Chapter I

Introduction - A review of literature

Somatic embryogenesis in conifers has in the recent years been used as a potential tool for the mass propagation of the forest trees. At the same time, it provides an ideal experimental process for investigation of differentiation in the organism and understanding the expression of totipotency in plant cells. Embryogenic cell lines maintain their competence for a long time and provide a necessary medium for the study of the developmental process associated with somatic embryogenesis.

Tissue culture techniques have found wide use in the commercial propagation of plants including forest trees. Somatic embryogenesis has been considered as a potential tool for propagation of conifers for afforestation programmes (Tautorus et al., 1991; Charest et al., 1995; Pullman et al., 2003; Von Arnold, 2003) mainly because:

- It offers an inexpensive, large scale propagation system for superior genotypes
- Production of somatic embryos throughout the year, reducing the risk where seed production is limited and uncertain
- Other applications which have been foreseen for somatic embryogenesis include provision of establishing cell lines for genetic engineering and
somatic hybridization, long term gene storage, and also use in research to gain an understanding of somatic genetics and development.

Somatic embryogenesis is the process of embryo formation from somatic cells to give rise to whole plants without the fusion of gametes. Somatic embryogenesis may be *direct*, when the embryo develops directly from the somatic or vegetative cells of the explant without the intervening callus phase. It may be *indirect*, when the embryos develop from an undifferentiated mass of cells. Embryogenesis can be induced very easily in some cells like cells of zygotic embryos and these are called *competent* or *pre-embryonic determined* cells. However, there are also a wide variety of cells which behave as embryos only when they are subjected to major manipulations, like the somatic cells in culture. These cells are called as the induced embryogenic determined cells or *potentially competent* cells. Besides, there are some cells which are so highly differentiated that it is almost impossible to induce embryogenesis in them and are designated as the non-competent cells. There are two kinds of growth in tissue culture, *unorganised growth* (forming undifferentiated tissues) and *organised growth* (characterised by development into embryos) are mediated by the exposure of responsive explants to critical concentration of exogenously applied plant growth regulators during the initial culture phase (Gupta and Grob, 1995; Bozhkov et al., 2002). In the absence of auxin, cells grow in an organised way and develop into embryos. Presence of exogenous auxin stimulates cell
enlargement, which disrupts the cellular organisation in the developing embryos and results in the proliferation of callus (Steward et al., 1964).

It has however been observed that the developmental genetic programme in the embryogenically competent somatic cells under the influence of specific phytohormones, proceeds in the same or closely similar fashion as would in a zygote for the development of a zygotic embryo. But somatic embryos are not very precisely organised like zygotic embryo. The suspensor region may consist of loosely associated cells, and many embryos may share a common suspensor system (Hakman and Fowke, 1987b). In contrast, organogenesis shows sequential shoot and root differentiation on different media.

There are three different methods for initiation of embryogenic cultures in conifers:

a) through the continuation of natural cleavage polyembryony of embryonal heads of explanted immature embryos (Durzan and Gupta, 1986b).

b) through cell division in the epidermal and subepidermal layers of hypocotyl, cotyledons or needles resulting in calli which then rapidly organise to form embryonal suspensor masses (Nagmani et al., 1987).

c) through cell division of small cells within the suspensor system of explanted immature embryo (Gupta and Durzan, 1987).
Somatic embryogenesis involves development of embryos from somatic tissues of the plant by redirecting the morphogenetic fate of the cells in the explant to form embryogenic cellular masses or embryoids. Most conifers undergo one of the two types of polyembryony which is a common and natural phenomenon in conifers, and could be either simple or cleavage (Fig.1). 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. Cleavage polyembryony is common in pines, where the cleavage embryos result from a separation of the apical tier cells of an individual proembryo into four files of cells, each of which may develop into a separate embryo. The resulting embryos are therefore genetically identical. One of these embryos becomes dominant and the other embryos cease development. The mechanism by which the successful embryo inhibits the growth of other embryos is unknown, although factors such as mechanical, nutritional and growth inhibiting influences of the dominating embryo, probably have some role to play (Dogra, 1967; Owens & Blake, 1985; Tautorus et al., 1991; Von Aderkas et al., 1991).

Of the conifers, pines constitute the most divergent, and economically important group of species. They provide valuable natural resources and though they are known to be pioneer species and active colonizers of
Figure 1. Simple and cleavage polyembryony in conifers
degraded sites, their population in the recent years have been fast depleting. The fact that they protect watersheds and sustain and regulate water supply in the Himalayan river basin in India, should be reason enough for us to try and sustain their diminishing/ dwindling population.

Ninety species or more of the pine population (genus: *Pinus*, family: Pinaceae, order: Coniferales, Gymnosperms) are known to be distributed in the Northern Hemisphere from the polar region to Guatemala, North Africa and Indonesia. Of these, six pine species are indigenous to India and are found scattered in the Himalayan region from Jammu to the North-east. These species are *P. roxburghii* Sarg. (syn. *P. longifolia* Roxb., *P. serenagensis* Maddeu, chirpine) *P. wallichiana* A.B. Jacks (syn. *P. excelsa*, Blue pine, Kail), *P. gerardiana* Wall. Ex Lamb. (chilgoza pine, Neoza pine), *P. insularis* Endl. (sys. *P. kesiya* Royle ex Gord., khasi pine), *P. merkusii* Jungh (Merkus pine) and *P. armandii* Franchlet (Armandi's pine). Of these *Pinus kesiya* is the most widespread in the north eastern states of India (Fig. 2).

*Pinus kesiya* (Royle ex Gord.) commonly known as the khasi pine is an economically important timber-yielding tree found in the North-east India and extends up to Myanmar, Phillipines and Vietnam. It grows mostly on the hills at an elevation of 750m to 2000m thriving best at 1350-1500m in sub-temperate and fairly moist regions, while it is distributed in Phillipines at elevations of 450m to 2450m above sea level, in upper Burma between 800m to 1900m above sea level, Yunnan and North Thailand and Malay
Figure 2. Distribution of subtropical pine forests in Meghalaya
Archipelago. In Meghalaya, *P. kesiya* is confined to higher reaches (800m to 2000m above sea level) of the Shillong plateau in Khasi and Jaintia hills, in a narrow belt running in east-west direction. Due to variation in altitude from 60m to 2000m above sea level and associated changes in rainfall and temperature, moist tropical forests occur below 1000m and the subtropical semi evergreen forests are found between 1000m and 2000m elevation in the state. In the upper limits it forms pure stand (Fig. 3) while lower down it is mixed with broad leafed species like *Quercus* spp., *Schima wallichii*, *Myrica esculenta*, *Rhododendron arboreum* and *Exbucklandia populnea*. Age old practice of shifting cultivation and other anthropogenic activities such as cutting trees for timber and construction of building, collection of fuelwood during the past several decades have destroyed the climax subtropical broadleafed forests at higher elevations in Meghalaya and elsewhere in the North-east India paving the way for invasion and successful growth of *P. kesiya* (Puri et al., 1989) and according to them, khasi pine was introduced in this region in prehistoric times. The shrub (including small trees) species growing in the forest strands include *Rubus ellipticus*, *R. khasianus*, *Myrsine semiserrata*, *Osbeckia crinita*, *Nellia thyrsiflora*, *Eupatorium* sp., *Lantana camara*, *Artemesia* spp., *Viburnum foetidum*, *Leptodermis* spp., besides others. During the monsoon the forest floor is covered with a profuse herbaceous undergrowth of annuals and perennial flowering plants and ferns, including *Lindenbergia hispida*, *L. racemosa*, *Paspalam* sp., *Ophiopogon wallichii*, *Hedychium coccineum*, *Eurya*
Figure 3. A *Pinus kesiya* strand Shillong, Meghalaya

Figure 4. Decrease in girth of pine trees
acuminate, Rubus monogynus, Senecio cappa, Galinsoga parviflora and Melastoma malabathricum. However, much of the ground flora lies dormant during winter, giving a barren look to the forest floor.

The wood of this tree is moderately hard, pale brown to red in colour and is used for building of houses because of its high resin content. The resinous wood is much used as firewood, the production of which cause considerable injury to the trees. This and deforestation to meet the ever increasing demand for timber, slash and burn cultivation and unplanned developmental activities such as new townships, road and dams, and clearing of land for permanent agriculture are the main reasons for the dwindling populations of *Pinus kesiya* in the recent years. Even the girth of the trees now shows reduction in many areas (Fig. 4). The problem has attained such a magnitude that Supreme Court of India has banned felling of all trees in the North-eastern region.

The trees are evergreen and monoecious, generally 25-30m tall with straight cylindrical bole and 2m girth bearing branches in pseudowhorls, crown on mature trees are broad (Fig.5). Bark is 2-4 cm thick, fissured and scale like in younger trees while dark brown in mature trees variously furrowed and plated. Shoots are light brownish, and are of two kinds, dwarf or spur shoot of limited growth, and long shoot of unlimited growth. Leaves are dimorphic, the small, thin, brown scale leaves which occur on long as well as dwarf shoots, from the axis of these scaly bracts arise the needle like
Figure 5. Morphology of *Pinus kesiya*

a. Complete tree
b. A branch with immature male and female cones
c. Mature female cones (before seed dispersal)
d. Mature female cones (showing dehiscence)
green foliage leaves 15-20 cm long which develop in cluster of three at the apex of the dwarf shoot. The tree generally flowers in April-May and bears ovulate cones after two years. Staminate cones are numerous and small, a mature cone is about 20-30 cm long, mostly ovoid to cylindrical in shape, yellow, orange or light red in colour and composed of many spirally arranged microsporophylls with two pollen sacs, seen as a dense cluster around the base of the current year growth. Pollen grains are shed and dispersed by wind during February to April. The female cones are purplish to deep green in colour and borne solitary or in clusters of two, three or five on the apices of new shoots. The young cones are erect and ovoid with fertile scales of inflorescence spirally arranged, with a bract scale above each of them. The scales on the cone close immediately after pollination and the cones turn inverted or horizontal on the branch to hibernate for the cold winter. The following season the cones again show active growth, turn green, fertilization occurs during this time, a year after pollination, and the cones are pushed to the lateral position on the branch due to the growth of new shoots. The following winter the cones gradually turn hard and brown, and attain their full development. In the third season, i.e., 24 - 26 months after appearance of the female cones, they open during the last week of December and dispersal of seeds continue up to March, the dry weather aids in the dispersal of the winged seeds, two in number attached at the base of each
woody scale. On an average, seeds are 5.7mm long and 3.9mm wide, the wing being about 18-20 mm long and the cotyledons being 3.5-4.0mm long.

Most pines are fire adapted, so in *P. kesiya*, the seedlings are fairly resistant to fire once over 3m tall, and mature trees are immune to fire damage owing to their extremely thick outer bark. However short cycles of shifting cultivation and regular annual fires prevent satisfactory regeneration and can lead to the elimination of pine. The pines remain dominant so long as fires occur at intervals of about 5 to 20 years. Pines are a frost tolerant species and can grow on well drained nutrient-poor soils on moderately sloping hillsides and landslide effected areas, all of which they colonize as typical pioneers. Its natural regeneration is best on mineral soils as well as porous soils with partially exposed to fully exposed rocks, primarily limestones. Soil development is minimal where recurrent fire has consumed litter and under growth vegetation. There being very little organic matter left on the surface, which sometimes is a bare rock, the underlying rocks are overlaid with a thin layer of poor soil, which serves as the rooting medium for pine. The species is preferred by foresters and the Forest departments of the North-eastern states have been involved in forest plantations over the years particularly for regeneration of areas affected by shifting cultivation. However, all these plantation programmes are achieved through conventional methods that appear to be inadequate to keep pace with the
ever increasing demand for timber and also lack of uniformity of planting material with desired characteristics.

Propagation of this tree by conventional methods faces constraints as natural regeneration occurs through seeds and the seed orchards show great variations and at times seed germination is very poor. Factors influencing natural regeneration of pines include, seed viability, light, moisture, soil condition, undergrowth, fire, grazing. A large number of seedlings are destroyed by fire, low winter temperature, heavy rainfall, cattle grazing, caterpillars and moths. Moreover, pine trees bear seeds in a cyclic manner and every third year is a good seed year and in between the trees bear only small crop reducing the seed reserves considerably. On the other hand multiplication through seeds gives rise to plants which are not true to type. Weed infestation is another factor adding to the cause of seedling mortality. Progress in large scale improved forest production through conventional methods is usually very slow and time consuming. The vegetative propagation methods used for multiplication from economically favoured genotypes through stem cuttings is rather difficult as this pine reaches sexual maturity at an early stage after which rooting ability of cutting decreases resulting in poor regeneration. In addition, as has been reported in other pine species, with increase in the age of mother tree, the rooting ability of cuttings is reduced considerably. It has been recommended that acreage under forest cover needs to be doubled. But *P. kesiya* is also susceptible to
different types of infection by pathogens at various stages of growth and development i.e., seedlings, nursery, plantations, and in natural strands. Many fungi are responsible for diseases like stem rot, needle rust and seed borne diseases, which results in tremendous loss to annual yield of timber in plantation as well as in natural pine stands. To meet the predicted requirements of timber and to conserve forests, there is an urgent need for mass propagation of *P. kesiya* using unconventional methods of propagation. Somatic embryogenesis through tissue culture has developed into an important tool through which plantation programmes could be implemented, produce plantlets on mass scale in a short period of time, besides establishing a system where the developmental mechanism of the plant under tissue culture can be studied.

Somatic polyembryogenesis has been described by many workers and have termed the embryogenic tissues as ‘embryonal suspensor masses’ (ESMs) due to their high degree of organization. Most embryogenic cultures of conifers have been induced from members of family Pinaceae and are similar in appearance. An embryo from a seed is placed on a medium containing appropriate nutrients, hormones etc. First a callus culture grows then becomes embryogenic and made of ESMs which are usually white, translucent in appearance and appears to glister due to production of mucilage when cultured on semi-solidified medium. They anatomically consist of a variable mixture of elongated cells, early stage embryos, which
have embryonal head and a suspensor system and sometimes later stage embryos (Gupta and Durzan, 1987a, and b; Finer et al., 1989; Laine and David, 1990). On the other hand non embryogenic callus appears opaque, friable and green when exposed to light. This type of callus may or may not have an anatomical organization. The somatic embryos differentiate on the callus, these embryos can then be germinated and grown into somatic emblings.

The embryogenic tissues induced in pines are generally glossy, translucent, white, mucilaginous cellular mass containing a mixture of elongated densely cytoplasmic clumped cells, embryo initials and sometimes older stage embryos (Gupta and Durzan, 1987b; Finer et al., 1998; Laine and David, 1990).

Considerable efforts have been directed towards somatic embryogenesis of conifers for many years. Studies have been conducted on the growth, metabolism and developmental patterns that characterize callus and cell suspension of conifers (Durzan and Steward, 1968; Durzan et al., 1976; Durzan 1980). Somatic embryogenesis and plant regeneration in conifers was first reported in *Picea abies* (Chalupa, 1985 and Hakman et al., 1985). The development of somatic embryogenesis in conifers has shown much progress since then (Steward et al., 1964; Attree and Fowke, 1993; Jain et al., 1995; Bozhkov et al., 2002). Somatic embryogenesis or organogenesis of tissues in *in vitro* cultures are influenced by environmental conditions of the
culture medium (Williams and Maheswaran, 1986) and besides basal medium, induction and development of somatic embryogenesis is influenced by various other factors like light, temperature, relative humidity, pH of the medium and organic carbon source.

The proper explant selection is critical for successful induction of somatic embryogenesis in vitro. Various tissues from the same plant or even tissues at different development stages have shown difference in their response when cultured in vitro (Attree and Fowke, 1991; Attree et al., 1991, Deb and Tandon, 2002; 2004). Mostly zygotic explants have so far been used to initiate embryogenic cultures in conifers. These tissues include megagametophytes containing developing zygotic embryos (immature embryos), mature zygotic embryos dissected from stored seeds, tissues from hypocotyls, cotyledons, leaf needles, apical domes and also recycled cotyledonary somatic embryos. Explants from various conifers that have been induced to form somatic embryos include explants from genera like *Abies, Larix, Picea, Pinus, Pseudotsuga* and *Sequoia*. *Pinus* is by far the largest and most important genus of conifers comprising approximately 95 spp. widely distributed over the Northern Hemisphere (Preston, 1989). While the process of somatic embryogenesis and its technique has been sufficiently well refined for commercial application in many species of spruce and larch, it is still not available for all conifers. In pine species, it was found much
more difficult to obtain somatic embryogenesis and even mass propagation of a large number of clonal lines is not yet possible.

Induction of somatic embryos in conifers have been obtained from excised tissues from seedlings, mature and immature zygotic embryos (Jain et al., 1989; Laine and David, 1990). In addition, megagametophytes of several conifers have been cultured with varying success. Large scale production of somatic embryos through embryonal suspensor masses by cleavage polyembryony in liquid culture with high concentration of hormones than that used for induction has been reported in conifers (Boulay et al., 1988; Gupta et al., 1991). In pines, somatic embryogenesis could be induced from explants at early stages of zygotic embryo development (Gupta and Durzan, 1987b), while Laine and David (1990) reported from early stages of polyembryony. Finer et al. (1989) found response from immature zygotic embryos just prior to cotyledon development in Pinus strobus, with increase in induction frequency with increase in age of zygotic embryos i.e., before cotyledonary primordia appeared. In contrast Jones and van Staden (1995) found response and embryogenesis at all stages of embryo development except at very young and cotyledonary stage in P. patula.

Many physiochemical factors have been reported to influence the initiation of embryogenic callus initiation in pines species. These factors include: basal media composition (Liao and Amerson, 1995a; Li and Huang, 1996; Deb and Tandon, 2002; 2004) plant growth regulators composition
(Becwar et al., 1988a; Nagmani et al., 1993) gelling agents (Li et al., 1997a, b, 1998) meso-inositol and silver nitrate concentration (Li and Huang, 1996), organic carbon source (von Arnold, 1987; Becwar et al., 1988a, b), organic nitrogen (Barett et al., 1997), and pH of the medium. Generally pH value 5.5 to 6.0 was found to be effective. Besides basal media composition and its supplements, induction and development of somatic embryogenesis has been found to be influenced by some other factors like the cultural conditions including pretreatment, light, temperature and the relative humidity.

Culture conditions play a vital role in tissue culture raised plants and initiation of somatic embryogenesis in cultures is largely regulated by the cultural conditions. Many environmental factors have been reported to which plant cells respond, and hence divide, elongate, polarize and differentiate. Light was found to be inhibitory for the induction of somatic embryogenesis in conifers and cultures incubated in the dark showed profuse callusing. Cultures grown in light tend to turn green and instead showed organogenesis. At the same time temperature effected the rate of embryogenesis in cultures.

Modification of basal medium components and culture conditions can significantly affect induction of embryogenic tissues and play a major role in enhancing initiation for more explants (von Arnold, 1987; Attree et al., 1990a; Tautorus et al., 1990a). Several media have been used by the workers in the
original as well as in various modified forms. Media requirements have thus not been very specific. It has been observed, however, that a certain basal media was best suited for the species of a particular genera. Thus embryonal suspensor masses have been initiated on several media such as LV (Litvay et al., 1981), DCR (Gupta and Durzan, 1985), DCR₁ (Becwar et al., 1995), modified Murashige and Skooge (mMS) (Gupta and Durzan, 1986b), BLG (Verhagen and Wann, 1989), P₆ (Gupta and Pullman, 1990), BM₁ (Gupta and Pullman, 1991), LP (Quoirin and Lepoivre, 1977), WTC (Gupta and Pullman, 1991) besides others. In all these cases it was found that the main modification appeared in the nitrate salt concentration especially ammonium nitrate and potassium nitrate. Barrett et al. (1997) has reported that removal of organic nitrogen sources like casein hydrolysate (CH) and L - glutamine is beneficial for P. glauca:

Saccharides are known to serve as carbon and energy sources, osmotic agents, stress protectants and signal molecules in plants. A few reviews have focused on deeper studies of carbohydrate metabolism, enabling insight into the physiological background of the crucial effects of carbohydrates by collecting and critically discussing the experimental data on exogenous saccharide applied, resulting endogenous levels and key enzyme activities obtained. The most thoroughly described genus in conifers is Picea (Lipavska and Kondradova, 2004). Low percentage of sucrose (1-2%) resulted in more ESMs formation (von Arnold, 1987; Becwar et al., 1988a, b). In the
maintenance medium too, concentration of the carbon source effected the formation and development of the proembryonal masses in media supplemented with 6% sucrose along with 10mgl\(^{-1}\) abscisic acid (ABA). Again with decrease of sucrose concentration from 20gl\(^{-1}\) to 5gl\(^{-1}\), there was a decrease in proembryo formation and loss of embryogenic potential in \(P.\) \textit{caribaea} (Laine and David, 1990). The physiological and osmotic roles of sucrose has been investigated by Trembley and Trembley (1995) in black spruce (\(P.\) \textit{mariana}), while a comparative study was carried out to understand the role of maltose and sucrose on the total number of mature somatic embryo formation in \(P.\) \textit{strobes} (Garin \textit{et al.}, 2000).

L-Glutamine has been used in the maintainance medium in concentrations lower than in the induction medium for the development of sugar pine (Gupta and Durzan, 1986a, b) and loblolly pine somatic embryos (Gupta and Durzan, 1987). Besides, role of polyethylene glycol (PEG) in the maturation medium was reported by Li \textit{et al.} (1997, 1998) in \(P.\) \textit{taeda} cultures which developed into mature somatic embryos successfully.

In most conifers, somatic embryos undergo morphologically similar development to zygotic embryos (Misra, 1994). Conifer somatic embryo usually has to be stimulated by ABA. A combination of ABA and a suitable osmoticum (for eg., non permeating osmoticum like PEG or permeating like higher percentage of carbohydrates like sucrose, maltose, mannitol)
promotes the normal development of somatic embryos in conifers and are essential for their gene expression.

Among the gelling agents gelrite was reported to be a superior compared to agar for initiation of ESMs in *P. strobes* cultures (Finer *et al.*, 1989; Puchooa *et al.*, 1999), while phytagel was effective for *P. taeda* (Li *et al.*, 1998).

Plant growth regulators appear to control all the main developmental events in somatic embryogenesis starting from induction of embryogenic cultures to germination of somatic embryos, and majority of the workers have unambiguously consented to the crucial role of plant growth regulators in the regulation of somatic embryogenesis. The effect of exogenously added growth regulators in media was extensively studied by Vagner *et al.* (1998), but about endogenous state of the growth regulators, very little is known (Dunstan *et al.*, 1995). Usually both auxins and cytokinins are necessary for somatic embryogenesis and amongst the various auxins, 2,4-dichlorophenoxy acetic acid (2,4-D) was the most preferred for the initiation of ESMs in most conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). α-naphthaleneacetic acid (NAA) also has been used for ESMs induction in *P. abies* (Verhagen and Wann, 1989) while no significant difference was reported in ESMs or development of embryos in conifers with NAA versus 2,4-D as the sole auxin source (Gupta and Grob, 1995). Again with 2,4-D as the sole auxin source, somatic embryos were induced in *P. taeda* (Li *et al.*, 1998).
1998), and incorporation of either 6-benzylaminopurine (BAP) or kinetin in the medium was found to be beneficial in most cases. Auxin proved to be inhibitory for the initiation of embryogenic cultures in *A. nordmanniana* (Norgaard and Krogstrup, 1991; Norgaard *et al.*, 1992). Development of ESMs was observed by application of different concentrations of hormones, like 2,4-D (2-10mg l\(^{-1}\)) and BA (0.5-5.0mg l\(^{-1}\)) in *Larix* (Cornu and Geoffrion, 1990; Bonga *et al.*, 1995), NAA and BAP (2mg l\(^{-1}\) each) and 2,4-D (10-110mg l\(^{-1}\)) in *P. abies* (Gupta *et al.*, 1991), and 2,4-D (2-10mg l\(^{-1}\)) along with BAP (0.5-2.5mg l\(^{-1}\)) in *P. palustris* (Nagmani *et al.*, 1993). A higher frequency of embryogenic callus was reported at lower concentrations of phytohormones in *P. serotina* (Becwar *et al.*, 1988) and *P. taeda* (Li *et al.*, 1998). Nagmani *et al.* (1993) on the contrary reported an increased frequency at higher level of plant growth regulators in *P. palustris*.

Embryo maturation and plant development have been significantly influenced by different concentration of ABA in the medium. Cleavage polyembryony is inhibited by ABA allowing singulation and continued growth of individual embryos. Several studies resulted in improved embryo maturation after treatment with ABA prior to transfer to phytohormone free medium for final germination (Backs and Reinert, 1970; Durzan and Gupta, 1987; Roberts *et al.*, 1990a; Dunstan *et al.*, 1994). Promotive effect of ABA on maturation of somatic embryos was reported in *P. sylvestris* and *P. pineaster* (Lelu *et al.*, 1999). Different species and genotypes show different frequency
of proliferation of embryogenic cultures in the medium. While some species and genotypes proliferate readily into embryogenic cultures on the induction medium, in some others, reformulation of medium is required (Gupta and Grob, 1995). Maintenance and proliferation of embryogenic culture is important to increase its availability for regeneration and genetic manipulation. Proliferation is usually done on medium with lower concentrations of growth regulators. The final step in conifer somatic embryogenesis is the successful germination of embryoids into emblings which is generally achieved on media free of any growth regulators. There has been limited report of successful establishment of regenerants in conifers and so far, successful germination and subsequent transfer of somatic embryos in *Pinus* is restricted to a few species, viz. *P. patula*, *P. taeda* (Gupta and Durzan, 1987; Gupta and Pullman, 1990; Tang et al., 1998a,b), *P. caribaea* (Laine et al., 1992), *P. patula* (Jones et al., 1993; Jones and Van Staden, 1995; Ford et al., 2000), *P. sylvestris* (Lelu et al., 1999), *P. elliottii* (Tang et al., 1997), *P. strobes* (Klimaszewska and Smith, 1997; Garin et al., 1998; Klimaszewska et al., 2000), *P. nigra* (Salajova et al., 1999) and *P. pinaster* (Lelu et al., 1999).

In spite of constantly growing knowledge, there is a lack of proper understanding of the biochemical and physiological events involved in somatic embryogenesis. To develop a deeper understanding of the regulation of embryogenesis, it is necessary to conduct detailed biochemical
studies of the complex, highly conserved developmental events which lead to the formation of somatic embryos.

Somatic embryogenesis was first clearly described in domestic carrot (*Daucus carota* L.) and till date the carrot system is the most comprehensively studied with respect to culture conditions and developmental physiology and biochemistry of somatic embryogenesis. Hence, it has been a useful model for investigation of the mechanisms controlling somatic embryogenesis. It was way back in 1970 that the study of biochemical aspects of somatic embryogenesis began to be studied and till date a lot of work has been done in this aspect.

The synthetic auxin 2,4-D has been shown to be the most efficient inducer of embryogenic pathway (Ammirato, 1983; Sung *et al.*, 1984). Embryo specific genes and proteins have been intensely searched for and studied (Backs and Reinert, 1970; Mc William *et al.*, 1974; Nomura and Komamine, 1985; Zimmerman, 1993; Donga and Dunstan, 1994; Paques, 1993). From an ultrastructural study of embryogenesis in carrot cell suspension, it was concluded that embryogenic induction probably occurs during isolation and growth of tissues in auxin containing medium, although formation of more organised structures reminiscent of zygotic embryos is prevented as long as auxin is present in the medium. At the same time, the profile of newly synthesized proteins of nonembryogenic and embryogenic cultures of carrot was made by two-dimensional gel electrophoresis (Sung
and Okimoto, 1981). In this work using $^3$H-methionine, to label the proteins, the workers have identified two proteins in 12 day old embryogenic cells. The surprising finding was that regardless of the presence or absence of 2,4-D in the medium, these proteins were synthesized by cells during the early days of their growth in fresh medium, but in the presence of 2,4-D, the proteins gradually diminished and completely disappeared after 12 days. Hence, it seems that synthesis of embryogenic proteins is triggered by auxin, its very presence in the medium also prevents the continued synthesis of these proteins necessary for embryogenesis coming to fruition.

The mechanism of the regulation of somatic embryo development of broad leafed woody flora remained largely unknown. Nevertheless, these systems have begun to be investigated (Gavish et al., 1991, 1992; Puupponen et al., 1993). Gene expression during seed development, maturation and germination has been examined in several angiosperm species and distinct subsets of developmental regulated genes that respond to distinct regulatory signals have been identified (Goldberg et al., 1989); much less is known about conifer somatic embryogenesis. The study has been limited due to the long reproductive cycle and inaccessibility of developing seeds from conifer species. A biochemical marker may be useful for early identification of embryogenic cultures before any morphological changes occur. Its use would help to optimize culture conditions necessary for embryogenesis and to discriminate cultures following the multiplication process. Some of the
reasons for the lack of information was unavailability of data on the isolation and characterization of markers for embryogenic cells. Efforts were made to find specific molecular markers for somatic embryos and several biochemical variables have been shown to discriminate between embryogenic and non-embryogenic tissues in cultures (Sung and Okimoto, 1981; Choi and Sung, 1984; Nomura and Komamine, 1986). Somatic embryogenesis in _Abies alba_ Mill was even induced using SH medium (Vooková et al., 1998), though other conifers did not respond in this medium. Macromolecule accumulation and synthesis (proteins, polysaccharides and nucleic acids) are indicators of cell growth and physiological change (De Vries _et al._, 1988; Neilson _et al._, 1992; Coutos-Thevenot _et al._, 1993; Uchiyama _et al._, 1993). Proteins as indicators of differentiation have been investigated as markers (Sung and Okimoto, 1981; Komamine _et al._, 1992; Paques _et al._, 1993; Kormut _et al._, 2003) while they could be useful to identify specific stages of development of somatic embryos (Menendez _et al._, 1994). Enzyme patterns also change during developmental stages and peroxidase patterns have been known to indicate embryogenecity of cultures (Kochba _et al._, 1977; Hrubcova _et al._, 1994; Egertsdotter, 1998; Bagnoli _et al._, 1998; Kormut _et al._, 2003).

In carrot, results from two dimensional gel electrophoresis detected three embryogenic proteins ‘a’, ‘b’, ‘c’, throughout the process of totipotency i.e., from single competent cells to the globular embryo stage via, heart shaped and torpedo shaped embryos, but disappeared during the process of
losing totipotency. Two mRNAs showed the same pattern as the proteins. Additionally, protein 'd' disappeared during the single cell stage. In stages of cell clusters and in globular stage embryos, the pattern of in vitro translated products of mRNA extracted from embryogenic and nonembryogenic cultures (in the presence of auxin) were exactly similar except for four proteins, two appeared and two disappeared during these phases. This indicates that a few proteins accumulate during maturation and can identify somatic embryos that have completed the phase of embryogenesis. Donga and Dunstan (1994) observed that intracellular protein content is maximum during the early stage (at day 9) of culture and decreased at a later stage of culture. Against the general assumption that soluble storage proteins are similar in zygotic and somatic embryos of conifers (Hakman et al., 1990; Hakman, 1993). Kormut et al. (2003) indicated the presence of a higher number of storage proteins in somatic embryos of silver fir than in their zygotic counterparts. Among six super-numerary fractions revealed in somatic embryos, the 53 kDa fraction was the most conspicuous that marked the divergent nature of somatic embryos. The 16 and 19 kDa fractions were detected in the embryogenic callus only; not in the nonembryogenic calli. The transition between these tissues represents the initial stage of somatic embryogenesis in conifers. Paques et al. (1993) detected both nonembryogenic callus-specific and embryogenic callus-specific polypeptides to distinguish the two types of calli of P. abies.
Smith et al. (1988) reported a nuclear protein associated with cell division, an antigen against 21D7 monoclonal antibody. Komamine et al. (1992) applied 21D7 to the carrot system and analysed by Western blotting and immunocytochemical method to examine whether antigen 21D7 (21D7 protein) can be a candidate for molecular marker of totipotency. 21D7 could be detected throughout the process of expression of totipotency, which disappeared within 48 hrs during the process of losing the totipotency, i.e., when the single cell were cultured in the absence of auxin. Furthermore, when the single cells were microinjected with 21D7, they elongated and no longer divided nor differentiated even if cultured in the presence of auxin. These results indicate that expression of 21D7 protein may be essential for expression of totipotency.

In zygotic embryo development, secretion of extracellular proteins has been considered a physiological event regulated by embryo specific genes. These extracellular proteins have various functions during embryo differentiation and development, such as metabolism, nutrient storage, phytohormone synthesis and transportation. There is increasing evidence to indicate that the secretion of extracellular proteins in vitro in embryogenic suspension cultures is also developmentally regulated by genes, reflective of normal requirements of the embryo development.

In one instance, an extracellular protein (EP1) that is only secreted by nonembryogenic cells (Van Engelen et al., 1991). Sterk et al. (1991) reported
that another extracellular protein (EP2) identified as lipid transfer protein, was only synthesized by embryogenic cells and somatic embryos. From these and other extracellular proteins described (Satoh and Fujii, 1988), it emerges that the developmental state of carrot suspension cells is reflected in the type of secreted proteins synthesized by these cells.

Extracellular proteins have various functions during embryo differentiation and development such as metabolism, nutrient storage, phytohormone synthesis and transportation. There is increasing evidence to indicate that genes reflective of normal requirements of embryo development also developmentally regulate the secretion of extracellular proteins in vitro in embryogenic suspension cultures (Van Engelen et al., 1991), they also identified an extracellular protein which is only secreted by nonembryogenic cells. Sterk et al. (1991) have identified a second protein which acts as lipid transfer protein and is synthesized by only nonembryogenic cells and somatic embryos. Egertsdotter et al. (1993) observed that the extracellular protein profiles from P. abies embryogenic suspension cultures were different between those cultures from which cotyledonary somatic embryos could be matured and those not capable of maturation. All embryogenic cultures consisting of somatic embryos with densely packed cells in their embryogenic region secrete proteins of 28 and 85 kD. Besides, concentrated extracellular proteins from embryogenic cell line stimulate another nonembryogenic cell line to develop further
(Egertsdotter et al., 1993). And since extracellular proteins of 28, 66 and 85 kD are also secreted by the induced cell line once they have attained embryogenic potential, it is assumed that these proteins, which are not normally secreted by this cell line, are involved in the stimulation of this induction process. This protein 28 kD is a member of protein family that appear to act by changing the membrane permeability.

The gene of a similar protein zeamatin isolated from Zea mays has been characterised (Malehorn et al., 1994) the polyclonal antiserum of which recognizes two extracellular proteins of 18 and 28 kD which is absent in the induced tissue. Zeamatin-like proteins, whose only known functions in the plant is to inhibit fungal growth, are stored in high concentrations in seeds (Roberts et al., 1990).

SDS-PAGE of soluble proteins from extracts of harvested tissues of sandalwood revealed the presence of 15 major bands ranging in molecular weight from 14–18 kD. From the profile study it was observed that two low molecular weight proteins of 15 and 30 kD, which could not be detected in the callus stage appeared as major bands in extracts from tissues of later stages. There was a consistent increase in the peak area of the polypeptides with progressive embryogenesis. Polypeptide pattern alteration analysed in the course of somatic embryogenesis of carrot (Sung and Okimoto, 1981; Choi and Sung, 1984; De Vries et al., 1988; Komamine et al., 1992), Nicotiana plumbaginifolia and Digitalis lanata (Reinbothe et al., 1992), pea cultivars (Stirn
and Jacobson, 1987) and Cinchorium intybus (Bayer et al., 1993) similarly revealed only minor adjustments to the pre-existing gene expression programme, and it is likely that many of the molecular processes of embryogenesis are already established in polyembryonal masses (PEMs) in the presence of auxin. Komamine et al. (1992) suggested that only a few proteins play an important role during embryogenesis, and these proteins are stage specific.

Using sandalwood somatic embryo cultures, a 48-50 kD intracellular glycoprotein was detected in extracts from tissues of PEMs and globular embryo stage, in gels stained for glycoproteins. The level of protein apparently decreased with transition of embryos to bipolar embryos. However, when globular embryos were cultures in 2,4-D containing MS medium, they disorganized into spheroidal and elongated cells, and the level of glycoproteins was higher than in the first two stages (Shankara Rao et al., 1996).

Endochitinases have been identified (De Jong et al., 1993) that has been able to rescue the development of somatic embryos of D. carota in a temperature-sensitive mutant, the main function being formation of the embryo protoderm. An assay system was presented based on the observation that the phenocritical period in temperature sensitive (ts) arrest at globular stage in carrot cell mutant ts11 coincided with the period of sensitivity to replacement of the conditioned medium by fresh growth
medium. When the medium conditioned by the wild type cell line was added to the ts11 culture medium, arrest at the globular stage under nonpermissive temperatures was lifted and embryo development in ts11 was completed up to torpedo stage, resulting in the formation of plantlets. This effect was found to be protease sensitive, suggesting that the secreted proteins were the causative component of the conditioned medium (Lo Schiavo et al., 1990; Anke et al., 1992). Purification of the secreted protein and partial protein sequences obtained from it, as well as biochemical characterization, identified this extracellular protein, designated EP3, as a 32 kD glycosylated acidic endochitinase. These results indicate that, apart from their postulated role in the plant defense response, at least the family of plant proteins with chitinase activity has a function in somatic embryo development. Bacterial signal proteins have been shown to have the same effect on embryo development in *D. carota* as the 32 kD endochitinase (Goldberg et al., 1989). In that study it was concluded that embryogenic cell lines of *P. abies* contain somatic embryos which have reached different stages of development reflected in the presence of extracellular proteins.

Peroxidase activity in embryogenic cells and its somatic embryos have been studied by many workers. Egertsdotter (1998) working on peroxidases and chitinase activity of *P. abies* has shown them to differ between developmental stages of somatic embryogenesis (Kormut et al., 2003). According to Bagnoli et al. (1998) reported that the antioxidant enzymes
superoxide dismutase and catalase could be convenient markers to define the developmental stages in *Aesculus hippo-castanum* somatic and zygotic embryogenesis. The same role was also postulated for peroxidase and esterase, whose isoenzyme patterns were shown to reflect the embryogenic potential of *Medicago sativa* and *Dactylis glomerata* suspension cultures (Hrubcová et al., 1994). The peroxidase activity of mature somatic embryos was triple the corresponding enzyme activity of dormant zygotic embryos of silver fir. Starting with the early cotyledonary stage, a decline in peroxidase activity was registered throughout zygotic embryogenesis, and the situation was similar during somatic embryogenesis. However, peroxidase activity changed abruptly during two stages of somatic embryogenesis. The first stage was the transition of nonembryogenic to embryogenic callus, accompanied by a conspicuous decline in specific enzyme activity. The second stage was that of regenerated seedlings, which had seven times higher peroxidase activity than mature somatic embryos (Kormut et al., 2003). Tunicamycin, a fungal antibiotic, which prevents N-glycosylation of proteins was found to inhibit somatic embryo development at an early preglobular stage in *D. carota*. This inhibition could be overcome by simultaneous addition of correctly glycosylated proteins to the culture medium (De Vries et al., 1988). This glycoprotein was purified and identified as a cationic peroxidase of horse radish (Cordewener et al., 1991). Thus, they have isolated and purified a cationic peroxidase that actively prevented cell
expansion in the preglobular stage embryos. The enzyme has been shown to be responsible for development of somatic embryos in carrot cell cultures. Based on the observed expansion of small embryogenic cells in the presence of tunicamycin and the identification of a peroxidase activity that prevents this expansion, a model was presented that identifies the peroxidase-mediated restriction of cell size as an important prerequisite for successful somatic embryogenesis to occur (Van Engelen and De Vries, 1992). It has also been observed that peroxidase activity is significantly higher in areas surrounding wounds and necrosis followed by rise in soluble protein content (Johansson et al., 2004) besides playing a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic compounds cross-link (Saroop et al., 2002).

Ethylene is a phytohormone that plays an important role in every phase of plant growth and development (Abeles et al., 1992). Its biosynthetic pathway has been well established (reviewed by Yang and Hoffman, 1984; Kende, 1993). In higher plants, ethylene is synthesized from methionine through S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC). In plant cell, tissue and organ culture, the influence of ethylene on the regulation of different physiological processes occurring in in vitro culture, particularly during somatic embryogenesis, is not fully understood. This led to studies on the effects of ethylene on different steps of somatic embryogenesis and have yielded conflicting results and conclusions
(Gahagan et al., 1968), in conifers (Yang and Hoffman, 1984; Tan and Thimann 1989; Li and Huang, 1996; Selby et al., 1996; El Meskaoui and Tremblay, 1999; El Meskaoui et al., 2000). Silver nitrate being an ethylene antagonist was used to investigate and determine if the ability of an embryogenic cell line to produce mature embryos, i.e. maturation capacity, could be associated with its patterns of ethylene production (Meskaoui and Tremblay, 2001).

An upward shift in the concentration of calcium (Ca²⁺) present in the medium during somatic embryogenesis increased the number of embryos produced, approximately to two folds (Jansen et al., 1990). It was found that at elevated concentrations of calcium, the synthetic auxin was not able to completely prevent somatic embryogenesis, suggesting that calcium partially counteracts the inhibitory action of 2,4-D on somatic embryogenesis. On the contrary reducing the concentration of calcium to one fourth in the maturation medium improved embryo development (Pullman et al., 2003), this trend however varied when experimented with differing levels of boron, iron, potassium and copper ion concentrations.

Somatic embryos of carrot contain low levels of abscisic acid during early stages of development, the levels then reach a peak and decline during maturation (Kamada and Harada, 1981; Dunstan et al., 1991). Exogenous application of ABA to immature zygotic embryos of carrot suggests that ABA specifically inhibits precocious germination and promotes maturation
and accumulation of storage proteins (Barret, 1986; Kuhlemeier et al., 1987). Before them, Sung and Okimoto (1981) found that the light treatments that promote maturation (formation of cotyledons) also increase levels of exogenous ABA in carrot somatic embryos, and aberrant embryo structures formation is suppressed (Kamada et al., 1981). Some more recent experiments have suggested that ABA may play an important role in regulating the expression of some classes of embryo specific genes (Goldberg et al., 1989; Kermode, 1990). Besides, the maturation medium of conifers commonly contains ABA which is necessary for embryo maturation (Jain et al., 1995).

The influence of the levels of ethylene in the embryogenic cultures and maturation of somatic embryos in experiments on black spruce embryogenic cell lines (El Meskaoui and Tremblay, 2001) can be significantly observed and thus, it may be that somatic embryogenesis is regulated by the interaction between endogenous ABA and ethylene metabolism. A link between ethylene and ABA has been reported in oat leaves and in apple slices, where ABA decreases ethylene production through a decrease in ACC synthesis (Tan and Thimann, 1989). Moreover, the endogenous ACC level in embryogenic tissues of black spruce and white spruce growing on maintenance medium, without ABA but with 2,4-D and BA, was very high compared to those in the embryogenic tissues cultivated on maturation medium (El Meskaoui and Tremblay, 2001). In white spruce, ABA reduced ethylene production and promoted maturation (Kong and Yeung, 1994), and
it is important to note that ABA has been found to be both promotive and inhibitory on somatic embryos maturation depending on its concentration, besides auxin/cytokinin ratio that may be responsible for reduced ACC synthesis in cultures during maturation. It has also been indicated that ethylene can negatively affect the quality of white spruce somatic embryos by forming large intercellular spaces in the shoot apex that can be partly responsible for the low conversion rate of the somatic embryos into plants (Kong and Yeung, 1994). However, ethylene did not affect the quality of somatic embryos in the study of El Meskaoui et al. (2000) as in the previous study (Kong and Yeung, 1994). From these studies, it may be observed that the optimal somatic embryo maturation capacity does not depend only on the sensitivity of the embryogenic cell line to ABA but many other factors, and this requires more studies.

Keeping in mind the importance of *Pinus kesiya* need for its mass multiplication through somatic embryogenesis and the complex interplay of physiological and biochemical processes during somatic embryogenesis, the following studies were carried out resulting into the current doctoral work:

- Effect of culture conditions, media composition and plant growth regulators on the induction of somatic embryogenesis.
- Quantitative and qualitative estimations of intra- and extra cellular proteins in nonembryogenic and embryogenic cultures.
• Profile study of stage specific soluble proteins (i.e., glycoproteins) if present and their role during embryogenesis.

• Qualitative and quantitative assay of peroxidase activity and its relationship with different stages of somatic embryo induction and development.

• Relation and effect of abscisic acid and calcium to the proteins formed during somatic embryogenesis.