Chapter 5

Discussion

Somatic embryogenesis provides an ideal experimental system for investigation of the process of plant differentiation as well as the mechanism of expression of totipotency in plant cells. It is a process analogous to zygotic embryogenesis, but in somatic embryogenesis, a single cell or a small group of somatic cells are the precursors of somatic embryos. Unlike organogenesis, somatic embryogenesis recapitulates the events of zygotic embryogenesis with the production of a bipolar structure. Dunstan (1988), Wann (1988) and Gupta et al. (1991) have emphasized the potential of somatic embryogenesis as a method for rapid in vitro multiplication of conifers. Somatic embryos are also an important source of totipotent protoplasts useful for genetic transformation. They are useful for long-term germplasm storage using artificial seeds and cryopreservation (Chen and Kartha, 1987; Taurantus et al., 1991).

Proper explant selection is critical to achieving successful induction of somatic embryogenesis in conifers. This is important
because various tissues of the same plant or tissues at various developmental stages can differ in their response in vitro (Roberts et al., 1989; Arya et al., 2000). Four explants viz., female gametophytes (with immature zygotic embryo), mature zygotic embryos, secondary needles and apical dome sections (shoot discs) from mature trees of *Pinus kesiya* were used for initiation of embryogenic cultures. It was possible to induce somatic embryogenesis using female gametophytes (with immature zygotic embryos) and mature zygotic embryos as explants whereas it was not possible when other explants were used. Frequency of induction of somatic embryogenesis with mature zygotic embryos of *P. kesiya* was found to be very low. The developmental stage i.e. age of the explant is a critical factor that decides its embryogenic potential. In the present study, the embryogenic potential of the explants declined with an increase in the age. This decrease is due to the progressive specialization of the tissues, reducing the plasticity and capacity of the cells to dedifferentiate. Abdullah et al. (1987b) had shown a lower response of explants with increasing age in *P. brutia*.

Majority of the reports regarding initiation of embryogenic cultures in *Pinus* species is restricted to immature zygotic embryo (Salajova and Salaj, 1992; Gupta and Grob 1995; Arya et al., 2000, Tandon and Choudhury, 2002), while only few have reported induction
of embryogenesis from mature zygotic embryos (Bozhkov et al., 1998). No reports are available in pines regarding development of mature somatic embryos from explants other than the zygotic embryos. However, in other conifers, somatic embryogenesis has been reported from immature and mature embryos (von Arnold and Hakman, 1986; Verhagen and Wann, 1989), cotyledons of germinated seedlings (Krogstrup, 1986; Lelu et al., 1987), 20-30-day old seedlings (Attree et al., 1990b), 14-month-old somatic emblings (Ruaud et al., 1992) and buds and needles of 7-year-old trees (Westcott, 1992). Development of non-embryogenic callus from mature zygotic embryos in pines have been reported by Jain et al. (1989) and Salajova and Salaj (1992).

The importance of time of collection of explants in the present study suggested that there is a developmental period in which zygotic embryos are highly responsive towards somatic embryogenesis. Further, determination of optimum stage based on the time of season is ineffective because seed development may vary from year to year and influenced by latitude and altitude (Arya et al., 2000). In the present investigation, immature zygotic embryos 10-12 weeks after fertilization were suitable for initiation of embryogenic cultures. Embryos 4-5 weeks after fertilization were most responsive in loblolly pine (Gupta and Durzan, 1987b). Although post fertilization period can serve as an
effective marker, the precise time of fertilization is very difficult to ascertain. This necessitated the need for identification of the optimum stage of explant for induction of somatic embryogenesis on the basis of size and morphology of the zygotic embryo. The advanced pre-cotyledonary stage i.e. stage-c (Figure 4b) with head size 0.2-1.1 mm was highly responsive and was found to be the most appropriate stage for induction of somatic embryogenesis in *P. kesiya*. This observation conforms to the report on suitability of pre-cotyledonary stage for initiation of somatic embryogenesis in *P. taeda* (Becwar *et al.*, 1990). The embryogenic potential has been found to decline with mature zygotic embryo in *P. kesiya* (Table 5). Jain *et al.* (1989) suggested that right stage of zygotic embryos for embryogenic callus induction is based on embryo length. They also suggested that embryos with attached suspensors were necessary for embryogenic culture production. In general, in *Pinus* species, pre-cotyledonary zygotic embryos are the best for inducing somatic embryogenesis (Gupta and Grob, 1995), whereas in *Picea* species cotyledonary zygotic embryos are better (Becwar *et al.*, 1989; Tautorus *et al.*, 1991).

The pre-cotyledonary stage embryos were too small to be dissected out and were prone to desiccation during excision. Thus, intact
female gametophytes containing immature zygotic embryos were used as explants in *P. kesiya*. Female gametophytes containing immature zygotic embryos have been used as explants in many pine species (Becwar *et al.*, 1988b, 1990; Salajova and Salaj, 1992; Nagmani *et al.*, 1993). The female gametophytes with embryogenic extrusions (Figure 4c), when cut open longitudinally, showed that embryogenic extrusions were initiated from the suspensors of the developing zygotic embryo in most of the explants examined rather than the embryonal head. This examination revealed that the induction of embryogenic callus was in fact a continuation of the process of cleavage polyembryony that already exist in the suspensors of developing zygotic embryo. The stage ‘a’, ‘b’ and ‘d’ embryos with no or few cleavage embryos were responsive to a limited extent towards embryogenesis. A comparative study on the frequency of somatic embryogenesis with pre-cotyledonary zygotic embryos and those possessing cotyledonary primordia showed a decline in the embryogenic response with the cotyledonary stage embryo (Table 5). Tatorus *et al.* (1991) reported that somatic embryos could arise by the process similar to cleavage polyembryony occurring in nature.

The culture of immature zygotic embryos poses a very serious time constraint, because of the seasonal availability of the material
restricting its culture only for a short duration annually. The induction of somatic embryogenesis from mature embryos from stored seeds on the other hand extend the period from just a few months to more than a decade (Gupta and Durzan, 1986a; Tatorus et al., 1990). But frequency of embryogenesis using mature zygotic embryos was very low after 3½ months in culture (Table 5). Mature seeds of *P. kesiya* during December to early February produced better embryogenic cultures (Table 5). The seeds collected during March resulted in poor response, as the embryos in these seeds were not healthy. The pre-cotyledonary to pre-germinating embryos were found suitable for initiation of embryogenic culture in *P. abies* (Hakman et al., 1985), black and white spruce (Hakman and Fowke 1987a), *P. strobus* (Finer et al., 1989), black and white spruce (Attree et al., 1990b), *P. palustris* (Nagmani et al., 1993), *L. decidua* (Lelu et al., 1994; Bonga et al., 1995). Finer et al. (1989) reported that the best stage of embryo of *P. strobus* 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 the cotyledonary embryos of *P. kesiya* could be due to two reasons- (i) biochemical and molecular events may have set in which lead to germination and (ii) termination of cleavage embryony to a great
extent due to negligible amount or total absence of suspensor region in mature cotyledonary embryos.

Several reports are available on the formation of embryogenic cultures using secondary needles from *Picea* species. 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 to 10 year old stored seeds (Attree *et al.*, 1990b). Ruaud *et al.* (1992) reported induction of somatic embryogenesis from *P. abies* using needles of 14 months old somatic seedlings grown in a green house. Westcott (1992) induced embryogenic callus from buds and needles of seven-year-old trees of *P. abies*. Deb and Tandon (2002) reported somatic embryogenesis from secondary needles of 5-6 week-old glasshouse-raised seedlings of *P. kesiya*. In the present study, mature secondary needles of *P. kesiya* collected during March to June produced healthy, white and soft callus and were much better in appearance than those collected during the other parts of the year. A heavy rainfall and moderate temperature are experienced which are ideal during this period. From July onwards the secondary needles become harder and show poor initiation of callus, which is mostly hard in texture. In this investigation, secondary needles from mature trees
though produced white, soft callus, but no somatic embryogenesis was recorded (Table 14).

The sections of apical dome of *P. kesiya* collected during May to July (during second flushing) formed very healthy, white and soft callus (Table 18). Both secondary needles and apical dome sections did not produce somatic embryos in all the media with different combinations and concentrations of plant growth regulators and organic carbons tried. Therefore, the callus obtained from these two explants could be regarded as non-embryogenic. These non-embryogenic calli so obtained, appeared opaque, friable and turned light green in the light as also reported by Tautorus *et al.* (1991). The non-embryogenic callus may or may not have anatomical organization and composed of isodiametric as well as elongated cells of variable size. The cells divided in disorganized fashion and no bipolar structures typical of embryogenic calli were present. A similar observation was made by Jasik *et al.* (1995) in *P. nigra*.

In the present study, five basal media viz., MS, mMS, DCR, BM$_{1}$ and Litvay's in full as well as half strength were tried (Figure 10). Amongst the different media tested, $\frac{1}{2}$Litvay's medium for female gametophyte (Table 6) and mMS medium for zygotic embryos (Table 10) were found suitable for induction of embryogenic cultures in
P. kesiya. MS medium for secondary needles (Table 14) and \( \frac{1}{2} \)DCR medium (Table 18) for apical dome sections were found better for initiation of soft, white non-embryogenic callus. Gupta and Durzan (1986b) used MS medium with modified levels of \( \text{NH}_4\text{NO}_3 \) (550 mg\text{l}^{-1}) and \( \text{KNO}_3 \) (4676 mg\text{l}^{-1}). A half strength modified MS medium supplemented with CH (500 mg\text{l}^{-1}) and sucrose (3\%) was used for somatic embryogenesis of P. abies. For mature zygotic embryos, comparison of full strength LP and half strength LM (Litvay et al., 1985) media gave similar induction frequencies when tested for both P. glauca (Tremblay, 1990) and P. mariana (Tatorous et al., 1990). Li et al. (1998) reported that BM\textsubscript{1} medium (Gupta and Pullman, 1991) was superior over other media tried for initiation of embryogenic cultures from immature zygotic embryos of P. taeda. Kim et al. (1999) used LM, LP and MS media for induction of somatic embryogenesis from immature zygotic embryos of L. leptolepis and recorded 60\%, 67\% and 59\% embryogenic tissue formation, respectively. Though LP medium was slightly superior at the initial stage, with time LM medium was found to be more effective in this species as embryogenic tissues failed to proliferate on LP medium.

For initiation of somatic embryogenesis in conifers, generally, lower concentrations of organic carbon sources were more effective
(von Arnold and Hakman, 1986; von Arnol, 1987, Becwar et al., 1988; Nagmani et al., 1993; Bonga et al., 1995; Li et al., 1998). In the present investigation, a comparative study on different media, carbohydrate types and their concentrations was studied on initiation of embryogenic cultures (Figure 10 and 11). Of various concentrations and types of carbohydrates, sucrose 3% was found to be most effective for embryogenic response using female gametophytes (35%) and zygotic embryos (12.5%) (Figure 11). 3% sucrose was also very effective in initiation of soft, white callus from secondary needles and apical dome sections. However, these two explants failed to produce embryogenesis. Maltose at 3% level was not very effective and could initiate embryogenesis only in female gametophytes and zygotic embryos. Lactose and fructose (3% each singly) were least effective, producing embryogenic cultures only in case of female gametophyte explants (Figure 11), which turned brownish in subsequent sub-culture and could not be maintained. Nagmani et al. (1993) also reported similar observations. They used maltose, glucose and sucrose as organic carbon source for initiation of embryogenic cultures in P. palustris from zygotic embryos and female gametophytes and found better initiation of embryogenic cultures on medium containing 3% sucrose. Kim et al. (1999) reported that 2% sucrose in the medium resulted in better
initiation of embryogenic cultures in *L. leptolepis*. To induce somatic embryogenesis in conifers, the most preferred and widely used organic carbon was sucrose at 3% level (Mathur *et al.*, 2000; Lelu *et al.*, 1999; Nagmani *et al.*, 1993; Becwar *et al.*, 1990).

Usually both an auxin and a cytokinin are used for initiation of embryogenic cultures in most of the conifer species (Attree and Fowke, 1991). 2,4-D generally has been the preferred auxin for the induction of ESMs in conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). NAA has also been successfully used in some cases. Verhagen and Wann (1989) found 2,4-D and NAA equally effective in initiation of somatic embryogenesis from mature embryos of Norway spruce. von Arnold (1987) found slightly higher frequency of embryogenesis with 2,4-D (at 20 µM) as compared to NAA at the same level, but NAA was more effective at lower concentrations (5 µM). Initiation of embryogenic culture has been achieved with different concentrations of plant growth regulators such as 10-110 mg l\(^{-1}\) 2,4-D (Gupta *et al.*, 1991). In *P. kesiya* 2,4-D and NAA were the preferred auxins like other conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991; Gupta *et al.*, 1995a,b; Norgaard, 1997; Li *et al.*, 1998; Kim *et al.*, 1999). In the present study, female gametophyte explants exhibited optimum embryogenesis in the medium containing 5 mg l\(^{-1}\) 2,4-D and 2.5 mg l\(^{-1}\) NAA. 2,4-D and NAA at 5 mg l\(^{-1}\)
each were effective in initiation of embryogenic cultures from zygotic embryo explants and same concentration of auxins was promotive in soft, white callus induction from apical dome sections. For secondary needles, optimum callus induction was recorded using 2,4-D and NAA each at 3 mg/l. 2,4-D and NAA combination was highly beneficial in culture initiation compared to use of either alone. Incorporation of BAP in auxin rich medium was found to enhance embryogenic culture initiation. The present investigation is in conformity with the observation of other reports on conifer somatic embryogenesis (Gupta et al., 1991; Nagmani et al., 1993; Bonga et al., 1995; Guevin and Kirby, 1997). The balance between auxin and cytokinin was highly beneficial than the absolute concentration of auxins. Li et al. (1998) in *P. taeda* found higher rate of extrusion and proliferation on lower auxin concentrations (3 mg/l 2,4-D) in combination with cytokinin (0.5 mg/l BA). In *P. kesiya*, the media containing only cytokinins (BAP and kinetin) were found to be ineffective in embryogenic culture initiation from female gametophyte and zygotic embryo. Secondary needles (Table 26 and 27) and apical dome sections (Table 28 and 29) as such did not produce any embryogenic culture in BAP and kinetin containing medium. However, BAP along with auxins in the medium promoted culture formation. BAP (2.5 mg/l) along with auxins (2,4-D and NAA;
Table 23 and 25) was found to accelerate embryogenic culture formation from female gametophyte and zygotic embryo explants. However, higher concentrations resulted in hard non-embryogenic cultures. In some conifers, cytokinins alone were found effective in initiation of embryogenesis. Norgaard and Krogstrup (1991) reported that the ESM cultures of *A. nordmanniana* were best initiated with cytokinins alone (BA and kinetin), while auxin was found to be inhibitory.

In the present study, induction of embryogenic cultures was done in the dark as also reported in most of the conifers (Tautorus *et al.*, 1991; Gupta and Grob, 1995). Embryogenesis is reported to occur in light in *P. abies* (Verhagen and Wann, 1989). Light was found to inhibit embryogenic culture initiation and resulted in hard and light-greenish cultures in *P. kesiya*.

The present investigation using female gametophytes indicated that higher rate of callus in the first one to one-and-half month of culturing could be due to the response of embryos towards injury or nourishment from female gametophytes. It was also recorded that although higher percentages of female gametophytes initiated the embryonal extrusion but only a limited number could survive sub-culturing over a longer period of time (Table 5). von Aderkas *et al.* (1990) and Becwar *et al.* (1988a) also suggested that megagametophytes
tissue supplied the nourishment necessary for embryogenic culture initiation. In *Picea* species also, only some embryogenic cultures that were initiated could be maintained for several months (Attree *et al.*, 1989; Webb *et al.*, 1989; Tautorus *et al.*, 1990).

In the present investigation, cultures on semi-solid medium from all the explants after few sub-cultures ceased to proliferate and started browning on growth regulator rich medium. Therefore, the cultures had to be sub-cultured on basal medium containing reduced level of growth regulators to sustain proliferation and multiplication. The cultures were transferred every 12-15 days on to fresh medium containing reduced level of growth regulators and maintained in the dark at 25±2°C. In the present investigation, optimal multiplication of cultures was achieved on 1/10th growth regulator containing medium along with 3% sucrose (Table 30, 32, 34 and 36). Durzan and Gupta (1987a,b) also reported the use of lower concentrations of growth regulators for maintenance of the ESMs. 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*.

For initiation of suspension cultures of *P. kesiya* using calli from different explants, 3% sucrose was found to produce best results (Table- 38 and 39). Increase in sucrose concentration beyond 3% was
found inhibitory. Low concentration (30 mM) of sucrose has been reported beneficial in suspension cultures (Hakman and von Arnold, 1988; Tautorus et al., 1992; Lulsdorf et al., 1992; Dunstan et al., 1993). Find et al. (1998) used 3% sucrose in suspension cultures of Norway spruce and Sitka spruce. Other organic carbon sources like maltose, lactose and fructose were found less effective in initiation of suspension cultures in *P. kesiya*.

In the present study, auxins and cytokinins at the same level of concentrations as used for initiation of cultures on semisolid medium were found equally effective in initiation of embryogenic suspension cultures from calli of female gametophytes and zygotic embryos in *P. kesiya* (Table 40 and 41). Healthy non-embryogenic suspension cultures from calli of secondary needles and apical dome sections were also initiated using the same growth regulator concentrations to that used in initiation of cultures in agar-solidified medium (Table 42 and 43).

Inoculum density exhibited a prominent bearing on initiation and establishment of suspension cultures. In the present study, 40 g l⁻¹ (2 g ml⁻¹) inoculum density was found to be optimum for initiation of suspensions (Table 44 and 45). An increase or decrease of inoculum density below 40 g l⁻¹ resulted in poor culture growth (Table 44 and 45).
Dunstan et al. (1993) found 40 g l\(^{-1}\) as optimum inoculum density in *P. glauca* suspension culture. Lulsdorf et al. (1992) reported the same inoculum density for *P. glauca-engelmannii*-complex and *P. mariana* suspension cultures.

In the present investigation, 10% SCV was optimum for proliferation of embryogenic suspension cultures with all the explants. An increase in SCV resulted in culture browning (Table 46 and 47). By decreasing the SCV (below 10%) a poor culture growth was recorded which may be due to inadequate culture density. Higher SCV (> 10%) slowed down the growth of cultures associated with browning, which may be attributed to competition for nutrients due to over crowding of cells. Low SCV (< 10%) slowed down the culture growth, which could be due to low cell density in the culture environment. Krogstrup (1990) reported that culture density was crucial and determined the quality of early stage embryos in suspension cultures in *P. sitchensis*. Find et al. (1998) found 20% (v/v)±1% SCV suitable for maintenance of suspension cultures of Norway spruce and Sitka spruce. Ingram and Mavituna (2000) also reported 100 ml SCV l\(^{-1}\) medium (i.e. 10% SCV) was suitable for proliferation of embryogenic suspension cultures in *P. sitchensis*. 
In *P. kesiya*, the suspension cultures from all the explants showed browning if maintained in the medium containing high concentrations of growth regulators. So the suspension cultures were required to be subcultured in basal medium containing reduced growth regulators. Optimum culture growth was found in 1/10th or 0 concentrations of growth regulators (Table 48, 50, 52 and 54) at 10% SCV and 3% sucrose. Durzan and Gupta (1987); Gupta *et al.* (1995a), reported the use of reduced growth regulator levels in the maintenance medium for *Picea* cultures.

A comparative study of sucrose and maltose (3% each) in maintenance medium showed that sucrose was superior over maltose in the increment of growth of suspension cultures. But browning of culture was found to set in rapidly in sucrose containing medium than maltose containing medium.

Microscopically, the embryogenic culture of *P. kesiya* showed the presence of somatic embryos at various developmental stages interspersed with elongated and spherical cell clusters. The present findings are similar to Gupta and Durzan (1987b) have discussed the similar constitution of embryogenic cultures in loblolly pine. The embryogenic cultures exhibited somatic embryos with fused suspensors and with subsequent culturing those fused parts cleaved into many
embryos connected to a common suspensor (Figure 12b-e and 13c-g). However, these embryos separated from each other on transfer to ABA containing medium (Figure 12f and 13h). These observations suggested that “cleavage polyembryony” occurred in embryogenic cultures that constitute a method of multiplication of somatic embryos. Similar results of polyembryony have also been reported by Gupta and Durzan (1986a,b), Durzan and Gupta (1987a,b), Arya et al. (2000).

Tautorus et al. (1991) reviewed the mode of origin of somatic embryos in vitro in conifers. Origin of somatic embryos may vary depending upon the type of explant material used (Nagmani et al., 1987; Finer et al., 1989). Three pathways have been suggested, which account for the origin of conifer somatic embryos (Hakman et al., 1987)- (i) somatic embryos may arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryonal apex and suspensor region (Hakman et al., 1987; Nagmani et al., 1987; Jain et al., 1989). In P. abies and P. glauca, the two-celled proembryos resulted from an unequal division of somatic cell, forming a dense cytoplasmic embryonal head-like cell and a vacuolated suspensor-like cell. (ii) somatic embryos may develop from small meristematic cells within the suspensor. Their initials could arise by asymmetric division of suspensor cells or from meristematic cells of the embryonal apex region.
that have failed to elongate while being integrated into the suspensor (Hakman et al., 1987). and (iii) somatic embryos could arise by a mechanism similar to cleavage polyembryogeny with the initial separation occurring in embryogenic region. A cleavage-like polyembryony has been described in somatic embryo cultures of *A. alba* (Schuller et al., 1989), *L. deciduas* (Nagmani and Bonga, 1985; von Aderkas and Bonga, 1988), *Pinus* and *Picea* species (Becwar et al., 1988a; von Arnold and Woodward, 1988) and *P. menziesii* (Durzan and Gupta, 1987).

In the present study, non-embryogenic callus appeared white, opaque, friable and turned light green in the light as also reported by Tautorus et al. (1991). The non-embryogenic callus may or may not have anatomical organization and composed of isodiametric cells of variable size. Non-embryogenic suspension cultures were composed of mixture of isodiametric and elongated suspensor like cells (Figure 14 and 15). The cells divided in disorganized fashion and no bipolar structures typical of embryogenic cultures were present. Similar observations were also made by Jasik et al. (1995) in *P. nigra*.

Abscisic acid has been used for cotyledonary embryo development in many plant species including conifers (Gupta and Durzan, 1987a,b). In the present study, the stage-I embryos (Figure 12d
and 13e) converted into stage-II (Figure 12f and 13h) and cotyledonary embryos (Figure 12g-i and 13i) in respective basal media containing higher concentrations of sucrose or combination of sucrose and mannitol (3% each) in conjunction with ABA (Table 56-63). Submerge culture method and filter paper base method were found to be superior over direct culture method of somatic embryo maturation (Figure 16 and 17). It was also found that neither sucrose nor ABA singly could promote maturation of somatic embryos. A concentration of 6% sucrose and 8 mg l\(^{-1}\) ABA was found to be quite effective in embryo development and maturation. A comparative study of sucrose and mannitol in maturation medium showed that use of sucrose and mannitol at 3% level each in conjunction with 8 mg l\(^{-1}\) ABA was most effective and resulted optimum embryo development and maturation from cultures of female gametophytes and zygotic embryos (Table 58) using submerge culture method. It was believed that the higher osmoticum helped in the development of embryos while ABA brought about desiccation stress resulting in maturation of somatic embryos. Sucrose, mannitol and ABA at higher concentrations probably increased the medium osmoticum, thus limiting the water uptake by the tissue and causing water stress, which led to embryo maturation in *P. kesiya*. It has been suggested that the favourable effect of ABA could be due to increase in storage
reserves, such as storage proteins, triglycerides and lipids. Another effect of ABA is prevention of precocious germination (Lelu et al., 1994). 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 could serve as an osmoticum and as a carbon and energy source in black spruce somatic embryo maturation. Li et al. (1998) reported the promotory effect of maltose on embryo maturation in P. taeda. Norgaard (1997) reported that maltose (3.4-4.3%) used singly was better organic carbon source than sucrose (3%) in maturation and germination of somatic embryos in A. nordmanniana. The combined effect of both maltose and sucrose was more satisfactory than either of these used singly. Carrier et al. (1997) studied the effect of exogenous sucrose on maturation and germination of somatic embryo in interior spruce. They 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 available sucrose in the medium. The exogenously supplied sucrose promoted the formation of linolenic acid, which participated in the maturation of embryos. Durzan and Gupta (1987a,b) reported that ABA inhibits cleavage polyembryony and allowed singulation of embryos and further development in Douglas fir.
Mannitol (6%) enhanced embryo maturation in interior spruce (Roberts, 1991). Webster et al. (1990) reported that 40 µM ABA was optimum for the production of mature embryos. Gupta et al. (1995a) reported that in *Picea abies*, embryo development occurred following the removal of auxins and cytokinins and addition of ABA.

In *P. abies*, an increase in exogenous ABA levels (5-40 µM) resulted in an increase in somatic embryo maturation both in semisolid and liquid medium (Vagner et al., 1998). In the absence of exogenous ABA, yield of embryos was negligible. Embryogenic cultures on auxin rich medium has been found to produce ethylene which act as inhibitor in somatic embryo development (Biddington et al., 1993). Further it was 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*. It was found that more ethylene was formed in non-embryogenic cultures. Ethylene interfered with the development of polarity. Ethylene inhibitors like ABA, when incorporated in the media, formation of ethylene was inhibited and that promoted somatic embryo maturation. Influence of ethylene and different ethylene inhibitors on somatic embryo maturation of white spruce was studied by Kong and
Yeung (1994). They reported that the presence of ABA in the development and maturation medium showed a decrease in ethylene production. Absence of any ethylene inhibitor resulted in very poor cotyledonary embryo formation. It was further reported that polarity development was better with decrease in ethylene production.

In the present study, the embryogenic cultures in BCBs from different explants produced stage-I somatic embryos. But these embryos could not develop further into cotyledonary embryo even in presence of ABA and sucrose or ABA and sucrose-mannitol in the medium. Failure in achieving cotyledonary somatic embryos could be attributed to problems linked to self-designed BCBs. In the present study, one of the most important reasons could be flow rate and dissolved oxygen in the medium, which might have affected and inhibited maturation of somatic embryos. Tautorus et al. (1992) aerated the embryogenic cultures of *Picea mariana* and *Picea glauca-engelmannii* at a rate of 0.2 to 0.6 vvm (volume air per volume culture per minute) and dissolved O₂ was maintained at above 80% saturation. Okamoto et al. (1996) reported the effect of oxygen concentration in the aeration gas on plantlet regeneration from rice cells in bioreactor cultures. They showed that the efficiency of regeneration in cultures aerated with gas mixture of over 40% oxygen was higher than that in a SF culture. In the present BCB
culture, though not severe, sedimentation occurred with the embryo cultures to some extent suggesting that the mixing in the BCB was insufficient to agitate the cultures thoroughly. Tanaka (1982) found accumulation of sedimented cells and dead zones in *C. tricuspidata* (*Capsicum*) culture at high cell densities and attributed that to the configurations of airlift bioreactors and bubble bioreactors. Ingram and Mavituna (2000) reported sedimentation of embryos of *P. sitchensis* within the bubble bioreactors. They reinforced that bioreactors could be used successfully for large-scale somatic embryogenesis so long as the bioreactor configuration, design and operating conditions are carefully chosen to suit the physiological, metabolic and morphological characteristics of the culture.

Measurement of cell growth and assessment of growth kinetics are important to optimize the methods for large-scale somatic embryo production (Ryu *et al*., 1990). In the present investigation, growth kinetics were studied and analysed using an array of parameters like sedimented culture volume, packed culture volume. These were compared with fresh weight, dry weight and somatic embryo number in order to determine the parameters that were highly correlated with growth and embryo number. The embryogenic cultures of female gametophytes showed increased biomass in the media with sucrose and
mannitol (each at 3% level) compared to sucrose alone in the medium of female gametophytes (Table 64). In case of cultures from female gametophytes, a maximum dry weight of 1.5 g l\(^{-1}\) (in 10-days time) and 150 embryos ml\(^{-1}\) suspension (in ½ Litvay's medium with 3% each of sucrose and mannitol and 8 mg l\(^{-1}\) ABA containing medium) was obtained (Table 64). Similarly, with cultures of zygotic embryo, a maximum DW of 0.88 g l\(^{-1}\) and 35 embryos ml\(^{-1}\) suspension was found in mMS medium with 3% each of sucrose and mannitol (Table 65). The effect of sucrose concentration on culture growth and number of somatic embryo formation was studied by Tautorus et al. (1992) in black spruce and interior spruce. They reported an increase of FW and DW with 60 mM and 90 mM sucrose in 10-12 days time in the culture medium. Fresh mass, dry mass and corresponding number of embryo formation was also correlated by Dong and Dunstan (1994) in P. glauca. Higher sucrose concentration has been found essential in somatic embryo formation and maturation. Lulsdorf et al. (1992) found that SF suspensions of interior spruce and black spruce became carbohydrate-limited in medium with low concentration of sucrose (30 mM). They also reported that sucrose depletion caused suspension cultures to enter a stationary growth phase, which was characterized by a decline in biomass and browning of embryos. In the present study of growth
kinetics, a lag phase was found with zygotic embryo suspension cultures but no lag phase was found in female gametophyte suspensions and exhibited a linear phase, stationary phase and decline phase (Figure 19 and 20). Similar growth patterns have been reported in embryogenic suspension cultures of *P. sitchensis* (Krogstrup, 1990), *P. glauca-engelmannii* (Lulsdorf *et al.*, 1992), *P. glauca-engelmannii* and *P. mariana* (Tatorus *et al.*, 1992), *P. glauca* (Dong and Dunstan, 1994).

Comparative study of growth parameters of callus culture and growth kinetics of suspension culture in the present study revealed the advantages and superiority of suspension culture over callus culture in somatic embryogenesis. This was evident from the fact that, from suspension culture, a maximum of 150 somatic embryos could be obtained from just 1 ml of suspension whereas the number of somatic embryo formed from 1 g callus was just 15 (Figure 22). Therefore, suspension culture method could be considered as the best potential method for mass-multiplication of somatic embryos and their use in clonal forestry.

Culture technique and media type (i.e. liquid culture and semi-solid culture) have been found to affect maturation of somatic embryos in conifers to some extent. In *P. kesiya* maturation of somatic
embryos were possible in all the culture methods tried i.e. *submerge culture method*, *filter paper base method* and *direct culture method*. There have been no reports of successful maturation of conifer somatic embryos and plantlet recovery following submerged liquid culture method (Attree *et al.*, 1994). Somatic embryo maturation has been restricted to solid medium (Tautorus *et al.*, 1994) or to solid supports soaked with liquid medium (Attree *et al.*, 1994). Ingram and Mavituna (2000) reported maturation of somatic embryos of SS03 *P. sitchensis* genotype using both solid medium and submerged culture method and that conforms to our result.

Development, maturation, germination and conversion of somatic embryos into emblings has been a persistent problem and remains as the limiting factor in regeneration of conifers through somatic embryogenesis (Gupta and Grob, 1995). In the present study, germination of somatic embryos was found better in a low strength medium with low concentration of carbohydrates. Somatic embryos obtained from cultures of female gametophytes showed optimum germination of 56.66% in ¼Litvay’s medium containing 1% sucrose. 50% germination of somatic embryos was recorded in case of cultures derived from zygotic embryos and sucrose was found superior to maltose. Germination of somatic embryos with both the types of
cultures mentioned above were found to be better when cultured directly on semi-solid germination media (Figure 23 and 25). However, our study revealed that, though germination percentage was low (20% and 6.66% with female gametophyte and zygotic embryo cultures respectively) but it was possible to achieve germination using liquid-submerge culture method (Figure 24 and 26). But the germinated embryos from submerged cultures showed thick, fleshy hypocotyls and could not be converted into emblings. No successful reports are available on somatic embryo maturation and germination in liquid medium and have been restricted to solid medium (Tautorus et al., 1994) or to solid supports soaked with liquid medium (Attree et al., 1994). But in P. sitchensis, somatic embryo maturation has been reported in both solid medium and submerged culture (Ingram and Mavituna, 2000). Tremblay (1990) reported germination of P. glauca somatic embryos on ¼SH medium. Bomal and Tremblay (1999) studied the effect of desiccation on germination and plantlet regeneration from black spruce (P. mariana). They reported 93.3-100% germination rate and 33.3-40% germinated plantlets with epicotyls in black spruce. Kim et al. (1999) reported germination of L. leptolepis somatic embryos on ½LM medium with about 2% sucrose. 72% of P. sylvestris and 80% of P. pinaster somatic embryo germination was reported by Lelu et al.
(1999). In the present study, somatic embryos germinated well in light (1900 lux) at 12h photoperiod (Table 66) and a maximum of 56.66% and 50% germination was recorded from female gametophyte and zygotic embryo explants respectively. The embryos of *P. abies* germinated best at 7 days in the dark followed by continuous light and 80-90% germination was recorded (Gupta *et al.*, 1995b). Cryopreserved somatic embryos of *P. patula* were allowed to germinate in the dark before being placed in the light (Ford *et al.*, 2000). Ramarosandratana *et al.* (2001) obtained about 47.8% germination of somatic embryos in maritime pine with 2% sucrose under 16h photoperiod (80 µE m⁻² s⁻¹).

In the present study, gradual decrease of humidity was found better in hardening of the emblings. When the emblings were hardened at higher RH value of 95±5%, there was about 95% survivability of emblings (Table 67) in the hardening experiment. When the emblings were not hardened gradually and RH value was 50±5%, the survivability declined to 15% in both female gametophyte and zygotic embryo derived emblings.

The substrate of vermiculite: peat: pumice (1: 1: 1) as well as soil obtained from pine forest were found equally suitable for transplantation of emblings. Transfer of emblings needed around 1½ months hardening treatment before exposure to natural condition.
In the present study, growth parameters of emblings and seedlings of same age group were evaluated. Measurements of shoot length and root length showed variations in their growth and emblings showed better growth and an increment of 1.4-fold and 1.13-fold in shoot and root length respectively in 180d old emblings over seedlings of same age (Figure 29). Similarly biomass evaluation of 40d, 90d, 180d old (i.e. DW of shoot and root) emblings and seedlings revealed significant difference in the growth performances. The comparative study showed that morphology and growth rates of emblings were better than the seedlings. 45d and 180d old emblings showed 1.14-fold and 1.3-fold increase in biomass respectively as compared to the seedlings of the same age groups (Figure 30). One of the possible reasons for better growth performance of emblings over seedlings could be the capture of ‘+’ characters from the parent. Gupta et al. (1995b) reported normal range of growth rates and morphology of emblings of *P. menziesii*. Normal emblings with normal growth rates to that of seedling was also reported by Webster et al. (1990) in interior spruce. Morphological similarity of somatic embryo-derived plantlets and the control plantlets obtained from seed germination was reported in *P. glauca* (Tremblay, 1990).
In the present study, no significant difference was noted in the survivability performance of emblings to that of seedlings. The survivability of the emblings was about 73.53% with 45d old emblings and it was 50% with 180d old emblings (Figure 31). The survivability of 75% and 51% was recorded in 45d and 180d old seedlings respectively. The potted regenerants showed satisfactory growth and performance in the natural conditions (Figure 32). Plantlets from somatic embryos have been successfully established in soil for *P. menziesii* (Gupta and Durzan 1987a), *P. taeda* (Gupta and Durzan, 1987b), *P. abies* (von Arnold and Hakman, 1988), *L. decidua* × *L. leptolepis* (Klimaszewska, 1989), *P. glauca* (Tremblay, 1990), *P. abies* (Gupta *et al.*, 1991), *P. jezoensis* (Ishii, 1991), *L. occidentalis* (Thompson and von Aderkas, 1992), *P. glauca* (Dunstan *et al.*, 1993), *A. nordmanniana* (Norgaard, 1997), *P. strobus* (Garin *et al.*, 1998), *L. leptolepis* (Kim *et al.*, 1999), *P. sylvestris* and *P. pinaster* (Lelu *et al.*, 1999).

In the present study, the initiation, development, maturation and subsequent conversion of somatic embryos to emblings have been achieved using both semisolid and suspension culture methods. Emblings were established in the pots under natural conditions. Enhancement in number of somatic embryo production through suspension cultures opens up a great potential for large-scale
propagation of this species. Further studies are essential for better understanding of the developmental processes in order to ascertain the best media supplement and other factors to enhance the number of somatic embryo formation in suspension culture and bioreactors. Germination and conversion frequency of SEs to emblings and their successful establishment in soil needs to be worked out in detail in future studies.