a carbon and energy source. Many cultures, especially embryo and some shoot cultures, require a medium with high osmotic potential (Tremblay & Tremblay 1995). Large and available carbon amounts are necessary for structural growth, differentiation and development.

The effect of temperature too can be explained in relation to auxin action and it is believed that the flux or quantity of auxin transported per unit time increases with temperature. This can be correlated with our study on incubating cultures under different temperature conditions. It was found that callusing was inhibited at lower (4-12°C) and also at a higher range of 36°C while cultures kept between 22-24°C showed profuse callusing and conversion.

The effect of light in the induction of somatic embryogenesis is not very clear. Cell polarity and asymmetric cell division are involved in the initiation of somatic embryogenesis. In alfalfa, stimulations by auxins
promote asymmetric cell divisions to form embryogenic protoplasts (Bogae et al., 1990; Dudits et al., 1991), while protoplasts with nonembryogenic lines divide symmetrically. The first asymmetric cell division is generally initiated by a gradient of light, the plane of division always being perpendicular to the light axis. In zygote and female gametophyte (immature fertilized organs that are protected from light, the first division plane is predetermined. Abscisic acid production peaked at the heart stage of embryogenesis and synthesis was most pronounced in the dark (Michler and Lineberger, 1987). Since in the present study on *P. kesiya*, callus initiation and proliferation occurred in the dark, while light turned the explants green, light may not be a decisive factor in somatic embryogenesis.

Effect of light could be related to cytokinin activity from the observation in oats leaf culture (Thinmann, 1980; Thinmann et al., 1982) that cytokinins delay senescence by keeping the stomata open and allowing CO₂ to enter the cell. The CO₂ is believed to inhibit competitively the strongly promotive action of ethylene on senescence. Interestingly, seeds are the highest producers of ethylene, and auxins are present in high amounts in seeds, and therefore, it is believed that auxins greatly stimulate ethylene production. In dark culture conditions and in the presence of cytokinin (BAP) the activity of the CO₂ is probably diverted towards competitively controlling ethylene levels and this could probably be related to the present aspect of study in some way.
Ethylene in many reports has been found to be associated with induction of somatic embryogenesis even in many conifers. Ethylene in the ambient microenvironment was observed to be lower in the embryogenic culture environment compared to nonembryogenic tissues of Picea abies (Wann et al., 1987). Silver nitrate in the culture medium is known to be a potent ethylene action inhibitor. In conflicting reports, AgNO₃ was found to stimulate induction of somatic embryogenesis in Pinus taeda (Li and Huang, 1996), but inhibited somatic embryo induction and/or maturation in P. pungens (Afele et al., 1992) and P. pinaster (Bercetche and Paques, 1995) suggesting that ethylene may be beneficial for somatic embryogenesis in these species. In P. glauca ethylene inhibited growth of embryogenic tissue (Kumar et al., 1992).

In the present work, the influence and effect of ethylene on induction of somatic embryogenesis was carried out by incorporating silver nitrate into the medium. The idea was to find out if at reduced level of ethylene in the ambient microenvironment of the cultures could indeed enhance somatic embryogenic cell formation, silver nitrate being a potent ethylene inhibitor. It was found that there was inhibition of embryogenic callus initiation in lower concentration of AgNO₃ while in higher concentrations, there was no callus formation at all (Table 25). However, study of the effect of ethylene on embryogenic system does not end here. More work needs to be done in this aspect which could not be covered here. For now, on the basis of the study
carried out it can only be assumed that it is possible that in case of *Pinus kesiya*
presence of ethylene may enhance induction and maturation of embryogenic
culture in the medium. Another possibility of artificially increasing the
ambient ethylene level in the culture microenvironment may speed up the
physiological process.

Application of increased concentrations of Ca$^{2+}$ in the maturation
medium increased the number of embryogenic cell lines (Table 29 and 34).
BM and 1/2 Litvay's medium contain, CaNO$_3$ and CaCl$_2$ respectively. An
increase or decrease in their concentrations reduced the number of cell
cultures that converted to ESMs. Insoluble Ca$^{2+}$ salts may play an important
role as pools of Ca$^{2+}$ and buffering pH, callose formation or cell wall
stabilization (Pedroso and Pais, 1995). Without organic nitrogen (glutamine
and casein hydrolysate) i.e., with only inorganic nitrogen in the medium, the
fresh weight increase of tissue masses was significantly less than with organic
nitrogen in both initiation and maturation medium. No differences were
found between the dry/fresh weight ratios obtained with the various
nitrogen treatments (Barret *et al.*, 1997).

Macromolecule accumulation and synthesis (proteins, polysaccharides
and nucleic acids) are indicators of cell growth and physiological state that
have proved to be useful in characterization of embryogenic cultures in
conifers. Intracellular protein content has been positively correlated with
changes in growth of embryogenic cultures in white spruce *P. glauca* (Donga
and Dunstan, 1994). There are several studies on changes in protein content and protein pattern during somatic embryogenesis in conifers.

In *P. kesiya* the protein profile study revealed 98, 65, 42 and 25 kDa doublets under nonreduced conditions, and 65, 63, 35, 30, 23 and 22 kDa proteins under reduced conditions (Fig. 26). In all these studies, the vast majority of the resolved proteins remain constant between unorganised cell clusters of the stages I and II differentiated when somatic embryos (or proembryonal masses) were formed (Fig. 29). Roberts *et al.* (1989) and Flinn *et al.* (1991) reported that there was no significant changes in the protein pattern in the early stages of induction of somatic embryogenesis in *P. glauca*. Storage protein accumulation took place during the later stages of maturation following the onset of cotyledonary embryo development i.e., stage III in the present studies. A 55-57 kDa doublet was observed under nonreduced conditions while reduced extract showed 41, 35, 33, 30, 27.5, 24 and 22 kDa proteins in stage III calli. It is believed that somatic embryos closely resembling zygotic embryos not only morphologically but biochemically would display more vigorous growth.

In the present study, SDS-PAGE protein profile revealed that at stage I, the proteins were the same as compared to that in mature zygotic embryos (Fig. 27). Major proteins with apparent molecular weights of 66, 65, 63, 45, 32, 30, 28 kDa, and minor proteins at 37 and 35 kDa were observed.
Comparison of protein profile of calli at different stages of somatic embryogenesis from stage I undifferentiated calli to stage III calli having proembryonal masses showed major proteins of 45, 43-41, 32-35, 30, 28, 25, 18 and 15 kDa molecular weights. These proteins remained constant between the stages, but intensity of the bands increased with age of calli (Fig. 28).

It has been reported that there was an appearance of a 41 kDa protein during the later stages of somatic embryogenesis in interior spruce (Flinn et al., 1991). In somatic embryos this 41 kDa protein showed an initial rapid accumulation that continued to accumulate during the entire cotyledon/embryo maturation and differentiation period. When compared, in zygotic embryos, this 41 kDa protein initially accumulated more rapidly over a 3 week period and thereafter the protein levels appeared to remain relatively constant or increase only slightly.

Comparison of protein patterns between early and mature somatic embryos with initial zygotic embryo explants showed differences as well as similarities as reported in interior spruce (Flinn et al., 1991) and in silver fir (Kormut et al., 2003). In silver fir, compared to zygotic embryos, the protein pattern of somatic embryos involved additional protein fractions of 36, 35, 32, 31 and 19 kDa size. As reported, in contrast to zygotic embryos, a 14 kDa fraction was rather faint in somatic embryos, while the main difference was a major protein 43 kDa size distinct in cotyledonary stage of somatic embryos, which was absent in dessicated mature zygotic embryos.
In the present study, three phases in the growth cycle were observed during the study period, an initial steady phase (stage I) following the second subculture in initiation medium, followed by a rapid linear increase during the next two subcultures (stage II calli). This was followed by a declining phase (stage III) when the protein content went low. Similar pattern of changes in intracellular protein content has been shown as a positive correlation with fresh mass accumulation of total DNA content and embryo number in *P. glauca* (Dong and Dunstan, 1994). While the first two phases could be correlated with proliferation, comprising elongated suspensor vacuolated cells undergoing cleavage polyembryony. This involves asymmetric rapid cell divisions in single cells and within cell clusters (Tautorus et al., 1991) resulting in repetitive somatic embryo production. Several possible explanations for this have been proposed, like nutrient mostly carbohydrate depletion, which has been found to coincide with maximum cell mass followed by browning of cultures (Fowler et al., 1982; Lulsdorf et al., 1992).

Several studies for the presence of proteins in the growth medium (extracellular proteins) have been carried out to reveal their function in somatic embryogenesis (Egertsdotter et al., 1993; Dong and Dunstan, 1994). In the present study, carried out, the protein content in the spent medium showed a linear increase with maturation of cultures (Fig. 37). Initiation of somatic embryogenesis can be related with a marked change in the pattern of
secreted proteins (deVries et al., 1988). There have been aberrant protein patterns in suspension cultures that have lost embryogenic potential and in nonembryogenic mutant cell cultures. Somatic embryogenesis could be restored in some of the cell lines by the addition of wild type extracellular proteins, thus indicating that one or several extracellular proteins are involved in somatic embryogenesis.

When mature zygotic embryo explants of *P. kesiya* were raised in semisolid medium, followed by callus initiation and proliferation in semisolid medium, then transferred to liquid medium from stage II calli onwards till the end of the study period. SDS-PAGE profile showed a gradual accumulation of some low molecular weight proteins in the spent medium of 35, 32, 20, 15, 13 kDa size. Spent medium from stage II cultures showed a faint protein band of 15 kDa size while following 2 more subcultures on the maturation medium, there were 15 and 13 kDa protein size. Stage III cultures on spent medium showed intense protein bands of 20, 15 and 13 kDa size (Fig. 39).

Secretion of proteins into the growth medium do not appear to result from leakage or lysis of cells *in vitro* as observed in *P. abies* embryogenic suspension cultures, and differed in those cultures from which cotyledonary somatic embryos could be matured and those not capable of maturation (Egertsdotter et al., 1993). There is increasing evidence to indicate that this is regulated by genes reflective of normal requirements for embryo development.
A number of gaseous or volatile compounds have been found to be accumulated in the culture flasks (Thomas and Murashige, 1979; Kumar et al., 1989), some of these compounds have been shown to affect growth and differentiation of cells in vitro including ethylene, CO₂ (Kumar et al., 1989), oxygen (Kessel and Karr, 1972) and ethanol (Perrata et al., 1986). Such compounds might inhibit culture growth rate, affect cell structure or accelerate degradation or lysis of living cells or tissues and the decline phase observed could be a reflection of cell lysis into the suspension medium. This was also reflected in the protein content in the nonembryogenic calli of *P. kesiya* which was comparatively much less and showed a decline as the calli matured (Fig. 36).

Peroxidases take part in processes occurring inside and outside the cell. Their activity is regulated developmentally by changes in the level of Ca^{2+} ions (Pullman et al., 2003). Plant tissue necrosis and subsequent cell death are usually observed during in vitro regeneration in conifers, especially in plant regeneration via somatic organogenesis in pine species. Cell death is correlated with the elevated levels of peroxides. The effects of antioxidants on in vitro regeneration of Virginia pine (*Pinus virginiana* Mill) showed that antioxidants, PVP and DTT were found to improve callus formation. In this case higher peroxidase activity of tissue cultures during subculture from callus proliferation medium to shoot differentiation medium and to rooting
medium was observed. The addition of antioxidants reduces and inhibits browning by reducing the accumulation of peroxidase (Pullman et al., 2004).

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) also reported that the peroxidase activity decreased prior to visual manifestation of embryoids and also synthesized several isoperoxidases. This phenomenon was not observed in the nonembryogenic cultures. However, in the present study, an increase in peroxidase activity could be related to the development and maturation of cultures following organogenesis. This was indicated by the increase in activity of peroxidase and protein content in the intracellular tissues as well as in the spent medium in embryogenic cultures.

In the present studies, it was observed that the changes in the protein levels as well as changes in their profile could be used as parameter to recognize cultures with embryogenic potential. Peroxidase activity also indicated the embryogenic potential in the cultures.