

MORPHOGENETIC STUDIES OF TWO INDIAN PINES
***IN VITRO: Pinus roxburghii* Sarg. and *Pinus wallichiana* A.B. Jacks**

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CERTIFICATE

This is to certify that the work incorporated in this thesis entitled, **“Morphogenetic studies of two Indian pines in vitro: *Pinus roxburghii* Sarg. and *Pinus wallichiana* A.B. Jacks”** submitted by **Mr. Gaurav Mathur**, was carried out under my supervision at Tissue Culture Pilot Plant, National Chemical Laboratory, Pune. Such material, as has been obtained from other sources, has been duly acknowledged in the thesis.

Pune

Dr. (Mrs.) R. S. Nadgauda

Date:

Research Guide

KEY TO ABBREVIATIONS

° C	Degree centigrade
μE	Microeinstein
μg	Mirograms
μl	Microlitre
μM	Micromoles
μm	Micrometres
@	At the rate of
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ,γ-dimethylallylaminopurine)
ABA	Absciscic acid
ANOVA	Analysis of variance
BA	N ⁶ -benzyladenine
BFC	Bud forming capacity
CH	Casein hydrolysate
cm	Centimeter
DCR	Douglas fir cotyledon revised medium
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DPX	DPX-4 1889-(2-chloro-N-(methoxy-6-methyl-1,3,5-triazin-2yl aminocarbonyl) benzene sulfonamide)
EDTA	Ethylenediaminetetraacetic acid
FAA	Formaldehyde: acetic acid: alcohol
FDA	Fluorescein diacetate
Fig	Figure
GA ₃	Gibberallic acid
g/l	Grams per litre
HCl	Hydrochloric acid
HgCl ₂	Mercuric chloride
h	Hours
IAA	Indole-3-acetic acid

IBA	Indole-3-butyric acid
IZE	Immature zygotic embryo
kin	Kinetin
LN	Liquid nitrogen
LPm	Leaf protoplast medium
m	Metres
M	Moles
mg	Milligrams
mg/l	Milligram per litre
min	minutes
mM	Millimoles
mm	Millimeters
MZE	Mature zygotic embryo
N	Normality
NAA	α -Naphthalene acetic acid
NaOH	Sodium hydroxide
PEG	Polyethylene glycol
PEM	Proembryogenic mass
PGR	Plant growth regulator
rpm	Revolutions per minute
RT	Room temperature
S	seconds
S.D.	Standard deviation
SCV	Settled cell volume
SE	Standard error
SH	Schenk and Hildebrandt medium
TBA	Tertiary butyl alcohol
TDZ	Thidiazuron, 1-phenyl-3-(1,2,3-thidiazol-5yl) urea
v/v	Volume/volume
WPM	Woody plant medium
zea	Zeatin {6-(4-hydroxy-3-methyl-but-2-enyl) amino purine}

SYNOPSIS

The genus *Pinus* finds its importance not only for its timber and high quality resin, but also for the fact that it has been associated with the vibrant but fragile ecosystem of the Great Himalayan ranges in the Indian sub-continent. A number of indigenous pine species find home in India and considering their economic and ornamental value, recently exotic tropical pine species have also been planted in different parts of the country. The amount of indiscriminate felling of trees and clearing of hills for agricultural purposes, timber, different forest products and mining, that has taken place during the 19th and 20th centuries, has put the complete Himalayan ecosystem under tremendous pressure. The pines have been mainly exploited for their timber and resin.

Despite reserving natural forest stands and putting in large efforts to intensify afforestation, the forest departments have not been completely successful in providing enough planting material using conventional propagation techniques. This certainly has opened up the avenues for the newer biotechnological approaches that can be tapped to supplement the demand for good quality planting material.

Clonal propagation of gymnosperms, or for that matter, any other forest tree species, use of mature plant material has met with little success due to the recalcitrant nature of the tissue. Among the conifers, pine tissues have shown even greater rigidity in responding in vitro. Adventitious bud proliferation and somatic embryogenesis are the two methods that have been used successfully in other pine species for propagation.

The work included in the present thesis titled 'Morphogenetic studies of two Indian pines in vitro: *Pinus roxburghii* Sarg. and *Pinus wallichiana* A. B. Jacks' is an attempt to develop regeneration system in these two indigenous pine species using adventitious bud proliferation and somatic embryogenesis. The objective of the study was to optimize the media and culture conditions to develop an effective protocol that can be utilized in the future for the improvement of these two important Indian pine species.

THESIS ORGANIZATION

This thesis has been divided into five chapters followed by summary and bibliography.

Chapter 1: General introduction

This chapter gives a general introduction about the genus *Pinus*, its position and importance in Indian forestry and, the role of biotechnology in its improvement and propagation. The chapter includes a survey of tissue culture studies on pines done over the years giving special emphasis to adventitious bud proliferation and somatic embryogenesis. It also includes the objectives with which the present studies were intended and the approach with which the experiments have been planned and conducted.

Chapter 2: General materials and methods

In this chapter, general methodology that is followed throughout the course of study has been explained. The source of various chemicals, glassware, plastic-ware and other materials utilized during the course of the work, glassware cleaning and preparation, basic media compositions and preparation, culture conditions, etc., have been discussed.

Materials and methodology related to specific experiments and studies have been included separately in chapters 3, 4 and 5.

The following chapters (3, 4 and 5) are further sub-divided into Introduction, Specific materials and methods and, Results and discussion.

Chapter 3. Morphogenetic response of *Pinus wallichiana* A. B. Jacks. (Blue pine) in vitro

Pinus wallichiana A. B. Jacks (blue or Bhutan pine) is an elegant tree reaching 30-90m in height and is found in the Himalayas from Kashmir to Bhutan. Large natural forest areas of blue pine and associated trees have lately been cleared up for various reasons like agriculture, mining, timber, etc. It is possible now to supplement the conventional propagation methods with newer biotechnological approaches to provide good quality planting material. On these lines, present studies with blue pine were initiated. In this chapter the morphogenetic response of tissue culture and development of a micropropagation protocol for *P. wallichiana* is discussed.

Plantlet regeneration was achieved by organogenesis of mature zygotic embryos of blue pine. The effect of various basal media and cytokinins were tested

for adventitious bud induction, development and elongation were investigated. Among the different concentration and combinations of media tested, DCR basal medium supplemented with 2.5 μ M BA and 0.025 μ M TDZ was found to be most effective in inducing adventitious buds from mature zygotic embryos. The effect of pulse treatment of BA was tested and the bud forming capacity was quantified. Histological examination of adventitious bud induction was also carried out to ascertain the origin of the buds.

The elongation of adventitious buds was achieved on half strength DCR basal medium with 2% sucrose and 0.5% activated charcoal. Experiment of rooting the elongated shoots showed that a 6-h treatment of IAA-IBA (1mM each) was most effective among the different methods examined. Rooted shoots were successfully hardened and transferred to greenhouse and showed a survival percentage of 45.7 after 6 months.

Chapter 4. Somatic embryogenesis in *Pinus roxburghii* Sarg. (Chir pine)

P. roxburghii Sarg. or chir pine is the other important Indian pine species belonging to the lower altitudes of western Himalayas. It has been mainly exploited for its high quality resin that is used in the production of turpentine, paints, varnishes and also has medicinal properties. Reckless tapping for resin and lopping for fuel-wood has made it necessary to look into aspects of selection and propagation of chosen elite varieties.

In this chapter, method of propagating this species through somatic embryogenesis has been discussed. Among the gymnosperms, *Pinus* has been the most recalcitrant genus in terms of inducing somatic embryogenesis. Induction was achieved only using the immature zygotic embryos at a particular stage of development that appears only for a couple of weeks in a year.

Initial work includes standardization of the most responsive explant and pinpointing the very narrow 'window' or the exact developmental stage of the immature zygotic embryo for initiation, from two different provenances in the western Himalayas. Different basal media and other parameters have been tested for initiation and proliferation of embryogenic tissue. A study of the development of somatic embryos, effect of pre-maturation, maturation treatments on germination of somatic

emblings has been tested. Embryogenic cell lines are now being maintained on semi-solid as well as suspension cultures, for further studies and refinement of the regeneration protocol at a larger scale.

Chapter 5. Studies with the embryogenic cell lines of *Pinus roxburghii* Sarg. : (i) scaling-up in bioreactor and, (ii) cryopreservation

Due to the long life cycles of tree species, the assessment of desired characters in the clonally propagated plants becomes difficult. More so, when the initial explant for propagation is seed from open-pollinated natural stand. In such cases, cryopreservation of the embryogenic tissue becomes mandatory. Once the somatic embryos are germinated, hardened, established in the field, and are tested true to type for the desired characters, the cryopreserved, 'mother' embryogenic tissue can then be scaled-up and used for propagation.

This chapter discusses the experiments conducted to standardize the pre-treatment of embryogenic tissue with different cryoprotectants (DMSO, sorbitol, glucose, PEG) and the survival of embryogenic cell lines after storage in liquid nitrogen. Genetic fidelity of the cryo-stored cell lines was tested using ISSR primers.

Experiments conducted for scaling-up of the embryogenic tissue using bioreactor have also been discussed. Suspension cultures grown in flasks were inoculated in 1-liter bioreactor culture vessel to optimize the conditions for the growth of the tissue in continuous culture. Various factors like pH, temperature, have been tested for the optimal growth of the embryogenic cell lines.

Gaurav Mathur

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Research Guide

Chapter 1

General Introduction

1.1. Introduction

The genus *Pinus* maintains a very unique position of being an integral part of the vibrant, but fragile ecosystem of the Great Himalayan ranges in the Indian sub-continent. From time immemorial a number of indigenous pine species find home in India, and considering their economic and ornamental value, recently exotic tropical pine species have also been planted in different parts of the country as an alternative to bamboo for paper and pulp industry (Sahai 1996, Satish Chandra *et al.* 1996). The amount of indiscriminate felling of trees and clearing of hills for agricultural purposes, timber, different forest products and mining, that has taken place during the last century, has put the complete Himalayan ecosystem under tremendous pressure (Banerjee 1996). The Indian pines have been exploited for their timber and mainly, for their high quality resin (Maheshwari and Konar 1971).

Vegetative propagation of trees has been practiced for centuries in Japan for *Cryptomeria japonica* (Ahuja 1993), and elsewhere, generally for tree species that were considered easy for rooting, like *Populus* spp., and *Salix* spp. (Toda 1974). This practice has been extended in Germany and Sweden for establishing *Picea abies* forests (Talbert *et al.* 1993), Eucalypts in Brazil (Zobel 1985), *Pinus radiata* in New Zealand (Aitken-Christie and Jones 1987, Arnold and Gleed 1985, Horgan and Holland 1989), and *Pinus taeda* and *Pseudotsuga menziesii* in USA (Gupta *et al.* 1993, Timmis 1998). Traditionally, forests have been regenerated from seedlings derived from mixed seed lots collected from nature, or more recently from seeds collected from randomly pollinated plus-trees, resulting in large variations in growth form and vigour (Chen and Ahuja 1993).

Plant propagation of tree species through tissue culture is a recent development and may be accomplished by employing callus, organ, cell, and

protoplast cultures (George 1993). Initially callus cultures were used for plantlet regeneration, now preferably organ cultures are employed for clonal propagation, mostly to avoid the somaclonal variations. The long life cycles of trees and recalcitrancy of the tissue explants from mature tree species, poses tremendous complexities for their clonal propagation (Bonga and von Aderkas 1993). Despite having a clear advantage over callus and organ culture, cell suspensions and protoplast cultures have not yet been successfully employed for large-scale plant regeneration in clonal forestry. The success stories of *in vitro* propagation of trees have so far come mainly from conifers like spruce and pines (Adams *et al.* 1994, Gupta *et al.* 1991, Gupta and Grob 1995, Pâques *et al.* 1995). Large-scale production of somatic embryos in bioreactors, their selection and subsequent production of synthetic seeds for plantation has now become a reality (Attree and Fowke 1993, Gray *et al.* 1995, Handley 1995, Redenbaugh *et al.* 1991, Tautoris and Dunstan 1995). This also incorporates the efforts taken in genetic manipulation for variety improvement (Ellis 1995).

1.2. Classification of genus *Pinus*

The genus *Pinus* L. belongs to the family Pinaceae or Abietaceae of the order Coniferales. The pines have been traced back in the geological history to Jurassic period (150 million years) reaching their climax in the tertiary period (60 million years). The two distinct groups viz. Haploxylon (soft pines) and Diploxylon (hard pines) had emerged by the end of lower Cretaceous period (125 million years) (Maheshwari and Konar 1971, Mirov 1967).

The other genera belonging to family Pinaceae are, *Abies* Mill., *Cathaya* Chun & Kuang, *Cedrus* Link., *Keteleeria* Carr., *Larix* Mill., *Picea* A. Dietr., *Pseudolarix* Gord., *Pseudotsuga* Carr., and *Tsuga* Carr. Some characteristic features shared among this family are as follows: evergreen and monoecious plants; spirally arranged microsporophylls with two abaxial sporangia each; female cones with numerous spirally arranged pairs of scales (bearing seeds); ovules adaxial with their micropyles directed towards the cone-axis and seeds generally winged (Sporne 1965).

The pines are represented by 105 species (Mirov 1967), whereas Dallimore and Jackson (1966) mention 80 valid species that are cosmopolitan and distributed mainly in the Northern hemisphere. Being a dominant genus of the temperate region, pines are also abundant in the subtropical and the Mediterranean regions and few species also make an appearance in the tropics. The cosmopolitan genus covers almost all of North and Central America, Northern Europe, Bahamas, British Honduras, the subtropics of North Africa, the Canary Islands, Afghanistan, Pakistan, India, Burma, Indonesia, Philippines and spreading over to New Zealand.

1.3. Distribution, general morphological characters, importance and uses of pines

1.3.1. Distribution in India

Four out of the six indigenous pine species of the Indian subcontinent, viz., *P. roxburghii* Sarg. (chir pine), *P. wallichana* A. B. Jacks (blue pine or kail), *P. gerardiana* Wall. ex Lamb. (chilgoza or nioza pine) and *P. kesiya* Royale ex Gord. (khasi pine) (*P. insularis* Endl.), are distributed in the Himalayas. Whereas, *P. armandii* Franch., occurs in the North-eastern provenances and *P. merkusii* Jhung and de Vriese are found in the hills of Burma (Maheshwari and Konar 1971).

Specific morphological characteristics, distribution, habits and uses of *P. wallichana* A. B. Jacks and *P. roxburghii* Sarg., have been discussed separately in chapters 3 and 4 respectively.

1.3.2. General morphological characters

Young pine trees are generally pyramidal in shape with horizontal branches disposed as regular whorls and they lose this symmetry on maturity with the rounding, flattening or spreading of the crown. The lower branches also tend to fall off exposing the long bole due to the closeness of plantations. The stem bears two types of branches, long and dwarf shoots. Long shoots arise as

lateral buds on main stem ending in an apical bud surrounded by bud scales. The lateral buds grow more or less horizontally producing one or more internodes every year. The dwarf shoots develop on the long shoots and bear needles. The number of needles per dwarf shoot is one of the features that aid in identification of different pines. The other external characters that facilitate the identification of different pine species are, the position of umbo on apophysis and, shape, size and colour of the resting bud.

The male and female cones are borne on different branches of the same tree. The male cones (modified dwarf shoots) appear in clusters on lower branches whereas the female cone replaces the terminal bud of the long shoots (modified long shoots). In most species, including *P. roxburghii* and *P. wallichiana*, the mature female cones open and release seeds but in some species the seeds are dispersed only after the cone falls to the ground and rots.

The pines are generally light demanders and do not thrive in areas that remain hot and humid throughout the year. A changeover of seasons is essential for normal development of pines. Only a few tropical pine species like *P. taeda* grow well in wetlands (Maheshwari and Konar 1971, Mirov 1967)

1.3.3. Importance and uses

The Indian pines are mostly exploited for their resin, than they are for timber. In general, the yield of oleoresin is more in the species found in warmer than in temperate zones. *Pinus roxburghii*, *P. wallichiana* and *P. kesiya* are the three common pine species tapped for resin with chir pine leading the way (Maheshwari and Konar 1971). The growth of the resin industry was a result of the research work conducted by the forest departments in the late- 19th century (Tewari 1994). Even with the advent of newer technologies, numerous methods of tapping and distilling resin from pines that are still prevalent were introduced and devised during the last century (Choudhari *et al.* 1996). The products and by-products of oleoresin distillation range from turpentine, charcoal, lamp-black, pine oil and wood-rosin. Turpentine extracted from *P. roxburghii* is devoid of pinene and cannot be used in the camphor industry

whereas, turpentine from *P. wallichiana* and *P. kesiya* contains pinene and is useful in production of synthetic camphor (Coppen John *et al.* 1988). Turpentine is mainly used as thinner in paints and varnishes and a solvent for rosin and lacquer. It is an ingredient in shoe polishes, lubricants, sealing waxes, etc. Rosin is chiefly utilized by the paper and soap industries and a number of other oil based products. Pine needle oil is used for medicinal purposes, perfumery (Dayal *et al.* 1989) and also insect repellents (Zaheer K *et al.* 1986).

Pine timber constitutes the major forest wealth of the countries where they grow. The hardwood pines, the heartwood of which is darker than the sapwood, are mostly preferred over the softwood pines when it comes to the wood quality. Timber is mainly used for construction purposes and furniture. In Europe, especially Norway and Sweden, where pines form a bulk of the vegetation, the wood is used to make very high quality writing and kraft paper (Guha 1958, Maheshwari and Konar 1971).

The edible kernel of *P. gerardiana*, commonly known as ‘Chilgoza’ or Nioza’, is one of the 18 species of pines that has a seed large enough and attractively flavored, to be edible. ‘Chilgoza’ is very highly priced ‘dry-fruit’ in the Indian subcontinent and is consumed after roasting. Moreover, the ‘Chilgoza’ pine is the most endangered pine species in India, found at high altitudes in the inaccessible North-western Himalayas, occurring on the borders of Tibet, Kashmir and Pakistan. It also occurs in some patches of Kinnaur and Pangi districts of Himachal Pradesh (Maheshwari and Konar 1971). With the number of trees dwindling each year, there is a constant effort being put in by the forest departments for the protection and propagation of this pine species. Due to the difficult terrain, scarcity and the high price that the seed fetches in the market, procuring *P. gerardiana* plant material, especially in the form of immature cones for clonal propagation studies, is presently not possible.

The seeds of all pines are rich in fat and proteins (Sehgal *et al.* 1989), but in only 18 species the kernels are large enough and attractively flavored to be edible. The kernels of *P. pinea*, pignolia or pignoli, are mostly collected in Italy and Spain, are used in many Italian preparations and also eaten raw,

roasted or salted. In east Europe the edible seeds of *P. cembra* are used for culinary purposes and so are the seeds of *P. cembroides* in North America. *P. lambertiana*, *P. monticola*, *P. sabiniana*, *P. coulteri*, *P. torreyana* and *P. jeffreyi* are also important sources of edible pine seeds (Maheshwari and Konar 1971).

1.4. Propagation of pines

In a country where tree breeding and hence, the availability and assurance of good quality hybrid seeds has not been practiced, nor is it foreseen in the near future, plantations raised from a mixed seed lot would not be sufficient enough to solve the problem of propagating elite varieties of Indian pines. However, work on vegetative propagation of pines in the past two to three decades by various research institutes and universities in India have demonstrated success in using traditional methods like grafting, air-layering and rooting of stem cuttings, yet the favored method for propagation remains to be through nursery-raised seedlings (Dhiman *et al.* 1996, Singh *et al.* 1996, Tewari 1994). The need for consistency in quality of planting material has now opened up avenues for newer methods to be used for propagation.

State forest departments, Government and private forest agencies/nurseries have been instrumental in the large-scale plantations of indigenous, as well as exotic pines at different locations in India (Sahai K. 1996, Satish Chandra *et al.* 1996). The seeds of various pine species are collected and preserved every year and used for propagation, except for *P. gerardiana*, most of the seeds of which are edible and fetch a good price. On the whole, the major disadvantage of the existing propagation system is inconsistency in the quality of the seeds due to the open pollination in the natural stands.

Tissue culture and molecular biology techniques can not only aid in supplementing the planting material, but also can help in improvement, selection, breeding and typifying the indigenous species (Ravikumar 2001). In fact, for many conifers cloned propagules still cannot be obtained from cultured

tissue of mature trees, indicating the large-scale application of these techniques to tree improvement programmes of conifers is still a long way from being achieved. Although, in some species, it seems that the mass propagation of selected trees or, of a superior hybrid is more likely to be achieved through tissue culture than by any other method.

1.5. Morphogenetic studies in *Pinus* spp.

The demand for wood and forest products as an industrial raw material has increased at such an alarming rate, that the traditional methods used for forest tree propagation and improvement (cutting, grafting) are not adequate to meet them completely. This phenomenon has been observed worldwide, more so in the developing countries, where indiscriminate clearing of forests is widely reported. It is therefore an obvious choice to supplement the traditional methods with the more recently developed techniques like, tissue and organ culture. This is a potential technique to clone and vegetatively produce plants rapidly on a large scale (George 1993, Merkle and Dean 2000, Ravikumar G. 2001)

Last two decades has witnessed pioneering work in the advancement of *in vitro* culture of a large number of economically important gymnosperm species (David 1982, Talbert *et al.* 1993, Thorpe *et al.* 1991). Initial studies with clonal propagation using mature and juvenile tissues led to breakthrough in induction of somatic embryogenesis and it became a preferred method of obtaining large number of clonal propagules (Gupta and Grob 1995). Vegetative propagation from physiologically mature tissue tends to be quite difficult (Greenwood 1995). Ideally, clonal propagation of trees old enough to have expressed their desirable characteristics is an effective means of rapidly obtaining improved planting stock. On the other hand, vegetative propagation from physiologically juvenile tissue has been successful in a number of conifer species and appears to result in plants that are true to type (Thorpe *et al.* 1991). Three methods of juvenile tissue propagation have been widely used as mass-

propagation alternatives: rooted cuttings, organogenesis and somatic embryogenesis.

As rooting was the major hurdle with adventitious and axillary shoots (Girouard 1974), maturation and conversion of somatic emblings into healthy plantlets is the major bottleneck with somatic embryos of pines (Attree and Fowke 1993). This has been highlighted in a number of reports in various conifer genera (Tautorus *et al.* 1991). Another impediment most commonly faced by tissue culturists is the genotypic dependence of the *in vitro* morphogenetic response, thus making it impossible to follow strict rules for micropropagation. Two pathways that have been preferably and successfully used for micropropagation of conifers are, via adventitious shoot proliferation and somatic embryogenesis. It is usually observed that the quality of the vegetatively propagated clones is heavily subjected to the quality or genetic material of the population or the mother explant (George 1993, Vasil and Vasil 1980)

Micropropagation of pines has mostly been practiced using juvenile explants like embryos or young seedling tissues, however there are few reports of propagation using differentiated tissues (Abdullah *et al.* 1987, Baxter *et al.* 1989, Bonga 1981, Gupta and Durzan 1985, Lapp *et al.* 1996, Nadgauda *et al.* 1993). The problems in tissue culture of pines, for that matter, all conifers, are numerous. Even propagules produced from donor as young as mature zygotic embryo, may not ultimately be phenotypically true-to-type (Bergmann and Stomp 1994a, 1994b). But, Goto and co workers (1998) have recently used random amplified polymorphic DNA (RAPD) markers to determine the genetic stability of more than 10-year old micropropagated shoots of *P. thunbergii* Parl and observed no aberration in banding patterns in these studies. The problem of rejuvenation of physiologically mature tissue can be solved through somatic embryogenesis (Ruaud and Pâques 1995). Induction of somatic embryogenesis re-sets the developmental clock of the cells, consequently following a step-wise pattern of development starting from the embryo stage.

In order to develop protocols for propagation using adventitious bud proliferation or somatic embryogenesis with precise control over the developmental scheme, a basic understanding of the biochemical and molecular processes that underline the formation of buds and somatic embryos is essential. A compilation of the significant documented work carried out in the field of micropropagation of pines, is presented below.

1.5.1. Adventitious bud proliferation

During the last two decades, comprehensive studies were conducted to find out the possibilities of propagation of conifers using tissue culture methods. Due to the recalcitrancy of the mature or differentiated tissues of pines like, needles, axillary buds, male and female cones *in vitro* (Bonga 1981), attention was shifted to seedling and cotyledonary stage, i.e. juvenile or immature tissues (Abdullah *et al.* 1989, Bergmann and Stomp 1992 & 1994a, Bonga 1981, Capuana and Giannini 1995, Halos and Go 1993, Lapp *et al.* 1995, McKellar *et al.* 1994, Mott and Amerson 1981, Murithii *et al.* 1993, Pulido *et al.* 1990, Saborio *et al.* 1997). These reports are mainly concerned with the adventitious bud proliferation from mature zygotic embryos and cotyledons of pines, but only a notable few could be successfully exploited at a commercial scale (Aitken-Christie *et al.* 1981, Baxter *et al.* 1989, Horgan and Holland 1989). Although, developing a reproducible regeneration protocol in recalcitrant tree species has its own advantage of being able to be utilized for genetic improvement programmes in future. As a result, a number of *Pinus* species as well as other economically important conifers are now documented having a complete regeneration protocols using adventitious bud proliferation.

1.5.1.1. General aspects:

Organogenesis involves induction of adventitious buds and growth of shoots and roots separately, originating either from unorganized callus, or from pre-formed induced buds (George 1993). Induction of adventitious buds is mainly achieved using explants like mature zygotic embryo or seedling tissues

such as cotyledons. Shoot meristems of adventitious buds arise randomly in abnormal positions and can either be formed directly from the explants or indirectly through intermediary callus. It is however advisable to avoid callus establishment because, it is preferable to induce adventitious buds directly from the explant and eliminating the probable risk of genetic variation (George 1993). Propagation via adventitious bud proliferation can be divided into four stages:

- (i) Initiation of shoot buds
- (ii) Development of buds into shoots
- (iii) Rooting of shoots
- (iv) Preparation of plantlets for planting out (hardening)

Rooting of elongated shoots has been the major impasse after the induction of adventitious buds (Girourard 1974), which in fact, could be achieved relatively easily using a number of cytokinins. Presently, there are different methods documented that are being utilized to induce rooting in elongated shoots. Apart from shoot and root formation, extensive studies have also been carried out with respect to media, culture conditions and light regimes needed for *in vitro* culture.

1.5.1.2. Organogenesis in pines:

The organogenic response in many pines has been observed to be light dependant, with light quality, intensity, photoperiod and temperature having different effects in different pine species (Ellis and Webb 1993). The given parameters that are required for optimal response will depend not only on the species and explant, but also on the morphogenetic response desired. It is therefore not unreasonable to expect that for optimal culture response, one set of conditions (media, growth regulators, light, temperature) may be needed for each phase of micropropagation, whether it is via organogenesis or somatic embryogenesis.

A list of reported studies on a number of *Pinus* species successfully propagated using adventitious bud induction during the last two decades, is

presented in Table 1.1. Adventitious bud proliferation has been reported in more than 30 different pine species belonging to different geographical locations, clearly illustrating the importance of the genus. It is to be noted that the starting material for all is mature zygotic embryo (MZE) or seedling tissue, and also the fact that, not all the references report a complete regeneration system. The embryonic explants like, mature zygotic embryos (Calixto and Pais 1997, Chang *et al* 1991, Chesick *et al.* 1991, Halos and Go 1993, Harry and Thorpe 1994, Lambardi *et al.* 1991, Lapp *et al.* 1995, Tang *et al.* 1998) and, cotyledons (Abdullah *et al.* 1987, Garcia-Ferriz *et al.* 1994, Kaul 1987, Murithi *et al.* 1991, Saborio *et al.* 1997, Saravitz *et al.* 1991) have been utilized by most of the researchers. Investigations for the choice of explant, medium components, culture conditions, growth regulators have been extensively carried out (Bergmann and Stomp 1992, Flinn *et al.* 1986, McKellar *et al.* 1994, Perez-Bermudez *et al.* 1987, Pulido *et al.* 1994).

Table 1.1. Micropropagation of *Pinus* spp. through adventitious bud proliferation: explants used and recovery of plantlets

	<i>Species</i>	<i>Common name</i> ¹	<i>Explant</i>	<i>Result</i>	<i>Reference</i>
1	<i>Pinus ayacahuite</i> var <i>ayacahuite</i> (Ehremb)	Mexican white pine	cotyledon	Plantlets	Saborio <i>et al.</i> 1997
2	<i>P. banksiana</i> Lamb.	Jack pine	MZE	Plantlets	Harry & Thorpe 1994
3	<i>P. brutia</i> Tenore	Calabrian pine	Seedling tissues	Plantlets	Abdullah <i>et al.</i> 1985, 89
4	<i>P. canariensis</i> Sweet ex K. Spreng.	Canary Island pine	cotyledon	Plantlets	Pulido <i>et al.</i> 1990, 92, 94
5	<i>P. caribaea</i> Morelet	Caribbean pine	MZE	Plantlets	Halos & Go 1993
			MZE	Adv. Buds	Webb & Santiago 1983
6	<i>P. caribaea</i> var. <i>hondurensis</i>	Caribbean pine	MZE	Plantlets	Go <i>et al.</i> 1993
7	<i>P. contorta</i> Dougl. Ex Loud.	Lodgepole pine	MZE	Shoots	von Arnold & Eriksson 1981
8	<i>P. coulteri</i> D. Don	Coulter pine	MZE	Shoots	Patel & Berlyn 1983
9	<i>P. echinata</i> Mill.	Shortleaf pine	MZE	Plantlets	Bergmann & Stomp 1992
			cotyledon	Plantlets	Jang & Tainter 1991

Table 1.1. continued

10	<i>P. eldarica</i> Medw.	Afghan pine	MZE	Shoots	Bergmann & Stomp 1992
			seedling	Plantlets	Gladfelter & Phillips 1987
11	<i>P. elliottii</i> Engelm.	Slash pine	cotyledon seedling	Plantlets	Sen <i>et al.</i> 1994
			MZE	Axillary shoots	Burns <i>et al.</i> 1991
				Adv. Buds	Perez-Bermudez & Sommer 1987
12	<i>P. halepensis</i> Mill.	Aleppo pine	MZE	Plantlets	Lambardi <i>et al.</i> 1991
13	<i>P. monticola</i> Dougl.	Western white pine	MZE	Plantlets	Tzfira <i>et al.</i> 1999
			MZE	Plantlets	Lapp <i>et al.</i> 1995
			MZE, cotyledon	Plantlets	Mott & Amerson 1981
14	<i>P. nigra</i> Am.	Austrian pine	MZE	Plantlets	Salajova 1992, 1993
15	<i>P. omorika</i> (Panc.) Purk.		Seeding		
			MZE ,	Plantlets	Kolevska-Pleticapiæ & Buturovic-Deric 1995
			Seedling shoot tip		
16	<i>P. oocarpa</i> Scheide	No English name	MZE	Shoots	Bergmann & Stomp 1992
17	<i>P. palustris</i> Mill.	Longleaf pine	MZE	Plantlets	Sommer <i>et al.</i> 1975
18	<i>P. patula</i> Scheide <i>et</i> Deppe	Jelescote pine	Seedling	Shoots	McKellar <i>et al.</i> 1994
19	<i>P. pinaster</i> Ait.	French maritime pine	MZE	SHoots	David <i>et al.</i> 1982
20	<i>P. pinea</i> L.	Stone pine	MZE	Shoots	Dimantoglou <i>et al.</i> 1990
			Cotyledon	Shoots	Garcia-Ferriz <i>et al.</i> 1994
			Cotyledon	Plantlets	Capuana & Giannini 1995
21	<i>P. ponderosa</i> Laws.	Ponderosa pine	MZE	Multiple Buds	Ellis & Bilderback 1984, 89
22	<i>P. radiata</i> D. Don	Monterey pine or radiata pine	MZE, cotyledon	Plantlets	Aitken <i>et al.</i> 1981; Horgan & Aitken 1981
			MZE	Plantlets	Bergmann & Stomp 1992, 1994a, 1994b
23	<i>P. resinosa</i> Ait	Red pine	MZE	Shoots	Noh <i>et al.</i> 1988
24	<i>P. roxburghii</i> Sarg.	Chir pine	cotyledon	Plantlets	Muriithi <i>et al.</i> 1993
25	<i>P. serotina</i> Michx.	Pond pine	MZE	Shoots	Bergmann & Stomp 1992

Table 1.1. continued

26	<i>P. strobus</i> L.	White pine	MZE	Shoots	Bergmann & Stomp 1992
			MZE	Plantlets	Chesick <i>et al.</i> 1991
			MZE	Shoots	Flinn <i>et al.</i> 1986
			Cotyledon	Plantlets	Kaul 1987
			MZE	Shoots	Minocha 1980
			MZE	Plantlets	Schwarz <i>et al.</i> 1988
27	<i>P. sylvestris</i> L.	Scots pine	MZE	Plantlets	Jain <i>et al.</i> 1988
			Seedling callus	Plantlets	Supriyanto & Rohr 1994
28	<i>P. taeda</i> L.	Loblolly pine	MZE	Plantlets	Bergmann & Stomp 1992
			MZE	Plantlets	Tang <i>et al.</i> 1998
			callus	Plantlets	Tang 2001
29	<i>P. tecunumanii</i> (Schwd.) Equiluz & Perry		MZE	Plantlets	Bergmann & Stomp 1992
30	<i>P. virginiana</i> Mill.	Virginia pine	cotyledon	Plantlets	Chang <i>et al.</i> 1991
			cotyledon	Plantlets	Jang & Tainter 1991
31	<i>P. wallichiana</i> A.B. Jacks	Blue pine	cotyledon	Plantlets	Saravitz <i>et al.</i> 1991
			MZE	Plantlets	Mathur and Nadgauda 1999
			MZE	Plantlets	Bastola <i>et al.</i> 2000

¹ Reference: Maheshwari and Konar 1971, Mirov 1967

1.5.2. Somatic embryogenesis

1.5.2.1. General aspects of somatic embryogenesis:

Somatic embryogenesis is a non-sexual propagation process where somatic cells differentiate into somatic embryos (George 1993, Ronchi and Giorgetti 1995). The original description of somatic embryogenesis came from the observations of *Daucus carota* cell cultures (Steward *et al.* 1958). The first reproducible report of diploid somatic embryogenesis in conifers was from immature embryos of *Picea abies* (Hakman *et al.* 1985) and the embryogenic cultures regenerated plantlets (Hakman and von Arnold 1985). Nagmani and

Bonga (1985) were first to report haploid embryogenesis and plantlet regeneration from megagametophytes of *Larix decidua*.

Plant regeneration through somatic embryogenesis includes four different stages:

- (i) Initiation of embryogenic cultures
- (ii) Proliferation of embryogenic cultures
- (iii) Maturation of somatic embryos
- (iv) Conversion into plantlets

Each and every cell of the plant contains all the genetic information necessary to create a complete and functional plant (totipotency). The differentiation of various organs and organ systems during the development of the plant is achieved through very tight regulation of spatial and temporal expression of genes (De Jong *et al.* 1993). The induction of somatic embryogenesis must then be a termination of existing gene expression pattern in the explant tissue, and its replacement with the embryogenic gene expression program (Wilde *et al.* 1995, Zimmerman 1993).

In conifers, once the embryogenic cells are determined, they continue to proliferate forming pro-embryogenic masses (PEMs). Auxin is required for the switching from single cells to PEMs, but it is inhibitory for the development of PEMs into embryos (Nomura and Komamine 1995). The depletion of auxins stops the proliferation of PEMs and embryo development progresses with the formation of protoderm and the root and shoot meristems. During maturation somatic embryos accumulate storage products that exhibits the same biochemical characteristics as those of their zygotic counterparts. However, the amount of the storage product may differ from that of the zygotic embryos (McKersie *et al.* 1995, Yeung 1995).

One of the most interesting aspects of somatic embryogenesis is the similarity between the somatic and zygotic developmental patterns, despite the differences in the physical and chemical environments of both somatic and zygotic embryos (Attree and Fowke 1993). The fact that structurally and developmentally normal embryos can develop from somatic cells, clearly

demonstrates that the genetic information for embryogenesis and the development of somatic embryos is contained within the cell and can function without the maternal gene products. There is a certain possibility of culture components to mimic some of the maternal signals involved in the regulation of embryogenesis (Lo Schiavo 1995).

A basic understanding of the developmental processes that underline the formation of somatic and zygotic embryos is necessary to develop stage specific molecular markers, which can be used to optimize somatic embryogenesis protocols by the fine tuning of easily identifiable, developmentally regulated events (Serk and de Vries 1993). When integrated with conventional breeding, and supported by molecular techniques, somatic embryogenesis can be extremely useful for faster genetic improvement of forest trees (Ravikumar 2001, Serk and de Vries 1993, Walter *et al.* 1998).

1.5.2.2 Somatic embryogenesis in conifers:

The success in the field of *in vitro* propagation of gymnosperms throughout the world has been mostly through somatic embryogenesis, and achieved in a number of cycads and conifer species (Attree and Fowke 1993). Somatic embryogenesis in conifers involves the reactivation of the developmental processes of normal embryogeny (Ruaud and Pâques 1995). The mass of proliferating conifer embryogenic tissue is referred to as “embryonal-suspensor mass (ESM)” due to its organized nature and thus use of the term “callus” is generally avoided. This proliferating embryogenic tissue or mass, continuously initiates immature somatic embryos by cleavage polyembryony (Gupta and Durzan 1987a). The immature somatic embryos are polarized structures organized into an embryonic region subtended by elongate suspensor cells. Thus proliferation of this “embryogenic tissue” can continuously provide unlimited numbers of identical somatic embryos that ultimately grow into trees (Attree and Fowke 1993). This system of proliferating conifers has been commercially accepted and tested successfully by timber and paper pulp industries in countries like USA, Canada and New

Zealand (Adams *et al.* 1994, Chandler and Young 1995, Gupta *et al.* 1993, Timmis 1998).

In genus *Pinus*, however relatively fewer species have been successfully propagated at a large scale. This is mainly due to the recalcitrant nature of the tissue and the limitation of the explants that are responsive to induction of somatic embryogenesis (Attree and Fowke 1993). The most responsive explant in pines has been observed to be the immature zygotic embryo that is extruded out of the megagametophyte *in vitro* and proliferates as embryogenic mass. It is only at a particular developmental stage of the immature zygotic embryo that it gives rise to embryogenic tissue. To be able to obtain the correct stage of immature zygotic embryo in culture is the first major hurdle in the initiation of somatic embryogenesis in pines. This particular responsive period in the seed development in pines is known as the “window” and only the immature zygotic embryos from this stage show embryogenesis (Jones *et al.* 1993). Other conifer genera like *Abies* and *Picea*, have been reported to be less demanding in terms of their *in vitro* response for embryogenesis, as embryogenic cultures could be induced from mature zygotic embryos and even differentiated tissues like needles (Attree *et al.* 1990a & 1990b, Boulay *et al.* 1988, Bourghard and Favre 1988, Chavez *et al.* 1992, Ishii 1991, Tremblay 1990, von Arnold 1987). Table 1.2. lists in brief, the documented work on somatic embryogenesis in *Pinus* species, describing the explant used for induction and the media used for maturation studies.

Table 1.2. Somatic embryogenesis in *Pinus*: explants used for induction and media supplements used for maturation (plant growth regulators and osmoticum)

<i>Species</i>	<i>Explant used</i>	<i>Media supplements for maturation</i>	<i>Reference</i>
<i>Pinus caribaea</i> Morelet.	IZE	15µM ABA, 2-3% sucrose	Laine & David 1990, Laine <i>et al.</i> 1992 David <i>et al.</i> 1995
<i>P. elliotii</i> Engelm.	IZE	Not described	Jain <i>et al.</i> 1989, Newton <i>et al.</i> 1995
	IZE	30µM, 6% maltose	Liao & Amerson 1995b

Table 1.2. continued

<i>P. koraiensis</i> Sieb <i>et</i> Zucc	MZE	Not described	Bozkov <i>et al.</i> 1997
<i>P. lambertiana</i> Lamb.	MZE	No PGR, 1-3% sucrose	Gupta & Durzan 1986
<i>P. nigra</i> Am.	IZE	0.38-31µM ABA, 6-9% maltose	Jasik <i>et al.</i> 1995 Salajova & Salaj 1992, Salajova <i>et al.</i> 1995, 1999
<i>P. palustris</i>	MZE	Not described	Nagmani <i>et al.</i> 1993
<i>P. patula</i>	IZE	1.3mg/l ABA, 3% sucrose	Jones and van Staden 1995
<i>P. pinaster</i>		15µM ABA, 6% sucrose	Bercetche & Paques 1995
<i>P. radiata</i> Don.	IZE	6% sucrose, 2mg/l ABA	Chandler & Young 1995
<i>P. roxburghii</i> Sarg.	IZE	Not described	Arya <i>et al.</i> 2000
	IZE	30µM ABA	Mathur <i>et al.</i> 2000
<i>P. strobus</i> L.	IZE	38µM ABA, 12% sucrose	Finer <i>et al.</i> 1989
	IZE, MZE	80µM ABA, EMM amino acids, 3% sucrose, 1% gellan gum	Garin <i>et al.</i> 1998
	IZE	80-120µM ABA, 1% gellan gum	Klimaszweska & Smith 1997, Klimaszweska <i>et al.</i> 2000
	IZE	37.84µM ABA, 6% sucrose	Kaul 1995
<i>P. sylvestris</i> L.	IZE	32µM ABA, 5% PEG, 6% sucrose	Haggman <i>et al.</i> 1999
		30µM ABA	Hohtola 1995
<i>P. taeda</i> L.	IZE	2.6µM ABA, 6% sucrose	Becwar <i>et al.</i> 1990, 1991 Becwar & Pullman 1995
	IZE	2 µM KIN, 2µM BA, 5µM 2,4-D, 3% sucrose	Gupta & Durzan 1987a, b Gupta 1995
	IZE	Not described	Li & Huang 1996

1.6. Growth studies of embryogenic cultures

The full potential of somatic embryogenesis will only be realized if this system of propagation could be made into a functional, continuous and affordable process that would ensure a supply of good quality somatic embryos whenever required. Suspension cultures have this advantage over the cultures growing on solid medium. Faster and uniform growth rates, continuous culture, less variation in chemical and physical parameters, excellent source of protoplasts and automation, makes culture of cells or tissue in suspension, a

much preferred method (Denchev *et al.* 1992, Gupta *et al.* 1991, Krogstrup 1990, Tautorus and Dunstan 1995).

Before venturing into large-scale production of somatic embryos, it is essential to study the various growth parameters quantitatively for the optimum growth of the cultures in a bioreactor. However, a variety of non-embryogenic plant cultures have been grown in a range of bioreactor types (Panda *et al.* 1989, Scragg and Fowler 1985), there have been fewer reports of use of bioreactors for culture of somatic embryos, for example, carrot (Ammirato 1983), alfalfa (Chen *et al.* 1987, Stuart *et al.* 1987) and sandalwood (Bapat *et al.* 1990). Among conifer species, embryogenic tissue of *Picea abies* has been reported to grow in bioreactors (Tautorus *et al.* 1992).

The present investigation was conducted with the aim of initiating suspension cultures and studying growth pattern and subsequently standardizing the different growth parameters like, initial inoculum: media ratio, pH, temperature, etc., necessary for the optimal growth of the embryogenic cultures, before testing the growth and biomass yields at a larger scale, in a bioreactor.

1.7. Cryopreservation of embryogenic tissues

1.7.1 General aspects

Cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196°C), is the only method currently available to ensure the safe and cost-effective long-term conservation of genetic resources of species that have recalcitrant seeds or are vegetatively propagated (Cyr 2000, Park *et al.* 1998, Pence 1995). This *ex situ* approach of conserving the plant genetic resources includes seed storage, *in vitro* storage, DNA storage, pollen storage, field gene banks and botanical gardens. *Ex situ* conservation implies, conserving outside their natural habitats (Bajaj 1995a & 1995b, Cyr 2000).

Classical cryopreservation techniques involve slow cooling to a defined pre-freezing temperature, followed by rapid immersion in liquid nitrogen. As the temperature reduces during slow cooling, the cells and the external medium initially supercool, followed by ice formation in the medium (Mazur 1984). The cell membrane acting as a physical barrier and prevents the ice from seeding the cell interior and the cells remain unfrozen but supercooled. As the temperature is further decreased, the extracellular solution is further converted into ice, thus concentrating the intracellular solutes. The aqueous vapour pressure of the supercooled cells exceeds that of the frozen extracellular compartment, and the cells equilibrate by loss of water to external ice. In optimal conditions, most or all the intracellular water is removed. This depends on the rate of cooling and pre-freezing temperatures and thus prevents intracellular ice formation on subsequent immersion of specimen into liquid nitrogen. The re-warming or thawing should be as fast as possible to avoid re-crystallization (Bajaj 1995a, Engelmann 2000).

Cell and callus cultures are prone to inadvertent genetic changes resulting from selection pressure imposed on the system through various culture conditions including routine subcultures. This *in vitro* somaclonal variation is being widely used to create additional variability for various biotechnological applications. Cryopreservation strategies could be successfully employed to preserve both, genetic fidelity as well as induce variability (Kantha *et al.* 1988).

1.7.2. Cryopreservation of conifer embryogenic tissues

The importance of clonal forestry lies in the fact that it offers larger genetic gains from selected elite individuals than are possible using sexual offsprings. Due to the long life cycles of tree species, the assessment of desired characters in the clonally propagated plants becomes difficult. More so, when the initial explant for propagation is a seed from open-pollinated natural stand. Somatic embryogenesis has provided means of mass propagating superior clones that are genetically uniform and can be used for production of synthetic

seeds. In this manner, somatic embryogenesis can become a powerful tool, especially when coupled with cryopreservation (Sakai 1995a & 1995b).

Liquid nitrogen based cryopreservation of embryogenic tissue, therefore becomes mandatory since it is considered as means of avoiding the loss of embryogenic potential during long-term subculture (Engelmann 2000, Gupta *et al.* 1987, Kartha *et al.* 1988). Once the somatic embryos of a particular embryogenic cell-line are converted to plantlets, hardened, established in the field and, tested true-to-type for the desired characters, the cryopreserved, 'mother' embryogenic tissue of the selected cell-line can then be proliferated and used for propagation. Another advantage of cryopreservation of embryogenic tissue is in limiting contamination and also evading possible somaclonal variations that may be caused by the long-term maintenance of actively growing tissue. It also cuts down the costs incurred during regular subculturing. Cryopreservation is a very useful technique for preserving genetic resources as well as providing means of storing genetically altered material while field trials are being conducted (Bajaj 1995a, Gupta *et al.* 1991, Gupta *et al.* 1993, Klimaszewska *et al.* 1992, Sakai 1995b).

Most of the work on cryopreservation of cell/callus cultures is confined to angiosperms and only a few reports of successful cryopreservation of embryogenic tissue in conifers based on the storage in liquid nitrogen, which includes only four species of *Pinus* (Table 1.3.). The important aspects of cryopreservation of conifer embryogenic tissue is the pre-treatment with cryoprotectants, slow freezing and fast thawing. Cryoprotectants like DMSO have been commonly used with a combination of sorbitol pre-treatments for conifer embryogenic tissues. The different pre-treatments that have been used to cryo-preserve conifer embryogenic tissue in liquid nitrogen, have been briefly listed in Table 1.3.

Table 1.3. Cryopreservation studies with conifer embryogenic tissue: different pre-treatments and recovery of plantlets

<i>Species</i>	<i>Pre-treatment</i>	<i>Recovery of plantlets</i>	<i>Reference</i>
<i>Abies cephalonica</i>	10% DMSO; 10% PEG, 10% glucose, 10% DMSO	Not reported	Aronen <i>et al.</i> 1999
<i>A. normanniana</i>	0.4M sorbitol, 5% DMSO	Not reported	Nørgaard <i>et al.</i> 1993)
<i>Larix X eurolepis</i>	0.4M sorbitol, 10% DMSO	Plantlets recovered	Klimaszweska <i>et al.</i> 1992
<i>Picea abies</i>	0.4M sorbitol, 10% DMSO	Not reported	Nørgaard <i>et al.</i> 1993
	10% PEG, 8%glucose, 10% DMSO	Plantlets recovered	Gupta <i>et al.</i> 1987c
<i>P. glauca</i> Engelm.	5% DMSO, 0.4M sucrose	Not reported	Cyr <i>et al.</i> 1994
<i>P. glauca</i>	0.4M sorbitol, 5% DMSO	Plantlets recovered	Kartha <i>et al.</i> 1988
<i>P. glauca</i> (Moench) Voss		Plantlets recovered	De Verno <i>et al.</i> 1999
<i>P. mariana</i>	0.4M sorbitol, 10% DMSO	Plantlets recovered	Klimaszweska <i>et al.</i> 1992
<i>P. sitchensis</i>	5% DMSO, 0.4M sorbitol	Plantlets recovered	Find <i>et al.</i> 1993
<i>Pinus caribaea</i>	5% DMSO	Plantlets recovered	Laine <i>et al.</i> 1992
<i>P. patula</i>	0.3M sorbitol, 5% DMSO	Plantlets recovered	Ford <i>et al.</i> 2000
<i>P. sylvestris</i>	10% PEG, 10% glucose, 10% DMSO	Not reported	Haggman <i>et al.</i> 1998
<i>P. taeda</i>	10% DMSO; 10% PEG, 8% glucose, 10%DMSO	Plantlets recovered	Gupta <i>et al.</i> 1987c

1.8. Objectives of the present study

1.8.1. Selection of species

Chir pine and blue pine are the two most widely distributed, economically and ecologically important pine species of the western Himalayas. Together, the distribution of these species dominates region between 800-2500 m above sea level. Intermixed with other gymnosperm and angiosperm tree species, they contribute greatly to the immense biodiversity of the Himalayas (Sahai K. 1996).

Population explosion has caused inhabitation of even the most inaccessible regions in the Himalayan belt, naturally resulting in clearing of forests for agricultural as well as construction purposes. This is in addition to ongoing exploitation of forests for natural products and timber. The efforts taken by the forest departments for afforestation are commendable considering the immensity of the task. Where conventional methods of propagation are already steadily progressing, it is but obvious that newer techniques will give an additional boost to clonal forestry.

The ongoing work by our group on Indian pine species in developing workable protocols for clonal propagation was the basis of the choice of these two pine species for further studies following similar lines. Our group had been instrumental in multiplication of shoots from axillary buds of *Pinus caribaea* (tropical pine introduced in India) and *P. kesiya* (pine species from the eastern Himalayas) (Nadgauda *et al.* 1993), thus *P. wallichiana* and *P. roxburghii* were selected for the present investigations.

The limitations of working with mature or differentiated tissues of pines for clonal propagation, it was decided to work with juvenile tissues, and, seeds were the most obvious choice for initial studies on organogenesis. Additionally, due to inaccessibility because of sheer distance, using good quality starting material for micropropagation, mainly mature buds, was almost impractical. Since the *in vitro* response of any explant is very much dependant on the quality of the starting material. Also, as one of the major constraints in developing genetically engineered trees is the non-availability of a proper regeneration system, it was essential to work with the juvenile material and develop a workable regeneration protocol.

During the course of the studies, a plan was worked out for the collection of immature female cones of chir pine to initiate embryogenic cultures. The sites for collection were identified with the help of Conifer Research Centre, Shimla, and Institute of Himalayan Bioresource Technology, Palampur. Since the induction of embryogenesis in pines is reported to be extremely stage (developmental stage of the immature zygotic embryo) and genotype specific, it was very important to coordinate the collection of correct plant material for the work, thus avoiding any loss of time and effort that was spent in this process of collection.

1.8.2. Aim of the present investigation

Keeping in mind the importance and necessity of developing alternative methods to supplement the ongoing propagation techniques for Indian pines, the present work was initiated with the following aims:

1. To study *in vitro* morphogenetic response in *Pinus wallichiana* for its micropropagation by developing a workable regeneration protocol through organogenesis.

- Optimization of media for initiation of adventitious buds
- Elongation of initiated adventitious buds
- Rooting of elongated shoots
- Hardening and acclimatization of regenerated shoots
- Histological examination of initiated adventitious buds

2. To study *in vitro* morphogenetic response in *Pinus roxburghii* for its micropropagation by developing a workable regeneration protocol through somatic embryogenesis

- Optimization of media and culture conditions for the initiation of embryogenic cultures
- Finding the correct stage of immature zygotic embryos for induction of embryogenic tissue
- Standardization of conditions for proliferation of embryogenic cell lines
- Maturation of somatic embryos
- Conversion and germination of somatic embryos

3. To initiate embryogenic cell suspension cultures of *P. roxburghii* for scaling-up in bioreactor and, cryopreservation of embryogenic cultures.

- Initiating embryogenic suspension cultures

- Growth studies – growth curve, physical and chemical parameters
- Scaling-up of embryogenic cultures in bioreactor
- Testing different pre-treatments with cryoprotectants for cryopreservation of embryogenic cell lines
- Re-growth of cryopreserved embryogenic tissue and checking their viability and genetic fidelity

CHAPTER 2

General materials and methods

General materials and methods have been described in this chapter, and the specific materials and methodologies employed for various experimental procedures during this study, are described separately in chapters 3 - 5.

2.1. Glassware and plasticware

Test tubes (25 x 150 mm), conical flasks (125, 150, 250, 500, 1000 ml capacity), pipettes (1, 2, 5 and 10 ml capacity), measuring cylinders, funnels, etc. used in various experiments were made of Corning or Borosil glass. Locally manufactured jam bottles with polypropylene screw caps were also used. All glassware was cleaned using liquid soap and thoroughly washed in running tap water. Washed glassware was rinsed with distilled water and oven dried before use. Flasks and bottles containing infected cultures were decontaminated by sterilization for 1 h, prior to washing. Culture tubes and flasks were plugged tightly with absorbent cotton prior to autoclaving.

Sterile/disposable transparent polypropylene petri-plates (55 X 15 mm and 85 X 15 mm) from Laxbro®, India Ltd. were used throughout the studies. Auto-pipettes from Tarsons aquipet®, India (0-20µl, 20-200µl and 200-1000 µl) were utilized for accurate addition of fine chemicals in the medium. Autoclavable filter assembly units (Laxbro®, India) were used with 0.22 µm MF Millipore™ filter membranes (mixed cellulose acetate and nitrate) for filter sterilization of small quantities of heat labile chemicals like L-glutamine, abscisic acid (ABA), etc.

2.2. Glassware sterilization

All the empty glassware to be used for media preparation (flasks, bottles, test tubes, pipette tips) and instruments needed for sterile dissection and subculturing (forceps, spatula and scalpel etc.) were autoclaved at 15 lb. in.⁻² (121°C) pressure for 1 hour.

2.3. Chemicals

Various chemicals used and their source is listed in the table 2.1 below. Any other specific chemical used in the present investigation has been mentioned separately in chapters 3 – 5.

Table 2.1. Chemicals and their source

Chemical	Source
Macro - and Micro –nutrients (AR grade)	Qualigens, India.
Vitamins and amino acids.	Sigma Chemical Co., USA.
Plant Growth Regulators	Sigma Chemical Co., USA.
Amino acids	Sigma Chemical Co., USA.
Caesin hydrolysate	Sigma Chemical Co., USA.
Myo-Inositol	Sigma Chemical Co., USA.
Phytigel (gellan gum)	Sigma Chemical Co., USA.
Bacto agar	Difco, USA
Sucrose (ExelAR grade)	Qualigens, India
Activated charcoal	Sigma Chemical Co., USA.
Labolene ®	Qualigens, India
Cetrimide*	ICI, India

* Cetrimide solution contained 20% IP Cetrimide.

2.4. Tissue culture media

For all the experiments during the investigations, DCR basal medium (Gupta and Durzan, 1985) was fortified with different auxins and/or cytokinins or any other test chemicals as mentioned in the following chapters. The other basal media used in the studies were SH (Schenk and Hildebrandt 1972), WPM (Lloyd and McKown 1981), LPm (von Arnold and Eriksson 1981) and BM₁ (Gupta and Pullman 1990). The concentrations of the macro- and microelement salts, organic constituents and amino acids of the basal media used are given below in Table 2.2.

Table 2.2: Composition of basal media: DCR (Gupta and Durzan 1985), SH (Shenck and Hildebrandt 1972), WPM (Lloyds and McKown 1981) and LPm* (von Arnold and Eriksson 1981)

(mg/l)	DCR	SH	WPM	LPm*
Macro-nutrients				
KNO ₃	340	2500	400	1900
NH ₄ NO ₃	400			1200
Ca(NO ₃) ₂ .4H ₂ O	556		556	
CaCl ₂ . 2H ₂ O	85	200	96	180
MgSO ₄ . 7H ₂ O	370	400	370	370
KH ₂ PO ₄	170	300	170	
K ₂ SO ₄			990	340
Micro-nutrients				
MnSO ₄ . 7H ₂ O	29.432	10.0	29.43	22.0
ZnSO ₄ . 7H ₂ O	8.6	1.0	8.6	
H ₃ BO ₃	6.2	5.0	6.2	0.63
KI	0.83	1.0		0.75
Na ₂ MoO ₄ . 2H ₂ O	0.25	0.2	0.25	0.025
CuSO ₄ . 7H ₂ O	0.25	0.1	0.25	0.0025
CoCl ₂	0.025	0.1		0.0025
NiCl ₂	0.025			
FeSO ₄ . 7H ₂ O	37.3	15	27.8	14.0
Na ₂ . EDTA	37.3	20	37.3	19.0

Table 2.2. continued

Vitamins				
Thiamine – HCl	1.0	5.0	1.0	5.0
Nicotinic acid	0.5	5.0	0.5	2.0
Pyridoxine – HCl	0.5	0.5	0.5	1.0
Glycine	2.0		2.0	2.0

* Lpm also contained the following amino acids (mg/l): alanine (0.05), arginine (0.01), cysteine (0.02), glutamine (0.4), leucine (0.01), phenylalanine (0.01), tyrosine (0.01).

2.4.1. Stock and media preparation

All the stock solutions and media were prepared in glass distilled water or Milli-Q (Millipore water purification system) water. Stock solutions of different elements were prepared as 10X or 100X depending on the final concentration of the element in the medium (Table 2.3.). Sucrose, myo-inositol, CH, L-glutamine were always weighed and added at the time of preparation of the medium. Specific growth regulators (apart from those mentioned in table 2.3.) and other media constituents were filter sterilized (if necessary) and added at the time of media preparation.

Table 2.3. Stock solutions

Stock	Concentration	Storage
Major salts	10 X	Refrigeration
Minor salts	100 X	Refrigeration
Na ₂ FeEDTA*	100 X	Refrigeration
Vitamins	100 X	- 20 °C
L-glutamine	25 mg/ml	- 20 °C
2,4-D	5 mM	RT
BA	2 mM	RT
kin	2 mM	RT

* The Na₂FeEDTA stock (total volume 500ml) was prepared by dissolving Na₂EDTA (100X) in 200 ml hot water and to this, 200 ml solution of FeSO₄ (100X) was added drop-wise. The resultant solution was mixed thoroughly and the final volume was made to 500 ml.

After dissolving all the ingredients except for solidifying agent, the pH of the medium was adjusted between 5.8 (unless otherwise stated) using 1.0N

HCl/NaOH. The final volume was adjusted and gelling agent was added for semi-solid media before sterilization. If the media were to be poured in test tubes, bottles or flasks before autoclaving, gelling agent was melted by steaming the medium and then dispensed in the appropriate container. Media was dispensed in petri-plates in laminar airflow only after sterilization.

2.4.2. Media sterilisation

All culture media were autoclaved at 15 lb. in.⁻² pressure (121°C) for 20 minutes. Heat labile chemicals were added in the media in the required quantity after filter sterilization into the autoclaved media.

2.5. Culture incubation conditions

Cultures were incubated in the dark or under illumination of cool white fluorescent light for different duration, depending on the aim of the experiment. The illumination of the light was 10 to 20 $\mu\text{E m}^{-2}\text{s}^{-1}$. The temperature of incubation was $22^{\circ}\pm 2^{\circ}\text{C}$ or $25^{\circ}\pm 2^{\circ}\text{C}$, as required for the optimal growth of the cultures. The suspension cultures were incubated on a rotary shaker at 100 rpm.

2.6. Statistical Analysis

All the experiments were repeated at least 2 – 3 times (depending on the availability of the plant material) with a minimum of 10 - 20 replicates per treatment before conducting the statistical analysis. Various statistical methods for data analysis have been used, like, standard error (S.E.), standard deviation (S.D.), ANOVA, Chi-square analysis, Tukey's HSD Test, and have been described separately in the experimental procedure section of Chapters 3, 4 and 5.

CHAPTER 3

Morphogenetic response of *Pinus wallichiana* A. B. Jacks (blue pine) *in vitro*

3.1 Introduction

3.1.1. Distribution, specific morphological characters and uses of blue pine

Pinus wallichiana A. B. Jacks, the blue or Bhutan pine, commonly known as *Kail*, is found at 1500-3500 m in the Himalayas widely distributed in Kashmir, Himachal and Gharwal hills in the west, spreading to Bhutan and sporadic distribution in Arunachal in the east. It is frequently associated with other conifer genera like, *Cedrus deodara* (Roxb.) G. Don, *Abies pindrow* (Royle) Spach and *Picea glauca* (Moench) Voss. The species is found on a variety of soil types and geographical formations but grows luxuriantly on well-drained moist soil, needing an annual rainfall of 100-200 cm (Maheshwari and Konar 1971).

The blue pine is an elegant tree reaching 30-90m in height on maturity with horizontally spreading branches (Fig.3.1a) and can be identified by the following morphological features. The dwarf shoot has five needles (12.5-20.0 cm long) and the young shoots are glaucous-green in colour. The leaf sheaths (scale leaf, cataphyll and prophyll) are non-persistent. The winter buds are cylindric-conic and may be 0.6-1.2 cm long. The male cones are initiated during October-November and are visible only by April. The male cones, which replace the dwarf shoots, are spirally arranged c. 15 per cluster. The pollen is shed during May-June and carried away by wind. The female cones are narrowly cylindrical, 15-30 cm long with rounded ovuliferous scales that bear winged seeds, the wings are almost thrice as long as the seed. The female cones, 4-5 per cluster, are borne at the tip of a shoot of unlimited growth (Maheshwari and Konar 1971).

Since the yield of resin and oleoresin in the pines from warmer regions is more than the temperate pines, *P. wallichiana* along with the other Indian pine species are used for the resin industry. Turpentine is used as a thinner in the paints and varnishes and a solvent for rosin and laquer. It is also an ingredient in shoe polishes, lubricants, sealing waxes and other products. Rosin, rich in pinene, forms the base material for production of camphor. Turpentine from the resin of *P. wallichiana* contains pinene, which is not present in other Indian pine species. Being a softwood pine, its timber is not preferred for long-term construction purposes but is used in packaging industry. Lopping of lower branches to be used for fuel wood is very common by the locals (Maheshwari and Konar 1971).

3.1.2. Propagation

Blue pine is propagated through nursery-raised seedlings from seeds collected in the natural forest stands. As the species depends on wind for its pollination, it is obvious that not all the seeds would express the desired traits of the mother. Although, the seedlings are selected before planting during their initial growth in the nursery, but that does not overcome the major problem of genetic variation. Conventional methods like cutting, air layering are presently being employed for some of the Indian pine species for the sole purpose of clonal propagation of elite trees (Tewari 1994). These methods were earlier not successfully utilized for propagating gymnosperms. This clearly illustrates an urgent need to develop effective protocols for plantlet regeneration for important Indian pines by tissue culture, not only to supplement the nursery derived seedling transplantation, but also for propagation of selected elite trees.

Due to the inherent recalcitrancy of the mature and differentiated tissues of pines, juvenile tissues like mature zygotic embryos, seedling tissues and cotyledons have always been favored for the in vitro propagation (Chapter 1, Table 1.1.). However, there are a few reports where young axillary buds have been used for shoot multiplication, but they have been faced with the problem of rooting of the shoots. Regeneration protocols for a number of pine species

have been developed using juvenile tissues, mainly mature zygotic embryos, cotyledonary leaves and seedling (Chapter 1, Table 1.1.). Though rooting has been reported in most of regeneration systems, but the protocols are yet to be tested for the feasibility of large-scale production, with the exception of radiata pine (Aitken-Christie 1984, Aitken-Christie and Thorpe 1984, Aitken-Christie and Jones 1987).

3.2 Experimental procedure

3.2.1. Plant material

Mature seeds of *Pinus wallichiana* A. B. Jacks (Fig. 3.1b) were procured from the Conifer Research Centre, Shimla. The seeds were collected from open pollinated trees of a natural stand from the neighboring areas (Conifer Research Centre Forest Nursery at Shillaru; altitude: 2200 m above sea level) and were stored in screw cap jam bottles at 6–8°C (refrigerated) until utilized for the experiments. The germination percentage of the fresh lot of seeds was tested and re-checked at regular intervals. Since the seeds were from a mixed lot collected from open pollinated trees, it was not possible to categorize them by any available method therefore each mature zygotic embryo (MZE) (Fig. 3.1c) from the seed could thus represent a unique genotype.

3.2.2 Surface sterilization of seeds

The soiled seeds were first cleaned with a blade to remove the wing remnants and other dirt sticking to it and then rinsed in running tap water. Surface sterilization treatment was given with the laboratory detergent, (Labolene® diluted to 1% solution for 10 min) and HgCl_2 (0.1%, 5 min) and washed 3-4 times with sterile distilled water subsequently. The HgCl_2 and washing treatments were given in laminar airflow. Before dissecting out the MZEs, the seeds were soaked in sterile distilled water overnight and kept in the refrigerator (4–5°C) to soften the seed coat and to eliminate the non-viable, empty seeds.

Fig. 3.1. (a) *Pinus wallichiana* A. B. Jacks (Blue pine) tree (35-40 year old)
 (Conifers Research Centre, Forest Nursery, Shillaru, H.P.)
 (b) Mature seeds of *P. wallichiana* (bar = 1 cm)
 (c) Megagametophyte (meg) and mature zygotic embryo (mze)
 of *P. wallichiana* (bar = 1 cm)



Fig. 3.1.

3.2.3. Germination

The seed coat of the overnight soaked seeds was aseptically removed and whole megagametophyte (Fig 3.1.c.) was placed in petri-plates containing half strength DCR basal medium (Gupta and Durzan 1985, see Chapter 2, Table 2.2.) with 2% sucrose and 0.6% agar (Qualigens, India) for germination. The pH of the medium was adjusted to 5.8 prior to autoclaving. A total of 100 seeds (four sets of twenty five seeds each) were tested with 12-15 megagametophytes per petri-plate. Percentage germination was noted after 6-8 weeks.

3.2.4. Culture conditions

For all the other experiments, the seeds were dissected in sterile conditions in laminar airflow and only the intact embryos were pre-cultured on half-strength DCR basal medium for 3–4 days to eliminate the damaged and non-viable embryos. Half-strength DCR basal medium refers to 50% concentration of macro- and micro- nutrients. Only the healthy and viable embryos were selected for the induction of adventitious buds. This differentiation between a healthy and non-viable MZE was conducted on the basis of the immediate response of the MZEs in the basal medium. Non-viable embryos turned yellow and the cotyledons remained closed, whereas, the healthy embryos were white, showing slight opening of cotyledons.

Ten MZEs were inoculated per petri-plate (55mm X 15mm). All media were gelled with 0.8% Difco® Agar and pH adjusted to 5.8 before autoclaving. Cultures were incubated at $23^{\circ} \pm 2^{\circ}\text{C}$ under 16 h photoperiod, at $10\text{-}20 \mu\text{E m}^{-2} \text{s}^{-2}$, from cool white fluorescent tubes.

3.2.5. Effect of basal media on adventitious bud induction

Three different basal media were tested during the initial studies: DCR (Gupta and Durzan 1985), SH (Schenk and Hildebrandt 1972) and WPM (Lloyd and McKown 1982). The compositions are listed in Chapter 2, Table 2.2. For this particular experiment, all media contained 2.5 μ M BA, 2% sucrose and 0.8% Difco Bacto Agar. Five sets of experiments were conducted with 50 healthy embryos (selected as mentioned above) for each treatment and the average of the percentage of bud induction in each set was taken as the final observation. Ten MZEs were inoculated per petri-plate (55mm X 15mm). The results were collected after 4-6 weeks of incubation. A set of MZEs dissected out directly from un-soaked (un-hydrated) seeds, were also kept on DCR basal medium with 2.5 μ M BA (as above) to test the effectiveness of the soaking of seeds.

3.2.6. Effect of cytokinins on adventitious bud formation

DCR basal medium was used in all the following experiments, as it showed the best results among the three basal media tested (Table 3.1.). Five cytokinins were tested for the induction of adventitious buds: N⁶-benzyladenine (BA), kinetin (kin), 2-isopentyl adenine (2iP), zeatin (zea) and thidiazuron (TDZ), singly, and in combinations (Table 3.2.). The inoculation, culture conditions, maintenance of culture and analysis of results was conducted as mentioned previously (Section 3.2.4.).

The MZEs were also subjected to BA pulse treatment for bud induction. For this experiment, filter sterilised solution (non-buffered, pH 5.8) of BA at four concentrations (200, 400, 600 and 800 mM) was utilized. MZEs were aseptically dissected out from surfaced sterilized seeds (as mentioned above, section 3.2.4.) and after selection of the healthy MZEs, they were immersed in filter sterilized BA solutions for 30 min., 3 h and 6 h, for each of the concentrations mentioned above. The embryos were then cultured on half-strength DCR basal medium supplemented with 2% sucrose and 0.8% Difco Bacto agar, containing no growth regulators. A control experiment was carried

out by using sterile distilled water in place of BA, for each pre-treatment time (Table 3.4.). Thirty embryos were subjected to each pulse treatment and the experiment was repeated thrice.

3.2.7. Bud elongation

After the induction of buds on the surface of the mature embryos, they were subcultured on to hormone free DCR basal medium (half-strength), with or without 0.05% activated charcoal (Sigma Chemical Co., USA) for elongation. Multiple buds (0.5-1.0 cm) originating from the surface of MZEs were separated out before they were subjected for elongation treatment. The buds were transferred to test tubes for this purpose. DCR basal medium supplemented with GA₃ (3-10 µM) and kinetin (5-15 µM) was also tested for their effects on shoot elongation.

3.2.8. Rooting

Elongated adventitious shoots, approximately 1-1.5 cm long, were then subjected to the following three types of treatments for rooting:

- (i) Pre-treatment with sterile 1mM indolebutyric acid (IBA), 1mM indoleacetic acid (IAA) and a combination of both for a period of 6 h and, subsequent culture on half-strength DCR basal medium containing 1% sucrose and 0.05% activated charcoal, solidified with 0.15% gellan gum (PhytageI™, Sigma Chemical Co., USA),
- (ii) Cultured on semi-solid (gelled with 0.15 % gellan gum) half-strength DCR medium supplemented with 1% sucrose, 0.05 % activated charcoal and different concentrations of IAA and IBA (10, 25 and 50 µM), and,
- (iii) Cultured on half-strength DCR medium (liquid) with 1% sucrose and auxins, IAA and IBA for 3 weeks and subsequent transfer to half-strength DCR medium (liquid) with 1% sucrose, devoid of growth regulators.

(Table 5)

3.2.9. Hardening

After the induction of roots, the adventitious shoots were then transferred for hardening to the sterile sand: soil mixture (garden soil mixed with river sand in the ratio 1:1) moistened with DCR salts (1/4 strength, pH 5.8), in jam bottles. The plantlets were then transferred to polybags, containing the same potting mixture, after 5-6 weeks and kept in the greenhouse for acclimatization at $25 \pm 1^\circ\text{C}$ and $80 \pm 5\%$ humidity.

3.2.10. Histology

MZEs producing multiple adventitious buds on their surface were selected fixed in FAA (formaldehyde: alcohol: acetic acid) in small glass vials. They were then subjected to dehydration treatment using TBA (tertiary-butyl alcohol) series. Then the dehydrated MZEs were embedded in paraffin wax ($58^\circ\text{--}60^\circ\text{C}$) using brass block makers. Sectioning of the samples preserved in wax was carried out using microtome and sections of 8-10 μm thickness were cut and placed on clean glass slides that were smeared with a drop of egg albumin for ensuring that the section remains sticking to the slide during the xylene series and staining procedure. The clear and relevant sections were then selected under the microscope and stained with hematoxylin and eosin after passing them through the xylene series to remove the wax. The slides were then mounted with DPX mountant (Qualigens, India) and a cover slip was placed on the sections. The edges of the slides were sealed with colourless nail paint. The sections were examined and photographed under Zeiss Axioplan 2 microscope.

3.2.11. Quantitative and statistical analysis of data

The MZEs subjected to bud induction were assessed after 4-5 weeks in culture. Each experiment was done at least thrice using a minimum of five replicates with 30-50 MZEs per treatment. The percent MZEs showing bud induction was calculated for each treatment. The mean number of buds per embryo and, standard deviation (S.D.) was also calculated. Based on the

percent MZEs responding and mean number of buds per embryo, the bud forming capacity (BFC) was calculated as follows (Capuana and Giannini 1995, Harry *et al.* 1994, Pulido *et al.* 1990 & 1994, Saborio *et al.* 1997).

$$\text{BFC} = \frac{(\text{Average number of buds per explant}) \times (\% \text{ explants forming buds})}{100}$$

Data regarding percent MZEs responding to each treatment was subjected to Chi-square analysis for significance. The statistical analysis of the discrete data was carried out by analysis of variance (ANOVA) and Tukey HSD test at $P \leq 0.05$ to compare the means.

3.3 Results and discussion

3.3.1. Germination

The viability of the *Pinus wallichiana* seeds was checked at regular intervals by germination. The seed coat (along with the papery nucellus) was removed and the megagametophytes were kept for germination. This facilitated quick response as compared to the seeds germinated without removing the seed coats. It was observed that the percentage germination decreased with time and also, the relatively fresh seeds showed better response to bud induction. Seeds, stored for over a year after their collection, showed drastic decline in percent germination and even in the ability to form adventitious buds, as it can be clearly observed in Fig. 3.2. The bud forming capacity (BFC) index was calculated after culturing the MZEs on DCR basal medium containing 2.5µM BA. It was therefore, necessary to always work with the fresh lot of seeds every year to be able to get the best response of adventitious buds from each mature zygotic embryo.

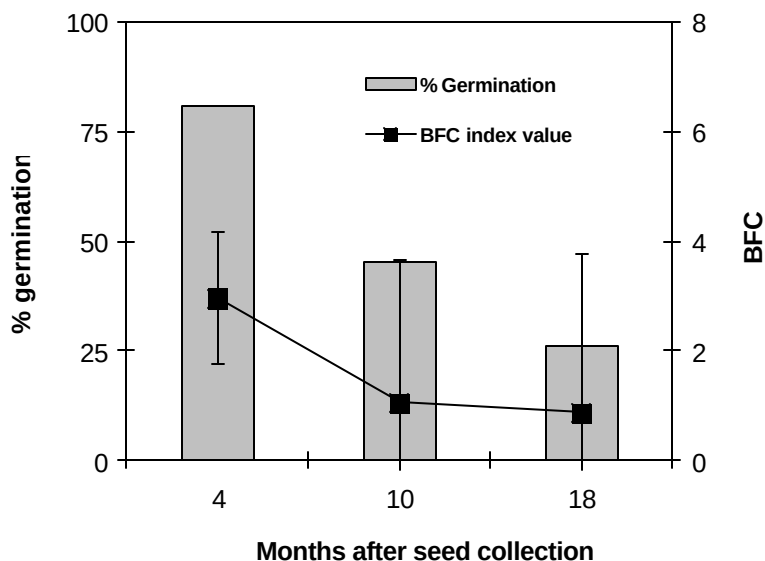


Fig. 3.2. Percent germination of *Pinus wallichiana* seeds - 4, 10 and 18 months after collection. Bud forming capacity (BFC) index of the MZEs tested on DCR basal medium containing 2.5 μ M BA and 2% sucrose observed at the same interval.

3.3.2. Pre-treatment of mature zygotic embryos

The seed of mature *P. wallichiana* is approximately 1.0 X 0.5 cm in size with a hard seed coat and a papery wing that assists in the dispersal of the seeds after the cones have dried and opened up. It was necessary to clean the seeds before the surface sterilization treatment is administered for minimizing the contamination. After the surface sterilization treatment with HgCl_2 , the seeds were soaked in sterile distilled water for soaking overnight. Soaking the *P. wallichiana* seeds overnight in sterile distilled water served the following purpose; it not only softened the hard seed coat and hydrated the embryo, but also eliminated the non-viable/empty seeds as they floated on the surface of the water. The floating empty seeds were removed, the rest were used for the pre-culture treatment after dissecting out the MZE, on half-strength DCR medium for 3–4 days. This also helped in elimination of the non-viable zygotic embryos. The non-viable embryos turned yellowish, showing no indication of

germination and opening of cotyledons. Only the healthy embryos that remained white and showed slight opening of cotyledons, were then transferred to various media combinations for adventitious bud induction, thus ensuring that all the MZEs used in the experiments were healthy and viable. A 10- day old MZE can be seen in Fig. 3.3.a.

Unhydrated (unsoaked) seeds and MZEs without the pre-culture were observed to be much less responsive to the induction of adventitious buds (Table 3.1.). One of the reasons for this was that, the non-viable embryos were not being eliminated, thus decreasing the percentage of the viable and responsive embryos. Another observation was that the response time for bud induction of the unsoaked seeds was longer (4 - 6 weeks) than that of the soaked seeds (3 - 4 weeks). This shows the effectiveness of soaking the seeds and a very efficient method of eliminating the un-responsive MZEs. This closely resembles the work of Flinn *et al.* (1986), who had exposed the MZEs, and not the whole seeds, to liquid culture medium (and not sterile water) before subjecting them for bud induction treatments.

3.3.3. Effect of basal media on adventitious bud induction

Before conducting experiments at a large-scale to test the bud inducing capacity of cytokinins, it was essential to find out, among the few basal media that have been previously used for gymnosperms, which would be most conducive for sustaining the normal growth and would elicit the required effect for the blue pine MZEs. Among the three basal media tested in the initial experiments (Table 3.1.), it was observed that DCR basal medium gave the best results for bud induction in blue pine. This basal medium was then used for all further experiments. A good bud forming capacity (BFC) of 3.11 with 2.5 μ M of BA (Fig. 3.3.b.) was obtained on DCR basal medium as compared to the other two basal media tested (SH and WPM) (Table 3.1.). The advantages of using BFC as an indicator for testing the effect of different media on MZEs, has been highlighted in the following section (3.3.4.).

Various other basal media have also been tried for the induction of adventitious buds in pines. Modified GD basal medium for *P. elliotii* Engelm. (Burns *et al.* 1991), *P. echinata* Mill. and *P. virginiana* Mill. (Jang and Tainter 1991); MCM for *P. roxburghii* Sarg. (Murithi *et al.* 1993) and *P. banksiana* Lamb. (Harry and Thorpe 1994); and, SH for *P. eldarica* Medw. (Sen *et al.* 1994), have been found to be generally suitable for most of the pine species.

The adventitious buds initiate from almost anywhere on the surface of the MZEs, but are mostly concentrated around the base of the cotyledons (Fig 3.3.d.) Although, excised cotyledons from seedlings have been separately utilized successfully as explants in a number of pine species for bud induction. A few notable examples in pines where cotyledons have been successfully used for plantlet regeneration after inducing buds, are as follows: Murithi *et al.* (1993) for chir pine, Saborio *et al.* (1997) for Mexican white pine, Pulido *et al.* (1990, 1992, 1994) for Canary Island pine and Capuana & Giannini (1995) for stone pine.

There are only two other published reports on in vitro propagation of blue pine. In the earlier work, Konar *et al.* (1980) reported bud initiation in blue pine from callus obtained by treating zygotic embryos with NAA (0.1mg/l) and transferring them to medium containing BA (1.0mg/l). However, there was no mention of direct organogenesis, root initiation and transfer of regenerated plantlets to the soil in *P. wallichiana*. In a recent report by Bastola *et al.* (2000), germinating embryos of blue pine were used as explants placed in an inverted position in LP medium containing BA for the induction of adventitious buds. The authors observed that by placing the germinating seedlings in an inverted position, more number of buds per embryo could be produced.

Table 3.1. Effect of different basal media on adventitious bud induction in *P. wallichiana* A. B. Jacks mature zygotic embryos (MZE)

Basal Media ¹	% MZE showing bud induction	Mean no. of buds per MZE $\pm$ S.D. ²	BFC ³ index
DCR (soaked seeds)	61.3 ^a	5.08 $\pm$ 0.30 ^a	3.11
DCR (Un-soaked seeds)	24.4 ^b	2.15 $\pm$ 0.35 ^b	0.52
SH (soaked seeds)	26.6 ^b	2.02 $\pm$ 0.07 ^b	0.53
WPM (soaked seeds)	44.0 ^c	2.46 $\pm$ 0.02 ^b	1.08

¹ All media supplemented with 2% sucrose and 2.5 μ M BA

² Values followed by the different letter are significantly different at $P \leq 0.05$

³ BFC = bud forming capacity (section 3.2.11.)

3.3.4. Effect of cytokinins

The role of cytokinins in adventitious bud induction and shoot differentiation is very well established. There are ample reports in a number of conifers, and also in other gymnosperms and angiosperms, where the positive effect of cytokinins is documented for adventitious bud induction, axillary shoot multiplication and caulogenesis from callus cultures. This aspect of the *in vitro* effect of cytokinins, has been discussed in great detail by George (1993). Conifers generally produce adventitious buds in response to exogenous cytokinins alone, with BA being most commonly used for the purpose. Synthetic cytokinins like BA and 2iP have been observed to be more effective and widely used, as compared to the natural cytokinins (viz. kinetin and zeatin), mainly because of their low cost. Only in a few cases auxins appeared to have superior effects on bud induction in pines. Mott and Amerson (1981) reported bud initiation from excised cotyledons of *P. monticola* on GD basal medium with BA and NAA. Chang *et al.* (1991) also obtained the same results in *P. virginiana* Mill.

Table 3.2. Effect of different cytokinins on adventitious bud induction in *P. wallichiana* A. B. Jacks mature zygotic embryos (MZE)

Cytokinins ¹		Conc. (mM)	% MZE showing bud induction	Mean no. of buds per MZE $\pm$ S.D. ²	BFC ³ index
1)	BA	2.5	61.3 ^a	5.08 $\pm$ 0.30 ^a	3.11
		4.5	59.9 ^b	3.12 $\pm$ 0.06 ^b	1.85
		15.0	73.3 ^a	2.56 $\pm$ 0.03 ^c	1.87
		25.0 μ M - no bud induction			
2)	Kin	2.5	41.3 ^b	2.25 $\pm$ 0.12 ^c	0.93
		4.5	31.7 ^{bc}	2.44 $\pm$ 0.03 ^c	0.76
		15.0 and 25 μ M – no bud induction			
3)	2iP	5.0	29.3 ^c	2.53 $\pm$ 0.10 ^c	0.74
		2.5, 15.0 and 25.0 μ M – no bud induction			
4)	zea	5.0	45.9 ^b	2.09 $\pm$ 0.03 ^c	0.95
		0.25, 0.5, and 15.0 μ M – no bud induction			
5)	TDZ	0.025	80.6 ^d	5.61 $\pm$ 0.02 ^a	4.53
		0.05	64.2 ^a	4.47 $\pm$ 0.02 ^e	2.89
		0.15	58.7 ^a	4.35 $\pm$ 0.23 ^e	2.55
		0.25	24.3 ^c	5.17 $\pm$ 0.67 ^a	1.27

¹ DCR basal medium (half-strength) with 2% sucrose

² Values followed by different letter are significantly different at $P \leq 0.05$

³ BFC = bud forming capacity (section 3.2.11.)

In the present studies, we exposed the blue pine MZEs to growth regulators immediately after dissecting out from seeds. Thus ensuring that the explant is in relatively undifferentiated state at the time of bud induction treatment. As it has earlier been proposed by Yeung *et al* (1981), that high bud forming capacity of cotyledons of *P. radiata* was related to the undifferentiated state of cotyledonary cells at the time of the culture. During the micropropagation studies of *P. ayacahuite*, Saborio *et al* (1997) also demonstrated that the most of the cotyledonary cells kept in culture for more than a week, may lose the ability to differentiate and therefore are unable to respond to bud induction conditions. They observed that the older cotyledons produced shoots only from their apical region, which is very similar to our observations with blue pine MZEs.

Fig. 3.3. Adventitious bud induction from MZEs of *P. wallichiana* (bar = 5mm)

- (a) 10 day old *P. wallichiana* MZE on DCR basal medium containing 2.5mM BA, 2% sucrose and 0.8% agar**
- (b) Adventitious bud induction from MZE of *P. wallichiana* after 3-4 weeks on DCR basal medium with 2.5mM BA, 2% sucrose and 0.8% agar**
- (c) Bud-like structures on the surface of hypocotyl of *P. wallichiana* MZE that did not develop into normal adventitious buds**
- (d) Appearance of adventitious buds clustered around the base of the cotyledons of MZE of *P. wallichiana***
- (e) Abnormal needle-like structures appearing on the surface of *P. wallichiana* MZE, that did not develop into adventitious shoots**

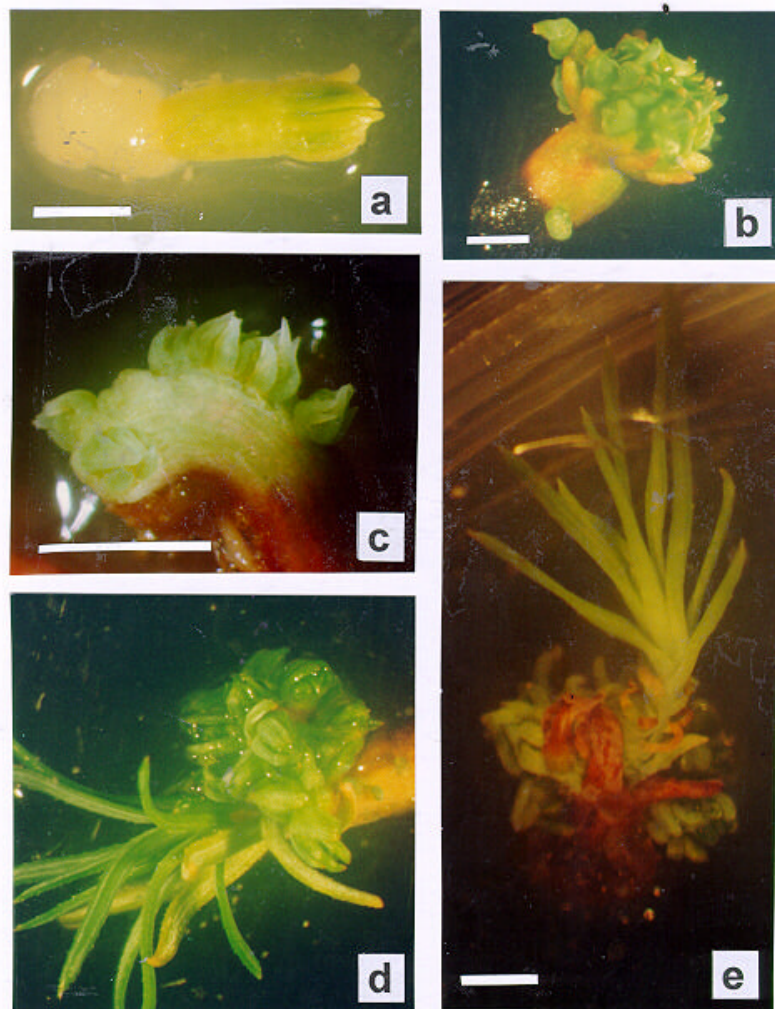


Fig. 3.3.

Among the different cytokinins used during our studies, the effect of kin, 2iP and zeo on the MZEs for bud induction was observed to be very low when used singly, as compared to that of BA and TDZ (Table 3.2.). Most of the MZEs treated with Kin, 2iP and zeo showed initial enlargement and callusing, finally turned brown and died. This is very similar to the other reports, except in *P. canariensis*, where 2iP effectively induced adventitious buds (Pulido *et al.* 1992). TDZ at 0.025 μ M showed maximum bud induction with BFC index of 4.53, followed by BA at 2.5 μ M with a BFC of 3.11. Though there was no significant difference between mean number of buds formed in medium containing 0.025 μ M TDZ, 0.25 μ M TDZ and 2.5 μ M BA, the percent MZEs responding differed significantly (Table 3.2.).

The use of a mixture of cytokinins in tissue culture studies with conifers was first reported by Cheng (1975) and later by Rumary and Thorpe (1984) in white and black spruces. The type of cytokinins used in combination significantly affected the number of buds produced, and for blue pine, it was observed that morphogenesis was not much improved by addition of other cytokinins to BA-containing media. Although, a combination of TDZ (0.05 μ M) and BA (2.5 μ M) also gave equally good adventitious bud induction as compared to the treatment of a single cytokinin (BA or TDZ) (Table 3.3.). It has been observed in pines that cytokinins when used singly, are sufficient to induce adventitious buds (Flinn *et al.* 1986, Pulido *et al.* 1992) but, cytokinin combinations have also proven to be effective. Pulido and co workers (1994) could induce shoots from cotyledonary explants of *P. canariensis* in Bommann's basal medium containing BA, kinetin and even zeatin. They also observed a high BFC index in medium containing BA + kin (BFC = 8.3), BA + zeo (BFC =9.5) and BA + 2iP (BFC = 9.5).

Table 3.3. Effect of different combinations of cytokinins on the adventitious bud induction in *P. wallichiana* A. B. Jacks mature zygotic embryos (MZE)

Cytokinins (mM) ¹	% MZE showing bud induction	Mean no. of buds per MZE $\pm$ S.D. ²	BFC ³ index
BA 2.5 + kin 1.5	57.30 ^a	3.59 $\pm$ 1.00 ^a	2.05
BA 2.5 + kin 2.5	11.24	1.05 $\pm$ 0.52	1.18
BA 4.5 + kin 1.5	34.67 ^b	3.81 $\pm$ 0.12 ^a	1.32
BA 4.5 + kin 2.5	No bud induction, callusing		
BA 2.5 + 2iP 1.5	Callusing		
BA 2.5 + 2iP 2.5	Callusing		
BA 4.5 + 2iP 1.5	Callusing		
BA 4.5 + 2iP 2.5	Callusing, browning		
BA 2.5 + zea 1.5	Callusing		
BA 2.5 + zea 2.5	Callusing		
BA 4.5 + zea 1.5	Callusing		
BA 4.5 + zea 2.5	Callusing		
BA 2.5 + TDZ 0.025	63.30 ^c	4.56 $\pm$ 0.46 ^b	2.88
BA 2.5 + TDZ 0.05	78.21 ^c	5.58 $\pm$ 0.01 ^c	4.36
BA 4.5 + TDZ 0.05	48.10 ^a	2.97 $\pm$ 0.05 ^a	1.42
BA 4.5 + TDZ 0.75	No bud induction		

¹ DCR basal medium (half-strength) with 2% sucrose

² Values followed by different letters are significantly different at $P \leq .05$

³ BFC = bud forming capacity (section 3.2.11.)

In the present study with blue pine, most cytokinin combinations failed to induce adventitious buds in the MZEs. The effect of BA and TDZ when used singly was much better than any other cytokinin used singly. The combination of BA and TDZ produced more buds as compared to kin, zea and 2ip when used singly. This also is the first report showing the effect of TDZ on the adventitious bud induction in any pine species. Although, the BFC index of MZEs treated with TDZ was observed to be higher than BA, but considering the cost analysis for the long-term feasibility of the protocol, BA may be chosen as a cheaper alternative. This work has been presented in symposia and also is published as a research article (Mathur and Nadgauda 1995, 1998 & 1999).

BFC index gives a very definite indication of the efficiency of bud induction (Capuana and Giannini 1995, Pulido *et al.* 1990), as it not only takes into consideration the number of embryos showing induction of buds, but also the number of buds per MZE. The combination of these two factors gives a more realistic determination of the efficacy of a given treatment.

Abnormal bud and needle like structures were also frequently observed among some of the cytokinin combinations that were tested (Fig. 3.3.e). These pseudo-bud-like structures (Fig. 3.3.b.) did not give rise to normal adventitious buds or shoots thereafter. Vitrification or hyperhydration of buds and shoots was also observed in a number of cultures and mostly when there was accumulation of water droplets in the petri-plates due to unexpected rise in temperature by the fluorescent tube lights illuminating the cultures in the lower culture racks. This problem was soon overcome by rearranging the cultures such that, there was no more direct heating and collection of water droplets in the petri-plates.

The tremendous variation observed in the response of the MZEs can be attributed to the genetic variation among the plant material. The fact that the seeds were obtained from a mixed lot of an open-pollinated natural stand implies that every embryo may represent a different genotype. A study among the different selected genotypes would finally and clearly demonstrate the effect of genetic variability on the adventitious bud forming capacity in this species. Since studies involving breeding of trees is still in its infancy in India, there is a non-availability of seeds even from half-sib families and, genotype specific *in vitro* studies are not possible at present. For a tree improvement programme to succeed it is imperative that there is coordination between, and incorporation of the knowledge of traditional breeders, silviculturists and the newer advancements in the field of tissue culture and molecular techniques.

3.3.4.1. Histology of adventitious buds:

MZEs with initiating adventitious buds were histologically examined to observe the development of the buds from its surface (Fig. 3.4.a.). Developing

Fig. 3.4. (a) Longitudinal section of multiple adventitious buds arising from the surface of MZE of *P. wallichiana* after 4 weeks in culture (bar = 0.5 mm)

(b) Longitudinal section of a single adventitious bud arising from the surface of MZE of *P. wallichiana* showing the densely nucleate apical meristematic dome (apm) and over hanging initial needles (n) (bar = 0.5 mm)

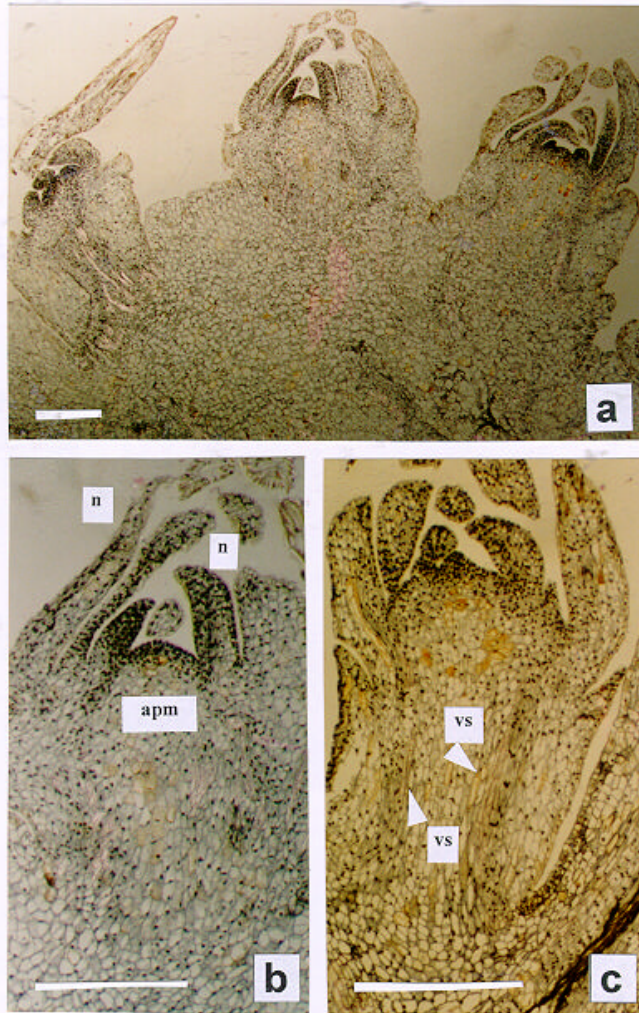


Fig. 3.4.

shoot apical meristems can be observed in the MZE explant after 3-4 weeks culture in cytokinin containing medium. All the cells in the apical dome have a prominent nucleus and cytoplasm was stained densely (Fig. 3.4.b). The apical meristem is surrounded by well-developed overhanging needle-like leaf primordial (Fig. 3.4.b). The developing vascular connection of each bud with the MZE can also be seen in the section (Fig. 3.4.c).

3.3.5. BA pulse treatment

This experiment was conducted with an aim to reduce: i) exposure of the explant (MZE) to growth regulators, ii) period of bud induction and, iii) material and labour involved in the maintenance of the culture. Pulse treatment with BA also promoted equally good bud induction at 400mM for 30min with a BFC of 3.78 (Table 3.4.). Bud induction was also observed at other concentrations tested in the experiment, but results were not very efficient in terms of BFC index. The control with sterile distilled water did not show any bud induction.

All the three objectives, for which the pulse treatment was tried, were met in this experiment. Since the best observed BA pulse treatment given to the MZEs was of half an hour, their exposure time to cytokinin reduced drastically. Also, the appearance or the induction of buds on the MZEs was observed within 15 days of culture as compared to 3-4 weeks where BA was administered in the medium. Thirdly, as the treated MZEs were directly placed on the hormone-free medium, the subcultures did not require any further addition of growth regulators in the medium. The adventitious buds induced by this method were subjected to elongation and gave rise to normal shoots that converted to plantlets after rooting treatment.

In a similar study with *Pseudotsuga menziesii*, Goldfarb *et al.* (1991) did achieve better results with pulse treatment in as compared with the long exposure to cytokinins when given in the medium, whereas, Pulido *et al.* (1992) did not observe any significant difference in bud induction within these two different approaches of administering cytokinins in *Pinus canariensis*.

Pulsing methods have been attempted with *Picea abies* (Bomman 1983, von Arnold and Eriksson 1985, von Arnold *et al.* 1988), *Pinus strobus* (Webb *et al.* 1988), and *Picea engelmannii* (Patel and Thorpe 1986). Vacuum infiltration has also been tried with Norway spruce, and not surprisingly the results from all these different methods have been conflicting. Positive results are only reported for Douglas fir and Norway spruce, using both excised cotyledons and whole embryos.

Table 3.4. Effect of BA pulse on adventitious bud induction in *P. wallichiana* A. B. Jacks mature zygotic embryos (MZE) cultured on half-strength DCR basal medium with 2% sucrose.

	Pre-treatment time	BA conc. (mM)	% MZE showing bud induction	Mean no. of buds per MZE $\pm$ S.D. ¹	BFC ² index
1	30 minutes	200 - no bud induction			
		400	70.4 ^a	5.38 $\pm$ 0.01 ^a	3.78
		600	46.6 ^b	5.20 $\pm$ 0.29 ^a	2.42
		800 - no bud induction			
2	3 hours	200	50.6 ^b	4.46 $\pm$ 1.20 ^b	2.25
		400	40.2 ^c	4.80 $\pm$ 0.21 ^b	1.92
		600 – no bud induction			
		800 – no bud induction			
3	6 hours	no bud induction at any concentration			

¹ Values followed by different letters are significantly different at $P \leq 0.05$

² BFC = bud forming capacity (section 3.2.11.)

These promising initial studies of pulse treatment in blue pine have paved ways for re-defining of the existing micropropagation protocol. The incorporation of pulse-treatment method into the regeneration protocol of blue pine and its refinement for maximum production of plantlets is still in progress as we are sure enough that this would certainly cut down the production cost drastically.

3.3.6. Bud elongation

Bud development and shoot formation phase in micropropagation of conifers involves the development of nodular tissue formed during bud induction into shoots with primary needles (Pulido *et al.* 1990). The formation of true apices with juvenile leaf primordial requires transfer onto a medium devoid of any growth regulators. Thorpe and Biondi (1984) demonstrated this in *P. roxburghii* where bud growth was promoted by transferring the cotyledons after 2 weeks on cytokinin-containing medium to half strength basal medium devoid of any hormones.

In the present investigation, elongation of the adventitious buds was the best observed while using half-strength DCR medium with 2% sucrose and 0.05% activated charcoal. Multiple buds (0.5-1.0 cm in length) of blue pine were separated out and transferred to elongation medium in test tubes, jam bottles and flasks. Incorporation of GA₃ and kin in the medium in different concentrations had no positive effect on the elongation of the buds. Fig 3.5.a. shows multiple buds arising from MZE, before separating them for elongation. This observation is very much similar to the other reports (Murithii *et al.* 1991, Pulido *et al.* 1990) where low concentration of activated charcoal has proven to be effective for elongation of adventitious buds by adsorbing phytohormones and other phenolic substances (Fridborg *et al.* 1978). Murthii *et al.* (1993) transferred the well formed and developing adventitious buds of *P. roxburghii* (5 weeks after induction) to a medium containing 0.05% activated charcoal, 0.8% agar and 3% sucrose to facilitate the elongation of buds. While studying the control of shoot elongation in *P. ponderosa*, Lanner and Connor (1988) could deduce that, it was the number of fascicles that determined the amount of basipetal auxin transport and ultimately, the elongation of shoots.

Separating the buds before giving the elongation treatment proved beneficial for *P. wallichiana*, as it was observed that it allowed faster shoot elongation but, in few cases it also lead to necrosis. Allowing the buds to elongate without dividing them was observed to be completely detrimental for

Fig. 3.5. **(a) Multiple buds (un-separated) arising from *P. wallichiana* MZE kept on DCR basal medium supplemented with 2% sucrose, 0.05% activated charcoal and 0.6% agar, for elongation**
(b) Elongated adventitious buds of *P. wallichiana* on DCR basal medium with 2% sucrose, 0.05% activated charcoal and 0.6% agar

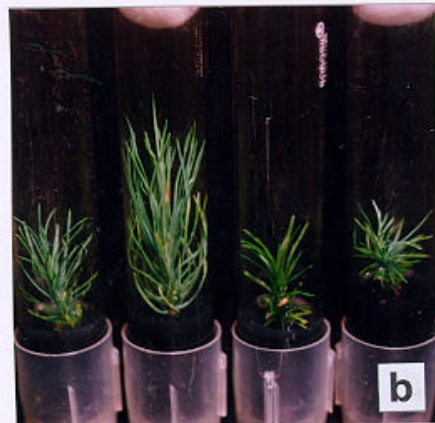


Fig. 3.5.

further development of the shoots. Elongated shoots, without root development can be observed in Fig 3.5.b.

3.3.7. Rooting and hardening

Elongated shoots, 1-2 cm in height, were subjected to various rooting treatments (Table 3.5.). A pulse of IAA and IBA solutions (1mM each) for 6h and subsequent transfer to semi-solid half-strength DCR medium containing 1% sucrose and 0.05% activated charcoal gave the best rooting frequency. IBA was clearly observed to be the preferential growth regulator for the induction of rooting. Fig 3.6.a. shows root initiation from the base of the adventitious bud. Addition of activated charcoal seemed to be beneficial for the rooting either by inhibiting the light at the shoot base or by adsorbing the rooting inhibitors, as suggested by Dumas and Monteuiis (1995). Most commonly, a single root developed from each of the adventitious shoot and in some explants secondary roots were also observed.

The adventitious shoots with initiated roots were transferred to sterile sand: soil (1:1) mixture and were kept in the green house for hardening after 5-6 weeks. The survival percentage was 64.4 after 5 weeks and 45.7 after 6 months in the green house. An elongated, well-developed root system can be observed in Fig. 3.6.b, where the plantlet has been removed from the sand: soil mixture after 5 weeks, for photography. Supriyanto and Rohr (1994) have reported marked improvement and survival of regenerated plantlets in which mycorrhizal fungus was inoculated with perlite mixture in *Pinus sylvestris*. Similarly, Normand *et al.* (1996) have also used ectomycorrhizal fungus *Hebeloma cylindrosporum* for inducing in vitro rooting of micropropagated cutting of *P. pinaster* and *P. sylvestris*.

Rooting in conifers is a slow and problematic procedure as compared to rooting of herbaceous plants. Exogenous auxin application and a reduction of minerals and sucrose concentrations are some requirements for successful rooting (Rumary and Thorpe 1984). Agar-solidified medium has been a

Fig. 3.6. (a) Root induction in adventitious shoots of *P. wallichiana* after 6 h pulse treatment with 1mM each of IAA and IBA and subsequent culture on DCR basal medium (half strength), 1% sucrose and 0.15% gellan gum
(b) Rooted plantlet of *P. wallichiana* originating after adventitious bud induction from MZE

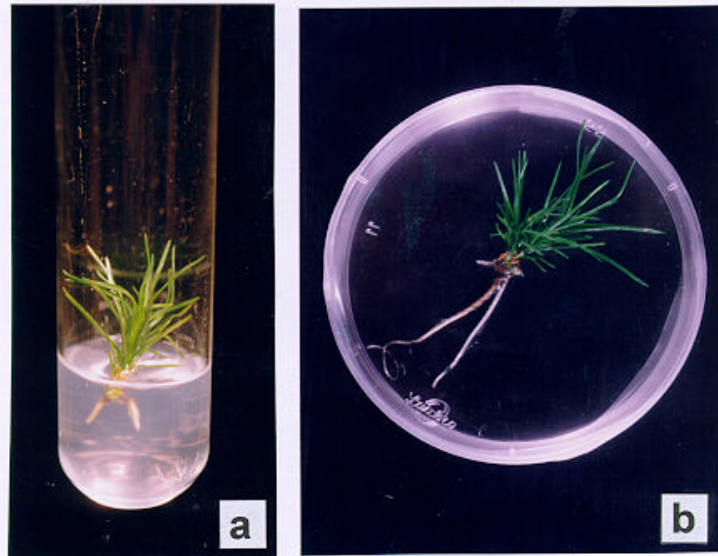


Fig. 3.6.

commonly used substrate, but it probably impedes gas exchange as well as production of root hairs (Pulido *et al.* 1990).

Table 3.5. Effect of IAA and IBA on root formation in *P. wallichiana* A. B. Jacks adventitious shoots

	Treatment type	Concentration	% shoots with well formed roots
1	Pulse		
i	IAA - 6 h.	1mM	--
ii	IBA - 6 h.	1mM	27.4
iii	IAA + IBA - 6 h.	1mM each	79.5
2	Semi-solid medium (DCR 1/2, sucrose 1%, gellan gum 0.15%, activated charcoal 0.05 %)		
i	+ IAA	(10 µM, 25 µM, 50 µM)	--
ii	+ IBA	(10 µM, 25 µM)	--
		50 µM	38.9
3	Liquid Medium (DCR 1/2 + 1% sucrose)		
i	+ IAA	(10 µM, 25 µM)	--
		50 µM	19.7
ii	+ IBA	10 µM	--
		25 µM	32.5
		50 µM	45.2

Murithii *et al.* (1993) achieved high percentage and multiple rooting in adventitious shoots of *P. roxburghii* after incubation in 0.5µM NAA in semi-solid medium, than with a pulsing treatment of 100µM IBA. In Virginia pine, culturing on IBA-containing medium about 50% rooting was observed (Saravitz *et al.* 1991). NAA has been also been used for rooting in *P. elliotii* (Bronson and Dixon 1991). With many conifers auxin pulse followed by *ex vitro* rooting has been successfully managed, as in, *Larix occidentalis* (Harry *et al.* 1991), *P. canariensis* (Pulido *et al.* 1990), *P. halepensis* (Lambardi *et al.* 1993) and *P. pinea* (Capuana and Giannini 1995). These results are very similar to our observations with the rooting treatment and development of

plantlets in *P. wallichiana*. McAfee and co workers (1993) could induce rooting in adventitious shoots (from MZEs) of *P. monticola* and *Larix laricina* by co-cultivation with *Agrobacterium rhizogenes*.

This protocol, starting from MZEs as explants, has proved to be efficient for regenerating plantlets of blue pine and, can be effectively utilized in the genetic transformation studies in the future. Pulse treatment can be effectively used to cut down the production cost if incorporated in the present regeneration protocol. With the availability of high quality hybrid seeds, this method can also be standardized for large-scale propagation of other Indian conifer species.

3.3.8. Conclusions

During the present studies of morphogenesis in *Pinus wallichiana* A. B. Jacks mature zygotic embryos aimed towards developing a protocol for regeneration, the following notable observations were made:

- Mature zygotic embryo (MZE) is a very suitable explant for inducing adventitious buds.
- The bud forming potential of MZEs extracted from seeds that have been collected and stored for more than 6-8 months show a drastic decline. It is therefore very essential to always utilize fresh seeds for bud induction.
- Soaking of seeds in sterile water and a pre-treatment of MZEs was found to be an efficient method to eliminate the non-viable MZEs.
- Using MZEs and not the germinated seedling or cotyledon as an explant saved that much time for adventitious bud induction.
- Administering exogenous cytokinins either in the medium or as pulse treatment was observed to be effective for adventitious bud induction.
- Single cytokinin was found to be sufficient for adventitious bud induction as compared to the application of cytokinin combination.
- Both BA and TDZ were effective in eliciting bud induction in MZEs.
- BA was a preferred choice over TDZ considering the cost effectiveness of the protocol, although the bud forming capacity (BFC) index of TDZ was more than BA.

- BFC index gives an accurate indication and effectiveness of a particular treatment as it takes into consideration both the number of embryos showing bud induction and number of buds formed per embryo.
- Incorporation of pulse-treatment method to the regeneration protocol of blue pine will surely reduce the time taken for inducing buds on MZEs.
- Pulsing the MZEs with 400mM BA for 30 min was enough for eliciting bud induction after transferring to hormone-free medium.
- Bud formation observed within 3 weeks when cytokinin was administered as a pulse as compared to 4 –5 weeks when given in the medium.
- A change from bud induction medium to a medium lacking growth regulators and containing activated charcoal was essential and helped in elongation of the adventitious buds.
- Pulsing with auxins IBA and IAA was observed to be efficient method for root formation in the elongated adventitious shoots.
- Approximately 50% of the hardened plantlets survived after six months in greenhouse conditions.

Part of this work has been presented and published as:

Mathur G., Nadgauda R.S.: Adventitious bud induction in Chir pine (*Pinus roxburghii* Sarg.) and Blue pine (*P. wallichiana* A. B. Jacks)

Poster presented In: All India Symposium on Forest Advances in Biotechnological Applications of Plant Tissue and Cell Culture, CFTRI, Mysore, June 22-24, 1995

Mathur G., Nadgauda, R.S.: *In vitro* plantlet regeneration of Blue Pine (*Pinus wallichiana* A. B. Jacks).

Paper Presentation and Abstr. In: National Symposium on Commercial Aspects of Plant Tissue Culture, Molecular Biology and Medicinal Plant Biotechnology, Centre for Biotechnology and Department of Botany, Jamia Hamdard University, New Delhi, February 25-27, 1995

Mathur G. and Nadgauda R.S.: *In vitro* plantlet regeneration from mature zygotic embryos of *Pinus wallichiana* A. B. Jacks.

PLANT CELL REPORTS (1999) 19: 74-80

Somatic embryogenesis in *Pinus roxburghii* Sarg. (chir pine)

4.1 Introduction

4.1.1. Specific morphological characters, distribution and uses of chir pine

Pinus roxburghii Sargent or chir pine, forms an important feature of the major flora in the lower altitudes of western Himalayas. Apart from timber and fuel-wood, indiscriminate tapping for resin and lopping for fuel-wood from chir pine, is adding on to the already existing pressure on the ecology of the area (Chaturvedi 1998). Elite trees producing high quality resin are mostly selected, propagated and tapped for resin by the forest department and other non-government agencies by the conventional methods (Maheshwari and Konar 1971, Tewari 1994). Chir pine is the primary source of oleoresin in India. The value of oleoresin as a raw material in resin and turpentine industries viz. paints and varnishes, perfumery, pharmaceuticals, paper, soap, textiles and shoe polish etc. is well documented. Over the years the resin production has been on a decline, with over 74,000 tonnes in 1975-76 to 28-30,000 tonnes in 1990-91. The reason for this drastic fall in resin production has been attributed to unscientific and indiscriminate blazings by traditional methods, frequent forest fires in chir pine forests and clearing of forests for various reasons (Choudhari *et al.* 1996).

4.1.1.1. Morphology:

Pinus roxburghii Sarg. is the most important among the principal pines found in India. It's an evergreen tree with a crown that spreads on maturity into umbrella shape with massive branching system and the tree may reach 30 – 55 m in height with a 6-10 m girth (Fig 4.1.a). The young shoots are pale brown and the winter buds are ovoid and non-resinous. In the older trees the plates of the bark measure up to 60 cm in length, 10 cm in breadth and 3 cm in

- Fig. 4.1.**
- (a) *Pinus roxburghii* Sarg. tree (30-35 year old) (Conifers Research Centre, Forest Nursery, Solan, H.P.)**
 - (b) Immature female cones of *P. roxburghii* (bar = 5 cm)**
 - (c) Mature seeds of *P. roxburghii* (bar = 1 cm)**
 - (d) Megagametophyte (meg) and mature zygotic embryo (mze) of *P. roxburghii* (bar = 0.5 cm)**
 - (e) White glistening non-embryogenic callus (nec) (marked with arrow) from MZE of *P. roxburghii* on DCR basal medium with 2% sucrose 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 20mM NAA and 0.6% agar.**

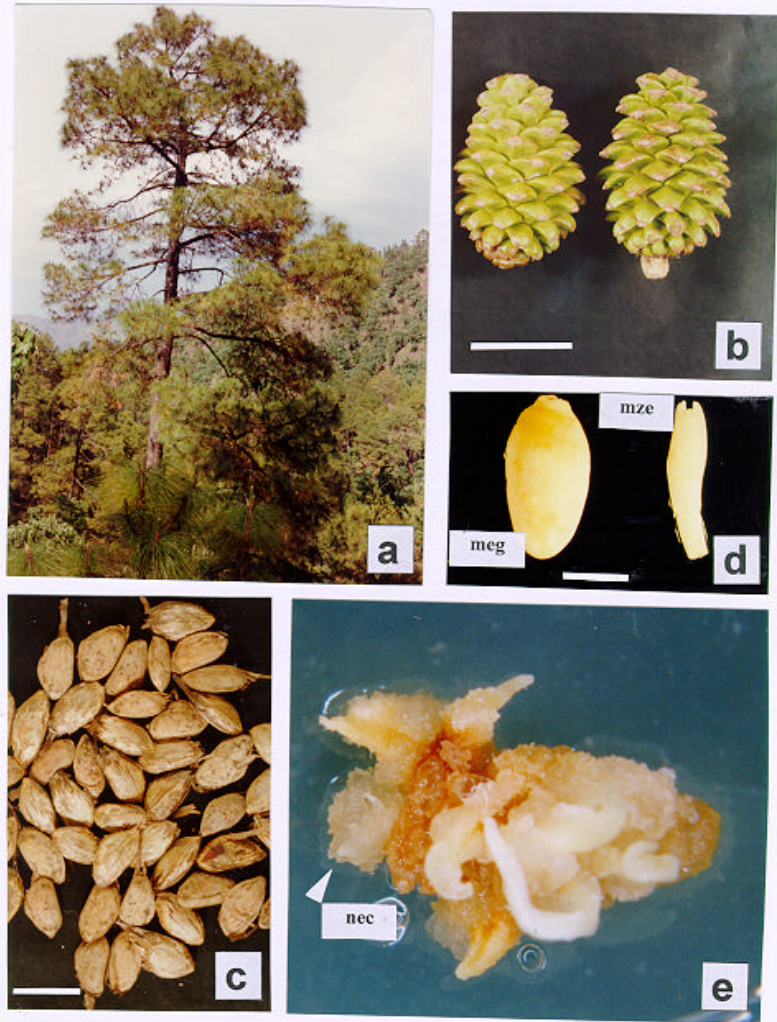


Fig. 4.1.

thickness. The leaves or needles are acicular, 1.5 – 6.5 cm long in young seedling and are glaucous–green, sharply serrulate. Dwarf shoots bear three needles that may grow up to 15-30 cm long (Maheshwari and Konar 1971, Tewari 1994).

Chir pine, like all other gymnosperms is a sporophyte and is monoecious, bearing male and female cones or strobili on different branches of the same plant. Male cone is 1.3-1.8 cm long and consists of a cluster of 100-140 spirally arranged microsporophylls. The female cones are borne on the tip of the long shoot mostly on the upper branches of the tree. Two to four cones may be found in a group. Each cone comprises 80-90 ovuliferous scales or megasporophylls in the axil of the bract arranged spirally on the cone axis (Fig. 4.1.b.). The ovuliferous scale and the bract scale are collectively called as seed-scale-complex. Cones are 10-20 cm long and 7.5-15 cm wide, ovoid, short stalk and stout (Fig 4.1.b.). The scales are woody, thick and hard and bear seeds that are 8-16mm long and winged (Fig. 4.1.c.) (Maheshwari and Konar 1971, Tewari 1994).

4.1.1.2. Distribution:

Pinus roxburghii Sarg. is a native of outer ranges and valleys of the Himalayas from Afghanistan in the east to Arunachal Pradesh in the North Eastern India between 450-2300 m altitude, where the full force of the monsoon is felt. It covers wide areas as pure forests and is also associated with other species like *Cedrus deodara*, *Pinus wallichiana*, *Quercus leucotricophora*, *Rhododendron arboreum*, *Lyonia ovalifolia*, *Myrica esculenia*, etc. in the upper limits. In the lower limits the important associates are *Shorea robusta*, *Anogeissus latifolia*, *Ougenia oogeinensis*, *Buchanania lanzan*, *Bauhinia semla*, etc. The undergrowth of chir pine forests varies with the locality (Tewari 1994).

The female cones for the present studies were collected from Simla and Palampur in the state of Himachal Pradesh. In this region, pure chir pine forests

are abundant in Kangra valley (450-1050m), Chamba (900-1350m), Dalhousie (1200-1500m), Kullu valley (1050-1800m) and Simla (450-1300m).

4.1.1.3. Uses:

Chir pine is mainly exploited for its non-wood products than for the timber. It has a limited usage as timber due to its non-durable nature and the wood also is not suitable for manufacture of plywood due to the large amount of extractives present in it. Although after various preservation treatments the wood has been shown to be useful for specific applications (Tewari 1994). Unlike the developed countries where coniferous wood is the chief source of pulp and paper production, in India only limited investigations have been carried out at the Forest Research Institute, Dehradun, with respect to pulp and paper making characteristics from chir pine (Guha 1958).

In pines, the oleoresin is formed and translocated in resin canals occurring in the root, wood, inner bark of stem and leaves (Mutton 1962). Chir pine yields commercially important oleoresin, which forms the raw material for resin and turpentine industry in India. Resin is obtained by tapping standing pine trees. Numerous improved methods have been devised by Forest Research Institute, Dehradun, for efficient tapping of resin (Tewari 1994). Turpentine is used in preparation of paints and varnishes, shoe and other polishes and as a solvent for resins. Pine oil is used in perfumery industry, as antifoaming agent, solvent and thinner. Rosin and its derivatives are extensively used in soap making and paper industry. It is also required in manufacture of linoleum, sealing wax, lubricants, paints, cosmetics etc. Pine needles are a very good source of essential oil, which is obtained after distillation and finds numerous uses (Maheshwari and Konar 1971, Tewari 1994).

4.1.2. Embryology of chir pine

The embryology of chir pine has been studied in great detail by Konar and Ramchandani (1958), Konar (1960, 1963) and, Maheshwari and Konar (1971). A brief mention of the embryology of chir pine is important at this juncture, since it will not only provide a reference comparison to somatic embryogenesis, but also will aid in understanding the importance of the stage specific response of the immature zygotic embryos in the induction of somatic embryogenesis. In Fig 4.2., a flow diagram, based on Konar (1960) of the life cycle of *Pinus roxburghii* Sarg., can be seen indicating the important stages of the of micro- and megasprogenesis, male and female gametophyte development, up to the seed dispersal.

According to our findings during the present investigations, we have also incorporated in the flow diagram an indication of the 'window' that appears for 2-3 weeks in the life cycle of three years, and is the stage of the immature zygotic embryos that is most responsive for induction of somatic embryogenesis. This has been described separately in section 4.1.2.1.

In the lower altitudes, the pollination in chir pine occurs during March-April while it occurs a little later in the higher regions. Large amounts of pollen are shed and carried away by wind. After the pollen is deposited in the nucellus and the tube nucleus migrates in to the pollen tube, the ovuliferous scales of the female harden indicating the start of the resting phase. Fertilization takes place only in the next year after the activity of the male gametophyte is renewed.

A diagrammatic representation of the developmental stages of the chir pine zygotic embryo can be seen in Fig 4.3. (after Konar 1960, Owens and Molder 1985). Embryo development in *Pinus* is described as free nuclear, because the zygotic nucleus divides after fertilization, to form 2, 4, or 8 nuclei that migrate to the base of the fertilized archegonium.

During the embryonal development, formation of rosette tier takes place, followed by suspensor and embryonal tiers. The cells of suspensor tier elongate and thrust the embryonal cells into the gametophytic tissue resulting in formation of 'corrosion cavity'. Proembryos appear due to the division of

embryonal initials. It is normally observed that the terminal embryo grows faster and its development takes about six weeks from the fertilization. The growing embryo is smooth paraboloid structure, with a suspensor at the proximal end. The active division the suspensor cells finally gives rise to the root cap. This is shortly followed by the development of the cortical tissue, cotyledons and the shoot apex. Most commonly, a solitary embryo matures, and rarely two embryos may reach maturity (polyembryony).

4.1.2.1. The 'window':

In addition to the genotype dependence, one of the major bottlenecks in the induction of somatic embryogenesis in pines is the stage specificity of the explant. Immature zygotic embryo, only at a particular stage of development, has been observed to be capable of giving rise to embryogenic mass. This stage of zygotic embryo development occurs approximately 2-3 weeks after fertilization for 10-15 days, and is known as the 'window' (Fig. 4.2.). At this stage of development, the embryonal head is approximately at the 16 - 32 cell stage. A smooth paraboloid structured embryo is too late a stage to give rise to the embryogenic tissue. However, there have been a couple of reports of induction of embryogenesis from mature zygotic embryos in pines, but that seems mostly an exception rather a rule.

Fig. 4.2.

**TIME
SCALE**

***Pinus roxburghii* Sarg.: LIFE CYCLE SHOWING PERIODS OF MICRO & -
MEGASPOROGENESIS, MALE & FEMALE GAMETOPHYTE DEVELOPMENT
AND, EMBRYONIC DEVELOPMENT**

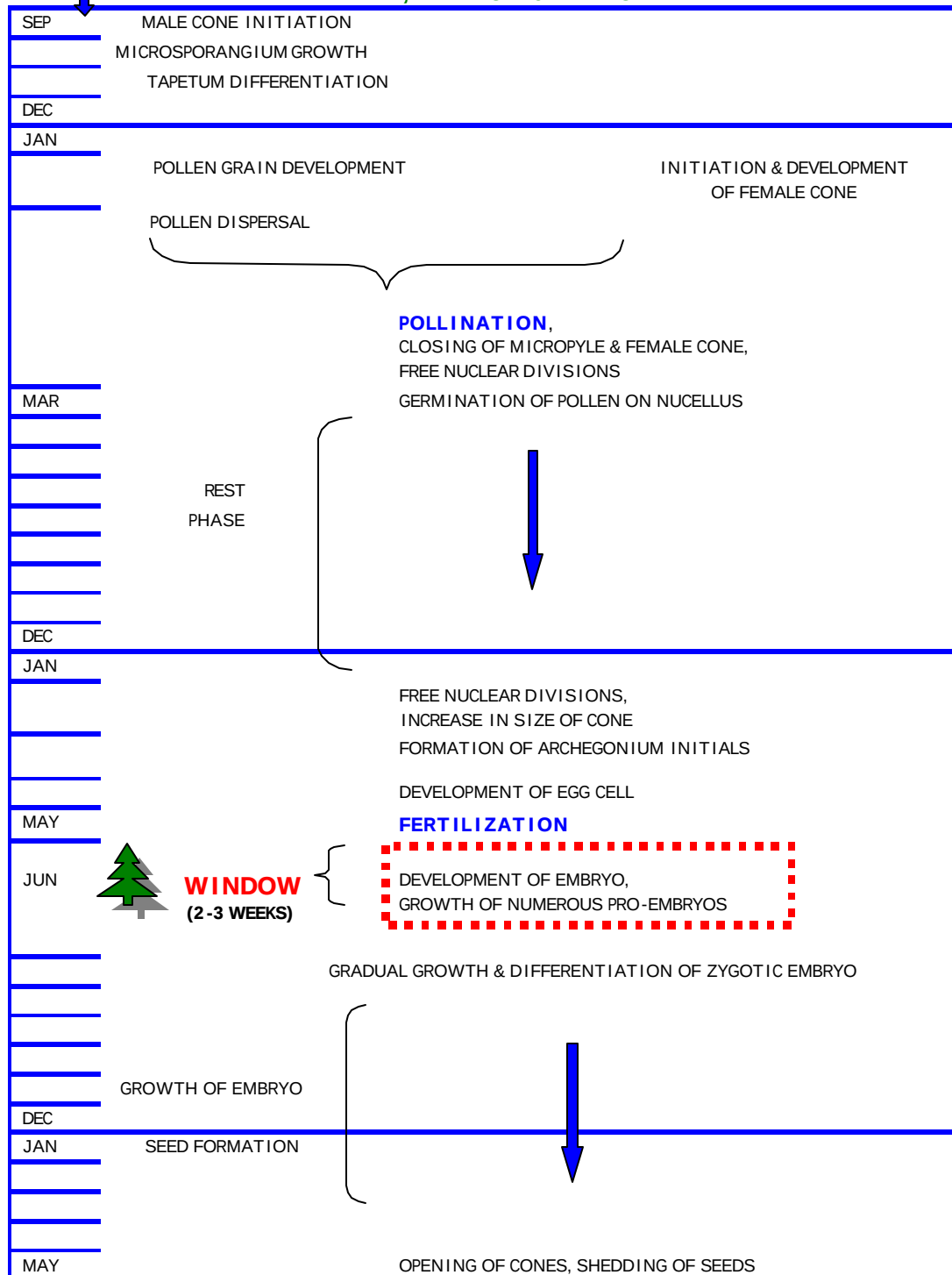
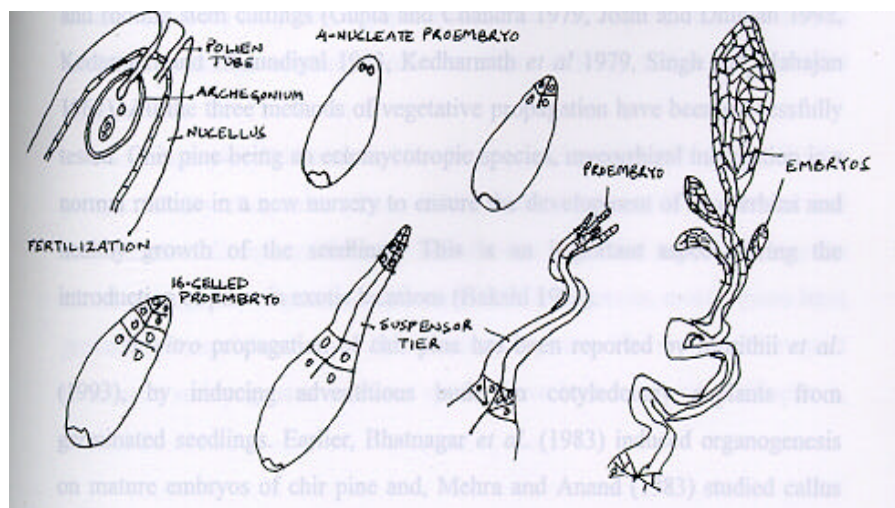


Fig 4.3. Diagrammatic representation of the developmental stages of immature zygotic embryo of *Pinus roxburghii* Sarg. (after Konar 1960, adaptation from Owens and Molder 1985)



4.1.3. Propagation

Chir pine is a strong light demander and requires well-drained soils. It is one of the least exacting species among Himalayan trees and grows on bare areas, and the most favored geological formation is the quartzite rock. Natural regeneration from seeds dispersed on the forest floor occurs if sufficient rain is received and conditions are favorable. Being a pioneer species, it is less demanding on the soil and nutrients than other conifers (Tewari 1994). Artificial regeneration by nursery-germinated seedlings is the most common and by far the most successful method of propagating chir pine. Seeds are collected during the good-seed years as they show better response than the seeds from lean-seed years.

The realization of the importance of vegetative propagation of chir pine for yield and variety improvement, and for the establishment of clonal seed orchards and clonal plantations, careful selection of elite trees is important. Species like chir pine, blue pine and khasi pine, which occur in vast areas, are differentiated into many provenances and races. To exploit the natural variability, selection of desirable seed source is the first step to tree improvement. Efforts have been taken by Forest Research Institute, Dehradun,

to vegetatively propagate elite varieties of chir pine by grafting, air layering and rooting stem cuttings (Gupta and Chandra 1979, Joshi and Dhiman 1992, Kedarnath and Dhaundiyal 1963, Kedhamath *et al* 1979, Singh and Mahajan 1967). All the three methods of vegetative propagation have been successfully tested. Chir pine being an ectomycotrophic species, mycorrhizal inoculation is a normal routine in a new nursery to ensure the development of mycorrhiza and healthy growth of the seedlings. This is an important aspect during the introduction of pines in exotic locations (Bakshi 1974).

In vitro propagation of chir pine has been reported by Murithii *et al.* (1993), by inducing adventitious buds on cotyledonary explants from germinated seedlings. Earlier, Bhatnagar *et al.* (1983) induced organogenesis on mature embryos of chir pine and, Mehra and Anand (1983) studied callus induction from different seedling tissues. Our group has also been instrumental in clonal propagation of chir pine using axillary buds from 25-30 yr old trees. Rooting of shoots has been achieved and plantlets have been kept for hardening in the green house (unpublished results).

4.1.4. Somatic embryogenesis

Somatic embryogenesis in conifers has been regarded as a model for large-scale propagation of tree species throughout the world. Although, clonal multiplication using vegetative methods has been successful to an extent, the end results are still far from convincing. The need for propagating superior clones at a large scale is ever increasing and the efforts in these lines are mostly impeded by various factors like genetic variability and recalcitrance of mature tissues (Jain 1999, Ravikumar 2001). Propagation of pines by somatic embryogenesis has to a very large extent proven its credibility by overcoming these hurdles and is presently the only method being employed by a number of groups around the world for large-scale multiplication (Attree and Fowke 1993, Gupta *et al.* 1993a & 1993b, Gupta and Timmis 1999, Handley 1995). Somatic embryogenesis clearly provides a number of production advantages: i) large number of plants can be produced rather inexpensively ii) both root and shoot

development occur in the same process step iii) scaling up is quicker using liquid culture iv) long term cryopreservation can be used v) emblings can be established through synthetic seeds or direct delivery system vi) provides rejuvenation.

Radiata pine field trials in New Zealand and loblolly pine in the USA by private forestry companies (Carter Holt Harvey, Rotorua, New Zealand and Weyerhaeuser Company, Tacoma, USA) are a couple of success stories for clonal propagation of pines (Gupta *et al.* 1991). However, mostly pines have proved more recalcitrant than any other conifer genera. The results with respect to somatic embryogenesis in *Pinus* species have been tabulated in Chapter 1, Table 1.2.

4.2 Experimental procedure

4.2.1. Explant source

Immature female cones (Fig. 4.1.b.) of chir pine were procured from two different locations; the Conifers Research Centre Forest Nursery, Solan (altitude: 800m) and Institute of Himalayan Bioresource Technology, Palampur (altitude: 1500m). The weekly collections were made through the months of May till July in the years 1996 to 2000. The cones were transported to the laboratory in Pune, personally, or sent by post and were stored in cardboard boxes at 8 – 10 °C till utilized. The material was used up within 10-15 days of collection after which the immature cones generally decayed.

The developmental stage of the zygotic embryo was determined by histological examination (as described in section 4.2.6. in this chapter) of the immature megagametophytes removed from 2 - 3 cones picked out randomly from each collection.

For the studies of induction of somatic embryogenesis using mature zygotic embryos, the seeds of *Pinus roxburghii* Sarg. were purchased from Pratap Nursery, Dehradun,.

4.2.2. Surface sterilization

4.2.2.1. *Mature seeds*

The seeds were individually cleaned with a blade to remove the wing remnants and then washed in running tap water. Surface sterilization included washing with 1% solution liquid detergent (Labolene®, Qualigens, India) for 5 - 6 min, followed by 70% alcohol (2 - 3 min) and then with 0.075% HgCl_2 (10 min), ending with 5 - 6 washes with sterile distilled water in the laminar airflow. The seeds were then soaked in sterile distilled water and left overnight in the refrigerator (5°-8°C). This was to eliminate the non-viable seeds that floated on water.

4.2.2.2. *Immature seeds from megagametophytes*

Immature seeds were removed from the cones, cleaned by scraping with a blade and washed thoroughly under running tap water. Surface sterilisation included washing with 1% solution liquid detergent (Labolene®, Qualigens, India) for 5 - 6 min, followed by 70% alcohol (2 - 3 min) and then with 0.05% HgCl_2 (5 min), ending with 5 - 6 washes with sterile distilled water in the laminar airflow. The megagametophytes were aseptically dissected out of the seed coat using a forceps and sterile surgical blade (mounted on a blade holder) and placed horizontally on various initiation media. The number of explants tested for each treatment varied according to the number of cones collected and the number of seeds removed from each cone. Efforts were taken to equally distribute the megagametophytes on all media formulations.

4.2.3. Studies with mature zygotic embryos

The mature zygotic embryos (Fig. 4.1.d.) were dissected out of the seeds and megagametophytes (Fig. 4.1.d) in sterile conditions (in laminar airflow) and were placed horizontally on various media combinations (Table 4.1.) for the induction of somatic embryogenesis. The cultures were incubated at $22^\circ \pm 2^\circ\text{C}$ in dark. The response was observed after 3-4 weeks and the callus (if formed) was maintained by sub-culturing every three weeks.

4.2.4. Induction of somatic embryogenesis from immature zygotic embryos and culture conditions

Three different basal media, DCR (Gupta and Durzan 1985), LPM (von Arnold and Eriksson 1981) and BM₁ (Gupta and Pullman 1990), were tested for the initiation of embryogenic cultures in 1996 studies. The effect of growth regulators, organic nitrogen (casein hydrolysate and L-glutamine), myo-inositol and gelling agents (Difco® Bacto agar and gellan gum) was also tested (Table 4.2.). The pH of all media was adjusted to 5.8 with 1N NaOH/HCl prior to autoclaving.

Among the three different basal media tested initially, only DCR basal medium with gellan gum (0.18%) as gelling agent, were used in the experiments in the subsequent years (1997 onwards) (Table 4.4.). An average of 15-20 megagametophytes were cultured in each sterile disposable Laxbro® petri plate (85 mm x 15 mm) and all cultures were incubated at 22° ± 2°C in dark for the extrusion of embryogenic mass. Approximately 60-100 explants were totally inoculated per treatment depending on the availability of cones and the harvest of immature seeds from each cone. The different treatments included various concentrations and combinations of different growth regulators to extrusion, growth and proliferation of the extruded immature zygotic embryos.

4.2.5. Embryogenic culture establishment and maintenance

The extrusion of immature zygotic embryos from within the megagametophytes started as early as 8-10 days of culture in some explants depending mostly on the developmental stage of the immature zygotic embryo. The extruded masses were initially sub-cultured every week on the same initiation medium even after it's detachment from the megagametophyte. The proliferation of the established embryogenic cultures was carried out on DCR basal medium containing the same supplements as the initiation medium. Embryogenic callus that has proliferated to 1-1.5 cm in diameter was considered as an established culture and was further multiplied by sub-

culturing every three weeks. Every seed represented a unique genotype and only the established cultures that finally gave rise to cotyledonary stage somatic embryos were referred to as embryogenic cell line (with a given number).

4.2.6. Maturation and germination

4.2.6.1. *Pre-treatment:*

The proliferating embryogenic cultures of chir pine were shifted to hormone-free medium prior to exposing the cultures to different maturation treatments. For this purpose the embryogenic cell lines were cultured on DCR basal medium (half strength) containing 2% sucrose, 1.0g/l myo-inositol, 0.18% gellan gum (with and without 0.5% activated charcoal), for, 1, 2, 3 and 6 weeks. Additionally, the cultures were also kept for 3 weeks on DCR basal medium (full strength) supplemented with 3% sucrose, 1000 mg/l myo-inositol, 5 μ M 2,4-D, 2 μ M BA and 2 μ M kinetin, and 0.18% gellan gum (Table 4.9.). The proliferating embryogenic tissue clumps were transferred to a sterile filter paper disc (Whatman No. 1) cut to a size that would fit in the petri-plate (55mm X 15mm sterile disposable Laxbro® petri-plates), and 3-4 small clumps were cultured in each plate. All cultures were incubated at 22° $\pm$ 2°C in dark.

4.2.6.2. *Maturation treatments:*

The established embryogenic cell lines were subjected to different maturation treatments (Tables 4.8. and 4.10.) after passing them through the medium devoid of growth regulators (Table 4.9.). The effects of ABA, maltose, PEG (polyethylene glycol) and gellan gum in different combinations and concentrations were tested for the maturation of the somatic embryos. Data was collected by, counting the number of embryogenic clumps giving rise to the smooth paraboloid shaped somatic embryos and the number of cotyledonary stage somatic embryos formed for each given treatment after and incubation period of 8-10 weeks. The cultures on maturation medium were sub-cultured

every 10 days by shifting the filter paper disc with the embryogenic cultures. All cultures were incubated at $22^{\circ} \pm 2^{\circ}\text{C}$ in dark.

4.2.6.3. Germination of somatic embryos:

Cotyledonary stage somatic embryos with developing root initials were given a partial desiccation treatment in laminar airflow for 2-3 hours and then shifted to DCR basal medium (half strength) containing 2% sucrose, 0.1g/l myo-inositol, 0.25% gellan gum and 0.5% activated charcoal for complete development of the somatic embryos before transferring them for hardening treatment. The somatic embryos were incubated at $24^{\circ} \pm 2^{\circ}\text{C}$ under cool fluorescent lights at $10\text{-}20 \mu\text{E m}^{-2} \text{s}^{-2}$, with a 16 h photoperiod for germination.

4.2.7. Histology and histochemical staining

The immature megagametophytes at different stages of development were fixed in FAA (formaldehyde: acetic acid: alcohol), passed through a TBA (tertiary-butyl alcohol) series for dehydration, embedded in paraffin wax ($58^{\circ}\text{--}60^{\circ}\text{C}$ congealing point) and sectioned (8-10 μm thickness). The sections were then stained with hematoxylin and observed under light microscope to study the stage of the immature zygotic embryo.

Histochemical staining of embryogenic cultures in suspension was done by 1% acetocarmine (Sigma) for few minutes, washed and observed directly under Zeiss light and stereo-microscopes.

4.3. Results and discussion

The immense surge in the field of conifer biotechnology during the past two decades has been reviewed by several authors (Adams *et al.* 1994, Attree and Fowke 1993, Dunstan 1988, Gupta *et al.* 1991, Gupta and Grob 1995, Misra 1994, Tautorius *et al.* 1991, Walter *et al.* 1998) and somatic embryogenesis is suggested and proven to a great extent, as a powerful tool for

successful clonal propagation as compared to other tissue culture propagation procedures.

Partial or complete somatic embryogenesis has been reported in various species of *Pinus*, viz., *P. caribaea* (Laine and David 1990, Laine *et al.* 1992, David *et al.* 1995), *P. elliotii* (Jain *et al.* 1989, Liao and Amerson 1995a & 1995b, Newton *et al.* 1995), *P. lambertiana* (Gupta and Durzan 1986), *P. nigra* (Salajova and Salaj 1992, Salajova *et al.* 1995 & 1999), *P. patula* (Jones and van Staden 1995), *P. pinaster* (Bercetche and Pâques 1995), *P. sylvestris* (Haggman *et al.* 1999, Hohtola 1995), *P. strobus* (Finer *et al.* 1989), *P. taeda* (Becwar *et al.* 1990 & 1991, Becwar and Pullman 1995, Gupta and Durzan 1987a & 1987b, Li and Huang 1996) describing the importance of the genus world wide. In *P. serotina* Michx., embryogenic cultures were initiated (Becwar *et al.* 1988), but further maturation was not obtained. In *P. strobus* L. and *P. sylvestris* L. cotyledonary stage somatic embryos were obtained without complete plant regeneration (Webb and Flinn 1991). With *P. ellioti*, partial embryogenesis was first reported by Jain *et al.* (1989) and later, Liao and Amerson (1995) and Newton *et al.* (1995) have reported embryo maturation and plantlet production. In other pines the regeneration of complete plantlets via somatic embryogenesis has been reported for *P. lambertiana* Lamb. (Gupta and Durzan 1986), *P. taeda* L. (Gupta and Durzan 1987a), *P. radiata* D. Don. (Smith *et al.* 1988) and *P. caribaea* Morelet var. *hondurensis* (Laine and David 1990).

With the objective of propagation of selected elite varieties for high quality resin production, by initiating studies using somatic embryogenesis, in continuation with the ongoing work on other Indian pine species in our lab (Mathur and Nadgauda 1995, 1998 & 1999, Nadgauda *et al.* 1993) the present investigation was undertaken. This part of the chapter discusses the results, describing the explant, media composition, conditions for extrusion and proliferation of embryogenic mass in *P. roxburghii* Sarg. and, successful maturation and development of somatic emblings.

4.3.1. Studies with mature zygotic embryos of chir pine

Liao and Amerson (1995a) have elegantly described the recalcitrance of the genus *Pinus* in terms of selection of suitable explant for initiating somatic embryogenesis and the difficulty in getting successful plantlet regeneration through tissue culture as compared to other conifers. The suitable explant for giving rise to embryogenic cultures of pines has been limited to immature zygotic embryos (Attree and Fowke 1993). It was extremely essential to obtain a sound knowledge of the embryology of chir pine so as to be sure about the correct stage during the collection. Thus, while studying the embryology of chir pine, it was an obvious choice to make use of the mature zygotic embryos for the initiation of embryogenic tissue.

A number of combinations and concentrations of various growth regulators were tested for the purpose (Table 4.1.). As can be seen from the Chapter 1, Table 1.2., there are only four species of pines that have positively responded in giving rise to embryogenic cell lines from mature tissues. *P. lambertiana* yielded embryogenic cultures from mature zygotic embryos (Gupta and Durzan 1986), whereas, *P. caribaea* produced embryogenic cultures from both mature zygotic embryos and secondary needles (Berlyn *et al.* 1991).

Chir pine mature zygotic embryos were unable to give rise to embryogenic cultures. Profuse callusing (Fig. 4.1.e.) was observed in a number of cultures using different growth regulators, but none of the cultures were found to be embryogenic. Some of the cultures that appeared glistening white (Fig. 4.1.e.), were initially suspected of being able to give rise to embryogenic tissue, also turned out to be negative. The observations of this experiment have been tabulated in Table 4.1. Even after repeated sub-culturing, none of the cultures could be termed as embryogenic tissue. Apart from the recalcitrant nature of the mature tissue, another reason for this non-responsiveness could be attributed to improper culture conditions. Indicating that more efforts are needed for the optimization of media and other conditions in inducing embryogenesis from mature zygotic embryos.

Table 4.1: Effect of different growth regulators in the medium, on mature zygotic embryos of *Pinus roxburghii* Sarg. for induction of somatic embryogenesis

	DCR basal medium + 2% sucrose, 1g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 0.6% agar supplemented with growth regulators (μ M):		Observations
1	2,4-D	5	Callusing
2		10	”
3		20	”
4		50	”
5		100	”
6	2,4-D + BA	5 + 1	”
7		5 + 5	”
8		10 + 1	”
9		10 + 5	Profuse callusing
10		20 + 1	Yellow, watery callus
11		20 + 5	Callusing
12		50 + 5	Callusing
13		50 + 20	Cotyledons elongate
14		100 + 5	Callusing
15		100 + 20	Cotyledons elongate
16	NAA	1	Swelling of explant
17		5	“
18		10	Callusing
19		20	Glistening white callus
20		50	Callusing
21	NAA + BA	5 + 1	White, friable callus
22		10 + 5	“
23		20 + 5	Callusing
24		50 + 5	“
25		50 + 20	“

Mature zygotic embryos of *P. lambertiana* and *P. caribaea* are reported to give rise to that the embryogenic cultures arising from the radicle area, but it is still uncertain whether the point of origin of the embryogenic tissue, are the root cells or residual suspensors attached to the radicle. Bozkhov *et al.* (1997) have also described two alternative pathways of somatic embryo origin from polyembryonic mature seeds of *P. koraiensis* Sieb *et* Zucc. The first pathway was associated with production of friable, translucent callus in the hypocotyls-

cotyledon region and the second pathway related to proliferation of translucent, moist and mucilaginous tissue in the suspensor region of zygotic embryo. Recently, Garin *et al.* (1998) have also been successful in obtaining plantlets from embryogenic tissue of *P. strobus* L. from mature zygotic embryos.

4.3.2. Induction of somatic embryogenesis from immature zygotic embryos

The major bottleneck in initiating embryogenic cultures of pines has been the identification of the correct developmental stage of the immature zygotic embryo, known as the ‘window’ (Fig. 4.2.) followed by, successful maturation and conversion of somatic embryos to plants. Thus the preliminary studies (years 1995 and 1996) were totally concerned with the aim of identifying the critical developmental stage for establishing embryogenic cultures of *P. roxburghii* Sarg., followed by developing an efficient regeneration protocol.

The choice and response of initial explants for the induction of somatic embryogenesis in coniferous species has been very limited. Mature zygotic embryos, seedling cotyledons, cotyledonary stage immature zygotic embryos and haploid megagametophytes are some of the sources used for raising embryogenic tissue in a number of species (Attree *et al.* 1990a & 1990b, Boulay *et al.* 1988, Bourghard and Favre 1988, Chavez *et al.* 1992, Ishii 1991, Nagmani and Bonga 1985, Tremblay 1990, von Arnold 1987). In genus *Pinus* however, the most suitable explant for successful induction of embryogenesis has been observed to be from the immature zygotic embryos that are extruded out from micropylar end of the intact megagametophytes. The embryogenic cultures from immature zygotic embryos of pines appear to have originated from proliferating cells in suspensor mass (Becwar *et al.* 1988, Jain *et al.* 1989) rather than directly from the embryonic axes, as mostly reported in spruce species (Lu and Thorpe 1987, von Arnold and Woodward 1988, Webb *et al.* 1989). At this stage the ovule, as a result of cleavage polyembryony, contains more than one viable zygotic embryo, including the dominant one. Culturing the zygotic embryos at this stage leads to an “embryo rescue” situation (Jones

et al. 1993), which may result in somatic embryogenesis. This process has been repeated and confirmed by Becwar *et al.* (1988) who have observed multiple embryos attached to a common suspensor, suggesting that in *Pinus*, somatic embryogenesis may be a re-initiation of the cleavage process.

4.3.2.1. Embryology – study conducted in the year 1995:

The studies with respect to the time of fertilization and development of zygotic embryo of chir pine were undertaken not only to pin-point correctly the developmental stage of the zygotic embryo that is most responsive in giving rise to embryogenic culture, but also as a reference to the developing somatic embryo. In Fig. 4.4.a., pre-fertilized megagametophyte with developing archegonia can be seen and the egg and neck cells are also visible. Fig. 4.4.b. shows the corrosion cavity in the megagametophyte containing the developing zygotic embryos. Very early stage (2-4 cell) zygotic embryo with entwined suspensor cells (Fig. 4.5.a.), a 32-64 cell stage small globular shaped zygotic embryo can be seen in Figs. 4.5.b. & c. with an elongated suspensor. This stage of zygotic embryo is most responsive to produce embryogenic mass and in Fig 4.5.d. a cotyledonary stage zygotic embryo can be observed.

4.3.2.2. Studies during the year 1996:

On determination of the post-fertilization period and the development of the zygotic embryo from the studies conducted during the previous year, an experimental plan was worked out in the year 1996 with the aim of optimizing the media most suitable for induction and proliferation of embryogenic mass. The collection plan was devised such that megagametophytes from pre- to 5-6 weeks post-fertilization were procured and the collection was conducted only from Solan, Himachal Pradesh.

On basis of these studies, the megagametophytes showed best response of extrusion and proliferation of embryogenic tissue on DCR basal medium (48.31% extrusion and 13.5% cultures established) among the three basal media tested (Fig. 4.6. and Table 4.3.), and was used in the experiments in the

Fig. 4.4. (a) Longitudinal section of immature megagametophyte (meg) of *P. roxburghii* (pre-fertilization) showing archegonia (ar), archegonial neck cells (nc), micropylar end (me), nucellus (nu), egg cell (ec)

(b) Longitudinal section of immature megagametophyte (meg) of *P. roxburghii* (post-fertilization) showing corrosion cavity(cc), developing immature zygotic embryos (ize), nucellus (nu)

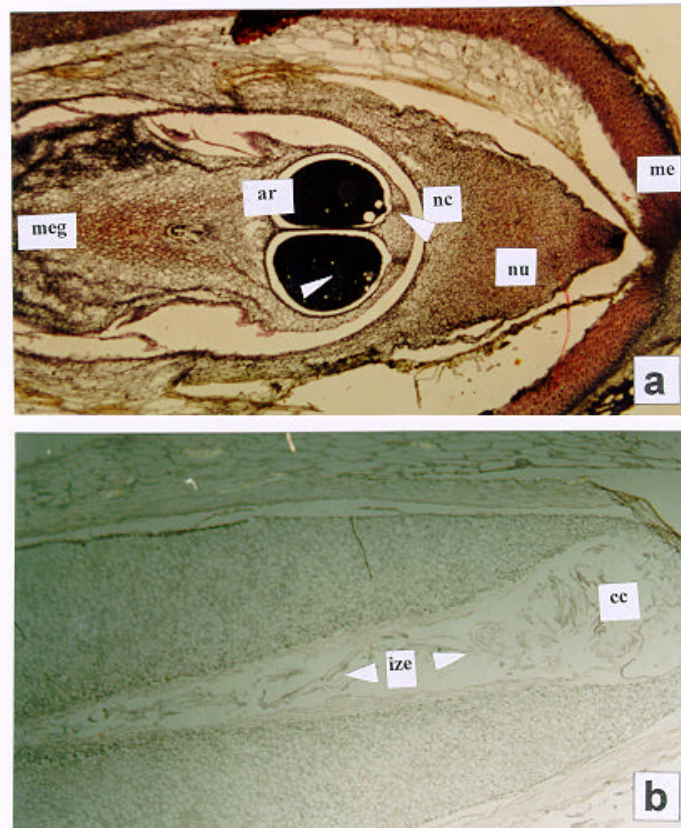


Fig. 4.4.

- Fig. 4.5.**
- (a) Magnified view of immature zygotic embryo of *P. roxburghii* [2-4 cell stage embryo head (emb)] with entwined suspensor cells (sus) (bar = 1mm)**
 - (b) Globular stage zygotic embryo (32-64 cell stage) of *P. roxburghii* with a long suspensor (bar = 1 mm)**
 - (c) Magnified view of globular shaped zygotic embryo head (emb) of *P. roxburghii* with attached suspensor cells (sus) (bar = 0.5 mm)**
 - (d) Cotyledonary stage zygotic embryo of *P. roxburghii* showing well developed embryo (emb) with cotyledonary bumps (cot) and suspensor (sus) (bar = 2 mm)**

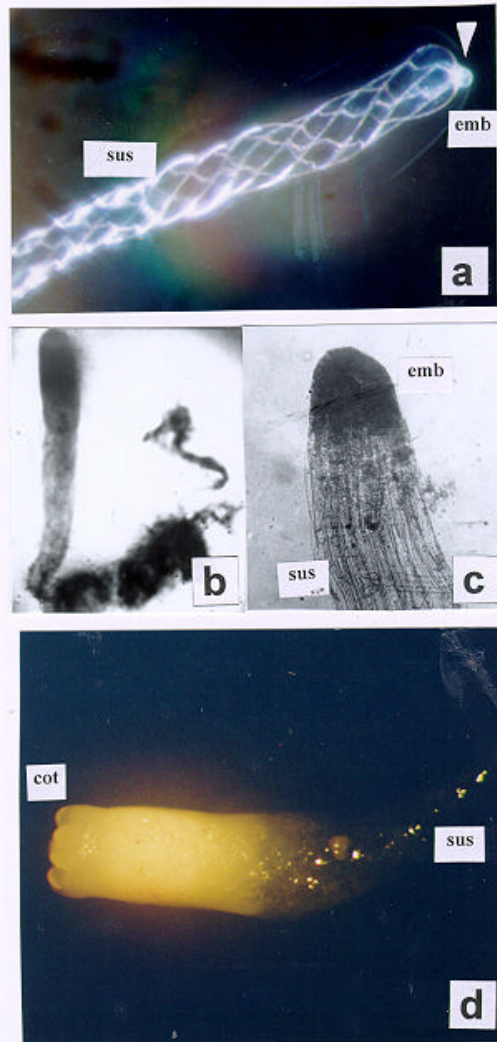


Fig. 4.5.

subsequent years. Due to extreme limitations in procuring the female cones, it was possible to test only three most widely used basal media in similar studies in pines. The limitation in the number of megagametophytes procured in each collection also restricted the different media treatments (Tables 4.2.). Despite these shortcomings, efforts were made to fully utilize the available resources for as many investigations as possible. Since the standardization of surface sterilization of immature megagametophytes, inoculation, culture techniques and conditions were also to be conducted along with the optimization of the medium, a large number of explants were damaged/wasted in the process.

The method of surface sterilization that was being used initially, i.e., -treating of the entire cones with NaOCl (sodium hypochlorite) before aseptically dissecting them in laminar airflow, had to be changed since it proved extremely time consuming and impractical. After working with a few other surface sterilization methods (that also proved unsuccessful), it was decided to switch over to extracting the seeds individually from the cones and then giving the surface sterilization treatment as mentioned in section 4.2.2.2. A number of different concentrations and combinations of growth regulators were also tested for the extrusion of the embryogenic mass (Table 4.3.) and only DCR basal medium supplemented with a combination of 2,4D, BA and kinetin, was found to induce embryogenic response in chir pine. Although recently, Arya *et al.* (2000) have also reported induction of embryogenesis in chir pine using DCR basal medium supplemented with 2,4D, NAA and BA.

In other conifer species, various other basal media have been tested for the induction and proliferation of embryogenic cultures. It will not be possible to describe in detail the effects of different basal medium on induction of embryogenesis in all conifer species, but a brief mention about reported work on pines will be presented. DCR basal medium or Douglas fir cotyledon revised medium, was formulated by Gupta and Durzan (1985) and has been by far the most widely utilized successfully for a number of pine species, viz., *P. strobus* L. (Finer *et al.* 1989, Garin *et al.* 1998, Kaul 1995), *P. palustris* (Nagmani *et al.* 1993), *P. patula* (Jones and van Staden 1993, Jones *et al.*

1995), *P. nigra* Arn. (Salajova and Salaj 1992) and *P. sylvestris* L. (Hägmann *et al* 1999). Arya *et al.* (2000) have also used DCR basal medium to initiate embryogenic cultures of chir pine. Other basal media that have been successfully used for initiating embryogenesis in pines are, MSG for *P. taeda* L. (Becwar *et al.* 1990) and *P. palustris* (Nagmani *et al.* 1993); LP for *P. caribaea* (Laine and David 1990); GD for *P. pinaster* (Bercetche and Pâques 1995); BM₁ for *P. sylvestris* L. (Hohtola 1995) and *P. taeda* (Li and Huang 1996). From the studies regarding the media and culture conditions that were conducted in the year 1996, a number of very useful observations were made that helped in scheduling the collections and further fine-tuning of inoculation and extrusion protocols for future experiments. With these results it was possible to:

- Correctly identify and collect the most suitable stage of the explant (immature zygotic embryo) for best response *in vitro*.
- Use the least harmful but most effective surface sterilization procedure.
- Inoculate maximum number of megagametophytes for statistical analysis.
- Study other physical and chemical parameters for the establishment and proliferation of embryogenic cultures.
- To identify other locations for a comparative study.
- To establish as many embryogenic cell lines of chir pine.

Table 4.2. Different basal media and other media constituents tested for the extrusion of embryogenic tissue from immature zygotic embryo of *Pinus roxburghii* Sarg. during the 1996 collection from Solan.

Media no.	1	2	3	4	5	6	7	8	9	10	11
Macro nutrients	DCR half strength										
Micro nutrients	DCR half strength										
Vitamins	DCR full strength										
Sucrose (%)	3	3	3	3	3	1	1	6	6	3	3
myo-inositol (g/l)	1	1	1	1	1	1	1	1	1	1	1
CH (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		
L-glutamine (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		
2,4-D (mM)	3	3	5	5	50	5	3	5	3	5	3
BA (mM)	0.5	1	2	2	20	2	0.5	2	0.5	2	0.5
Kinetin (mM)		1		2	20	2	0.5	2	0.5	2	0.5
Gellan gum (%)	0.18										

Table 4.2 continued

Media no.	12	13	14	15	16	17	18	19	20	21	22
Macro nutrients	DCR half strength										
Micro nutrients	DCR half strength										
Vitamins	DCR full strength										
Sucrose (%)	2	2	2	2	2	3	3	3	3	3	3
myo-inositol (g/l)	0.5	0.5	0.5	0.5	0.5	1	1	1	1	1	1
CH (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-glutamine (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2,4-D (mM)	5	5	10	10	11.0	50					
BA (mM)	2	2	4	4	4.5	20					
Kinetin (mM)	2		4		4.3	20					
NAA (mM)							1	3	5	10	0.5
Difco agar (%)						0.6					
Gellan gum (%)	0.18						0.18				

Media no.	23	24	25	26	27	28	29	30	31	32
Macro nutrients	DCR half strength								DCR full	
Micro nutrients	DCR half strength								DCR full	
Vitamins	DCR full strength								DCR full	
Sucrose (%)	3	3	3	3	3	3	3	3	2	2
myo-inositol (g/l)	1	1	1	1	1	1	1	1	1	1
CH (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-glutamine (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2,4-D (mM)					50	50	50	50	50	5
BA (mM)	0.2	1	4	5	20	20	20	20	20	2
Kinetin (mM)					20	20	20	20	20	2
NAA (mM)	1	3	10	20						
Ca-pantothenate (g/l)							0.5			
D-biotin (g/l)								0.5		
CM (%)					10	20				
Gellan gum (%)	0.18									

Table 4.2. continued

Media no.	33	34	35	36	37	38	39	40	41	42
Macro nutrients	LP full strength									
Micro nutrients	LP full strength									
Vitamins	LP full strength									
Amino acids	LP full strength									
Sucrose (%)	3	3	3	3	3	2	6	6	6	3
myo-inositol (g/l)	1	1	1	1	1	1	1	1	1	1
CH (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-glutamine (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2,4-D (mM)	50	50	20	10	5	50	20	10	5	50
BA (mM)	20			4		20		4	2	20
Kinetin (mM)	20			4		20		4	2	20
Difco agar (%)										
Gellan gum (%)	0.18									

Media no.	43	44	45	46	47	48	49	50	51	52
Macro nutrients	BM ₁ full strength									
Micro nutrients	BM ₁ full strength									
Vitamins	BM ₁ full strength									
Sucrose (%)	3	3	3	3	3	2	6	6	6	3
myo-inositol (g/l)	1	1	1	1	1	1	1	1	1	1
CH (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-glutamine (g/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2,4-D (mM)	50	50	20	10	5	50	20	10	5	50
BA (mM)	20			4		20		4	2	20
Kinetin (mM)	20			4		20		4	2	20
Difco agar (%)										
Gellan gum (%)	0.18									

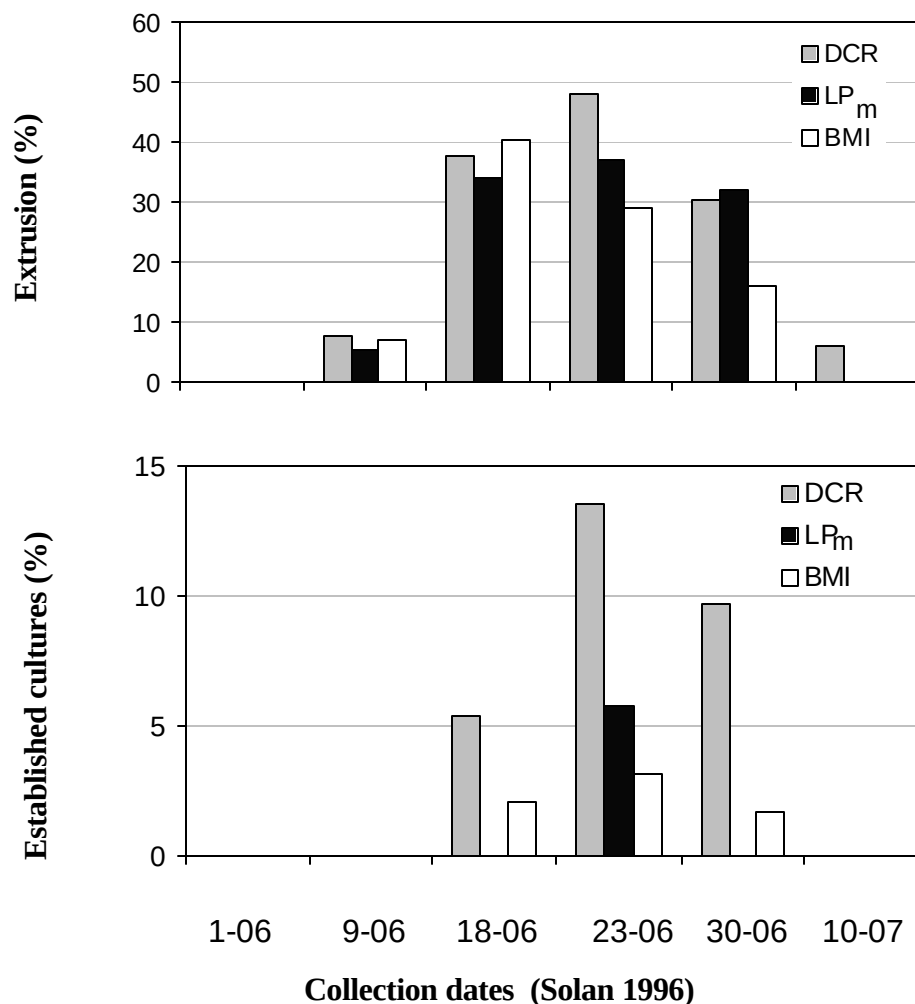


Fig 4.6. Effect of three different basal media (DCR, LP_m and BM₁) on the extrusion and establishment of embryogenic cultures of *P. roxburghii* Sarg. All three basal media contained 3% sucrose + 1.0g/l myo-inositol + 0.5g/l L-glutamine + 0.5g/l caesin hydrolysate + 50μM 2,4-D + 20μM BA + 20μM Kin + 0.18% gellan gum; i.e. media nos. 5, 33 and 43, from Table 4.2. (Data from collections made in Solan during May-June 1996)

Table 4.3. Effect of different media tested (Table 4.2.) on the extrusion and establishment of embryogenic cultures of *Pinus roxburghii* Sarg. from immature zygotic embryos, during the 1996 collections from Solan.

Media no.	Collective data of total extrusions (%) from all stages of development of the immature zygotic embryo	Total no. of established embryogenic cultures
1	0	0
2	0	0
3	0	0
4	9.52	3
5	48.31	8
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	11.25	5
13	0	0
14	15.67	3
15	0	0
16	21.33	0
17	6.80	1
18	0	0
19	0	0
20	0	0
21	0	0
22	0	0
23	0	0
24	0	0
25	0	0
26	0	0

Table 4.3. continued

Media no.	Collective data of total extrusions (%) from all stages of development of the immature zygotic embryo	Total no. of established embryogenic cultures
27	0	0
28	0	0
29	0	0
30	0	0
31	13.27	2
32	16.35	2
33	19.33	1
34	0	0
35	0	0
36	0	0
37	0	0
38	15.34	2
39	0	0
40	21.90	1
41	0	0
42	11.45	0
43	21.25	2
44	0	0
45	0	0
46	22.15	3
47	0	0
48	17.70	4
49	0	0
50	0	0
51	0	0
52	9.46	1

4.3.2.3. Studies during the year 1997:

On basis of the studies conducted in the year 1996 regarding the basal medium and the choice of growth regulators, other physical and chemical parameters for the induction of embryogenesis were tested in the year 1997. DCR basal medium was used for all the experiments and a limited number of growth regulator combinations were chosen from the 1996 studies (Table 4.4.). During this year, the collections were made from two different locations (Solan and Palampur, Himachal Pradesh) at varying altitudes. A difference was clearly evident in the developmental stages of immature megagametophytes of *P. roxburghii* Sarg. collected from these two provenances (Tables 4.5. – 4.7.). This had a direct effect on the extrusion 'window' in the two provenances. In addition to the variation observed among the developmental stages of two different locations, variation was also observed between two trees in Palampur (Tables 4.6. & 4.7.). Despite these variations in the developmental stages of the maturing megagametophyte, the correct stage for extrusion (Type 1, Fig. 4.8.a.), or the 'window' appeared quite uniformly within 10-15 days of the fertilization.

4.3.2.4. Extrusion of embryogenic tissue and initiation of embryogenesis:

The first indication of culture initiation is the extrusion of immature zygotic embryos through the micropylar end of megagametophyte within 8-10 days of culture, which was glistening, translucent to white (Figs. 4.7.a & b.). The morphology of the extrusions varied and was classified according to Liao and Amerson (1995a). No single type of extrusion was observed to be prominent on one particular medium. Thus implying that the variation in extrusions is related only to the developmental stage of the immature zygotic embryo. Four different types of extrusions were observed during the study (Figs. 4.8. a. - d.) and among these, type II, III and IV extrusions did not give rise to embryogenic cultures (Figs. 4.8.b. - d.). Embryogenic callus that had proliferated to 1-1.5 cm in diameter

Fig. 4.7. (a) Extrusion of immature zygotic embryos from the micropylar end of megagametophytes of *P. roxburghii* on DCR basal medium containing 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kinetin and 0.18% gellan gum. The extruding tissue forms the pro-embryogenic mass (bar = 5 mm)

(b) Magnified view of *P. roxburghii* megagametophyte (meg) showing extrusion of immature zygotic embryos (ize) through the micropylar end (mc) (bar = 5 mm)

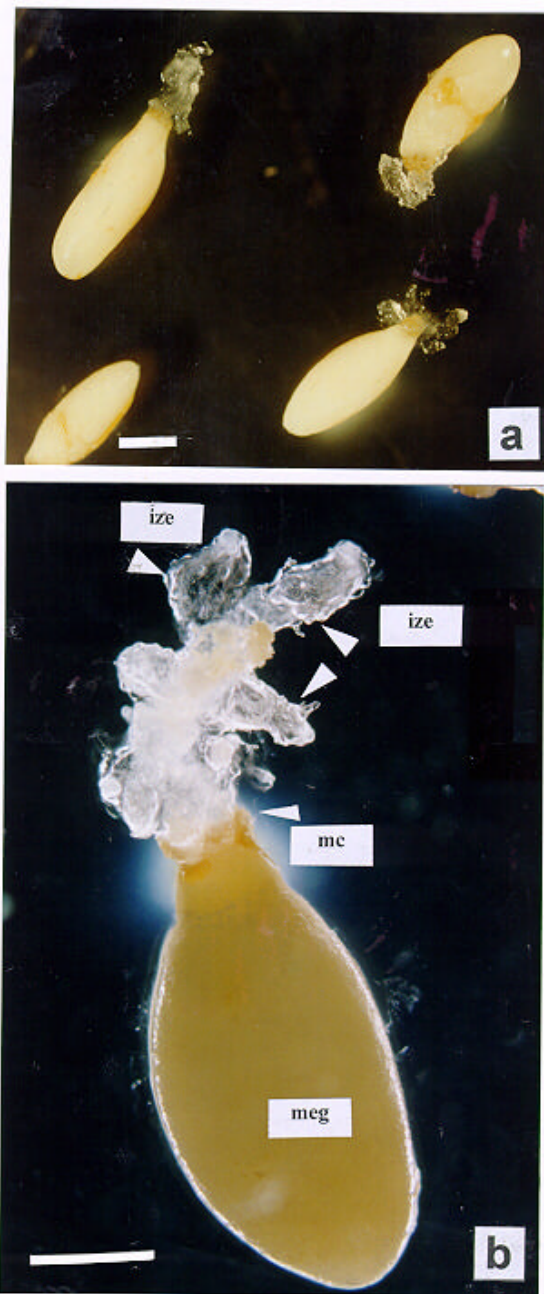


Fig. 4.7.

(Fig. 4.9.a.) was considered as an established culture and was further multiplied by subculturing every two weeks.

Table 4.4. Various media tested for the extrusion and establishment of embryogenic cultures of *Pinus roxburghii* Sarg. during the 1997 collections from Solan and Palampur.

Media*	D-1	D-2	D-3	D-4	D-5 [#]
CH (g/l)	0.5	0.5	-	0.5	0.5
L-glutamine (g/l)	0.5	0.5	-	0.5	0.5
2,4-D (μM)	50	50	50	50	-
BA (μM)	20	20	20	-	-
Kin (μM)	20	20	20	-	-
Gellan gum (%)	0.18	-	0.18	0.18	0.18
Difco Agar (%)	-	0.8	-	-	-

* Media D-1 to D-5 contain DCR basal salts + 3.0% sucrose + 1.0 g l⁻¹ myo-inositol with different concentrations and combinations of growth regulators, organic nitrogen (L-glutamine and CH) and gelling agents (agar and gellan gum).

[#] Medium D-5 is the control, devoid of growth regulators.

Table 4.5. Effect of growth regulators, organic nitrogen and gelling agent on extrusion and establishment of embryogenic cultures of *P. roxburghii* Sarg. (Collection – Solan, 1997)

Collection Dates (1997)	Stage of zygotic embryo ¹⁹	Culture medium (See Table 4.4.)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion ± S.E. (% Established cultures) [#]				
23-May	No fertilization	- No extrusions observed -				
31-May	No fertilization	- No extrusions observed -				
13-June	Early cellular	41.12 ± 2.2 (3.8)	18.6 ± 1.54 (2.7)	24.22 ± 1.39 (0.0)	18.94 ± 3.13 (4.4)	18.04 ± 2.98 (0.0)
24-June	Mid cellular	46.64 ± 4.58 (20.6)	25.45 ± 1.37 (11.8)	21.43 ± 0.67 (0.0)	30.6 ± 0.76 (10.4)	16.07 ± 1.81 (0.0)
8-July	Smooth paraboloid	14.22 ± 1.06 (0.0)	22.62 ± 0.98 (0.0)	16.4 ± 2.2 (0.0)	12.43 ± 1.06 (0.0)	0.0 (0.0)

[#] The data is given as % extrusion ± S.E. and the data within parentheses represents % established cultures from the extruded embryogenic tissue

Table 4.6. Effect of growth regulators, organic nitrogen and gelling agents on extrusion and establishment of embryogenic cultures of *P. roxburghii* Sarg. (Collection - Palampur: Tree – 1 (T1), 1997)

Collection Dates (1997)	Stage of zygotic embryo (after Konar 1960)	Culture medium (See table 4.4.)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion $\pm$ S. E. (% Established cultures) [#]				
2-June	Early cellular	30.03 $\pm$ 1.68 (6.8)	39.80 $\pm$ 1.44 (7.7)	25.26 $\pm$ 2.24 (0.0)	5.70 $\pm$ 1.16 (0.0)	0.0 (0.0)
10-June	Mid-cellular	49.10 $\pm$ 3.14 (26.3)	35.88 $\pm$ 1.3 (16.6)	22.03 $\pm$ 1.64 (0.0)	26.32 $\pm$ 1.17 (0.0)	32.94 $\pm$ 1.53 (0.0)
17-June	Smooth paraboloid	27.46 $\pm$ 0.72 (9.5)	33.3 $\pm$ 3.85 (7.3)	30.75 $\pm$ 0.67 (0.0)	23.31 $\pm$ 0.85 (0.0)	14.76 $\pm$ 1.13 (0.0)
24-June	Pre-cotyledonary bumps	29.48 $\pm$ 9.78 (0.0)	33.46 $\pm$ 1.01 (0.0)	0.0 (0.0)	23.73 $\pm$ 0.82 (0.0)	0.0 (0.0)
1-July	Developing cotyledons	No extrusions observed				
8-July	Developing cotyledons	No extrusions, only germination of zygotic embryo observed				

[#] The data is given as % extrusion $\pm$ S.E. and the data within parentheses represents % established cultures from the extruded embryogenic tissue

Table 4.7. Effect of growth regulators, organic nitrogen and gelling agents on extrusion and establishment of embryogenic cultures of *P. roxburghii* Sarg. (Collection - Palampur: Tree – 2 (T2), 1997)

Collection Dates (1997)	Stage of zygotic embryo (after Konar 1960)	Culture medium (See Table 4.4.)				
		D-1	D-2	D-3	D-4	D-5 (control)
		% Extrusion $\pm$ S. E. (% Established cultures) [#]				
2-June	No fertilization	No extrusions observed				
10-June	No fertilization	No extrusions observed				
17-June	Early cellular	39.16 $\pm$ 0.56 (8.5)	41.83 $\pm$ 0.80 (7.3)	12.6 $\pm$ 1.08 (0.0)	26.3 $\pm$ 0.15 (0.0)	22.12 $\pm$ 1.06 (0.0)
24-June	Mid cellular	41.40 $\pm$ 1.29 (14.6)	35.44 $\pm$ 1.11 (14.2)	24.80 $\pm$ 2.07 (0.0)	37.96 $\pm$ 2.47 (2.4)	20.91 $\pm$ 1.89 (0.0)
1-July	Smooth paraboloid	9.03 $\pm$ 1.78 (0.0)	14.90 $\pm$ 0.95 (3.3)	0.0 (0.0)	15.23 $\pm$ 1.0 (0.0)	0.0 (0.0)
8-July	Developing cotyledons	No extrusion				

[#] The data is given as % extrusion $\pm$ S.E. and the data within parentheses represents % established cultures from the extruded embryogenic tissue

Fig. 4.8. Different types of extrusions observed during embryogenic culture initiation of *P. roxburghii* (bar = 2 mm)

- (a) Type I extrusion: proliferates and gives rise to embryogenic cultures**
- (b) Type II extrusion: with individually proliferating embryonal heads**
- (c) Type III extrusion: cell mass pushed away by the cord-like formation of suspensor**
- (d) Type IV extrusion: showing early cellular stage zygotic embryo with thread like suspensor**

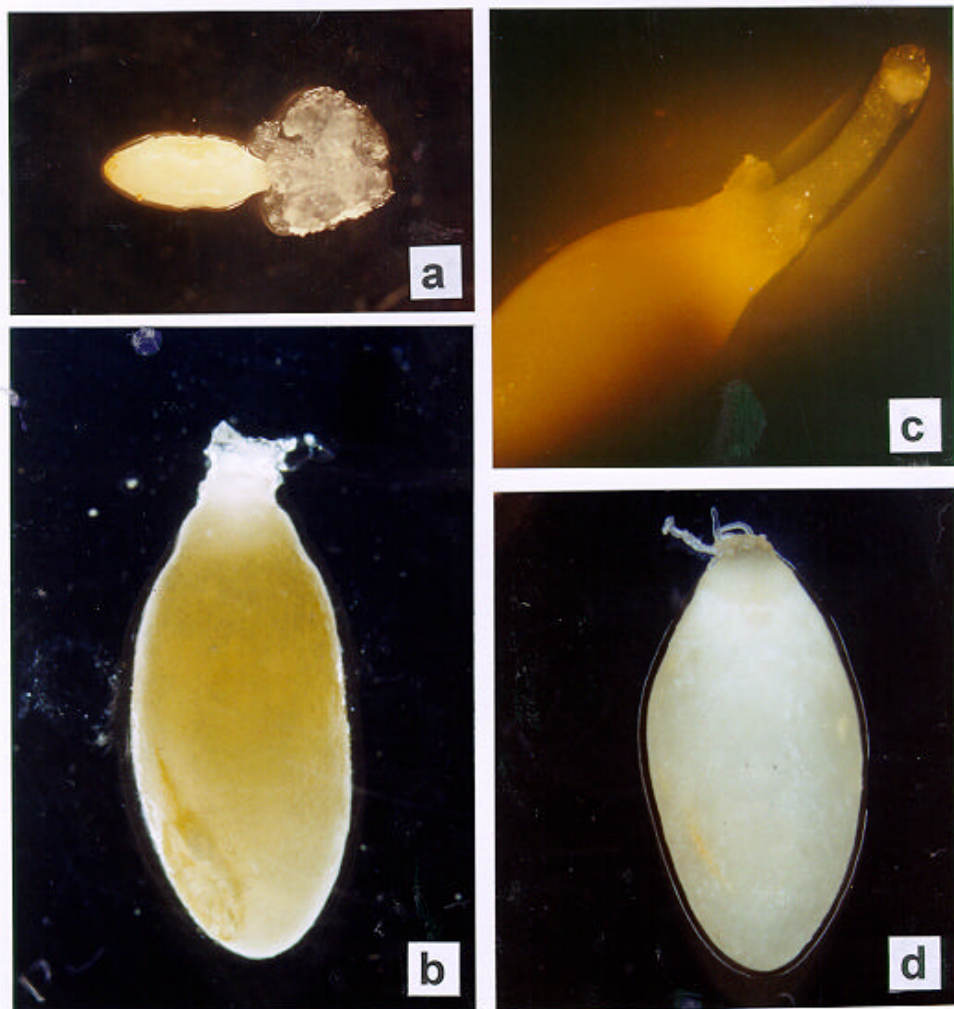


Fig. 4.8.

It is notable that only the type I extrusions (Fig. 4.8.a.) survived, proliferated and established as embryogenic cultures. The proliferating cells in the embryogenic mass in pines are generally the suspensor cells (Attree and Fowke 1993, Becwar *et al.* 1988, Gupta and Durzan 1987a). Fig. 4.9.b shows initial somatic embryos separated out from embryogenic mass. Apart from a suitable media composition (basal salts, growth regulators, organic nitrogen, and gelling agent), the induction and proliferation of embryogenic mass was observed to be sensitive to temperature. The proliferation was best observed at $20^{\circ}\pm 2^{\circ}\text{C}$ and an increase in temperature by $3^{\circ}\text{--}4^{\circ}\text{C}$ was absolutely detrimental for the proliferating cultures (details in chapter 5, section 5.3.1.2.)

The extrusion and establishment of embryogenic mass is completely dependent on the collection of cones at the correct stage of the immature zygotic embryo. As it can be clearly observed from the present study that the time of maturation of cones varies not only within the locations, but also within the trees in one location (Tables 4.5.–4.7. and Fig. 4.10.). The collection schedule has to be very well regularized to obtain maximum number of well-established embryogenic cultures. From the 38 established embryogenic cultures from the 1996 collections (Table 4.3.) and a total of 84 established cultures from the 1997 collections (Tables 4.5.-4.7.), only three cell lines viz., R-1, R-3 and R-29, were selected among the different cell lines, that are presently being maintained on the same initiation medium and cultures are being utilized for maturation studies. These three cell lines proved most responsive to maturation treatments (Table 4.8.).

The advanced cellular stages of zygotic embryos only (Type I extrusion) produce embryogenic cultures and even the type of extrusion is very much similar among different locations. In the Type IV extrusions, the zygotic embryo is of very early cellular stage, and unable to give rise to the embryogenic cultures. Similarly, in Type III extrusion, the zygotic embryo has matured to an early smooth paraboloid structure and thus unable to respond and give rise to embryogenic tissue.

Fig. 4.9. (a) Proliferating embryogenic cultures of *P. roxburghii* (bar = 1 cm)
(b) Embryogenic cultures of *P. roxburghii* kept for pre-treatment
(prior to the maturation treatment) on DCR basal medium with
2% sucrose, 0.1g/l myo-inositol, 0.5% activated charcoal and
0.18% gellan gum

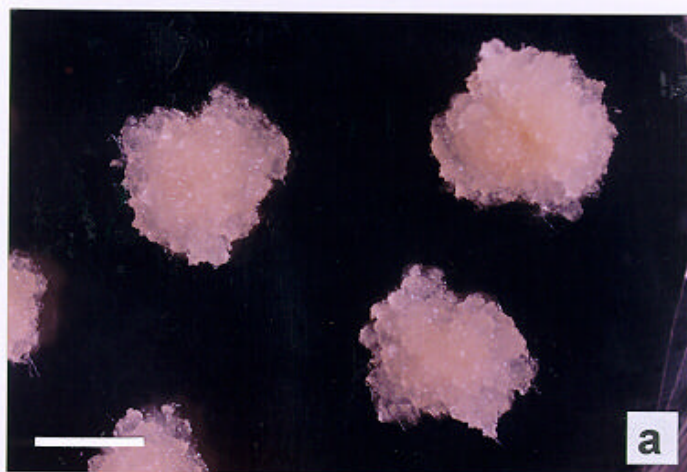


Fig. 4.9.

4.3.2.5. Studies in the year 2000:

In the year 2000, collections were done from three selected trees (T1, T2 and T3) in Palampur, Himachal Pradesh, and the extrusion data is presented in Fig. 4.10. Only seven out of the 410 extrusions, established as embryogenic cell lines. Majority of the extruded cell masses failed or quickly ceased to proliferate in subsequent incubations as observed in the previous studies also. This indicates that a general low efficiency (12.35% extrusion and 0.211% embryogenic culture establishment in the year 2000 studies) of culture initiation in chir pine is not due to lack of suitable culture conditions, instead it signifies stage specificity and the genotypic dependence of the response. The seven cell lines were labeled as follows: T10614, T10620, T10622, T10704, T20617, T20627 and T30622.

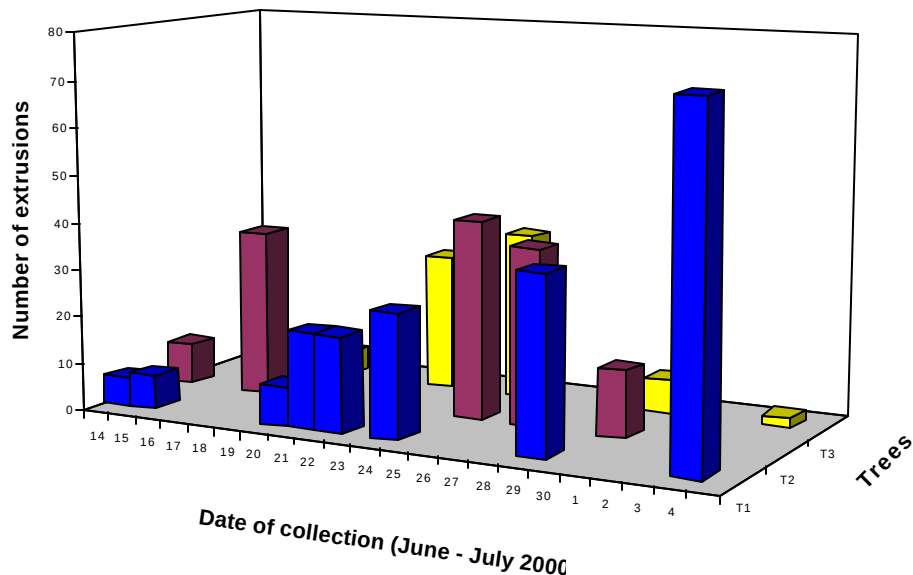


Fig 4.10. Number of extrusions observed from megagametophytes among three trees (T1, T2 and T3) during the collection from Palampur, Himachal Pradesh (from June 14, 2000 to July 4, 2000). Out of a total of 3305 megagametophytes inoculated, 410 extrusions were observed and only 7 embryogenic cell-lines were established.

A similar decline of growth in the initiated cultures of slash pine has also been reported by Liao and Amerson (1995a) and Newton *et al.* (1995). Simple and cleavage polyembryony results in formation of multiple zygotic embryos in pines (Singh 1978). Simple polyembryony results in fertilization of more than one embryo and formation of genetically different proembryos. Cleavage polyembryony on the other hand, produces identical proembryos (Maheshwari and Konar 1971). Pines typically have two to five egg cells per ovule. If an ovule has two egg cells that are fertilized and the resulting zygotic embryos undergo cleavage, then the ovule will contain eight developing embryos with two different genotypes. Becwar *et al.* (1991) studied this aspect and showed that it is possible to initiate embryogenic tissue from more than one zygotic embryo of a loblolly pine seed and that the resulting cell lines may be genetically different.

All the cell lines that were initiated during our studies are now being tested for efficient development of somatic embryos and scaling-up in bioreactor. For the full utilization of the efforts taken to establish embryogenic cell lines, it becomes very much essential to preserve the embryogenic cultures not only by continuous sub-culturing, but also by cryo-storage. These aspects will be discussed in detail in the next chapter.

4.3.3. Pre-maturation and maturation treatments

For promoting maturation of somatic embryos in conifer embryogenic cultures, the immature somatic embryos must be shifted from an environment that promotes cleavage polyembryony (auxin, cytokinin and low osmotic concentrations) to one containing ABA and, ideally raised osmotic concentrations (Attree and Fowke 1993). Effects of ABA and gellan gum were tested in the initial studies (in the years 1996-97, 1997-98) with three cell lines (R-1, R-3 and R29) on the maturation of somatic embryos. Initial experiments suggest that a combination of 30 μ M ABA with 0.25% gellan gum, among the various combinations tested, was best suited for the maturation of somatic embryos (Table 4.8.). Klimaszweska

- Fig. 4.11.**
- (a)** Embryogenic culture of *P. roxburghii* on maturation medium containing DCR basal salts, 0.1g/l myo-inositol, 3% maltose, 30mM ABA and 0.25% gellan gum (bar = 5 mm) (developing somatic embryos are marked with arrows)
 - (b)** Stage D somatic embryo of *P. roxburghii*: globular head (h) and suspensors (sus) (bar = 1mm)
 - (c)** Cotyledonary stage somatic embryos of *P. roxburghii* on maturation medium (bar = 2 mm) showing developing cotyledonary bumps (cot) on the embryo head (emb)

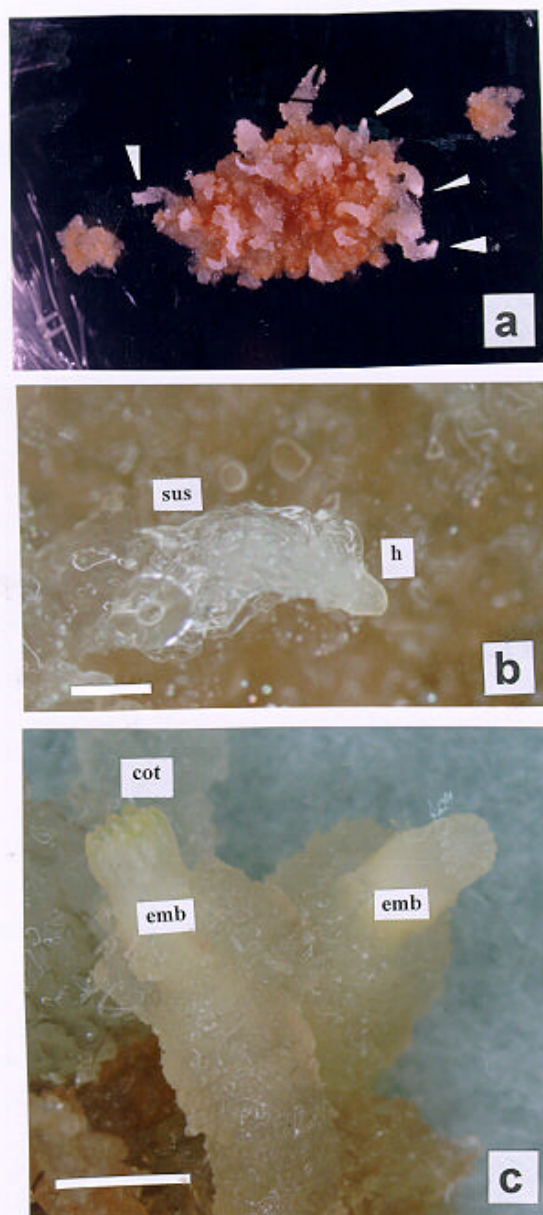


Fig. 4.11.

and Smith (1997) reported the use of very high concentration of gellan gum (1%) and ABA (120 μ M) suitable for *P. strobus* somatic embryo maturation. There are mixed reports for the medium suitable for maturation in *Pinus* species, but the role of ABA in the normal development is fully established (Attree and Fowke 1993). Somatic embryos resembling stage D zygotic embryos (with smooth paraboloid shaped head) can be seen developing in Figs. 4.11.a. and b. The prominent head with pre-cotyledonary bumps or small cotyledons (Fig. 4.11.c. and Fig 4.12.a.), and hypocotyl with a developing radicle can be clearly observed in Figs. 4.12.b. and d. Fully developed somatic embryos are formed on separating out from the callus and culturing them on medium devoid of growth regulators (Fig. 4.12.d.).

It was later realized that for the normal development of somatic embryos, it is absolutely essential to first create conditions unsuitable for proliferation of the embryogenic culture before subjecting them to maturation treatments. For this purpose, various pre-maturation treatments were given to the embryogenic cultures (Table 4.9.). The cultures were incubated on media devoid of any growth regulators for different periods. The best pre-treatment response for the normal development of somatic embryos was found to be culturing for 3 weeks on DCR basal media supplemented with very low concentrations (5 μ M 2,4-D, 2 μ M BA and 2 μ M kin) of growth regulators and 3% sucrose. Only cell line T20627 was tested for this experiment and the results can be observed in Table 4.10. McKersie *et al.* (1995) have discussed in detail the importance of tissue culture procedures necessary to produce dry quiescent somatic embryos to achieve synthesis of storage reserves and acquisition of desiccation tolerance.

It was generally noted for the cell line that had been continuously sub-cultured from the 1997 studies (R-29), required 6-7 weeks of pre-treatment before giving the maturation treatment. Whereas, the cell line (T20627) from the 2000 studies showed best response after culturing for 3 weeks on media with low levels of hormones (Table 4.9.). Inclusion of maltose and PEG in the maturation medium did improve the efficiency and quality of the somatic embryos as compared to the

- Fig. 4.12.**
- (a) Magnified view of cotyledonary stage somatic embryo of *P. roxburghii* (bar = 2 mm)**
 - (b) Well formed cotyledons (cot) and developing root initials (ri) in the somatic embryo of *P. roxburghii* (bar = 2 mm)**
 - (c) Abnormal somatic embryo of *P. roxburghii* showing development of only the cotyledonary ring (cot) (bar = 1 mm)**
 - (d) Well formed somatic embryo of *P. roxburghii* on germination medium – DCR basal medium (1/4), 2% sucrose and 0.5% activated charcoal (bar = 5 mm)**

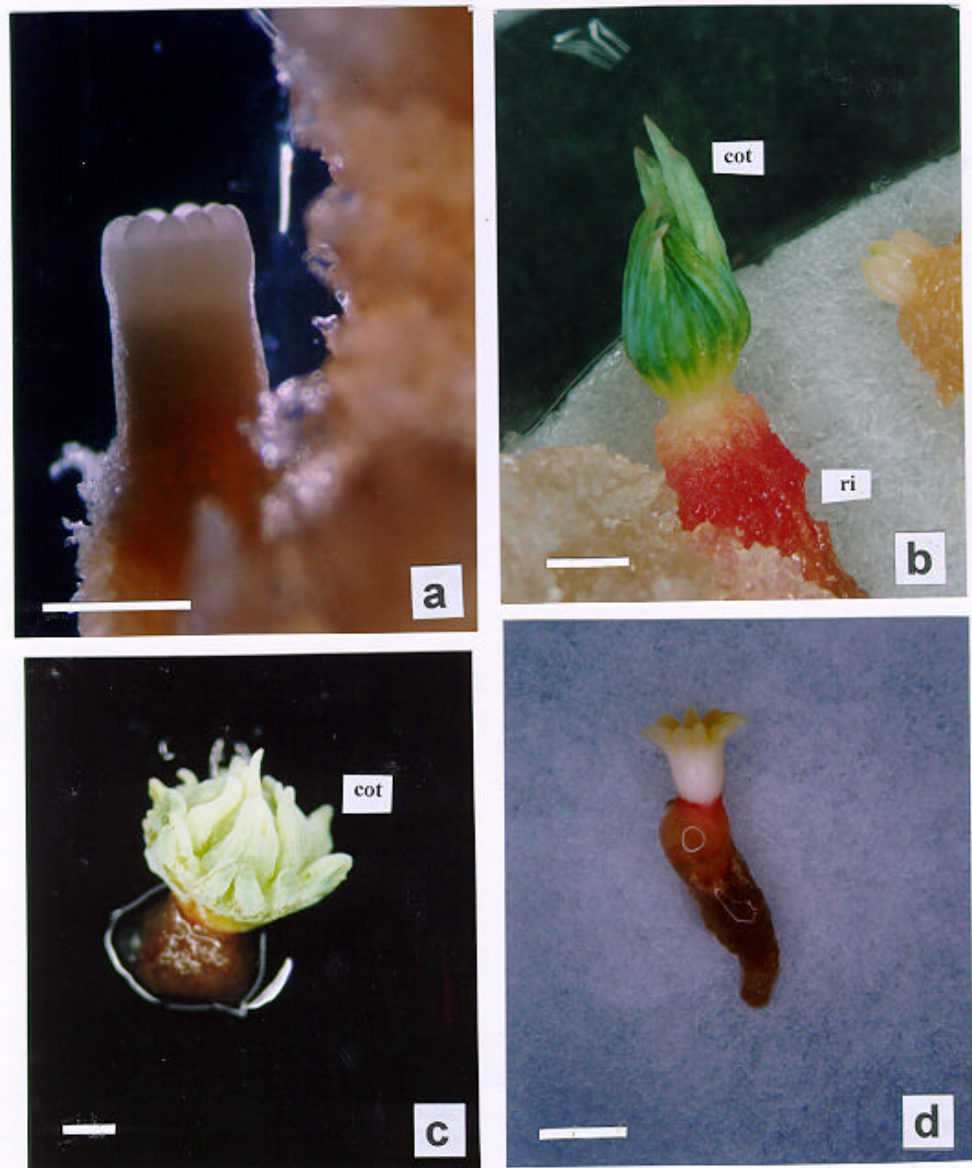


Fig. 4.12.

maturation studies conducted in 1997. The best response was achieved on DCR basal medium (half strength) supplemented with 3% maltose, 1.0g/l myo-inositol, 30 μ M ABA and 0.25% gellan gum (Table 4.9.). This was after a pre-treatment for 3 weeks on low hormone medium. ABA has been found to be inhibiting cleavage polyembryony and promoting the synchronous maturation of somatic embryos of many conifer species (Attree and Fowke 1993).

It has also been observed that the type of osmoticum used in conjunction with ABA profoundly affects the development of conifer somatic embryos. Thus simple sugars and salts are not always effective as high molecular weight osmotica such as polyethylene glycol (PEG) and dextrans (Attree *et al.* 1991). In conifer somatic embryogenesis it is a common practice to restrict the water availability by exposing embryogenic tissue to maturation media of high osmolality (low ψ). However, the responses of different conifer species vary when exposed to the same solute. Maltose is reported to be superior to sucrose and glucose in maturation of *Abies nordmanniana* and *Pinus taeda* somatic embryos (Norgaard and Krogstrup 1995, Li and Huang, 1996). On the contrary, high sucrose levels (0.2-0.4 M) have been used effectively to increase osmolality in *Picea glauca*, *Picea mariana*, *Larix* hybrids and *Larix laricina* cultures (Lu and Thorpe 1994, Cheliak and Klimaszewska 1991, Lelu *et al.* 1994a & 1994b, Klimaszewska *et al.* 1997). PEG (molecular weight 4000 and higher) has also been used by Attree *et al.* (1995) for *P. glauca* somatic embryo maturation.

In *Pinus taeda* and *Abies normanniana*, a combination of maltose and PEG was most beneficial in exerting somatic embryo maturation frequency (Norgaard 1997, Li *et al.* 1998). However, the use of PEG in maturation of *Larix laricina* (Klimaszewska *et al.* 1997) and *Picea abies* (Bozhkov and von Arnold 1998) was detrimental to embryo germination. In chir pine too, the use of PEG in maturation did not have any positive effect on the maturation or germination of the somatic embryos (Table 4.10.).

Table 4.8. Effect of various maturation treatments on three established embryogenic cell lines (R-1, R-3 and R-29) of *P. roxburghii* Sarg. from 1997 collections. The embryogenic culture clumps were first transferred to hormone free half-strength DCR basal medium for one week before transferring them to maturation media.

Medium*	Embryogenic cell lines		
	R-1	R-3	R-29
10µM ABA + 0.18% gellan gum	54 (4) [#]	46 (0)	38 (2)
30µM ABA + 0.18% gellan gum	45 (7)	38 (3)	47 (4)
30µM ABA + 0.25% gellan gum	38 (13)	47 (8)	52 (18)
0.30% gellan gum	28 (0)	35 (0)	42 (1)
Control	0 (0)	0 (0)	0 (0)

* Media contains DCR basal salts (half strength) + 2% sucrose + 1.0 g/l myo-inositol

[#] Data represents number of clumps of embryogenic cell lines cultured on different media combinations and data within parentheses represents the number of clumps of embryogenic tissue showing smooth paraboloid-shaped somatic embryo formation. (Weight of each clump of the embryogenic mass varied from 320-550 mg)

Table 4.9 Various pre-treatments given to the embryogenic cultures of *Pinus roxburghii* Sarg. (cell line T20627 from year 2000 collections from Palampur) before transferring to maturation medium

S.No.	Pre-treatment medium*	Incubation period (weeks)
1	*	1
2	”	2
3	”	3
4	”	6
5	* + 0.5% activated charcoal	1
6	”	2
7	”	3
8	”	6
9	* + 5µM 2,4-D + 2µM BA + 2µM kin	3
10	”	6

* DCR basal medium (half) + 2% sucrose + 1.0g/l myo-inositol + 0.25% gellan gum

Table 4.10. Different maturation treatments tested after the pre-treatments (Table 4.9.) to the T20627 embryogenic cell line of *Pinus roxburghii* Sarg.

Maturation Medium @	Pre-treatments	1	2	3	4	5	6	7	8	9	10
1	30µM ABA + 3% maltose	0*	0	0	24	0	0	0	15	33	9
2	30µM ABA + 3% maltose + 5% PEG	0	0	0	7	0	0	0	11	12	0
3	30µM ABA + 6% maltose	0	0	0	0	0	0	0	0	0	0
4	30µM ABA + 3% maltose + 10% PEG	0	0	0	0	0	0	0	0	6	3
5	60µM ABA + 3% maltose	0	0	0	0	0	0	0	0	0	4
6	60µM ABA + 6% maltose	0	0	0	0	0	0	0	0	0	7
7	30µM ABA + 5% PEG	0	0	0	0	0	0	0	0	0	0
8	30µM ABA + 10% PEG	0	0	0	0	0	0	0	0	0	0
9	30µM ABA + 3% sucrose	0	0	0	0	0	0	0	0	0	0
10	3% sucrose	0	0	0	0	0	0	0	0	0	0

@ DCR basal medium (half strength) + 1.0g/l myo-inositol + 0.25% gellan gum + supplements.

Data represents number of clumps (out of a total of 48 clumps per treatment) forming somatic embryos with emerging cotyledonary bumps, after 8-10 weeks of incubation.

4.3.4. Germination of somatic embryos

After the appearance of the cotyledonary bumps and the root initials, the somatic embryos were subjected to partial desiccation treatment by leaving them open in sterile petri-plate in the laminar airflow for 2-3 hours. The somatic embryos were kept for germination on DCR basal medium (half strength) with 2% sucrose, 0.5% activated charcoal and 0.25% gellan gum. The cotyledons developed within 3 weeks on germination medium and root formation took place within 5-6 weeks (Fig. 4.13.a.). The elongation of the shoot apex was observed in 2 out of 6 somatic embryos that germinated (Fig. 4.13.b.). The percentage of germination of the somatic emblings was found to be low. Only 6 out of 37 somatic embryos were able to germinate. Aberrant embryo germination patterns such as formation of cotyledonary whorls, absence of shoot apical meristems and

Fig. 4.13. (a) Somatic embling of *P. roxburghii* (after 6 weeks on germination medium)
(b) Somatic emblings of *P. roxburghii* (bar = 1 cm)



Fig. 4.13.

root initials were also visible (Fig. 4.12.c). Precocious germination of somatic embryos was also observed to be a common feature.

This may be caused due to improper desiccation or maturation treatments. Desiccation of somatic embryos has been reported to play a major role in normal development and growth of the somatic emblings (Roberts *et al.* 1990, Attree *et al.* 1991). These treatments aim at reducing the water content in the embryo tissues and/or completing the maturation process. In *P. strobus*, it has been proved (Klimaszewska and Smith 1997, Klimaszewska *et al.* 2000) that high concentration of gellan gum or agar was beneficial for reducing the water content of the mature somatic embryos, thus eliminating the need of partial desiccation. The embryogenic capacity of cell lines that have been continuously sub-cultured for over three years was observed to be diminishing, which may be another cause of abnormal embryo structures apart from somaclonal variations.

4.3.5. Conclusions

From our studies it can be collectively concluded that it is possible to induce somatic embryogenesis from immature zygotic embryos of chir pine and is similar to the cleavage polyembryony observed in nature. The 'window' or the correct stage of the zygotic embryo is the middle cellular stage, where the embryo has not developed into a smooth paraboloid structure. It is also possible that the time when a number of embryos are competing and only one outgrows the others, this 'embryo rescue' like situation leads to induction of somatic embryogenesis *in vitro*. Further studies for better understanding of the developmental process are essential to fully ascertain the best media supplements and culture conditions for the maturation of somatic embryos, in order to obtain the optimum conversion percentage, to maximize the embryogenic potential and normal development of somatic embryos into plantlets. The pre-maturation and desiccation treatments are critical stages after successful establishment of embryogenic tissue for the development of somatic embryos to plants.

The following important observations were made during the present studies:

- Immature zygotic embryos of chir pine were observed to be suitable for the induction of somatic embryogenesis.
- Mature zygotic embryos gave rise to non-embryogenic callus
- The induction of somatic embryogenesis from immature megagametophytes is extremely specific to the developmental stage of the immature zygotic embryo.
- The development of embryo differs within different locations and as well as among trees within the same location.
- DCR basal medium was observed to be most suitable for the induction of embryogenesis.
- A combination of 2,4-D (50 μ M), BA (20 μ M) and kin (20 μ M) with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 0.18% gellan gum at a pH of 5.8 before media sterilization was found to be best for the induction as well as proliferation of embryogenic tissue of chir pine.
- The morphology of the extrusion of embryogenic tissue from within the megagametophyte was observed to be dependent on the stage of the zygotic embryo development.
- Four types of extrusions were observed and only Type I extrusion was capable of proliferation.
- Apart from the media, the proliferation of embryogenic cell lines was also observed to be sensitive to temperature.
- Three cell lines from the years 1996 and 1997 studies (R-1, R-3 and R-29) and seven from the year 2000 studies () are being maintained.
- Generally, the frequency of extrusion and embryogenic culture establishment was observed to be very low.

- Shifting the embryogenic cultures from proliferation medium to hormone free medium (or a medium with low levels of growth regulators) was essential for healthy development of the somatic emblings.
- The somatic embryos germinated after partial desiccation on DCR basal medium with 2% sucrose, 0.5% activated charcoal and 0.25% gellan gum.
- Aberrant somatic embryos were also observed during the germination.
- Regularization of collection schedules for optimal culture establishment is a must and for that a sound knowledge of the embryology of pines is essential.

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Studies with the embryogenic cell lines of *Pinus roxburghii* Sarg:

(i) Scaling-up in bioreactor and, (ii) Cryopreservation

5.1 Introduction

5.1.1. Growth studies of embryogenic cultures

For economical, large-scale propagation of plants, somatic embryos of many woody species have been grown as suspensions, where the growth is more rapid than on agar solidified medium (Tautorus and Dunstan 1995). The full potential of somatic embryogenesis will only be exploited by developing reliable methods for suspension cultures of embryogenic cell masses (Nishimura *et al.* 1995). The unique ability of coniferous embryogenic cells for repetitive formation of proembryos and their genetic and embryogenic stability, make these systems particularly suited for suspension culture (Krogstrup 1990). The experimental time in suspension cultures is less compared to solid medium culture and it even reduces the variations caused by gradients of chemical and physical factors. Additionally, suspension cultures are an excellent source of protoplasts, and can also be used in bioreactors for low-cost multiplication process (Denchev *et al.* 1992, Nishimura *et al.* 1995)

Embryogenic suspension cultures have been reported in a number of conifers like *Picea abies*, *P. glauca*, *Pseudotsuga menziesii*, *Pinus sitchensis*, *P. strobus* and *P. glauca* but only a few of these studies contain quantitative data (Krogstrup 1990, Lulsdorf *et al.* 1992, Tautorus *et al.* 1992). It is essential to be able to quantify the parameters for growth and development in order to utilize the embryogenic suspension cultures as a consistent and reproducible regeneration system in a bioreactor.

In the present study, efforts have been made to establish embryogenic suspension cultures of *P. roxburghii* Sarg. Various growth parameters were investigated for optimal growth of the embryogenic tissue in flasks and in bioreactor. The maintenance of homogeneity in the culture medium, larger working volume and, control over cultural and physical environment for optimal growth, are few of the many advantages of growing plant tissues in bioreactors.

5.1.2. Cryopreservation

Maintaining embryogenic cultures of pines or any other plant species, for long a period may cause the tissue to lose the embryogenic capacity and also may induce somaclonal variations during subculturing. These problems can be easily overcome by cryopreservation of cultures in liquid nitrogen (Bajaj 1995a & 1995c).

Along with the progress in somatic embryogenesis studies in pines (Attree and Fowke 1993, Gupta and Grob 1995, Timmis 1998), there has also been significant work conducted and reported for the cryopreservation of the following pine species: *Pinus caribaea* (Laine *et al.* 1992), *P. patula* (Ford *et al.* 2000), *P. sylvestris* (Häggman *et al.* 1998) and *P. taeda* (Gupta *et al.* 1987). In all the above mentioned reports, the re-growth capacity of the cryo-stored cultures and their morphology has been compared to the non-frozen cultures, except for the work by Häggmann and co-workers (1998) who have also checked the genetic fidelity of the cryopreserved *P. sylvestris* embryogenic tissue. Cryopreservation and the use of biochemical and molecular markers for testing the genetic homogeneity has also been reported earlier in other conifer genera viz. *Picea* (Eastman *et al.* 1991, Fourre *et al.* 1997, Isabel *et al.* 1993) and *Larix* (De Verno *et al.* 1994).

The present studies were undertaken with the aim of optimizing the cryoprotectant treatments necessary for the embryogenic chir pine cultures to survive the cryo-storage and for this purpose, initially the cultures were stored for

only 72 h in liquid nitrogen. These results are important for the planning of the long-term cryopreservation studies that are presently underway in our laboratory.

5.2 Experimental procedure

5.2.1. Growth studies for scaling-up embryogenic cultures of *P. roxburghii* Sarg. in bioreactor

5.2.1.1. Suspension culture initiation and maintenance:

Vigorously growing embryogenic cultures of *Pinus roxburghii* Sarg. were utilized for initiating suspensions. Liquid proliferation medium (40 ml) (DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50 μ M 2,4-D, 20 μ M BA and 20 μ M kin, pH 5.8) was taken in jam bottles and it was inoculated with 8-10 g of 2 wk old embryogenic tissue. The cells of the tissue were separated by pumping, using a 1 ml auto-pipette with a wide cut tip (to ensure the separation of cells aggregates without any damage). Before using this suspension for inoculating the 250 ml Erlenmeyer flasks, the settled cell volume (SCV) was measured in a sterile measuring tube. SCV was noted after a fixed time period of 20 min throughout the studies. Embryogenic suspension was added at a culture density of 25:100 (25 ml of settled cells made up to 100 ml v/v).

For initiating suspension cultures, 30 ml proliferation medium was taken in 250 ml flasks and additional 30 ml medium was added to it after 14 days to make up the total volume to 60 ml. After another 14 days, 30 ml medium was replaced with fresh medium and this continued till the SCV increased by 400-500 percent. These suspensions were used for maturation studies, bioreactor inoculation and growth studies. Growth curve was plotted, by checking SCV every 5 days, for 7 weeks. The morphology of the embryogenic suspension cultures was also continuously monitored.

5.2.1.2. Effect of initial inoculum:

Different amounts (ml. SCV v/v) of embryogenic tissue were cultured in 250 ml Erlenmeyer flasks as mentioned above and the initial suspension culture densities were adjusted to a final volume of 30 ml as follows: 5:100; 15:100; 25:100 and 40:100 (v/v). The growth of suspensions was measured as above and readings were noted every 5 days for 4–5 weeks.

5.1.2.3. Effect of temperature and pH:

Embryogenic cultures growing on semi-solid medium (DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50 μ M 2,4-D, 20 μ M BA, 20 μ M kin and 0.18% gellan gum with a pH 5.8) in petri-plates were utilized for this study. The growth of embryogenic tissue was monitored as percent increase in fresh weight. The tissue was placed on a sterile Whatman paper disc in the petri-plate containing proliferation medium to facilitate in the weighing of the proliferating embryogenic culture.

To observe the effect of temperature on the proliferation of the embryogenic tissue, it was incubated at two different temperatures, 22 $^{\circ}$ ± 2 $^{\circ}$ C and 25 $^{\circ}$ ±2 $^{\circ}$ C. The increase in fresh weight of the tissue was checked every 5 days for 4 weeks. The effect of pH on the growth of the embryogenic tissue of *Pinus roxburghii* was observed by culturing on proliferation medium (as above) at different pH, 4.6, 5.0, 5.4, 5.8, and 6.0. The pH was adjusted before sterilization of the medium. The growth was monitored as increase in fresh weight (same as above). Five sets embryogenic cultures were used for each treatment and the experiments were repeated twice before the percent (average) increase in fresh weight and standard deviation (S.D.), were calculated.

5.2.1.4. Bioreactor setup and maintenance:

New Brunswick stir tank fermentor was utilized for the purpose, which had a stainless steel jacket and a one litre glass vessel agitated by flat blade impellers.

Filtered (through 0.22µm filter) air was pumped into the vessel by a compressor to control the dissolved oxygen (DO) concentration. Probes for dissolved oxygen, pH and temperature were used for monitoring these parameters throughout the studies (Fig. 5.6.b.).

A circulating water bath was connected to the jacket of the culture vessel for maintaining the temperature at $22^{\circ}\pm 2^{\circ}\text{C}$. Initially, only batch cultures were used for studying the growth kinetics and biomass yields. The bioreactor along with the pH probe was sterilized (at 15 lb. in.⁻² (121°C) pressure for 1 hour.) before the addition of sterile proliferation medium and the inoculum.

Embryogenic cultures growing in flasks were used as inoculum (25:100 culture density) for the bioreactor and constant monitoring of the pH, temperature and DO was conducted. Culture samples were routinely drawn out to observe the morphology of the growing somatic embryos.

5.2.2. Cryopreservation of *P. roxburghii* Sarg. embryogenic cultures

5.2.2.1. Plant material:

Embryogenic cultures of *Pinus roxburghii* Sarg. were initiated by culturing immature megagametophytes as described in Chapter 4. Embryogenic cell line T20627 from the year 2000 collection (Chapter 4, Section 4.3.2.5.), was utilized for all cryopreservation experiments. The cultures were maintained on DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50µM 2,4-D, 20µM BA, 20µM kin and 0.18% gellan gum. The pH of the medium was adjusted to 5.8 prior to autoclaving. The cultures were incubated at $22^{\circ}\pm 2^{\circ}\text{C}$ in dark and subcultured every three weeks.

5.2.2.2. Pre-treatments:

The main aim of this experiment was to determine the optimum pre-treatment of cryoprotectants for the embryogenic cultures to survive the freezing in liquid nitrogen. Different concentrations of sorbitol and dimethyl sulfoxide

(DMSO) were tested for this purpose. Three to four grams of freshly subcultured embryogenic tissue (2 weeks after last subculture) was precultured in 40 ml liquid proliferation medium (same as in section 5.2.2.1.) in culture bottles. These suspension cultures were placed on shaker (100 rpm) in dark at $22^{\circ}\pm 2^{\circ}$ °C. For the cryopreservation studies, only vigorously growing embryogenic culture (exponential growth phase) were utilized, thus embryogenic tissues were used after 2 weeks of last subculture (normal subculture time being 3 weeks).

Six pre-treatments with cryoprotectants were tested using sorbitol and DMSO along with the controls:

- (a) 0.3 M sorbitol + 5 % DMSO
- (b) 0.3 M sorbitol + 10 % DMSO
- (c) 0.5 M sorbitol + 5 % DMSO
- (d) 0.5 M sorbitol + 10 % DMSO
- (e) no sorbitol + 5 % DMSO
- (f) no sorbitol + 10 % DMSO.

The controls included:

- (i) no pre-treatment (P-T), no slow freezing (SF), no liquid nitrogen (LN)
- (ii) no P-T, SF conducted, no LN
- (iii) no P-T, no SF, kept in LN
- (iv) P-Ts (a – f), conducted SF, no LN
- (v) P-Ts (a – f), no SF, kept in LN
- (vi) P-T (a), SF conducted, kept in LN, re-growth without washing off cryoprotectants
- (vii) embryogenic cultures that were not subcultured for more than 5 weeks, P-T (c), conducted SF, kept in LN.

Ten replicates were tested for each pre-treatment and five each for the controls and the experiment was conducted twice.

For the pre-treatment involving sorbitol and DMSO, cultures were first primed with sorbitol for 24 h before adding DMSO. From 4M filter sterilized stock solution, sorbitol (Hi-media, India) was added ten times over a period of 30 min to give a final concentration of 0.3M or 0.5M. The cultures were continuously and gently agitated during the addition of cryoprotectants and the cultures were then incubated on shaker. On day two, culture bottles were kept on ice during the treatment with DMSO (Sigma Chemical Co., USA), which was also added to the cultures ten times over a period of 30 min to give a final concentration of 5% or 10% (v/v) (Ford *et al.* 2000). All pretreatments were done in sterile conditions in laminar airflow.

5.2.2.3. Cryopreservation:

Cryomed™, a programmable controlled-temperature cooling chamber was used for slow freezing the tissues till -50°C . The pre-treated tissues were dispensed (1.5 ml each) in Nalgene™ cryo-vials and were kept on ice for 2 h before slow freezing. Thereafter, the vials were transferred to cryo-caness just before slow freezing protocol, which was as follows: hold at -1°C for 5 min, slow freezing @ $-0.3^{\circ}\text{C}/\text{min}$ to -16.5°C , hold at -16.5°C for 15 min, slow freezing @ $-0.3^{\circ}\text{C}/\text{min}$ till -35°C , freezing @ $-25^{\circ}\text{C}/\text{min}$ to -50°C (personal communication with Prof. Sara von Arnold). The canes containing the vials were then plunged immediately in liquid nitrogen and removed after 72 h.

5.2.2.4. Thawing and re-growth:

Thawing of the cryo-stored tissue was done after removing the cryo-vials placing the cryo-vials for 2-3 min in Magenta™ jars containing sterile water at 45°C . The vials were then washed in 70% ethanol and transferred to jars containing sterile water kept on ice, till they were poured out for re-growth. The thawed tissue was poured on Whatman filter paper no. 1 discs (45 mm diameter),

and excess supernatant was filtered out using vacuum. The tissue was washed with liquid DCR basal medium (without growth regulators) before placing the discs on fresh solid proliferation medium in Laxbro® sterile petri dishes (55 mm X 1.5 mm). The cultures were transferred to fresh medium after 24 h, then subcultured every week.

5.2.2.5. *Viability assay:*

Fluorescent stain, fluorescein diacetate (FDA) (Sigma Chemical Co., USA) was used to test the viability of the cryopreserved tissue (Häggmann *et al.* 1998, Widholm 1972). A 0.5% stock solution of FDA was prepared in acetone and stored in refrigerator. Some of thawed culture (20-30 mg) was harvested immediately after thawing in a 1.5 ml Eppendorf tube containing 800µl liquid medium, to which 20µl FDA was added from the stock (final concentration of 0.05 % FDA) and the stained tissue was observed under UV light using Zeiss Axioplan2 microscope.

5.2.2.6. *Analysis of growth:*

The data was collected as the increase in fresh weight of the cryopreserved tissue kept for re-growth on proliferation medium. The readings were taken, starting on day zero and then at weekly intervals for 5 weeks. The re-growth of the tissue was calculated as % increase in fresh weight (W_i) (Laine *et al.* 1992):

$$W_i = \frac{w_i - w_0}{w_0} \times 100 \quad (w_i = \text{weight on day } i, w_0 = \text{weight on day zero})$$

Standard deviation (S.D.) was calculated for the % increase in fresh weight for each treatment and the controls.

5.3 Results and discussion

5.3.1. Growth studies for the scaling-up of embryogenic suspension cultures of *Pinus roxburghii* Sarg. in bioreactor

Somatic embryogenesis of conifers has opened up new avenues for realizing and implementing biotechnological advancements for clonal forestry (Dunstan 1988, Gupta 1988, Gupta *et al.* 1991, Gupta *et al.* 1993a, Gupta *et al.* 1993b, Walter *et al.* 1998). Today in several countries clonal propagation of conifers is mainly carried out at a very large scale by using good quality somatic embryos (Adams *et al.* 1994, Attree and Fowke 1993, Gupta *et al.* 1991, Gupta *et al.* 1993b). Tissue culture of pines is still in its infancy in India, and only few reports of research in this area are available since the initial research reports by (Bhatnagar *et al.* 1983, Konar 1980, Mehra and Anand 1993) and recently, by two groups, each from National Chemical Laboratory, Pune (Mathur and Nadgauda 1999, Mathur and Nadgauda 2000, Nadgauda *et al.* 1993) and, Forest Research Institute, Dehradun (Arya *et al.* 2000). Somatic embryogenesis has been proved to be an indispensable technique for mass propagation and improvement of a large number of plant species, notably for conifers (Bajaj 1995b, Jain 1999, Kendurkar *et al.* 1995, Gupta and Grob 1995).

Embryogenic cultures of chir pine are being maintained and initial studies were conducted with respect to physical and chemical parameters of growth, necessary for scaling-up of these cultures in bioreactor. Growing embryogenic cultures in bioreactor will not only be used for producing large numbers of somatic embryos, but also will ensure a continuous harvest (Tautorius and Dunstan 1995). Physical parameters like pH and temperature, initial inoculum density and chemical constituents of the medium, need to be optimized before the culture is to be grown in a bioreactor. The present studies highlight the importance of some

parameters essential for optimal growth and scaling-up of embryogenic cultures of chir pine in bioreactor.

It is necessary to develop reliable methods of suspension cultures of embryogenic tissues in order to obtain maximum advantage of somatic embryogenesis technique and scale-up the technology for mass production. The repetitiveness and stability of the conifer embryogenic mass makes it an ideal system for growing as suspension culture. There are a number of advantages that suspension cultures offer over culturing on semi-solid medium. Firstly, it accelerates the experimental response time and reduces the variation caused by gradients of chemical and physical factors. Secondly, it provides an ideal system for protoplast isolation and also can be used for lost cost clonal propagation using bioreactors and automation (Krogstrup 1990, Tautorus and Dunstan 1995).

Bioreactors are now being widely used for scaling-up of conifer somatic embryos as it clearly offers the capability of rapid growth, maturation, conversion and delivery of somatic emblings (Gupta *et al.* 1993b, Gupta and Timmis 1999, Tautorus and Dunstan 1995). Introduction of bioreactor culture for scaling-up of sandalwood somatic embryos by Bapat and co-workers (1990) has already given the needed impetus and opened up new vistas by demonstrating the potential of this technique for clonal forestry in India (Bapat 1995). Mostly, the physical parameters like pH and temperature, initial inoculum density and media: vessel ratio etc., need to be optimized before the culture is to be grown in a bioreactor and keeping this objective in mind, the present investigation was undertaken.

5.3.1.1. *Effect of initial inoculum on the growth of suspension culture*

Studies with the growth of cell suspensions among conifers have mostly been conducted with embryogenic cultures of spruce (Attree *et al.* 1994, Krogstrup 1990, Lulsdorf *et al.* 1992, Tautorus *et al.* 1992 & 1994). Whereas, reports on growth studies with embryogenic suspension cultures of pines is sparsely documented (Finer *et al.* 1989, Smith *et al.* 1991).

The growth curve of embryogenic cultures of chir pine was plotted by checking the settled cell volume (SCV) of the growing suspension, every 5 days, for 7 weeks. This is a non-destructive method of quantification of biomass and provides an excellent indication of the growth of suspension cultures. Although, it may not be as accurate as packed cell volume or dry weight methods, but it did serve our purpose in this experiment. The readings of the SCV were taken after 20-35 min of settling down of the embryogenic cell aggregates/tissue, to minimize any discrepancy in observations. Other methods of evaluating growth kinetics in cell cultures of interior spruce and black spruce have been described by Lulsdorf and co-workers (1992).

The morphology of the embryogenic suspension cultures was also continuously monitored. The suspension cultures of chir pine mostly contain organized masses or the initial stage somatic embryo aggregates (Fig. 5.6.a). These cell aggregates consists of cells that will give rise to the embryonal head and suspensor cells. The single cells in the chir pine suspension normally do not give rise to somatic embryos, it is only the cells from the aggregates that proliferate, thus making the suspension culture a very attractive system for large-scale proliferation.

It was necessary to find out the optimal initial inoculum needed to initiate suspensions cultures to ensure a minimal lag phase before its proliferation. The inoculum culture density had a direct effect on the growth rate of the embryogenic culture of *P. roxburghii* Sarg. as it can be observed from Fig. 5.1.

An initial inoculum density of 25:100, i.e. approximately 25 % (v/v) cell density (6.0 – 7.5 ml SCV of cell culture in 30 ml liquid medium) was observed to be optimal for the relatively good growth rate of suspensions in flasks with a minimal lag phase. Suspension cultures showed a typical sigmoid growth curve – a lag phase followed by exponential growth and stationary growth. Lower inoculum densities (5:100) showed a longer lag phases, before achieving the log phase of growth and so did the higher inoculum density (40:100) (Fig. 5.1.).

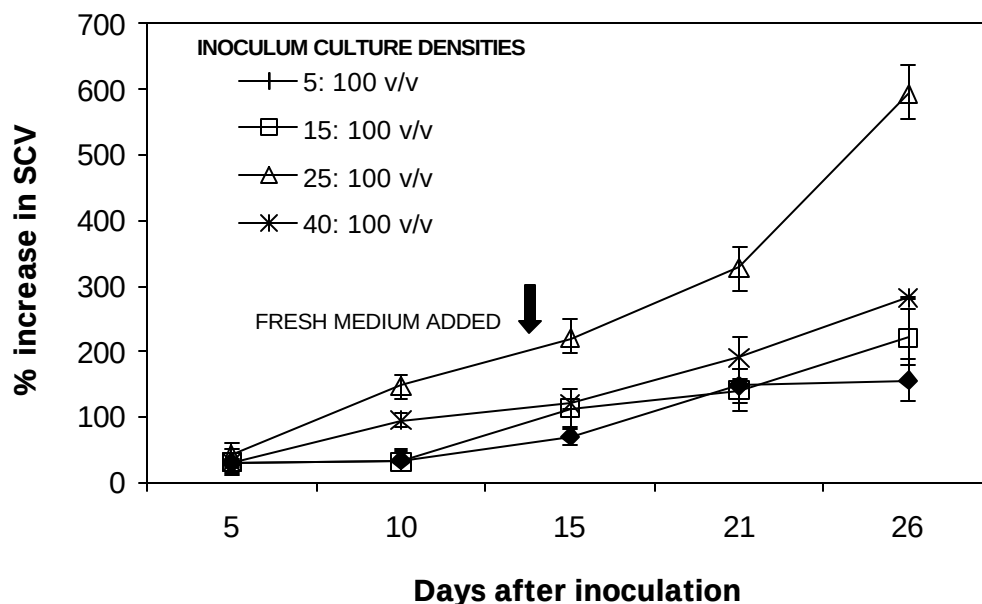


Fig. 5.1. Effect of initial inoculum densities (5:100, 15:100, 25:100 and 40:100 v/v) on the growth embryogenic suspension cultures of *Pinus roxburghii* Sarg. in 250 ml Erlenmeyer flasks. The growth of embryogenic tissue is calculated as % increase in Settled Cell Volume (SCV). DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inisitol, 0.5g/l L-glutamine, 0.5g/l CH, 50mM 2,4-D, 20mM BA and 20mM kin (pH 5.8 of the medium before autoclaving) was utilized for the studies.

Thus for initiating the bioreactor batch culture studies, the initial inoculum density was always adjusted to 25:100 for the optimal growth of the embryogenic culture of chir pine. In a similar study with *Picea sitchensis* suspensions Krogstrup (1990) found a culture density of 20:100 (v/v) suitable for the optimal growth of the embryogenic cultures. Additionally, effect of total volume of a suspension culture per flask was also tested (Krogstrup 1990), where the lower proliferation rates could be attributed to lower medium availability and a direct correlation of proliferation rate per ml SCV with the gaseous atmosphere in the culture flasks could be made.

5.3.1.2. Effect of temperature and pH on the growth of embryogenic cultures

It was clearly observed that temperatures above 24°C were completely detrimental for the growth and survival of the chir pine embryogenic tissue (Fig. 5.2.). Acceleration in the growth rate was observed in the cultures that were kept at 25° - 27°C when they were incubated at lower temperature after 4 weeks. As it can be observed in fig. 5.2., the embryogenic cultures growing at 25°-27°C had almost reached the plateau of their growth phase before the cultures were transferred to 21°-23°C. The revival of growth was immediate on incubation at lower temperature. This study proved to be very important during the scaling-up experiment in bioreactor, where it was absolutely essential to maintain the temperature of the culture vessel to an optimal level, since there was a rise in the temperature of the culture due to the constant stirring by the impellers.

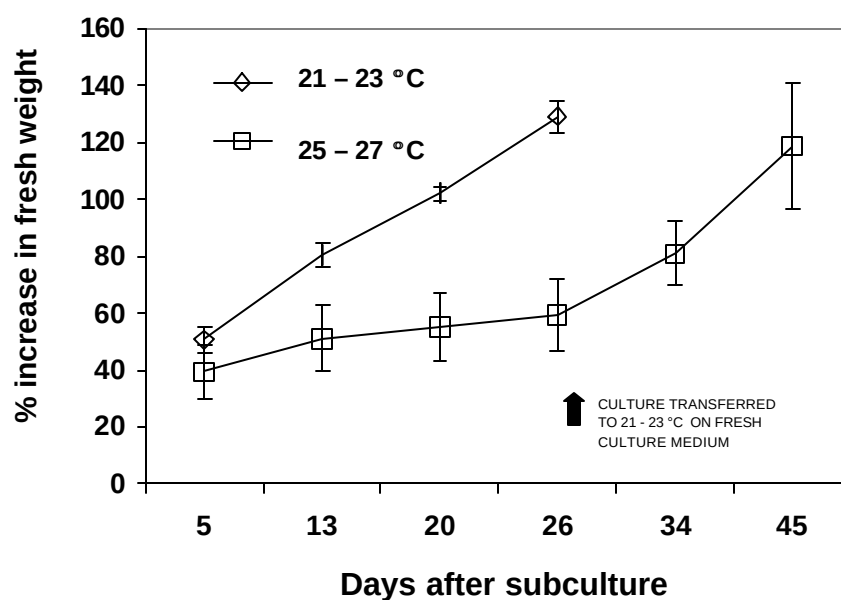


Fig. 5.2. Effect of temperature on the growth of embryogenic cultures of *Pinus roxburghii* Sarg. on semi solid medium DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l L-glutamine, 0.5g/l CH, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum (pH 5.8). The growth was calculated as the percent increase in fresh weight of the cultures.

A pH of 5.8 before sterilization of the medium was found to be most conducive for the optimal growth of the embryogenic cultures of chir pine (Fig. 5.3). This is been observed in studies with other pine species, where the pH of medium is adjusted to 5.8 prior to autoclaving. Even for the studies with the bioreactor the pH of the medium was adjusted to 5.8 and the change in pH during the growth of the cultures was observed. Studies where the pH of the medium could be controlled and maintained in bioreactor cultures of non-embryogenic alfalfa cells resulted in inhibition of growth (McDonald and Jackman 1989).

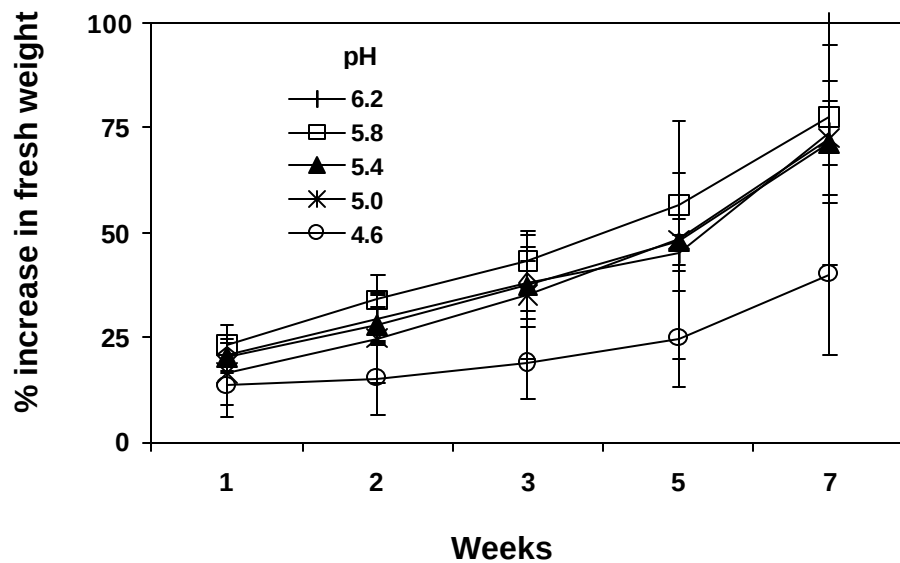


Fig 5.3. Effect of pH of the medium (before sterilization) on the proliferation of embryogenic cultures of *Pinus roxburghii* Sarg. DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l L-glutamine, 0.5g/l CH, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum was used for this experiment.

5.3.1.4. Batch culture of embryogenic suspension of chir pine in bioreactor

All the growth parameters studied with the suspensions in flasks were used in setting up and running of the bioreactor (Fig. 5.6.b.) for producing embryogenic cultures at a larger scale. The embryogenic chir pine cultures were grown as batch culture in the bioreactor for the present growth studies and these observations will be utilized for continuous cultures in the future. Although constant monitoring of the increase in the biomass (fresh weight/SCV/packed cell volume) of the growing cultures was not possible but the final SCV was observed only after running the bioreactor continuously for three weeks. On an average, a 600-650 percent increase in the SCV was observed after growing the embryogenic suspensions in 1 litre bioreactor with 25-40/ml of early smooth-paraboloid shaped somatic embryos (Fig. 5.6.a.)

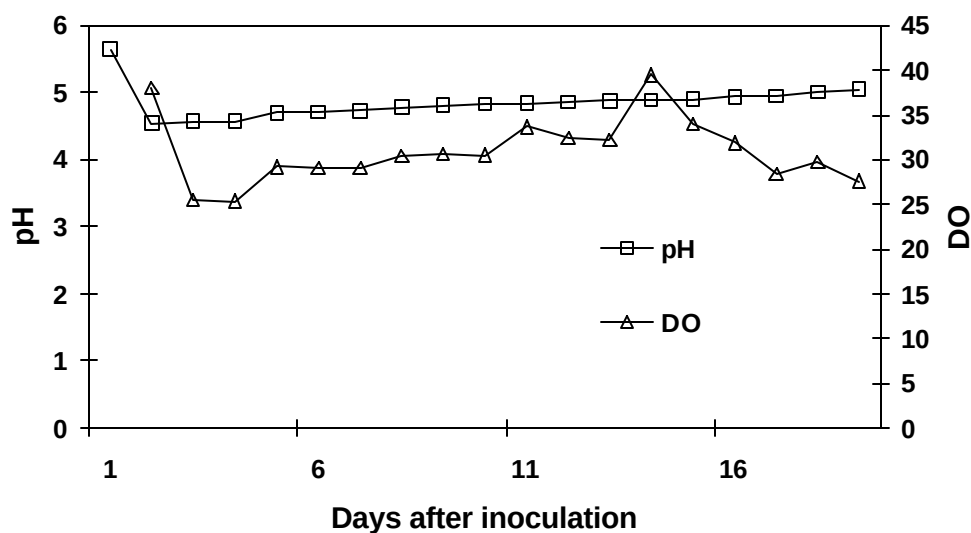


Fig 5.5. Monitoring of pH and dissolved oxygen (DO) concentration (%) during the culture of embryogenic suspensions of *Pinus roxburghii* Sarg. in 1 litre bioreactor. DCR basal medium supplemented with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l L-glutamine, 0.5g/l CH, 50mM 2,4-D, 20mM BA and 20mM kin was utilized for the experiment (initial pH was adjusted to 5.8 before autoclaving).

- Fig. 5.6.**
- (a) Embryogenic suspension culture of *P. roxburghii*:**
Somatic embryo aggregate in suspension showing embryonal heads (emb) stained with acetocarmine and suspensors (sus) attached to the embryo heads
- (b) New Brunswick bioreactor set-up for scaling-up embryogenic cultures of *P. roxburghii*:**
g – 1 litre glass vessel
pH – pH probe
DO – dissolved oxygen probe
T – temperature probe
WB – circulating water bath for temperature control
I – impellers
A – filtered compressed air inlet

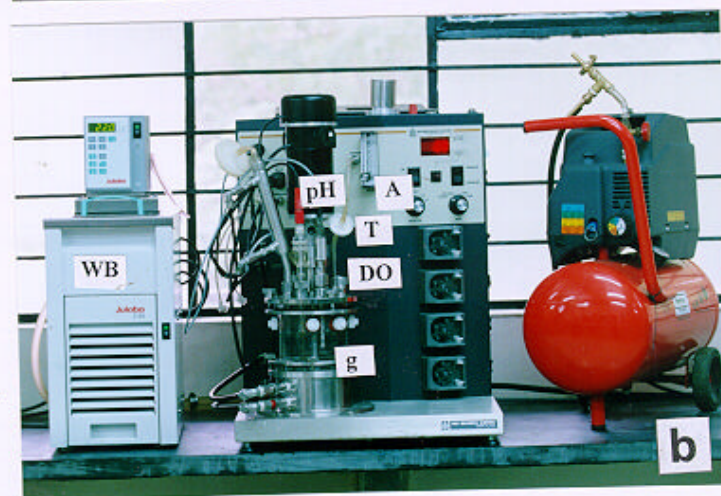
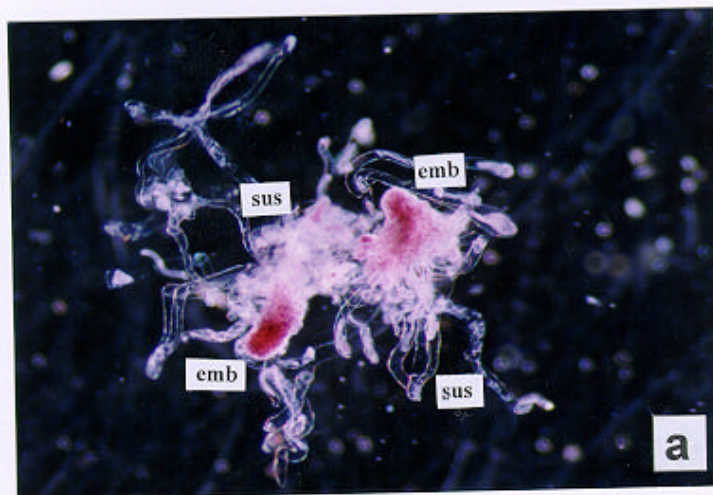


Fig. 5.6.

Other methods that have been used to monitor the growth of embryogenic suspension cultures of interior spruce and black spruce in a bioreactor (Tautorus *et al.* 1992) are pH, conductivity of the medium and osmolarity. The pH and dissolved oxygen (DO) was monitored throughout the growth phase and the temperature was maintained at $22^{\circ} \pm 2^{\circ}\text{C}$ using a circulating water bath attached to the jacket of the bioreactor vessel (Fig. 5.6.b.).

The pH of the medium drops to around 5.55 to 5.60 after autoclaving and a further drop in pH (4.4 to 4.65) was observed after the initial inoculum was added in the medium. The pH was then observed to increase to 4.9 – 5.1 by the third week and remained stable. These changes in pH are common with most of the plant cells and related to the balance between ammonium and nitrate uptake. The initial drop in pH is due to preferential uptake of ammonium at high ammonium levels, and subsequent increase in pH is due to nitrate uptake after ammonium is depleted (Tautorus and Dunstan 1995, Treat *et al.* 1989). There are a number of reports where pH has been monitored during the growth of suspension culture, but little information is available on the effect of growth of fixed pH levels. Lulsdorf *et al.* (1992) could not find any correlation of pH with any of the growth factors checked and deduced that it was not a suitable method for growth analysis and, reported that osmolarity is the best method of monitoring the growth of cultures growing in bioreactor.

The level of aeration and dissolved oxygen (DO) concentration in the medium has been shown to affect culture growth in bioreactors (Taticek *et al.* 1991, Preil *et al.* 1988). The levels of not only oxygen, but also carbon dioxide and ethylene have been reported to improve cell growth and metabolite synthesis and thus, the levels of these gases have to be evaluated during the optimization of bioreactor culture conditions (Bisaria and Panda 1991). We have monitored and maintained the levels of dissolved oxygen (DO) to approximately 25% during our studies and have observed positive effects on the growth of the culture. There are reports where $\text{DO} > 20\%$ have been shown to be detrimental for the growth of

plant cells and a DO concentration of < 16% was used for carrot cultures (Kessel and Carr 1972). Conflicting reports on the requirement for DO have been published for *Medicago sativa* (Chen *et al.* 1987, Stuart *et al.* 1987) and *Daucus carota* (Jay *et al.* 1992, Kessel and Carr 1972).

5.3.2. Cryopreservation

The technique of cryopreservation, in which the cells or tissue are frozen under controlled conditions and stored in liquid nitrogen, is a reliable method and has been an important tool for long term preservation of germplasm (Bajaj 1995a & 1995c, Sakai 1995a & 1995b). The methods tested for cryopreservation in the present investigation, including pre-treatment, slow freezing and fast thawing after cryo-storing in liquid nitrogen, proved successful for the *P. roxburghii* Sarg. embryogenic cultures. Considering the importance of propagating this pine species using somatic embryogenesis, developing a workable method of cryopreservation was very much essential. The long life cycle of trees delays the outcome of clonal propagation results. The embryogenic cell lines can be cryo-stored and used when the desired elite characters are observed during field trials (Gupta *et al.* 1993b).

Cryo-storing is not only beneficial for preserving the germplasm, but it also prevents any somaclonal variation that may ensue during the long-term maintenance of the embryogenic tissue (Kantha *et al.* 1988). Morphological differences and loss of embryogenic potential has been observed in some of the cell lines that we have maintained and subcultured continuously since their induction in 1997. This makes it even more essential to cryopreserve the freshly induced embryogenic cell lines. It has also been observed that, the ability of embryogenic cultures to tolerate cryopreservation can very well be genotype dependent, this has been reported by several workers in the past (Charest and Klimaszewska 1995, Nørgaard *et al.* 1993a, Nørgaard *et al.* 1993b; Häggman *et al.* 1998)

The present results, the first for any Indian pine species, clearly indicate the feasibility of the cryopreservation technique to store the embryogenic cultures. In our attempts to determine an effective cryopreservation treatment to enable the embryogenic cultures to sustain their viability, we observed that the embryogenic cultures survived the liquid nitrogen treatment for 72 h and could successfully recover as evident from their re-growth.

5.3.2.1. Effect of different pre-treatments on cryopreservation of embryogenic cultures of chir pine

A pre-culture or priming of the cells in a medium enriched with an osmoticum like sorbitol, has been shown to enhance the tolerance to freezing (Karthi *et al.* 1988, Klimaszewska *et al.* 1992). In the present study, when DMSO as the sole cyoprotectant, was unable to preserve the embryogenic tissue, it was necessary to use the sorbitol pre-culture strategy. The cytotoxicity induced by DMSO is reversed by the priming of the cells with sorbitol, prior to their exposure to DMSO. This is in concurrence with studies conducted with different tissues by various groups (Aronen *et al.* 1999, Cyr *et al.* 1994, Find *et al.* 1993, Ford *et al.* 2000, Karthi *et al.* 1988). The untreated embryogenic cultures of chir pine were unable to survive and recover after their exposure to freezing temperatures in liquid nitrogen (Fig. 5.7.).

The pre-treatment that was observed to be most suitable for the re-growth of embryogenic cultures of chir pine after their cryo-storage in liquid nitrogen, was a combination of 0.3 M sorbitol and 5 % DMSO. This is in agreement with observations made by Ford and co-workers (2000) in successfully cryopreserving the embryogenic cell lines of *P. patula*, that were frozen to -70°C in a freezer, before transferring to liquid nitrogen. Among the six pre-treatments tested in our studies, a combination of 0.5 M sorbitol with 5 % DMSO also proved useful for the re-growth of the cryopreserved cultures, although the re-growth was slower as compared to the other pre-treatment (Figs. 5.8. and 5.10.). Gupta and co-workers

(1987) have reported a lag phase of 5 weeks before the re-growth of cryopreserved cultures of *Picea abies* and *Pinus taeda*, after thawing. In our studies the lag phase was very short and observed to be of 5-7 days, which is similar to observations of Find *et al.* (1993).

Fig. 5.7. shows the re-growth data of the control cultures, where it can be clearly observed that the cultures subjected to slow freezing and/or storage in liquid nitrogen without any pre-treatment with cryoprotectants, did not show any re-growth. This emphasizes the necessity of treating the tissue with cryoprotectants without which the survival of the tissue is not possible.

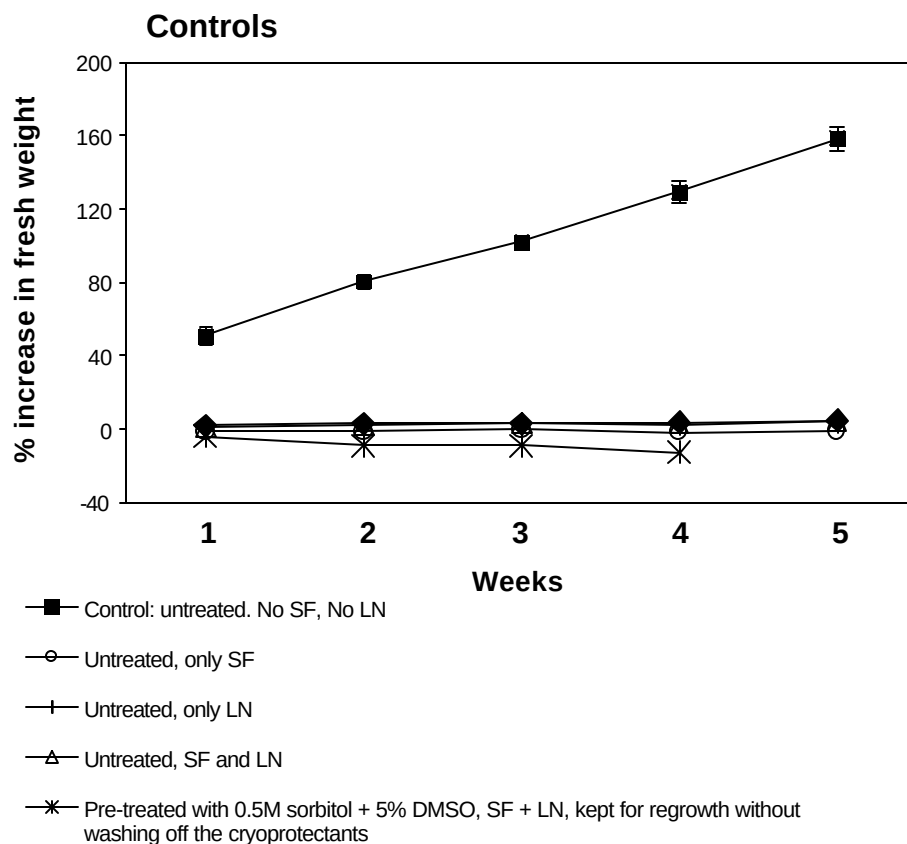


Fig. 5.7. Re-growth data of *Pinus roxburghii* Sarg. embryogenic cultures showing results of different controls conducted during the cryopreservation studies (P-T – pre-treatment; SF – slow freezing; LN – liquid nitrogen). All tissues were re-grown on DCR basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum

The pre-treatment of cryoprotectants found to be most conducive for the cryopreservation of embryogenic cultures of chir pine was observed to be a combination of sorbitol (0.3 M) and DMSO (5 %). The cultures pre-treated with this combination of cryoprotectants showed re-growth (Fig. 5.8.) comparable (69.94 % increase in fresh weight after 5 weeks) to the control (un-treated, un-stored) (158.53 % increase in fresh weight after 5 weeks). There is an initial lag phase in the re-growth of the cryopreserved cultures. One major factor influencing the re-growth of the embryogenic cultures after cryopreservation, was the state of the cultures at the time of pre-treatment. Vigorously growing cultures (2 weeks after last subculture) were best suited for cryopreservation.

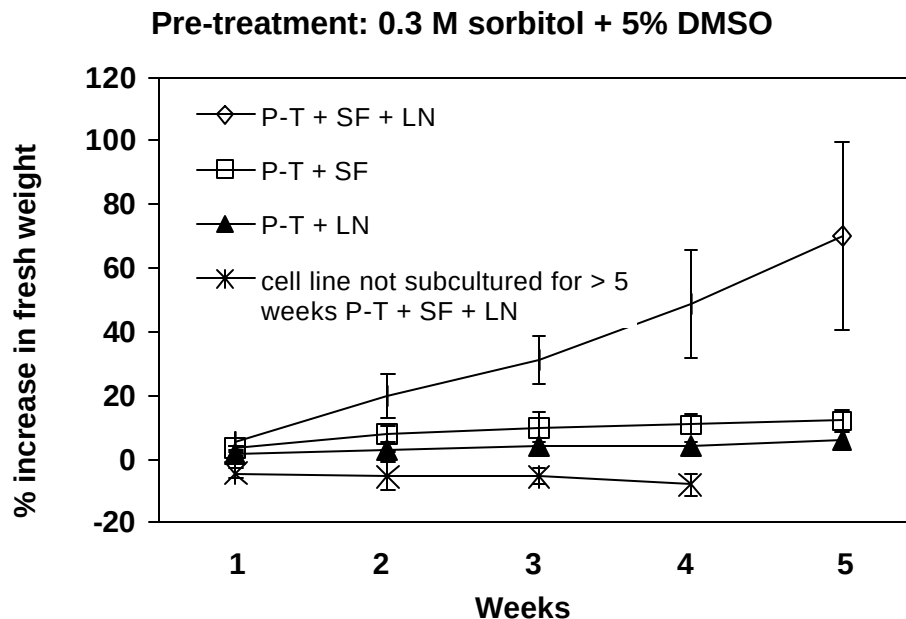


Fig. 5.8. Re-growth data of *P. roxburghii* Sarg. cryopreserved embryogenic cultures pre-treated with 0.5 M sorbitol and 5 % DMSO. This graph also shows re-growth data of a cryopreserved embryogenic culture that had not been subcultured for more than 5 weeks, before it was pre-treated with cryoprotectants. (P-T – pre-treatment; SF – slow freezing; LN liquid nitrogen) All tissues were re-grown on DCR b basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum

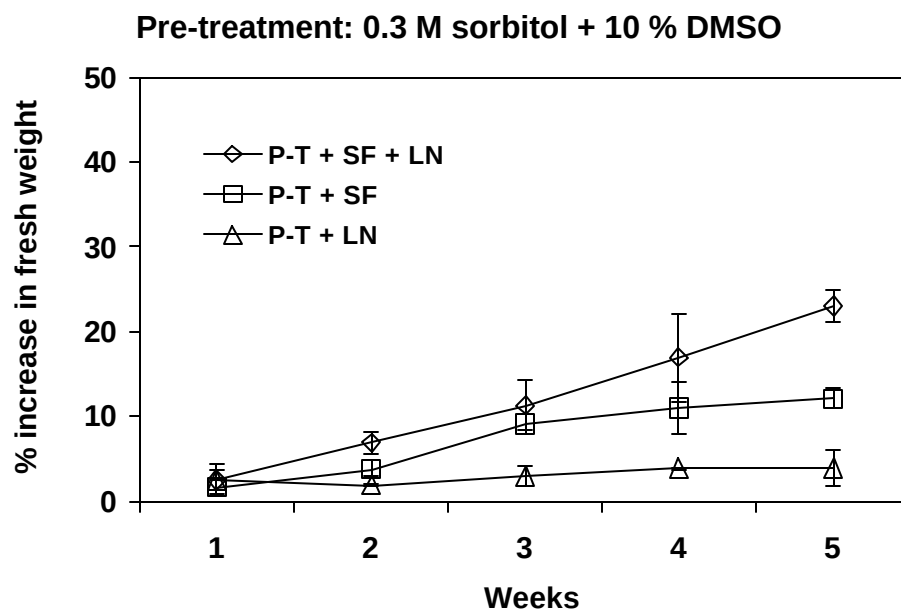


Fig. 5.9. Re-growth data of *P. roxburghii* Sarg. cryopreserved embryogenic cultures pre-treated with 0.5 M sorbitol and 10 % DMSO. (P-T – pre-treatment; SF – slow freezing; LN – liquid nitrogen) All tissues were re-grown on DCR basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum

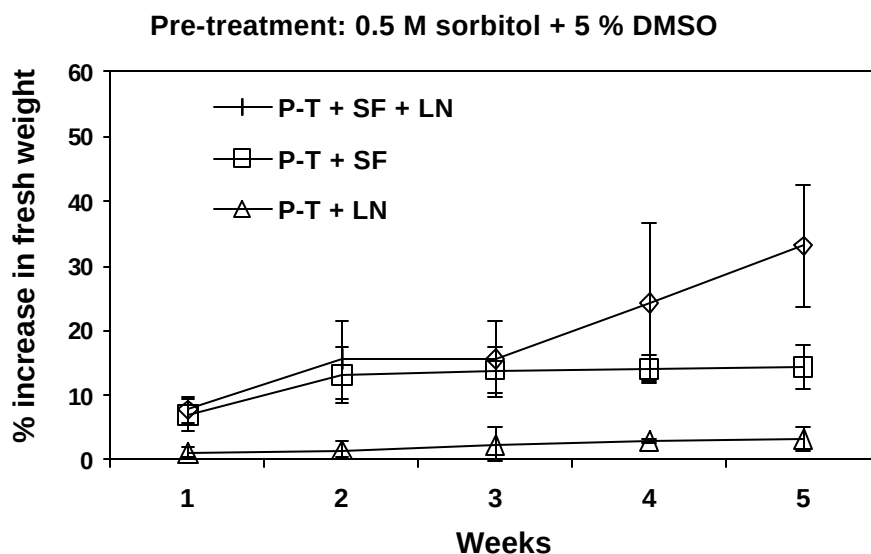


Fig. 5.10. Re-growth data of *P. roxburghii* Sarg. cryopreserved embryogenic cultures pre-treated with 0.5 M sorbitol and 5 % DMSO. (P-T – pre-treatment; SF – slow freezing; LN – liquid nitrogen) All tissues were re-grown on DCR basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum

Pre-treatment: 0.5 M sorbitol + 10 % DMSO

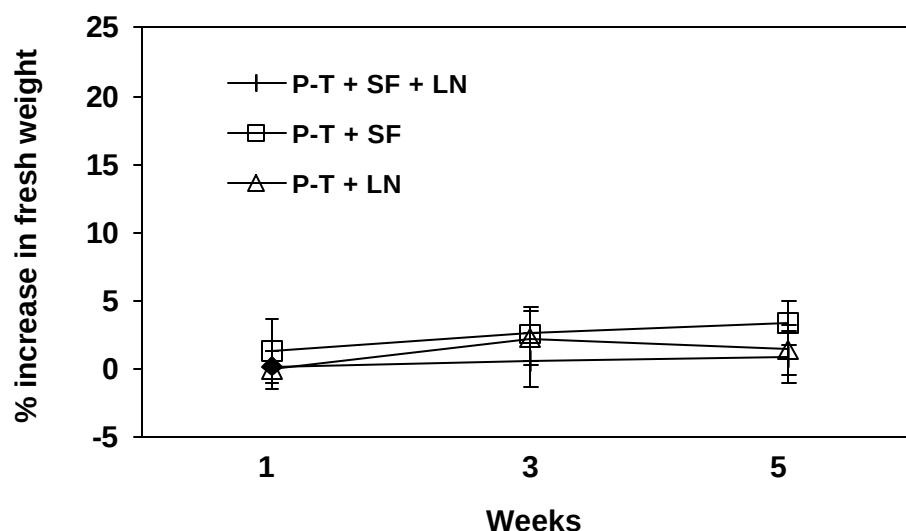


Fig. 5.11. Re-growth data of *P. roxburghii* Sarg. cryopreserved embryogenic cultures pre-treated with 0.5 M sorbitol and 10% DMSO (P-T – pre-treatment; SF – slow freezing; LN – liquid nitrogen). All tissues were re-grown on DCR basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin and 0.18% gellan gum

Pre-treatment: 5 % and 10 % DMSO

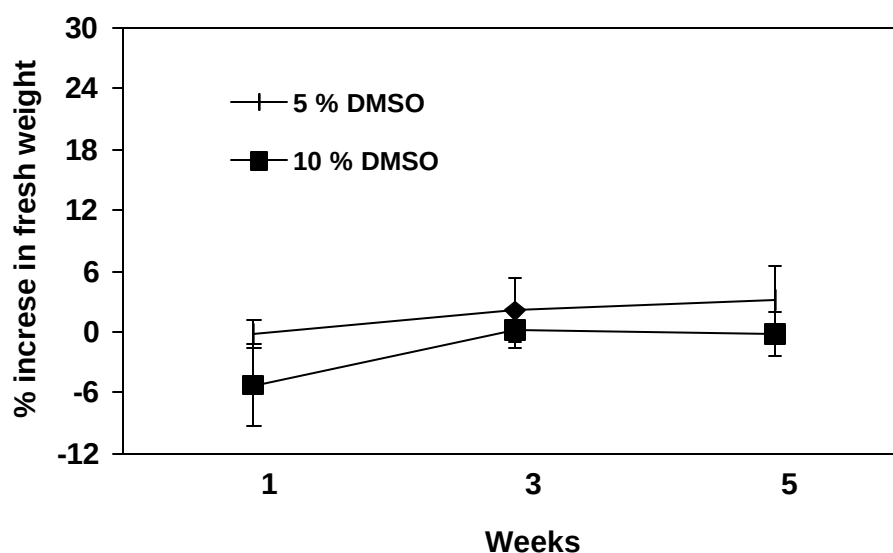


Fig. 5.12. Re-growth data of *P. roxburghii* Sarg. cryopreserved embryogenic cultures after thawing. The cultures were pre-treated with 5 % and 10 % (v/v) DMSO and passed through slow freezing process (up to -50 °C) before plunging in liquid nitrogen. All tissues were re-grown on DCR basal medium with 2% sucrose, 1.0g/l myo-inositol, 0.5g/l CH, 0.5g/l L-glutamine, 50mM 2,4-D, 20mM BA, 20mM kin & 0.18% gellan gum

It can also be seen from the results that the slow freezing step (gradually bringing down the temperature to -50°C) before plunging the culture in liquid nitrogen, is very much essential. Since all the cultures that were cryo-stored without undergoing the slow freezing protocol, did not show any re-growth (Figs. 5.8. to 5.11.). The cultures that were pre-treated with 0.5M sorbitol and 5% DMSO, also have survived and recovered (33.1% average increase in fresh weight after 5 weeks). It may be possible that embryogenic cultures are unable to tolerate DMSO at a concentration of 10 % and, 5 % DMSO is suitable for the survival of the tissue. Treatment with DMSO without priming with sorbitol, is also found to be lethal for tissues as can be seen from Fig. 5.12., since there was no re-growth observed in the cultures. However, there are reports that suggest that the DMSO alone, and even at a concentration as high as 20% (v/v), is not cytotoxic to the cells and recovery of cells is possible (Kantha *et al.* 1988).

5.3.2.2. Vital staining of cryopreserved embryogenic cultures

Vital staining with FDA and microscopical observations revealed that the cells that retained viability and capacity for re-growth were the embryonal head cells and some of the closely associated suspensor cells. Among the embryogenic mass, several dead cells that have not taken up the fluorescent dye can also be clearly seen (Fig. 5.13.a.). In Fig. 5.13b, the cryopreserved embryogenic culture (using 0.3 M sorbitol and 5 % DMSO) can be seen on proliferation medium. A number of normal somatic embryos were observed on subjecting to maturation treatment.

The microscopic examinations revealed that it is only the embryonal head and the associated suspensors that survive the cryopreservation and are capable of proliferating after thawing (Fig. 5.13.a.). Kantha *et al.* (1988) observed that the suspensor cells are eliminated during the pre-treatment with sorbitol and DMSO, even before the freezing. Washing the cryoprotectants off from the cryopreserved cultures after thawing was found to be very much essential. The unwashed

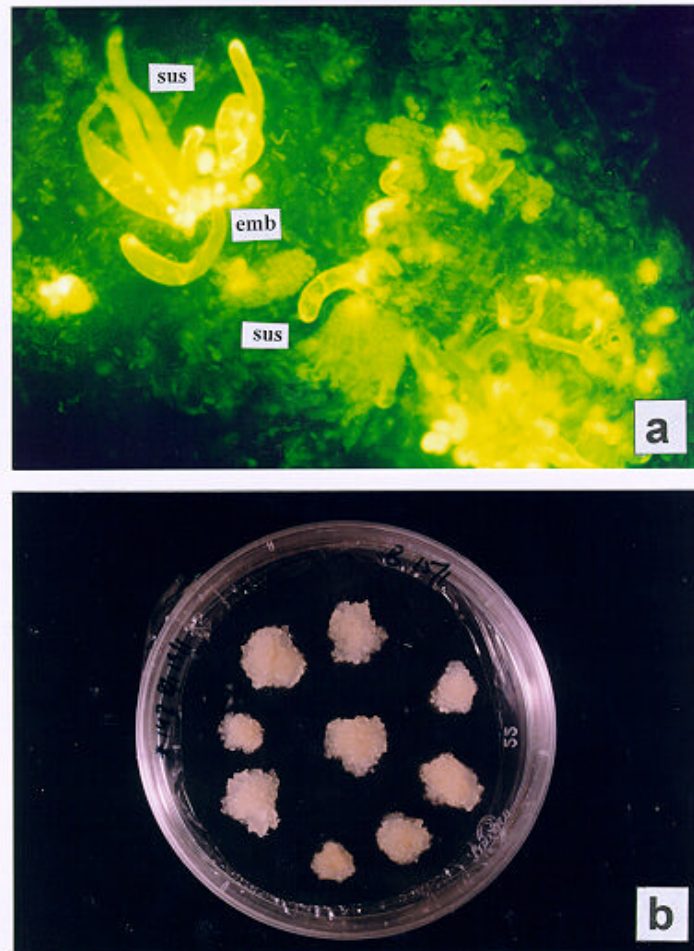


Fig. 5.13.

cultures did not recover even after several subcultures (Fig. 5.7.). Once the cryopreserved cultures are recovered from freezing, the proliferation is comparable to the untreated controls, specially in the cultures that were pre-treated with 0.5 M sorbitol and 5 % DMSO (Fig. 5.13.b.).

Another notable observation made during the studies was that only the vigorously growing cultures were suitable for cryopreservation. Slow proliferating cultures that had not been sub-cultured for more than 5 weeks, did not survive the cryopreservation despite pre-treatment with cryoprotectants. From fig. 5.7., it can be seen that, slow growing, un-subcultured embryogenic cultures pre-treated with 0.5M and 5 % DMSO and cryopreserved, did not show re-growth after thawing. This suggests the importance of the physiological state of growth of the culture that is to be utilized for cryopreservation.

Data analysis for the re-growth of cryopreserved cultures was conducted using an average of percentage increase in fresh weight. This was to avoid the discrepancies that would have surfaced if the weights were taken directly before placing them for re-growth, as there was a loss of culture during cryopreservation as well as during washing.

An optimization of pre-treatment with cryoprotectants is very much essential for any cryopreservation protocol. It is due to the effect of cryoprotectants that the embryogenic cultures of *P. roxburghii* Sarg. tolerate slow freezing and cryo-storage. The post-cryopreservation recovery, with a minimum lag phase, also depends very much on it. These results will definitely prove to be very useful for the ongoing studies regarding long-term cryopreservation of the embryogenic cultures of various pine species.

5.3.3. Conclusions

Significant observations from the growth and cryopreservation studies of embryogenic cultures of *Pinus roxburghii* Sarg. are presented below:

5.3.3.1. Growth studies for scaling-up embryogenic cultures of chir pine in bioreactor

- A normal growth curve was observed with a lag, log and plateau phases of growth with the embryogenic suspension cultures of chir pine
- An initial culture density of 25:100 (i.e. 25% culture v/v) optimal for the growth of embryogenic suspension in flasks as well as bioreactor.
- Embryogenic cultures in their exponential growth phase in flasks were used as inoculum for bioreactor.
- Embryogenic cultures of chir pine were observed to be very sensitive to change in temperature and the best growth was observed at $22^{\circ}\pm 2^{\circ}\text{C}$. Temperatures higher than 24°C were detrimental for the tissues.
- A pH of 5.8 (before sterilization of medium) was most conducive for normal growth of the embryogenic cultures of chir pine.
- An increase in 400-500 percent SCV was observed within two weeks of culture in 1 litre bioreactor.
- Normal small paraboloid shaped somatic embryos could be observed from culture samples in bioreactor.

5.3.3.2. Cryopreservation

- Embryogenic cultures of chir pine could be successfully cryopreserved in liquid nitrogen for 72 h as normal growth was observed in the cultures after thawing.
- A pre-treatment with suitable cryoprotectant is essential for the survival of the embryogenic cultures.

- Only the embryogenic tissue in the exponential phase of growth was found to be most suitable for surviving the cryopreservation.
- A pre-treatment of 0.5M sorbitol and 5% DMSO was most effective in preserving the embryogenic cultures of chir pine in liquid nitrogen.
- Slow cooling (up to - 50°C) before plunging the cultures in liquid nitrogen and fast thawing after the cryo-storage, were also observed to be essential for the normal re-growth of the embryogenic tissue of chir pine.
- Washing off the cryoprotectants after thawing of embryogenic cultures was also an important step, as the unwashed cultures did not show any re-growth.
- This protocol for cryopreservation of embryogenic cultures of chir pine can be utilized for long-term storage of embryogenic cell lines of pines.

Mathur G., Alkutkar V. A., Nadgauda R. S. (2001)
Cryopreservation of embryogenic culture of *Pinus roxburghii*
Sarg. (manuscript communicated)

Mathur G., Alkutkar V. A., Nadgauda R. S. (2001) Studies with
embryogenic cultures of chir pine: Optimization of growth
conditions for scaling-up in bioreactor
(manuscript communicated)

Mathur G., Alkutkar V.A. and Nadgauda R.S. (2001) Plantlet
formation from somatic embryos, and cryopreservation of
embryogenic cell lines of chir pine
(manuscript submitted).

SUMMARY

In India, pines form a group of trees having a very unique yet important position. Although, the distribution of pines is largely restricted to the Himalayan belt, but its usefulness has been shared throughout the country. With the population explosion in the last century, the need for growing food has also increased proportionally. The clearing of forests for agricultural and other purposes has put tremendous pressure on the ecological system, specially the Himalayas, with its rich and diverse flora that needs to be protected.

The innumerable uses, for which pines are valued, have been described at length. It is now the responsibility of the forest departments and other government agencies to ensure that the pine forests be replenished with the selected varieties. The forest departments have been instrumental in large-scale plantations of indigenous as well as exotic pines in several parts of the country. These efforts need to be complemented with the advancements in biotechnology that has proved to be of great potential.

Techniques like micropropagation and genetic engineering are already being fruitfully utilized in clonal forestry programmes in various countries. It is necessary now to incorporate these newer methods of variety improvement and propagation for conservation of the rich bio-diversity that our country possesses. The present investigations described in this thesis are initial steps in this direction.

Our lab has been instrumental in devising workable micropropagation protocols for a number of important forest tree species. This part of the work concerns with the propagation of two Indian pine species: *Pinus roxburghii* Sarg. and *Pinus wallichiana* A.B. Jacks. *In vitro* propagation has been successfully attempted using two methods, viz. adventitious bud induction and somatic embryogenesis, one for each of these pine species.

The results and outcome of this present investigation have been summarized as follows:

(a) Morphogenetic response of *Pinus wallichiana* A. B. Jacks (blue pine) *in vitro*

Mature zygotic embryos (MZE) dissected out of the seeds of blue pine were utilized for these studies. The response of MZE for inducing adventitious buds on the surface

was initially tested using three basal media (DCR, SH and WPM) containing BA. It was observed that DCR basal medium gave the best response among the three and was used for all further experiments involving the effects of different cytokinins on bud induction from MZEs. The bud forming capacity (BFC) index was calculated and it gave a clear indication of the number of explants and the number of buds per explant. It was observed that BA and TDZ gave the highest BFC as compared to the other cytokinins tested (2iP, kin and zeo). A combination of BA and TDZ was also effective in eliciting similar response. Administering BA through a pulse treatment was attempted and was found to be useful since it reduced the response time, exposure to growth regulator and also was cost effective. The adventitious buds were separated and elongated in DCR basal medium containing sucrose and activated charcoal. These elongated buds were then subjected to various rooting treatments with auxins (IAA and IBA). A high percentage of rooting was observed when the adventitious shoots were treated with a pulse treatment of IAA and IBA. On transferring the rooted plantlets to soil, the survival percentage was found to be low.

(b) Somatic embryogenesis in *Pinus roxburghii* Sarg. (chir pine)

The explants specificity in the induction of somatic embryogenesis was clearly observed as it is documented for the other pine species. It was only the immature zygotic embryo of chir pine, at a particular stage of development that was responsive in producing proliferating embryogenic tissue. The mature zygotic embryos formed non-embryogenic callus. The embryology of chir pine was studied with respect to the time of fertilization and development of the immature zygotic embryo so as to regularize the collection schedules. It was observed that the time of fertilization varied within locations and among trees of the same locations. DCR basal medium supplemented with 2,4-D, BA, kin, CH and L-glutamine was found to be most effective for extrusion and proliferation of proembryogenic mass. The morphology of the extrusion and, subsequently the embryogenic capacity however, mainly depended on the stage of development of the embryo. The percentage of extrusion and establishment of embryogenic cultures was very low. Very few embryogenic cell lines were finally selected and are being maintained for maturation studies. A pre-

treatment of embryogenic cultures on hormone-free medium prior to the maturation treatment was observed to be essential. Partial desiccation was also beneficial for the normal development of the germinating somatic embling.

(c) Studies with embryogenic cultures of *Pinus roxburghii* Sarg.:

(i) Scaling-up in bioreactor (ii) Cryopreservation

The potential of somatic embryogenesis will only be realized if it is coupled with large-scale production methods and cryopreservation, especially when working with tree species having long life cycles. Initial studies concerning various growth parameters of chir pine embryogenic suspension cultures were conducted. The growth curve was plotted and the effect of initial inoculum, pH and temperature was tested on the proliferation of embryogenic cultures. Cryopreservation is an essential component of long-term clonal propagation programmes. Various pre-treatments with cryoprotectants (DMSO and sorbitol in combination) were tested for the survival of the chir pine embryogenic cultures in liquid nitrogen. A pre-treatment of 5% DMSO and 0.5M sorbitol was found to be most effective when coupled with slow freezing (up to - 50°C) before plunging the tissues in liquid nitrogen. Fast thawing and washing off the cryoprotectants was an important step for the normal re-growth of the embryogenic cultures. Vital staining with FDA tested the viability of the cultures.

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