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"Plantlet Regeneration and Bud Developmental Anatomy of *Pinus roxburghii* Sargent"

by

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ABSTRACT

The aim of the study was to develop a protocol for the regeneration of plantlets of *Pinus roxburghii* Sargent *in vitro* and to study the anatomical events culminating in the formation of shoots. In the first of this two part study, various factors affecting morphogenesis including media, age of explant, phytohormones and environmental factors were tested.

The highest bud-forming-capacity (7.8) was realised when cotyledons were excised from one-day-old embryos. Half-strength Medium for Conifer Morphogenesis (MCM) was superior to all the other media tested. Although N^6-Benzyladenine (BA) at 10^{-5} M was the optimum for bud induction, bud development was enhanced when cotyledons cultured on 10^{-5} M BA for 7 days were transferred onto medium containing 10^{-5} M zeatin (Z) for another 7 days. The cotyledons were subcultured in cytokinin-free half-strength MCM for three weeks after the induction treatment. Half-strength Schenk and Hildebrandt (SH) medium was superior to half-strength MCM for bud development and was used from the sixth week of culture to the thirteenth to fifteenth week of culture. Excised shoots elongated faster on half-strength MCM solidified with gelrite compared with half-strength SH either solidified with gelrite or Difco bacto agar. Excised shoots were elongated for 13 to 15 weeks before rooting and shoot multiplication.

Four axillary shoots were obtained by decapitation of 28-week-old shoots. Eighty one percent rooting was obtained by incubating 28-week-old shoots in 1/4 MCM containing 5.37 x 10^{-7} M naphthalene acetic acid (NAA) for 4 weeks. The plantlets were hardened using plastic bags for 2-3 weeks. Eighty five percent survival was obtained after transfer of the plantlets to the greenhouse.

In the second part of the study, shoot-forming and non-shoot-forming cotyledons were harvested on day 0, 1, 2, 3, 4, 5, 8, 11, 21 and day 30, and embedded in paraffin wax. Sections (7μm in thickness) were stained and observed under the microscope. Plantlet parts were also embedded in paraffin wax, stained and observed under the light microscope.

Cytokinins were necessary for shoot formation and both periclinal and anticlinal mitotic figures were observed on the second day of culture. Other anatomical events culminating in shoot formation included meristemoid formation, meristematic domes and juvenile leaf primordium. After 30 days of culture a fully developed apical meristem was observed. The plantlets had a solid root-shoot junction which was necessary for the survival of the plantlets in the *in vivo* environment. The anatomical events were similar to those described for some other conifers.

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This thesis is dedicated to my parents, Muriithi and Njoki.

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(1.0) INTRODUCTION

The forests account for 29 -34% of the land area on earth (FAO, 1963). Of this area approximately 60% are gymnosperms or softwoods; some 38% are angiosperms or hardwoods, with remaining being made of mixed forests. The forests are Canada's most important natural resource, covering 4.53×10^8 ha or 45% of the total land base of the country (Brand, 1990). While most of the harvested material is used industrially, a significant portion of the hardwoods are utilised for fuel on a worldwide basis (Keays, 1974). Other values of the forests related to consumptive and nonconsumptive uses include fish and wildlife, tourism and recreation and water resources (Brand, 1990). It is generally accepted that the forests are being harvested at a faster rate than they are being regenerated, either naturally or artificially, hence, a shortage of wood and wood products has been forecasted for the end of this century (Keays, 1974). In addition, the rapid and disastrous effects of diseases, pests, and fires may jeopardise the very existence of certain tree species. Thus, there is an urgent need for larger numbers of improved, fast growing trees (Thorpe and Biondi, 1984). At present the tree improvement programs underway and the clonal propagation methods available offer only limited possibilities of achieving this goal.

Micropropagation is an integral part of any tree improvement programme and can play a role in producing superior planting stocks required for reforestation (Thorpe and Hasnain, 1988). At least 10% gain can be expected from planting selected clonal propagules rather than selected seeds (Kleinschmit, 1974). Cell culture as a tool in breeding programmes and mass clonal propagation has the main advantage of potentially enormous multiplication rates (Thorpe and Biondi, 1984). Thus, while a rooted cutting can produce a single plant from which several years later more cuttings are

available, even the most limited cell culture system, that of resting buds, with today's techniques can produce several axillary as well as adventitious shoots. Cost analyses on the application of tissue culture in forestry in New Zealand by the Forest Research Institute (FRI) (Smith, 1986) the second by Weyerhaeuser Corporation in Tacoma, Washington (Timmis, 1985), and the third in Canada by Natural Research Council (Hasnain et al., 1986) concluded that micropropagation was feasible. However, to achieve the maximum possible genetic gain for forest improvement both sexual reproduction and vegetative multiplication must be used (Hasnain and Cheliak, 1986).

Sexual reproduction is important for both introducing new genes to prevent inbreeding and for achieving those characteristics controlled by additive gene effects. Asexual reproduction allows the multiplication of elite full-sib families or individuals in a family, that exhibit significant gain due to additive gene effects. Plantlet regeneration can be achieved by organogenesis and somatic embryogenesis. Somatic embryogenesis leads to the formation of a bipolar structure with a root/shoot axis whereas organogenesis involves production of a unipolar shoot primordia (Thorpe, 1988). Although large-scale regeneration is not possible for a majority of species, over 70 angiosperms and 30 gymnosperms have been regenerated *in vitro* mostly by organogenesis (Thorpe et al., 1991). Regeneration in species such as *Pinus radiata* has been commercialised, whereas in other species acclimation of regenerants was not possible (Saravitz et al., 1991).

Organogenesis requires four distinct stages namely: induction of buds, development and multiplication of shoots, rooting of shoots and hardening of the plantlets. Plantlet regeneration involves the use of juvenile tissue in most cases, and the manipulation of the environment. The environment includes the medium, temperature, light etc. The medium typically includes phytohormones

to promote initiation of buds, but after bud formation, a medium lacking phytohormones allows elongation and shoot multiplication. Rooting of shoots and acclimation require different conditions and the optimum requirement of each stage must be experimentally determined (Thorpe and Biondi, 1984).

The gross anatomy of bud formation in conifers was first described in 1974 (Ishikawa, 1974). Since then more detailed studies have been done in other conifers (Villalobos et al., 1985). These structural investigations have contributed significantly to plant tissue culture research in areas such as organogenesis (Thorpe, 1980; Yeung, 1984). A complete understanding of the developmental anatomy of micropropagation is a useful aid in the optimization of any tissue culture system. However, the choice of the fixatives, processing and staining conditions is empirical (Yeung et al., 1981).

To the best of our knowledge *Pinus roxburghii* Sargent. an important pine in South Asia (Kumar, 1988; Rana et al., 1988) has not been regenerated *in vitro.* Therefore the aim of the research was to develop a protocol for the regeneration of *Pinus roxburghii* Sargent. Thereafter, investigation on the anatomical events culminating in the formation of shoots was to be conducted.

(2.0) LITERATURE REVIEW

Tree improvement involves three distinct, but related phases: conservation, selection and breeding, and propagation. Propagation of trees can be accomplished *in vitro* and *ex vitro* (Cheliak, 1990). The traditional methods for the vegetative propagation of forest trees are rooted cuttings or rooted needle fascicles (also known as brachyblasts, short shoots, dwarf spurs) and grafting. *Cryptomeria japonica* has been propagated by cuttings in Japan, and *Populus* and *Salix* in Europe since the last century. However, for the majority of trees, rooted cuttings are often characterised by a rapid loss of rooting ability of the ramet (cuttings) with increased age of the ortet (parent plant) (Thorpe and Biondi, 1984). Furthermore, for many rooted branch cuttings plagiotropic growth precedes orthotropic growth.

Our understanding of tissue culture can be traced back to the cellular theory which postulated that the cell is capable of autonomy and even totipotency (Schleiden, 1838; Schwann, 1839). Haberlandt and later his co-workers verified the cellular theory when they cultured vegetative cells in simple solutions, although the cells did not expand to form aggregates they stayed alive for prolonged periods (Krikorian and Berquam, 1969). Other significant events in the history of tissue culture include the discovery of auxin in 1926 (Went, 1927) and kinetin in 1955 (Miller et al., 1955). The importance of auxins and cytokinins was emphasised by Skoog and Miller's postulate of 1957 in which they demonstrated that organogenesis is controlled by a balance between auxins and cytokinins (Skoog and Miller, 1957). Our ability to culture species was further facilitated by the development of a high salt synthetic medium by Murashige and Skoog in 1962.

Pioneering work on the culture of woody species started in 1934 when callus was obtained from cambial tissues of *Acer pseudoplatanus* (Gautheret, 1934). However, the first adventitious buds of woody species was reported for *Ulmus campestris* in the early 1940s (Gautheret, 1985). The first dicot woody plantlet to be obtained by tissue culture was triploid aspen (*Populus tremuloides*) (Winton, 1968) whereas the first gymnosperm plantlet was obtained from *Pinus palustris* (Sommer et al., 1975). Today organogenesis which is a multistaged process of micropropagation is a major route used for the regeneration of woody plants *in vitro* (Thorpe et al., 1991). However, the biggest drawback in the regeneration of woody species is the inability to culture mature trees which have already demonstrated their superiority (Thorpe and Hasnain, 1988).

The distinct phases of shoot formation which include bud induction, shoot elongation and multiplication, rooting and acclimatisation require intensive experimentation in order to maximise the production of regenerants (Thorpe and Biondi,1984).

(2.1) BUD INDUCTION

The formation of adventitious buds involves an interplay between the inoculum, the media and the culture conditions. In conifers, buds are usually induced directly on the explant and the callus stage is bypassed. Juvenile tissues respond more readily to *in vitro* treatments leading to organogenesis (Thorpe and Hasnain, 1988). Explants are of two fundamental types of tissues namely meristematic types of tissues (shoot apices, lateral and axillary buds) and non meristematic tissues of newly germinated seeds (Bornman, 1983).

Fourteen day old cotyledons of *Picea abies* had better response than cotyledons excised from older seedlings (Bornman, 1983). Cotyledons from

germinated seeds were found to produce more rootable shoots than cotyledons derived from whole embryos for *Pinus radiata* D. Don (Aitken et al., 1981). Explants from black spruce consisted of epicotyls and intact cotyledons whereas for white spruce the hypocotyl and cotyledonary tips had to be removed to give the best perfomance (Rumary and Thorpe, 1984). For *Pinus contorta* the frequency of bud formation was higher on isolated embryos than cotyledons, hypocotyl segments and excised intact seedlings (Von Arnold and Erickson, 1981). For *Pinus radiata* the point of excision of the cotyledons was found to be critical and cotyledons excised too far from the point of attachment to the epicotyl did not survive and therefore an explant might require specific manipulations (Aitken et al., 1981).

Although many media differ only in degree, the medium is a critical part of tissue culture. Often dilution of standard media in proportional strength is used for certain phases such as culture initiation, organ induction and development (Cheng, 1975). Sometimes a medium combines the macronutrients of the standard medium with the vitamins of a second medium. The critics of the rationalisation of the media using the broad spectrum approach argue that the experiment becomes unmanageble (De Fossard, 1976). For *Picea abies,* Litvay's medium (LM) (Litvay et al., 1981), Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) favoured non differentiated cell and tissue growth, whereas the Medium for Conifer Morphogenesis (MCM) (Bornman, 1983) was the best for induction of shoot buds (Bornman, 1983). SH was used for the production of one hundred and eighty shoots of *Pinus radiata* D. Don. QP (Quoirin and Le Poivre, 1977) was found to be the best for the induction of shoot buds of eastern white cedar *Thuja occidentalis* (Harry et al., 1987). QP medium was also used for bud induction of lodgepole pine *Pinus*

contorta Dougl. ex Loud (Patel and Thorpe, 1984a). Dilution of media is also ideal for bud induction. At half-strength medium, 92% of the embryos formed shoots whereas a decrease in the number of shoot-forming embryos (81%) was observed at full strength media. Doubling of the composition of the media also culminated in a sharp decline to about 13% in the shoot-forming embryos (Harry et al., 1987).

Plant growth regulators are traditionally incorporated in the medium; however, they can also be administered by vacuum infusion of the explant tissues (Bornman, 1983). N^6-Benzyladenine (BA) elicited maximum response in terms of bud induction when applied singly in *Picea abies* (Bornman, 1983). However, BA can also be applied in combinations with other growth regulators such as N^6-(Δ^2-isopentenyl)-adenine (2iP), kinetin (K) and zeatin (Z). The use of mixed cytokinins was first reported for Douglas fir in 1975 (Cheng, 1975), and subsequently by others, e.g., in white and black spruces (Rumary and Thorpe, 1984). K and Z were found to produce more shoot-forming embryos. However the shoots induced were of poorer quality than shoots induced with BA and 2iP for eastern white cedar (*Thuja occidentalis)* (Harry et al., 1987). BA in combination with auxins induced callus in *Pinus canariensis* (Martinez Pulido et al., 1990).

Another critical factor for bud induction is the time of exposure to the cytokinin. For eastern white cedar exposure to BA at 10^{-6} M for 20-25 days gave the best results (Harry et al., 1987). For *Pinus canariensis* fourteen days exposure to BA gave the optimum number of buds (Martinez Pulido et al., 1990). Temperature may affect the time course and frequency of bud formation (Patel and Thorpe, 1984a). Bud formation in the micropropagation of conifers involves the development of nodular tissue initiated during bud induction into shoots with primary needles (Thorpe and Hasnain, 1988).

(2.2) BUD DEVELOPMENT AND SHOOT FORMATION

The formation of true shoot apices with juvenile leaf primordia may require transfer onto media with altered nutritional levels often with inclusion of activated charcoal (Thorpe and Biondi, 1984). For *Pinus contorta,* bud development was enhanced by transfer onto media of decreasing strength (Patel and Thorpe, 1984a). High salt media may be detrimental to the shoots (Harry et al., 1987). Although shoot elongation on the initial explant can be enhanced by dividing the explants into two to three parts when the cut surface is placed in contact with the medium (Aitken et al., 1981; Martinez pulido et al., 1990). Some species produce phenolics which cause browning of buds and therefore other techniques for bud elongation have to be used (Rumary and Thorpe, 1984). For western larch 1/2 MCMSH or 1/2 SHSH was used for the growth of shoots attached to the original explant even though 1/2 QP was the best for the first five weeks from culture initiation (Harry et al., 1991).

It is often necessary to elongate shoots detached from the original explant before rooting and shoot multiplication. After excision of shoots from initial explant, reduced strength of media, addition of activated charcoal and lower levels of sucrose have been found to enhance shoot elongation (Patel and Thorpe, 1984a). When activated charcoal of different origin, namely, bone, coconut and conifer were incorporated into the media 48 days from culture initiation, only conifer-derived charcoal (Sigma No. C4386) enhanced bud development in black and white spruce. In the presence of charcoal 50% of black spruce and 63.6% of the white spruce shoots elongated, compared with 11.4 and 0%, respectively in absence of activated charcoal (Rumary and Thorpe, 1984). Activated charcoal was also critical for shoot elongation for loblolly pine (Mehra-Palta et al. 1978), *Sequoia sempervirens* (Boulay, 1979), *Pinus pinaster* (David, et al., 1978) and lodgepole pine (Patel and Thorpe,

1984). Although, 3% sucrose was the best for elongation of eastern white cedar shoots (Harry et al., 1987), reduced sucrose of 2 % enhanced shoot elongation in black and white spruce (Rumary and Thorpe, 1984), *Pinus canariensis* (Martinez Pulido et al., 1990) and lodge pole pine (Patel and Thorpe, 1984a). Browning of shoots can be alleviated by subculture into fresh media (Harry et al., 1987). Although (1/2 QP) was superior for bud induction of western larch, it was inferior to 1/2 MCM and 1/2 SH for shoot elongation (Harry et al., 1991). Therefore, the medium for shoot elongation may be different from the basal medium.

(2.3) SHOOT MULTIPLICATION

The main aim of shoot multiplication is to produce the maximum number of rootable shoots in repeatable cycles. Primary shoots are multiplied in order to realise the potential for large-scale clonal propagation. Axillary buds are derived from buds formed in the axil of leaves. Axillary buds can be formed directly from the apex or away from the apex. For *Hydrocharis*, the axillary bud originates from the apical bud itself (Cutter, 1964). For *Taxus bacata* some cells on the axil of the needle remain meristematic. These buds originate from the apex and only divide on removal of correlative inhibition and form axillary buds (Fink, 1984). Axillary buds can also be initiated at a distance from the apex, from cells on the leaf axil that are already partially or completely differentiated (Majumdar, 1942).

In angiosperms, the growing shoot apex is known to exert correlative influence over a range of developmental events including axillary buds, the orientation of laterals, and the development of rhizomes and stolons. This concept of apical dominance may be manifested in various ways. In *Phaseolus*, a species with incomplete dominance, axillary bud growth continues at a slow

rate even in the presence of an intact shoot apex (Hillman, 1984). In plants with complete dominance such as *Tradescantia*, bud development is arrested at an early stage and mitotic activity is inhibited (Naylor, 1958). However reproductive structures may modify the level of dominance expressed by the shoot apex. For example, in *Perilla,* flowering decreases the level of dominance expressed by the shoot apex with subsequent increase in number of axillary shoots (Beever and Woolhouse, 1975). The shoot apex exerts its influence through the release and subsequent transport of indole-3-acetic acid. Decapitation and all the major classes of growth regulators have an effect on axillary bud growth, with cytokinin playing a key role (Tamas, 1987).

Although BA, Z and K could be used to produce axillary buds on primary *in vitro* shoots of *Pinus canariensis* most of them were vitreous and stunted. However an average of 3.1 non vitreous axillary buds were obtained when 1.5-cm shoots were decapitated and cultured on basal media for 6 weeks (Martinez Pulido et al., 1990). For *Pinus radiata,* a four-fold multiplication was possible every three months using decapitated shoots (Aitken-Christie and Thorpe, 1984). For *Thuja occidentalis* only two to four adventitious buds were formed on the initial explant, but when an average of 20 axillary buds excised from 6- to 7- month-old shoots were cultured for 5 months, each bud generated an average of 5 new buds and therefore 100 shoots could be obtained from one primary explant in one year. Although GA_3 was used to produce axillary buds in eastern white cedar, browning of the shoots was a major problem (Harry et al., 1987). Although secondary shoot multiplication could not be achieved by culturing longitudinal sections of shoot-forming clumps of black and white spruce in absence of antioxidants, ascorbic acid and glycine enhanced secondary shoot multiplication in black and white spruce respectively when they were incorporated in the media (Rumary and Thorpe,

1984). Other techniques useful in axillary bud breaking include incorporation of elevated levels of sucrose in the medium (6-9%). When 1-year-old-shoots of western larch were incubated in high levels of sucrose for two weeks and subsequently subcultured on basal media more buds (6-12 buds per primary explant) were formed compared to 2-5 axillary buds when 2iP was used (Harry et al., 1991). Recently, the "meristematic nodule multiplication" approach has been used successfully in radiata pine (Aitken-Christie et al., 1988) and to some extent poplar (McCown et al. 1988) in shoot multiplication. Meristematic tissue was induced from embryos of radiata pine using high BA concentrations, and from several poplar explants using a high auxin/cytokinin ratio. The meristematic tissue could be maintained, multiplied and induced to produce shoots.

(2.4) ROOTING

Rooting of angiosperms has been relatively easy while rooting of gymnosperms has been problematic (Sommer and Caldas, 1981). Although spontaneous rooting may occur, generally an auxin treatment, usually with indole butyric acid, is required for successful root formation. However other factors such as salt concentration and sucrose should be tested (Thorpe and Biondi, 1984). Rooting procedures can be broadly categorised into, *in vitro* rooting and *in vivo* rooting (Dunstan and Turner, 1984). *In vitro* rooting is done in sterile conditions whereas *in vivo* rooting is done under greenhouse conditions. *In vitro* rooting includes, long periods of incubation of shoots in media containing low concentrations of auxins (Jain et al., 1988), pulsing of shoots overnight in media containing high concentrations of auxins (Patel and Thorpe, 1986) and pulsing shoots in high concentrations of auxins in liquid or agarified medium (Martinez Pulido et al. 1990). *In vivo* treatments usually

involve application of commercial rooting powder and subsequent transfer into a rooting substrate (Rumary and Thorpe, 1984; Kurz et al., 1989). Although *in vivo* rooting is more desirable, because rooting and acclimatisation can be done in one step, some species root better under *in vitro* conditions, for example western larch (Harry et al. 1991).

The rooting substrates also have an effect on rooting, for example, sand was the best for rooting black and white spruce shoots (Rumary and Thorpe, 1984) whereas for *Pinus canariensis* Peat: vermiculite 1:1(v/v) produced the best results (Martinez Pulido et al., 1990). For *Pinus contorta*, the inclusion of activated charcoal in the rooting substrate, which consisted of agar-solidified medium, improved rooting by 36% (Patel and Thorpe, 1984a). Thermoperiodism was found to be critical for rooting in black and white spruce, with the realisation of higher rooting at 20 C:18 C and photoperiod of 12 hours, compared with 20 C:15 C with the same photoperiod, when charcoal was included in the rooting substrate (Rumary and Thorpe, 1984). Although a lower temperature was found to be necessary for *Pseudotsuga menziesii* shoots (Cheng and Voqui, 1977), for eastern white cedar, 60% of the shoots rooted at higher temperature (25 C) compared to 10% at a lower temperature (10 C). Different species seem to root better under different environmental cue, isolated empirically, which drastically slows down the rooting process. For some species like *Pinus resinosa* the environmental cue has not yet been determined, and only 15 plantlets were obtained out of an attempt to root 677 shoots (Noh et al., 1988). Another problem when rooting is how to partition effects due to an induction treatment and spontaneous rooting. Spontaneous rooting as high as 2% was reported for *Pinus contorta* (Patel and Thorpe, 1984a).

(2.5) ACCLIMATISATION

Acclimatisation is a process controlled by humans to adapt an organism to environmental change (Brainerd and Fuchigani, 1981). Acclimatisation of the plantlets is necessary because *in vitro*-grown plants are not adapted to the *in vivo* environment. Several authors have indicated that the waxy cuticle and stomata on leaves of *in vitro* grown plants are inadequate or inoperative and therefore incapable of preventing water loss that occurs in the variable humidity of the *in vivo* environment (Fuchigani et al. 1981). High sucrose and salt medium that is often used *in vitro* cultures also limits the photoautotropic capacity of the leaves (Wetzstein and Sommer, 1982). Several procedures have successfully been used for acclimation among others: maintenance of high humidity and a temperature gradient (from cool at the tip to warm at the shoot base after transplanting plantlets into soilless mixes (Dunstan and Turner, 1984) and hardening of shoots at 20 C for 3 weeks (Harry et al., 1987).

(2.6) DEVELOPMENTAL ANATOMY

There are many factors which affect the sequence of events leading to shoot formation. Light, BA and the age of the explant determine the locus for shoot initiation (Aitken-Christie et al., 1985; Villalobos et al., 1984a; Villalobos et al., 1985). In cotyledons of *Pinus radiata* excised 1 day post germination meristematic tissue formed along the entire length of the cotyledons in contact with the media after 21 days; whereas in cotyledons excised 2 to 3 days post germination meristematic tissue formed only at the tips. Cotyledons excised 5 days post germination did not form meristematic tissue but elongated. Unlike young cotyledons, older cotyledons were not active in division and represented a higher level of differentiation because they contained well developed

stomatal complex and epicuticular wax. The cells also had thicker cell walls and were more vacuolated. The shoot-forming tips of cotyledons 2-3 days post germination were in the same developmental state as those 1 day post germination, which formed meristematic tissue along the entire explant. These features of older cotyledons reduced the ability of the cells to undergo dedifferentiation. Older cotyledons also had reduced metabolite levels (Aitken-Christie et al., 1985). Organogenic processes usually have a high energy requirement (Thorpe, 1980).

Cotyledons cultured in BA containing media had a developmental sequence distinct from cotyledons cultured on BA-free media. Those cultured in BA-free medium elongated several times more than BA-treated explants. Unlike shoot-forming cotyledons, non-shoot-forming cotyledons formed stomatal complexes and also large intercellullar air spaces. There was cessation of cell division in non-shoot-forming cotyledons and cells became vacuolated. In contrast shoot-forming cotyledons swelled with subsequent formation of shoot buds (Yeung et al., 1981). Light was also found necessary for differentiation and cotyledons cultured in BA media in darkness failed to form shoots and elongated. Three days exposure to light was necessary for shoot formation and increased period of light inhibited cotyledonary elongation (Villalobos et al., 1984a).

Although the developmental sequence leading to shoot formation in conifers, which includes, formation of meristemoids, bud primordia, and adventitious shoots are similar, differences in sites and timing of shoot formation have been observed. Cotyledonary explants of *Pinus radiata* had actively dividing cells, whereas embryonic explants of *Pinus rigida* had quiescent cells. Epicotyl explants (including the shoot apex, primary needles cotyledonary bases and hypocotyl stub) of spruces were highly differentiated and contained

various cells, tissues and organs. Differences were also observed in sites of shoot initiation. *Pinus radiata* cotyledonary explants formed shoots on the surface in contact with the media, whereas *Pinus rigida* embryonic explants formed shoots on the surface away from the media, while the spruces formed shoots on the bases of the cotyledons. However, irrespective of the explant type or the species, adventitious shoots formed directly on the explant without concomitant callus formation (Thorpe and Patel, 1986). For the spruces secondary shoot formation was observed and accounted for the majority of shoots formed in culture. Whereas the primary shoots had vascular connections to the explant vascular tissue the secondary shoots were devoid of such connections (Rumary and Thorpe, 1984).

For *Pinus radiata,* histological examinations indicated that cytological changes leading to shoot formation begin 24 h after culture in BA-containing media. Unequal periclinal divisions were observed near the cotyledonary surface in contact with the media (Yeung et al., 1981). Other cellular events apparent in the shoot forming cotyledons included suppression of stomatal development and restriction of intercellular air space formation (Villalobos et al., 1985). For radiata pine, six to eight cell structures, promeristemoids, were formed after five days in culture (Villalobos et al., 1985). The promeristemoids formed meristemoids which appeared as nodular structures and later shoots with primary needles developed (Villalobos et al., 1985). It is therefore apparent that bud formation had a unicellular origin which was activated by physiological gradients of material moving out of the medium (Thorpe, 1980).

Histochemically the cells of the initial explant contained large starch and protein reserves (Yeung et al., 1981). They also contained lipid droplets (Douglas et al., 1982) and were rich in RNA (Patel and Thorpe, 1984b). DNA synthesis was also at a maximum in the initial explants (Villalobos et al.,

1984b). Although protein reserves of cotyledons cultured with and without BA declined significantly during the first 3 days of culture in shoot-forming and non-shoot forming cotyledons, the epidermal and subepidermal layers of shoot forming cotyledons in contact with media showed intense nuclear and cytoplasmic staining for proteins (Patel and Thorpe, 1984b). Localisation of DNA, RNA, and enzymes was observed on the epidermal and subepidermal layers in contact with the media.

Autoradiographically, it was confimed by incorporation of various labelled precursors that during cotyledonary elongation, macromolecular synthesis occured throughout the cultured tissues and was greatest during the first 3 days of culture, whereas in shoot forming cotyledons macromolecular synthesis was confined in epidermal and subepidermal cells (Vilalobos et al., 1984b). Histochemically, although starch grains generally disappeared from cells of BA-treated explants, the cells of shoot-forming layers contained abundant starch. An increase in enzymatic activity namely peroxidases, lipases, ATPases was also observed in differentiating cotyledons relative to elongating cotyledons indicating that there is a high energy requirement for differentiation (Patel and Thorpe, 1984b). These observations reinforced the view that organ formation *in vitro* has a high energy requirement and were thus in agreement with the thesis that the initiation of organised development *in vitro* involves a shift in metabolisim, which precedes and is coincident with initiation of the process and takes place in organogenic regions (Thorpe, 1980). Although there are morphogenic differences between conifers, a high number of shoots can be obtained by exposing the maximum surface area of the explant to cytokinin-containing medium (Thorpe and Patel, 1986). The distinct phases of shoot formation in conifers involve the induction of cell division in target cells, formation of meristemoids and finally formation of shoot buds.

In several conifers lack of a proper vascular connection between the root and the shoot was considered to be the main reason for the high mortality of tissue cultured plantlets upon transplanting. High mortality may be due to *in vitro* rooting within the basal callus (David, 1982). Initially the understanding of root initiation leading to *in vitro* plantlet formation was superficial and based on the observation of seedlings (e.g., Smith and Thorpe, 1975; Montain et al., 1983). During root induction, of *in vitro* shoots of spruces, some swelling occured at the base of the shoot, and some callus formed below the base under the influence of IBA, an active ingredient of the rooting powder used. Some of the cells within the base of the shoot and in the vicinity of the vascular system differentiated into cambium-like cells which later produced tracheid nests and resin canals. The tracheid nests composed of irregularly arranged tracheids of various sizes surrounded by the cells of the cambium. Some of the derivatives of the cambial initials differentiated into root meristemoids. The cells of these meristemoids were small and contained densely staining cytoplasm and large nuclei. Later in culture, these cells differentiated into the root primordia which then assumed the configuration of a root. These roots were connected with the vascular system of the shoots through the tracheid nest as confirmed by clearing with sodium hydroxide and subsequent staining (Patel et al., 1986b). However, for *Thuja occidentalis* the vascular tissue of shoots was directly connected with the roots with no apparent disruptions in the tissue culture plantlets. Although more rays were found in the root-shoot junction of the plantlet than in the seedlings, the root-shoot junctions were similar (Bender et al., 1987).

(3.0) EXPERIMENTAL METHODS

(3.1) PLANT MATERIAL

Seeds of *Pinus roxburghii* Sarg. were obtained from Mistletoe Seeds, California, U.S.A., and were stored at 5 C before use. Before use, seeds were scarified for 0, 1, 2, 3, 4, 5 and 6 minutes in concentrated sulphuric acid, rinsed thoroughly and subsequently washed for two nights in running tap water. Floating seeds were discarded after one day and the remainder sterilised for 30 minutes in 50% Javex^{R} bleach (6%) containing 3 to 4 drops of Tween per 100 ml and followed by five 5-minute rinses with sterile distilled water. The seeds were treated with hydrogen peroxide (10%) and again rinsed with sterile distilled water. The sterile seeds were stratified at 5 C for two days. The seed coats were cracked with sterile pliers and the intact megagametophyte was re-sterilised for 15 minutes in 15% bleach and for 5 minutes in hydrogen peroxide, each procedure was followed by 5x5 minute rinses with sterile distilled water (Martinez Pulido et al., 1990). Embryos were isolated from megagametophyte which had been plated on 1% sucrose agar for one day and plated on 1% sucrose agar. Cotyledons were only isolated from white, firm, undamaged embryos and plated on media.

In order to determine the seed quality of the experimental material , sterile seeds were germinated in jars containing 100 ml vermiculite moistened with 70 ml sterile double distilled water under two experimental conditions namely light and dark. Each experiment consisted of 6 jars containing 15 sterile seeds each. The incubation temperature was maintained at 27±1 C.

(3.2) CULTURE MEDIA

Initially four different media, namely, Bornmans MCM (Bornman, 1983), von Arnold and Erickson AE (von Arnold, 1981), Murashige and Skoog MS (Murashige and Skoog, 1962) and Gupta and Durzan (DCR) (Gupta and Durzan, 1985), were tested for bud induction at half strength and contained 10^{-5} M BA(Table 1-4). The minerals were supplemented with sucrose 3%, thiamine-HCl 5 mg/l, asparagine 100 mg/l, nicotinic acid 5 mg/l, pyridoxine-HCl 0.5 mg/l, Difco Bacto agar at 0.8% and media adjusted to pH 5.7 to 5.8 before autoclaving at a temperature of 121 C with a pressure of 15 p.s.i. (103.4×10^{10} Pa) for twenty minutes. After two weeks of culture the cotyledons were transferred to BA-free media for three weeks.

(3.3) BUD INDUCTION

For the selection of the best medium, cotyledons from day 0 to day 4 megagametophytes were used. All media were supplemented with BA (10^{-5} M). The cotyledons were subcultured into BA free media after fourteen days. Three replicates containing 10 cotyledons each were used per treatment. The incubation temperature was maintained at 24±1 C. under 16h photoperiod with light intensity of ca. $80 \mu E.m^{-2}s^{-1}$ from Gro Lux Ws lamps (Patel and Thorpe, 1984a). After the selection of the best medium and age of cotyledons the optimum BA concentration was determined from a range (5×10^{-6} M, 10^{-5} M ,2.5×10^{-5} M and 5×10^{-5} M for 7, 14, 21 and 28 days. The experiment was repeated twice. The best exposure time and concentration was used for determining the best explant, i.e. whole embryos and their cotyledons (Martinez Pulido et al. 1990). The influence of other cytokinins namely, zeatin (Z), kinetin (K), and 2-isopentyl adenine (2iP) were tested in combination with BA or singly. All treatments were evaluated using the responding explant and bud

Table 1. **MCM** medium (Bornman, (1981). Physiol. Plant., 57: 5-16)

salt	Conc. (mg/l)
Major salts	
Urea	150.0
$CaNO_3.4H_2O$	500.0
$(NH_4)_2SO_4$	400.0
KCl	150.0
KNO_3	2000.0
KH_2PO_4	270.0
$MgSO_4.7H_2O$	250.0
Minor salts	
H_3BO_3	1.5
$ZNSO_4.7H_2O$	3.0
$MnSO_4.H_2O$	0.17
$Na_2MoO_4.2H_2O$	0.25
KI	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron	
$Na_2FeEDTA.2H_2O$	37.5
Organics	
Myoinositol	90
Glycine	2
Thiamine.HCl	1.7
Nicotinic acid	0.6
Pantothenate	0.5
Pyridoxine-HCL	1.2
Folic acid	1.1
Biotin	0.125

Table 2. **MS** medium (Murashige and Skoog, (1962). Physiol. Plant. 15: 473-497)

salt	conc. (mg/l)
Major salt	
NH_4NO_3	1650.0
KNO_3	1900.0
KH_2PO_4	170.0
$CaCl_2.2H_2O$	440.0
$MgSO_4.7H_20$	370.0
Minor salts	
H_3BO_3	6.20
KI	0.83
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron	
$Na_2EDTA.2H_2O$	74.5
$FeSO_4.7H_2O$	55.7
Organics	
Thiamine-HCL	5.0
Nicotinic acid	5.0
Pyridoxine-HCL	0.5
Asparagine	100.0
Myo-inositol	100.0

Table 3. **DCR** medium (Gupta and Durzan, (1985). Plant Cell Rep., 4: 177-179)

salt	Conc. (mg/l)
Major salts	
$Ca(NO)_3.4H_2O$	556.0
$(NH_4)NO_3$	400.0
KNO_3	340.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
$CaCl_2.2H_2O$	85.0
Minor salts	
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$MnSO_4.H_2O$	22.3
$Na_2MoO_42H_2O$	0.25
KI	0.83
$CuSO_4.5H_2O$	0.25
$CoCl_26H_2O$	0.025
$NiCl_2$	0.025
Iron	
$Na_2EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
Organics	
Thiamine-HCL	1.0
Nicotinic acid	0.5
Pyridoxine-HCL	0.5
Glycine	2.0
Myo-inositol	200.0

Table 4. **AE** medium (von Arnold and Eriksson, (1981). Can. J. Bot. 59: 870-874)

salt	Conc. (mg/l)
Major salts	
$CaCl_2.2H_2O$	180.0
NH_4NO_3	1200.0
KNO_3	1900.0
KH_2PO_4	340.0
$MgSO_4.7H_2O$	370.0
Minor salts	
H_3BO_3	0.63
$MnSO_4.4H_2O$	2.20
$Na_2MoO_4.2H_2O$	0.025
KI	0.75
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025
Zn-EDTA	4.05
Iron	
$Na_2EDTA.2H_2O$	19.0
$FeSO_4.7H_2O$	14.0
Organics	
L-Glutamine	0.40
L-Alanine	0.05
L-Cysteine-HCL	0.02
L-Arginine	0.01
L-Leucine	0.01
L-Phenylalanine	0.01
L-Tyrosine	0.01
Glycine	2.0
Thiamine-HCL	5.0
Nicotinic acid	2.0
Pyridoxine-HCL	1.0
Meso-inositol	100.0

forming capacity as the parameter (Harry et al., 1987). Although, during bud induction the aim is to increase the number of explants responding and also the mean number of buds per explant, often the two parameters are negatively correlated and therefore decisions based on one parameter are inappropriate. Bud-forming-capacity is the product of the mean number of buds per cotyledon and the percentage of explants responding, divided by one hundred. Bud forming capacity is a parameter reflecting response per explant cultured and is often used as a criterion for selection.

(3.4) BUD DEVELOPMENT AND SHOOT FORMATION

To enhance elongation of buds attached on the initial explant, cotyledons which had been cultured for five weeks, were transferred to deeper petri dishes (100x25mm) containing 30 ml of either 1/2 MCM or 1/2 SH (Schenk and Hildebrandt, 1972)(Table 5) media containing 3% sucrose and 0.05% activated charcoal (Sigma, St. Louis, Mo, No. C4386). Three replicates containing ten cotyledons each were used per treatment and cotyledons were subcultured twice at 4 weeks intervals. After 13-15 weeks shoots longer than 1 cm could be excised from the original explant. In order to determine the best media for elongation of excised shoots, shoots longer than 1.5 cm were excised from the initial explants and distributed in (100x80mm) glass jars containing 100ml of either MCM or SH media at half strength and solidified with 0.5% gelrite. The best media for elongating shoots attached on the explant was used as a control. All the media used, contained 2% sucrose and 0.05% activated charcoal (Sigma, St. Louis, Mo, No. C4386). Three replicates containing five shoots each were used per treatment. The shoots were subcultured on the same

Table 5. **SH** medium (Schenk and Hildebrandt, (1972); Can. J. Bot., 50: 199-204)

salt	Conc. (mg/l)
Major salts	
$CaCl_2.2H_2O$	200.0
$NH_4H_2PO_4$	300.0
KNO_3	2500.0
$MgSO_4.7H_2O$	400.0
Minor salts	
H_3BO_3	5.0
$ZnSO_4.7H_2O$	1.0
$MnSO_4.H_2O$	10.0
$Na_2MoO_42H_2O$	0.1
KI	1.0
$CuSO_4.5H_2O$	0.20
$CoCl_2.6H_2O$	0.10
Iron	
$Na_2FeEDTA.2H_2O$	20.0
$FeSO_4.7H_2O$	15.0
Organics	
Thiamine-HCL	5.0
Nicotinic acid	5.0
Pyridoxine-HCL	0.5
Myo-inositol	1000.0

media four times after 3-4 weeks of subculture. After twenty eight weeks from culture initiation the height of the shoots was measured.

(3.5) SHOOT MULTIPLICATION

For shoot multiplication decapitated shoots were cultured in 1/2 MCM containing 0.05% activated charcoal (Martinez-Pulido et al., 1990). In another experiment, shoots were cultured in half-strength MCM containing 10^{-5} M BA and 2% sucrose for 14 days before transfer onto BA-free medium.

(3.6) ROOTING

In order to identify a method of rooting many preliminary rooting methods were evaluated. The methods of rooting included liquid pulse treatment ,which involved the immersion of the base of the shoots in filter-sterilised IBA at 10^{-3} M for 2, 3, 4, 5 and 6 hours and subsequent transfer to a mixture of peat and vermiculite 1:1 (v/v) after the appropriate period. The rooting substrate was moistened with 70 ml of medium to 100 ml of substrate. The medium consisted of 1/4 MCM containing 1 % sucrose. Some shoots were also incubated in 1/4 MCM containing 1 % sucrose and three levels of NAA, these were, $0.5x10^{-6}$ M, $5x10^{-6}$ M and 10^{-5} M. Scoring for rooting was done every month. An attempt was also made to root shoots by incubating them in 1/4 MCM containing 1 % sucrose and 10^{-4} M IBA for 5 days after which they were transferred into soilless mixture. In another experiment, shoots were cultured in 1/4 MCM media solidified with gelrite containing $5.37x10^{-7}$ M NAA and 1% sucrose and 0.5 % gelrite until rooting before transfer into soilless mixtures. The best method of root induction was confirmed by repeating the experiment.

(3.7) ACCLIMATISATION

After rooting the plantlets were hardened by gradually removing the organics from the media. High humidity was maintained by covering the shoots with transparent plastic bags. Low humidity was maintained by opening the transparent plastic bags. The plantlets were watered when they showed signs of wilting.

The potted plantlets were placed on a bench in greenhouse in summer after successful hardening. In the greenhouse the temperature was maintained at 25 C to 28 C under natural photoperiod. The plantlets were ferti-irrigated twice a week with water containing 250 ppm nitrogen (Peter's brand 20:20:20 or 20:10:20).

(3.8) DEVELOPMENTAL ANATOMY

The aims of these experiments were to study the cell structure before and after induction of the explants, and to identify the mitotic pattern leading to the formation of meristemoids. The paraffin method of embedding was used. Cotyledons were cultured for one week on 1/2 MCM containing 10^{-5} M BA and then transferred to 1/2 MCM containing 10^{-5} M Z for one week before subculture into Cytokinin-free medium. The explants were harvested at day 0 (initial explants), 1, 2, 3, 4, 5, 8, 11, 21, and day 30 and fixed in formalin-acetic-acid-alcohol FAA (50% ethanol : glacial acetic acid : formalin, 90 : 5 : 5) and dehydrated in tertiary butyl alcohol (TBA) series, after which they were embedded in paraffin wax. At each period of sampling 10 cotyledons were used. Serial 7μm thick sections were obtained with a rotary microtome. The sections were mounted on slides which had been coated with a chrome alum gelatin solution. The subbing solution was prepared as follows: 5.0 grams of U.S.R. gelatin, Knox unflavoured was dissolved completely in 1.0 litre warm

distilled water and 0.5 g of chrome alum (Chromium potassium sulphate, $CrKSO_4.12H_2O$) was added. After cooling the solution was filtered through Whatman #1 filter paper. The solution was stored at 5 C for a maximum of 48 h. After dipping slides into the subbing solution they were allowed to dry vertically in a dust free environment (Pappas, 1971).

In order to observe the root shoot junction, plantlets were cut longitudinary and fixed in formalin-acetic-acid-alcohol FAA, dehydrated in TBA series, and embedded in paraffin wax. Other plant parts which included root pieces and stems were also prepared for light microscopy. All sections were stained with a solution containing safranin O, basic fuchsin and crystal violet (0.5%,0.2%,0.2%, respectively, in 50% EtOH) and counterstained with fast green (Yeung et al., 1981; Yeung, 1984).

(4.0) RESULTS

(4.1) PLANT MATERIAL

Seeds of *Pinus roxburghii* Sarg. are big and have a tough seed coat which contains a fair amount of contamination. The intact embryo occupies a major portion of the seed cavity. The embryos contain 8-16 cotyledons, which measure from 4 to 6 mm. A reasonable asepsis was obtained by scarifying the seeds for 5 minutes in concentrated sulphuric acid. Scarification of the seeds did not affect the embryo vigour and was useful both as a sterilant and as an excoriating agent which allowed faster imbibition. Both hypochlorite and peroxide were necessary for elimination of most of the contamination. A thorough rinse with sterile double distilled water was useful for good asepsis. Embryos were plated on 1 % sucrose agar after excision from sterile megagametophytes which had been plated on 1% sucrose agar for one day, and the procedure not only facilitated selection of white, firm and undamaged embryos, but also reduced dehydration. The cotyledons were cut at the cotyledon epicotyl junction (Fig. 1).

In order to determine the seed quality of the experimental material , 6 jars containing 15 sterile seeds each were germinated under two experimental conditions namely light and darkness. The number of seedlings were counted after 49 days (Table 6). The seeds germinated under both the dark and light conditions. Seedlings growing under the two conditions were vigourous. However, the sterile seeds of *Pinus roxburghii* Sargent had poor germination of 7.2 %.

Table 6. Effect of the environment on the germination of *Pinus roxburghii* seeds after 49 days.

Treatments	% germination
Dark	6.6
Light	7.7

When 1-day-old whole excised embryos were plated on 1/2 MCM containing 10^{-5} M BA only a small part of the embryo was in contact with the media and therefore, only a few shoots were produced and also the embryo had a tendency to root inspite of cutting the hypocotyl (Fig. 2).

Usually explants are derived from germinated seeds or whole embryos plated for some time on sucrose agar. In order to determine the best source of explants namely, intact megagametophyte or whole excised embryos, one-day-old cotyledons from both sources, were plated on half-strength MCM containing 10^{-5} M BA for two weeks (Table 7). Organogenesis was higher when cotyledons were derived from megagametophyte plated on 1% sucrose agar (bud response 8.0±0.8) B.F.C. 7.2 than when derived from plated whole excised embryos (bud response 6.3± 0.9) B.F.C. 4.2.

Table 7. Response of cotyledons of *Pinus roxburghii* from whole excised embryo and megagametophyte after 9 weeks of culture.

Treatment	% cotyledons forming buds	Avg. no of buds/ cotyledon±SE	BFC Index*
Embryo	67	6.3±0.9	4.2
Megagametophyte	90	8.0±0.8	7.2

*BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) / 100.

(4.2) BUD INDUCTION

Both the basal media and concentration are very important for obtaining a high yield of adventitious buds (Von Arnold and Erickson, 1981; Patel and Thorpe, 1984). In order to determine the best media and age of cotyledons, Cotyledons isolated from the first day to the fourth day of sterilisation were plated on media containing MCM, DCR, AE and MS mineral salts each at half-strength and containing 10^{-5} M BA (Table 8). The mean number of shoots/cotyledon was highest on 1/2 MCM (12.5) on day 0 and lowest on 1/2 AE (1.0) on day 4. 1/2 MCM produced the highest number of shoot buds on day 0, 1, 2 and 3. The mean shoot bud differentiation was lowest for 1/2 AE on all days post sterilisation. The percentage cotyledonary response was lowest on day 0 and increased to a maximum of 87% on day 2 for 1/2 DCR and then declined in day 4. However bud forming capacity was highest on day 1 for all the media. Although the highest mean number of buds per cotyledon was realised on day 0, the percentage response was low, i.e, less than 55%, and hence reduced the bud-forming capacity. A decline in bud-forming capacity was observed after day 1 due to declining mean number of buds per cotyledon.

Table 8. Effect of age of explant and media on bud induction of *Pinus roxburghii* after 9 weeks.

Time(Days)	Media	% cotyledons forming buds	Avg. no. of buds/ cotyledon±SE	BFC Index*
0	1/2MCM	40	12.5±2.5	4.9
	1/2DCR	45	7.6±1.2	3.4
	1/2AE	53	6.0±0.7	3.2
	1/2MS	40	6.4±1.0	2.6
1	1/2MCM	77	10.2±0.8	7.8
	1/2DCR	77	5.9±0.2	4.5
	1/2AE	73	5.2±0.4	3.8
	1/2MS	70	8.0±0.7	5.6
2	1/2MCM	73	7.3±0.5	5.4
	1/2DCR	87	4.8±0.4	4.2
	1/2AE	80	3.6±0.3	2.9
	1/2MS	80	5.7±0.6	4.6
3	1/2MCM	80	5.2±0.7	4.2
	1/2DCR	80	3.5±0.4	2.5
	1/2AE	70	5.0±0.5	3.5
	1/2MS	70	5.6±0.6	3.9
4	1/2MCM	37	4.7±0.6	4.2
	1/2DCR	33	5.3±0.9	1.8
	1/2AE	30	3.4±0.5	1.0
	1/2MS	33	5.8±1.2	1.9

*BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) / 100.

Therefore, of the four basal media used, 1/2MCM on day 1 produced the best response and was used as the basal medium for bud induction.

Table 9. Effect of various concentrations of MCM on bud induction of one-day old cotyledons of *Pinus roxburghii* after 9 weeks

Media Dilution	% cotyledons responding	Avg. no. of buds /cotyledon±SE	BFC Index*
1/4MCM	73	5.8±0.6	4.2
1/2MCM	70	8.9±0.7	6.2
1MCM	0	0	0
2MCM	0	0	0

*BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) / 100.

After selecting the basal medium for bud induction it was necessary to determine the best salt concentration. MCM containing 10^{-5} M BA was tested at various concentration (x2, x1, x1/2, and x1/4). Each treatment contained three replicates (Table 9). Half-strength MCM produced a higher bud forming capacity and more vigorous shoots (Fig. 3) than other concentrations and was therefore used in subsequent experiments.

(4.2.1) INFLUENCE OF BA ON ORGANOGENESIS

Conifers generally produce adventitious buds in response to exogenous cytokinins alone (Thorpe and Hasnain, 1988), BA being the most commonly used cytokinin. The period of exposure to cytokinins is also known to affect bud induction. One-day-old cotyledons were used to test BA concentrations at 0, $5x10^{-6}$ M, 10^{-5} M , $2.5x10^{-5}$ M and $5x10^{-5}$ M for 7, 14, 21 and 28 days; followed by transfer to BA-free medium. Three replicates, containing ten cotyledons each, were used for each treatment and mineral salts were used at

Table 10. Effect of BA concentration and time of exposure on bud forming capacity of 1-day-old cotyledonary explants of *Pinus roxburghii* after 12 weeks

Time days	BA Molarity µM	% cotyledons responding	Avg. no of buds/ cotyledon±SE	BFC Index*
7	5	43	4.5±0.5	1.9
	10	55	5.2±0.8	2.8
	25	57	8.3±0.6	4.7
	50	73	10.4±0.6	7.6
14	5	63	5.5±0.6	3.5
	10	70	10.2±0.8	7.1
	25	70	11.5±1.1	8.1
	50	80	15.8±0.9	13.1
21	5	63	6.6±0.7	4.2
	10	77	8.5±0.8	6.5
	25	83	16.1±1.1	13.4
	50	73	13.6±0.9	10
28	5	63	5.0±0.7	3.1
	10	57	8.2±0.8	4.7
	25	77	12.4±0.8	9.5
	50	70	14.7±0.9	10.3

*BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) / 100.

half-strength. The performance of the cotyledons was evaluated after 12 weeks (Table 10).

When 1-day old cotyledons of *Pinus roxburghii* were cultured in the absence of cytokinin they continued to elongate, but in the presence of BA they formed numerous buds. The concentration and duration of exposure to BA affected the number of buds and the quality and subsequent growth rate of shoots produced. When the cotyledons were cultured for one week on BA very few shoots were formed whereas four weeks of exposure to BA produced stunted shoots. When very low concentrations of BA were used few shoots

formed, while very high concentrations produced numerous stunted shoots. Therefore, the optimum concentration of BA and time of exposure was based on both qualitative and quantitative determinants. The optimum level of BA for bud induction and shoot elongation was 10^{-5} M , and the optimum period of culture in the presence of BA was 14 days based on percentage forming buds 70 % and the average number of buds 10.2. Although the highest bud forming capacity was obtained at 2.5 x 10^{-5} M for 21 days (BFC 13.4) these shoots did not develop normally and appeared stunted. The second highest BFC (13.1) was realised at 5.0 x 10^{-5} M but the shoots appeared stunted.

(4.2.2) CYTOKININ COMBINATION

The use of mixed cytokinins in conifers was first reported in conifers by Cheng (1975) and subsequently others, e.g., in white and black spruces (Rumary and Thorpe, 1984). In order to study the effect of mixtures of cytokinins on bud induction 1-day-old cotyledons were cultured in basal media containing BA, Z, K and 2iP each at 10^{-5} M or BA in combination with other cytokinins at $5x10^{-6}$ M each. Three replicates containing ten cotyledons each were used per treatment. Morphogenesis was not improved by addition of other cytokinins into BA-containing media (Table 11).

BA alone produced a higher BFC (8.4) than Z (4.1), K (3.6) and 2iP (2.2). Addition of BA and other cytokinins in equimolar concentration (5.0 x 10^{-6}) into the media did not result in improved morphogenesis. However shoots were found to elongate faster in Z-containing media (Fig. 4).

Fig. 1. Initial explants of *Pinus roxburghii*. The cotyledons were excised from the cotyledon-epicotyl junction (arrow head).

Fig. 2. Whole excised embryos of *Pinus roxburghii* after 12 weeks of culture. Roots (arrow head) regenerated from the hypocotyl during culture.

Fig. 3. Cotyledons of *Pinus roxburghii* cultured in x1/4 (a), x1/2 (b), x1 (c) and x2 (d) MCM after nine weeks of culture.

Fig. 4. Effect of BA in combinations with other cytokinins on bud induction of *Pinus roxburghii* after 12 weeks BA (a), K (b), Z (c) and 2iP (d) at 10^{-5} M BA in combination with K (e), Z (f) and 2iP (g) each at $5x10^{-6}$ M.

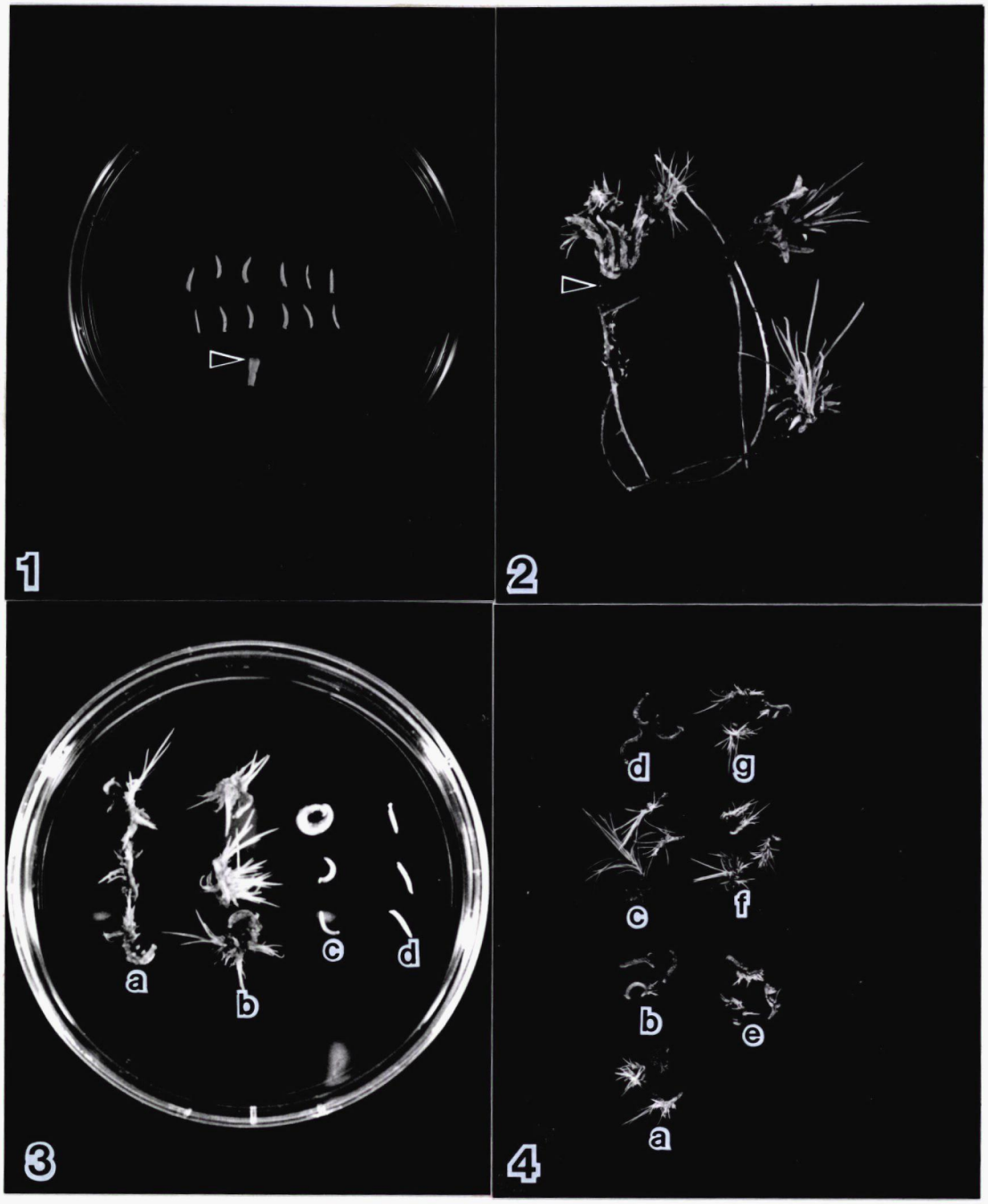
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Table 11. Effect of BA in combination with other cytokinins on 1-day-old cotyledons of *Pinus roxburghii* on bud induction after 12 weeks of culture.

Induction treatment	%cotyledons forming buds	Avg. no. of buds per cotyledon±SE	BFC Index*
Each at 10^{-5} M			
BA	60	13.9±0.8	8.4
K	37	3.6±0.5	1.3
Z	53	7.7±1.0	4.1
2iP	33	2.2±0.2	0.7
Each at $5x10^{-6}$			
BA-K	60	7.9±0.9	4.7
BA-Z	67	10.0±1.0	6.6
BA-2iP	50	5.1±0.6	2.7

* BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) /100.

An attempt was made to improve morphogenesis by subculturing 1-day-old cotyledons previously cultured on 10^{-5} M BA for seven days into other cytokinins of the same concentration. Three replicates were used per treatment. The percentage cotyledons responding, average number of buds and excisable shoots after 15 weeks were used as parameters (Table 12).

When cotyledons cultured on 10^{-5} M BA for 7 days were transferred into Z for 7 days, a higher shoot elongation index of 15.8 was obtained, compared with shoot elongation index of 12 when cotyledons remained in 10^{-5} M BA for 14 days. However transfer into K and 2iP produced lower shoot elongation index than cotyledons cultured for 14 days in half-strength MCM media containing 10^{-5} M BA.

Table 12. Effect of other cytokinins on 1-day-old cotyledons of *Pinus roxburghii* cultured initially on 10^{-5} M BA for 7 days after 15 weeks.

Induction treatment	% cotyledons forming buds	Avg. no of buds /cotyledon±SE	BFC Index*	SEC Index#
BA	60	12.4±0.8	7.5	12
BA-Z	63	10.5±1.0	6.6	15.8
BA-K	36.7	9.1±1.4	3.3	3.7
BA-2iP	46.7	7.4±0.9	3.5	4.7

* BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) /100.
# SEC (shoot elongation capacity) index = (no. of shoots > 1.5 cm after 15 weeks) x (% cotyledons forming buds) / 100.

(4.3) BUD DEVELOPMENT AND SHOOT FORMATION

This phase in the micropropagation of conifers involves the development of nodular tissue formed during bud induction into shoots with primary needles (Thorpe and Hasnain, 1988). In general the formation of true apices with juvenile leaf primordia requires transfer onto a medium with altered nutrition levels (Biondi and Thorpe; 1982; Thorpe and Biondi, 1984). With this species, bud growth was promoted by transferring the cotyledons onto petri dishes containing 30 ml half-strength basal media devoid of hormones, after 2 weeks. By the fifth week buds had started to develop and formed clusters on the cotyledons (Fig. 5). After the fifth week cotyledons were transferred into bigger petri dishes (100x25mm) containing 30ml media and 0.05% activated charcoal (Sigma, St. Louis, MO, no. c4386), 0.8% agar and 3% sucrose. The intact cotyledons were subcultured every 4 weeks into fresh media.

Table 13. Effect of transferring 1-day-old cotyledons of *Pinus roxburghii* from 1/2 MCM to 1/2 SH salt formulation after 13 weeks.

Media	% cotyledons forming buds	Avg. no. buds/ /cotyledon±SE	BFC Index*	SEC Index#
1/2 MCM	57	8.1±0.8	4.6	8.6
1/2 SH	63	7.6±0.6	4.8	20.8

* BFC (bud forming capacity) index = (avg. no of buds per cotyledon) x (% cotyledons forming buds) / 100.

SEC (shoot elongation capacity) index = (no. of shoots > 1.5 cm after 13 weeks) x (% cotyledons forming buds) /100.

The basal media (1/2MCM) was compared with 1/2 SH for shoot elongation on the initial explants. After five weeks from initiation of culture three replicates containing ten cotyledons each were transferred into either 1/2 MCM or 1/2SH containing 3% sucrose, 0.8% agar, and 0.05% activated charcoal (Table 13). After 13 weeks of culture 33 shoots were excised from cotyledons cultured on 1/2 SH whereas only 15 shoots were excised from cotyledons cultured on half-strength MCM. Unlike for *Pinus canariensis* division of explant into pieces resulted in necrosis therefore media which allowed a faster elongation was necessary. Shoots greater than 1.5 cm were separated in 13-15 weeks. The excised shoots were cultured individually.

Shoots excised from the initial explants after 13-15 weeks of culture were too small for rooting or shoot multiplication, therefore, it was necessary to determine the best medium for elongation of the shoots. Excised shoots were transferred to storage jars containing 100 mL of MCM and SH minerals, each at one-half strength and containing 2% sucrose, 0.05% activated charcoal, and 0.5% gelrite. The control consisted of half strength SH solidified with Difco

bacto agar instead of gelrite. Sixteen shoots were used in each treatment. The average height of the shoots after 28 weeks were 4.2 cm for half-strength MCM, 2.9 cm for half-strength SH solidified with Difco bacto agar and 3.6 cm for half strength SH solidified with gelrite. The shoots also browned in half strength SH solidified with Difco bacto agar. Therefore both media and gelling agent had an effect on shoot elongation. The excised shoots elongated well on half-strength MCM and therefore 1/2 MCM was selected as basal medium for elongation (Fig. 6).

(4.4) SHOOT MULTIPLICATION.

When 28-week-old shoots were decapitated in region containing upright leaves the apical meristem regenerated and no axillary shoots were formed. Also when when the shoots were decapitated too far from the upright leaves the shoots died. However, when the shoots were decapitated just below the upright leaves, four axillary shoots were obtained after one month of culture of the decapitated shoots.

(4.5) ROOTING

In the initial rooting experiment 24-week-old shoots were pulsed for 2, 3, 4, 5 and 6 hours in 10^{-3} M IBA and subsequently transferred into peat and vermiculite mixture 1:1 (v/v). In each treatment 6 shoots were used. Only one shoot rooted for 2 and 3 hours pulse treatment each (total rooting 6.7%) (Table 14)(Fig. 7).

An attempt to root 26-week-old shoots of *Pinus roxburghii* in peat : vermiculite 1:1 (v/v) substrate containing two levels of NAA; $5x10^{-7}$,$5x10^{-6}$ M and 10^{-5} M was unsuccessful. However when sixteen 26-week-old shoots were

Table 14. Effect of pulsing 6 month old in vitro shoots of *Pinus roxburghii* for various periods of time with subsequent transfer into peat : vermiculite 1:1 (v/v) substrate.

Pulsing time(h)	%rooting
2	16.7
3	16.7
4	0
5	0
6	0

incubated in 5.37×10^{-7} M NAA in 1/4 MCM media containing 1% sucrose, solidified in gelrite, 25% rooting was realised after 4 weeks of incubation. Therefore, a higher rooting was realised, and, up to four roots/plantlet was obtained using this method. (Fig. 8).

Another interest was to test the effect of microenvironment on rooting. Two treatments namely reduced light and rooting in normal light were tested. For the light reduction, sterilised aluminium foils which had been trimmed to size were placed on sterile media and the bottom was also covered with aluminium foils. Two replicates containing six, 28-week-old shoots each were used per treatment. The shoots were incubated in 5.37×10^{-7} M NAA in 1/4 MCM media containing 1% sucrose solidified in gelrite (Table 15).

After one month of culture 50% rooting was obtained under reduced light conditions whereas under free light 66.7% rooting was obtained. In another experiment run concurrently 70% rooting was obtained from 20 shoots cultured in reduced light. Under optimal conditions (Bud induction in 10^{-5} M BA for one week and 10^{-5} M Z for one week) 81 % rooting was obtained.

Fig. 5. 5-week-old non-shoot-forming (top row) and shoot-forming (bottom row) cotyledons of *Pinus roxburghii* .

Fig. 6. 28-week-old shoots of *Pinus roxburghii* elongated on 1/2 SH solidified with Difco bacto agar (a), 1/2 SH solidified with gelrite (b) and 1/2 MCM solidified with gelrite (c).

Fig. 7. 30-week-old plantlet of *Pinus roxburghii* rooted in 10^{-3} M IBA. The regenerant had one root (arrow head).

Fig. 8. 32-week-old plantlets of *Pinus roxburghii* rooted in 5.37×10^{-7} M NAA. The regenerants had multiple roots (arrow heads).

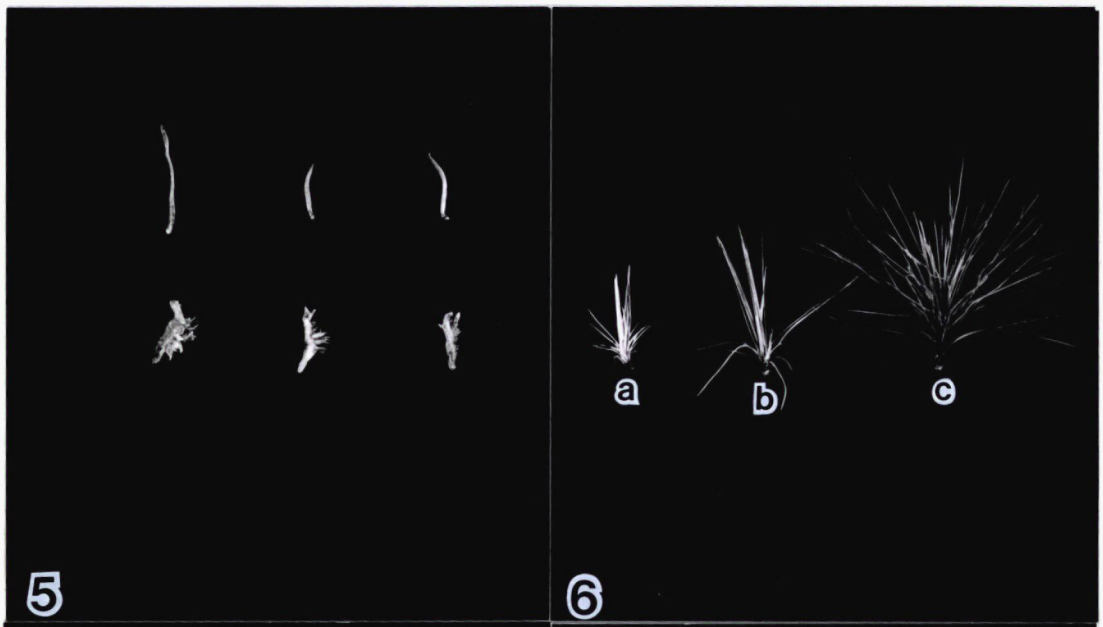
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Table 15. Effect of reduced light on rooting of 28 weeks old shoots of *Pinus roxburghii* after 28 days.

Treatment	% rooting	range	Avg. no. of roots/plantlet
Reduced light	50	1-4	2.8
Direct light	67	1-4	2.6

(4.6) ACCLIMATISATION

After rooting, the plantlets were put in peat:vermiculite 1:1 (v/v) moistened with 1/4 MCM devoid of sucrose for continued root development. From previous observation plants browned when cultured in the presence of sucrose after rooting. After 28 days plantlets were transferred singly into 1/2 L pots containing the peat:vermiculite 1:1 (v/v) moistened with 1/4 MCM mineral salts and devoid of all organic supplements. The pots were covered with transparent plastic bags to prevent dessication and left in the growth chamber for three weeks. The plantlets were transferred from the growth chamber after three weeks into growth cabinets maintained at 24 C to 18 C (light:dark) with a 16h:8h light:dark) photoperiod. The plastic bags were removed for 1 to 8 hours daily in order to enhance transpiration. Watering was done at least once a week as necessary. Only 25 ml of sterilised double distilled water was used. After three weeks of hardening in the growth cabinet the plantlets were left uncovered for one week after which they were transferred to the greenhouse (Fig. 9). After removal of the plastic bags the plants were watered every other day. The potted plantlets were transferred to the greenhouse after being hardened successfully.

(4.7) GREENHOUSE PERFORMANCE

The plantlets of *Pinus roxburghii* Sargent increased in both height and girth (Fig. 10). After one month in the greenhouse the plantlets increased by twenty four millimetres to an average height of 77 millimetres. The plantlets grew orthotropically and had green leaves. Some leaves grew upright and protected the apical meristem while other leaves grew plagiotrophically. The plantlets had a survival rate of about 85%. The plantlets also branched and up to five branches were found on one specimen which was one hundred and thirty five millimetres.

(4.8) SUMMARY OF THE PROTOCOL

Seeds of *Pinus roxburghii* Sarg. were scarified for 5 minutes in concentrated sulphuric acid, rinsed thoroughly and subsequently washed for two nights in running tap water. Floating seeds were discarded after one day and the remainder sterilised for 30 minutes in 50% Javex^{R} bleach (6%) containing 3 to 4 drops of Tween per 100ml and followed by five 5-minute rinses with sterile distilled water. The seeds were treated with hydrogen peroxide (10%) and again rinsed with sterile distilled water. The sterile seeds were stratified at 5 C for two days. The seed coats were cracked with sterile pliers and the intact megagametophye was re-sterilised for 15 minutes in 15% bleach and for 5 minutes in hydrogen peroxide, each procedure was followed by 5x5 minute rinses with sterile distilled water. Embryos were isolated from megagametophytes which had been plated on 1% sucrose agar for one day and plated on 1% sucrose-agar to facilitate selection. Cotyledons were only isolated from white, firm, undamaged embryos and plated on induction

Fig. 9. 40-week-old plantlets of *Pinus roxburghii* being hardened in the growth cabinet after rooting.

Fig. 10. 44-week-old plantlets of *Pinus roxburghii* growing in the greenhouse after hardening.

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media. Cotyledons which were white, firm and undamaged were cultured in half-strength MCM containing 10^{-5} M BA, 3% sucrose, asparagine 100 mgL^{-1}, thiamine-HCl, 5 mgL^{-1}, nicotinic acid, 5 mgL^{-1}, pyridoxine-HCl 0.5 mgL^{-1}, Difco Bacto agar at 0.8% and media adjusted to pH 5.7 to 5.8. After 7 days of culture, the cotyledons were transferred onto half-strength MCM of the same composition except that, 10^{-5} M BA was replaced by 10^{-5} M Z. The cotyledons were subcultured after 7 days.

After 5 weeks of culture cotyledons were transferred into bigger petri-dishes(100x25 mm) containing 30 ml media. The medium consisted of half-strength SH containing, 3% sucrose, 0.05% activated charcoal (Sigma, St. Louis, MO, no. C4386) and solidified with 0.8% Difco bacto agar. The intact cotyledons were subcultured after 3-4 weeks onto fresh media. Shoots longer than 1.5 cm were excised after 13-15 weeks.

Excised shoots were cultured in glass storage jars containing 100 ml half-strength MCM mineral salts, 2% sucrose, 0.05% activated charcoal, and 0.5% gelrite. The shoots were subcultured after 3-4 weeks onto fresh medium. After 28 weeks from culture initiation the shoots were ready for rooting.

The shoots were cultured in glass storage jars containing 100 ml. The medium consisted of 1/4 MCM, 5.37×10^{-7} M NAA, 1% sucrose, and 0.5% gelrite. Plantlets were transferred into glass storage jars containing peat and vermiculite 1:1 (v/v) and moistened with 1/4 MCM devoid of sucrose for 28 days.

After 28 days plantlets were transferred onto 1/2 litre pots containing peat and vermiculite 1:1 (v/v) and moistened with 1/4 MCM mineral salts and devoid of all organics. The pots were covered

with plastic bags to reduce transpiration. After 3 weeks the potted plantlets were transferred into the growth cabinet which was maintained at 24 C:18 C (light:dark) with a photoperiod of 16h:8h (light:dark). The plantlets were hardened by removing the plastic bag for 1 to 8 hours daily for 2 to 3 weeks after which, they were transferred to the greenhouse.

In the greenhouse the temperature was maintained at 25 C to 28 C under the natural photoperiod. The plantlets were ferti-irrigated twice a week with water containing 250 ppm nitrogen (Peter's brand 20:20:20 or 20:10:20).

(4.9) DEVELOPMENTAL ANATOMY OF *PINUS ROXBURGHII.*

(4.9.1) DEVELOPMENTAL SEQUENCE

At the time of excision (day 0), cotyledons generally measured ca. 3-6 mm (Fig. 11) . Those placed in shoot-initiation medium began to swell and increased in length up to day 21. By day 11, the cotyledon surface in contact with media acquired a pronounced nodular appearance. By day 21, leaf-like projections were visible on the cotyledon surface. The leaf-like projections later developed into shoots with primary needles. In contrast, cotyledons cultured without BA, elongated and reached a maximum by day 21, at which time they were twice as long as the BA-treated explants.

(4.9.2) STRUCTURE OF COTYLEDONS AT THE TIME OF EXCISION

The most noticeable cells in the cotyledons were those in the epidermis, these cells were tightly packed against each other and were smaller than adjacent epidermal and subepidermal cells (Fig. 12). In these explants the cotyledons had large prominent nuclei and the cytoplasm stained densely. At the time of excision mitotic figures were not observed.

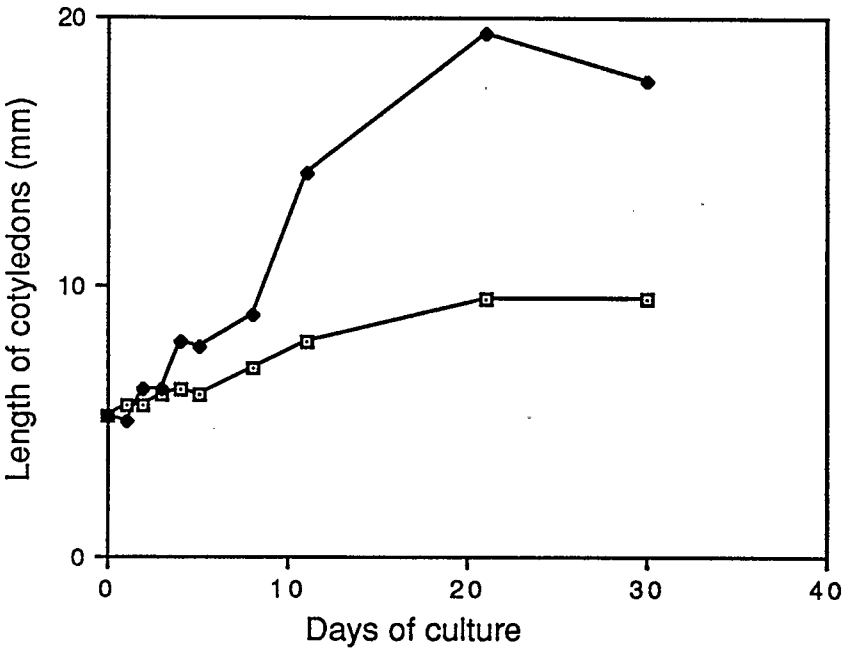


Fig. 11. Growth of cotyledons excised from 1-day-old embryos of *Pinus roxburghii* in half-strength MCM containing 10^{-5} M BA for 7 days, and 10^{-5} M Z for another 7 days and subsequently transferred to BA-free medium (open squares) and in cytokinin-free medium (dark squares). Each data represents the mean of five cotyledons.

The vascular initials were smaller in width but were longer than all the other cells. Most of the cells of the vascular initials had thicker cell walls than the neighbouring mesophyll and had small nuclei. The cells which differentiated into tracheids had the thickest cell walls when compared with the other cells of the vascular initials and were delineated easily. Unlike the cells of the mesophyll, the vascular initials were tightly packed against each other.

(4.9.3) STRUCTURAL CHANGES IN COTYLEDONS CULTURED IN CYTOKININ-CONTAINING MEDIUM

During day 1, the cotyledons remained highly cytoplasmic. Although mitotic figures were absent, the nuclei were enlarged and highly visible. The cells of the epidermis were more densely staining than the cells to the inside. The first cell divisions were observed on day 2 (Fig. 13). Both periclinal and anticlinal cell divisions were observed extending from the epidermis to the vascular initials. In day 3, more cell divisions were observed. Sometimes neighbouring cells divided at the same time (Fig. 14). Cell differentiation was also observed, especially in the vascular initials which formed tracheids. These tracheids appeared to be well developed and had secondary cell wall thickening.

By day 8, the cells of the epidermis remained highly cytoplasmic compared with mesophyll cells in which the cytoplasm was reduced to the peripheral layer. A group of densely staining cells, the meristemoid, could also be seen a few layers below the epidermis(Fig. 15). These cells were also small and could be delineated from the neighbouring non-meristematic cells of the mesophyll which were bigger and more vacuolated. The cells of the meristemoid divided both periclinally and anticlinally. The cells of the epidermis also divided anticlinally so that the increasing size of the meristemoids was

accommondated and therefore, the epidermal layer appeared uneven. Meristematic activity extended to the vascular initials.

Prolific cell division resulted in the protrusion of a meristematic dome by day 11 (Fig. 16). The meristematic dome appeared as a pronounced nodular structure which extended from the cotyledonary surface. The cells of the meristematic dome were small and full of densely staining components. Visually the domes appeared like bumps on the cotyledonary surface. These cells were tightly packed together and were devoid of airspaces. The cotyledonary epidermis was incorporated into the meristematic dome.

A developing apical meristem could be seen surrounded by leaf-like structures on day 21 (Fig. 17). Both periclinal and anticlinal cell division could be seen in the apical meristem. The leaf-like structures were also active in cell division. Although densely staining cells could be seen at the apex, cells below it appeared less densely staining and therefore less mitotically active. Tracheids developed both in the vicinity of the apical dome and below it towards the cotyledonary vascular system. Some of the non-meristematic cells of the initial explant directly below the apical meristem also developed into tracheids.

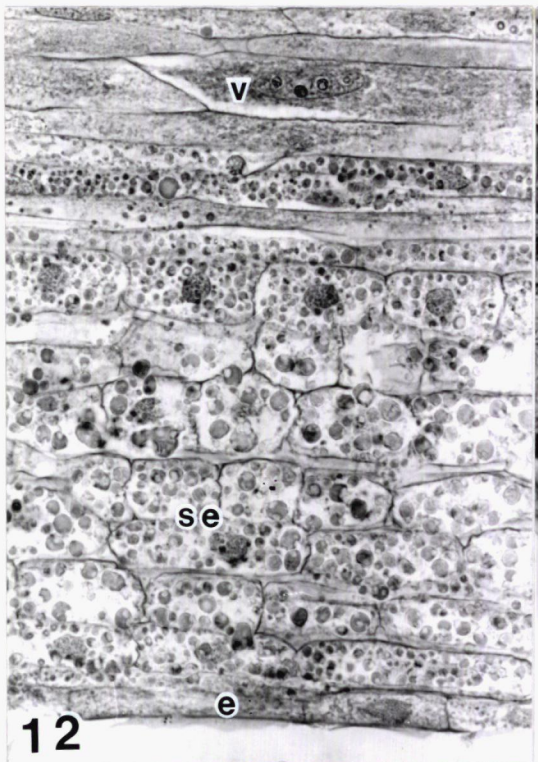
By day 30, many buds developed close to each other. These buds formed an extended layer along the cotyledonary surface in contact with the medium. The shoot apex was in its advanced stage of development and was dome-shaped (Fig. 18). Located at the top of the apical dome were densely staining small cells with centrally located nuclei. Proximal to these small cells lay larger cells that were also densely staining. Below the large cells lay less densely staining large cuboidal cells. These cells were arranged in vertical rows and had small nuclei that were located to the periphery. These cells extended well into the pith. Anticlinal cell division was visible to the periphery of the apical

Fig.12. Initial explants of*Pinus roxburghii* showing epidermal (e), subepidermal cells (se) and vascular tissues (v). The cotyledonary cells had dense cytoplasm and large amounts of storage products. Mitotic figures were not observed at this time; (x314).

Fig. 13. Cotyledons of *Pinus roxburghii* after two days of culture. Mitotic figures, anticlinal (arrow head) and periclinal (double arrow head) could be seen. Other cells had prominent nuclei which suggested that they were preparing for cell division; (x375).

Fig. 14. Cotyledons of *Pinus roxburghii* on day 3. Cells in the second sub-epidermal layer could be seen dividing periclinally (arrow heads) judging from the alignment of the chromosomes; (x417).

Fig. 15. Meristemoids (asterisk) could be seen on cotyledons of *Pinus roxburghii* after 8 days of culture. Epidermal cells below the meristemoid (arrow head) also appeared meristematic and had prominent nuclei compared to epidermal cells away from the meristemoid (double arrow heads); (x382).



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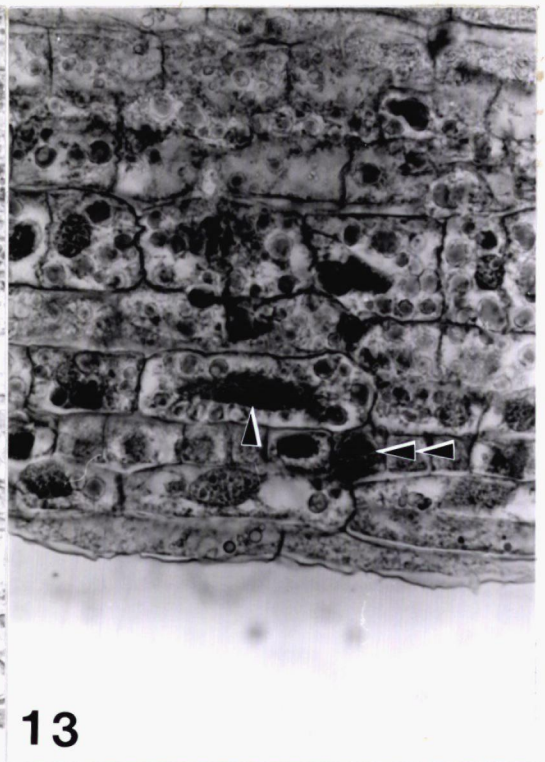

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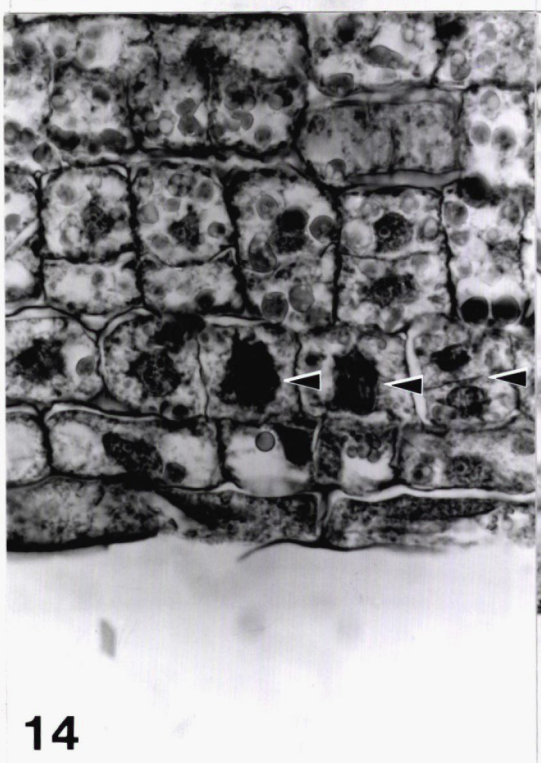

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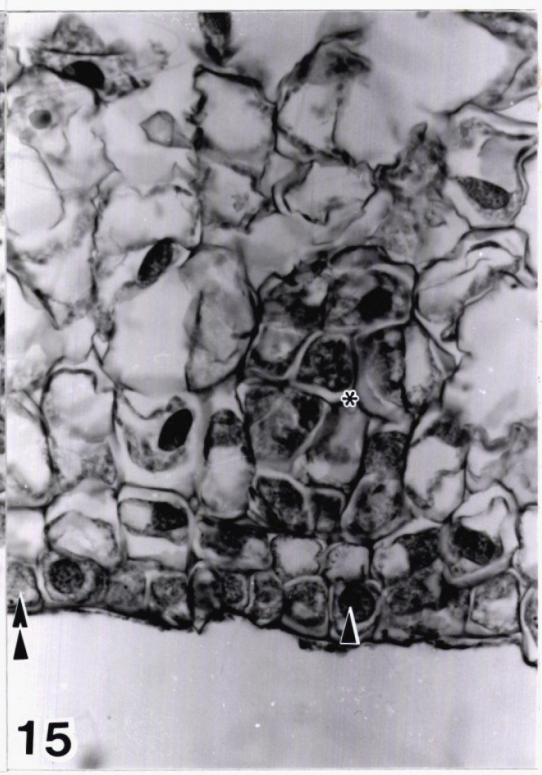

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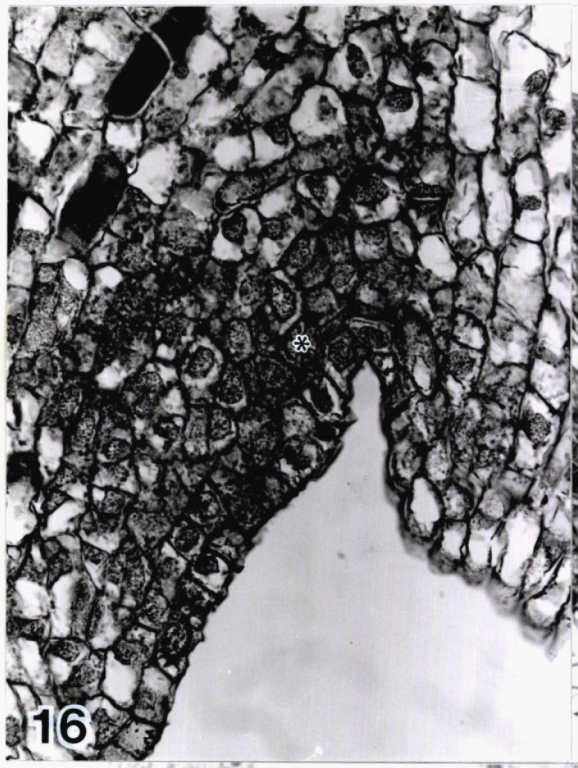
Fig. 16. Meristematic domes could be seen after 11 days from culture initiation of the cotyledonary explants of *Pinus roxburghii* . The cells of the meristematic dome were densely staining and were tightly packed together (asterisk); (x233)

Fig. 17. *De novo* apical meristems after 21 days of culture of cotyledonary explants of *Pinus roxburghii.* Developing leaf primordia surround the young apical meristem (arrow heads); (x120).

Fig. 18. *De novo* apical meristems after 30 days of culture of cotyledonary explants of *Pinus roxburghii* . Cells at the apex were more densely staining and smaller than cells proximal to the apex. The apical meristem was surrounded by well developed leaves; (x134).

Fig 19. Non-shoot-forming cotyledons of *Pinus roxburghii* after 8 days of culture. The cells were highly vacuolated and the cytoplasm was reduced to the peripheral layer (arrow heads); (x360).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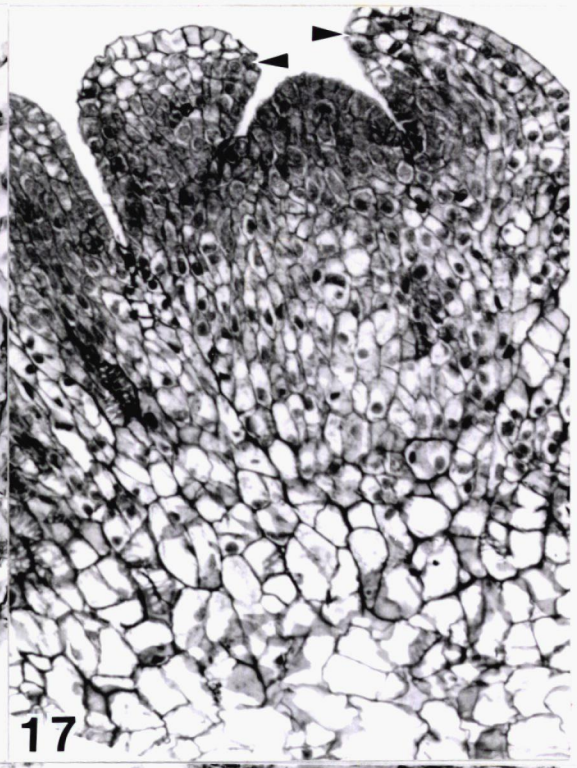
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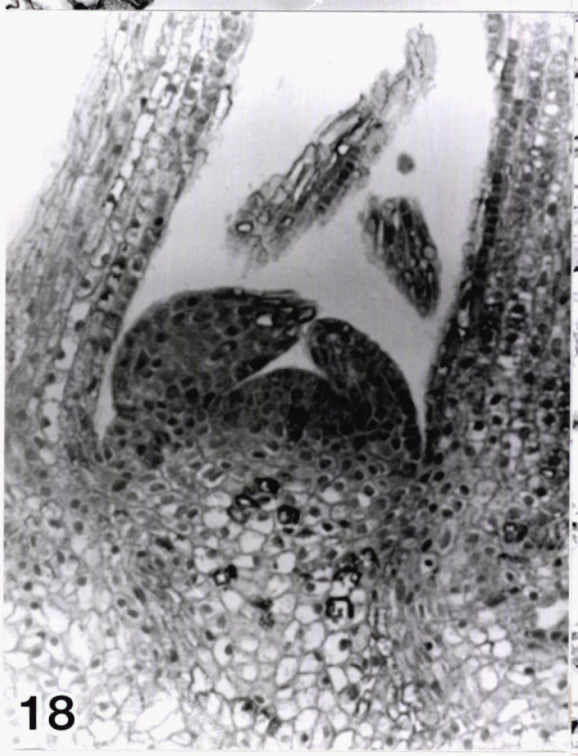
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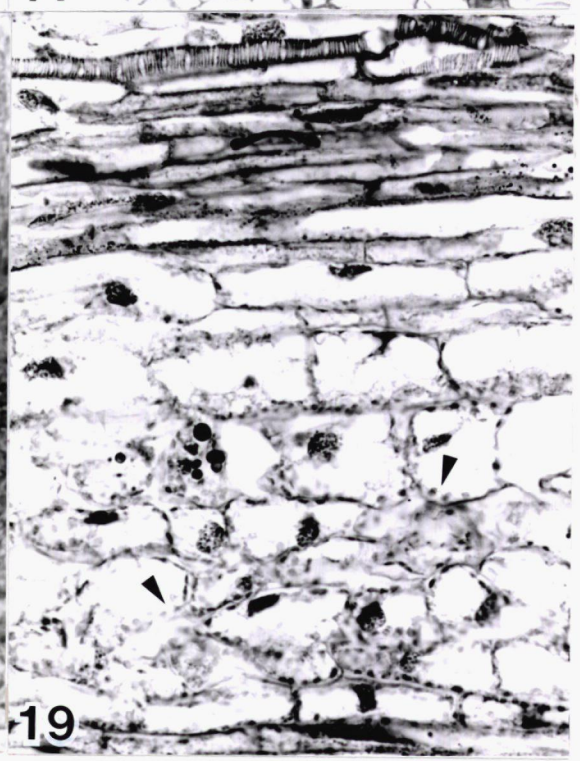
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dome which also had small densely staining cells. The vascular system was well developed and long tracheids were arranged vertically in the pith and extended into the leaves.

(4.9.4) STRUCTURAL CHANGES IN COTYLEDONS CULTURED IN CYTOKININ-FREE MEDIUM

In contrast to the BA-treated cotyledons, cell division was not observed in the cotyledons cultured in non-shoot-forming media on day 2. The cells stained uniformly and the nuclei were less prominent. Day 4, cotyledons stained less than younger cotyledons and only a few cell divisions were observed. The epidermis was not prominent. The vascular initials differentiated into tracheids. By day 5, the cells had little cytoplasm and had large intercellular airspaces. By day 8, the cells had lost their configuration and appeared to lose contact with each other (Fig. 19).

(4.9.5) ROOT-SHOOT JUNCTION

During the root induction treatment the base of the shoot became swollen and a small amount of callus was formed. The central core of the pith began from a distance below the shoot apical meristem and extended into the vascular cylinder. The cortex comprised of parenchyma cells and surrounded the vascular cylinder. The cells of the cortex extended into the leaves . The vascular cylinder comprised of xylem to the inside and phloem to the outside. The vascular tissue of the stem was directly connected with the root with no apparent disruption in the tissue culture plantlet. Tracheids could be traced from the stem into the roots (Fig. 20). The parenchyma of the cortex was also directly connected with the parenchyma of the root. The root apical meristem consisted of actively dividing cells that had large prominent nuclei (Fig. 21).

These cells were smaller than cells of the root cortex. Distal to the cells of the apex lay large elongated cells which had small nuclei. Proximal to the apex lay well developed tracheids. In the cross section of the root, four protoxylem poles lay scattered among the pith cells (Fig. 22). The pith cells had a nucleus whereas the protoxylem had big prominent cell walls. The pith cells were smaller than the cells of the cortex. The apical meristem of the plantlets had densely staining cells. These cells were small and had prominent nuclei. The cells divided frequently, both periclinally and anticlinally. Developing leaf primordia were seen at the periphery of the apical meristem. Below the apex, lay large less staining cells of the pith (Fig. 23). In a cross section of the stem, the cortex comprising of large cells lay underneath the tightly packed layer of the epidermis.

In a cross section of the stem, the epidermis was seen to the outside. The cells of the epidermis were tightly packed against each other and contained a thick cell wall. Below the epidermis lay the cortex which had large cells with small nuclei (Fig. 24). Resin canals could be seen in the cortex. Also many leaf traces could be seen scattered in the cortex. The vascular cylinder lay in the centre of the stem and comprised of xylem to the inside and phloem to the outside. The cortex and the pith were connected by pith rays which disrupted the vascular cylinder. The leaves were wide in the centre and tapering to the sides (Fig. 25). Below the tightly packed cells of the epidermis lay large cells of the mesophyll which were loosely packed with large intercellular airspaces. Resin canals were scattered among the cells of the mesophyll cells. Well developed vascular bundles lay in the centre of the leaf.

Fig. 20. Root-shoot junction of the plantlets of *Pinus roxburghii* . The tracheids could be traced from the stem (arrow head) into the root (double arrow head). The cortical cells formed a continuum from the shoot to the root; (x100).

Fig. 21. Longitudinal section of adventitious roots of*Pinus roxburghii* showing small densely staining cells of the root apex (asterisk). Distal to the apex the vacuolated cells of root cap could be seen; (x107).

Fig. 22. Cross section of adventitious roots of*Pinus roxburghii.* Four protoxylem poles could be seen in the cross section of the root (arrow heads); (x100).

Fig. 23. Shoot apical meristem of the plantlets of *Pinus roxburghii.* The apex was conical in shape. Two developing leaf primordia could be seen at the apex; (x114).

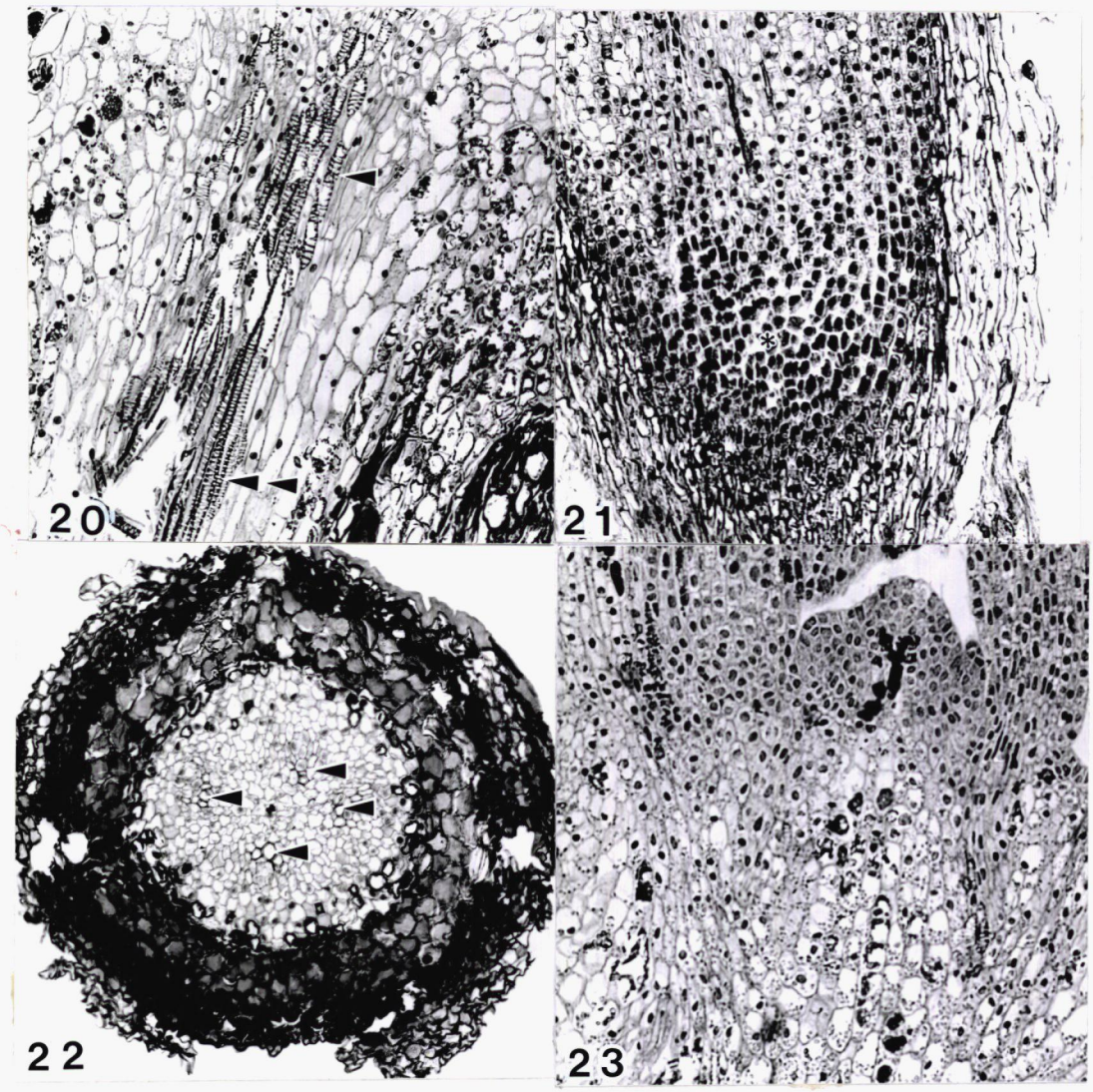
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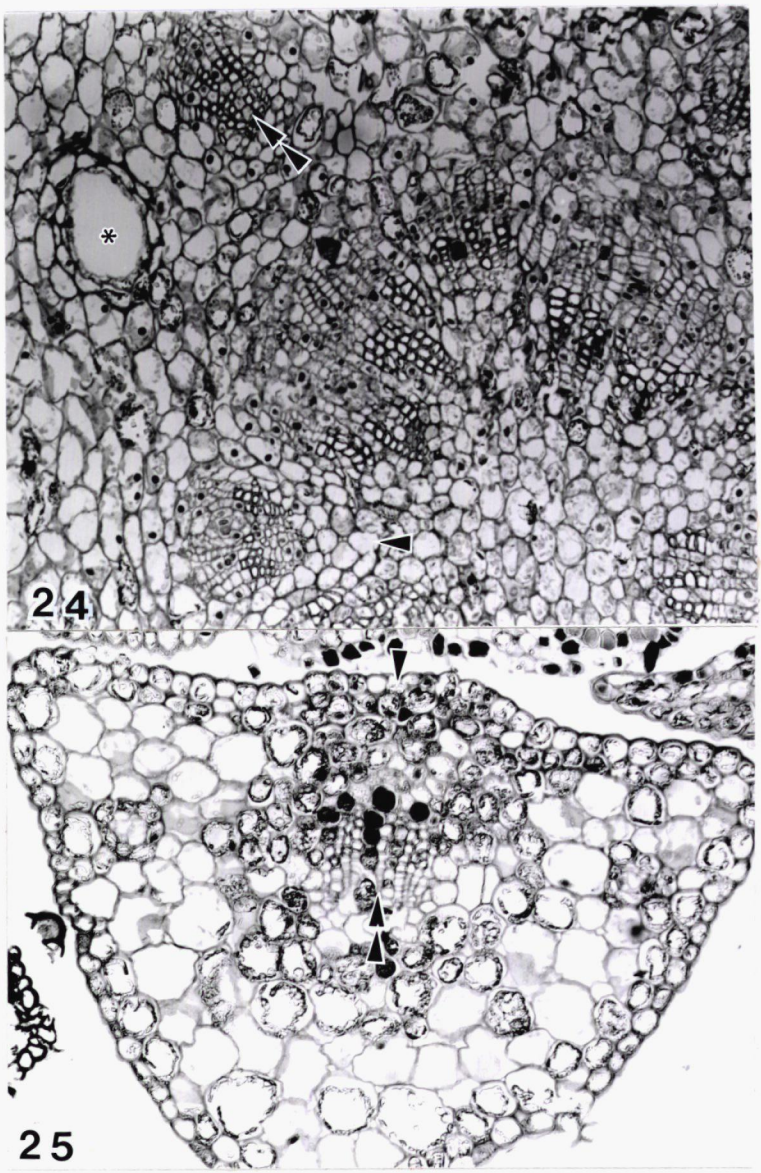
Fig. 24. Cross section of adventitious stems of *Pinus roxburghii*. The cortical cells were larger than pith cells. A leaf gap (arrow head), leaf trace (double arrow head), and resin canal (asterisk) could be seen; (x113).

Fig. 25. Cross section of adventitious leaves of *Pinus roxburghii* Sargent. The leaves were wide in the centre (arrow head) and tapering to the sides. Below the tightly packed cells of the epidermis lay the large cells of the mesophyll which were loosely packed together and had large intercellular air spaces. The vascular bundle (double arrow head) was located close to the base of leaf and very close to the epidermis; (x98).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(5.0) DISCUSSION

(5.1) PLANT MATERIAL

The question of germination percentage in tissue culture systems which utilise explants of seedling origin is a critical one. The ability to produce a maximum number of plantlets is directly dependent on germination percentage since a low yield of the explant material (poor germination) would undermine any improvements made in shoot-forming capacity of the explant. The germination percentage also indicates that the material being used for tissue culture is viable and is of good quality. Non-viable material would not be expected to respond in culture because tissue culture is a living system. Practises during and after the processing of the seeds may affect the quality of the seeds. There is also a variety of factors known to affect the germination of seeds, for example, pH (Konar, 1958), and germination substrate (Biondi, 1980).

Although the sterile seeds of *Pinus roxburghii* had a low germination percentage of about 7.2%, many factors affecting seed germination had not been evaluated. The seeds germinated both in light and darkness and therefore, neither of these two factors was limiting. A high germination percentage of about 50.1% was obtained for *Pinus radiata* using vermiculite soaked in distilled water and therefore the germination substrate had no adverse effect on germination of *Pinus roxburghii* seeds (Biondi, 1980). A higher germination percentage would have been attained under non-sterile conditions but a reasonable asepsis is necessary in tissue culture. The cones of *Pinus roxburghii* have a tendency to disperse the seeds leaving empty cones on the tree and therefore cones must be collected before seed dispersal (Dogra et al., 1983). Whereas it is possible to select cones with many ripe seeds, the seeds on the cone represent different levels of maturation which

might affect the germination percentage. Germination percentage among different conifers may be expected to vary depending on difficulties during harvesting and may be one of the reasons for the wide variation in morphogenic response observed during culture. However, the germination percentage indicated that the material was viable and was therefore, amenable for tissue culture.

(5.2) BUD INDUCTION

This phase of micropropagation included, the selection of a suitable explant, age of explant, culture medium and, cytokinin type and concentration. The best treatment combination was used in subsequent experiments.

Excised embryos of *Pinus roxburghii* are relatively large and contain 8-16 cotyledons. Although a good bud response can be expected by exposing maximum surface of explant to induction medium (Aitken et al., 1981), only a small portion of embryos of *Pinus roxburghii* were in contact with the induction medium. The embryos also had a tendency to germinate on the induction medium thereby reducing the surface area of contact. However, plantlets have been obtained from embryo-derived callus (Wagley et al., 1987), directly from cultured embryos (Kurz et al., 1989, Chang et al., 1991 Chesick et al., 1991) and seedlings (Abdullah and Grace, 1986).

Excised cotyledons are homogenous initial explant consisting, except for vascular initials, of one cell type, therefore plantlets derived from excised cotyledons may be expected to be similar. Buds induced from cotyledons cultured on induction-medium arise directly from the cells without an intermediate callus phase. The occurrence of a callus phase may result in somaclonal variation which is not desirable during clonal propagation. The cotyledons represent a juvenile phase of the embryo and have a high

regeneration capacity especially when they are derived from germinated seeds. For *Pinus radiata*, 23-fold multiplication of shoot-forming capacity index of cotyledons over whole excised embryos was obtained after 12 to 13 weeks of culture (Aitken et al., 1981).

The megagametophyte-derived cotyledons had a better bud response than cotyledons derived from whole excised embryos. The megagametophyte, therefore played a role in morphogenesis. The cotyledons derived from the megagametophyte may have been in a different physiological state than those derived from whole excised embryos. The megagametophyte is known to be a source of nutrients for the developing embryo. In the course of germination there is evidence of *de novo* enzyme synthesis (Borris, 1977). Although, the megagametophyte and the embryo had similar patterns of RNA and protein synthesis for lodgepole pine, the megagametophyte was found to contain higher levels of buffer-soluble proteins which decreased in the course of seed germination. The embryos had a lower level of buffer-soluble proteins which increased in the course of seed germination and therefore, the megagametophyte contained reserve proteins for the young embryo. Synthesis of different subsets of proteins occurred during germination and post germination development of the mature lodgepole pine seeds in both the megagametophyte and embryo (Gifford et al., 1991). In the megagametophyte, translation of mRNAs may contribute to the synthesis of hydrolyases that are involved in limited hydrolyases of storage protein reserves (Gifford and Tolley, 1989). Increased mRNA synthesis in both the megagametophyte and embryo has also been observed in Jack pine (Durzan et. al., 1972) and Douglas fir (Ching, 1966).

Of the four media used for bud induction half-strength MCM produced the highest number of buds. MCM medium was developed for the culture of *Picea*

abies by Bornman in 1983. Recently, MCM was used for the regeneration of plantlets of *Pinus canariensis* (Martinez-Pulido et al., 1990). The reason for the superior induction of buds on half-strength MCM may lie in the high level of reduced nitrogen present in MCM medium. However, morphogenic capacity of *Pinus ellioti* Engelm. (slash pine) was limited by ammonium and high nitrogen concentration. The percentage of surviving embryos of slash pine increased when NH_4NO_3 was drastically reduced or omitted and the frequency of shoot-forming embryos was inversely correlated with NH_4NO_3 concentration (Perez-Bermudez and Sommer, 1987). For *Pinus strobus* , when embryos were cultured in medium high in NO_3 :NH_4 ratios caulogenesis was observed (Flinn et al., 1988). For *Pinus wallichiana* (Konar and Singh, 1980) and *Pinus palustris* Mill. (Sommer et al., 1975) the best results was obtained when ammonia was omitted or reduced in the medium. The four media used for bud induction in this research, differ in the relative quantities of nutrients (including total N and NO_3^- to NH_4 ratios) and may account for differences in morphogenic response.

When medium dilutions were compared, half-strength MCM was superior to higher concentrations or lower dilutions. At higher concentrations cotyledons died whereas at lower concentrations the buds elongated slowly. Although full-strength media have been used for bud induction of yellow cedar (Kurz et al., 1989), *Picea abies* (Bornman, 1983), radiata pine (Aitken et al., 1981), some reports indicate that high concentrations of major salts could be detrimental to young explants (Erickson et al., 1977). Reduced strength of basal media have been used for the regeneration of the majority of species including western larch (Harry et al., 1991),

eastern white cedar (Harry et al., 1987) and red pine (Noh et al., 1988). The death of the cotyledons in high MCM concentration may have been due to osmotic stress and/ or ion toxicity whereas at very low concentrations the nutrients may have been limiting.

In the micropropagation of a majority of species the original formulations have been modified in various ways, for example the microsalts of Schenk and Hildebrandt were elevated during the micropropagation of calabrian pine (Abdullah and Grace, 1986), and the organics of Schenk and Hildebrandt substituted for the organics of the original formulations for the micropropagation of *Pinus canariensis* (Martinez Pulido et al., 1990), western larch (Harry et al., 1991) and eastern white cedar (Harry et al., 1987). The organics of Schenk and Hildebrandt (except myo-inositol which was added at 100 mg per litre) were included in the medium used for the micropropagation of *Pinus roxburghii* Sargent. These organics include thiamine-HCl, nicotinic acid and pyridoxine-HCl. Myo-inositol may not be essential but is added as a routine. Thiamine is an essential vitamin, whereas nicotinic acid and pyridoxine may enhance growth (Gamborg et al., 1976).

In the micropropagation of *Pinus roxburghii* the medium was solidified with Difco Bacto agar or gelrite. Although liquid media is advantageous in that it can be replaced or replenished and monitored easily in tissue culture (Hassig et al., 1987; Ingestad and Lund, 1986) a majority of regeneration protocols developed for various species have utilised solidified medium. However, for *Pinus caribaea* liquid medium was found to be superior to solidified medium (Skidmore et al., 1988).

During bud induction 3% sucrose was added to the induction medium. Although other carbon and energy sources such as fructose can be added to the media, sucrose is preferred by most cells (Gamborg et al., 1976). Sucrose at 3% was used in the regeneration of among others, yellow cedar (Kurz at al., 1989) and Engelmann spruce (Patel and Thorpe, 1986).

Although explants on day 0 produced higher average number of buds per cotyledon than older explants, the bud-forming-capacity was reduced by lower percentage response realised on day 0. Poor response of explants on day 0 may be due to injury during excision and the rigorous sterilisation procedure required for asepsis (Martinez-Pulido et al., 1990). The capacity to form large number of buds decreased with age of the explants. Day 1 cotyledons produced the highest bud forming capacity and were used in all subsequent experiments. Seedling developmental stage dramatically affected the number of buds formed for Douglas fir. The number of buds formed decreased with age in both BA-containing media and thidiazuron-containing media (Goldfarb et al., 1991). Decreased bud formation has been observed with increasing age of explant for several conifers including radiata pine (Aitken et al., 1981), *Picea abies* (Bornman, 1983) and *Pinus rigida* (Patel et al., 1986a).

Biochemical and anatomical changes are associated with age of explants and may account for the large response observed in the culture of those explants. For *Pinus radiata*, general features associated with state of differentiation such as immature stomatal complexes, thinner cell walls, absence of epicuticular waxes and unhydrolyzed lipid reserves in cotyledon cells were correlated with

explant morphogenesis (Aitken-Christie et al., 1985). Older cotyledons of *Pinus radiata*, had reduced metabolite levels which reduced the morphogenic potential (Biondi and Thorpe, 1982).

Cotyledons cultured in absence of cytokinin did not produce buds but elongated. Exogenous cytokinins are required for bud induction and the time of exposure to the phytohormone and its concentration varies (Thorpe and Patel, 1984). Both the time of exposure and the BA concentration affected the bud-forming capacity of cotyledons of *Pinus roxburghii* Sargent. At high BA concentration many buds formed but they did not elongate normally, whereas at very low concentrations only a few buds formed. For short periods of exposure to cytokinin, very few buds formed whereas longer periods produced many stunted buds. It appears that conditions which stimulate maximum bud production also inhibit continued growth of the buds (David, 1982). Although, high concentration BA (44μM) was found effective for the micropropagation of *Larix decidua* (Diner et al., 1986), for *Pinus strobus* the time of exposure and the cytokinin concentration affected bud induction. High cytokinin concentration promoted the largest number of shoots but these shoots showed less and slower development than those induced with lower concentration of cytokinins. Time of exposure of the explants to cytokinin was found to be critical, and longer periods of exposure produced more shoots than lower concentrations but the shoots were smaller (Perez-Bermudez and Sommer, 1987).

During bud induction both the number of buds and time of plantlet regeneration are important. It is therefore, necessary to produce the optimum number of buds in the shortest time possible.

N^6-Benzyladenine (10^{-5} M) for two weeks was found to be optimal in terms of number of buds produced and bud elongation for *Pinus roxburghii* Sargent. Cytokinins at 10^{-5} M and an exposure for two weeks was also found to be effective for western larch (Harry et al., 1991).

N^6-Benzyladenine was found to be more effective than other cytokinins for the bud induction of *Pinus roxburghii* Sargent. N^6-Benzyladenine was more effective for the induction of buds of *Pseudotsuga menziesii* (Cheng, 1977) and *Pinus contorta* and *Picea sitchensis* (Webb and Street, 1977). Although relatively more buds were produced on BA-containing medium (mean 13.9±0.8, BFC 8.4) than Z-containing medium (mean 7.7±1.0 BFC 4.1) the buds developed faster in the latter. BA in combination with other cytokinins at equimolar concentration produced fewer buds than BA added singly. However BA in combination with other cytokinin was more effective than BA alone for black, and white spruce (Rumary and Thorpe, 1984).

Since buds were found to elongate faster on Z-containing medium than in BA-containing medium an experiment was done to test whether buds would elongate faster when cultured on BA and Z for one week each. Shoot elongation index of 15.8 was obtained when cotyledons were cultured on BA and Z one week each compared to shoot elongation index of 12 when cotyledons remained in BA for 14 days. Histologically, periclinal and anticlinal cell divisions culminating in the formation of buds was observed two days from culture initiation and meristemoids were observed eight days from

culture initiation, therefore, Z may have been acting on these meristemoids stimulating their rapid development.

(5.3) BUD DEVELOPMENT AND SHOOT FORMATION

Although an average of ten buds per cotyledon were produced in BA-containing medium, only a few buds formed excisable shoots. It was therefore, necessary to increase the number of excisable shoots per explant.

Unlike *Pinus radiata,* when explants of *Pinus roxburghii* Sargent were divided into small pieces during bud development and placed on media, necrosis was observed; therefore, medium which allowed faster elongation was necessary. Half-strength SH was found to be superior to 1/2 MCM for bud development. MCM formulation differs from SH formulation both qualitatively and quantitatively. Unlike SH, MCM formulation has urea. SH has higher levels of potassium chloride, magnesium sulphate, boric acid and manganese sulphate than MCM. Clearly, there are differences between the two formulations, but it is not known what made SH superior to MCM for the development of buds of *Pinus roxburghii* Sargent. For western larch the medium for bud induction was also found to be inferior to the medium for bud development (Harry et al. 1991).

Shoots longer than 1.5 cm were excised from initial explants 13-15 weeks from culture initiation and were cultured individually. When these shoots were left to elongate on the initial explants they grew slowly and it was necessary to excise and culture them individually. The explants' efficiency for nutrient uptake decreased with age of cultured tissues and histologically, four weeks from

culture initiation the cytoplasm of non meristematic cells of the explant was reduced to the peripheral layer. The multitude of developing shoots required a bigger surface area for nutrient uptake than the surface area provided by the explant. Shoots attached on the original explant were found to have different orientations, therefore it was necessary to expose the shoots to direct light by maintaining them in an upright position.

Shoots excised from the initial explant had long needles and very minute stems. The shoots were elongated in order to increase the size of the turgid stem. Shoots with a turgid stem are known to produce a higher rooting response than smaller shoots. Also bigger shoots contained more axillary buds which could be induced to develop and hence enhance micropropagation. Tall shoots are expected to produce big plantlets which are more likely to survive the *in vivo* environment. For Douglas fir tall plantlets (41-60 mm) had a higher survival rate (53%) than smaller shoots (21-30) which had a survival rate of 20% (Mohammed and Vidaver, 1990).

Both the media and the gelling agent had an effect on the growth of shoots of *Pinus roxburghii* Sargent. Half-strength SH solidified with Difco Bacto agar was the best treatment in previous experiments and was therefore, used as the control treatment during shoot elongation. Shoots elongated on 1/2 SH solidified in Difco Bacto agar elongated slowly and had many brown leaves whereas shoots elongated on 1/2 SH solidified with gelrite remained green. Half-strength MCM solidified with gelrite was the best medium for the elongation of excised shoots of *Pinus roxburghii* Sargent. The nutritional requirements for the elongation of excised shoots of

Pinus roxburghii Sargent. was found to be different from those of shoots attached on the original explants. For *Pinus canariensis,* both media and gelling agent had an effect on shoot elongation and the requirements for elongation of shoots attached to original explants were different from those of excised shoots (Martinez Pulido et al., 1990).

The shoots of *Pinus roxburghii* Sargent. required three to four subcultures of 3-4 weeks in 1/2 MCM in order to obtain rootable shoots. The shoots obtained after 28 weeks were also big enough for shoot multiplication.

(5.4) SHOOT MULTIPLICATION

Shoot multiplication was essential for the realisation of the potential for large-scale clonal propagation. For *Thuja occidentalis* only two to four adventitious buds were obtained on the initial explant, however, up to 100 axillary buds could be obtained in one year and the potential for large-scale clonal propagation was realised (Harry et al., 1987). Although many researchers report on plantlet regeneration without evidence of shoot multiplication some reports indicated that shoot multiplication was feasible for *Pinus radiata* (Aitken-Christie and Thorpe, 1984) and *Pinus canariensis* (Martinez-Pulido et al., 1991). For *Pinus roxburghii,* shoot multiplication was achieved when shoots were long enough to contain many axillary buds.

After twenty eight weeks from culture initiation the shoots were tall enough for both rooting and shoot multiplication. In preliminary studies, shoot multiplication could not be achieved by

incubating shoots in BA-containing medium, however shoots were obtained by decapitation of 28-week-old shoots. The point of decapitation was found to be critical for shoot multiplication. Axillary buds were not obtained when shoots were decapitated in the region containing young upright leaves, therefore the young leaves and the apical bud were sources of axillary bud inhibition. Decapitation of the shoots too far from the apex resulted in shoot death, probably due to elimination of the photosynthetically active leaves. Although the lower leaves remained green they were less photosynthetically active. Micropropagation process can be enhanced by decapitation of the shoots after at least 28 weeks of culture.

Although very little histological research has been done to document the ontogeny of axillary buds (Thorpe et al., 1991), research in this area may not only elucidate the ontogeny of axillary buds, but may also assist in the isolation of sources of recalcitrance of the shoots to shoot multiplication. Although shoot multiplication was achieved for *Pinus roxburghii*, research on correlation of shoot height and axillary buds per shoot may be necessary in order to maximise the number of buds per explant.

(5.5) ROOTING

Rooting of conifers is usually a big problem, and the rooting conditions are highly empirical. In order to achieve rooting within a short time only methods popular with other conifers were screened. These methods included long period of incubation in gelrite-

solidified medium containing 5.37×10^{-7} M NAA (Jain et al., 1988), long period of incubation in soilless mixtures containing NAA (Mohammed and Vidaver, 1990), incubation in medium containing high levels of IBA (Martinez Pulido et al., 1991) and incubation of shoots in very high levels of IBA for a few hours.

Plantlets were not obtained by incubation of shoots in soilless mixtures containing low concentrations of auxins. Incubation of shoots for short period in gelrite-solidified medium was also not effective in rooting. When shoots of *Pinus roxburghii* Sargent. were rooted by pulsing in 10^{-3} M IBA for 2 to 6 hours only two plantlets were obtained out of an attempt to root 30 shoots. This method of rooting did not produce a reasonable rooting and only single roots were produced. However, when shoots were incubated in 5.37×10^{-7} M NAA a higher percentage rooting was obtained in four weeks. The plantlets also had multiple roots. Plantlets with multiple roots have been found to survive the *in vivo* environment better than plantlets with one or a few roots (Mohammed and Vidaver, 1990). The plantlet were expected to survive the *in vivo* environment because the roots of plantlets were directly connected to the shoot without any disruption.

An attempt was made to test the effect of reduced light on rooting. In preliminary experiments higher rooting (50%) was obtained in medium containing a dye (i.e., indian ink) compared with rooting in direct light (12.5%). Although indian ink created darkness in the medium other effects of indian ink could not be ruled out. Another attempt was made to root shoots in reduced light by placing sterilised aluminium foils trimmed to size on sterile medium and

covering the bottom of glass jars with aluminium foil. Under reduced light 50% rooting was obtained compared to direct light 67%. However, the plantlets obtained in reduced light had a slightly higher average number of roots per plantlet than those rooted in direct light. In another rooting trial run concurrently 70% rooting was obtained in reduced light. Under optimal conditions (see the summary of the protocol) 81% rooting was obtained. When entire plantlets of *Pinus strobus* were rooted in darkness 12.5% rooting was obtained compared to 35% rooting in direct light (Chesick et al. 1991). When entire shoots are rooted in dark, a poor response would be expected because the photosynthetic apparatus is affected. When shoots were rooted in reduced light most of the leaves were exposed to direct light and the photosynthetic apparatus was not affected.

Although, plantlets produced in reduced light had more roots than those produced in direct light more research needs to be done to confirm these findings. Success in rooting of conifers ranges from as high as 80% for western larch (Harry et al., 1991) to as low as 2% for red pine (Noh et al., 1988), and therefore, the rooting response for *Pinus roxburghii* was considerably high.

(5.6) ACCLIMATISATION

Plantlets need to be hardened before transfer to the greenhouse because the greenhouse and field have substantially lower relative humidity, higher light levels and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions (Preece and Sutter, 1991). Techniques that are most satisfactory for acclimatisation address the change from a relatively less

stressful environment to a stressful one. These techniques include, maintenance of high humidity for the first few days, low temperatures, reduction of light intensity, suitable pH, application of antitranspirants and control of disease-causing fungi and bacteria. A temperature gradient from high at the bottom to low at the top was found to be effective for the acclimatisation of some species (Dunstan and Turner, 1984).

There are many anatomical, morphological, and physiological differences between *in vitro* grown plants and *in vivo* grown plants. Epicuticular wax on cauliflower and cabbage leaves grown *in vitro* was only 25% of that of plants grown in the greenhouse (Sutter and Langhans, 1982). Differences have also been found in the chemical composition of greenhouse-grown plants and those grown *in vitro* (Sutter, 1984). Plantlets of raspberry grown *in vitro* were more slender and had considerably less collenchyma and sclerenchyma than those grown in the field (Donnelly et al., 1985). Uptake of CO_2 was found to be lower in cultured raspberry plants compared with field grown plants (Donnelly and Vidaver, 1984). These differences between *in vitro* grown plants and those grown *in vivo* may account for the high mortality when acclimatisation was not properly done.

After rooting, plantlets were too small for hardening, and therefore a lag phase between rooting and hardening was necessary. Hardening was essential for the survival of the plantlets *ex vitro.* After rooting the plantlets were put in peat:vermiculite 1:1 (v/v) moistened with 1/4 MCM medium devoid of sucrose. The rooting substrate was only moistened with media once to prevent ion toxicity due to mineral salt accumulation in the pots. Plantlets have

roots for the absorption of nutrients and shoots for photosynthesis and therefore an energy source was not necessary. From preliminary experiments the plantlets browned when sucrose was included in the medium. High sucrose is known to reduce the photoautotrophic capacity of the leaves (Wetzstein and Sommer, 1982)

After 28 days the plantlets were transferred into 1/2 litre pots containing the rooting substrate which was moistened with 1/4 MCM salts devoid of all organics. The organics were omitted to reduce fungal growth under the non-sterile conditions of the greenhouse. The plantlets were left in the pots for three weeks. The root system was expected to increase and hence to ensure rapid water absorption during hardening.

During hardening in the growth cabinet the plastic bags were removed for a period of 1 to 8 hours. The plantlets were covered when they showed signs of wilting. For the first few days of hardening the plantlets showed signs of wilting after a short time and the period in which the plastic bags were removed was increased gradually for three weeks. After covering the pots, the humidity in the bags increased and droplets of water could be seen on the inside of the bags and the roots absorbed water from the pots. In the following morning before the pots were uncovered the plants did not show symptoms of stress and recovered from wilting.

Water was lost by evapotranspiration from the pots during hardening and was replenished at least once a week. From previous observation *Pinus roxburghii* Sargent plantlets grew better under low moisture regime, therefore only 25 ml of sterile distilled water was added to the rooting substrate. During the hardening period the

plantlets remained green and increased in height. The growth cabinet was maintained at 24 C:18 C (day:night) with a photoperiod of 18h:6h (day:night). High temperature during the day enhanced transpiration whereas low temperatures at night lowered the evaporative demand and facilitated rehydration.

(5.7) GREENHOUSE PERFORMANCE

Morphological features such as multiple roots, upright needles and shoot height were associated with the survival of Douglas fir plantlets in the greenhouse (Mohammed and Vidaver, 1990). Plantlets with more than 10 roots per plantlet had 52% survival compared those with plantlets with 1-3 roots which had 25% survival under high stress environment. Plantlets taller than 40 mm had 53% survival compared with plantlets less than 30 mm which had 20% survival. Plantlets with upright needles also had a survival rate of 42% compared with plantlets with drooping needles which had 22% survival. On transfer to the greenhouse the plantlets of *Pinus roxburghii* were about 53 mm and had green leaves.

The tissue-cultured plantlets were transferred to the greenhouse where large-scale management was instituted. The greenhouse environment was also comparable to the field conditions; and the plantlets were found to grow faster in the greenhouse than in the growth chambers.

In the greenhouse the plantlets of *Pinus roxburghii* Sargent had upright and horizontal leaves which increased the photosynthetic efficiency of the plantlets. The upright leaves also protected the apical meristem. Branching was also necessary for increasing the

biomass of trees and therefore, tissue-cultured plantlets can be used for soil conservation. The increase in height was primarily due to the activities of the primary meristem, the shoot apical meristem whereas the increase in girth was due to the activities of the secondary meristem, the cambium. Both orthotropic growth and increase in girth are essential for the production of lumber in the forest industry, therefore, tissue-cultured plants can supply useful lumber.

The plantlets of *Pinus roxburghii* had a survival rate of 85%. None of the variation associated with organogenesis was observed among the tissue cultured plantlets growing in the greenhouse (Swartz, 1991). A high survival rate was necessary in order to reduce the overhead costs of tissue-cultured plantlets (Standaert-de Metsenaere, 1991). The overhead costs of commercial regeneration are high and can only be reduced by high efficacy of the regeneration process.

(5.8) DEVELOPMENTAL ANATOMY

Structural investigation give insight into the behaviour of cultured explants and, of the plantlets after transfer to the greenhouse. The structural investigations may be used in making tissue-culture related decisions.

The initial cotyledonary explants of *Pinus roxburghii* Sargent were a fairly homogenous population of cells except for the vascular initials. The cells of the epidermal and subepidermal layer had thinner cell walls than the vascular initials and were less differentiated. Unlike the epicotyl explants of black and white

spruce which were highly differentiated and contained various types of cells, tissues and organs (Rumary et al., 1986), the cotyledons of *Pinus roxburghii* Sargent consisted of a few types of cells. Unlike the initial explants of radiata pine which had mitotically active cells (Yeung et al., 1981) the initial explants of *Pinus roxburghii* Sargent consisted of quiescent cells. The cells of the initial explants were juvenile and had large prominent nuclei, small vacuoles, and densely staining cytoplasm.

Only BA-treated explants formed shoots and in the absence of BA the cells of the cotyledons underwent the process of cell maturation which is usually observed in the process of development, therefore, an external pertubation was necessary to alter the developmental pathway of the cells of the explants. The role of exogenous phytohormones in regulation of organ formation *in vitro* has been recognised for over thirty three years (Skoog and Miller, 1957). In gymnosperms, cytokinins have been found to be necessary for shoot formation (Biondi and Thorpe, 1982; Rumary et al., 1986; Thorpe and Patel, 1986; Villalobos et al., 1984b).

In the absence of BA, cotyledons of radiata pine (Yeung et al., 1981) and the cotyledons of *Pinus roxburghii* Sargent elongated, due to increase in cell size during cell maturation. Cytokinins are known to prevent cell elongation and induce shoot formation (Villalobos et al., 1984b). The metabolism in shoot-forming cotyledons was different from the metabolism of the initial explants and non-shoot forming cotyledons therefore, the cytokinins caused a shift in metabolism (Villalobos et al., 1984b). The increase in size of BA-treated explants up to day 21, was due to increase in size of

organised structures underneath the epidermis (Villalobos et al., 1985). Swelling of BA-treated explants with time in culture is a common phenomenon (Thorpe and Patel, 1986). For radiata pine the first asymmetrical periclinal cell division were observed after 24 hours from culture initiation (Yeung et al., 1981), however, for *Pinus roxburghii* Sargent both periclinal and anticlinal cell divisions were observed in BA-treated explants after two days of culture.

For radiata pine asymmetrical cell division was subsequently followed by two anticlinal cell divisions leading to a four cell structure which subsequently formed a six to eight cell structure (Villalobos et al., 1985). These studies used epon whereas in this study paraffin method of embedding was used and therefore such structures, if present, could not be observed.

Eight days from culture initiation meristemoids were observed a few layers below the epidermis. Shoot formation has been associated with meristemoid production in conifers (Cheah and Cheng, 1978, Coleman and Thorpe, 1977). Meristemoids have also been termed meristematic bud centres (Mott, 1981) and meristematic tissue (Reilly and Brown, 1976). The meristemoids of *Pinus roxburghii* Sargent. were close to the epidermis and were derived from subepidermal cells of the cotyledons. Although cells of the meristemoids were densely staining and had many mitotic figures, the neighbouring cells were non-meristematic and therefore, the meristemoids were derived from a few cells of the subepidermal layer. The epidermal layer was meristematic in the region adjacent to the meristemoids and was incorporated into the developing shoot primordia. For radiata pine the epidermal layer

was incorporated into developing shoot primordia and became part of the epidermis (Yeung et al., 1981).

Tracheids developed in the vicinity of the apical meristem and below it towards the cotyledonary vascular system. Some tracheids developed from non-meristematic cells of the explant. Such connections were observed in black and white spruce and presumably allowed for increased transfer of water and nutrients to the rapidly growing shoot (Rumary et al., 1986).

The apical meristem observed 30 days from culture initiation appeared well developed and was similar to the apical meristem of the plantlets, therefore the apical meristems developed very early in culture. Those apical meristems had a typical cytohistological zonation commonly observed in conifers (Pillai et al., 1980; Mauseth, 1987).

The cells of the non-shoot-forming cotyledons at day 8 were similar to the non-meristematic cells of the shoot-forming cotyledons and had undergone cell maturation. In both non-shoot-forming and shoot-forming cotyledons the tracheids were found to continue their development, therefore BA was not required for differentiation of the vascular initials.

Some callus observed during root induction was not part of the root-shoot junction, and the vascular tissue of the root was directly connected with that of the stem with no apparent disruption in the plantlet. Since the vascular tissue of the root and the shoots were similar it was difficult to delineate the region that connected the shoot and the root. The cells of the root cortex were also similar to the cells of shoot cortex. The root-shoot junction of *Pinus*

roxburghii Sargent was similar to those described for *Thuja occidentalis* (Bender et al., 1987) and for *Pinus eldarica* Medw. (Wagley et al., 1987). Photosynthate from the green leaves was translocated into the root through the solid connection between the root and shoot and, conversely, minerals absorbed through the root were transported to the shoot through the connection. The adventitious roots had an active apical meristem which produced cells in the proximal and distal regions. Some cells were visible distal to the apical meristem, presumably these formed the root cap. In a cross section of the root, protoxylem poles were scattered among the pith cells. These juvenile characteristics of the root were indicative of a functional organ.

The plantlets had green leaves that were connected to the roots through the leaf trace visible in a cross section of the stem. In a cross section of the stem secondary growth could be seen and the cambium appeared active. The apical meristem was active and had many mitotic figures. The vascular system lay a few cells below the apex.

Those features of the plantlet, including a solid root-shoot junction, juvenile roots, green leaves and an active apical meristems were necessary for survival of the plantlet in the *in vivo* environment. Lack of a solid root-shoot junction is a major cause of death of tissue-cultured plantlets upon transplanting. (David, 1982).

(6.0) CONCLUSION

Using the protocol described in this thesis (see the summary of the protocol), plantlets of *Pinus roxburghii* Sargent can be obtained after 32 weeks and grown in the greenhouse after 41-42 weeks. The shoots were rooted after only one month of root induction and about 81% rooting was obtained. The plantlets had a solid root-shoot connection which was necessary for the survival of the plantlets in the *in vivo* environment. Hardening was easily carried out so that on transfer to the greenhouse, a high survival rate of about 85% was obtained.

Shoot multiplication was demonstrated by decapitation of the shoots after 28 weeks of culture and up to four axillary shoots were obtained after one month of culture of decapitated shoots. Shoot multiplication is necessary in order to realise the potential for large-scale clonal propagation.

Cytokinins were necessary for shoot formation and cellular activities culminating in shoot formation were observed after two days of culture induction. Those cellular activities lead to the formation of meristemoids and subsequently bud primordia and finally adventitious shoots with an apical meristem and needle primordia. Inspite of the differences observed in the sites and timing of shoot development, the patterns of shoot development were similar to those described for other conifers.

The tissue-cultured plantlets of *Pinus roxburghii* Sargent had characteristics similar to those of seed-derived plantlets. Although, the lab-scale protocol described is useful in itself, the

protocol needs to be scaled-up to make it feasible for large-scale clonal propagation.

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