CRYOPRESERVATION OF GERMPLASM OF PINUS KESIYA ROYLE EX. GORD. - AN IMPORTANT TREE OF NORTH - EAST INDIA

BY
VARJINIA KALITA

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

PLANT BIOTECHNOLOGY LABORATORY
DEPARTMENT OF BOTANY
NORTH - EASTERN HILL UNIVERSITY
SHILLONG - 793022
1998

Telefax Work : +91 364 25 0300 (Direct)/ +91 364 25 0108/ +91 364 25 0076
E-mail : ptandon@nehus.ren.nic.in ☎Home: +91 364 25 0100 ; Work: +91 364 25 0106 *222

North-Eastern Hill University

Dr. PRAMOD TANDON
Professor of Plant Physiology

Plant Biotechnology Laboratory
Department of Botany
School of Life Sciences
Shillong 793 022, India

CERTIFICATE

I certify that the thesis entitled "Cryopreservation of germplasm of *Pinus kesiya* Royle ex. Gord.- an important tree of North-East India" submitted by Ms. Varjinia Kalita for the degree of Doctor of Philosophy in Botany of the North-Eastern Hill University, Shillong, embodies the record of original investigation carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other university.

Pramod Tandon 26.5.'98

Supervisor

Head
Department of Botany
School of Life Sciences
N. E. H. U., Shillong-14
26/5/98

ACKNOWLEDGEMENTS

I offer my heartiest gratitude to my guide and supervisor, Prof. P. Tandon, Plant Biotechnology Laboratory, Department of Botany, North -Eastern Hill University, for his able guidance, correcting my inadiquaries and advising me whenever needed, for spending much of his valuable time for guidance and continued keen interest during the course of present investigation. I would always remain grateful to him.

I am grateful to Prof. R. R. Mishra, Head, Department of Botany, North-Eastern Hill University for providing me the required facilities.

I am also grateful to Principal, Union Christian College for all the co-operation given to me.

I am grateful to Dr. Suman Kumaria Khatri, Lecturer, Department of Botany, North-Eastern Hill University, whom I could always turn to for help. I thank her for assisting in correction.

Words are inadequate to express my thanks to my lab. mate Hiranjit Choudhury who was always there to help me . I would always remain grateful to him for all hard work he took to put together this work.

I also offer my sincere thanks to my other lab. mates- Chittaranjan Deb, Amary Salam, Eugenia Kyndiah, Sanghamitra Purkayastha, and Mrs. S. Purkayastha and our lab. assistant T.B. Tamang who always gave me their whole-hearted co-operation.

I express my sincerest thanks to my friends Debashis, Prathima, Nora, Banita and Sunil for their generous help in innumerable ways throughout my research period and during preparation of the manuscript.

I am thankful to Mr. Babu John and Rupam Kataki for helping me in many ways.I am also thankful to Bablu for understanding me and encouraging me to complete this work.

I am also grateful to Subhasish, Sanjiban, Atanu, Gopa, Vineeta, Sushama Lipika, Juri, Namita, Sangita, Geetashree and others for their kind help.

I do not have words to express my gratitude and indebtness to my parents for their blessings and inspiration and to my sisters, brothers, sister-in-law and my little nephew for their encouragement and support.

Lastly I wish to thank the staff of National Bureau of Plant Genetic Resources (NBPGR), New Delhi, for all possible help and suggestions they offered during my visit there.

Shillong.
May 26, 1998

Varjinia Kalita.
(VARJINIA KALITA)

CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

Cryopreservation is the most promising approach to secure long-term conservation of valuable germplasm at cryogenic temperature in liquid nitrogen (LN). At the temperature of LN, (- 196^{0}C) almost all the metabolic activities of cells are at a stand still and they can be preserved in such a state for extended periods. The development of methods of germplasm preservation at cryogenic temperatures, saves labour and space and complements current germplasm storage methods. It is an important tool for long-term storage of germplasm of vegetatively propagated plants, recalcitrant seeds and even orthodox seeds. It has been playing an important role in preservation of samples or experimental materials without genetic alteration (Kartha, 1985; Withers, 1985).

Long- term preservation of experimental materials using *in-vitro* culture a variety of problems. First and foremost is the alteration of the original genetic complement of the explant material, which may be manifested by chromosome breakage (loss), chromosome doubling in aneuploidy (Halperin,1986). Secondly, as a result of genetic

changes, due to *in-vitro* selective pressures regeneration competence through organogenesis (Murashige and Nakano, 1967) or embryogenesis (Smith and Street, 1974) decreases. Thirdly physiological and epigenetic factors in long-term cultures may also contribute to the lack of regeneration competence and possibly lead to necrosis of the cultures (Gould, 1986; Halperin, 1986). Lastly the requirement for frequent cultures becomes costly. To over come all these problems, there is a growing need for cryopreservation which enables us to achieve genetically stable long-term *in-vitro* storage of various types of plant genetic resources. All available evidences including data on chrmosome number, secondary production, and morphogenesis suggests that cryopreserved cell cultures are genetically stable (Withers, 1986). Moreover, with the recognition of somaclonal variation, callus and cell cultures are being widely used to create additional variability for various biotechnological applications related to agriculture, forestry and industry. Cryostratagies could be successfully employed to preserve not only genetic fidelity but also induced variability.

Cryopreservation has a relatively long history in microbiology and animal cell cultures. It is in routine use for the maintenance of type cultures of these materials and also for the storage of semen and embryos in live stock industry and human medicine (Ashwood-Smith and Farrant, 1980; Fuller, 1987). Attention has been given to the development of cryopreservation methods for plant materials during the last two decades (Withers, 1987a; 1991). One of the earliest attempts on cryopreservation of plant materials was made by Sun (1958) when he was partially successful in preserving the desiccated seedlings of *Pisum sativum*.

Slowly, attempts have been made to preserve other cells and plant materials. Since 1980, it has been possible to offer a routine cryopreservation method for all suspension cultures (Withers and King, 1980; Wither, 1990). The first success in the cryopreservation of a cell suspension was reported by Quatrano (1968), who was able to expose a culture of *Linum usitatissium* to- 50°C and recover viability. Nag and Street (1973) regenerated somatic embryos from a cell culture of *Daucus carota* frozen to -196°C. During subsequent years the number of species cryopreserved as cell suspensions has risen and an encouraging number of species have been frozen as protoplast.

It is found that only a few biological specimens can be successfully frozen to sub-freezing temperatures without affecting the viability of the cells. For successful cryopreservation it is necessary to avoid lethal intracellular freezing. Cells and tissues have to be sufficiently dehydrated or concentrated before being immersed in liquid nitrogen. In case of dormant buds and orthodox seeds cryopreservation can be carried out readily in most without any pretreatment (Standwood,1985).But some precautions may need to be taken to avoid the shattering of seeds upon warming (Marin *et al.*, 1990). In other organised tissues some form of protection is required to tolerate the ultralow temperature. The use of cryoprotectants becomes necessary which protect living organisms against freezing injury. Cryoprotectants reduce the amount of freezing water from the cells there by giving less chance to ice formation and ice-damage (Farrant, 1980; Meryman and Williams, 1982). The discovery of chemicals with cryoprotective properties paved the way for the development of effective

cryopreservation, the major break through being detection of glycerol as a cryoprotectant for freezing avian spermatozoa (Polge *et al.,* 1949). Over the years a number of low-molecular weight compounds have been identified as potential cryoprotectants, the most commonly used ones being dimethyl sulfoxide (DMSO) and glycerol. DMSO was originally used to prevent freezing damage to human and bovine red blood cells and bull spermatozoa (Lovelock and Bishop, 1959). It is used either alone or in combination with other cryoprotectants like mannitol, sorbitol, sucrose, glucose, glycerol etc. Sometimes a mixture of cryoprotectants shows better result than a single cryoprotectant (Finkle and Ulrich, 1979; Hauptman and Widholm,1982; Chen *et al.*, 1984 a,b). Mannitol was found to be beneficial in reducing mean cell volume of cells of *Acer pseudoplatanus* and *Capsicum annum* and increase post-freezing viability (Withers and Street, 1977). Ulrich *et al.*, (1979) reported considerable benefit of use of a mixture of cryoprotectants involving polyethylene glycol (PEG), glucose and DMSO in sugarcane callus. There is ample evidence that supplementation of medium with some cryoprotectant is beneficial (Kartha *et al.,*1982a,b; Chen *et al.,* 1984b; Bhandal *et al.,*1985). Nag and Street (1975) found DMSO and glycerol as most effective cryoprotectant while preserving suspension cultures of carrot, belladonna and Sycamore at freezing temperatures. Sometimes these chemicals with high osmoticum are used in preculture medium. Sorbitol has been used in preculture medium as an osmotic agent and also as the sole cryoprotectant during cryopreservation of cell culture of *Glycine max, Datura innoxia, Brassica napus* and *Daucus carota* (Weber *et al.*, 1983). A

successfull cryopreservation could be achieved with shoot primordia of shoot apices from protocorms of *Vanda pumila* following abscisic acid (ABA) preculture and desiccation (Na and Kondo, 1996). ABA was also used in preculture medium during cryopreservation of *Cassava* shoot tips (Escobar *et al*, 1997).

Currently three cryopreservation methods are available, namely desiccation , slow freezing and vitrification. They differ in the way the amount of free water is being reduced. Desiccation method removes free water by transferring it into the air. The basic principle of desiccation method involves induction of an intrinsic tolerance to desiccation by triggering the genes responsible for desiccation tolerance and reduction of water content to a sufficiently low level where there is little free water. Orthodox seeds undergo these processes spontaneously during the course of their maturation. Most higher plants seem to have the genes for tolerance to a totally desiccated state, since they tolerate such a state in a part of their life cycle, either at the seed stage or pollen stage. Similar desiccation processes need to be worked out for recalcitrant seeds, buds, meristems, somatic embryos etc. in order to preserve them in liquid nitrogen. Desiccation of plant materials can be carried out in different ways. In case of zygotic embryos dehydration is carried out normally in the air current of a laminar flow cabinet, without any cryoprotective treatment (Grout *et al.*, 1983; Normah *et al.,* 1986; Chin *et al.,* 1988; Abdelnour - Esquivel *et al.*, 1992). Where as somatic embryos are desiccated by culturing them in high sugar medium (Engelmann *et al.*, 1985). A combination of both treatments (high sugar and dehydration) proved successful in case of coconut embryos (Assy-Bah and Engelmann, 1992). In case of axillary buds of

asparagus desiccation was carried out using silica gel (Uragami *et al.*, 1990). Somatic embryos of melon seeds were desiccated aseptically in chambers at constant relative humidity by using special containers with membrane filters.With desiccation method various cultured materials have been cryopreserved including callus of carrot (Nitzsche,1980) embryos of alfalfa (Senarantna *et al.*,1990) buds of asparagus (Uragami *et al.*, 1990), lateral buds of *Solanum phureja* (Fabre and Dereuddre,1990), callus of rice (Shin *et al.,* 1991), embryo axes of tea (Chaudhury *et al.*, 1991), embryos of oil palm (Dumet *et al.*, 1993;1994).

Another method of desiccation is encapsulation dehydration which is based on successive osmotic and evaporative dehydration (Dereuddre *et al.*, 1990). It consists of encapsulation of the materials which is in most cases the shoot-tip or somatic embryos in alginate bead (Gray *et al.*, 1987; Bapat and Rao, 1988) or in water soluble plastic wafers (Kitto and Janick, 1985) which are then air-dried in a laminar flow cabinet. Encapsulation may be more practical for handling embryos and forms protection against excessive dehydration of embryos. Some times the beads are precultured on high osmoticum for few hours to few days. Somatic embryos of *Daucus carota* tolerated direct freezing in liquid nitrogen after encapsulation dehydration (Dereuddre *et al.*, 1991). There is a report of alginate coated *in-vitro* grown shoot tip cryopreservation of apple, pear and mulberry, (Niino and Sakai, 1992). Apices of *in-vitro* grown plantlets of sugarcane are also cryopreserved by encapsulation - dehydration (Paulet *et al.*,1993). Somatic embryos of coffee encapsulated in alginate beads could be directly regrown after cooling in liguid nitrogen (Hatanaka *et al.*, 1994).

In case of *Catharanthus* encapsulated cells were precultured in sucrose enriched medium for several days, dried over silica gel and directly cooled in liquid nitrogen (Bachiri *et al.*, 1995). Desiccation by encapsulation dehydration were also carried out in shoot tips of different forest trees like *Cedrela odorata*, *Guazuma crinita* and *Jacaranda mimosaefolia* (Maruyama *et al.*, 1997).

In slow prefreezing method freezing tolerance is conferred by the addition of cryoprotectants and dehydration of cells by extracellular freezing which is initiated by ice-inoculation. Slow cooling is found to be a better method in many cases, where there is induction of extracellular freezing which causes cellular dehydration. There is progressive decline in temperature where at first the external medium supercools and then ice formation takes place. The cell wall acts as a barrier and prevents the ice from forming inside the cells. Freezing injury by this method is comparetively less. It brings an optimum situation between under-and-over dehydration (Kartha,1985; Withers,1985). Chen *et al.*(1984 a,b) developed a method to circumvent dilution of cryoprotectant step by plating the cryopreserved cells on filter paper and later transferring the filters on to fresh medium. Although most commonly slow prefreezing is carried out in an expensive programme freezer, it can also be carried out in an ethanol bath cooled by manual addition of liquid nitrogen or dry ice.

Vitrification is a recently developed method which enables cells and meristems to be cooled to - 196°C without ice formation. It is a physical process by which a highly concentrated solution super cooles to very low temperature and finally solidifies into metastable glass without crystallization. A vitrified solution does not get

subjected to osmotic concentration. Here dehydration is induced by crystallization of the external medium during the slow cooling step to an intermediate temperature (-30°C or - 40°C). Further quick cooling to temperature of liquid nitrogen, the cell contents solidify (Meryman and Williams,1985). Sakai (1966) had examined the possibility of surviving liquid nitrogen temperature by vitrification of plant cell water in extremely cold-hardy species. However, after cryopreservation of animal cells by vitrification method was reported (Fahy *et al.,* 1984; Takahashi *et al.,* 1986), the application of vitrification in plant tissue cryopreservation has been actively promoted . Several vitrification solutions have been developed (Uragami *et al.,* 1989; Sakai *et al.,* 1990a; Langis and Steponkus, 1990; Towill, 1990). A vitrification solution composed of glycerol and sucrose was used while cryopreserving meristem of lily, where 80% survival was achieved after 4 weeks(Matsumoto *et al.*,1995), while 55% survibility was found from cryopreserved tobacco cell suspension by vitrification (Reinhoud *et al.,* 1995). About 80% recovery was achieved from *in vitro* grown shoot tips of *Colocasia esculenta* (Takagi *et al.,* 1997). Ishikawa *et al.* (1996) reported that the pretreatment of broom grass cells with a cryoprotectant solution (sucrose: DMSO: glycerol - 10:10:5 % w/v) before vitrification enhanced the survival and regrowth. Without this pretreatment, the cells did not survive LN exposure by vitrification with PVS2. Successive improvement in frequencies of embryogenic calli of *Zea mays* and their cryopreservation by vitrification has been reported (Upadhyay *et al.*, 1993).

Different explants used for cryopreservation include menistems, shoot tips, cells, protoplasts, somatic and zygotic embryos, anther/pollen and whole seed (Kartha, 1985; Withers,1985). Cell cultures seem to be the most widely studied system for cryopreservation and they are found to be genetically stable. Furthermore they are amenable to treatments that modify their structure and physiology to enhance freezetolerance (Pritchard *et al.*, 1986). A routine method can be recommended for cryopreservation of cell cultures in different species with minor modifications (Withers, 1985b; 1990; Withers and King, 1980). About 10 - 50% survibility was observed in cryopreserved cell suspension of soyabean, *Datura* and tobacco (Bajaj, 1977), where as high level of viability was reported from cryopreserved cell suspension of *Picea glauca* even after storage in liquid nitrogen for one year (Kartha *et al.*,1988). Plants could be regenerated from cryopreserved cell suspensions of napier grass (*Pennisetum purpureum*) (Wan and Vasil, 1996). Protoplast cryopreservation is also based on the routine method for cell suspension (Hauptman and Widholm, 1982; Takeuchi *et al.*, 1982; Bajaj, 1983; Withers, 1985a). From mesophyll cells and callus cell suspension of *Atropa belladonna, Datura innoxia* and *Nicotiana*, protoplast could be cryopreserved and regenerated where normal flowers developed which also set seeds (Bajaj,1988).

In the beginning less attention was given to cryopreservation of callus cultures, however, general principle established for cell-suspension could be used to cryopreserve callus by fractioning the callus and subjecting a filtered suspension (Watanabe *et al.*, 1983; Ziebolz and Forche,1985). Despite receiving considerable

amount of attention comparable to that given to cell suspension cultures, shoots have proven far more difficult to cryopreserve. No single procedure has emerged as superior and only the loosest of guidelines can be offered for the extrapolation of reported methods to untried specimens. Despite the difficulties, there are now several reports of successful cryopreservation of shoot-tips of many species. Bajaj (1977) reported initiation of shoots and callus from potato sprouts and axillary buds frozen to - 196°C. Successful cryopreservation of shoot-tips of *Cassava* (Kartha *et al.*,1982 b), apple (Katano *et al.*,1983), oil palm (Engelmann *et al.*, 1985), Pear (Moriguchi *et al.*, 1985), cherry and honeysuckle (Suzuki *et. al.*, 1988) and many others have been described. Niino *et al.* (1992) reported that dehydrated shoot tips excised from mulberry winter buds could be cryopreseved without any cryoprotectant. The cryopreservation of shoot tips from *in vitro* grown root fragments of chicory (Demeulemeester *et al.*, 1993), and shoot tips of *Cassava* (Escobar *et al.*, 1997) have been possible. For germplasm conservation, somatic embryos have also been used. The genetic integrity of embryo-derived plants may need confirmation. The cryopreservation of somatic embryos of carrot and oil palm illustrate important practical points that might be translated into use with other systems. In carrot, somatic embryos are relatively unresponsive to methods found successful for cell suspensions of the species. As the embryos mature, they become increasingly prone to viability loss and incur damage in an increasingly large proportion of the embryo structure (Withers,1979; Withers and Street, 1977). Eventually a point reached when recovery occurred only from secondary embryogenesis in superficial cells. But these

embryos if drained free from the cryoprotectant solution, blotted dry on filter paper, enclosed in foil envelope, and slowly cooled, the recovery can be achieved. On the other hand, the somatic embryos and embryogenic clusters of callus of oil palm have been cryopreserved very successfully after preculturing for two months in high sucrose containing medium (Engelmann,1986; Engelmann *et al.*,1985). De Boucaud *et al.* (1994) reported successful cryopreservation of somatic embryos of walnut .

There are few reports of cryopreservation of seeds mainly belonging to recalcitrant type. Horse radish seeds which are considered recalitrant gave high viability after storage for one month, in liquid nitrogen vapour. Lemon seeds survived immersion in liquid nitrogen after dehydration (Mumford and Grout, 1979). Seeds from *Populas deltoides* were also cryopreserved (Pence, 1996).

There are few reports of survival of zygotic embryos in liquid nitrogen and their subsequent regeneration. Cryopreservation of embryonic axes embryos from species producing recalcitrant seeds makes important contribution in genetic conservation (Grout, 1986). Even orthodox embryos and some germinating embryos are cryopreserved with some cryoprotection and re-drying (Grout,1979). Embryogenic axes of rubber, a trully recalcitiant species, can be cryopreserved if dessicated to a water content of 14-20 % , where 20-90% survival levels have been recorded (Normah *et al.*, 1986). The embryos of *Veitchia* and *Howea* palm, could not develop haustorium after cryopreservation by desiccation (Chin *et al.*, 1988). The cryopreservation of embryonic axes of almond (*Prunus amygdalus*) has been reported by Choudhury and Chandel (1995). The embryonic axes of tea has also

been tried for cryopreservation after desiccation (Kuranuki and Yoshida , 1996). Some attention has also been given to the cryopreservartion of anthers, and pollen embryos , but few generalisation can be made (Bajaj, 1982; 1984; Withers, 1985 b; 1987 a). It appears that the induction of androgenesis is beneficial, although pollen embryos show declining surivival potential with increasing development , as in the case of somatic embryos.

Cryopreservation is proposed as the best means by which one can preserve or prevent variation not only in angiosperms (Withers,1985; Kartha,1987), but also in gymnosperms (Binder and Zaerr,1980; Kartha *et al.*, 1988). But most of the work on cryopreservation of cells, callus cultures are confined to angiosperms. With increasing interest in genetic engineering of forest tree species , the preservation of cultured cells with unique attributes such as ability to form somatic embryos and plantlets is assuming greater importance. Although Binder and Zaerr (1980) were successful in cryopreserving cell culture of Douglas fir, this line apparently did not possess the afore-mentioned attributes. On the other hand , cell cultures established from immature embryo-derived callus of white spruce (*Picea glauca*) were capable of producing numerous somatic embryos (Hakman and Fowke, 1987 a, b) and these cell lines have became the primary experimental material for successful isolation and culture of protoplasts (Attree *et al.*, 1987; Bekkaoui *et al.*, 1987). In *Pinus taeda* embryo production decreased after cryopreservation (Gupta *et al.*, 1987). The embryogenic potential of cryostored material was found similar to that of unfrozen one in case of *Picea glauca* (Kartha *et al.*,1988) and *Picea abies* (Bercetche *et al.*, 1990) . The

buds of scot pine could be also preserved at - 80°C for six months where about 90% viability was obtained (Kuoksa and Hohtola, 1991). Embryogenic cell suspension cultures of *Pinus caribaea* have been cryopreserved for four months, where DMSO and sucrose was used as cryoprotectants (Laine *et al.*, 1992).

There has been considerable progress in application of tissue culture techniques in breeding programme and trees were among the first plants to be cultured *in vitro*. Evidences for morphogenetic activity in callus of *Sequoia sampervirens* carried through several subcultures was reported long back (Ball, 1950), which was the first continuous culture of a coniferous species. White and Risser (1964) and Risser and White (1964) established the nutritional requirement for optimal growth of normal and tumor tissues of *Picea glauca* Murasshige and Skoog (MS) (1962) medium was used for callus growth in *Pinus* species by Brown and Lawrence (1968). Formation of embryoids on cotyledons of *Thuja orientalis* which later formed shoots on culture indicated that buds in gymnosperms could form shoots (Konar and Oberoi, 1965). Konar (1963) and Bethal (1972) reported induction of roots on callus of *Pinus gierardiana* and *Pseudotsuga* respectively. The first complete *in vitro* grown conifer plantlet was obtained from adventitious buds formed along the cotyledons of embryo of *Pinus palustris* in culture (Sommer *et al.*, 1975). Since then lot of work has been carried out on forest *in vitro* trees. Plantlets have been regenerated from *Picea glauca* (Durzan and Campbell, 1976), *Pinus radiata* (Reilly and Washer, 1977), *Thuja plicata* (Coleman and Thorpe, 1977), *Pinus taeda*

(Mehra -Palta *et al.,* 1978), *Sequoia sempervirens* (Boulay, 1979 a), White pine (Webb *et al.,* 1988; Schwarz *et al.,* 1988; Chesick *et al.,* 1991) and many others.

There have been successful use of embryos, young seedling parts like cotyledons, hypocotyl, epicotyl etc. as explant sources for initiation of adventitious buds in culture (Sommer and Brown, 1979). Induction of shoots from meristematic tissues of shoot apices in *Pinus radiata* was reported by Aitken *et al.* (1985). The details are also available on the production of plantlets from seedling parts and embryos of *Pinus strobus* (Kaul, 1987 ; Schwarz *et al.,* 1988 ; Webb *et al.,* 1988) . It is generally assumed that shoot meristems offers great genetic stability and the probability of producing abnormal plants via adventitious shoots is intermediate between that of axillary and callus derived shoots (Hussey, 1978). Many callus cultures from various explants are also established for many conifers, but regeneration of plantlets through callus cultures is rather difficult. Some successful examples are Douglas fir (Cheng , 1975; Winton and Verhagen, 1977) , *Pinus wallichiana* (Konar and Singh, 1980). Thus tissue culture method in conifers include shoot culture with proliferation of axillary and adventitious shoot and callus cultures with regeneration of shoots which originated from embryos and shoots apices.

Micropropagation of juvenile trees from immature embryos, mature embryos and young seedling explants have been more successful with major advancement in embryogenesis, where large scale clonal production is possible through organogenesis (Farnum *et al.,* 1983). For the first time somatic embryogenesis was reported by Steward *et al.* (1958) from carrot callus and till to date somatic

embryogenesis has been reported to occur in over 100 species including cereals, grasses, legumes and conifers. Somatic embryogenesis in conifers was first reported in 1985 from immature zygoticembryos of *Picea abies* (Hakman *et al.* ,1985 ; Chalupa ,1985). Somatic embryogenesis was also estabhlished from female gametophyte of *Larix decidua* (Nagmani and Bonga , 1985). Since 1985 several papers have been published on successful regeneration of conifer plantlets produced via somatic embryogenesis including *Pinus lambertiana* (Gupta and Durzan, 1986), *Pseudotsuga menziesii* (Durzan and Gupta, 1987), *Pinus taeda*(Becwar and Pullman, 1994, Becwar et al., 1990), *Pinus plaustris* (Nagmani *et al.*, 1993), *Taxus brevifolia* (Chee, 1996), *Picea glauca* (Chris *et al.*,1996 ; Barrett *et al.*, 1997)and many others.

Though there have been number of reports on micropropagation of various conifers including a number of pine species, from juvenile tissues, very little success has been achieved with explants from mature trees (Boulay, 1987). It is evident that for successful cryopreservation of a plant germplasm the development of the protocol for its regeneration is essential for regrowth and regeneration after cryopreservation has been accomplished.

The North-Eastern region of India comprising the states of Assam, Meghalaya , Arunachal Pradesh, Mizoram, Nagaland, Manipur and Tripura accounts roughly 8% of the total geographical area of the country. The region lies between 21° 51′N to 29° 28′ N latitudes and from 89° 40 ′E to 97 °25 ′ E longitude and has a geographical area of 25.5. million hectares of which about 45 % is under forest cover. The population

is 31.13 million as per 1991 census. The area is land locked and is bordered on the North by China, on the South by Burma and Bangladesh, on the North East by Burma and West by Nepal and Bhutan. Physiographically the region is divided into three divisions viz., Meghalaya Plateau, North-Eastern Hills and Basin and Brahmaputra Valley. The region has great altitudinal differences, varied physiographical attributes and climatic variations representing temperate (Himalayan ranges in Arunachal Pradesh), subtropical (Imphal Valley) and tropical areas (Plains of Tripura and Assam) . North- Eastern region of India is perhaps the richest and most interesting floristic regions in the country, with about 50% of the flora hailing from the region. The richness and diversity of the flora is mainly due to wide ranges of variation in rainfall, temperature and altitude. The North-Eastern region is considered to be the primary and secondary sources of origin of many crops and trees.

The forest resources of the region are slowly depleting due to indiscriminate felling of forest trees, for age old practice of shifting cultivation, locally termed as "Jhum", for timber and fuel wood extraction, and above all because of unplanned human activities like hydroelectric projects, road construction etc. The impact of these have resulted in great threat to the plant germplasm resources of great significance. *Pinus kesiya* Royle ex.Gord. which is a dominant forest tree of Meghalaya constitutes about 30% of the total forest and may face a similar threat because of its high potential value as producer of resin and also for wood and wood products. It is pre-dominant in the sub-tropics between altitude of 800m to 2000m

of North-East India, Myanmar and Phillipines. *Pinus kesiya* commonly called as Khasi pine is a large evergreen tree with more or less whorled branches and a crown. The crown is oval in young trees and rounded in mature trees. The bark is reddish grey in colour deeply fissured and in moderate to large trees about 1.0 to 1.8cm thick . Adult needles are 15 -25 cm long, slender and present in fascicles of three, The base is enclosed in a persistant papery sheath of 1.5-2.5 cm long. Under favaurable conditions *Pinus kesiya* reaches a height of 35 m feet or more and a girth of 3 m.

Like most woody plants, *Pinus kesiya* is also genetically heterozygous, because of its allogamous nature. Therefore, its genetical intergrity is possible either through vegetative propagation or *in vitro* cultures. Otherwise the same gene combination cannot be recovered. The decrease in the size of girth of *Pinus kesiya* during long period of time is an example of variation occuring in the field which may be due to heterozygosity.

The present work deals with the development of a protocol for *in vitro* multiplication of *Pinus kesiya* together with suitable cryopreservation methods for long-term storage of its germplasm.