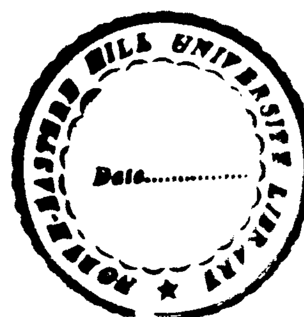
CRYOPRESERVATION OF GERMPLASM OF PINUS KESIYA ROYLE EX. GORD. - AN IMPORTANT TREE OF NORTH - EAST INDIA

BY
VARJINIA KALITA



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DOCTOR OF PHILOSOPHY IN BOTANY

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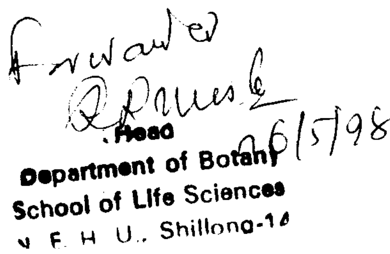
CERTIFICATE

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



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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$^{0\circ}$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 cryopresevéd 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.

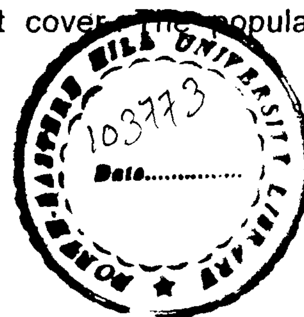
There have been successful use of embryos, young seedling parts like cotyledons, hypocotyl, epicotyl etc. as explant sources for initiation of adventitious buds in culture (Sommer and Brown, 1979). Induction of shoots from meristematic tissues of shoot apices in *Pinus radiata* was reported by Aitken *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 $21^\circ\ 51'$N to 29° $28'$ N latitudes and from $89^\circ\ 40'$E to $97^\circ 25'$ 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.

CHAPTER 2. *IN VITRO* MULTIPLICATION OF *PINUS KESIYA* ROYLE EX. GORD. USING DIFFERENT EXPLANTS

INTRODUCTION

The North-East India has 25.5 million hectares of geographical area, about 45% of which is under forests. The climatic conditions are ideal for the growth of diverse type of vegetations. The area has one of the richest reservoirs for genetic variability of great significance in a wide group of plants. Forests are also the rich source of plants for food, fodder, timber, fuel and other minor forest products. The forest wealth of this region is fast dwindling due to indiscriminate felling of forest trees for age old practice of shifting cultivation (locally known as "Jhum"), for timber and fuelwood extraction, and unplanned human activities like urbanization, hydroelectric projects, road and bridge construction and forest fires etc. Demand for forest products has increased sharply throughout the world over the last few decades. This is due to ever increasing need for wood for pulp, paper, timber and furniture industries

(Karnosky, 1981; Nagmani *et al.*, 1993). A large number of improved fast-growing trees with shortened rotation and disease-resistance are required. Trees of poor or heterogeneous quality and slow rate of growth are no longer acceptable. Moreover, the productivity of natural forests is insufficient to meet the epected future demand for wood products.

Trees are propagated both sexually and vegetatively but for maintaining superior genetical characters, the latter is preferred. In vegetative propagation, increase in productivity is immediate, whereas sexual propagation takes several generations. Also hybrids and polyploids with low sexual fertility can be vegetatively propagated (Bonga, 1977b; Bonga and Pond, 1991). Though propagation through selection over several generations brings some improvements, its gain is likely to be less than the vegetative propagation of the selected trees. The traditional methods of vegetative propagation are grafting and rooted cuttings or rooted needle fasicles. Grafting can generally be carried out with trees of any age. It is practical only where limited numbers of propagules are required. Also it is a slow and labour-intensive process. The advantage of grafting is that it can work where other methods of cloning have failed. Grafted clones have always been propagated in their mature form (Kester, 1976) which has the advantage of flowering and fruit set. Rooting of cuttings is an effective means of mass propagation, but for many species it can be carried out at juvenile stage only. It has been reported that rooting ability of cuttings from majority of coniferous species declines with increasing age of the parent plant (Hackett, 1988). Where it has been possible to analyse changes in rooting potential associated with

ontogenetic development, it has been shown that the upper and peripheral parts of a plant are the first to exhibit reduced rooting potential. On the other hand, one of the main aims of vegetative propagation is to multiply trees old enough to demonstrate their superior characteristics. Percent rooting, speed of rooting, root-length and number, and survival and growth in and after the year of rooting all decline, particularly when the parent plant is more than ten years old (Girouard, 1974). Moreover, vegetative propagules display slower height and diameter growth as the age of the parent increases (Libby, 1974).

Vegetative propagation of woody plants of a size large enough to have demonstrated their potential thus becomes difficult or impossible with the traditional methods of grafting and rooting of cuttings. Cloning by means of tissue culture is an alternative to the traditional methods, and has been successfully employed to obtain propagules. It produces uniform copies and thus provides a method for propagation of genetically improved specimens. However, the classical procedures have two major problems-low efficiency under nursery conditions, and difficulties in propagating mature trees. Tissue culture research has led to new, efficient methods of vegetative propagation of several angiosperms and a similar approach has been attempted with gymnosperms but with limited success. Different factors such as tree vigour and physiological conditions induced by season and by climatic conditions affects micropropagation of trees (Bonga, 1982).

Plant propagation through tissue culture may be accomplished by employing callus, organ, cell and protoplast cultures. Evidences for morphogenetic activity in

callus of *Sequoia sampervirens*, carried through several subcultures was reported long back (Ball, 1950). Jaquiot (1955) worked with cambial explants of *Abies nordmanniana* and established the shoot forming effect of adenine like substances, in contrast to the root forming effect of auxius. Callus was obtained from hypocotyls of *Picea abies* which later produced pro-embryo like structures in cell suspensions (Chalupa and Durzan, 1973). Buds and roots developed in cell colonies derived from hypocotyl cultures of *Pinus gerardiana* (Konar, 1975). Shoot were induced from cotyledons of *Pseudotsuga menziesii* (Cheng, 1975). Successful micropropagation has been reported in a large number of tree species viz *Picea glauca* (Durzan and Campbell, 1976), *Tsuga heterophylla* (Cheng, 1976) *Pinus radiata* (Reilly and Washer, 1977) *Thuja plicata* (Coleman and Thorpe, 1977), *Pseudosuga nziesii* (Cheng and Voqui, 1977; Boulay, 1979b), *Pinus taeda* (Mehra-Palta *et al.*, 1978), *Pinus pinaster* (David *et al.*, 1978), *Sequoia sempervirens* (Boulay, 1979a) etc. Callus and suspension cultures were also obtained from *Pinus strobus* (Finer *et al.*, 1989), *Pinus elliottii* (Valluri *et al.*, 1990), *Pinus pinea* (Diamantoglou *et al.*, 1990) and others. Boulay and Franclet (1977) cultured dormant buds of *Pseudotsuga menziesii* and obtained both callus and axillary buds in 2,4-D (2,4-dichlorophenonxyacetic acid) supplemented medium. Buds were also formed on the axils of cotyledons and juvenile leaves of *Pinus pinaster* (David and David, 1977). Induction of buds in hypocotyl sections with apical meristem and cotyledons from 20-days old seedlings of *Pinus pinaster* has been reported (David *et al.*, 1978; Isemukali, 1979), Boulay (1979a,b) studied axillary bud formation in stem sections of *Sequoia sempervirens* and found that

the origin of the material had a considerable effect. If explants were taken from branches of a tree more than 20 years old, no buds were formed. On the other hand, explants taken from stump sprout were found to be effective in plantlet regeneration.

In many trees plantlets are propagated from embryos. Sommer and Brown (1974) reported bud formation in cultured embryos of *Pinus palustris.* Buds were also produced on cultures of hypocotyl sections of *Cryptomeria japonica* (Isikawa , 1974). In *Picea abies*, sections of hypocotyl without the apical meristem formed buds on medium supplemented with benzyl amino purine (BAP) (Chalupa, 1975). Adventitious buds formed near the apex of the cotyledons of the embryos of *Pseudotsuga menziesii* after BAP treatment (Winton and Verhagen, 1977). Minocha (1980) reported formation of more than twelve buds per embryo from *Pinus strobus*, which grew at the apex of the cotyledons. Though work on bud formation has been discussed by several workers, the frequency of buds were found to be low (Biondi and Thorpe, 1982a,b, Patel and Thorpe, 1984). But Amerson *et al.* (1988) have reported production of thousands of plantlets from tissue culture of cotyledons. Successful work on plantlet production has also been carried on mature zygotic embryos of *Pinus sylvestris* (Zel *et al.*, 1988) and *Pinus caribaea* (Halos and Go, 1993).

Young needles excised from 10-12 weeks old plants of *Pinus radiata* developed meristematic zones which later on resulted in bud formation in hormone free medium (Reilly and Brown, 1976). Buds were also formed on needles from 2-3 months old plants of *Pseudotsuga menziesii* and *Picea abies* (Chalupa, 1977).

Formation of buds at the base of the needles was reported in cultures of *Pinus sylvestris* branchyblasts, with one pair of needles (Bornman and Jansson, 1980).

Success with explants from mature trees has been achieved for propagation of some hard woods by tissue culture (Mascarenhas *et al.*, 1982; 1988; Sommer, 1983). But there are very few reports on propagation using tissues from mature conifer explants (Bonga, 1981; Gupta and Durzan, 1985; Abdullah *et al.,* 1987). It has been reported that the shoot apical meristem can give rise to all aerial plant parts (Medford, 1992). Removal of meristem from mature surrounding tissues of older plants can release the meristems its juvenile potential (Monteuuis, 1988), Mature explants of *Larix decidua* formed adventitious shoots more rapidly than other conifers (Bonga and Pond, 1991; Bonga, 1995). Clonal propagules were produced from mature trees of *Larix eurolepsis* by adventitious organogenesis (Laliberte and Lalonde, 1988; Kretzschmar and Ewald, 1994; Diner, 1995).

Roots were initiated in plantlets developed from different explants of few trees. Roots were occasionally produced in cultures of *Picea abies* (Chalupa, 1975) and *Pseudotsuga menziesii* (Sommer, 1975) after 3-indole butyric acid (IBA) and α-naphthaleneacetic acid (NAA) treatments. Roots were also obtained on shoots of *Pinus palustris* after its transfer to auxin and cytokinin free medium (Sommer *et al.,* 1975). In few conifers use of vitamin, amino acid with IBA helped in formation of roots (David and David, 1977). Rooting of shoots in *Pinus radiata* was obtained by use of low minerals in medium (Reilly and Washer, 1977). Successful rooting has been accomplished on shoots of *Pinus brutia* (Abdullah *et al.*, 1987), *Pinus radiata*

(Horgan, 1987), *Pinus pinaster* (Dumas, 1987), *Pinus strobus* (Schwarz *et al.*, 1988; Chesick *et al.*, 1991), *Pinus caribaea* (Halos and Go, 1993) etc.

A major breakthrough in the micropropagation of forest trees occurred when somatic embryogenesis was reported from hypocotyls of *Liquidambar styraciflua* (Sommer and Brown, 1980). The formation of plantlets via embryogenesis is a multistaged process like organogenesis and consist of at least five distinct stages- viz., initiation of embryogenic tissue, multiplication of embryogenic tissue, development and maturation of somatic embryos, germination of embryos and acclimatization of plantlets. The first conifer plantlets obtained from somatic embryos were produced in *Picea abies* (Hakman and von Arnold, 1985; Chalupa, 1985). Somatic embryogenesis is also reported from haploid cultures of *Larix decidua* (Nagmani and Bonga, 1985), *Pinus lambertiana (Gupta* and Durzan, 1986), *Pinus taeda* (Gupta and Durzan, 1987a,b), *Picea glauca* and *Picea mariana* (Hakman and Fowke, 1987a; Lu and Thorpe, 1987) and many other tree species (Redenbough and Ruzin, 1989; Becwar *et al.*, 1990; Attree *et al.*, 1991; Tautorus *et al.,* 1991; Nagmani *et al.,* 1993; Halos and Go, 1993; Becwar and Pullman, 1994). Plantlet regeneration was accomplished using somatic embryos of *Taxus brevifolia* (Chee, 1996) and *Dalbergia sisso* (Das *et al.*,1997).

Pinus kesiya Royle ex. Gord. commonly called as Khasi pine is distributed in the sub-tropics between altitude of 800 m to 2000 m of North-East India, Myanmar and Phillipines. It is an important conifer of higher altitude of North-East India, which dominates the forest plants of Meghalaya, consisting of about 30% of the total forest

cover. It has high potential value as producer of resin and also for wood and wood products. The tree is large ever-green with branches that are more or less whorled. The crown is oval in young trees and rounded in mature trees. Needles are present in fascicles of three, enclosed in a persistant papery sheath. Adult needles are 15-25 cm in length. Under favourable conditions, *Pinus kesiya* reaches a height of 35 m or more and a growth of about 3 m or over.

Pinus kesiya being genetically heterozygous in nature. The genetical integrity can be maintained only through vegetative propagation or *in vitro* culture. Since *P.kesiya* attains sexual growth quite early its rootability declines and mass multiplication by *in vitro* culture becomes necessary. The following chapter aims at developing a protocol for its mass propagation using different explant sources to produce improved-fast growing plantlets from commercially favoured genotypes.

MATERIALS AND METHODS

Mature seeds of commercially favoured genotypes of *Pinus kesiya* Royle ex. Gord. were obtained from Agro-Forestry Division of Indian Council of Agricultural Research, Shillong, during January to February. The seeds were stored in plastic bags at 4°C for further use.

EMBRYO CULTURE

Embryo excision and culture for bud induction

The seeds were first washed with water containing a few drops of detergent and rinsed with pure (obtained from MiliQ water purification unit) water for 4-5 times. The seeds were soaked in sterilised pure water and kept at 4°C for 24 hr. They were then treated with 6% (v/v) hydrogen peroxide for 10 mins and rinsed with sterilised pure water 4-5 times and stratified at 4°C for for 24 hr. The seeds were again sterilised with 2% (w/v) mercuric chloride for 2-3 min and rinsed with sterilised pure water 4-5 times. The embryos were dissected out with the help of a needle and a forcep under aseptic conditions and cultured in diffrerent media viz., Lepoivre and Quoirin (LP, 1977) and Schenk and Hildebrandt (SH, 1972) (Table 1). Medium was supplemented with different concentrations of BAP and kinetin seperately in a range of 0.0 - 15.0 mgl^{-1} and also in combination with NAA and IBA. The different combinations tried were kinetin (5.0 mgl^{-1}) + NAA (0.1-0.5 mgl^{-1}) and kinetin (5.0 mgl^{-1}) + IBA (0.1-0.5 mgl^{-1}). The medium was gelled with 0.8% (w/v) agar before adjusting the pH to 5.8. 15ml of medium was poured in each culture tube and 25 ml of medium in each 100 ml flask and autoclaved for 20 min at 121°C. One excised embryo was cultured in each test tube /flask (Plate 1a). Thirty replicates were taken for each treatment and experiments were repeated thrice. Standard deviation was calculated following the method of Zar (1974). The cultures were incubated at 25 ± 2° C temperature and 12 hr. photoperiod of light intensity 150μ moles $m^{-2}sec^{-1}$. After 3-4 weeks, the embryos were transferred to fresh

Table 1. Composition of Schenk and Hildebrandt (SH), Leprovire and Quinone (LP) and Murashige and Skoog (MS) media.

Constituents	Media (amounts in mgl^{-1}) SH	LP	MS
Inorganics			
NH_4NO_3	-	400.000	1650.000
KNO_3	2500.0	1800.000	1900.000
KH_2PO_4	-	270.000	170.000
$MgSO_4.7H_2O$	400.0	360.000	370.000
$CaCl_2.2H_2O$	200.0	-	440.000
$Ca(N0_3)_2.4H_2O$	-	1200.000	-
$NH_4H_2PO_4$	300.0	-	-
KI	100.0	80.000	830.000
H_3BO_3	5.0	6.200	6.200
$MnSO_4.4H_2O$	20.0	1.000	22.300
$ZnSO_4.7H_2O$	1.0	8.600	8.600
$Na_2MoO_4.2H_2O$	0.2	0.250	0.250
$CuSO_4.5H_2O.5H_2O$	0.2	0.025	0.025
$CoCl_2.6H_2O$	0.2	0.025	0.025
$FeSO_4.7H_2O$	15.0	30.000	27.800
$Na_2EDTA.2H_2O$	20.0	40.000	37.200
Organics			
Thiamine HCl	5.0	0.400	0.100
Nicotinic Acid	5.0	-	0.500
Pyridoxine HCl	0.5	-	0.500
Glycine	-	-	2.000
Myo-inositol	1000.0	1000.000	1000.000
Sucrose	3%	3%	3%
Agar	0.8%	0.8%	0.8%

medium free of growth regulators but containing reduced sucrose (2% w/v) for shoot bud formation.

SHOOT APEX AND NEEDLE CULTURE

Dissection and culture for bud induction

The seeds were sterilised and stratified as described earlier and germinated aseptically on half-strength MS basal salts medium. Shoot apices of about 5.0 mm in size were dissected out aseptically with the help of a scalpel and a needle from 15 day-old *in vitro* grown seedlings. The needles were seperated out from 30-day old *in vitro* grown seedlings. The excised explants were cultured in MS and LP media (Table 1). Media were supplemented with different concentrations of BAP and kinetin seperately in the range of 0-10.0 mgl^{-1} concentrations and also in combination with NAA and IBA. The different combinations tried were BAP (4.0 mgl^{-1}) + NAA (0.1- 0.5 mgl^{-1}) and BAP (4.0 mgl^{-1})+IBA (0.1 - 0.5 mgl^{-1}). The medium was gelled with 0.8% (w/v) agar before adjusting its pH to 5.8. 15 ml of hot medium was poured into each culture tube and 25 ml into each 100ml flask and autoclaved for 20 min. at 121° C. One dissected shoot tip was cultured in each test tube/flask (Plate 2a) .Thrity replicates were taken for each treatment and the experiments were repeated thrice. Standard deviation was calculated following the method of Zar (1974). Cultures were incubated under 12 hr. photoperiod of light intensity of 150μ moles $m^{-2}sec^{-1}$ at 25±2°C. After 3-4 weeks, explants were subcultured on fresh medium deviod of growth regulators for shoot bud formation.

SHOOT MULTIPLICATION AND ELONGATION

For shoot multiplication and proliferation, the multiple shoot buds obtained from embryos were cultured in half-strength LP medium containing different concentrations of IBA (0.01, 0.1 and 1.0 mgl^{-1}) in conjuction with kinetin (1.0 mgl^{-1}) and 0.3% activated charcoal. In case of multiple shoot buds obtained from shoot apices half-strength MS with different concentrations of IBA (0.01, 0.1 and 1.0 mgl^{-1}) in conjuction with BAP (1.0 mgl^{-1}) and 0.3% activated charcoal was used. For multiplication and proliferation of shoot buds obtained from needles, they were cultured in half-strength MS medium supplemented with different concentrations IBA (0.01, 0.1 and 1.0 mgl^{-1}) along with kinetin (1.0 mgl^{-1}) and 0.3% activated charcoal. Shoots of about 5.0 mm size were dissected out carefully and grown for 2-3 passages at 3-4 weeks interval in fresh medium without growth regulator for further elongation till the shoots grew to a height of 20-30 mm.

ROOTING OF ELONGATED SHOOTS

For initiation of roots, the isolated shoots measuring 20-30 mm size derived from both embryos and shoot apices were cultured in half-strength LP and MS medium, respectively. Since shoot bud multiplication in needles was poor, root initiation was not attempted on such shoots. The media contained 4% sucrose and were fortified with NAA and IBA singly or in varying concentrations (0.1 - 0.5 mgl^{-1}). The cultures were incubated under 12 hr. photoperiod of light intensity of 150 μ moles m^{-2} sec^{-1} at 25±2 C. The shoots with root initials were removed from the rooting medium after 3-4 weeks

and transferred to half-strength respective medium free of growth regulators for elongation and subsequent growth of the roots.

In another experiment, shoot were given the pulse treatment of NAA and IBA. The basal end of shoots were dipped in NAA and IBA (10.0-30.0 mgl^{-1}) seperately for different duration of time (0-48 hr.) and then cultured in half-strength LP and MS medium (as required for shoots of different origin) or water agar (0.6%) and incubated under the same light intensity, photoperiod and temperature as mentioned above. They were subcultured every 3-4 weeeks in fresh medium for further elongation of the roots.

HARDENING OF PLANTLETS

The rooted plantlets were transferred to different potting mixtures namely. peat+ vermiculite+pumice (1:1:1), peat+pumice (1:1), vermiculate+pumice (1:1), pumice alone and soil mixture from pine forests in the thermocol glasses. The potted plants were fed with one-tenth MS salt- medium twice a week for the first 2 weeks and later every 15 days upto 3 months. The temperature of the hardening chamber was maintained at 25±2°C with 12 hr. photoperiod of light intensity 150 μmoles m^{-2} sec^{-1} and 80-85% humidity.

RESULTS

EMBRYO CULTURE

The results on multiple shoot bud induction in embryos precultured for 3-4 weeks in SH medium with different concentrations of BAP and kinetin and later

transferred to SH medium free of growth regulators are presented in Table 2. The optimum response of 10-15 shoot buds in about 50% cultures was recorded in case of 10.0 mgl^{-1} BAP treatment. Using 5.0 mgl^{-1} kinetin, 6-7 shoot buds were produced which did not show further development. Lower concentrations of both BAP and kinetin resulted in slight swelling of the embryos. However, higher concentrations were found to be inhibitory. In control without any growth regulator 96% of the embryos germinated without shoot bud development. As shown in Table 3, LP medium was found better than SH medium for shoot bud induction. In 5.0 mgl^{-1} BAP treatment, about 15 healthy shoot buds developed both from the base and apex of the cotyledons. A treatment of 5.0 mgl^{-1} of kinetin in LP medium esulted in the formation of 20-22 shoot buds in 90% cultures (Plate 1c,d). Both lower and higher concentrations of BAP and kinetin were inhibitory for shoot bud formation.

The findings on treatments comprising of kinetin in conjuction with either NAA or IBA used for multiple shoot bud formation on embryos are presented in Table 4. The use of auxins in combination with kinetin did not help in increasing both the frequency and number of multiple shoot bud formation. 10-12 and 9-10 shoot buds were formed in treatments of 5.0 mgl^{-1} kinetin with 0.1 mgl^{-1} each of NAA and IBA, respectively, The higher concentrations of auxins were found to be inhibitory . The responses varied from swelling of the embryos, opening up of the cotyledones to small bud formation.

Table 2. Multiple shoot bud formation on embryos* of *Pinus kesiya* cultured in SH medium devoid of growth regulators.

Growth regulators Concentrarion (mgl^{-1})	% Response	Remarks
Control	96.5±2.53	Embryos germinated, no shoot bud formed.
BAP		
2.5	15.5±1.58	Slight swelling of the embryos.
5.0	31.2±2.80	Initiation of few shoot buds.
10.0	50.6±1.92	About 10-15 shoot buds emerged from each cotyledon.
15.0	22.5±2.94	3-4 shoot buds formed on each cotyledon.
Kinetin		
2.5	49.8±2.80	Slight swelling of the embryos without further development.
5.0	35.8±2.23	6-7 shoot buds formed which showed no further development.
10.0	-	No response, explant dried.
15.0	-	-

± S.D.

- No response

* Embryos were precultured for 3-4 weeks on SH medium with different concentrations of BAP and kinetin as mentioned above.

Table 3. Multiple shoot bud formation on embryos* of *Pinus kesiya* cultured in LP medium devoid of growth regulators.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	96.5±2.53	Embryos germinated, no shoot bud formed.
BAP		
2.5	60.2±1.87	Few shoot buds developed on 2-3 cotyledons.
5.0	75.3±1.58	15-16 healthy shoot buds developed from the base and apex of each cotyledon.
10.0	50.5±2.77	7-8 shoot buds emerged from the base and apex of each cotyledon.
15.0	48.2±2.12	1-2 very small shoot buds with poor growth emerged from each cotyledon.
Kinetin		
2.5	61.2±1.58	Small shoot buds emerged on 1-2 cotyledons.
5.0	90.5±1.49	20-22 shoot buds emerged both at the base and apex of each cotyledon.
10.0	46.3±2.91	3-4 shoot buds developed on each cotyledon.
15.0	41.7±2.43	1-2 shoot buds developed, cotyledons turned brown.

± S.D.

* Embryos were precultured for 3-4 weeks on LP medium with different concentration of BAP and kinetin as mentioned above.

Table 4. Multiple shoot bud formation on embryos* of *Pinus kesiya* cultured in LP medium devoid of growth regulators.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	96.5 ± 2.53	Embryos germinated, no shoot bud formed.
Kinetin+NAA		
5.0+0.1	73.2 ± 2.40	10-12 adventitious and axillary shoot buds emerged.
5.0+0.2	60.3±2.88	Small buds formed only at the tip of the cotyledons.
5.0+0.3	51.5± 2.80	Opening of the cotyledons and formation of few shoot buds.
5.0+0.4	46.8± 2.58	Development of the existing cotyledons.
5.0+0.5	45.6± 2.96	Elongation of the cotyledons.
Kinetin+IBA		
5.0+0.1	85.5± 2.93	9-10 shoot buds developed which showed slow growth.
5.0+0.2	66.3± 2.58	Small buds formed on top of each cotyledon.
5.0+0.3	48.3± 2.76	Opening of the cotyledons and formation of few shoot buds.
5.0+0.4	42.4± 2.77	Embryos showed swelling.
5.0+0.5	40.5± 2.98	Slight swelling of the embryos.

± S.D.

* Embryos were precultured for 3-4 weeks on LP medium with different concentrations of BAP and kinetin as mentioned above.

SHOOT APEX AND NEEDLE CULTURE

About 15-16 axillary shoot buds developed per shoot tip in LP medium containing kinetin at 5.0 and 7.5 mgl^{-1}, out of which few developed into shoots (Table 5). With kinetin 2.5 and 10.0 mgl^{-1} in the medium there was reduction in number of shoot buds formed. Shoot apex cultured in LP medium supplemented with 5.0 mgl^{-1} BAP produced 7-8 very small shoot buds which failed to develop further. Lower concentration of BAP (2.5 mgl^{-1}) in the medium could initiate formation of only 1-2 shoot buds. Similarly at a higher concentration of BAP in the medium, 2-5 number of shoot buds were produced.

In MS medium containing 4.0 mgl^{-1} BAP about 22-25 axillary shoot buds were formed per shoot apex in 97% of its cultures (Table 6; Plate 2c,d). With 2.0 mgl^{-1} BAP in the medium, 10-12 very small shoot buds emerged. The increased concentrations of BAP (6.0, 8.0 and 10.0 mgl^{-1}) in the medium resulted in the reduction of number of shoot bud formation. A comparatively poor response was observed in the shoot apices cultured in MS medium supplemented with different concentrations of kinetin. Only about 8-10 shoot buds formed in cultures grown in MS medium containing kinetin (4.0 mgl^{-1}). A lower concentration of kinetin (2.0 mgl^{-1}) in the medium induced 4-5 number of shoot buds. However, in this case needle elongation was more pronounced.

Incorporation of NAA and IBA singly (0.1-0.5 mgl^{-1}) in conjuction with BAP (4.0 mgl^{-1}) in the medium reduced the number of shoot buds development (Table 7) as compared to cytokinin treatment alone (Table 6). Shoot apices cultured in MS medium supplemented with BAP (4.0 mgl^{-1}) in combination with IBA (0.1mgl^{-1}) resulted in

Table 5. Multiple axillary shoot bud formation on shoot apices of *Pinus kesiya* cultured in LP medium containing different concentrations of BAP and kinetin.

Growth regulator Concentration (mgl^{-1})	% Response	Remarks
Control	98.2±1.56	Needles elongated, no shoot bud formation.
BAP		
2.5	30.2± 2.81	1-2 shoot buds formed.
5.0	72.3±1.83	7-8 very small shoot buds emerged which failed to develop further.
7.5	60.5± 2.90	2-5 shoot buds formed which showed poor growth.
10.0	-	-
Kinetin		
2.5	43.7±1.58	3-4 shoot buds formed.
5.0	96.3± 2.91	15-16 shoot buds formed, out of which few developed into shoots.
7.5	90.5±2.58	15-16 shoot buds formed, few developed into shoots.
10.0	35.5±2.24	2-3 unhealthy shoot buds formed.

± S.D.

- No response.

Table 6. Multiple axillary bud formation on shoot apices of *Pinus kesiya* cultured in MS medium containing different concentrations of BAP and kinetin.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	98.2±1.56	Needles elongated, no shoot bud formed.
BAP		
2.0	84.2±1.22	10-12 very small shoot buds formed.
4.0	97.3±1.87	22-25 shoot buds formed, most of which developed into shoots.
6.0	95.5±1.58	8-10 shoot buds formed few developed into shoots.
8.0	51.3±1.31	6-7 shoot buds formed on each apex.
10.0	46.8±1.87	1-2 small shoot buds developed, which failed to grow further.
Kinetin		
2.0	61.4±2.91	4-5 shoot buds developed, needle elongation more pronounced.
4.0	72.3±2.87	8-10 shoot buds formed few developed into shoots
6.0	58.5±2.23	2-3 shoot buds formed at the apex.
8.0	37.1±2.01	1-2 shoot buds formed.
10.0	-	-

± S.D.

- No response.

Table 7. Multiple axillary bud formation on shoot apices of *Pinus kesiya* cultured in MS medium with cytokinin and auxins in combination.

Growth regulator Concentration (mgl^{-1})	% Response	Remarks
Control	98.2±1.56	Needles elongated, no shoot bud formation.
BAP+NAA		
4.0+0.1	70.2± 2.94	8-9 small shoot buds.
4.0+0.2	61.3± 2.88	Few shoot buds.
4.0+0.3	52.5± 2.58	Developing shoot buds showed poor growth.
4.0+0.4	28.3± 2.79	Slight swelling at the apex.
4.0+0.5	25.5± 2.23	-do-
BAP+IBA		
4.0+0.1	71.2± 2.91	9-10 small shoot buds.
4.0+0.2	62.3± 2.79	6-8 small shoot buds.
4.0+0.3	46.2± 2.44	2-3 shoot buds showing poor growth.
4.0+0.4	25.8± 2.98	Small protuberances at the apex.
4.0+0.5	21.3± 2.90	Swelling at the apex.

± S.D.

formation of 9-10 shoot buds (Table 7). A higher concentration of IBA (0.2 mgl^{-1}) in combination with BAP (4.0 mgl^{-1}) resulted in the development of 6-8 small shoot buds. With increasing concentrations of IBA in the medium a decline in shoot bud formation was observed. A similar response was observed in shoot apices cultured in MS medium containing higher concentrations of NAA (0.2- 0.5 mgl^{-1}) with BAP (4.0 mgl^{-1}). About 8-9 small shoot were emerged on the shoot apices cultured in the medium containing 0.1 mgl^{-1} NAA and 4.0 mgl^{-1} BAP.

Needles seperated from 30-day old seedlings were cultured in both MS and LP medium supplemented with different concentrations of cytokininis (BAP and kinetin) alone and in combination with auxins (NAA and IBA). Needles cultured in LP medium supplemented with 0.5 mgl^{-1} BAP developed 3-4 shoot buds at the base (Table 8). At 2.5 mgl^{-1} and 5.0 mgl^{-1} BAP in the medium there was a sharp decline in the shoot bud induction. Concentrations higher than these proved completely inhibitory. 1-2 small shoot buds were formed on needles cultured in LP medium supplemented with 2.5 mgl^{-1} and 5.0 mgl^{-1} kinetin. The increase in kinetin concentration (7.5 mgl^{-1} and 10.0 mgl^{-1}) in medium showed a very poor response.

About 7-8 small shoot buds were formed at the base of each needle, when cultured in MS medium supplemented with 5.0 mgl^{-1} of kinetin (Table 9, Plate 3). Needles cultured in medium with lower concentration of kinetin resulted in formation of 3-5 buds per needle. Higher concentration of kinetin (7.5 mgl^{-1}) in the medium showed formation of 2-3 shoot buds per needle, where as at 10.0 mgl^{-1} kinetin in the medium shoot bud development did not occur. Needles cultured in the medium with BAP (2.5

Table 8. Shoot bud induction on needles of *Pinus kesiya* cultured in LP medium with different concentrations of BAP and kinetin.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	-	-
BAP		
0.5	35.3± 1.81	3-4 shoot buds formed at the base of the needle.
2.5	21.2± 2.58	1-2 shoot buds formed.
5.0	12.4± 2.98	Only one shoot bud formed at the base.
7.5	-	-
10.0	-	-
Kinetin		
0.5	8.3± 1.58	A single shoot bud formed.
2.5	31.4± 2.91	1-2 shoot buds formed at the base.
5.0	32.6±1.49	1-2 shoot buds formed at the base.
7.5	12.3± 2.89	Slight swelling of the needle with no further development.
10.0	-	-

± S.D.

- No response.

Table 9: Shoot bud induction on needles of *Pinus kesiya* cultured in MS medium with different concentrations of BAP and kinetin.

Growth regulator Concentration (mgl^{-1})	% Response	Remarks
Control	-	-
BAP		
0.5	40.2± 1.81	Only one shoot bud formed at the base.
2 5	44.6± 2.45	1-2 small shoot buds with good growth.
5.0	15.8± 2.12	1-2 small shoot buds with poor growth.
7.5	-	No response, explant dried.
10.0	-	-do-
Kinetin		
0.5	41.3± 1.44	3-4 tiny shoot buds formed.
2.5	45.2± 2.90	4-5 small shoot buds which are dark green.
5.0	60.5± 2.91	7-8 small shoot buds formed at the base of the needle.
7.5	25.5± 2.58	2-3 shoot buds developed which did not grow further.
10.0	-	-

± S.D.

- No response.

and 5.0 mgl^{-1}) resulted induction of 1-2 small shoot buds. Higher concentrations of BAP (7.5 mgl^{-1} and 10.0 mgl^{-1}) were inhibitory.

Needles cultured in MS medium with 5.0 mgl^{-1} kinetin and 0.1 mgl^{-1} NAA in combination resulted in induction of 1-2 small shoot buds (Table 10). Similarly, about 1-2 small shoot buds developed per needle when cultured in MS medium supplemented with a combination of 5.0 mgl^{-1} kinetin and 0.1 mgl^{-1} IBA. Needles cultured in higher concentration of both NAA and IBA (0.2 - 0.5 mgl^{-1}) in combination with kinetin (5.0 mgl^{-1}) proved inhibitory.

SHOOT MULTIPLICATION AND ELONGATION

The different stages of shoot bud formation and multiplication using embryos, shoot apex and needle are shown in Plate 1(a-f), 2 (a-f) and 3 (a-c), respectively. Most of the developing shoot buds (derived from embryo) multiplied and proliferated in half-strength LP medium supplemented with 1.0 mgl^{-1} kinetin and 0.1mgl^{-1} IBA along with 0.3% activated charcoal in the medium (Table 11, Plate 1e). Further multiplication was accomplished by subculture of isolated shoots every 3-4 weeks in the same medium devoid of growth regulators for 2-3 passages (Plate 1f). Using this procedure a total of about 80-85 shoot buds were produced per embryo. Out of which about 20% of the shoots dried up on subculturing.

Shoot buds developed from shoot apices when cultured in half-strength MS medium containing 1.0 mgl^{-1} BAP and 0.1mgl^{-1} IBA in combination resulted in about 62% response. In about 4 weeks time the shoot buds multiplied with developed many

Table 10. Shoot bud induction on needles of *Pinus kesiya* cultured in MS medium with cytokinin and auxins in combination.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	-	-
Kinetin+NAA		
5.0+0.1	41.5± 2.77	1-2 small shoot buds showing poor growth.
5.0+0.2	35.5± 2.58	Small protuberances at the base.
5.0+0.3	18.2± 2.88	Slight swelling at the base.
5.0+0.4	-	-
5.0+0.5	-	-
Kinetin+IBA		
5.0+0.1	45.5± 2.53	1-2 small shoot buds formed.
5.0+0.2	25.2± 2.16	Slight swelling at the base of the needle.
5.0+0.3	17.3± 1.82	Swelling at the base of the needle.
5.0+0.4	-	-
5.0+0.5	-	-

± S.D.

- No response.

Table 11. Multiplication and proliferation of shoot buds obtained from different explants in respective media*.

Expalnt	Growthregulators Concentration (mgl^{-1})	% Response	Remarks
Contro		-	-
Shoot apex	BAP+IBA		
	1.0+0.01	45.3± 2.81	Shoot buds started multiplying.
	1.0+0.10	62.5± 1.35	Shoot buds multiplied and more needles were formed.
	1.0+1.00	20.5± 2.76	Multiplication of few buds occured.
Needles	Kinetin+IBA		
	1.0+0.01	38.4± 2.58	Shoot buds elongated became larger in size.
	1.0+0.10	56.5± 1.49	The buds developed more needles and elongated in size.
	1.0+1.00	25.1± 1.58	Developing buds failed to multiply.
Embryos	Kinetin+IBA		
	1.0+0.01	48.3± 2.77	Few buds multiplied and proliferated.
	1.0+0.10	72.5± 2.85	Most of the buds developed , multiplied and proliferated. Longer exposure resulted rooting in some cultures
	1.0+1.00	22.4± 1.68	Few buds proliferated.

± S.D.

- No response.

* For shoot apex and needle culture half strength MS medium and for embryo culture half strength LP medium were used.

Plate 1.(a-f). Regeneration of plantlets from embryos of *P. kesiya*.

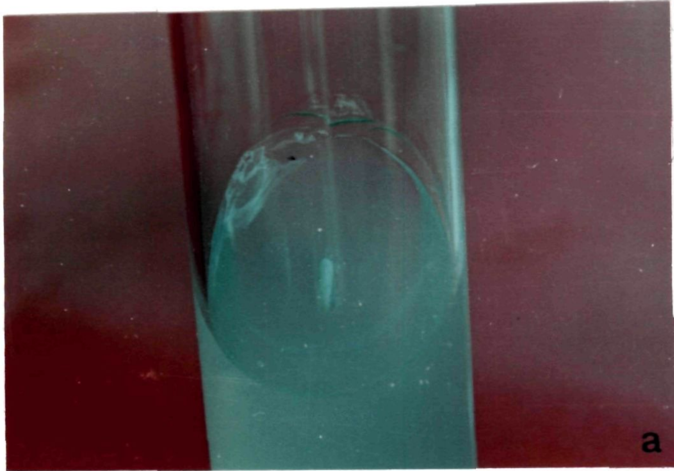
a. Dissected embryo cultured in LP + kinetin (5.0 mgl^{-1}).

b. Opening of cotyledons in culture.

c&d. Development of shoot buds in hormone free LP medium with reduced sucrose (2%).

e. Multiplication and proliferation of shoot buds in half-strength LP medium containing kinetin (1.0 mgl^{-1}) + IBA (0.1mgl^{-1}) + 0.3% activated charcoal.

f. Isolated shoot kept for elongation on half-strength LP medium with 0.3% activated charcoal.



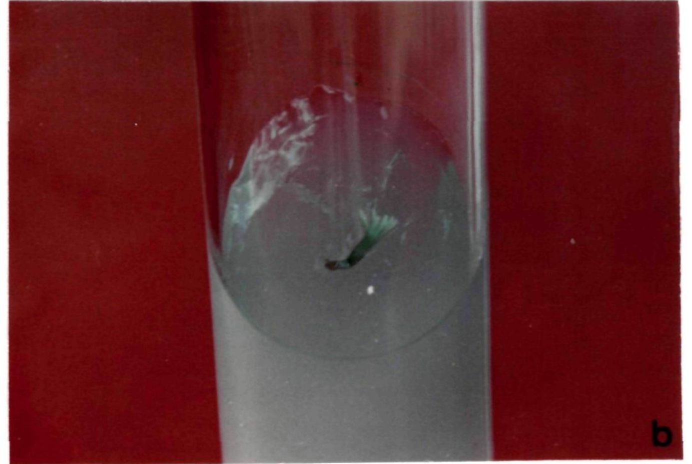


Plate 1. (a-f)

Plate 2. (a-f). Plantlet regeneration from shoot apices of *P. kesiya*.

a. Shoot apex cultured in MS + BAP (4.0 mgl^{-1}).

b. Growth of shoot apex in culture on the same medium.

c & d. Development of shoot buds.

e. Multiplication and proliferation of shoot buds in half-strength MS medium with BAP (1.0 mgl^{-1}) + IBA (0.1 mgl^{-1}) + 0.3% activated charcoal.

f. Isolated shoot showing elongation.

a

b

c

d

e

f

Plate 2. (a-f)

Plate 3. (a-c). Shoot bud formation on needles of *P. kesiya*.

a. Needle separated and cultured in MS + kinetin (5.0 mgl^{-1}).

b. Initiation of shoot bud at the base of the needles cultured in MS + kinetin (5.0 mgl^{-1}).

c. Formation of shoot buds at the base of the needle cultured on the same medium.

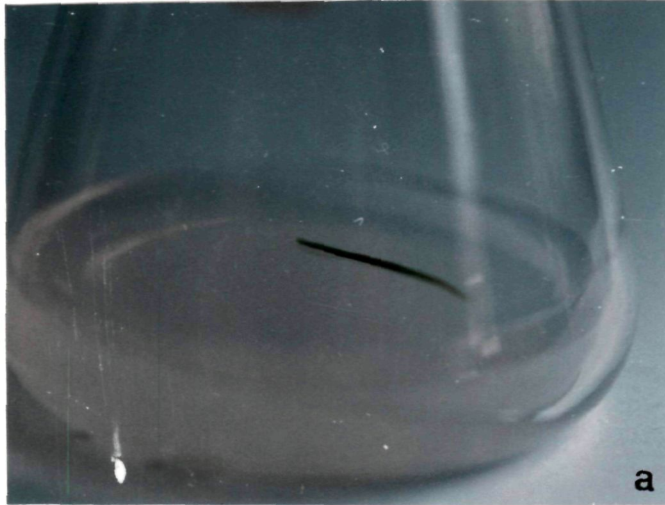


Plate 3.(a-c)

needles (Plate 2e). These shoots weer multiplied through subculture every 3-4 weeks in fresh half-strength MS medium devoid of growth regulators for about 2-3 passages. The shoots grew to a height of 20-30 mm (Plate 2f). Shoot buds developed from needles upon culture in half-strength MS medium with 1.0 mgl^{-1} kinetin and 0.1 mgl^{-1} IBA with 0.3% activated charcoal resulted in development of more needles. The response of shoot derived from needles was poor compared to embryo and shoot apex derived shoot multiplication.

ROOTING OF ELONGATED SHOOTS

About 64% rooting response was observed in shoots (derived from embryos) cultured in half-strength LP medium supplemented with 0.3 mgl^{-1} of NAA (Table 12). About 3-4 number of healthy roots emerged at the base of the isolated shoots. Use of lower concentration of NAA (0.2 mgl^{-1}) resulted in the formation of 2-3 roots. No root formation occured on shoots cultured in the medium with NAA (0.1mgl^{-1}). The number of root development declined with increase in concentration of NAA (0.4 and 0.5 mgl^{-1}). Shoots cultured in medium with 0.3 mgl^{-1} IBA resulted in formation of 2-3 roots per shoot with about 53% response. The rooting response declined with the increase in IBA concentration in the medium.

The rooting response of isolated shoots (derived from embryo) after pretreatment with NAA and IBA and subsequent culture in the water-agar medium is presented in Table 13. The optimum rooting response was observed in shoots treated with 10.0 mgl^{-1} NAA for 24 hr. About 3-4 number of roots developed in this treatment. Increase in

Table 12. Rooting response of isolated shoots (derived from embryos) of *Pinus kesiya* cultured in half strength LP medium supplemented with different concentrations of NAA and IBA.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	-	-
NAA		
0.1		-
0.2	42.5± 1.53	2-3 number of roots developed at the base.
0.3	64.2± 2.81	3-4 number of healthy roots emerged.
0.4	58.5± 2.58	1-2 number of large roots developed.
0.5	48.1± 1.82	1-2 number of roots developed.
IBA		
0.1	-	-
0.2	38.2± 1.98	1-2 roots developed.
0.3	53.6± 2.81	2-3 roots were formed.
0.4	25.5± 1.98	One small root developed which did not grow further.
0.5	-	-

± S.D.

- No response.

Table 13. Rooting response of isolated shoots (derived from embryos) of *Pinus kesiya* cultured in water-agar medium after pretreatment with different concentrations of NAA and IBA for different periods of time.

Growth regulators Concentration (mgl^{-1})	Period of Treatment (hr.)	%Response	Remarks
Control	-	-	-
NAA			
10			
	12	38.2± 1.58	One small root developed.
	24	70.1± 1.82	3-4 number of roots formed.
	48	62.5± 1.49	2-3 number of roots developed.
20			
	12	48.4± 2.58	3-4 number of roots developed.
	24	45.5± 1.46	1-2 number of roots developed
	48	-	The shoots became brown.
30			
	12	32.4± 2.82	1-2 number of roots developed.
	24	-	-
	48	-	-
IBA			
10			
	12	-	-
	24	58.6± 2.58	3-4 number of roots formed.
	48	48.1± 1.98	2-3 number of roots developed.
20			
	12	45.2± 2.83	One small root developed.
	24	56.5± 1.92	2-3 number of roots developed.
	48	-	-
30			
	12	-	-
	24	-	-
	48	-	-

± S.D.

- No response.

concenrations of NAA (20.0, 30.0 mgl^{-1}) and duration of treatment reduced the number of roots. Shoots treated for 24 hr. in 10.0 mgl^{-1} IBA formed 3-4 roots. Higher concentrations of IBA and duration of treatment were found to be inhibitory.

Shoots given pulse treatment with 10.0 mgl^{-1} NAA and 10.0 mgl^{-1} IBA for 24 hr. each when cultured in half-strength LP medium resulted in the formation of 3-4 roots per shoot (Table 14). With the increase in the concentration of NAA (20.0 and 30.0 mgl^{-1}) number of roots declined. Root initiation did not occur in treatments with higher concentrations of IBA.

Isolated shoots cultured in half-strength MS medium supplemented with NAA (0.2 and 0.3 mgl^{-1}) developed 3-4 healthy roots (Table-15). Increase in concentration of NAA (0.4 and 0.5 mgl^{-1}) in the medium reduced the number of roots. The rootings response of shoots was poor in the medium supplemented with different concentrations of IBA. About 1-2 roots formed on shoots cultured in medium with IBA (0.3 and 0.4 mgl^{-1}).

Basal end of shoots dipped in NAA (10.0 mgl^{-1}) for 24 hr. resulted in formation of 4-5 healthy roots upon culture in water-agar medium (Table 16; Plate 4a,b). When the period of treatment of NAA (10.0 mgl^{-1}) was decreased to 12 hr. initiation of only one root could be observed. However, with 48 hr. treatment, 3-4 roots were observed. Number of roots decreased when the concentration of NAA (20.0 and 30.0 mgl^{-1}) and period of treatment were increased. The response of shoots for rooting was poor when cultured in water-agar medium after pulse treatment in different

Table 14. Rooting response of isolated shoots (derived from embryos) of *Pinus kesiya* cultured in half strength LP medium after pretreatment with different concentrations of NAA and IBA for different periods of time.

Growth regulator Concentration (mgl^{-1})	Period of treatment (hr.)	% Response	Remark
Control	-	-	-
NAA			
10			
	12	-	
	24	68.1± 1.83	3-4 number of roots formed.
	48	62.2± 2.58	2-3 number of roots developed.
20			
	12	42.5± 2.53	2-3 number of roots developed.
	24	40.1± 1.92	1-2 number of roots developed.
	48	-	-
30			
	12	30.5± 2.83	1-2 number of roots developed.
	24	-	-
	48	-	-
IBA			
10			
	12	-	-
	24	55.5± 2.58	3-4 number of roots developed.
	48	45.6± 1.98	2-3 roots formed.
20			
	12	52.4± 2.58	One small root formed.
	24	-	-
	48	-	-
30			
	12	-	-
	24	-	-
	48	-	-

± S.D.
- No response.

Table 15.Rooting response of isolated shoots (derived from shoot apices) of *Pinus kesiya* cultured in half strength MS medium supplemented with different concentrations of NAA and IBA.

Growth regulators Concentration (mgl^{-1})	% Response	Remarks
Control	-	-
NAA		
[illegible]	-	-
0.2	68.1± 2.58	3-4 number of healthy roots formed.
0.3	61.3± 1.92	3-4 number of roots developed.
0.4	56.2± 2.53	1-2 number of roots formed.
0.5	42.5± 1.82	1-2 number of roots formed.
IBA		
0.1	-	-
0.2	-	-
0.3	36.4± 2.5	1-2 roots developed.
0.4	28.3± 1.96	One small root developed.
0.5	-	-

± S.D.

\- No response.

Table 16. Rooting response of isolated shoots (derived from shoot apices) of *Pinus kesiya* cultured in water agar medium after pretreatment with different concentrations of NAA and IBA for various periods of time.

Growth regulators Concentration (mgl^{-1})	Period of treatment (hr)	%Response	Remarks
Control	-	-	-
NAA			
10			
	12	45.1± 2.58	One small root initiated.
	24	72.3± 1.83	4-5 number of healthy roots developed.
	48	59.2± 1.82	3-4 number of roots formed.
20			
	12	48.4± 2.83	2-3 number of roots developed.
	24	45.3± 1.56	1-2 number of roots.
	48	-	-
30			
	12	36.1± 1.98	One small root initiated which further elongated.
	24	-	The shoot turned brown.
	48	-	-do-
IBA			
10			
	12	-	-
	24	25.3± 2.98	One small root developed.
	48	-	-
20			
	12	10.3± 1.58	Swelling of the base only.
	24	-	-
	48	-	-
30			
	12	-	-
	24	-	-
	48	-	-

± S.D.

\- No response.

Table 17. Rooting response of isolated shoots (derived from shoot apices) of *Pinus kesiya* cultured in half-strength MS medium after pretreatment with different concentrations of NAA and IBA.

Growth regulators Concentration(mgl^{-1})	Period of Treatmet (hr)	%Response	Remarks
Control	-	-	-
NAA			
10			
	12	45.3± 2.83	One small root developed.
	24	70.1± 1.56	3-4 number of roots developed.
	48	58.2± 2.56	2-3 number of roots developed.
20			
	12	48.3± 2.81	2-3 number of roots.
	24	46.4± 2.98	1-2 number of roots.
	48	-	-
30			
	12	35.1±1.86	1-2 small roots initiated.
	24	-	-
	48	-	-
IBA			
10			
	12	-	-
	24	25.3± 2.53	One small root developed.
	48	22.1± 1.58	One root initiated.
20			
	12	-	-
	24	-	-
	48	-	-
30			
	12	-	-
	24	-	-
	48	-	-

± S.D.
- No response.

Table 18. **Response of *in vitro* grown plantlets to different potting mixtures.**

Substrate used	% Survival	Growth
Peat+Vermiculture+Pumice (1:1:1)	49.5± 1.22	++
Peat+Pumice (1:1)	46.2± 1.29	++
Vermiculture+Pumice (1:1)	34.8± 2.11	+
Pumice	15.5± 1.83	-
Soil from pine forests	70.2± 1.33	+++

- Poor growth.
+ Fair growth.
++ Good growth.
+++ Best growth.

Plate 4. (a-c). rooting of isolated shoots of *P. kesiya*

a. Root formation on isolated shoot in water-agar (0.6%) medium after pulse treatment in NAA (10.0 mgl^{-1}) for 24 hr.

b. A rooted plantlet.

c. Rooted plantlet kept for hardening in soil from pine forest.

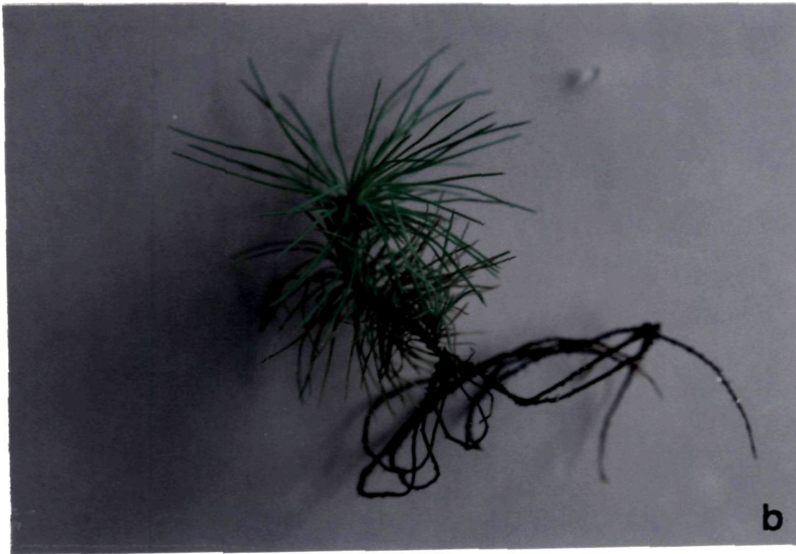


Plate 4.(a-c)

concentrations of IBA. Shoots treated with 10 mgl^{-1} IBA for 24 hr. resulted in formation of one small root.

About 3-4 roots developed on shoots derived from shoot apices when cultured in half-strength MS medium after pulse treatment with 10.0 mgl^{-1} NAA for 24 hr. (Table 17). Twelve hour pulse treatment with 10 mgl^{-1} NAA initiated formation of one small root per shoot. Higher concentration of NAA (20.0 and 30.0 mgl^{-1}) reduced the number of roots. Longer exposure of shoots (24 hr. and 48 hr.) on NAA (30.0 mgl^{-1}) did not result in any root formation. Pulse treatment of shoots with IBA did not give good rooting performance. With 24 hr. pulse treatment of shoot in 10.0 mgl^{-1} IBA there was development of one small root.

HARDENING OF PLANTLETS

After taking adequate care about 70% plantlets survived with good growth in soil mixture collected from the pine forests (Table 18, Plate 4b). Plantlets transferred to pumice alone resulted in poor growth. Where as a mixture of vermiculite and pumice in the ratio of 1:1 resulted in about 35% survival. Plantlets transferred to pots containing mixture of peat and pumice and also mixture of peat, vermiculite and pumice resulted in fair growth with 46 to 50% response, respectively.

DISCUSSION

Tissue culture of conifers could provide fast means of clonal propagation. It has been employed as a research tool for the development of procedures in basic research.

In vitro techniques are desirable as they can capture the non-additive traits that are difficult to retain by breeding (Bonga and von Aderkas, 1993). The causative factors controlling morphogenesis and regeneration from various explants are diverse. The nutritional requirements for the optimal growth of the plants *in vitro* vary from species to species. It has been reported that the tissues from different organs or/and parts differ in their growth substance requirements (Audus, 1972). For shoot initiation, the choice of explants and the concentration of growth regulators are more important than the nutrient medium composition. But for shoot elongation and multiplication and root initiation, the nutrient medium has a profound effect. In the present study, the embryos, shoot apices and the needles were found to be suitable explants for *in vitro* propagation of *P. kesiya*. The morphological changes that accompanies shoot development in *P.kesiya* embryo starts with nodular structures on the surface of the explant, which is similar to buds described earlier by Yeung *et al.* (1981) and Halos and Go (1993). Induction of about 20-22 shoot buds in 90% embryos of *P. kesiya* occurred when the embryos were transferred to plain LP medium devoid of growth regulators and reduced sucrose (2%) after 3-4 weeks culture in kinetin (5.0 mgl^{-1}) containing medium. In the present study, kinetin was found to be better than BAP. However, in *Pinus strobus* and *Pinus radiata* BAP was found to be suitable for bud induction on embryos (Chesick *et al.*, 1991) when cultured in LP medium. On the other hand Schwarz *et al.* (1988) reported the use of modified MS medium with BAP suitable for bud induction in embryos of *Pinus strobus*. The use of SH medium supplemented with 10.0 mgl^{-1} BAP could initiate production of only 10-15 shoot buds on each embryo in the present study

which is similar to the result obtained by Chesick *et al.* (1991) in *Pinus strobus*. The use of auxins was not found suitable for development of adventitious shoot buds on embryos of *P. kesiya* cultured in LP medium (Table 4). This is consistent with the findings of Sommer (1975) on Douglas fir embryos cultured *in vitro*. A wide variation in morphogenetic response of conifers has been reported by Mott *et al.* (1977) and Wochok and Abo El-Nil (1978). In case of *P. kesiya*, the number of adventitious shoot buds developed per embryo varied from 1 to 25 on different medium with various growth regulators. The shoot bud proliferation was recorded in cultures that were transferred to the same medium without growth regulators and with reduced sucrose (2%). The use of 2% sucrose has been recommended by Berlyn *et al.* (1991) for Caribbean pine shoot proliferation. The use of 3% sucrose for elongation and multiplication of shoot buds is of wide spread use in forest trees. Addition of 0.3% activated charcoal in half- strength LP medium showed a positive effect on shoot proliferation which is in line with the other reports on *Pinus pinea* (Diamantoglou *et al.*, 1990), *Pinus roxburghii* and *Pinus wallichiana* (Agrawal *et al.*, 1991). Transfer of the shoot buds to fresh medium also facilitated multiplication of the buds emerging out of the embryo of *P. kesiya* (Table 11). The use of half-strength LP medium for shoot proliferation was based on earlier reports of Patel and Thorpe (1984) where it was suggested that half-strength medium was most effective for inducing buds in embryos of *Pinus radiata*. In the present study, kinetin (1.0 mgl^{-1}) and IBA (0.01 mgl^{-1}) in combination together with 0.3% activated charcoal resulted in optimum multiplication and proliferation of shoot buds. The use of auxins and cytokinins in combination for

shoot bud formation and multiplication on embryos of *Pinus strobus* have already been reported by Schwarz *et al.* (1988). However, the kind of auxins and cytokinins and their concentrations may be different for various pine species.

In about 4 weeks time, about 22-25 axillary shoot buds were produced on shoot apex of *P. kesiya* cultured in MS medium containing 4 mgl^{-1} BAP (Table 6). A high percentage response of 97% was recorded. Burns *et al.* (1991) reported production of 18 buds on a single seedling of slash pine. Use of auxins for axillary bud production on shoot apex of *P. kesiya* seedlings was not suitable as it did not result in production of more shoot buds (Table 7). Similar response has been reported in radiata pine where the incorporation of auxin in MS medium did not promote shoot bud induction, but enhanced callus formation (Biondi and Thorpe, 1982b). However, in some cases double cytokinins and auxins are more effective than single growth hormone. Cheng (1975) reported that Douglas fir responds well to BAP plus 2-isopentenyl purine (2 ip) and IAA plus IBA in the medium.

In most cases formation of shoot buds from apices requires transfer of the buds into a medium with altered nutritional and hormonal level. In some, once the buds are formed, a change of medium is necessary to allow shoot elongation (Mehra-Palta *et al.*, 1978). In the present study, for multiplication and elongation of the shoot buds, subculture in half-strength MS medium supplemented with reduced concentration of BAP (1.0 mgl^{-1}) and low concentration of IBA (0.1 mgl^{-1}) together with activated charcoal (0.3%) was found to be effective. The use of half-strength medium for shoot elongation of Douglas fir and loblolly pine was also reported earlier (Cheng, 1975;

Mehra-Palta *et al.*, 1978). Use of activated charcoal (0.1-1%) was found beneficial for shoot elongation in loblolly pine (Mehra-Palta *et al.*, 1978), *Pinus pinaster* (David *et al.*, 1979), *Pseudotsuga menziesii* (Boulay, 1979 b), *Picea mariana* and *P. glauca* (Rumary and Thorpe, 1984). According to Fridborg *et al.* (1978) activated charcoal probably absorbs excess hormones or inhibitory substances. Though previous studies on conifers have confirmed that hoot elongation can be obtained on cytokinin free medium (Aitken *et al.* 1985), the present study, however, shows that use of auxins and cytokinins at low concentration is beneficial. The use of auxins at low concentrations along with cytokinins have been bound to promote elongation and multiplication of shoot buds in *Pinus strobus* (Schwarz *et al.*, 1988).

Needles obtained from 30-day old *in vitro* grown seedlings of *P. kesiya* when cultured in MS medium supplemented with kinetin (5.0 mgl^{-1}) resulted in initiation of 7-8 axillary shoot buds within two weeks time. Kumar and Tandon (1995) reported caulogenesis and direct regeneration in culture of needle of *P. kesiya* on MS medium supplemented with cytokinin. In the present study, however, the response of needles of *P. kesiya* was poor compared to shoot apices and embroys. von Arnold and Eriksson (1979) found that isolated needles of *Picea abies* could develop adventitious bud primordia when cultured in the medium containing a relatively high cytokinin-auxin ratio. But in the present study, the use of auxins (NAA and IBA) along with cytokinins (BAP) was found unsatisfactory. Swelling of the needles of *P. kesiya* occurred in culture with higher kinetin (7.5 mgl^{-1}) in LP medium where as in *Picea abies* parts of needles

swelled and formed multiple bud primordia in the presence of cytokinins (von Arnold and Eriksson, 1979).

The growth and elongation of shoot buds in *P. kesiya* was more rapid in case of embryos than shoot apices and needles. Adventitious bud production required a minimum of 12 weeks to elongate into shoots as compared to axillary buds from shoot apices and needles which took about 14-16 weeks and 24 weeks, respectively. Lesney *et al.* (1988) reported that the adventitious buds produced on slash pine embryo cotyledons required a minimum of 12 weeks to elongate into shoots. Browning and necrosis of explants viz., cotyledons, shoot apices and primary needles generally were observed within 1 week of their culture. Axillary and adventitious shoots elongated rapidly while still attached to original explants and reached a maximum height. These shoots were then removed and subcultured on fresh medium to encourage further elongation. It was observed that new buds were formed on these subcultured shoots which were similar to those already existing buds. These findings are consistent with the work of Zel *et al.* (1988) on *Pinus sylvestris*.

Roots were developed on isolated shoots derived from embryos and shoot tips. More than 72% root formation was observed on isloated shoots derived from shoot apices of *P. kesiya*, when cultured in water-agar medium after they were given pulse treatment of NAA (10.0 mgl^{-1}) for 24 hr. In about 3-4 weeks time, prominent roots numbering 4-5 could be observed on each shoot (Table 16). These grew to few mm in length upon subculturing in the same medium. In an earlier report, 64% rooting of isolated shoots of *P. kesiya* was reported (Kumar and Tandon, 1994). Earlier reports

have also shown pulse treatment of shoots with different concentrations of auxins for varying period of time to be beneficial for root initiation in many tree species (Hary and Thorpe, 1991). In *Pinus strobus,* roots were developed on shoots cultured in medium supplemented with NAA (Kaul, 1987), which was also true in many other tree species. The use of water- agar medium has already been reported by Horgan and Aitken (1981) while working on embryos and seedling shoot tips of radiata pine. In *P. kesiya* shoots a longer exposure to NAA resulted in development of fewer roots and browning of shoots. Pulse treatment of shoots of *Eucalyptus* resulted in successfull rooting (Blomstedt *et al.*, 1991). Incorporation of auxins in half-strength medium also resulted in formation of roots in shoots developed from different explants. Use of 4% sucrose for rooting of isolated shoots in *P. kesiya* was found suitable which is consistant with the earlier reports of Driver and Suttle (1987) and Horgan and Holland (1989). Half-strength medium has been used earlier for intiation of roots in *Pinus roxburghii* (Agrawal *et al.*, 1991). NAA treatments exhibited more roots than IBA treatment in *P. kesiya*. In the present study, the rooting percentage also varied according to the concentration of auxins used. The requirement of exogenous application of auxins in rooting of conifers has already been established (Mohammed and Vidaver, 1988). Auxins have also been reported to affect the quality of roots (Rancillac, 1979), Flinn *et al.* (1986), and Kaul (1987) reported that the requirements of growth regulators for caulogeneis and rhizogenesis in pine varies from species to species. The rooted plantlets were potted in pots with different potting mixtures. The transferr of plantlets from culture vessels to pots needs careful, stepwise procedure. The successful

transplantation depends on the suitable size and growth of the plantlets and the substratum used. The transferred plantlets had healthy roots which ensured better establishment and growth. The soil mixture from pine forest was found suitable for hardening of plantlets. The feeding of plantlets with diluted MS nutrient salt solution provided the essential nutrients to the developing plantlets. The substratum used was easily available and convenient for transfer and establishment of *in vitro* grown plantlets. The mychorrhizae available in the soil collected from pine forests helped in better establishment of *in vitro* grown plantlets.

From the study of *in vitro* propagation of *P. kesiya* it is found that embryos, shoot apices and needles can give rise to complete plantlets. But the time taken by each explant varied. The growth and elongation of shoot buds were rapid in case of embryos than shoot apices and needles. The buds from embryos take 12 weeks to elongate into shoots and another 3-4 weeks to develop roots whereas shoot buds from shoot apices take 14-16 weeks to elongate into shoots and 3-4 weeks to develop roots. The needles take the longest time in forming shoot where about 24 weeks are required to achieve its desired growth. Rooting of such shoots was quite difficult. Though mass propagation has been reported in some species of pines, the frequency and the number of shoot buds formed in such studies was comparatively less (Nadgauda *et al.*, 1993). In the present study, a total of about 80 shoot buds per embryo, 22-25 shoot buds per shoot apex, and 7-8 buds per needles could be produced which is relatively higher than the reports available so far from other conifers.

CHAPTER 3. CRYOPRESERVATION OF SHOOT APICES AND ZYGOTIC EMBRYOS OF *PINUS KESIYA* USING RAPID FREEZING, SLOW FREEZING AND VITRIFICATION METHODS.

INTRODUCTION

Germplasm, which is the source of variety of plant life provides the building blocks in agriculture, horticulture and forestry. Its preservation is important for the economy and ecology of human life. But conservation of important germplasm in field gene banks in the form of orchards, plantation etc. becomes problematic as it is costly to maintain. Moreover, there are risks of diseases and damages by extreme weather conditions, by fire and by animals.

Cryopreservation of plant cells, meristems and organs has become an important tool for the long-term preservation (usually in liquid nitrogen at -196^{0}C) of germplasm and experimental material without genetic alteration (Kartha, 1985;

Withers, 1985a). Through cryopreservation one can preserve or prevent variation not only in angiosperms (Kartha, 1987; Withers, 1985b) but also in gymnosperms (Binder and Zaerr, 1980; Kartha *et. al.*, 1988) . In the past, mostly vegetatively propagated plants were cryopreserved. But with rapid progress with plant transformation, cryopreservation is widely used in preserving the experimental materials of primary transformed tissues, secondary cultures etc. (Kendall *et al.*, 1993).

A typical cryopreservation procedure involves the stages of pregrowth, cryoprotection, freezing, storage, thawing and recovery. Pregrowth provides the opportunity for selecting or inducing the most freeze-tolerant growth phase. It covers the period of time in culture during which measures are taken to enhance freeze-tolerance through influence on the growth and metabolism of the culture and from which the most freeze tolerant stages of growth can be selected. Generally material in active growth is more resistant to freezing stress. For most cell suspension cultures the late lag phase or the exponential phase is the most freeze-tolerant phase. Plant tissues are pre-grown in presence of substances such as amino acid, mannitol, sorbitol, sucrose, ABA, DMSO etc. to enhance freeze-tolerance . The cultures become freeze-tolerant by changing their cell size, degree of vaculation and cell wall flexibility. In case of shoot tips, pregrowth also provides opportunity to recover from dissection damage. The requirement for pregrowth varies from culture system to culture system and.with species to species.

Cryoprotection which is the next step involves application of diverse compounds referred to as cryoprotectants during the period immediately preceding cooling. For

successful cryopreservation this step is necessary. It gives a protection against freeze damage. Application of cryoprotectants reduces the amount of freezing water from the cells; thereby giving less chance for ice formation and ice damage (Farrant, 1980; Meryman and Williams, 1982). Different hypertonic solutions are used as cryoprotectants. Glycerol was used for the first time in animal cells (Polge *et al.*, 1949). The most frequently used cryoprotectant chemical is DMSO singly or in combination with various sugars, sugar alcohols and other compounds, which may include mannitol, sorbitol, sucrose, glucose etc. Some times a mixture of cryoprotectants shows better result than a single one (Finkle and Ulrich, 1979; Hauptman and Widholm, 1982; Chen *et al.*, 1984 a,b). A considerable benefit of using a mixture of cryoprotectants involving polyethylene glycol (PEG), glucose and DMSO in sugarcane callus has been reported (Ulrich *et al.*, 1979; Finkle *et al.*, 1985) ABA has also been used in cultures of shoot primordia from shoot apices of cultured protocorms of *Vanda pumila* and others (Na and Kondo, 1996). These cryoprotective agents are usually with low molecular weight and are easily miscible, easily washable and can easily permeate the cells. Cryoprotectant mixtures are often prone to caramelisation during autoclaving and therefore must be sterilised by filtration. The concentration and the type of cryoprotectants suitable for successfull cryopreservation varies for different plants. The duration of exposure also varies. Generally a concentration of 5 to 10% of DMSO and 10 to 20% of glycerol is adequate for most materials. During cryoprotection step the materials are exposed to different cryoprotectant solutions and treated for

different periods (few min. to hr.) before cooling. The samples in cryoprotectants are next subjected to cooling/freezing.

The cooling rate has a very significant role in survival of tissues and organs. Cooling can be carried out at a range of relatively slow or rapid rates (Farrant, 1980; Meryman and Williams, 1982). Rapid freezing directly exposes the material to ultra low temperature at a rate of several hundred degrees centrigrade per minute. Rapid cooling causes relatively early intracellular freezing and little dehydration. Intracellular freezing leads to death of cells even in very hardy plants due to the mechanical destruction of biomembranes. In rapid freezing innocuous intracellular freezing occurs. Cryopreservation by rapid freezing results in very low survival percentage and some times even in complete loss of viability. But it is effective in some organised tissues. Damage by intracellular freezing depends on the amount, crystal size and location of the ice. But in many cases slow freezing is found to be a better method. Slow cooling is carried out at a constant, linear rate, where there is progressive temperature reduction. At first the external medium supercools and then there is ice formation. So extracellular freezing occurs, which causes cellular dehydration. The cell wall acts as a barrier and prevents ice from forming inside the cell. Freezing injury is comparatively less and it brings an optimum situation between under- and over-dehydration. Slow cooling is usually carried out in a programmed freezer where the rate of cooling can be varied for different specimens.

Storage is carried out in liquid nitrogen. The storage temperature should be low to prevent progressive deterioration resulting from ice recrystalization. Usually

below -100^{0} C is recommended which can be achieved by the use of liquid nitrogen cooled freezer in which the liquid phase is stable at -196^{0} C. Metabolism is suspended at this temperature but molecular changes due to ionizing effect of radiation, and consequent free radical activity may lead to cumulative damage in long term storage.

Warming or thawing is also as critical as cooling for the survival of specimens. It is essential to carry out thawing at a rate which prevents recrystalization of any ice that is present intracellularly. Warming is usually carried out rapidly by use of hot water bath (40^{0}C) or warm medium (20^{0}C or higher). The thawed specimens are cultured on fresh medium containing nutrients, vitamins and growth regulators for their recovery. Some times the composition of the recovery medium is modified. Viability can be recorded basing on the performance on recovery medium or by some viability tests such as TTC (trichloro tetrazolium chloride) and FDA (fluorescein diacetate) staining.

Though the cryopreservation has been carried out in almost all type of tissues and organs like cells, protoplasts, somatic and zygotic embryos, anthers, pollens and hole seed (Kartha , 1985 and Withers, 1985), shoot and embryo cultures are found to be more relevant to genetic conservation. In recent years, successful cryopreservation has been reported from embryonic axes of some truly recalcitrant seeds eg., *Hevea brasiliensis* (Normah *et al.*, 1986) and also from dissected embryos of seeds difficult to conserve like *Elaeis guineensis* (Grout *et al.*, 1983) and *Veitchia* and *Howea* (Chin *et al.*, 1988). The techinque involved dessication of the explant to about 10-16% moisture level followed by immersion in liquid nitrogen. In most cases the zygotic embryos are the primary tissue in transformation studied (Kartha *et al.*, 1989; Chibbar

et al., 1991; Christou *et al.,* 1991; Vasil *et al.,* 1992). Zygotic embryos are totipotent, genetically uniform, highly regenerative and culture conditions in many are well established. These features make zygotic embryos excellent candidates for cryopreservation. Cryopreservation of embryos in liquid nitogen has been suggested as the most promising method for storage in many woody species. The zygotic embryos and embryonic axes of tea and cocoa are successfully cryopreserved by Chandel *et al.* (1993). The embryonic axes of *Poncirus trifoliata* are preserved for one year (Radhamani and Chandel, (1992). A report is also available on different response of embryonic axes and cotyledons of tea seeds to desiccation and cryoexposure (Kuranuki and Yoshida, 1996). In most cases desiccation of embryos are carried out by air flow in laminar flow cabinet which is followed by immersion in liquid nitrogen (Grout *et al.,* 1983; Normah *et al.*, 1986; Chin *et al.*, 1988; Abdelnour *et al.*, 1992).

Though shoot cultures have received as much attention as other cells and organs it had proved far more variable in response and generally were more difficult to cryopreserve (Kartha *et al.*, 1982 a,b; Withers, 1987 a,b). But in the recent years a lot of work has been carried out on shoot tips of various plants. Over the years a thread of continuity and logic is seen in efforts to develop cryopreservation procedure for cell cultures and other organs. The shoot tips dissected from established plants or seedlings is more difficult to handle than those grown *in vitro*. But they are often chosen for cryopreservation being genetically stable intrinsically. The first report of successful cryopreservation of shoot tips was made by Seibert (1976). Grout and Henshaw (1978) reported successful regrowth of shoot tips of *Solanum goniocalyx* after cryoprotection

with 10% DMSO in liquid nitrogen. The meristem of potato could be cryopreserved and stored for 24 months (Bajaj , 1981). The shoot tips of asparagus (Kumur *et al.*, 1983), brussels sprouts (Harada *et al.*, 1985) and cold hardened woody species (Moriguchi *et al.*, 1985) were cryopreserved using 5 to 15% DMSO as cryoprotectant. Success of shoot tip cryopreservation of *in vitro* grown plantlets of potato (Towill, 1984) and apple (Kuo and Lineberger, 1985) were demonstrated. Shoot tips from *in vitro* grown plantlets of *Chamomilla recutita* (Diettrich *et al.*, 1990) from root fragments of Chicory (Demeulemeester *et al.*, 1993), shoot primordia of *Vanda pumila* (Na and Kondo, 1996), shoot tips of *Cassava* (Escobar *et al.*, 1997) have also been cryopreserved.

Though rapid and slow freezing were the common methods used in cryopreservation, recently some new methods have been developed which are cheaper and easier to perform. These are encapsulation dehydration and vitrification. Encapsulation dehydration is a method based on successive osmotic and evaporative dehydration (Dereuddre *et al.*, 1990). Here the material which is normally a shoot tip or somatic or zygotic embryo is encapsulated in sodium alginate beads. These encapusalted beads can be precultured in high osmoticum mainly in high sucrose solution and then air dried and plunged in liquid nitrogen. Encapsulation dehydration procedure has been successfully applied to many temperate and tropical species (Dereuddre, 1990). In sugarcane, the apices from *in vitro* grown plantlets were encapsulated and dehydrated, where between 38 and 91% survival was obtained in five varieties (Paulet *et al.*, 1993). Two weeks old microspore embryos of oil seed rape

(*Brassica napus*) were successfully cryopreserved after ca-alginate encapsulation, high sucrose preculture and air dehydration (Uragami *et al.*, 1993). Somatic embryos of *Coffea canephora* were precultured in different concentrations of sucrose before encapsulation and dehydration (Hatanaka *et al.*, 1994). Bachiri *et al.* (1995) were successful in cryopreserving suspension cells of *Catharanthus*. After preculture on sucrose enriched medium, the beads were dried over silica gel.

Vitrification which is a relatively new method enbales cells and meristems to cool to the temperature of liquid nitrogen without ice formation. It is a physical process by which a highly concentrated solution is supercooled to very low temperature and finally solidifies into metastable glass without crystallisation. Dehydration is induced by crystallisation of the external medium during the slow cooling steps. Successful vitrification becomes difficult in non-hardy and less hardy cultured plants cells and organs without partial freeze dehydration in the presence of cryoprotectant prior to ultra rapid cooling (Sakai, 1986; Uemura and Sakai; 1980). The cultured cells and somatic embryos of asparagus were first cryopreserved by vitrification (Uragami *et al.*, 1989), but the survival rate was not satisfactory. To improve the survival and to develop simpler technique many vitrification solution have been developed (Sakai *et al.*, 1990 ; Langis and Steponkus, 1990; Towill, 1990; Matsumato *et al.*, 1995). Good result was obtained when the vitrification solution (PVS2) was diluted and used at low concentration with shoot tips of sweet potato (Towill and Jarett, 1992). The nucellar cells of navel orange (*Citrus sinensis*) have been successfuly cryopreserved for 40

days by vitrification (Sakai *et al.*, 1991). Apical meristem from adventitious buds of lily were successfully cryopreserved (Matsumato *et al.*, 1995).

The cryopreservation of *P. kesiya* was attempted in view of the importance of the germplasm of a conifer of North-East India.

In the following chapter, attempts have been made to develop protocols for cryopreservation of zygotic embryos and shoot apices of *Pinus kesiya*. Influence of various parameters like pregrowth, cryoprotectant, cold treatments, osmotic treatments etc. on the survival of cryopreserved materials have also been studied.

MATERIALS AND METHODS

EXPERIMENTAL MATERIAL

The shoot apices and zygotic embryos of *P. kesiya* were used for cryopreservation . The procedure for obtaining the experimental material is described in Chapter 2.

Size of the shoot apices

The shoot apices 1-2 mm and 4-5 mm in size were used to assess the influence of size of the explant on cryopreservation. For cryopreservation experiments, 1-2 mm size shoot apices were used.

PRECULTURE

Shoot apices

DMSO, ethylene glycol, sucrose, glycerol, sorbitol and mannitol were incorporated singly at different concentrations ranging from 5-20% (w/v) in the MS basal medium (devoid of growth regulators) . 10 ml of the autoclaved agar medium while still hot was poured in each pre-sterilised 90mm disposable petri dish in a laminar flow cabinet. The petri dishes were covered after the cooling of the medium. The dissected shoot apices numbering 5 were cultured in each petridish and incubated at 25 ± 2^0C in light at an intensity of 150 µmoles m^{-2} sec^{-1} for 7, 14 and 21 days. Some shoot apices were also precultued in MS medium containing 10% (w/v) sucrose and incubated in the dark at 0, 4 and 8^0 C temperature for varying periods (0 - 15 days).

Embryos

Glycerol, sorbitol and mannitol were incorporated singly, in concentrations ranging from 0-20% (w/v) in basal LP medium (devoid of growth regulators). 10 ml of the autoclaved medium while still hot was poured in each 90 mm pre-sterilised disposable petri dishes in a laminar flow cabinet. The petri dishes were covered after the cooling of the medium. The excised embryos numbering 5 were cultured in each petri dish and incubated at 25 ± 2 C temprature and light intensity of 150 µ moles m^{-2} sec^{-1} for different periods ranging from 12-72 hr.

In another experiment, 10 ml autoclaved LP basal medium was poured into each 90 mm petri dish in a laminar flow cabinet while still hot. The petri dishes were covered after cooling of the medium. The excised embryos numbering 5 were inoculated in each petri dish and incubated in the dark at 0,4 and 8^0C temperature for varying period (0-15 days). They were also cultured at 25 ± 2^0C for the same period.

DESICCATION

The precultured embryos numbering 5 were placed on pre-sterilised aluminum foil (7.0x7.0 cm) in a laminar flow cabinet and desiccated by sterile air flow for 3 hr. The aluminum foil was then slowly folded by taking care not to damage the explant inside and then wraped with paraflim before directly plunging in liquid nitrogen.

Embryos were also cryopreserved without any preculture. For this the excised embryos numbering 5 were placed on aluminium foil (7.0x7.0 cm) inside a laminar flow cabinet and desiccated by sterile air flow for 0-5hr. The total moisture content of the embryos was determined at interval of every hr. of desiccation by drying 20 embryos for 48 hr. at 105 C. To get the per cent moisture content both initial weight (fresh weight) and the final weight (dry weight) were recorded. The desiccated embryos numbering 25 in each case were sealed as described above and plunged directly in liquid nitrogen.

Effects of hydration and dehydration

For studying the effect of hydration , the seeds were soaked in sterile pure water for 0-12 hr. The embryos were then asceptically dissected out and desiccated for 3 hr. in the same way as mentioned above in a laminar flow cabinet and then plunged directly in liquid nitrogen.

CRYOPROTECTION

Effect of DMSO concentration and duration of exposure

DMSO solutions at concentration ranging from 0-20% (w/v) were prepared in liquid MS and LP media (devoid of growth regulators) and sterilised by autoclaving at 121 C for 20 min. The shoot apices and embryos without preculture were then treated with this solution. The explants numbering 5 each were placed in 10 ml graduated glass stoppered test tube. To it, 0.5 ml of cryoprotectant solutions was added and incubated on ice for 1, 2 and 3 hr. in case of shoot apices and 1, 3 and 5 hr. for embryos.

Effect of sucrose, DMSO and glycerol in combination

A solution comprising sucrose, DMSO and glycerol at different concentration (0-10% w/v) was prepared in liquid MS medium and sterilised by autoclaving. The shoot apices precultured on 10% (w/v) sucrose for 14 days at 25 ± 2°C were used. 5 number of shoot apices were placed in each 10 ml graduated glass

stoppered test tube. To it, 0.5 ml of cryoprotectant solution was added. The test tubes were placed on ice for 1 hr.

FREEZING

Slow freezing method

The shoot apices and embryos numbering 5 each were placed separately in each pre-sterilised 10 ml graduated glass stoppered test tube, 0.5 ml cryoprotectant solution was poured into each tube. They were kept on ice for different periods as described above. The cryotubes were then placed in the freezing chamber of a programmable freezer (Planer Niomed Kryo 10 series Controller Model 10-20) and frozen using different cooling rates (0.0-1.0 C min^{-1}). The explants were cooled to various terminal temperatures (-20,-30,-40, and -80 C). The tubes were held at different terminal temperatures for 1 hr before plunging in liquid nitrogen.

Rapid freezing method

The desiccated embryos numbering 5 in aluminium foil as described above were plunged in liquid nitrogen rapidly. In other cases, the shoot apices and embryos numbering 5 each were placed in 10 ml graduated glass stoppered test tube. 0.5 ml of each cryoprotectant solution as mentioned above was poured in each tube and kept at 25 ± 2^0 C for 1,2 and 3 hr. The tubes were then rapidly plunged in liquid nitrogen.

Concise method

Shoot apices

The precultured shoot apices numbering 5 were placed in each graduated glass test tube. To it, 0.5 ml of a mixture of solution containing sucrose, DMSO and glycerol in various combinations ranging from 0-10% (w/v) were added slowly. The cryotubes were placed on ice for 1 hr. and then transferred to freezers held at different temperatures (-20,-30, -40, and -80 C) and stored there for 1 hr. before plunging in liquid nitrogen.

Embryos

The embryos precultured for 24 hr. in LP basal medium containing combinations of DMSO (5% w/v) and glycerol (5,10 and 15% w/v) for 24 hr. were used. Five embryos were placed in each pre-sterilised 10 ml graduated glass stoppered test tube. 0.5 ml of 5% DMSO was then slowly added to the tubes. The crytubes were then kept on ice for 1 hr. and transferred to freezers held at different temperatures (-20, -30, -40, and -80 °C). They were kept in the freezers for 1 hr. before plunging in liquid nitrogen.

Vitrification method

A vitrification solution designated as PVS2 (Sakai *et al.*, 1990) comprising of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO with 0.4M sucrose was prepared in respective basal medium as required for shoot apices and

embryos. The explants numbering 5 were placed in each cryotubes. Plastic, glass and aluminium cryotubes with 2.5 , 10 and 5 ml capacities, respectively were used for the experiments. 0.3 ml of PVS2 solution was added to each tube and explants were exposed for different periods of time ranging from 0-10 min. at 25+2 C and then rapidly plunged in liquid nitrogen. In other experiments, different volumes of vitrification solutions (0.0, 0.1, 0.3 0.5, and 1 ml) were added to the cryotubes and the explants were exposed for 2 min. before plunging in liquid nitrogen.

STORAGE IN LIQUID NITROGEN

The shoot apices and embryos frozen by different methods described above were stored in liquid nitrogen at least for 1 hr. before thawing. The explants were also stored upto 180 days in the dewar containing liquid nitrogen. The liquid nitrogen lost due to evaporation was replenished regularly.

THAWING

The cryotubes containing explants frozen solwly, rapidly and by concise method were removed from liquid nitrogen and were immediately plunged in a water bath held at varying temperatures (0-50 C). Soon after the ice melted the tubes were removed and sprayed with 70% alcohol and then taken to a laminar flow cabinet.

The vitrified explants in cryotubes were immediately thawed at 40^0 C for 2 min. by constantly shaking the tubes. The tubes were then taken to laminar flow cabinet after surface sterilisation.

WASHING

The embryos which were cryopreserved only after desiccation were not washed with any solution. The shoot apices and embryos were washed after thawing. To the cryotubes 5 ml of respective liquid basal medium was poured drop wise within a period of 20-30 mins. the tubes were intermittently shaken very gently. The washing solution was decanted and 1ml of fresh liquid basal medium was slowly poured. The washing medium was decanted and the explants were blotted dry on sterile filer paper (Whatman No. 42) in a 90 mm petri dish. Taking adequate care they were cultured in fresh medium.

The vitrified explants were washed using varying concentrations (0-4.8M) of sucrose solutions. 5 ml of the sucrose solution was added either dropwise within a period of 20-30 min. or added directly. While adding drop wise the tubes were shaken slowly after addition of every drop. This solution was poured out and 1 ml of the fresh sucrose solution was added and tubes were shaken gently. The washing solution was decanted. One batch of explants were dried over filter paper and cultured directly on fresh medium. To the other batch different volumes (0-5ml) of MS and LP liquid basal medium (as the case may be) were poured dropwise within a period of 20-30 min. and shaken gently intermittantly. After pouring out, another 1 ml of the medium was added dropwise. The explants together with the medium were expelled out on a petri dish and then blotted dry on filter paper in another petri dish before culturing in recovery medium.

REGROWTH

The washed and unwashed explants (from the experiments involving desiccation only without the use of cryoprotectant solutions) were cultured on respective recovery medium. The embryos were cultured in LP + kinetin (5 mgl^{-1}) and shoot apices in MS + BAP (4 mgl^{-1}). One explant each was cultured in a test tube/flask under the same culture conditions as described in chapter 2 for micropropagation.

The first sign of viability could be observed with the opening of the cotyledons in case of embryos and greening of the shoot apices. he responses of explants on regrowth media were recorded periodically. The viable explants were then cultured using various steps of mass propagation for getting plantlets.

For all the experiments described above 25 replicates were taken for each treatment and the experiments were repeated thrice. The standard deviation was calculated using the method of Zar (1974). The per cent response is based on the number of explants cryopreserved and the number of explants showing regrowth.

RESULTS

RESPONSE OF SHOOT APICES

Effect of DMSO concentration and period of exposure

The effect of DMSO concentration and duration of exposure both for treated controls and frozen shoot apices is presented in Table 19. With increase of concentration of DMSO from 5 to 15% for 1 hr. did not result in any decline in survivability of treated controls. On the other hand, increase in duration of exposure to

Table 19. Effect of DMSO-concentration and duration of exposure on per cent regrowth of shoot apices of *Pinus kesiya* after cryopreservation.

DMSO Conc. (%)	% Regrowth								
	Without LN			With LN					
				Rapid freezing			Slow freezing		
	1hr.	2hr.	3hr.	1hr.	2hr.	3hr.	1hr.	2hr.	3hr.
0*	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	-	-	-	-	-	-
5	100.0 ± 0.00	100.0 ± 0.00	98.2 ± 2.11	-	-	-	28.6 ± 1.14	38.3 ± 2.96	30.7 ± 1.98
10	100.0 ± 0.00	98.3 ± 1.08	96.3 ± 2.15	-	-	-	20.1 ± 1.93	25.3 ± 2.15	23.3 ± 2.88
15	100.0 ± 0.00	92.4 ± 1.98	89.7 ± 1.83	-	-	-	23.2 ± 1.99	15.9 ± 2.31	-
20	94.5 ± 1.28	86.2 ± 2.18	75.5 ± 2.56	-	-	-	22.4 ± 2.08	12.0 ± 2.86	-

* Untreated control

During rapid freezing shoot apices were directly plunged in LN and kept for 1 hr. before thawing and using slow freezing method they were cooled at the rate of 0.5°C/min till -30°C and then plunged in LN.

± S.D.

DMSO and its concentration resulted in a decline in survivability. The shoot apices frozen rapidly did not survive. Using slow freezing method, the maximum survivability and regrowth of 38.3% was recorded in 5% DMSO treatment for 2 hr. By increasing or decreasing the duration of treatment in this case exhibited a decline in regrowth. A similar picture was also recorded using 10% DMSO. Higher concentrations of DMSO were found to be inhibitory. In case of 15 and 20% DMSO treatment for 1 hr. the per cent survival and regrowth was higher than 2 hr. treatment. At this concentration there was no survival in 3 hr. treatment.

Effect of preculture

The shoot apices precultured in medium containing osmotic agents did not show any effect on the survivability and regrowth in most of the treated controls (Table 20). Higher concentration of sucrose, glycerol, ethylene glycol and DMSO were found to be inhibitory. Using sucrose and glycerol seperately at 10% (w/v) concentration in the medium resulted in higher survivability and regrowth (48.5% in case of sucrose and 46.8% in case of glycerol) after cryopreservation of shoot apices precultured for 14 days using slow freezing method. However, at 7 and 21 day preculture it showed lower survivabilty. In general 14 day preculture was found to be better for slow freezing of shoot apices in all the preculture treatments.

Table 20. Effect of preculture of *Pinus kesiya* shoot apices in the presence of DMSO and osmotic agents on per cent regrowth after cryopreservation.

Cryoprotectant % (w/v)	% Regrowth Without LN			With LN Slowly frozen		
	7 days	14days	21days	7days	14days	21days
Untreated *control*	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	-	-	-
DMSO						
5	100.0 ± 0.00	100.0± 0.00	96.1 ± 2.86	33.7 ± 2.82	43.3 ± 2.86	-
10	26.2 ± 2.36	16.7 ± 2.92	-	12.9 ± 2.53	-	-
15	-	-	-	-	-	-
20	-	-	-	-	-	-
Sucrose						
5	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	35.2 ± 2.96	38.6 ± 2.91	40.2± 2.76
10	100.0 ± 0.00	100.0± 0.00	92.3 ± 2.71	38.9 ± 2.86	48.5± 2.99	42.6± 2.78
15	60.2 ± 2.79	45.1± 2.78	-	35.3 ± 2.91	26.2 ± 2.98	-
20	23.6 ± 2.96	-	-	-	-	-
Glycerol						
5	100.0 ± 0.00	100.0 ± 0.00	98.4± 2.82	30.1± 2.86	39.2 ± 2.98	40.3± 2.98
10	100.0 ± 0.00	99.1 ± 2.98	97.7± 2.86	39.6± 2.98	46.8± 2.56	42.2± 2.23
15	78.6± 2.12	62.3± 2.93	48.1± 2.89	36.8± 2.82	32.9± 1.36	29.1± 2.48
20	-	-	-	-	-	-
Ethylene glycol						
5	98.9± 2.95	78.3± 2.84	47.7± 2.82	20.1± 2.88	21.8± 2.92	15.6± 2.96
10	89.4± 2.80	56.8± 2.96	15.2± 2.98	22.2± 2.96	20.2± 2.98	-
15	42.3± 2.96	-	-	-	-	-
20	-	-	-	-	-	-
Sorbitol						
5	100.0± 0.00	100.0± 0.00	100.0± 0.00	28.2 ± 2.93	23.2± 2.98	25.4± 2.98
10	100.0± 0.00	98.1± 2.98	97.3± 2.66	22.7 ± 2.89	30.5± 2.94	27.3± 2.96
15	98.7± 2.18	96.3± 2.92	96.5± 2.95	25.9 ± 2.64	26.3± 2.96	22.0± 2.50
20	92.8± 2.58	90.5± 2.42	90.3± 2.58	15.1± 2.96	21.7± 2.99	20.2± 2.99
Mannitol						
5	100.0± 0.00	100.0 ± 0.00	100.0± 0.00	26.3± 2.86	26.0± 2.48	22.9± 2.76
10	100.0± 0.00	100.0 ± 0.00	100.0± 0.00	28.7± 2.11	30.2± 1.98	20.3± 2.28
15	98.8± 2.99	97.3 ± 2.93	97.5± 2.82	21.9± 2.99	25.5± 2.86	19.8± 2.97
20	95.5± 2.68	93.7± 2.98	90.5± 2.88	22.6± 2.86	20.5± 2.97	23.2± 2.80

The precultured shoot apices were treated with 5% DMSO for 2 hr. and then cryopreserved by rapid and slow freezing method (rate 0.5°C/min. upto -30 °C) and then plunged into LN. No survival was observed using rapid freezing method.
± S.D.

FREEZING

Slow freezing

Effect of cooling rates

Shoot apices precultured on 10% sucrose for 14 days and treated with 5% DMSO for 2 hr. were frozen slowly with different cooling rates (Fig. 1). The optimum per cent regrowth of 48.5% was recorded in shoot apices frozen using the cooling rate of 0.5°C min^{-1}. The rate of cooling slower or faster than 0.5°C min^{-1} resulted in decrease in per cent regrowth in the recovery medium.

Effect of DMSO in combination with surcrose and glycerol

Concise method

A combination of DMSO with surcrose and glycerol resulted in better performance (Table 21) than DMSO alone (Table 19). A combination of sucrose 10% (w/v), DMSO 10% (w/v) and glycerol 5% (w/v) used as cryoprotectant solution for 1 hr. using concise method resulted in maximum per cent regrowth of 45.3% of shoot apices. Using sucrose and glycerol alone without DMSO did not show any survivability. The other combinations of the three cryoprotectants reduced the per cent regrowth of cryopreserved shoot apices.

Effect of prefreezing temperatures

Shoot apices kept at -30°C before plunge in liquid nitrogen showed the highest per cent regrowth after thawing (Fig. 2). A higher and lower than this prefreezing

Table 21. Effect of DMSO in combination with sucrose and glycerol on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya* by rapid freezing method.

Cryoprotectants % (w/v)	% Regrowth Without LN	With LN
Control	100.0± 0.00	-
Sucrose-DMSO-Glycerol		
10-10-0	100.0± 0.00	25.3± 2.77
10-10-5	98.3± 2.47	45.3± 2.91
10-0-10	100.0± 0.00	-
10-5-10	99.2± 2.52	25.2± 2.89
0-10-10	99.3± 2.96	20.3± 2.88
5-10-10	98.7± 2.58	25.3± 2.89
10-10-10	98.2± 2.86	30.1± 2.97

Shoot apices were precultured in the medium with 10% sucrose for 14 days at 25±2^{0}C and incubated for 1hr. in cryoprotectants and then kept for 1 hr. at -30° C before rapid plunging in LN.
± S.D.

Fig 1. Effect of various cooling rates on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya* .

± S.D.

Shoot apices were precultured in the medium with 10% sucrose for 14 days at 25± 2^{0}C and treated with 5% DMSO for 2 hr. before freezing slowly with various cooling rates to - 30°C and then plunged in liquid nitrogen.

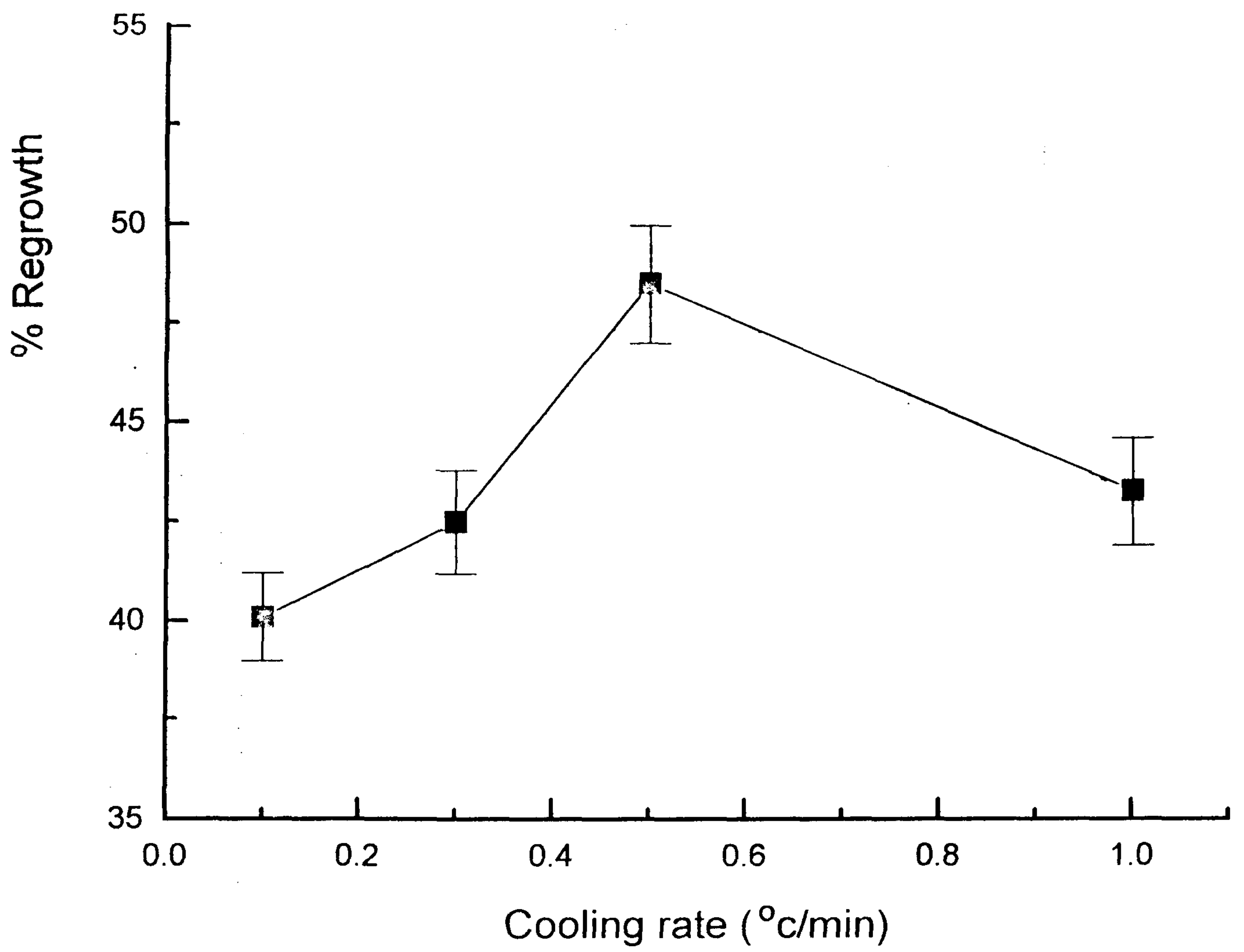


Fig. 1

Fig 2. Effect of different prefreezing temperatures on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya*.

± S.D.

Shoot apices were precultured in the medium with 10% sucrose for 14 days at 25± 2°C and incubated for 1 hr. in combination of sucrose (10%), DMSO (10%) and glycerol (5%) and followed by 1 hr. storage at different temperatures.

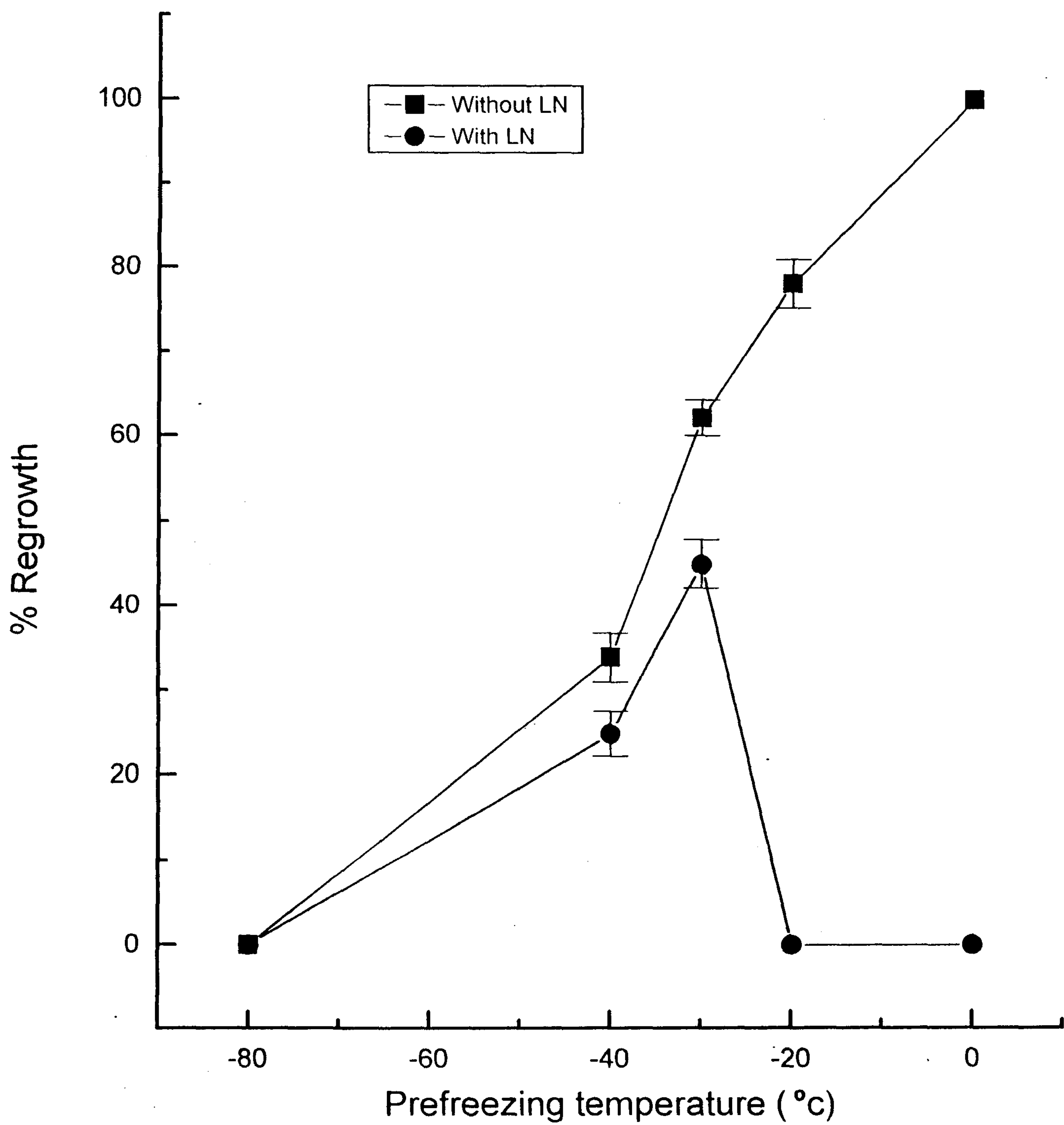


Fig. 2

temperautre was not suitable for cryopreservation. Samples prefrozen at -80°C did not give any survivability in both treated control and the one transferred to liquid nitrogen. In case of treated controls by decreasing the temperature, a sharp decline in regrowth was observed.

Slow freezing method

A regrowth of 52.6% was recorded in shoot apices treated with a combination of sucrose 10% (w/v) DMSO 10% (w/v) and glycerol 5% (w/v) for 1 hr. before freezing slowly to -30°C and the rate of 0.5°C min^{-1} (Table 22). A survivability of 40.5% was recorded in treatment with sucrose, DMSO and glycerol (5-10-10%). However, concentrations other than stated above exhibited lower survivabilty and regrowth.

Effect of the size of shoot apices

The smaller size (1-2 mm) of the shoot apices were better for cryopreservation. The maximum survivabilty was observed in 1 hr. treatment of cryoprotectants (Fig. 3). The per cent regrowth declined slowly in both treated control and cryopreserved material as the incubation period was increased. No survivability and regrowth occurred in shoot apices treated for 4 and 5 hr. The larger shoot apices (4-5 mm) did not survive after cryopreservation. Even treated controls showed no survivability after 3 hr. treatment.

Table 22. Effect of DMSO in combination with sucrose and glycerol on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya* using slow freezing method.

Cryoprotectants % (w/v)	% Regrowth Without LN	With LN
Untreated control	100.0± 0.00	-
Sucrose-DMSO-Glycerol		
10-10-0	100.0± 0.00	30.1± 2.96
10-10-5	98.9± 2.82	52.6± 2.89
10-0-10	100.0± 0.00	-
10-5-10	98.2± 2.91	36.3± 2.98
0-10-10	99.3± 2.92	28.3± 2.63
5-10-10	97.8± 2.53	40.5± 2.88
10-10-10	98.0± 2.62	33.3± 2.68

The shoot apices were precultured in the medium with 10% sucrose for 14 days at 25± 2^{0}C and treated for 1 hr. in cryoproctectant solutions and frozen slowly to -30°C (rate 0.5^{o}C/min) before plunging in LN.
± S.D.

Fig 3. Effect of size of shoot apices and incubation period in cryoprotective solution on the per cent regrowth of shoot apices of *Pinus kesiya*. ± S.D.

Shoot apices were precultured in the medium with 10% sucrose for 14 days at 25 ± 2°C and cryoprotected with combination of sucrose (10%), DMSO (10%) and glycerol (5%) for different periods of time and frozen slowly (rate 0.5°C min^{-1}) to -30°C before plunging in liquid nitrogen.

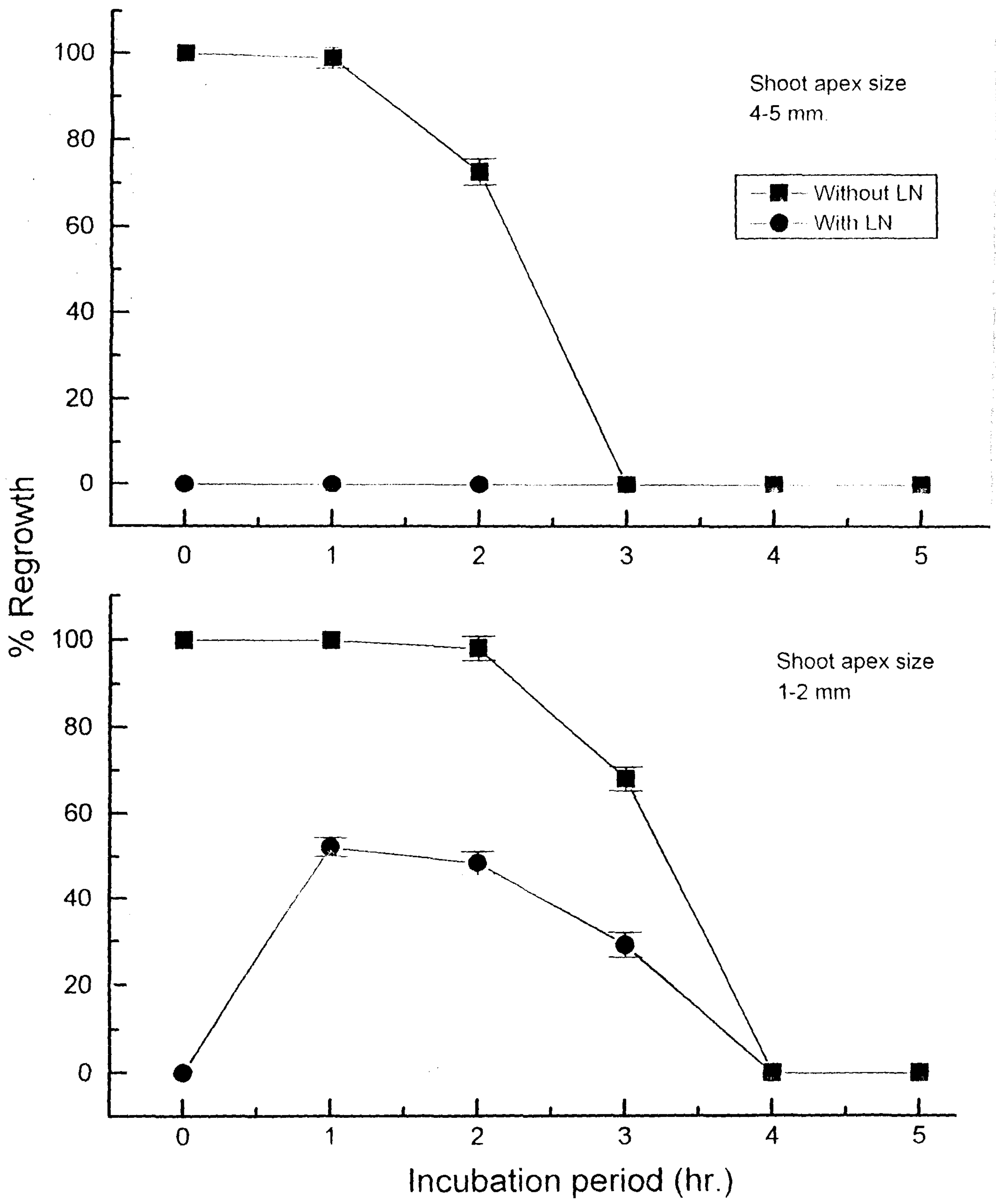


Fig. 3

Effect of preculture at different temperatures

Shoot apices cultured at 4°C for 10 days showed maximum per cent regrowth (55.3%) after cryopreservation (Fig.4). An increase in the period of treatment slightly decreased the per cent response at this temperature. Preculture of shoot apices at 8^0C showed poor response compared to the ones cultured at 4°C. The preculture for 5 days at 8°C was better compared to other periods of growth. By increasing the preculture period at 4 and 8°C the survivability of treated controls showed a decline. The cultures grown at 0°C exhibited a survivability and regrowth of about 40%. The duration of preculture did not alter this response significantly.

Effect of different terminal temperatures

Out of the various terminal temperatures at which the s ample were pre cooled, -30°C resulted in optimum per cent regrowth of 51.2% (Fig. 5). Lower and higher than this temperature resulted in decline in per cent regrowth. In case of treated controls a lower than -30°C resulted in a decline of per cent regrowth of the samples.

Effect of different thawing temperatures

The shoot apices thawed at 40°C water bath resulted in optimum per cent regrowth of 53.3% (Fig.6). The per cent regrowth of shoot apices was lower when thawed at 30, 35, 45 and 50°C temperatures. Shoot apices thawed at 0 and 25°C could not show any regrowth upon culture in recovery medium.

Fig 4. Effect of preculture at different temperatures on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya*.

± S.D.

Shoot apices were precultured in the medium with 10% sucrose at different temperatures and treated with combination of sucrose (10%), DMSO (10%) and glycerol (5%) for 1 hr. and then frozen slowly (0.5°C min^{-1}) to -30°C before plunging in liquid nitrogen.

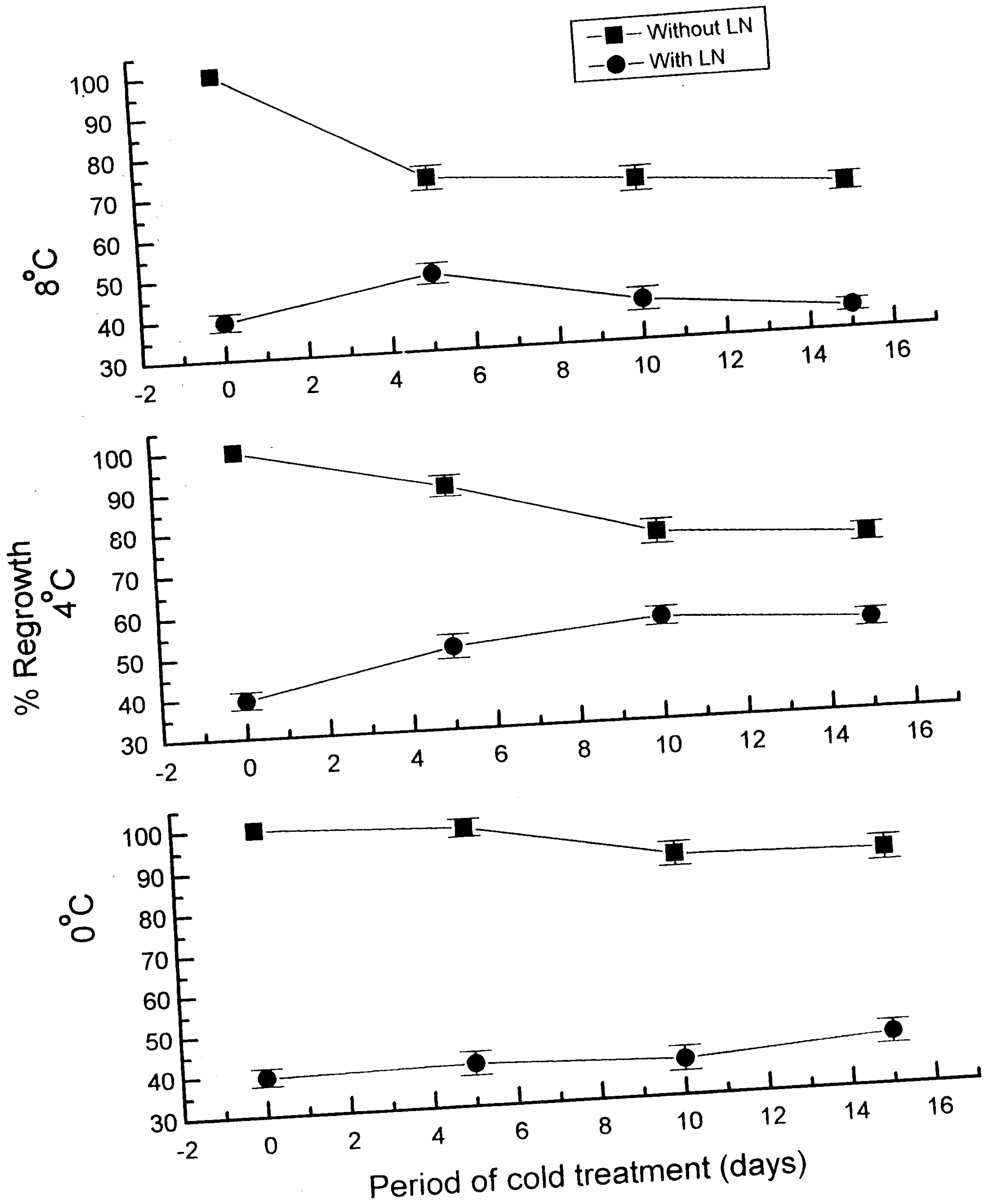


Fig. 4

Fig 5. Effect of different terminal temperatures on the per cent regrowth of cryopreserved shoot apices of *Pinus kesiya*.

±S.D.

Shoot apices were precultured in the medium with 10% sucrose for 14 days at 25±2°C, and treated with combinations of sucrose (10%), DMSO (10%) and glycerol (5%), for 1 hr. and then frozen slowly (rate 0.5°C min^{-1}) to different terminal temperatures before plunging in liquid nitrogen.

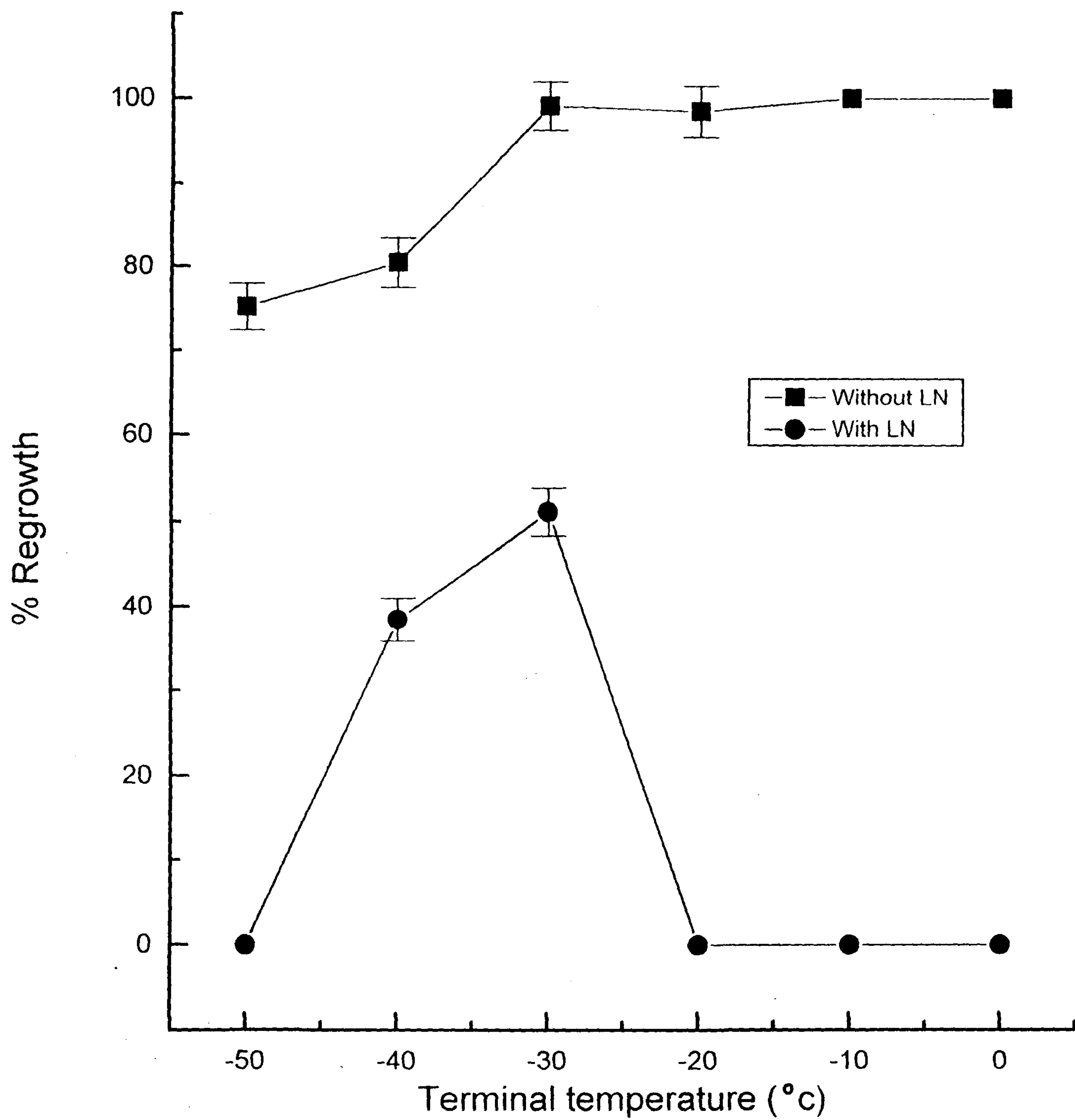


Fig. 5

Fig 6. Effect of different thawing temperatures on per cent regrowth of cryopreserved shoot apices of *Pinus kesiya*.

±S.D.

The shoot apices were precultured in the medium with 10% sucrose for 14 days at 25 ±2°C, and treated with combination of sucrose(10%), DMSO(10%) and glycerol (5%) for 1 hr. and then frozen slowly (rate 0.5°C min^{-1}) to -30°C before plunging in liquid nitrogen.

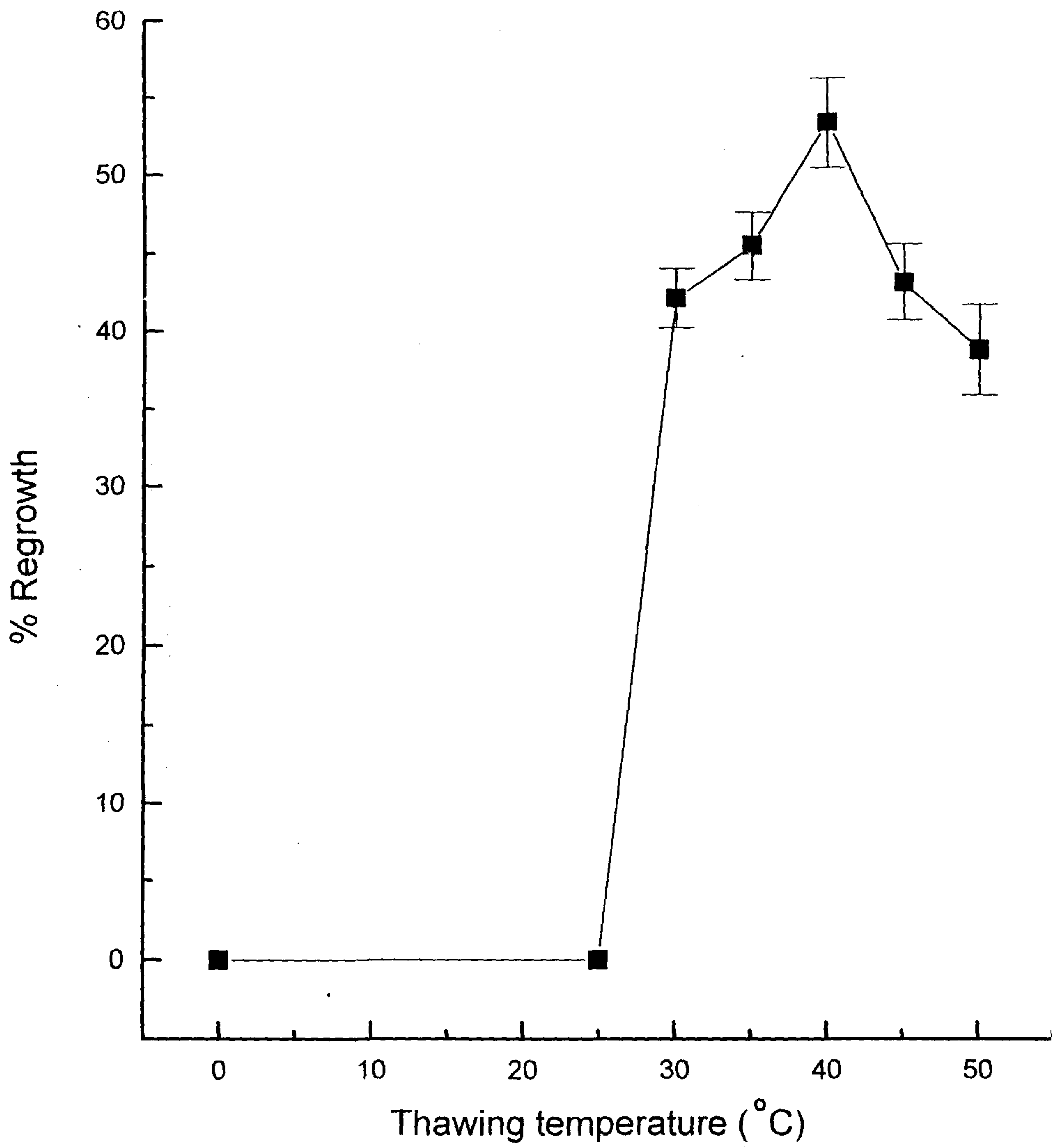


Fig. 6

Effect of long term storage

The per cent regrowth remained almost steady during the entire period of storage upto 180 days (Fig. 7). However slight decline in survivability and regrowth was observed in samples that were stored beyond 24 hr.

RESPONSE OF EMBRYOS

Effect of desiccation by air flow

The embryos cryopreserved rapidly after simple desiccation in a laminar flow cabinet for 3 hr. exhibted almost 80% plantlet regeneration (Fig.8). Moisture content after 3 hr. desiccation was 12%. With increase in desiccation period the moisture content decreased. The desiccation for shorter and longer duration than 3 hr. decreased the survivabilty and per cent plantlet regeneration. The unfrozen embryos (treated control) showed decline in per cent plantlet regeneration after 3 hr. desiccation.

Effect of age of culture

. The embryos without preculture exhibited better response after cryopreservation than those with preculture (Fig. 9). Using both rapid freezing and slow freezing methods survivablity and plantlet regeneration of embryos declined with increase in the age of cultures.

Fig 7. Effect of duration of storage in liquid nitrogen on per cent regrowth of shoot apices of *Pinus kesiya*.

± S.D.

The shoot apices were precultured in the medium with 10% sucrose at 4°C for 15 days and treated with combination of sucrose (10%), DMSO (10%) and glycerol (5%) for 1 hr. and frozen slowly ($0.5°C min^{-1}$) to -30°C before plunging in liquid nitrogen.

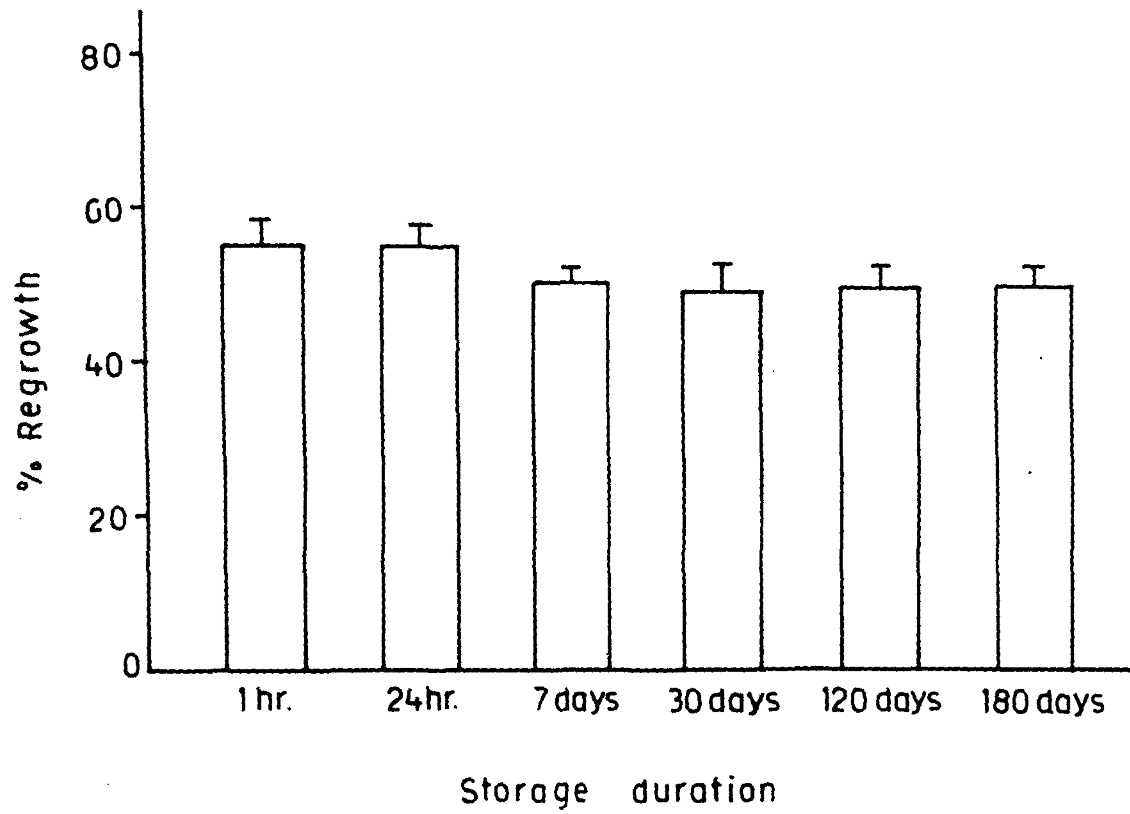


Fig. 7

Fig 8. Effect of desiccation period on per cent plantlet regeneration from unfrozen and rapidly frozen embryos of *Pinus kesiya*.

± S.D.

Embryos were desiccated with sterile air for different periods of time before plunging rapidly in liquid nitrogen.

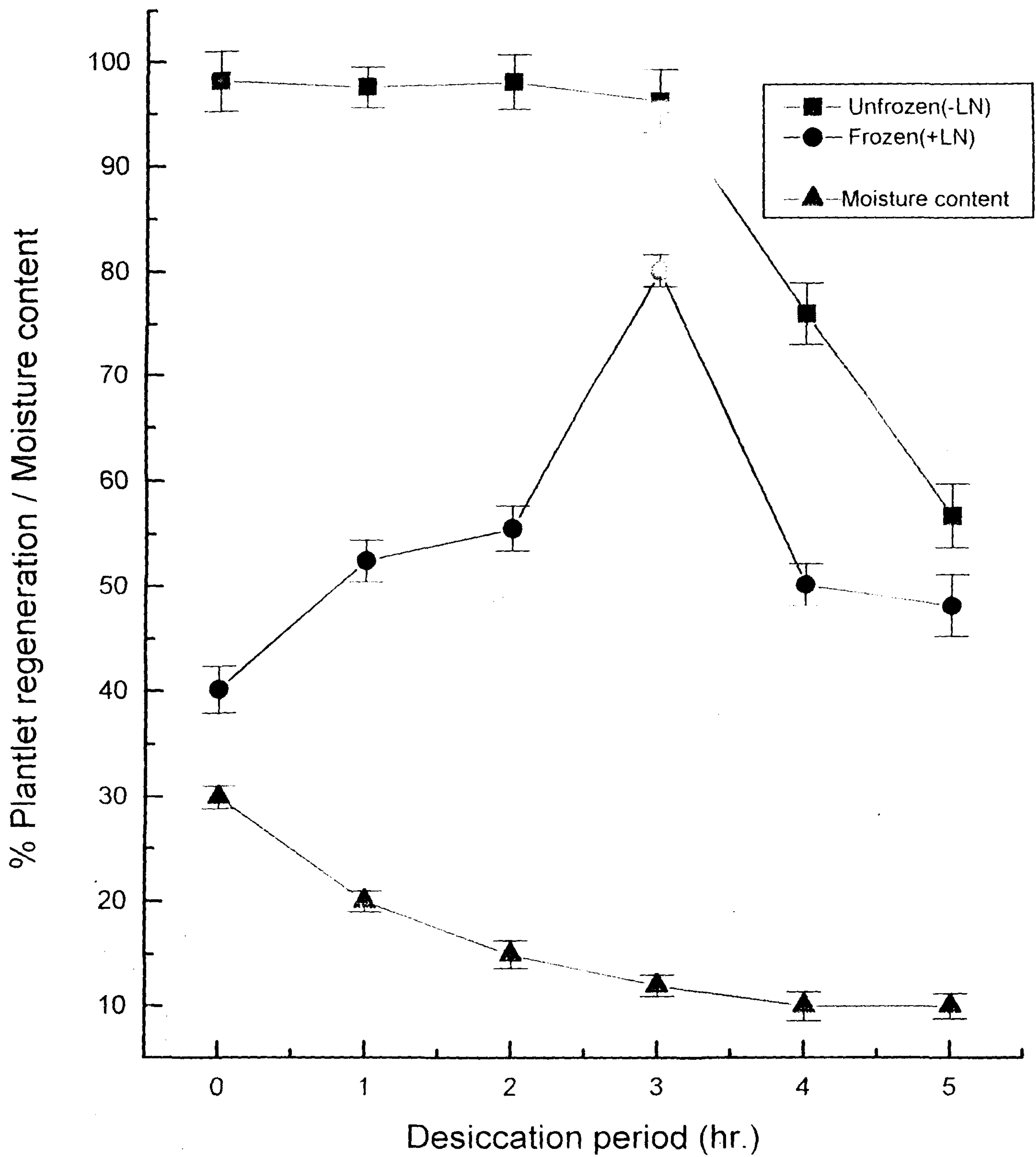


Fig. 8

Fig 9. Effect of age of culture in the medium on per cent plantlet regeneration from embryos of *Pinus kesiya* using rapid and slow freezing methods. $\pm$ S.D.

For rapid freezing, after 3 hr. desiccation the embryos were plunged in liquid nitrogen. In slow freezing method embryos (without desiccation) were cooled (rate 0.5° C min^{-1}) to -30°C before liquid nitrogen plunge.

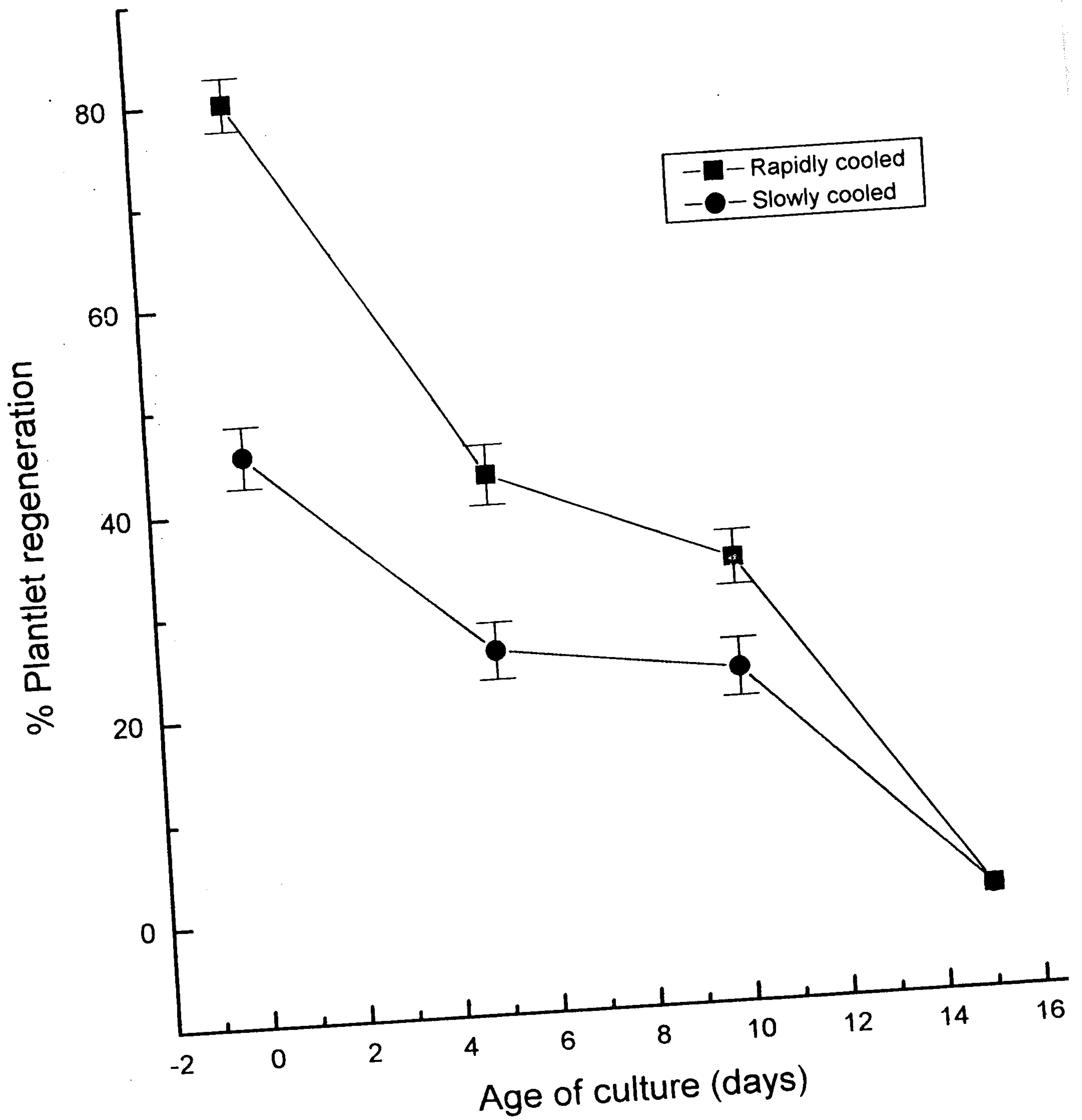


Fig. 9

Effect of cold treatment

The embryos precultured at 4°C for 5 days and desiccated for 3 hr. before rapid plunge in liquid nitrogen resulted in the maximum (76.5%) plantlet regeneration after cryopreservation followed by 8°C and 0°C treatments (Fig. 10). The embryos which were not desiccated before plunging in liquid nitrogen could not show survivablity at 5 ,10 and 15 days of culture at different temperatures. However, at 0 day there was a response of 32.4%. The treated controls showed a decline in per cent plantlet regeneration with increase in period of treatments.

Effect of DMSO concentration and period of exposure

Embryos treated with 5% DMSO for 1 hr. could result in 78% plantlet regeneration after their cryopreservation by slow freezing method (Fig. 11). A slight inecrease in per cent plantlet regeneration was observed in case of embryos treated for 3 hr. and 5 hr. with the same concentration of DMSO. As the concentration of DMSO and the period of exposure to it was increased, the per cent plantlet regeneration decreased. This was also true for treated controls. The sample slowly frozen without any cryoprotectant did not survive cryopreservation.

Effect of preculture

The embryos precultured in medium with different osmotic agents in case of treated controls exhibited better plantlet regeneration using upto 10% concentration of the osmotic agents except in case of mannitol where it was 5%. With increase in the

Fig 10.Effect of different cold treatments on per cent plantlet regeneration from embryos of *Pinus kesiya* before and after exposure to liquid nitrogen. $\pm$ S.D.

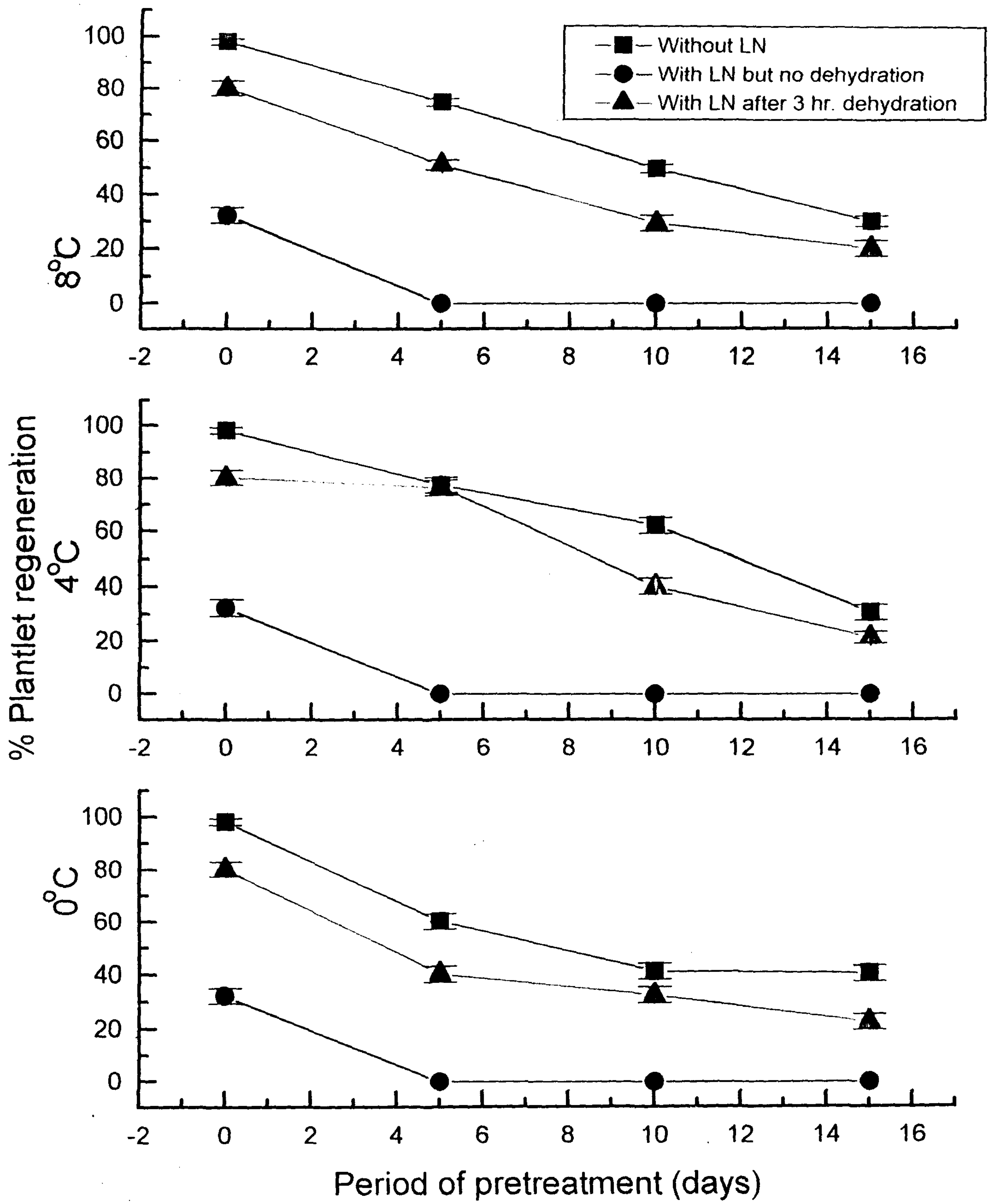


Fig. 10

Fig11. Effect of DMSO concentration and period of incubation on per cent plantlet regeneration from embryos of *Pinus kesiya* after exposure to liquid nitrogen.

± S.D.

Embryos were treated with DMSO and frozen slowly at the rate of 0.5 °C min^{-1} to -30°C before plunging in liquid nitrogen.

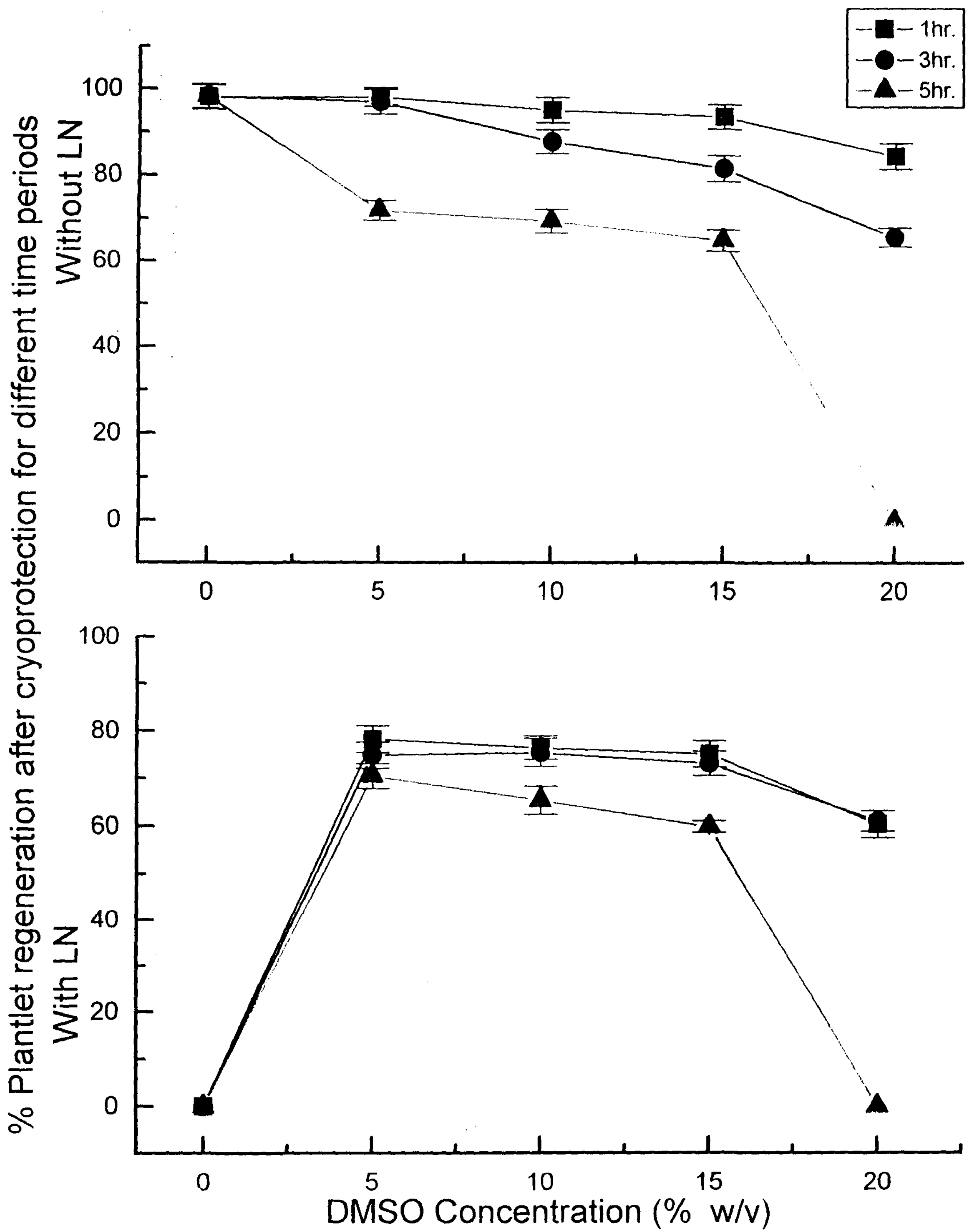


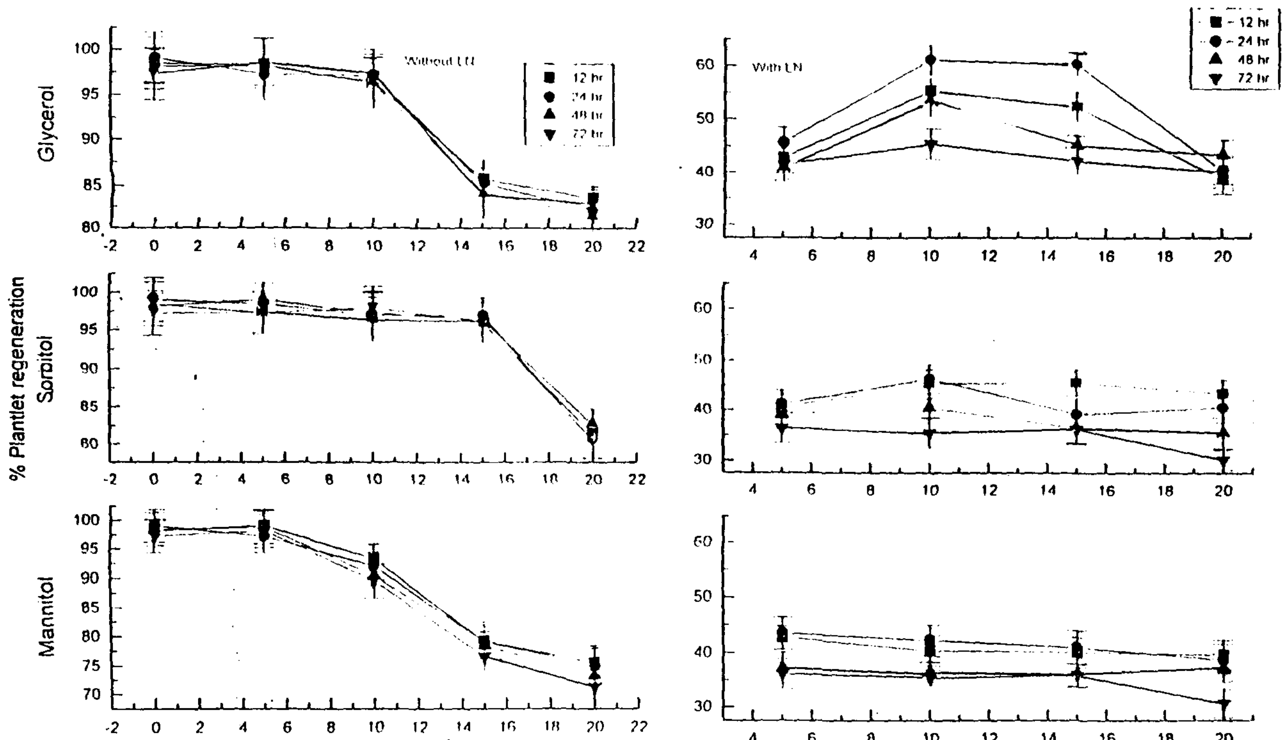
Fig. 11

concentration of osmotic agents and duration of preculture a decline in per cent plantlet regeneration was recorded (Fig. 12, without LN). The embryos precultured in 10% glycerol for 24 hr. exhibited 61.3% survival which was more or less the same in case of preculture in medium containing 15% glycerol (Fig. 12, with LN). Higher concentration of 20% in the medium was found to be inhibitory. A similar pattern was also observed in embryos precultured in glycerol at different periods of time. The application of mannitol and sorbitol at different concentrations in the medium and different periods of preculture did not show much of variation in terms of per cent plantlet regeneration after cryopreservation, except for the use of sorbitol at 10% for 12 hr. and 24 hr. of preculture of embryos. In general, there was a decline in plantlet regeneration with increase in sorbitol and mannitol concentrations in the medium and duration of preculture.

Effect of prefreezing temperature

The embryos precultured in medium containing 10% glycerol and 5% DMSO for 24hr. and frozen at -30^{0}C using concise method exhibited optimum plantlet regeneration of 80.3% (Fig. 13). Embryos precultured on 15% glycerol and 5% DMSO and frozen at -30^0 C showed 79.5% plantlet regeneration. With 5% each of glycerol and DMSO in the preculture medium, the per cent response was comparatively lower. At -80^{0}C freezer, all the embryos precultured in different treatments did not survive both with or without liquid nitrogen exposure.

Fig12. Effect of different osmotic agents and preculture time on per cent plantlet regeneration of unfrozen and cryopreserved embryos of *Pinus kesiya*. ± S.D.



Osmotic agents (% w/v)

Fig. 12

Fig13. Effect of different prefreezing temperatures on per cent plantlet regeneration of cryopreserved embryos of *Pinus kesiya*.

± S.D.

Embryos were precultured in the medium with combinations of glycerol and DMSO for 24 hr. and treated with 5 % DMSO for 1 hr. and then kept for 1 hr. in freezers held at different temperatures for before plunging in liquid nitrogen.

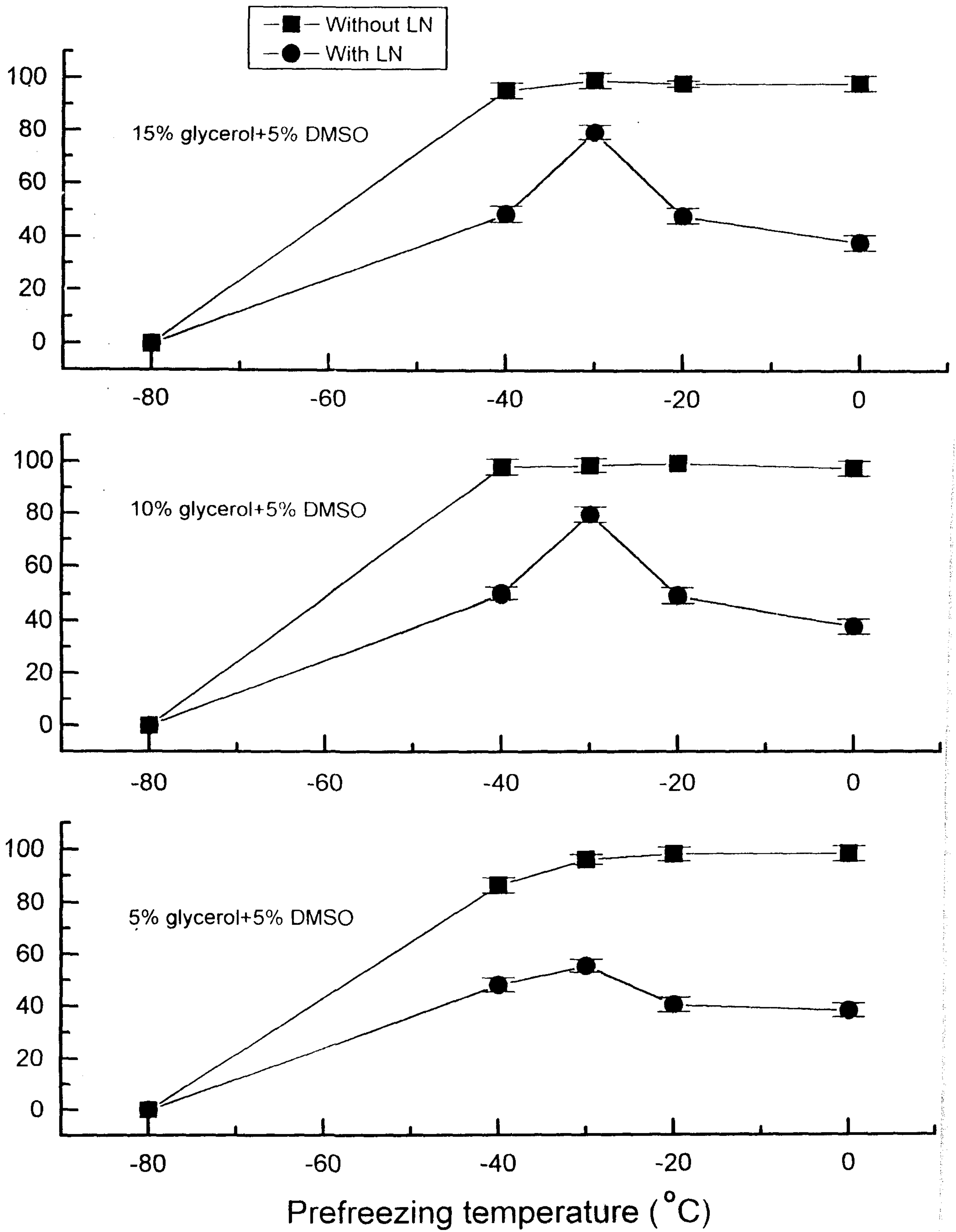


Fig. 13

Effect of different hydration and dehydration period

The per cent plantlet regeneration declined sharply with increasing hydration period followed by 3 hr. desiccation of the embryos before cryopreservation (Fig. 14). The hydration period for 4 hr. could result in more than 50% plantlet regeneration compared to above 80% in case of embryos cryopreserved without hydration. The controls without liquid nitrogen but with 3 hr. desiccation after hydration showed slight decrease in per cent plantlet regeneration as the period of hydration was increased.

Effect of thawing temperature

A thawing temperature of 30^0C resulted in optimum plantlet regeneration (Fig. 15). Lower and higher temperatures than this resulted in decline of plantlet regeneration of cryopreserved embryos. The temperature of 45 and 50^0C for thawing were completely inhibitory.

Effect of long term storage in liquid nitrogen

A slight decline in per cent plantlet regeneration was observed after 24 hr storage. In general, regeneration of plantlets from cryopreserved embryos upto 180 days remained steady (Fig. 16).

Fig 14. Effect of different hydration periods on per cent plantlet regeneration of embryos of *Pinus kesiya* after rapid freezing.

± S.D.

Embryos were hydrated and then dehydrated by sterile air flow for 3hr. before plunging in liquid nitrogen.

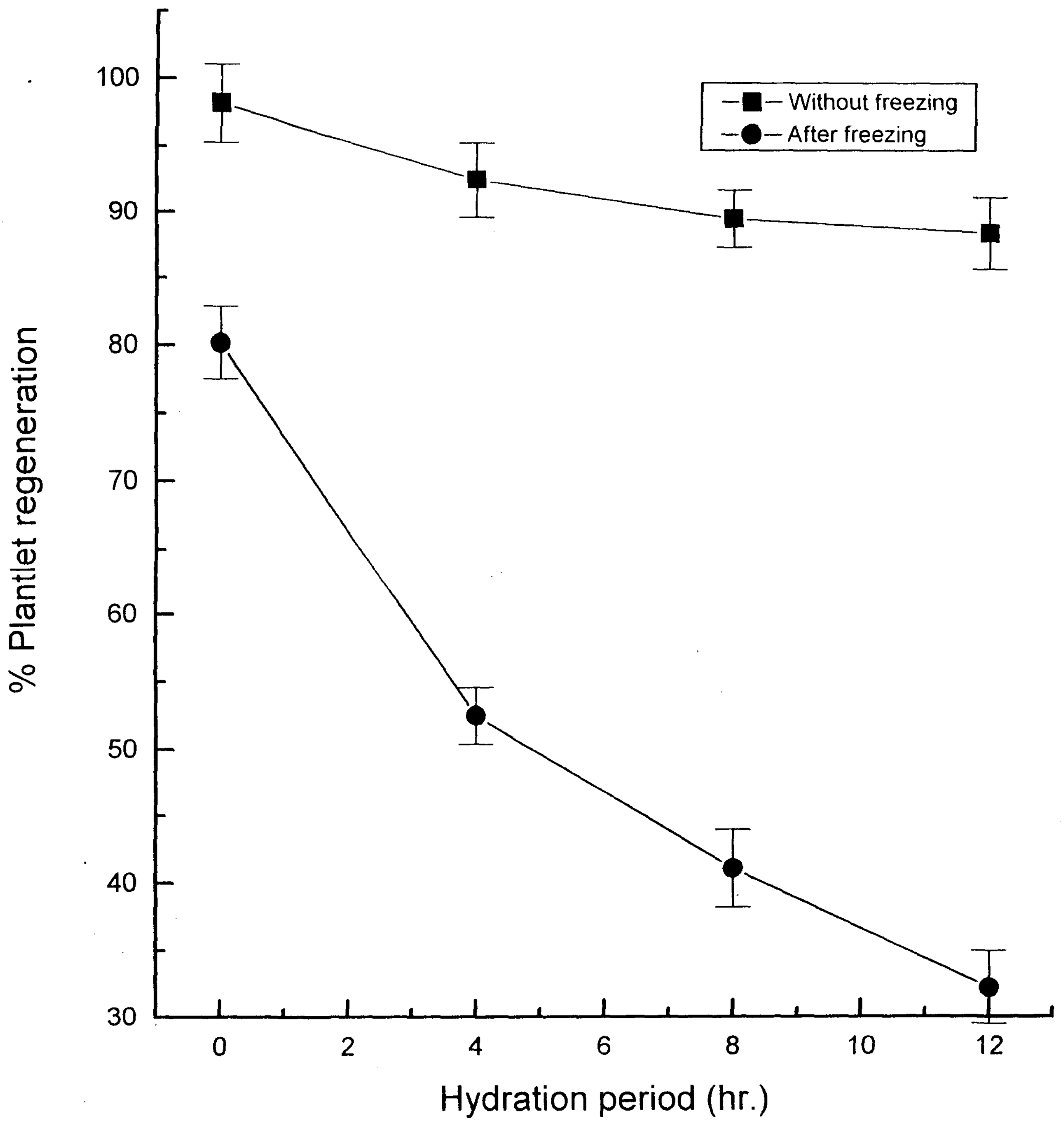


Fig. 14

Fig15.Effect of different thawing temperatures on per cent plantlet regeneration from cryopreserved embryos of *Pinus kesiya*.

± S.D.

Embryos were desiccated for 3 hr., rapidly plunged in liquid nitrogen and then stored in LN for 1 hr. before thawing.

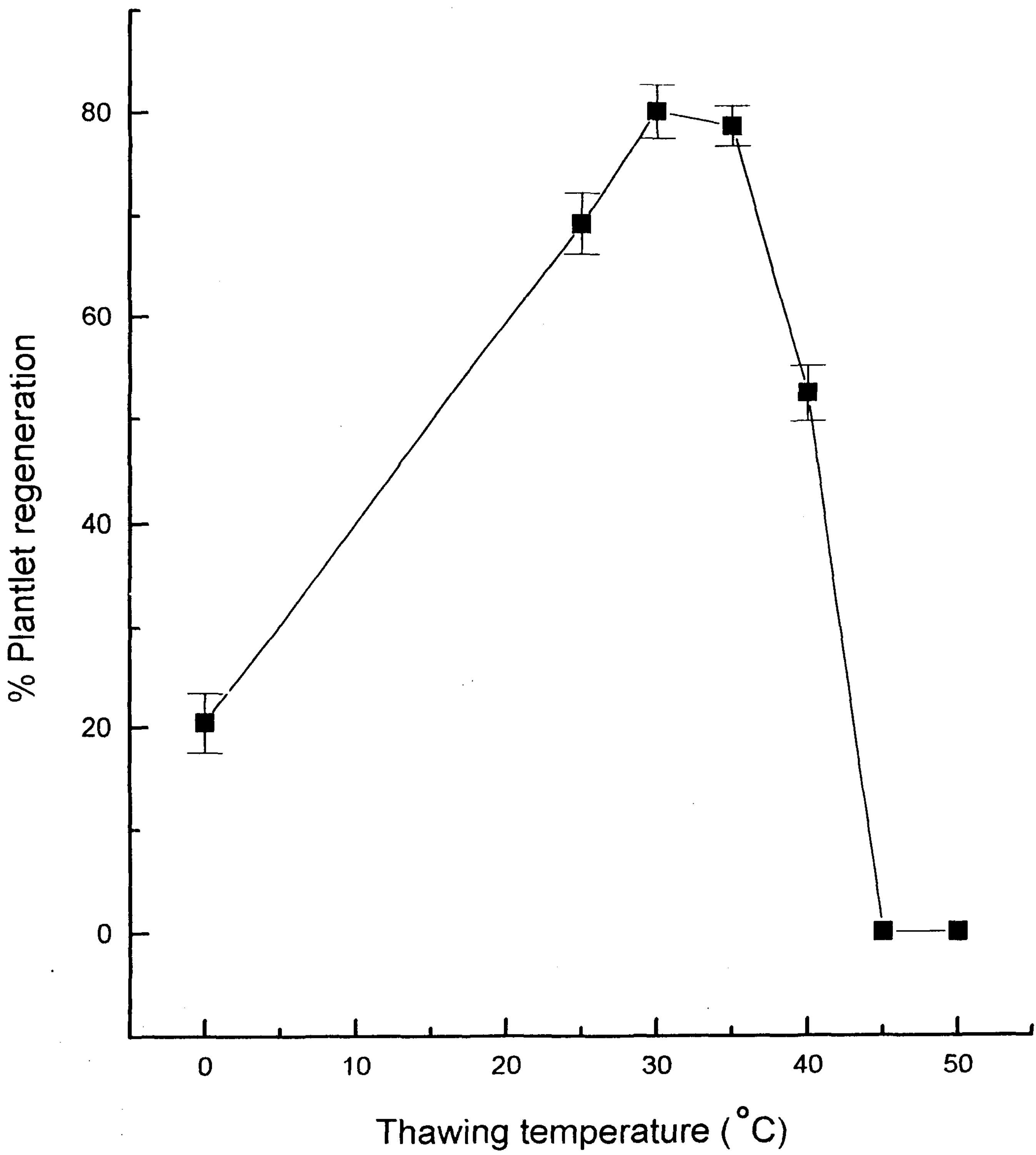


Fig. 15

Fig 16.Effect of different durations of storage in liquid nitrogen on per cent plantlet regeneration from embryos of *Pinus kesiya*.

± S.D.

After 3 hr. desiccation the embryos were rapidly plunged in liquid nitrogen and stored for various period.

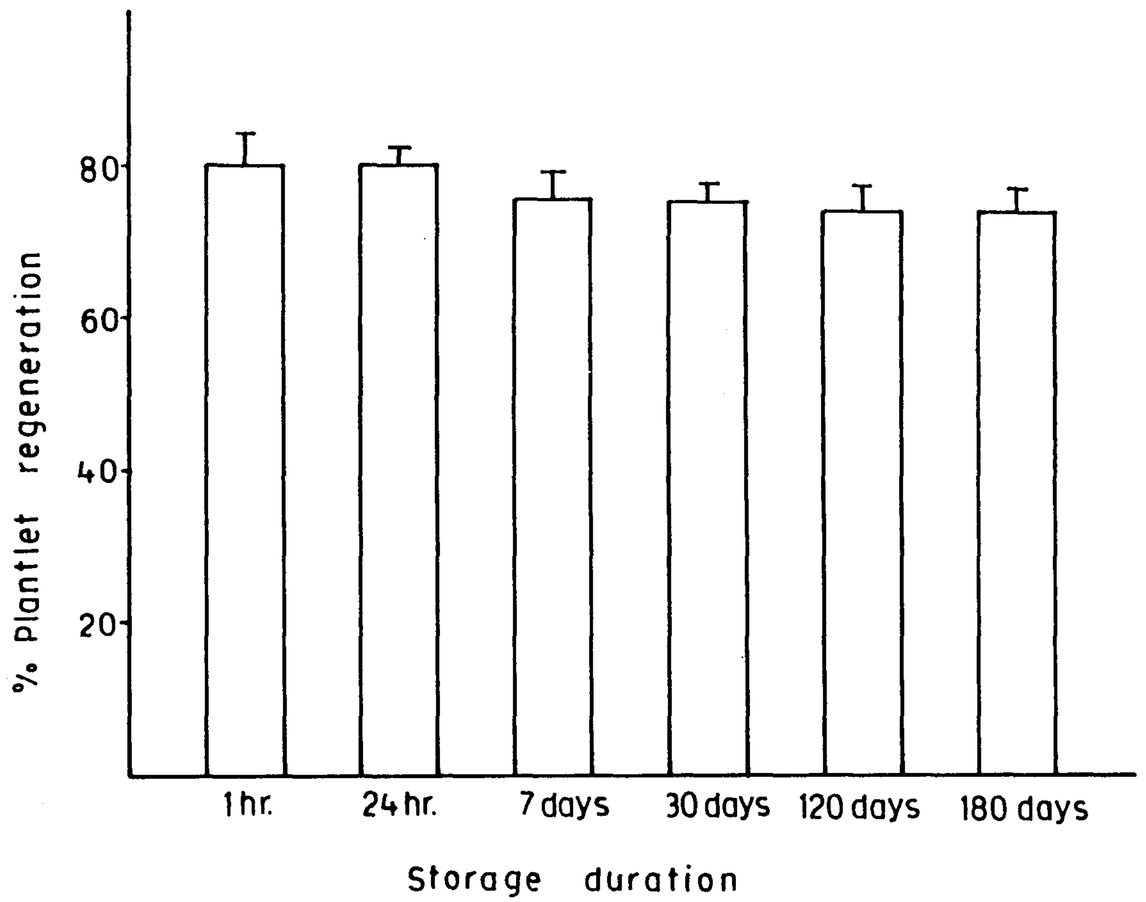


Fig. 16

Vitrification of shoot apices and embryos

Effect of exposure time

The best exposure period of shoot apices and embryos to PVS2 was found to be 2 mins. resulting in maximum of 42.3% and 56.3% survivability, respectively. (Figs. 17 and 18). As the exposure time was increased the regrowth declined in both treated control and vitrified samples. Beyond 6 min. exposure the solution seemed to be toxic to the explant.

Effect of volume of vitrification solution

Using 0.3 ml of vitrification solution a maximum of 42% shoot apices and 55.5% embryos survived after vitrification (Fig. 19). A volume of 0.5 ml of vitrification solution resulted in decline in survivability and regrowth. However, no survival was recorded using other volumes of vitrification solution.

WASHING

Effect of sucrose solution

The maximum survivability and regrowth of shoot apices and embryos resulted in samples washed with 1.2 M sucrose after vitrification either added dropwise or directly (Figs. 20 and 21). Using other concentrations of sucrose, the drop wise dilution was found better compared to direct addition in case of both shoot apices and embryos.

Fig 17. Effect of different exposure time to vitrification solution on the per cent regrowth of shoot apices of *Pinus kesiya* after cryopreservation.

± S.D.

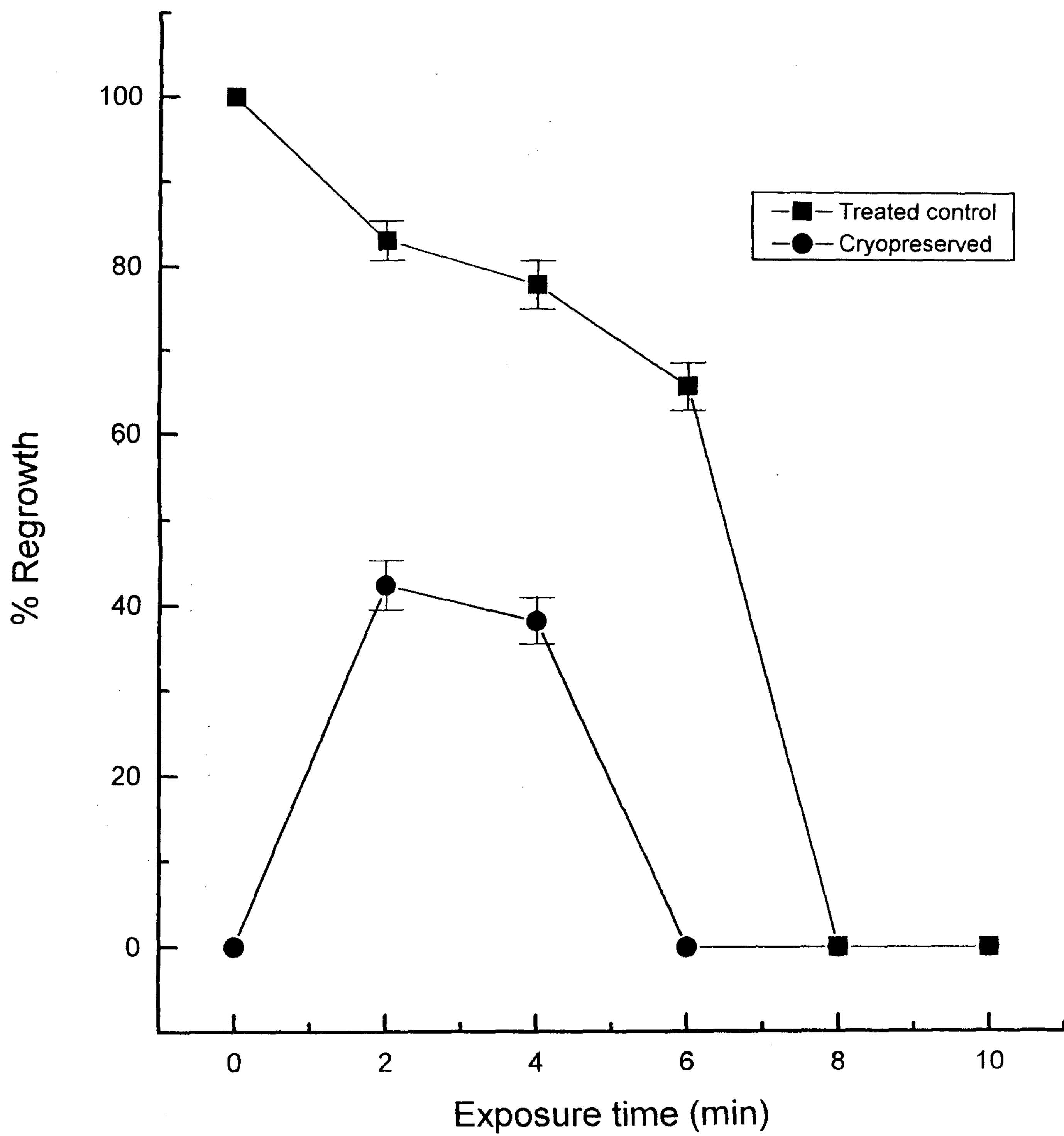


Fig. 17

Fig 18. Effect of exposure time to vitrification solution on per cent plantlet regeneration from embryos of *Pinus kesiya* after cryopreservation. ± S.D.

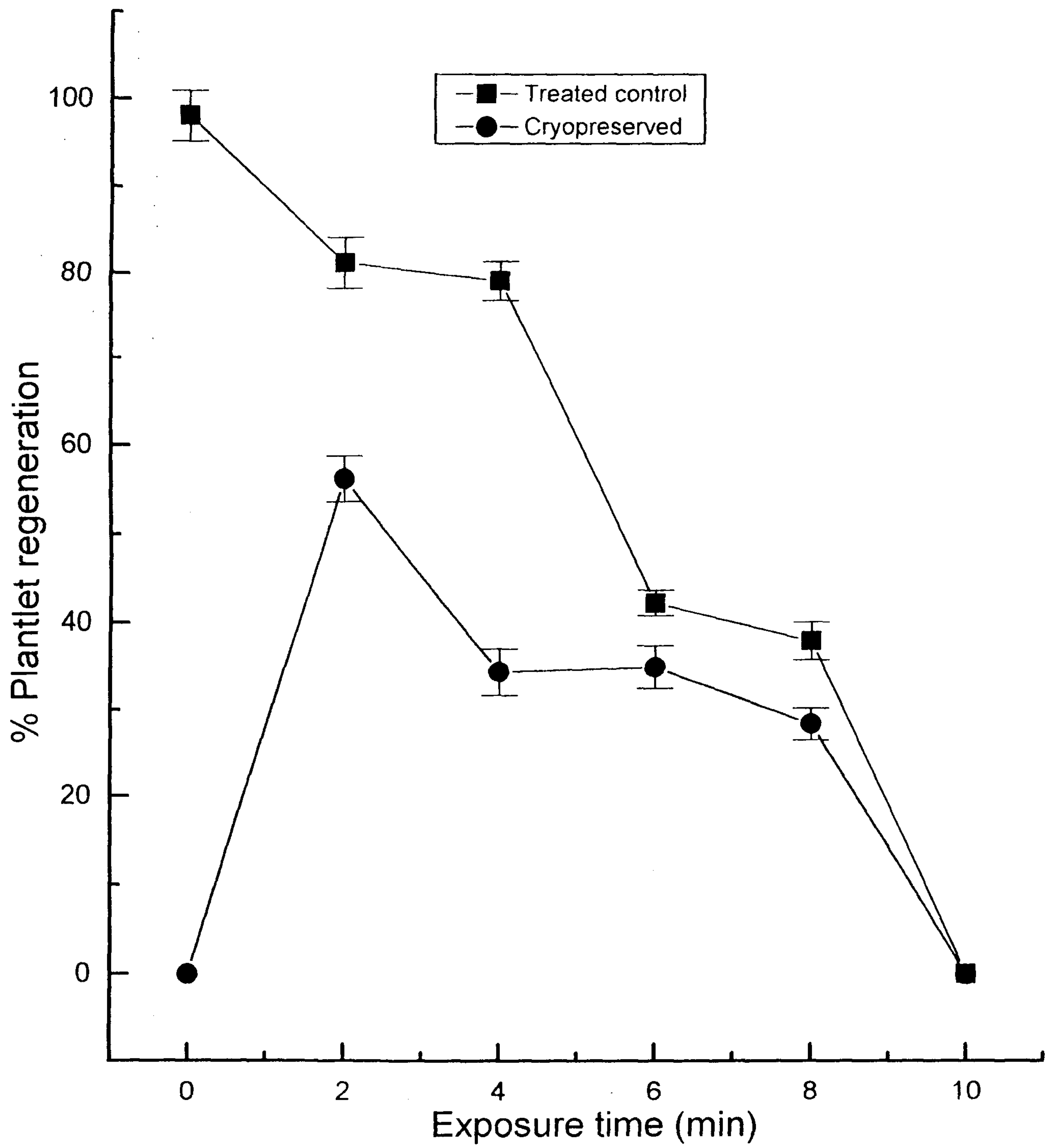


Fig. 18

Fig19. Effect of volume of vitrification solution on per cent growth of cryopreserved shoot apices and embryos of *Pinus kesiya.*

$\pm$ S.D.

The explants were exposed to vitrification solution for 2 mins. before plunging in liquid nitrogen.

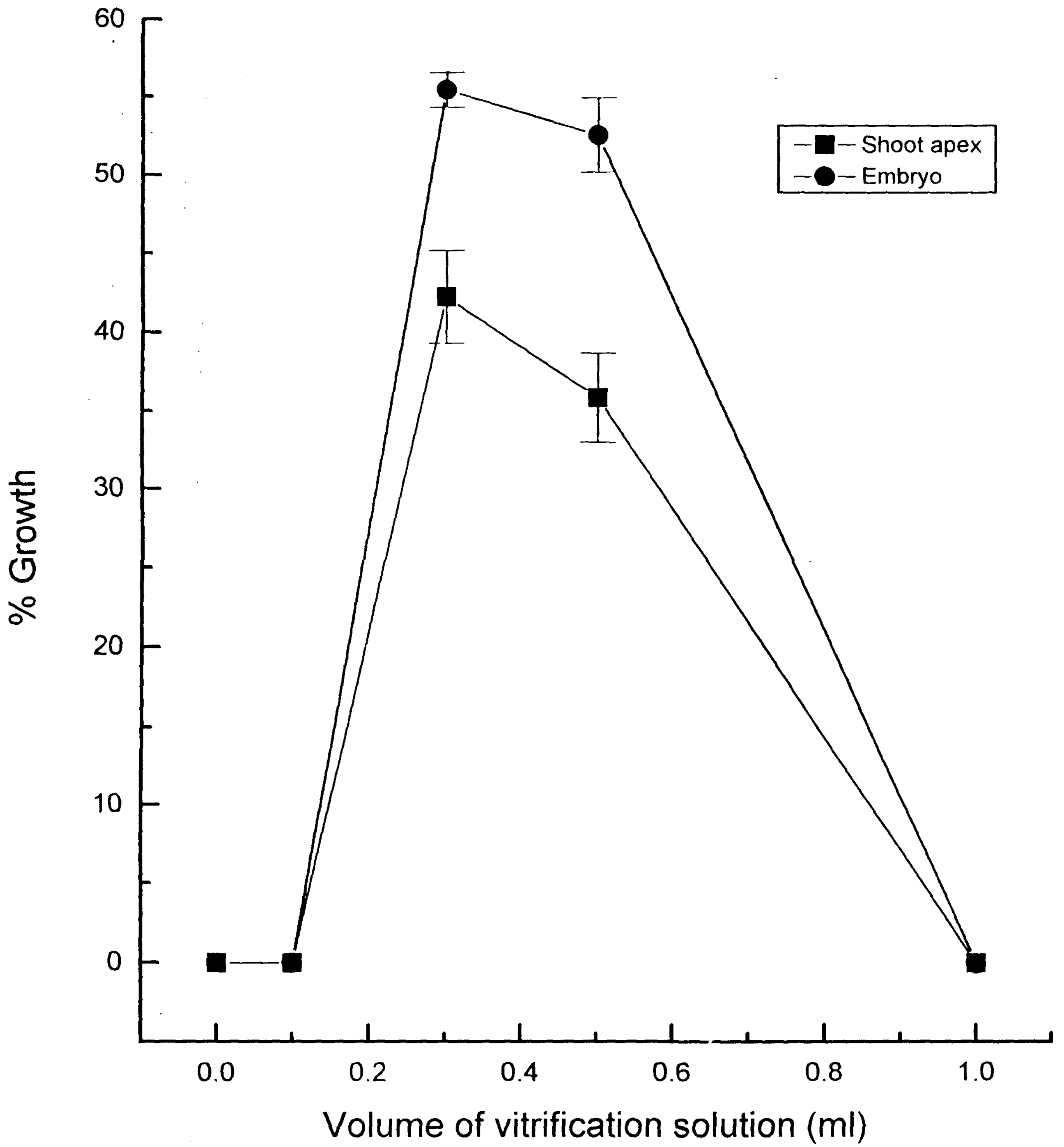


Fig. 19

Fig 20. Effect of osmolarity of sucrose solution in the diluent, on per cent regrowth from vitrified shoot apices of *Pinus kesiya* after thawing. ± S.D.

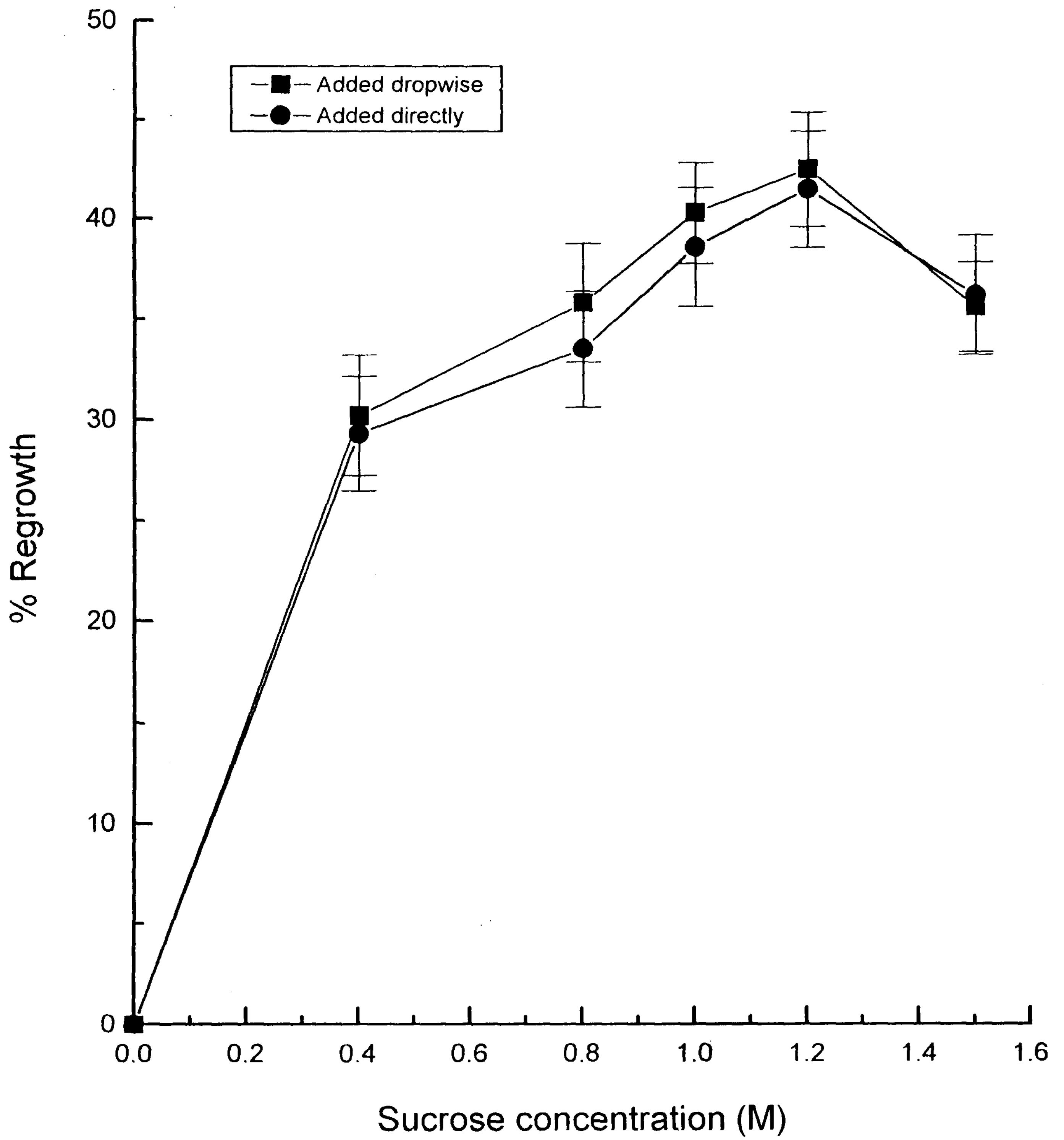


Fig. 20

Fig 21. Effect of osmolarity of sucrose solution in the diluent, on per cent plantlet regeneration from vitrified embryos of *Pinus kesiya* after thawing. ± S.D.

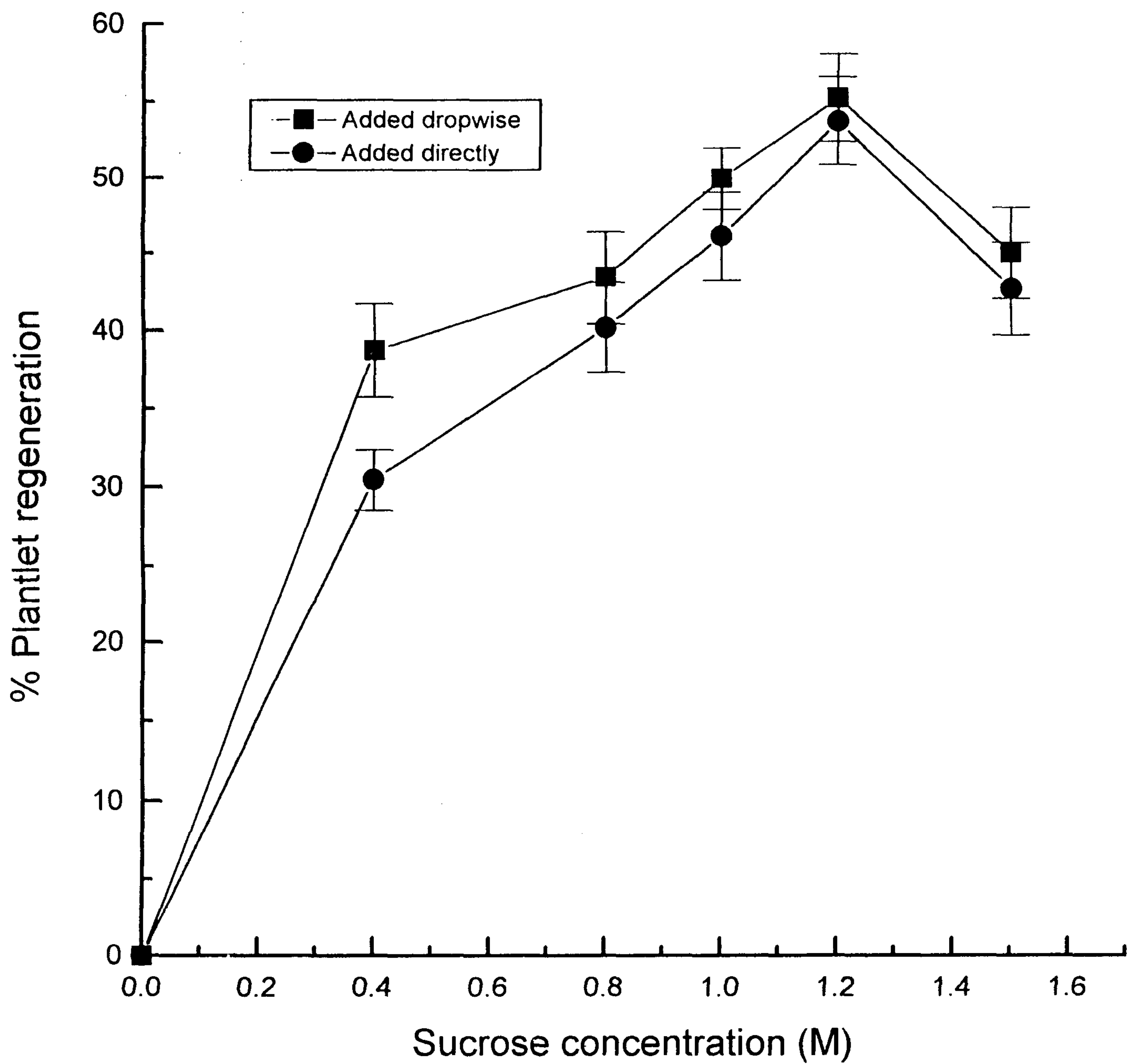


Fig. 21

Fig 22 .Effect of washing of vitrified samples with nutrient medium after thawing and washing with 1.2 M sucrose

± S.D.

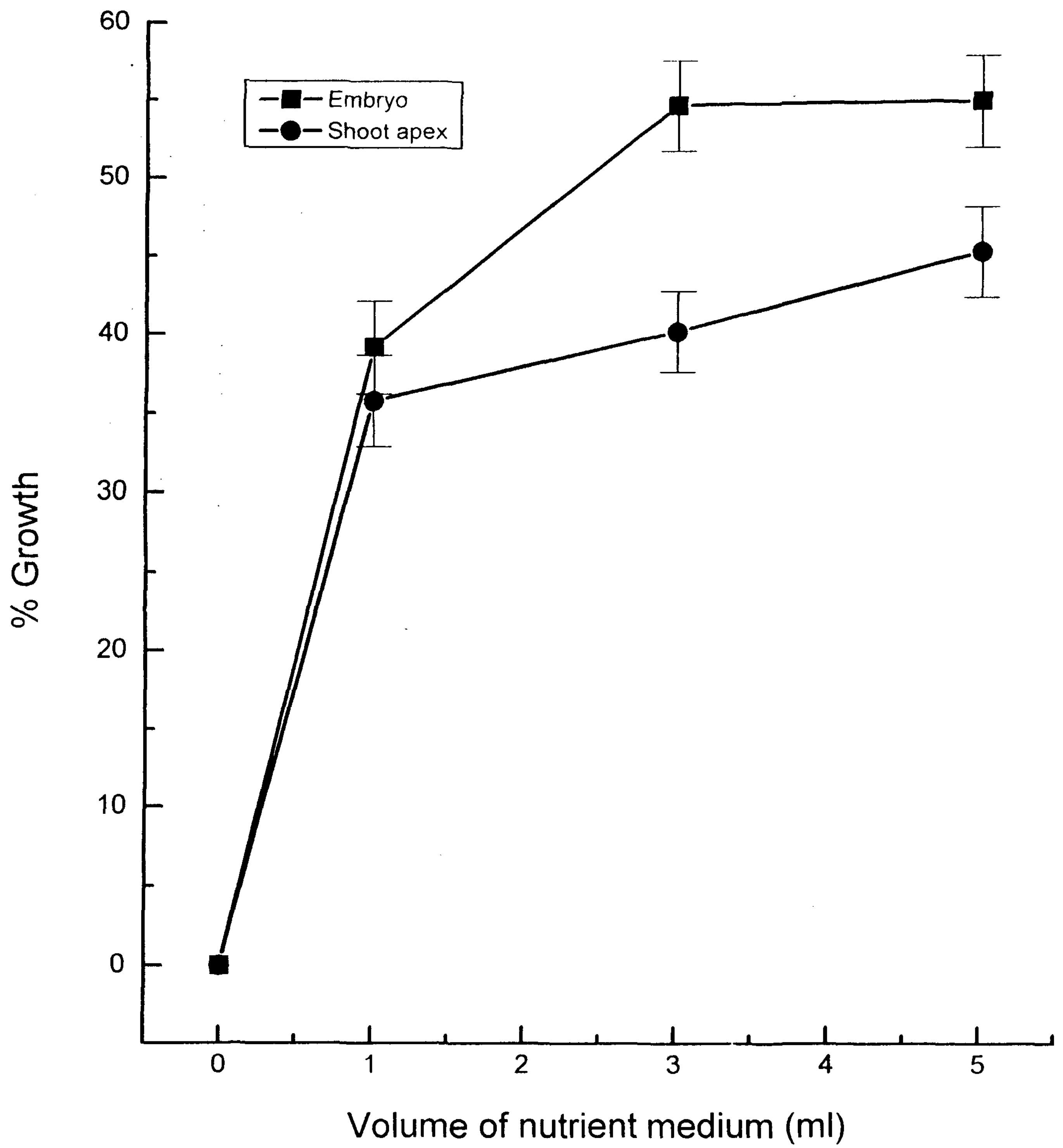


Fig. 22

Fig 23.Effect of different cryovials on per cent regrowth of vitrified shoot apices and embryos of *Pinus kesiya*.

± S.D.

0.3 ml of PVS2 solution was used for vitrification of shoot apices and embryos in different cryotubes before plunging in liquid nitrogen.

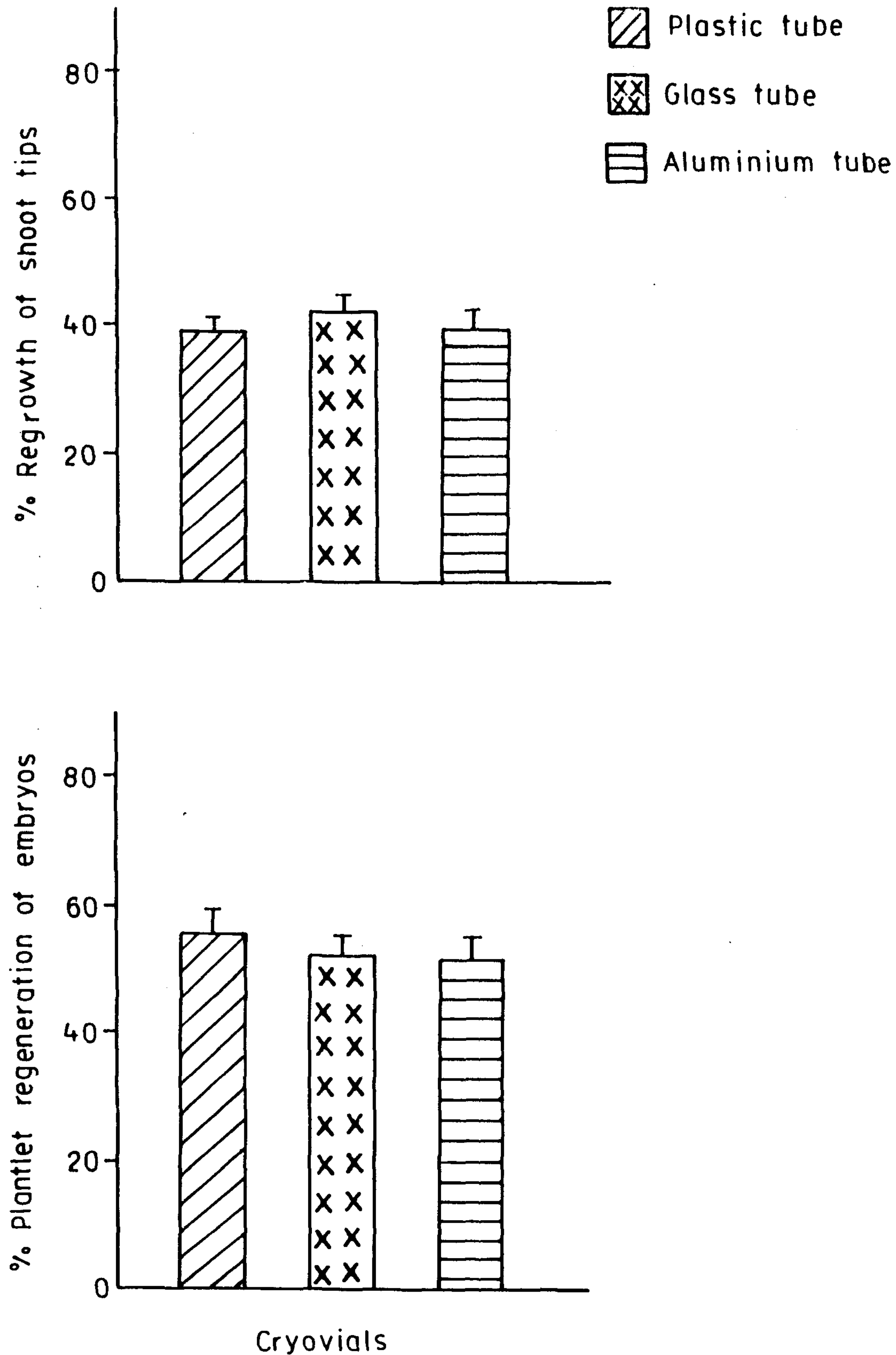


Fig. 23

Plate 5.(a-d). Different stages of shoot induction and multiplication from cryopreserved shoot apices of *P. kesiya*.

a. Shoot apex showing growth in MS + BAP (4.0 mgl^{-1}).

b & c. Formation of multiple shoot buds in MS (hormone free) medium.

d. Multiplication and elongation of shoots in half-strength MS medium with BAP (1.0 mgl^{-1}) + IBA (0.1 mgl^{-1}) + 0.3% activated charcoal.

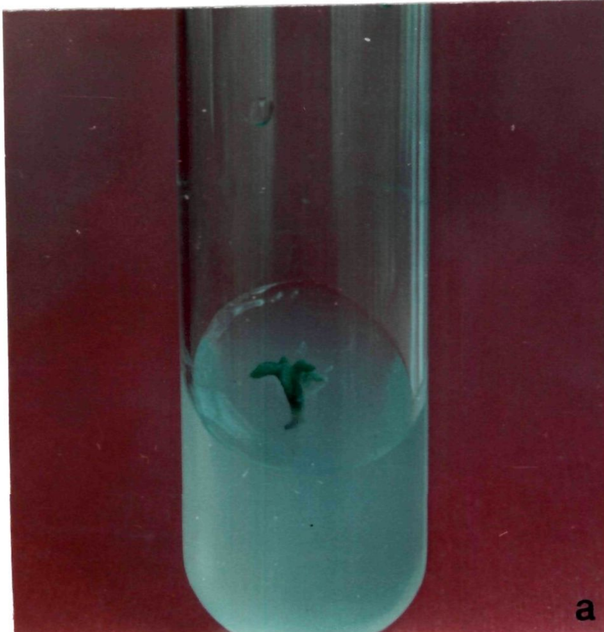
Plate 5. (a-d)

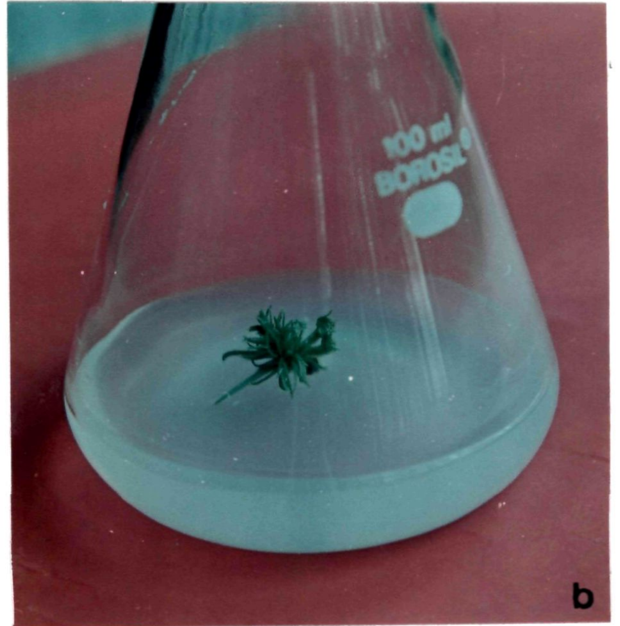
Plate 6.(a-d).Different stages of shoot bud induction and multiplication from cryopreserved embryos of *P. kesiya*.

a. Opening of cotyledons in LP + kinetin (5.0 mgl^{-1}) medium.

b & c. Development of shoot buds and their multiplication in hormone free LP medium with reduced sucrose (2%).

d. Isolated shoot showing elongation in half-strength LP medium with 0.3% activated charcoal.





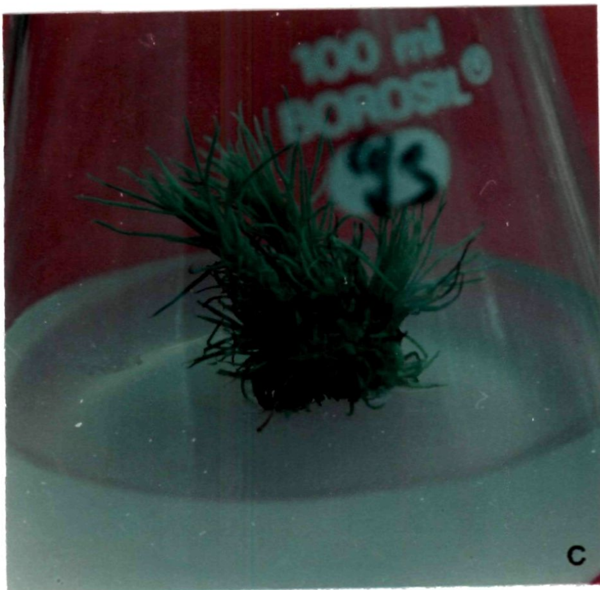


Plate 6. (a-d)

Plate 7. (a-b)

a. A rooted plantlet ready for potting.

b. A plantlet after hardening in a pot with soil from the pine forest.

Plate 7. (a-b)

Effect of washing with nurtient medium

With the increase of volume of nutrient medium added drop wise to the samples already washed with 1.2 M sucrose after vitrification, resulted in increase in survivability and growth. Using 5 ml of nutrient medium the maximum survival and growth of 55.2 and 45.5% was recorded in embryos and shoot apices, respectively (Fig. 22).

Effect of different cryotubes

The optimum survivability of vitrified shoot apices and embryos resulted using glass and plastic cryotubes, respectively (Fig. 23).

Regrowth

The different stages of development of cryopreserved shoot apices and embryos are presented in Plate- 5, 6 and 7.

DISCUSSION

In the present study on cryopreservation of *P. kesiya,* an analysis has been made on some of the critical components which have a direct bearing on the development of a successful cryopreservation protocol. This includes the type of cryoprotectant used and the probable toxitcity they exert on the tissues, optimal cooling rates which ensures maximum viability, temperature at which control freezing

is arrested (terminal freezing temperature) prior to plunge in liquid nitrogen, the pregrowth condition and the period of growth, strategies for inducing regrowth from cryopreserved tissues etc. The critical factors which determine the survival of tissues in freeze preservation are the cryoprotectant, the prefreezing techniques and the cooling velocity (Kartha *et. al.*, 1979). The commonly used cryopreservation methods necessitate either preconditiong with the cryoprotectant or preconditiong of the tissues at a low temperature. Cryopreservation of tissues with a higher water content is more difficult and the tissues have to be protected against ice crystals that can destroy the cells. Shoot apices of *P. kesiya* used in the present study has a moisture content of 78± 2% and embryos used have total moisture content of 30±2%. For cryopreservation shoot apices are generally excised from green houses grown plants, such as potato (Towill, 1981; 1984), asparagus (Kumu *et al.*, 1983), brussels sprouts (Harada *et al.*, 1985) etc. *In vitro* grown shoot apices have also been cryopreserved in apple (Kuo and Lineberger, 1985), carnation (Dereuddre *et al.*, 1988) etc. In the present study, *in vitro* grown shoot apices of *P. kesiya* were used for cryopresérvation. It is commonly admitted that *in vitro* cultured plantlets are a preferable source of material for germplasm preservation purposes because they are cultured under disease- free conditions and their growth can be easily controlled.

Shoot apices of *P. kesiya* treated with 5% DMSO for 2hr. resulted in about 38% shoot regrowth after cryopreservation by slow freezing method. In case of embryos also a concentration of 5% DMSO was found to be optimum. The DMSO level used in the present study did not seem to be toxic. 5 to 15% DMSO solution was earlier usec

for shoot tips of asparagus (Kumu *et al.*, 1983), brussels sprouts (Harada *et al.*,1985), carnation (Dereuddre *et al.*, 1988) and pear (Moriguchi *et al.*, 1985). In pea meristem, 1 hr. pretreatment with 5% DMSO before slow cooling to -40°C resulted in 60% viability (Kartha *et al.*,1979). Shoot apices of *P. kesiya* treated with different cryoprotectants (5 -20%) for different periods did not result in any survivablity when cryopreserved by rapid freezing method (Table 19). This may be due to intracellular freezing in the cells that brings about mechanical destruction to the biomembranes. The per cent regrowth of cryopreserved shoot apices on fresh medium could be increased to more than 48.5% when they were precultured before treating with DMSO. Use of sucrose and glycerol singly at varying concentration was found beneficial for shoot apices in the present study. Similar observation was recorded earlier by Dereuddre *et al.* (1988) where high concentration of sucrose in pretreatment was essential for successful cryopreservation of *in vitro* grown shoot tips of carnation. For the successful cryopreservation of axillary buds of asparagus, pretreatment on MS agar medium containing 0.7M sucrose for 2 days was effective (Uragami *et al.*, 1990). However, preculture of shoot primordia clumps of melon in standard medium for 3 days with low sucrose concentration increased survival after cryopreservation (Ogawa *et al.*, 1997). In the present study preculture in high osmoticum increased per cent survival of shoot apices when frozen slowly (Table 20). Preculture could not bring any success in cryopreservation of shoot apices using rapid freezing method. Use of glycerol in preculture medium was found to be beneficial for embryos of *P. kesiya* also (Fig. 12). During slow cooling the shoot apices were cooled upto a temperature of -

30°C with different cooling rates. A rate of 0.5°C min^{-1} showed the best result (Fig. 1). In case of melon somatic embryos it was reported that the best cooling rate with optimum survival was 0.3°C min^{-1} (Niwata *et al.*, 1991). Kartha *et al.* (1988) also reported optimum cooling rate of 0.3°C min^{-1} cryopreservation of cell culture of *Picea glauca*. But Sakai *et al.* (1991) and Nishizawa *et al.* (1992) found 0.5°C min^{-1} cooling rate optimum for frozen nucellar cells of navel or orange and asparagus. A combination of different cryoprotectants viz., sucrose, DMSO, and glycerol was found to be better for *P. kesiya* shoot apices than cryoprotection with DMSO alone. The precultured shoot apices of *P. kesiya* (in medium containing 10% sucrose for 14 days) were treated with a combination of sucrose, DMSO, glycerol (10-10-5% w/v) for 1hr. before cooling at -30°C for 1 hr., resulted in 45.3% regrowth. Low molecular weight compounds like DMSO and glycerol are thought to enter the cell and exert a colligative action with the cell preventing an excessive concentration of toxic electrolytes. Higher molecular weight compound like surcose do not penetrate the cell but may exert an osmotic action and consequently avoid intra cellular ice formation. A mixture of cryoprotectant was also found to be less damaging on cooling and rewarming than a single compound (Finkle *et al.*, 1985). Bajaj (1977) reported that a mixture of cryoprotectant containing DMSO, sucrose and glycerol resulted in higher viability after cryopreservation in embryos of *Atropa* and *Nicotiana* than when used singly. Glycerol has been often used in cryopreservation process in cultured cells (Nag and Street, 1973, 1975a). About 25% of cassava meristems survived after freezing to the temperature of liquid nitrogen in the presence of glycerol (10%) and sucrose (5%)

(Bajaj, 1977). The use of mixture of sucrose, DMSO and glycerol as cryoprotectant in *P. kesiya* shoot apices is consistent with the earlier reports of Ishikawa *et al.* (1991 and 1996) on cell cultures of broom grass and Ogawa *et al.* (1997) on melon shoot primordia. In case of embryos of *P. kesiya*, cryoprotection with glycerol (10%) and DMSO (5%) resulted in a maximum of 80.3% regrowth which is consistent with earlier reports on the use of glycerol and DMSO. Observations during experimentation on *P. kesiya* led to a simpler method of cryopreservation where the precultured shoot apices and embryos after treating with cryoprotectant mixture at room temperature can be kept in freezers at different temperatures(-20, -30,-40 and-80°C) for 1hr. and then plunged in liquid nitrogen.

Out of different prefreezing temperatures used, -30°C was optimum for cryopreservation of shoot apices and embryos of *P. kesiya*. This method is more convinient and less time taking than the conventional slow cooling method and also less costly. There are reports of pre freezing of shoots at -30°C before cryopreservation (Suzuki *et al.*, 1988; Oka *et al.*, 1991). The per cent regrowth of shoot apices of *P. kesiya* was increased to almost 52% when they were cryopreserved slowly after treating with cryoprotectant mixture comprising sucrose, DMSO and glycerol. This increase may be due to less freezing damage during slow freezing. In the present study, the small sized shoot apices (1-2 mm) gave better results than bigger sized shoot apices. This finding is consistent with the result obtained by Ogawa *et al.* (1997) on shoot primordia of melon. A cold treatment of shoot apices in conjuction with osmotic treatment increased the per cent regrowth of cryopreserved shoot apices of *P.*

kesiya. Optimum response was observed with shoot apices cultured at 4°C for 10 days before freezing (Fig. 4). Dereuddre *et al.* (1988) reported the use of cold treatment for several days in carnation to obtain high regrowth rate after freezing in liquid nitrogen. *Chamomilla recutita* shoots when precultured for 8 weeks at 4°C resulted upto 50% shoot survivability (Diettrich *et al.*, 1990). During the course of study on cryopreservation of *P. kesiya* the effect of terminal temperature prior to immersion to liquid nitrogen was also examined. The shoot apices frozen to a terminal temperature of -30°C showed the higher per cent regrowth of about 51%. This is consistent with several reports (Sakai *et al.*, 1991).

Cryopreservation of zygotic embryos were comparatively easier than shoot apices of *P. kesiya*. In general water present in the embryos shows different characteristics depending on whether it is above or below the so-called critical water content, which usually resides around 20% on fresh weight basis varying from 15 to 20% depending on species . The water content below this is bound water and is not freezable and the water content above this critical value is the free water which is freezable at low temperature. Embryos of *P. kesiya* have water content of about 30% which is slightly above the critical water content. Therefore, a simple desiccation with air flow in a laminar flow cabinet was found to be suitable to bring down the water level. Using this process, within 3 hr. continous air flow the embryos possessed 12% of moisture content and could be preserved successfully where almost 80% regeneration could be observed. Chin *et al.* (1988) reported successfull cryopreservation of excised embryos of *Veitchia* and *Howea* palm seeds after desiccation by air flow in laminar

flow cabinet, where after reduction of water level to 10% , 63 -64% recovery was observed. A similar sutdy was also carried out by Grout *et al.* (1983) on excised embryos of oil palm (*Elaeis guineensis*) . The age of culture of *P. kesiya* embryos showed difference in per cent regeneration after cryopreservation both by rapid and slow freezing methods (Fig.9). Embryos cryopreserved without culture in medium showed the optimum plantlet regeneration . The decrease in per cent regeneration with increase in days of culture is due to accumulation of more water by the embryos from the surrounding medium. In the present study, pregrowth of embryos at cold temperatures resulted in a lower survival rate than those cryopreserved by simple desiccation. This suggests that cold temperature treatment for embryos is not required which may be due to natural cold hardiness in *P. kesiya*. Optimum per cent regeneration of about 76% was observed when the embryos were precultured at 4°C for 5 days and then exposed to 3hr. desiccation. The use of only cold treatment was not suitable as those embryos which were not subjected to air flow dehydration could not regenerate after cryopreservation (Fig. 10) by rapid freezing method. It can be suggested that the excess water which was perhaps imbibed by the embryos during the period of preculture had to be removed by dehydration to achieve success. The effect of hydration period of embryos of *P. kesiya* was similar to embryos cultured in medium for 15 days. The decrease in per cent regeneration of embryos with increase in period of hydration, prior to cryopreservation was due to accumulation of water by the embryos which is detrimental to successful cryopreservation. The thawing temperature and the rate of thawing plays an important role in the performance of

cryopreserved tissues on regrowth medium. The specimens are rewarmed rapidly in water bath of different temperatures. In the present study a thawing temperature of 40°C was found to be most suitable for shoot apices which is consistent with the report of Diettrich *et al.* (1990) in shoot tips of *Chamomilla recutita*. The thawing temperature suitable for zygotic embryos of *P. kesiya* was 30°C whereas in embryos of *Veitchia* and *Howea*, a thawing temperature of 40°C was found suitable (Chin *et al.*, 1988). According to Sakai (1966), frozen specimens remain alive if warmed rapidly. Rapid warming of cryopreserved tissues is a well accepted fact except in some cases where warming at room temperature or slow warming is more beneficial. The cryopreserved shoot apices and embryos of *P. kesiya* survived a longer pierod of storage of 180 days in liquid nitrogen (Figs. 7 & 16). There was no significant decrease in survivablity of cryopreserved shoot apices and embryos of *P. kesiya* after long period of storage. Kartha *et al.* (1988) reported storage of cell suspension of *Picea glauca* for more than one year in liquid nitrogen.

Using the vitrification method, cells, tissues and organs are dehydrated by highly concentrated vitrification solution. The vitrification solution need to be a suitable solution that vitrifies during rapid freezing and which permeates the cells of the tissue to an extent that vitrification also occurs within the cells. Vitrification has been proposed to have benefits over conventional cooling strategies for tissues (Fahy, 1988). In the present study a vitrification solution termed as PVS2 (Sakai and Kobayashi 1990; Sakai *et al.*, 1990) comprising glycerol, DMSO, ethylene glycol with 0.4 M sucrose was used. PVS2 has been widely used for vitrification of nucellar cells of

navel orange, apical meristem of white clover (Yamada *et al.*, 1991) apple, pear and malberry (Niino and Sakai, 1992) etc. This solution was relatively toxic on increasing the exposure time that resulted in death of the tissues both in control and cryopreserved ones (Figs. 17 & 18). A 2 min. exposure to the vitrification solution was optimum for both embryos and shoot apices of *P. kesiya* which is consistent with the report of Sakai *et al.* (1991) on nucellar cells of navel orange. The volume of vitrification solution used also has a role in regrowth of vitrified cells (Fig. 19). In the present study, 0.3 ml of the solution found to be optimum for successful vitrification of embryos and shoot apices. 1.2 M sucrose solution used for washing of shoot apices and embryos was found suitable for vitrified tissues of *P. kesiya*. Similar findings have been reported by Sakai *et al.* (1991) on nucellar cells of navel orange and Ishikawa *et al.* (1996) on broom grass suspension cells. Use of drop wise and direct addition of sucrose solution at 1.2 M concentration did not have any significant effect. Addition of lower concentration of sucrose solution dropwise resulted in better survivability and regrowth than direct addition. By the drop wise addition of washing solution change in osmolarity is gradual and thus injury to tissues is avoided. In case of embryo cryopresevation better response was recorded using plastic cryotubes than glass and aluminium tubes. Plastic containers were advocated for cryostorage by Withers (1983). But Grout and Crisp (1985) preferred aluminium foil. For shoot tips cryopreservation, glass tubes were found better than plastic and aluminium tubes. There seems to be difference in the type of cryotubes to be used for vitrification of shoot apices and embryos. The cryopreserved tissues were later regrown in their respective recovery

medium. The cryopreserved shoot apices and embryos produced plantlets upon culture in respective medium identical to the untreated phenotypes. There was no intermediary callus formation in these cases. The plantlets developed fully within about 4 months after culture. The first sign of viability could be seen after 1 week in case of shoot apices when they start becoming green and in 3-4 days in case of embryos when cotyledons start opening.

From the study of cryopreservation of *P. kesiya*, it is found that the zygotic embryos could be cryopreserved with 80.3% regeneration using simply air drying of the embryos for 3hr. in laminar flow cabinet and then plunging in liquid nitrogen. For cryopreservation of shoot apices the best method was preculture of shoot apices in 10% sucrose at 4°C for 10 days and then pretreatment with 5% DMSO for 1hr. followed by slow cooling -30°C (with a rate of 0.5°C min^{-1}) before rapid plunge in liquid nitrogen.

From the above discussion it is evident that *P. kesiya* germplasm could be successfully cryopreserved, which is necessary in the light of poor regeneration through conventional means showing high rate of variability. It cannot be over emphasised that trees with heterogenous and poor growth and undesirable wood quality are no more acceptable. Together with micropropagation protocols of *P. kesiya* described in chapter 2, the cryopreserved material could be regenerated at will throughout the year. It is expected that procedures described in this thesis will help in clonal forestry which is the need of the hour for mass afforestation programmes using trees of superior qualities.

CHAPTER 4. SUMMARY

In vitro plantlets were produced from zygotic embryos, shoot apices and needles of *P. kesiya*. For multiple shoot bud production in embryos both SH and LP medium with different concentrations of BAP (0-15.0mgl^{-1}) and kinetin (0-15.0 mgl^{-1}) singly and also in combinations with (NAA and IBA) (0.1-0.5 mgl^{-1}) were used. 20-22 shoot buds were produced per embryo cultured in LP medium with 5.0 mgl^{-1} kinetin after its transfer to basal LP medium with reduced (2%) sucrose. The per cent response being 90.5%. Shoot apices and needles obtained from *in vitro* grown seedlings were c ultured in LP and MS medium, respectively, with cytokinins (0-10 mgl^{-1}) singly and incombination with auxins (0.1-0.5 mgl^{-1}). Using shoot apices, 20-25 buds per shoot apex were produced (with 97.3% response) in MS medium supplemented with 4.0 mgl^{-1} BAP. The shoot bud production was lower in the medium containing other concentrations. In case of needles cultured in MS medium supplemented with 5.0 mgl^{-1} Kinetin 7-8 small shoot buds developed. Use of auxins in

combination with cytokinins could not increase the number of shoot bud production in embryos, shoot apices and needles.

The multiplication and proliferation of the shoot buds produced from embryos could be achieved by culture in half-strength LP medium supplemented with 1.0 mgl^{-1} kinetin and 0.1 mgl^{-1} IBA along with 0.3% activated charcoal. Further miltiplication was accomplished by their subculture every 3-4 weeks in the same medium devoid of growth regulators for 2-3 passages. Using this procedure a total of about 80-85 shoot buds were produced per embryo. Shoot buds developed for shoot apices when culture in half-strength MS medium containing 1.0 mgl^{-1} BAP and 0.1mgl^{-1} IBA along with 0.3% activated charcoal resulted in about 62% response where in about 4 weeks time the shoot buds multiplied. They were subcultured every 3-4 weeks for 2-3 passages till the shoots grew to a height of 20-30 mm. Shoot buds developed using needles upon culture in half-strength MS medium with 1.0 mgl^{-1} kinetin and 0.1mgl^{-1} IBA along with 0.3% activated charcoal resulted in development of needles. The response of shoots derived from needles was poor compared to embryos and shoot apex derived shoot multiplication.

Isolated shoots derived from embryos and shoot apices were cultured for rooting. The isolated shoots derived from embryos were cultured in half-strength LP medium with different concentrations of NAA and IBA singly. Some shoots were given pulse treatment with high concentrations of NAA and IBA (10.0-30 mgl^{-1}) for varying period of time and then cultured either in half-strengh LP medium devoid of growth regulators or in water-agar (0.6%) medium. A maximum of 3-4 number of roots

emergbed from the base of the shoots cultured in half-strength LP medium supplemented with 0.3 mgl^{-1} NAA. Same number of roots were also produced on shoots precultured in 10.0 mgl^{-1} NAA and 10.0 mgl^{-1} IBA for 24 hr. and then cultured in both half-strength LP medium devoid of growth regulators and also in 0.6% water-agar medium.

The shoots derived from shoot apices were cultured in half-strength MS medium with different concentrations of NAA (0.1-0.5 mgl^{-1}) and IBA (0.1-0.5 mgl^{-1}) singly. Some shoots were given pulse treatment with NAA (10.0-30 mgl^{-1}) and IBA (10.0-30.0 mgl^{-1}) for 12, 24 and 48 hr. before culturing in either water-agar (0.6%) medium or in half-strength MS medium devoid of growth regulators. A maximum of 4-5 number of roots were produced in each shoot given pulse treatment with NAA (10.0 mgl^{-1}) for 24 hr. and cultured in water-agar (0.6%) medium. The response in this case was 72.3%. Since the number of shoots derived from needles were few and the time taken for elongation of these shoots were longer, rooting was not attempted on these shoots.

The rooted plantlets were tranferred to pots with different potting mixtures. After taking adequate care about 70% plantlets survived with good growth in soil mixture collected from the pine forest. The successful transplantation depends on the suitable size and growth of the plantlets and the sustratum used. The plantlets were fed with $1/10^{th}$ MS nutreint salt solution twice a week for the first 2 weeks and later every 15 days upto 3 months.

For cryopreservation experiments shoot apices and zygotic embryos were used. Shoot apices were cryopreserved both by rapid freezing and slow freezing method after

treating with DMSO (0-20% w/v) for 1, 2 and 3 hr. Shoot apices treated with 5% DMSO for 2hr. and frozen slowly (at the rate of 0.5^{0}C min^{-1}) resulted in 38.3% viability and regrwoth after cryopreservation, whereas, rapidly frozen shoot apices did not surviive liquid nitroegen exposure. The shoot apices precultered in 10% sucrose for 14 days and treated with 0.5% DMSO for 2 hr. before cooling slowly increased the per cent regrowth to 48.5%. Out of differnt cooling rates used in cryopreservation of shoot apices a rate of 0.5^{0}C min^{-1} was found to be the best.

Shoot apices when treated with a comibnation of cryoprotectant soultion comprising sucrose (10% w/v), DMSO (10% w/v) and glycerol (5% w/v) for 1hr. and then kept for 1hr. in a freezer held at -30^{0}C before plunging in liquid nitrogen (concise method) resulted in per cent regrowth of 45.3. The shoot apices treated for 1hr. with the above cryoprotectant mixture and then frozen slowly (at the rate of 0.5^{0}C min^{-1}) resulted in 52.6% viability and regrowth. The smaller size (1-2 mm) shoot apices gave better result than larger sized (4-5 mm) shoot apices after cryopreservation. Shoot apices precultered in the medium with 10% sucrose at 4^{0}C for 15 days and cooled slowly with 0.5^{0}C min^{-1} to -30^{0}C before LN plunge, resulted in 55.3% regrowth after cultture in recovery medium. The experiment conducted to find out the optimum terminal temperature showed that shoot apices cooled slowly to -30^{0}C (at the rate of 0.5^{0}Cmin^{-1}) before plunging in liquid nitrogen gives highest per cent survial and regrowth.

The cryopreserved shoot apices thawed at 40^{0}C water bath resulted in optimum survival. The shoot apices showed almost similar survival and regrowth during 180 days of storage in liquid nitrogen.

Zygotic embryos could be cryopreserved with a high percentage of plantlet regeneration using desiccation of excised embryos with sterile air flow for 3hr. in a laminar flow cabinet and then rapidely plunging them in liquid nitrogen. The level of mositure content was reduced to 12% during 3 hr. desication. About 80% regeneration of plantlets could be achieved by this process after its culture on recovery medium. The per cent regeneration of cryopreserved embryos decreased as the age of the culture in medium was increased before cryopreservation by rapid and slow freeezing methods. 76.5% plantlet regeneration was achieved in cryopreserved embryos that were precultered at 4^{0}C for 5 days, desiccated for 3 hr. and then plunged in liquid nitrogen rapidly.

Embryos treated with 5% DSMO for 1hr. could result in 78% plantlet regeneration after their cryopreservation by slow freezing method. Embryos precultered in 10% glycerol for 24 hr. exhibited 61.3% survival which was about the same as in the case of precultered in medium containing 15% glycerol. Embryos precultered in the medium containing 10% glycerol and 5% DMSO for 24 hr., treated with 5% DMSO in cryotubes for 1hr. and then kept for 1 hr. in a freezer held at -30^{0}C (using concise method) resulted in 80.3% plantlet regeneration. The per cent plantlet regeneration declined sharply with increase in hydration period of embryos followed by 3 hr. dehyration. The hydration period of 4 hr. resulted in a maximum of about 50%

plantlet regeneration. The thawing temperature suitable for embryos was 30^0C. Embryos, thawed after different period of storage in liquid nitrogen upto 180 days resulted in more or less steady per cent plantlet regeneration on recovery medium.

A vitrification solution PVS2 was used to cryopreserve shoot apices and embryos of *P.kesiya*. The optimum per cent regrowth of both shoot apices and embryos was obtained from cultures exposed to 0.3 ml of PVS2 solution for 2 min. Sucrose solution (1.2 M) either added drop wise or directly to dilute the PVS_2 solution after thawing resulted in highest survivability. The washing of vitrified samples with nutrients medium (5 ml) after washing with 1.2 M sucrose resulted in a bettter per cent growth of 55.2% in case of shoot apices and 45.5% in case of zygotic embryos. Out of different cryovials used optimum survivability and regrwoth of shoot apices and embryos resulted in glass and plastic tubes, respectively.

Using the procedures described here the cryopreserved explants of *P. kesiya* showed regeneration of plantlets in respective medium. The plantlets could be obtained at will throughout the year from cryopreserved material, and using the methods described here, could be made available for clonal forestry after scale up.

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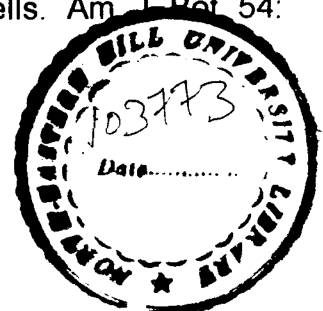

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