MICROPROPAGATION AND *in vitro* STUDIES OF *Pinus patula* Scheide *et* Deppe

by

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ABSTRACT

For the South African forestry industry, the patula pine (Pinus patula) is the most commercially important softwood species. A pine clonal programme has yet to be fully implemented in this country and at present much effort is being made to establish clonal plantings of selected trees. In order to accomplish this, it is essential that satisfactory commercially viable propagation technologies be developed for this species. This study examined the possibilities and constraints of three different in vitro systems for mass propagation of rare and important P. patula material.

Seed germination and sterilisation techniques were developed for adventitious bud and somatic embryogenesis experimentation. Adventitious buds were initiated from excised mature P. patula embryos cultured on LM medium containing 5 mg l^{-1} BA. Although, between 50 and 60% of the embryo explants produced adventitious buds, only 3-5 buds per explant actually developed further to form distinct shoots. The adventitious shoots elongated slowly (±8 mm in 2 months) on LM medium, containing 10 g l^{-1} activated charcoal. Axillary buds were induced on 10 week-old juvenile shoots, after the development of an effective surface sterilisation procedure, using 0.02% $HgCl_2$. The effect of removing the apex and trimming the needles on bud induction was significant. Dwarf shoots elongated at a rate of 25 mm in 5 weeks. Rooting studies conducted on juvenile P. patula shoots indicated that the most effective treatment was wounding the shoot base and placing the shoot

in composted bark growing medium, under a greenhouse mist regime. Rooting percentages were low (50%). Included in this study is the first successful production of somatic pro-embryos from mature Pinus patula embryos. Calli were produced on LM induction medium containing 2 mg l^{-1} 2,4-D. Cultures were first placed in the dark for 4 weeks and then transferred to a 16 h photoperiod for a further 2 weeks, after which Stage 1 embryogenic cells were observed. When calli were placed on LM maturation medium, containing 12 mg l^{-1} ABA, for a further 6-8 weeks, pro-embryo structures (maximum of 7 pro-embryos per callus) were detected embedded in the callus mass.

Hence, investigations into the development of protocols for the micropropagation of Pinus patula, were undertaken. Two major constraints for applying in vitro techniques to the commercial production of pine were identified: the poor yield of shoots and pro-embryos and the length of time taken for plantlets to be produced. This study, however, provides some fundamental knowledge and background work required by tree breeders who wish to implement biotechnological techniques in the selection and improvement of P. patula genotypes.

Plate 1 A stand of ten year-old *Pinus patula* in the Natal midlands.

I will set pines in the wasteland,
the fir and the cypress together,
so that people may see and know,
may consider and understand,
that the hand of the Lord has done this.
- Isaiah 41: 19-20

DECLARATION

I, David Stuart McKellar, declare that this thesis has not been submitted for a degree in any other university and is the result of my own investigations except where the work of others is acknowledged.

Signed .. ..

D. S. McKellar

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LIST OF ABBREVIATIONS

ABA	abscisic acid
BA	benzyladenine
BAP	benzylamino purine
°C	degrees Celsius
cm	centimetre
DCR	Gupta and Durzan (1985) nutrient formulation
2,4-D	2,4-dichlorophenoxyacetic acid
FAP	furfurylamino purine
g	gram
GA_3	gibberellic acid
GD	Mehra-Palta and Smeltzer (1978) nutrients
h	hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IEDCs	induced embryogenically determined cells
2iP	isopentenylamino purine
l	litre
LM	Litvay et al. (1981) nutrient formulation
LP	Aitken-Christie (1984) nutrient formulation
MCM	Bornman (1981) nutrient formulation
mg	milligram
mm	millimetre
MS	Murashige and Skoog (1962) nutrient formulation
NAA	∝-naphthaleneacetic acid
PGR	plant growth regulator
RO	reverse osmosis
s	second
SH	Schenk and Hildebrandt (1972) nutrient formulation
TIBA	tri-iodobenzoic acid

INTRODUCTION

It has been predicted by the forestry industry that the world demand for forest products will rise sharply over the next few decades (Thorpe and Biondi, 1984). This forecast can be confirmed easily if one considers the ever-increasing value given to wood by the pulp, paper, timber, and furniture industries, as well as the ecological need of reforestation (Burley, 1989). There is an urgent requirement for large numbers of improved, fast-growing trees with shortened rotation (Bonga, 1977). Present-day tree improvement and propagation methods offer only limited possibilities in achieving this goal. Historically, investment in forestry-related research has not been considered profitable. This was true as long as wood was a plentiful and cheap end-product. Wild stands of forest trees, however, are no longer able to supply the future needs of humanity for forest products (Thorpe and Biondi, 1984).

Today, trees of poor or heterogenous quality and slow or unreliable propagation methods are no longer acceptable (Thorpe and Biondi, 1984; Dunstan, 1988). There is an urgent need to make a broad and concerted effort in domesticating and breeding trees with superior wood quality, optimal stem form and uniformity, rapid growth rates, short rotations and a high production index (stem: total tree biomass), resistance to diseases and pests, the ability to adapt to new climates and extreme environmental variables, the ability to respond well to silvicultural practices (for example, fertilisation) and, ultimately, as

artificial fertilisers become increasingly uneconomical, the ability to fix nitrogen directly (Thorpe and Biondi, 1984; Nel, 1985). Forest tree improvement employs the basic concept of selecting individuals that would perform better under new environmental conditions. Outstanding phenotypes are identified in the best populations of uniformly aged and managed plantations; their genotypic values are estimated through some form of progeny testing and the best individuals are selected as the parents of the next generation (Burley, 1989). With each generation of trees, new gene combinations are being obtained that will produce improved trees (van Wyk, 1983). Since even small increases in yield due to tree improvement result in considerable increases in the commercial value of planted forests, these programmes are a good investment (Carlisle and Teich, 1971).

To date, breeding in forestry has lagged behind breeding in agriculture. Long life cycles, usually fifteen to twenty years from seed to flowering, are the main cause for this lag, since it is difficult to breed enough successive generations to develop both uniformity and superiority (Thorpe and Biondi, 1984; Cheliak and Rogers, 1990). For many species the main impact of time to sexual maturity is likely on the propagation phase and not on the improvement phase. For example, a second generation _Pinus taeda_ selection made in an eight year old progeny test in 1984, and grafted into an orchard in 1985 or 1986 would begin meaningful production of seed only in about 1995. With vegetative propagation, seed production would not be necessary in the production phase of the programme (Thorpe

and Biondi, 1984). Conifers have been introduced to a number of countries as exotic species, and many trials and selections have been carried out to find the particular species most suitable to the requirements of each country. For example, *Picea sitchensis* and *Pinus contorta* are grown in the United Kingdom and *Pinus radiata* is grown in New Zealand (John, 1983).

The introduction to South Africa of exotic forest tree species, with a much faster growth rate than indigenous species, brought a major advantage to the South African timber industry. Several exotic softwood species are being grown in South Africa, the most commercially important being patula pine. *Pinus patula* Schiede *et* Deppe was introduced to South Africa from Mexico in 1907 (Poynton, 1977). The species patula belongs to the Genus Pinus, Family Pinaceae, Order Coniferales, Class Gymnospermae which is part of the Division Spermatophyta (Foster and Gifford, 1959). Other genera found in the Abietineae (Fir tribe) are *Picea*, *Abies*, *Pseudotsuga*, *Cedrus* and *Larix* (John, 1983).

Pinus patula thrives best on cool, moist sites in the summer rainfall area, the largest concentration of stands being located in the eastern and south-eastern Transvaal mist-belt (Figure 1). *P. patula*, also known locally as the Mexican Weeping pine, is grown mainly for sawlog production, although it is also planted on an extensive scale as a pulpwood crop (Poynton, 1977). By 1953, patula pine was used as pulpwood in South African paper mills. The pulp obtained from patula pine is suitable either alone, or

in mixture with other species, for making most grades of paper (Wormald, 1975). Scott (1953) reported that the patula pine wood was used in South Africa for ceilings, internal joinery work and shelving. Dense P. patula timber is considered suitable for flooring (de Villiers and Perry, 1973).

Vegetative propagation can aid forest tree improvement programmes since trees with desirable characteristics can be multiplied to establish large stocks of genetically identical material with consistently high performance characteristics. Traditionally the vegetative propagation of pine has been by the rooting of stem cuttings and needle fascicles, but this technique has its problems (Reilly and Washer, 1977). For the majority of conifers, propagation by rooted cuttings is often characterized by a rapid loss of rooting capacity of the ramet (cutting) with increasing age of the ortet (parent plant)(Thorpe and Biondi, 1984). Research, investigating the commercial viability of propagating P. patula from cuttings (Osborne, 1992), is presently being undertaken in South Africa (Danks, pers. comm.)[1].

Currently, improved seed for commercial planting stock is produced by grafting scions from select trees into seed orchards (McKeand, 1981). However, incompatibility between root stock and scion may occur with time, resulting in the death of the graft (Reilly and Washer, 1977). Inherent

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[1] R. Danks, Mondi Forests, E. Tvl. (Personal communication).

Figure 1.1 Forestry in South Africa today.
Shaded areas show all the forestry plantation areas in the country. P. patula plantations are found mainly in the mist belts along the mountain ranges where there is an annual rainfall of about 900 mm upwards (Adapted from Anon., 1980).

factors - either chemical or anatomical - are the causes of incompatibility between the stock and scion, that is, graft failure or delay or partial inhibition of union formation (Nienstaedt et al., 1958). Experience in Africa has been that the most satisfactory technique for the vegetative propagation of P. patula is tip cleft grafting (Wormald, 1975). However, though a good proportion of grafts take, incompatibility between scion and stock has caused problems. The fact that the scion is incompatible with the stock may not become apparent for four or five years, and the incidence of incompatibility varies very much from clone to clone; some clones can be so bad that propagation by grafting is nearly impossible (Wormald, 1975).

Plant tissue culture techniques have been successfully applied to vegetative propagation of agricultural and horticultural crops (Boxus et al., 1977; Kochba and Spiegel-Roy, 1977; Krul and Myerson, 1980; Welander and Huntrieser, 1981), and they offer novel solutions to limitations imposed by classical methods of vegetative propagation (Mehra-Palta and Smeltzer, 1978). In vitro propagation offers an additional or alternative way of propagating selected pine genotypes. The main advantage of using cell culture as a tool in breeding programs and mass production is its potential for enormous (potentially unlimited) multiplication rates (Thorpe and Biondi, 1984). The financial benefits from realised genetic gains, eight to ten years earlier than with conventional seed orchards, would be enormous. Current seed orchard designs exploit only additive genetic effects while tissue culture propagation should ideally utilize all (additive and non-

additive) genetic effects. Tissue culture propagation could increase genetic gains by a third to a half but may even double genetic gains for some traits such as volume growth and disease resistance (Amerson et al., 1984). Another economic benefit of micropropagation is the increased uniformity of vegetative propagules. Variation in form, size and wood properties of trees results in a materials-handling problem at both pulp and saw mills. Although environmental variation would still exist, the elimination of genetic variation among cloned trees may be expected to increase uniformity and reduce stand management problems for foresters (McKeand and Weir, 1984).

Explants for cloning of superior individuals could come from seeds, buds, leaves, cotyledons, stem sections, or cells (Cheliak and Rogers, 1990). In vitro culture of such explants can provide a method of multiplying-up genetically superior seed or seedlings for planting in tree breeding providence/progeny trials or in seed orchards. Seed from controlled-pollinated crosses between proven superior parents is available only in small amounts. Yet, trees grown from such seed will be more valuable because they have been selected for consistently better performance in volume, growth, stem form, and, in some cases, disease resistance (Aitken-Christie, 1984).

General aims of this study

Biotechnological techniques are playing an important role in increasing the rate at which progress can be made in tree breeding. Clones of regenerated plants which are genetically almost identical to the original donar plants

can be produced from cultures of organs, tissues or cells (Nel, 1985). Clonal propagation through tissue culture offers the possibility of evaluating selected genotypes before bulking and distribution into national breeding populations (Burley, 1989).

In view of the urgent need for large numbers of superior Pinus patula clones in South Africa, the aim of this investigation was to establish protocols for obtaining plants using in vitro techniques. In vitro propagation was considered to be the most practical method for the implementation of clonal propagation, particularly if the superior clonal material desired was in very short supply. In this study, emphasis was placed on being able to produce in vitro plants from mature seed collected from a selected P. patula orchard. Available quantities of such selected seed can be extremely low, for example, a few grams in some instances. Because the use of organogenesis for in vitro propagation is considered to be the area in which the most progress in conifer biotechnology research has been made to date (Dunstan, 1988), most of the efforts in this study focused on this in vitro propagation route. In this regard, this study enabled protocols for the in vitro induction of adventitious shoots from mature embryos to be established. Methods were also developed for the in vitro propagation via the axillary bud route. Finally, the possibility of plantlet regeneration via somatic embryogenesis was addressed as there is much potential here for the production of large numbers of plants.

CHAPTER 1

GENERAL ASPECTS OF *in vitro* CULTURE OF PLANT CELL, TISSUES AND ORGANS.

The term, plant tissue culture, has generated into an all encompassing, convenient term to describe all types of sterile plant culture procedures pertaining to the growth of plant protoplasts, cells, tissues, organs, embryos and plantlets. The term *in vitro* is used to describe the sterile, artificial culture environment. Plant regeneration through tissue culture can be accomplished using somatic embryogenesis and organogenesis (Winton and Huhtinen, 1976; Jansson and Bornman, 1980; David, 1982; Tulecke, 1987; Smith and Drew, 1990)(Figure 2). Somatic or asexual embryogenesis is the production of embryo-like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin (Tisserat and Murashige, 1977). Such embryos can further develop and germinate into plantlets through the events that correspond with the zygotic occurrences (Tisserat, 1987). Embryogenesis can be either a) direct, where somatic embryos originate from an explant without a callus phase or b) indirect, where somatic embryos originate after a proliferation of callus tissue (Sharp *et al.*, 1980; Bhojwani and Razdan, 1983; Wann, 1988). Somatic embryogenesis occurs after the explant is exposed to critical concentrations of exogenously supplied hormones during the initial culture phase (Cheliak and Rogers, 1990). Plant development through organogenesis is

the formation and outgrowth of shoots from callus or initiation and outgrowth of axillary buds generated from cultured tips, and their subsequent adventitious rooting (Sommer and Caldas, 1981; David, 1982; Tisserat, 1987; Thorpe and Hasnain, 1988). The shoot is a unipolar structure and is physically connected to the tissue of origin (Tisserat, 1987). As for somatic embryogenesis, the organogenetic route may be indirect or direct (Hicks, 1980).

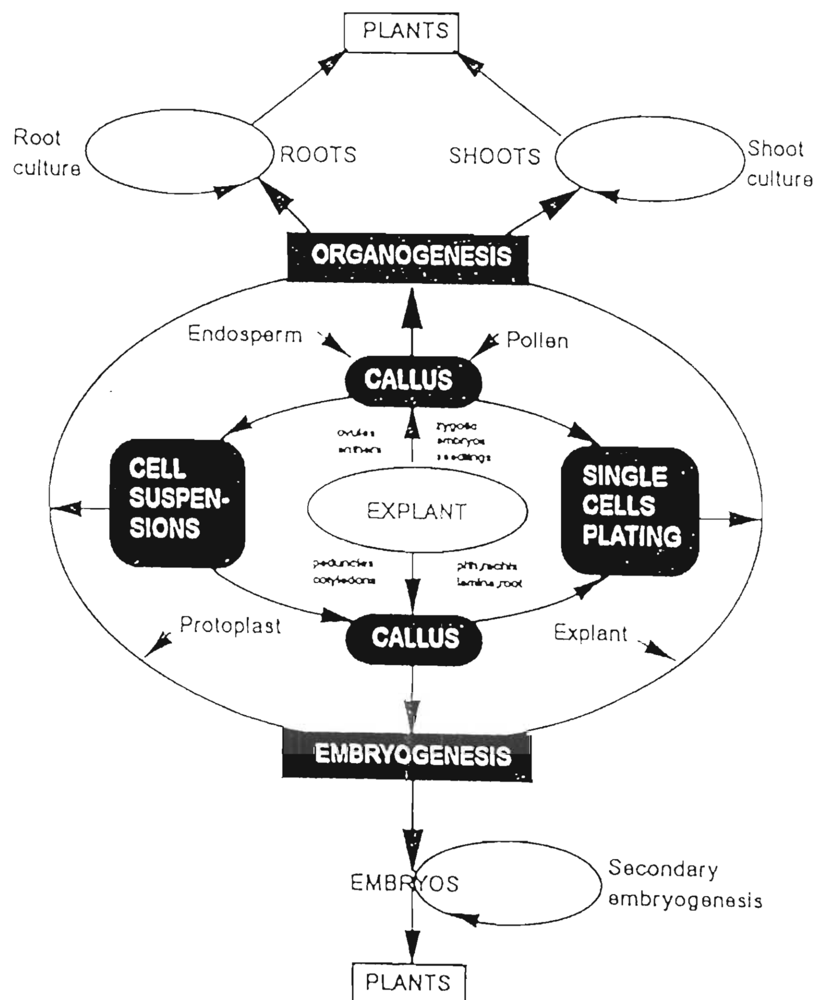

Figure 2 Cultural manipulations now possible through tissue culture techniques. (Adapted from Mantell <u>et al</u>., 1985 by Blakeway, 1992)

1.1 SELECTION OF EXPLANTS

The term explant is used to describe the initial piece of the plant introduced in vitro. Explant selection plays a paramount part in successful plant regeneration studies and for optimum success, explants must be obtained from healthy vigorous plants (Tisserat, 1987). Pre-culture plant conditions may greatly influence the subsequent growth of explants in culture (for example, rate and nature of chemical fertiliser treatment, season and environmental growth conditions)(Tisserat, 1987; Pierik, 1990).
Practically any part of the plant can be successfully cultured in vitro and can regenerate plantlets, provided the explant is obtained at the proper physiological stage of development. Immature tissues and organs are invariably more morphogenetically plastic in vitro than mature tissues and organs (Tisserat, 1987). Furthermore, meristematic tissues or organs should be selected in preference to other tissue sources because of their clonal properties, culture survival, growth rates and totipotentiality in vitro (Quak, 1977; Tisserat, 1987).

1.2 STRATEGIES FOR THE MAINTENANCE OF ASEPTIC CONDITIONS

Following explant selection, the sterilisation procedure becomes the primary consideration. Disinfection of tissues is necessary in order to eradicate surface microorganisms. Plant tissue culture media, which contain a high concentration of sucrose, support the growth of many micro-organisms. On reaching the medium these microbes generally

grow much faster than the cultured tissue and finally kill it. The contaminants may also give out metabolic wastes which are toxic to plant tissues. Fungal and bacterial explant contamination in plant cultures is usually detectable one to fourteen days after placing in culture. The incorporation of antibiotics into the medium or the dipping of explants in antibiotics has been advocated by some researchers (Katznelson and Sutton, 1951; Morgan and Goodman, 1955; Phillips et al., 1981; Pollock et al., 1983; Young et al., 1984; Cornu and Michel, 1987; Fisse et al., 1987; Falkiner, 1988; Ellis et al., 1989; Tsang et al., 1989).

According to Bhojwani and Razdan (1983) there are several possible sources of contamination of the culture medium: the explant, the medium itself and culture vessels, the environment of the transfer area, and the instruments used to handle the plant material.

1.2.1 Maintenance of transfer area and instruments

It is essential that all precautions are taken to prevent the entry into the culture vial of any contaminant when the vial is opened for subculture. To achieve this, all transfer operations are carried out under strict aseptic conditions under a laminar flow hood. An airflow of dust- and bacteria- free air, from high-efficiency particulate air (HEPA) filters, is maintained over the working area (Pelczar et al., 1986).

The instruments used for aseptic manipulations, such as forceps and scalpels, are normally sterilised by dipping in 95% ethanol followed by flaming and cooling. This is done at the start of the transfer work and several times during the operation (Bhojwani and Razdan, 1983). The combination of ethanol and flaming is essential as some instruments, especially those with sharp cutting edges, do not lend themselves well to sterilisation by heat alone, so "cold" sterilisation by means of a chemical solution is needed (Reddish, 1957). The ability to sterilise instruments by ethanol alone may be lessened because of the rough surfaces on some instruments which offer potential harbouring sites for microorganisms (Reddish, 1957). Some bacteria can develop in the alcohol used to flame the dissection instruments (Boxus and Terzi, 1988; Deberg and Vanderschaeghe, 1988). This source of contamination can be minimised or eliminated by regularly changing or eliminating the alcohol (Deberg, 1987). The latter is possible by replacing the Bunsen burner with an electric torch or with a heating device with glass pearls. The temperature reached with those devices is approximately 250°C (Deberg, 1987). Both Singha et al. (1987) and Kunneman and Faaij-Groenen (1988) observed the spread of a bacterial contaminant during subculture when ethanol (70%-96%) and flaming over an alcohol burner was used for instrument sterilisation. However, flaming over a bunsen burner for 12 seconds seems to provide effective sterilisation (Singha et al., 1987). Kunneman and Faaij-Groenen (1988) found that the addition of Burtan, Dettol and sodium hypochlorite to the ethanol reduced bacterial survival only partly.

1.2.2 Sterilisation of Growth Media

Moist heat in the form of saturated steam under pressure is the most dependable method known for the destruction of all forms of microbial life (Stanier et al., 1977; Pierik, 1989). Steam under pressure provides temperatures above those obtainable by boiling. In addition, it has the advantage of rapid heating, penetration, and moisture in abundance, which facilitates the coagulation of proteins (Pelczar et al., 1986). The autoclave is essential for the sterilisation of the nutrient media and containers (Pelczar et al., 1986). The medium is autoclaved at 1.06 kg cm^{-2} (121 °C) for 15-40 minutes (Bhojwani and Razdan, 1983; Pierik, 1989). The exposure time varies with the volume of liquid to be sterilised[1]. Certain compounds, such as antibiotics, zeatin, abscisic acid (ABA) and certain vitamins are thermolabile. These should not be autoclaved but rather be added to the cooled autoclaved medium (approximately 40 °C) through bacteria-proof filter membranes of pore size 0.45 μm or less (Bhojwani and Razdan, 1983).

1.2.3 Surface Sterilisation of the Explant

Surfaces of plant parts carry a wide range of microbial contaminants. To avoid this source of infection the tissue must be thoroughly surface sterilised before plating it on the nutrient medium; tissues with systemic fungal or bacterial infection are usually discarded in tissue culture

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[1] Sigma Chemical Company, St. Louis, U.S.A. - 1991 catalog pp. 1422.

studies (Bhojwani and Razdan, 1983). Not all contamination is necessarily of surface origin. Internal infection may occur either from organisms trapped within areas not reached by sterilising solutions or due to diseased or necrotic tissue (Sommer and Caldas, 1981). It is important to note that a surface sterilant is also toxic to the plant tissue. Therefore the concentration of the sterilising agent and the duration of treatment should be chosen to minimise tissue death. Table 1.1 gives an indication of the various sterilising agents that have been used to disinfect plant tissues. To increase tissue wettability a small amount (0.01% - 0.1%) of surfactant, such as Tween 20, Tween 80, Teepol, Mannoxol, or other types of laundry detergents can be added to the disinfecting solutions (Bhojwani and Razdan, 1983; Hartmann and Kester, 1983; Evans et al., 1986). Magnetic stirring, ultrasonic vibration, or a vacuum may be applied while soaking plant materials in disinfectants in order to reduce the possibility of trapping air bubbles on the surface of the plant materials (Evans et al., 1986). After surface sterilisation, the plant material must be rinsed three or four times in sterile distilled water to remove all traces of the sterilising agent (Bhojwani and Razdan, 1983).

Many researchers, working on conifer micropropagation, have used these chemical sterilants successfully. Mott and Amerson (1982) sterilised the female loblolly pine gametophyte in a sodium hypochlorite solution before removing the embryonic cotyledons. Other authors (Cheng, 1975; Bornman, 1983; Patel and Thorpe, 1984; Perez-Bermudez

Table 1.1 Common surface sterilants for treating explants prior to aseptic culture.

Sterilant	**Concentration (%)**	**Treatment Time (min)**	**Used Against**	**Reference**
Mercuric chloride	0.01 - 1.0	2 to 10	Bacteria & Fungi	Reddish, 1957. Hartmann & Kester, 1983.
Ethanol	50 -90	Rinse and allow to evaporate off.	Fungi	Reddish, 1957. Bhojwani & Razdan, 1983.
Hydrogen peroxide	1 - 10	5 to 15	Bacteria & Fungi	Pelczar *et al.*, 1986. Hartmann & Kester, 1983.
Hypo - chlorites	2-10	5 to 30	Bacteria & Fungi	Pelczar *et al*, 1986. Bhojwani & Razdan, 1983.

and Sommer, 1987; Ellis and Bilderback, 1989) sterilised the seed itself with sodium hypochlorite before aseptically excising the embryos. Von Arnold and Hakman (1988a) and Campbell and Durzan (1975) used calcium hypochlorite to sterilise their Picea abies and Picea glauca seeds, respectively, whereas Bornman (1983), Mudge (1986), and Martinez Pulido et al. (1990) used hydrogen peroxide (H_2O_2) in their seed sterilisation procedures. Von Arnold and Hakman (1988a) used 70% ethanol in addition to the calcium hypochlorite treatment.

1.2.4 The use of Antibiotics and Fungicides in Plant Culture

Internal infections, which can be a considerable problem, are caused by micro-organisms present inside the plant itself, and cannot be eliminated by external sterilisation (Pierik, 1989). Antibiotics have been considered for the control of bacterial contamination of plant tissue culture for many years (Katznelson and Sutton, 1951; Morgan and Goodman, 1955). However, according to some authors, the use of antibiotics, although often used in culture procedures, should not be relied upon to allow compromise in routine asepsis (Phillips et al., 1981; Falkiner, 1988).

Antibiotics are chemical compounds derived from microorganisms which have the capacity of inhibiting the growth of, and even destroying other microorganisms. Antibiotics can be classified on the basis of their mode of action, and examples of the most commonly used in plant

tissue culture are presented in Table 1.2.

Table 1.2 Mode of action of some well known antibiotics.
(Adapted from Falkiner, 1990)

Antibiotic	Mode of Action	Comments
Aminoglycosides Streptomycin Kanamycin Gentamicin Neomycin	Inhibit protein synthesis by interaction with 30S or 50S ribosomes	Bactericidal
Polymixins Rifampicin	Interferes with mRNA formation by binding to RNA polymerase	Resistance emerges readily
ß-Lactams Penicillin Ampicillin Novocillin Cefuroxime	Inhibit bacterial cell wall synthesis	Bactericidal
Chloramphenicol	Inhibit protein synthesis by acting on 50S ribosome	Bacteriostatic
Tetracyclines	Inhibit protein synthesis by acting on 30S ribosome	Bacteriostatic

Many antibiotics are bacteriostatic in low concentrations and bactericidal in higher concentrations (Pollock et al., 1983). To be effective in plant tissue sterilisation, the ideal antibiotic should be bactericidal in plant tissue culture medium, non-toxic to plant cells and have a broad spectrum of microbiological activity (Pollock et al., 1983; Pierik, 1989).

Antibiotics may be included in the growth medium but this often leads to a phytotoxic phenomena (Pierik, 1989). The high concentration of antibiotics necessary to control the bacteria may also inhibit the growth and development of the plant. For example, Tsang et al. (1989) found that the addition of antibiotics reduced the number of bud-forming embryos of Picea glauca. The immersion of the plant parts into antibiotic solutions results in excellent contact between the plant and the antibiotic and the exposure time, that is frequently critical, can be perfectly controlled. It has been shown that certain antibiotics are non-toxic and effective when used at specific concentrations (Young et al., 1984). Ellis et al. (1989) demonstrated that carbenicillin, ampicillin (500 and 750 $\mu g\ ml^{-1}$), and cefotaxime (250 and 500 $\mu g\ ml^{-1}$) cleared the in vitro environment of bacteria and had little effect on the elongation or bud formation from P. glauca embryos. In order to reduce the surface tension and thereby spread the antibiotic uniformly over the tissue surface, surfactants can be used with antibiotics (Goodman, 1959).

The wide range of microbial contaminants that can be detected in plant in vitro cultures was illustrated by

Leggatt et al. (1988) who isolated and characterised 31 microorganisms from 10 different plant cultures. Yeasts, Corynebacterium species and Pseudomonas species were the commonest isolates. Most isolates were able to utilise a wide variety of carbon sources and a number were resistant to antibiotics. Of the antibiotics tested, rifampicin, chloramphenicol and neomycin were the most inhibitory. Young et al. (1984) isolated bacteria from contaminated shoot cultures of 13 woody plant species. Many of these cultures contained more than one type of bacterial contaminant. No single antibiotic was effective against them all, but some antibiotics, notably tetracycline and rifampicin had a broader range of bactericidal activity than others. Katznelson and Sutton (1951) showed that aureomycin and terramycin were very effective against phytopathogenic bacteria. Streptomycin, neomycin and polymyxin were found also to be effective.

Although fungi are found repeatedly in plant tissue cultures, very few have been described in literature (Leifert and Waites, 1990). The ideal antifungal agent should be fungicidal in plant tissue culture medium, non toxic to plant cells and have a broad spectrum of fungicidal activity. Shields et al. (1984) examined various fungicides for their effectivity against yeast and other fungi. The best fungicides were the benzimidazoles MBC and FBZ (30 $\mu g\ ml^{-1}$) or imazalil (10-20 $\mu g\ ml^{-1}$) amongst the imidazoles.

Mites and thrips found in tissue cultures do not usually harm the plants directly but introduce other contaminants

such as fungi, yeasts and bacteria into sterile plant cultures (Blake, 1988; Leifert and Waites, 1990).

1.3 GROWTH MEDIA AND OTHER ENVIRONMENTAL CONDITIONS

Both direct and indirect morphogenesis is very dependent on the medium used for in vitro culture and on a correct balance of inorganic, organic and growth regulator constituents of the growth medium (George and Sherrington, 1984).

The inorganic nutrients of a plant cell culture are those required by the normal plant. The following are required in millimole quantities: nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), sulphur (S) and magnesium (Mg). As regards the nitrogen source, the cells may grow on nitrate alone but often there is a distinct beneficial and occasionally a requirement for ammonium or another source of reduced nitrogen. The essential nutrients required in micromolar concentrations include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo) (Bhojwani and Razdan, 1983). The mixture of macro- and micro-salts chosen is strongly dependent on the experimental plant (Pierik, 1989). The Murashige and Skoog (1962) medium is very popular, because most cultures react to it favourably. However, this nutrient solution is not necessarily always optimal for growth and development since the salt content is very high. To counteract salt sensitivity of some woody species, Lloyd and McCown (1981) developed the so-called WPM (woody plant medium). Commonly

used plant tissue culture media are those first formulated by Heller (1953), Murashige and Skoog (1962), White (1963), Nitsch (1969), Schenk and Hidebrandt (1972) and Gamborg et al. (1976).

The standard carbon source is sucrose or glucose (Bhojwani and Razdan, 1983; Pierik, 1989). It is essential to add an utilisable source of carbon to the culture medium as usually the plant cultures are autotrophic (Bhojwani and Razdan, 1983). Ball (1953, 1955) demonstrated that autoclaved sucrose was better than filter-sterilised sucrose for the growth of Sequoia callus. Autoclaving seems to bring about a small level of hydrolysis of sucrose into more efficiently utilisable sugars, such as fructose. Other carbohydrates which have been tested include lactose, maltose, galactose and starch, but these compounds are generally much inferior to sucrose or glucose as a carbon source (Gautheret, 1959; Gamborg and Shyluk, 1981). Normal plants synthesise the vitamins required for growth and development. However, when cells of higher plants are grown in culture, some vitamins may become limiting (Gamborg and Shyluk, 1981). Of these, thiamine (vitamin B_1) is an essential ingredient. Other vitamins, especially pyridoxine (vitamin B_8), nicotinic acid (vitamin B_3) and calcium pantothenate (vitamin B_5), and μ-inositol are also known to improve the growth of cultured plant materials (Gamborg and Shyluk, 1981; Bhojwani and Razdan, 1983).

The inclusion of organic nitrogen compounds is generally necessary only when a callus is being initiated (Gamborg and Shyluk, 1981). Common sources of organic nitrogen in

nutrient media include amino-acids, glutamine, asparagine and adenine (Straus, 1960; Risser and White, 1964).

Numerous complex nutritive mixtures of undefined composition, like casein hydrolysate, coconut milk, malt extract and yeast extract, have also been used to promote the growth of certain calli and organs (Bhojwani and Razdan, 1983). However, the use of these natural extracts should be avoided as different samples of these substances may affect the reproducibility of results because the quantity and quality of growth-promoting constituents in these extracts often vary with the age of the tissue and the variety of the donor organism (Bhojwani and Razdan, 1983).

Growth and organogenesis in vitro is highly dependent also on the interaction between naturally occurring endogenous growth substances and growth regulators of an analogous type, added to the medium. Growth regulators can bring about de novo morphogenesis on media containing suboptimal salt concentrations, and considerably enhance morphogenesis on an otherwise optimal medium (George and Sherrington, 1984).

In plant tissue cultures, the auxin growth regulators have been used to induce cell division and root differentiation (Abercrombie et al., 1978). The auxins commonly used in tissue culture are: indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and the naturally occurring indole-3-acetic acid (IAA). Cytokinins are incorporated mainly for cell

division and differentiation of adventitious shoots from callus and organs (Abercrombie et al., 1978). These compounds are used also for the release of axillary buds from apical dominance (Bhojwani and Razdan, 1983). More commonly used cytokinins are: 6-benzyladenine (BA), N-isopentenylamino purine (2iP), 6-furfurylamino purine (FAP) and zeatin which is a naturally occurring cytokinin (Bhojwani and Razdan, 1983). 6-benzyladenine (BA) is the nomenclature currently used for 6-benzylamino purine (BAP).

Gelling agents are used in tissue culture media to prevent the tissue from becoming submerged in the nutrient liquid medium and dying from lack of oxygen. The gel must be firm enough to support explants, but if it is too hard it may prevent adequate contact between the medium and the tissues, especially after some drying out (Pierik, 1987). Agar, a polysaccharide obtained from seaweeds, can be obtained in various forms (Bhojwani and Razdan, 1983). Gelrite is used extensively in preference to agar because its clarity helps the detection of microbial contamination (De Fossard and De Fossard, 1988). One striking gel-related problem is the occurrence of vitrification. Vitrification or hyperhydric transformation is the phenomenon of vitreousness or glassiness or waterlogging (Deberg, 1983). Raising the agar/Gelrite concentration avoids vitrification of cultured tissues but results in a drastically lowered propagation ratio (number of shoots and macroscopically visible buds at the end of the culture period divided by the number at commencement) (Brown et al.,1979; Deberg, 1983).

Plant tissue cultures grow differently depending on the environment to which they are subjected. The intensity, type and duration of light, temperature, oxygen/ carbon dioxide and other gas concentrations, and physical composition of the medium all play a role in the morphogenesis of the culture (Bhojwani and Razdan, 1983; Pierik, 1987; Deberg and Zimmerman, 1991).

Most plant tissue culturists will support the view that media need to be adapted according to the kind of culture or the species of plant that is to be studied (George et al., 1988b). The optimum culture conditions (including the formulation of the growth medium) strongly depends on the type of explant used and the particular in vitro route used to produce the end product. Explants for in vitro conifer culture have been derived from almost every tissue present during the life history of conifers: male and female gametophytic tissue; immature and mature embryos; hypocotyl, cotyledons and shoot apex of newly germinated seedlings; apical meristems; whole shoots; juvenile and mature needles; brachyblasts (needle fascicles) and various cambia in more mature shoots (John, 1983). There are incredible variations in protocols and media formulations for in vitro plant culture. A comprehensive review on all plant species that have been cultured through tissue culture, and their culture conditions, has been compiled by George et al. (1988a).

1.4 SUCCESSES IN CONIFER in vitro CULTURE

Achievements in regeneration of plants from gymnospermous tissues are similar to those from explants of woody angiosperms (Evans et al., 1981). In at least one example, Pseudotsuga menziesii regeneration has been accomplished from mature needles (Winton and Verhagen, 1977) and lateral branch shoot tips (Coleman and Thorpe, 1977). Regeneration is usually via organogenesis and over the past 15 to 20 years, procedures for the organogenic in vitro propagation of over 25 diverse species have been developed (Dunstan, 1988), including the well-known examples of Pinus radiata (Aitken-Christie et al., 1988) and Pseudotsuga menziesii (Cheng and Voqui, 1977). The possibility of clonally multiplying older trees and establishing field trials with in vitro derived plants has been demonstrated in species such as Sequoia sempervirens (Ball, 1987), Pinus pinaster (Franclet et al., 1980) and Pinus radiata (Horgan, 1987).

Substantial progress has been made also towards the development of systems for in vitro embryogenesis in conifers. Since the first report of somatic embryogenesis from zygotic embryos of Picea abies in 1985 (Hakman and Von Arnold, 1985), cultured explants of at least 18 different coniferous species have been induced to produce somatic embryos (Tautorus et al., 1991). Liquid medium has been used to propagate Pinus pinaster via axillary buds and Picea abies via somatic embryogenesis (Paques et al., 1992).

Other types of in vitro systems include protoplast and

haploid culture. Protoplast culture is used to incorporate potentially useful genes from one plant species to another by fusion of protoplasts and regeneration from the hybrid line (Smith and Drew, 1990). Protoplasts are cells from which the cell wall has been removed by mechanical and/or enzymatic methods. Because such cells lack a cell wall they are amenable to a number of techniques that are not possible with plant cell cultures (Carlson, 1973; Sheperd et al., 1983). Haploid culture is the culture of gametophytic tissue, that is the microspores (pollen) and the megagametophyte of conifers (Dunstan, 1988). This is of interest to tree breeders who want to use homozygous diploid plants in their study of inheritance. Reviews of conifer haploid culture have been made by Rohr (1987) and Bonga et al. (1988).

CHAPTER 2

MICROPROPAGATION OF *Pinus patula* VIA ADVENTITIOUS SHOOT PROLIFERATION

2.1 INTRODUCTION AND REVIEW OF THE LITERATURE

Organogenesis (morphogenesis) involves the creation of a new cellular form and organisation, where previously it was lacking. Certain *in vitro* cultural conditions can induce single cells or groups of cells to become centres of active cell division (meristems), each capable of producing an organ of one particular kind (Sommer *et al.*, 1975; Cheng, 1979; Mott, 1979; Aitken *et al.*, 1981; George and Sherrington, 1984). Such morphogenetic meristems can theoretically occur in either of two distinct ways: from the differentiated cells of a newly transferred piece of whole plant tissue, without the proliferation of undifferentiated tissue (direct organogenesis) and from the unspecialised and unorganised cells of callus tissues or suspension cultures (indirect organogenesis) (David, 1982; George and Sherrington, 1984; Von Arnold and Hakman, 1988b).

Under *in vitro* culture conditions, direct tissue/organ initiation begins with parenchyma cells that are located either in the epidermis (Tran *et al.*, 1974) or just below the surface of the organ used as the explant (Kato and Kawahara, 1972): some of these cells become meristematic and pockets of small, densely staining cells termed meristemoids develop. Indirect development of adventitious

shoots first involves the initiation of basal callus from excised shoot material in culture (Ross and Thorpe, 1973). Shoots arise from the periphery of the callus and initially they are not connected directly to the vascular tissue of the explant (Hartman and Kester, 1983). In both direct and indirect organogenesis, as cells come to comprise the new meristems, they adopt a different inherent programming which determines the subsequent pattern of development (George and Sherrington, 1984). Growth regulators, in the presence of the correct nutritional factors, can induce this programming (Von Arnold and Hakman, 1988b).

The process of plantlet formation via organogenesis requires four distinct stages (Thorpe and Patel, 1984): (a) initiation of shoot buds (directly or indirectly via a callus stage), (b) development and multiplication of these buds, (c) rooting of the shoots, and (d) hardening of the plantlets.

2.1.1 Choice of Explants for Adventitious Shoot Production

Until 1950, research in tree tissue culture had been insignificant; in vitro work with gymnosperms started seriously in the 1950s, probably because of promising results obtained by Ball (1950) with Sequoia sempervirens. Since then, much work has concentrated on obtaining cultures from various explants of Picea abies (Norway spruce), and this has formed the basis for the micropropagation of many other conifer species.

In _Picea abies_, successful micropropagation via adventitious shoot production has been achieved using isolated needles (Von Arnold and Eriksson, 1979), cotyledons (Bornman, 1983), as well as mature (Von Arnold, 1982b; Von Arnold and Eriksson, 1984 and 1985; van Staden _et al.,_ 1986; Von Arnold, 1987a and Von Arnold and Hakman, 1988b) and immature (Simola and Honkanen, 1983) embryos. Furthermore, _in vitro_ propagation has been also successfully undertaken from resting, vegetative buds of fifty to one hundred year-old clones (Von Arnold, 1982a and 1984) and needles of frost hardened (Jansson and Bornman, 1980) _P. abies_ plants.

Work on other _Picea_ species has also been reported. Rumary and Thorpe (1984) used epicotyl explants of black and white spruce. The explants were selected 27 to 29 days following germination. Campbell and Durzan (1975 and 1976) excised hypocotyl segments (5-7 mm long) from six to 12 day-old white spruce seedlings. Adventitious shoot production from white spruce seedling cotyledons was investigated by Toivonen and Kartha (1988). Lu _et al._ (1991) induced buds from mature embryo, cotyledon and hypocotyl explants of red spruce.

Another commercially important conifer, the Douglas-fir, has been also the focus of numerous _in vitro_ investigations. Douglas-fir cotyledons of two to four week-old seedlings derived from open pollinated seeds were used in adventitious bud studies by Hasegawa _et al._ (1979). Cheng (1975 and 1977) utilised fresh cotyledon explants obtained from two to eight week-old Douglas-fir seedlings,

whereas Kirby and Schalk (1982) cultured cotyledons obtained from four to five week greenhouse-grown seedlings of Douglas fir. Adventitious bud formation on Douglas fir cotyledons was also achieved by Cheah and Cheng (1978).

Of the pine species investigated most work has been done with Pinus radiata. Aitken et al. (1981) examined the influence of explant selection on the shoot-forming capacity of juvenile tissue of Pinus radiata. Three types of explant were used: whole embryos, cotyledons excised from whole embryos which had been cultured for one week on the shoot initiation medium, and cotyledons excised from one week-old aseptically germinated seeds. The average number of rootable shoots produced per seed in twelve to thirteen weeks was nine for excised embryos, 18 for their separated cotyledons, and 180 for the cotyledons from germinated seeds. Reilly and Washer (1977) and Aitken-Christie and Thorpe (1984) described a process whereby radiata pine was micropropagated from embryos of mature seed. Villalobos et al. (1984 and 1985), Brown and Sommer (1977), Aitken-Christie (1984) and Aitken-Christie and Jones (1987) used cotyledonary explants of P. radiata as an explant source. Working with this same species, Horgan and Aitken (1981) produced plantlets from embryos and seedling shoot tips. Table 2.1 lists other pine species that have been researched for adventitious bud production.

Table 2.1 Summary of published reports on successful adventitious bud initiation and subsequent shoot multiplication in *Pinus*. Induction media: MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrant, 1972), GD (Gresshoff and Doy, 1973), D (Campbell and Durzan, 1975), RW (Reilly and Washer, 1977), MCM (Bornman, 1981), LP (Aitken-Christie, 1984), C (Perez-Bermudez and Sommer, 1987), BLG (Saravitz *et al.*, 1991).

Species	Explant	Induction Media	Reference
P.carariensis	Cotyledons	MCM	Martinez Pulido *et al.*(1990)
P.contorta	Embryo parts	LP LP	Von Arnold & Erikkson (1981) Patel & Thorpe (1984)
P.coulteri	Embryo	D	Patel & Berlyn (1982)
P.echinata	Cotyledons Hypocotyl	MS	Kaul & Kochhar (1985)

(continued over the page)

Table 2.1 (continued)

Species	**Explant**	**Induction Media**	**Reference**
P.elliottii	Embryos	C	Perez-Bermudez & Sommer (1987)
	Cotyledons	GD	Brown & Sommer (1977)
P.mugo	Embryo Seedling explants	GD	Mudge (1986)
P.palustris	Embryo	GD	Sommer et al (1975)
P.pinaster	Cotyledons	RW	David et al. (1982)
P.ponderosa	Embryo	SH	Ellis & Bilderback (1984)
P.rigida	Cotyledons	GD	Brown & Sommer (1977)
P.sabiniana	Cotyledons	GD	Brown & Sommer (1977)

(continued over the page)

Table 2.1 (continued)

Species	**Explant**	**Induction Media**	**Reference**
P.strobus	Cotyledons Hypocotyl	MS	Kaul & Kochhar (1985)
	Embryo	MS	Minocha (1980)
P.taeda	Cotyledons	GD	Mott & Amerson (1982)
	Embryo parts	GD	Mehra-Palta (1978)
P.virginiana	Cotyledons	GD	Brown & Sommer (1977)
	Cotyledons	BLG	Saravitz et al. (1991)

2.1.2 Conifer Adventitious Bud Induction via Direct Organogenesis

Some interesting and informative work, on the phases and events that occur during the induction process for shoot buds, has been published. For example, Villalobos et al. (1985) undertook a scanning electron microscope study to gain insight into the developmental process leading to de novo shoot induction in radiata pine cotyledons. According to that study, shoot formation in radiata pine cotyledon explants can be divided into two developmental phases. One phase includes the events of induction and differentiation and the other involves development of the primordia (Villalobos et al., 1985). Cell division occurs at the periphery of the explant to yield typical meristematic cells which are small with large nuclei and dense cytoplasm (Sommer et al., 1975; Cheng, 1979; Mott, 1979; Aitken et al., 1981). Within three to six weeks in culture, the second phase starts with the appearance of increasingly definite "dome-like swellings" (Coleman and Thorpe, 1977), "meristemoids" (Yeung et al., 1981) or "tube-like protrusions" and "scale-like organs" (Von Arnold and Eriksson, 1979).

A number of factors affect the initiation of adventitious buds. These include the presence of growth regulators (Skoog and Miller, 1957; Von Arnold and Hakman, 1988b), explant positioning on the culture medium (Bornman and Vogelman, 1984), the composition and amounts of nutrients (Gamborg et al., 1976; Bornman, 1983; Perez-Bermudez and Sommer, 1987), the concentration of gelling agent used

(Bornman and Vogelman, 1984; Von Arnold and Eriksson, 1984) and light exposure (Amerson et al., 1984; Villalobos et al., 1984).

It is well established that cytokinins are essential for initiating bud formation. For example, Ellis and Bilderback (1989) found that in order to initiate buds, cotyledons of newly excised ponderosa pine embryos had to be exposed to the cytokinin, 6-benzylamino purine (BAP), for at least three days. Exposure to BAP for longer than seven days did not significantly increase the number of cotyledonary buds. Cotyledons elevated above the BAP-supplemented medium retained organogenic competence to form buds for up to fourteen days but did not form buds unless the cotyledons were placed in contact with the medium. When examining the effect of explant positioning, Bornman and Vogelmann (1984) found that when the primary explant of the fourteen day-old Picea abies seedling was positioned so that its cotyledons were flush with the BAP-incorporated gel the cotyledons, at ninety six hours, accumulated about one tenth of the ^{14}C-BAP of the hypocotyls as compared with the nearly equal distribution between cotyledons and hypocotyls in explants with their cotyledons erect. However, these cotyledons produced a nearly ten-fold greater number of bud-like adventitious structures. It was surmised that when cotyledons were in contact with the agar, ^{14}C-BAP was taken up only by certain "target" cells of the epidermis directly in contact with the medium.

Martinez-Pulido et al. (1990) found that the optimum level of BAP for Pinus carariensis bud induction and shoot

elongation was 2.3 mg l^{-1}, and the optimum period of culture in the presence of BAP was between two and three weeks. Longer exposures to BAP enhanced callus formation and led to the production of shoots that did not grow satisfactorily. Picea abies needles were cultured by Jansson and Bornman (1980) on media supplemented with BAP (0.23-2.3 mg l^{-1}) and 1-naphthylacetic acid (NAA) (9.3×10^{-4} - 9.3×10^{-2} mg l^{-1}). In that experiment adventitious bud formation was favoured by BA:NAA ratios of 100:1. In comparison to treatments lacking exogenous auxin, the response to NAA in the concentration range of 9.3×10^{-4} - 9.3×10^{-2} mg l^{-1} was twofold: pseudobulbils, frequently the precursors of shoots, differentiated acropetally along a part of the needle axis; and shoot regeneration itself occurred. Those researchers suggested that pseudobulbil and adventitious shoot bud induction might be triggered when endogenous and exogenously-applied levels of auxin and cytokinin together reach a certain critical concentration and ratio, the specific sites of meristemoidal activation being determined by the position in the needle axis where this balance is optimal. Cheng (1977) found that a substantial difference existed among the various phytohormones with respect to their stability, growth promotion and influence on Douglas fir organ formation. The presence of NAA and BAP in the culture medium was extremely effective in promoting adventitious bud formation. The observed lower frequency of morphogenic response, in that study, indicated that cytokinin, when used as the sole growth regulator in culture, was not sufficient to support the growth of cultured cells. In contrast, tri-iodobenzoic acid (TIBA), when used at 0.5-1.0 mg l^{-1}, induced the

formation of adventitious shoots at the tips of the white pine embryonic cotyledons (Minocha, 1980). According to that author, one possible explanation for the TIBA effect might have been that it caused the accumulation of an optimal level of endogenous auxin in the cotyledons by inhibiting its downward transport from the embryonic shoot apex. This may in turn have been responsible for the production of adventitious shoots from the cotyledons.

From these examples it can be stated that the most common method for adventitious bud formation is the culturing of the explants on a cytokinin-containing medium. However, various experiments have shown that the same effect can be achieved by treating explants with concentrated cytokinin solutions for short periods and subsequently culturing the material on media devoid of cytokinin (Bornman, 1983; Von Arnold and Eriksson, 1985). Von Arnold and Eriksson (1985) used half-strength SH basal medium for culturing *Picea abies* embryos. By comparing embryos cultured on basal medium supplied with BA (1.1 mg l^{-1}) for four weeks and embryos pulse-treated with BA (56.3 mg l^{-1}) for two hours, it was found that the pulse treatment was as effective after one month as was the conventional treatment after two months. Those researchers divided the process of adventitious bud formation after pulse treatment with BA into three stages. The first stage (approximately the first two weeks) included the formation of meristematic centres as reflected in the nodulated appearance of the embryos. During the second stage (approximately the third week), bud primordia developed from meristematic centres. The third stage (approximately the fourth to the eighth week)

included the formation of adventitious buds. Von Arnold and Hakman (1988b) induced Picea abies adventitious buds on medium containing cytokinin or pulse-treated with cytokinin and then cultured on medium lacking cytokinin. Those workers found that the final yield of adventitious buds was similar in both cases. However, the advantages of the pulse treatment technique were that bud differentiation was more synchronised, adventitious buds developed faster and the variation between different experiments was smaller. The number of embryos forming adventitious buds increased significantly when the embryos were treated with BAP for thirty minutes. Prolonged treatment of up to four hours had no significant effect on bud formation. The cytokinin that was released to the culture medium after the pulse treatment influenced embryo development. When the embryos were cultured on media containing released cytokinin for more than six days, elongation growth of cotyledons and roots was inhibited. In contrast, when the embryos were transferred, after the initial release of cytokinin (that is, after about 24 hours), to fresh medium lacking cytokinin, the cotyledons and roots elongated to various extents. The uptake of BA in explants of Picea abies and Pinus sylvestris was investigated by Vogelmann et al. (1984). According to that report, the coefficients of variation, that is, in adventitious bud induction between seedling explants grown in tissue culture, can be reduced when, instead of following the standard practice of incorporating BA in the agar medium, it is instead applied as a pulse in a liquid medium prior to culture on agar without plant growth regulators. When BA was included in agar, meristemoids usually originated in the subsidiary

cells of the stomal apparatus of cotyledons in surface contact with the agar, whereas after pulse treatments they often originate in subepidermal tissues as well. For bud induction, Bornman (1983) applied BA either as a short-duration (three hour), high concentration (28.1 mg l^{-1}) pulse or by vacuum infiltration and incubation in a BA-containing (1.1 mg l^{-1}) infusion medium. Pulse and infusion treatments resulted in the induction of greater numbers of adventitious buds over a three to four week shorter culture period than was the case with the conventional inclusion of growth regulators in the solid medium. Infusion of plant growth regulators appeared to be a more effective method, although more cumbersome, than pulse loading. Also, it was critical that the osmotic concentration of the infusion medium was not hypertonic to that of the tissue.

Besides being responsive to cytokinins, shoot initiation and subsequent development are both light-dependent events; neither cell divisions nor organizational activities occur in constant darkness (Amerson et al., 1984). A study by Villalobos et al. (1984) revealed that three days of exposure to light gave rise to some meristematic tissue, but longer exposure was needed for optimum shoot induction. The light period was not needed at the initiation of the culture, as was the requirement for cytokinin, but could be given at least after ten days in culture. After twenty one days in darkness transfer to light did not allow for any shoot formation. Adventitious bud production from Pinus contorta embryos was enhanced by a dark pretreatment prior to exposure to high light intensity. Embryos of Pinus sitchensis were less critical of the physical environment

of the culture (Webb and Street, 1977).

Another important factor affecting adventitious bud induction in conifer cultures is the media salt formulation. Adventitious bud induction appears to be influenced the most by the nitrogen concentration (NH_4 and/or NO_3) in the culture medium (Sommer et al., 1975; Konar and Singh, 1980; Chalupa, 1983). Perez-Bermudez and Sommer (1987) found that the high nitrogen content of the Murashige and Skoog (1962) (MS) formulation seemed to be deleterious for cultured adventitious buds of Pinus elliottii as the frequency of embryos forming shoots was inversely correlated with NH_4NO_3 concentration. In that study, significantly higher number of shoots were induced when ammonium was omitted. A study by David et al. (1982) concerning the morphogenic activity of Pinus pinaster cotyledons revealed a positive effect on adventitious budding when the NH_4^+/K^+ ratio was one. Experiments performed by Von Arnold and Eriksson (1981) with Pinus contorta embryos showed that optimal concentrations for all classes of nutrients, except for the sugars, were at about a quarter strength. Only the glycine concentration was found to be critical as regards to the stimulating effect of dilution of the amino acids. Other amino acids exerted only a small influence upon growth and were not critical.

Bornman (1983) screened four culture media for his Picea abies regeneration work. The media were those of Murashige and Skoog (MS) (1962), Schenk and Hildebrant (SH) (1972), Litvay et al. (LM) (1981) and Bornman (MCM) (1981). The

rationale was as follows: (1) MS and SH are popular media which, although developed specifically for rapid growth of angiospermous callus tissues, have also been applied in gymnospermous tissue culture. (2) LM, a medium developed at the International Institute of Paper Chemistry, Wisconsin, U.S.A., is intended specifically for cell culture studies. MCM medium was the product of intensive research on morphogenesis in Picea abies and Pinus sylvestris by that author. In that report (Bornman, 1983), the LM, MS and SH media favoured non-differentiated cell and tissue growth, whereas with MCM, morphogenesis was reproducibly better. Use of MCM not only resulted in the induction of larger numbers of adventitious shoot buds and roots, but also lower coefficients of variation. LM media proved to be less effective in morphogenesis. This could have been due to the low Ca^{2+} content (twenty times lower than in MS) since it is known that this element plays an important role in sustaining shoot apical meristematic activity (Bornman, 1983; George et al., 1988b). According to Bornman (1983), reasons for the better response to MCM appear to lie in the wider range of vitamins and other organic constituents, lower levels than normal of Mn^{2+} and I^- and, possibly, the addition of reduced nitrogen. In a similar investigation by Martinez-Pulido et al. (1990), of the four different basal media used (SH, MCM, QP and AE), SH and MCM produced the best response with respect to obtaining high yields of adventitious buds from embryo explants of Canary Island pine.

The type and concentration of the gelling agent used in the induction medium can also affect adventitious bud

development. The effect of gel medium rigidity on BA-induced adventitious bud formation and vitrification in Picea abies was investigated by Bornman and Vogelmann (1984). Significantly greater numbers of adventitious buds per explant were induced at low to medium levels of rigidity (2.5-10.0 g l^{-1} Tayio, 1-5 g l^{-1} Gelrite). However, this advantage was almost completely nullified at the lower levels (2.5 and 5.0 g l^{-1} Tayio and 1 and 1.5 g l^{-1} Gelrite) as a result of the high incidence of vitrification. Similarly, Von Arnold and Eriksson (1984) found that the development of adventitious shoots of Picea abies was affected by the agar concentration in the medium. Shoots grew faster and became more vitrified on lower agar concentrations. Very vitrified shoots on 5 g l^{-1} agar were dark green and the needles seemed to adhere to each other. Shoots on 20 g l^{-1} agar were light green and the needles had a dry appearance.

The protocols used for conifer adventitious bud induction have been quite varied. Examples of different strategies are discussed below. In an attempt to induce organogenesis in callus cultures of white pine, Kaul and Kochhar (1985) plated hypocotyl and cotyledon segments on MS media supplemented with 0.2 mg l^{-1} NAA and 2 mg l^{-1} BAP. Meristematic areas appeared in the callus two to four weeks after inoculation. Five to six weeks after inoculation 100% of the callus cultures became very dark green and had many protuberances on the surface, which probably resulted from meristematic activity just below the surface of the callus tissue. Kirby and Schalk (1982) cultured Douglas fir cotyledons on a modified MS medium containing BAP (1 mg l^{-1})

and NAA (9.3×10^{-3} mg l^{-1}). After one week in culture cells of the hypodermal region immediately below the epidermis began to elongate and divided, rupturing the epidermis. Apical domes of adventitiously produced primordia emerged from the ruptured epidermis after fourteen to twenty one days in culture. A modified MS medium containing 11.3 mg l^{-1} cytokinin was also used by Von Arnold (1982a) when culturing mature embryos and resting vegetative buds of mature Picea abies trees. Von Arnold (1982b) found that the addition of auxins to a cytokinin-containing medium had no significant effect on the induction of the adventitious Picea abies bud primordia.

Mott and Amerson (1982) plated Pinus taeda cotyledons on a modified GD medium containing BAP (10 mg l^{-1}) and NAA (0.01 mg l^{-1}). After approximately five weeks the swollen cotyledons were transferred to half-strength GD medium containing 10 g l^{-1} charcoal without growth regulators. After four weeks the explants were placed on half-strength GD without charcoal for an additional four weeks. Sommer et al. (1975) cultured longleaf pine embryos on modified GD medium supplemented with BA (5 mg l^{-1}) and NAA (2 mg l^{-1}). Adventitious buds were induced on excised loblolly pine cotyledons by Mehra-Palta and Smeltzer (1978) when plated on cytokinin-supplemented GD medium. There was an increase in bud-induction frequency with an increase in cytokinin/auxin ratio.

Pinus radiata embryos and cotyledons from germinated seeds were placed on a Quoirin and Lepoivre (LP) medium containing 5 mg l^{-1} BAP (Aitken-Christie and Thorpe, 1984).

The cotyledons of the excised embryos were cut from the embryos after one week and replaced on the same medium for greater surface area contact. After three weeks a nodular, yellow meristematic callus tissue formed on the side of the cotyledons in contact with the medium and gave rise to adventitious shoots (Aitken-Christie and Thorpe, 1984). Aitken-Christie (1984) and Aitken and Jones (1987) cultured radiata pine explants on modified LP containing 5 mg l^{-1} BAP. Patel and Thorpe (1984) cultured lodgepole pine explants on a modified LP medium containing BAP (2.25 mg l^{-1}). During the first few of days of culture in the presence of BAP, the embryos elongated slightly and the cotyledon and the hypocotyl became green. After about five days in culture, further elongation of the embryos stopped. After seven to ten days of culture the cotyledons and hypocotyl swelled and their upper surfaces acquired a nodular appearance. After 15 to 20 days the entire upper surface was covered with distinct shoot or needle primordia.

Pinus radiata embryos placed on SH medium containing BAP (at concentrations varying from 5 x 10^{-2} mg l^{-1} to 25 mg l^{-1}) became swollen and succulent after five days in culture (Reilly and Washer, 1977). Two to three weeks after embryos were placed on the agar, small buds and clusters of small primary leaves were visible on the cotyledons and on some hypocotyls. Cotyledons touching the medium became very swollen and often smooth-surfaced tissue proliferated from them. Some of this tissue gave rise to masses of tiny buds with clusters of small scale-like primary leaves. In contrast, cotyledons not in contact with the medium swelled

very little and only one or two individual buds at the tips of the cotyledons were formed. The initiation of adventitious shoots on P. radiata explants was carried out on a SH medium containing 30 g l^{-1} sucrose and supplemented with BAP (Aitken et al., 1981). For subsequent transfers, which were carried out every three or four weeks, the tissues were placed on the same nutrient medium but containing only 20 g l^{-1} sucrose. Rumary and Thorpe (1984) cultured black and white spruce epicotyl explants on SH media containing equimolar concentrations of BAP and 2iP. Initial shoots were formed between the cotyledons on the explant. Between days 40 and 60 in culture, much of the original explant, including the cotyledons, main axis, and epicotyl needles, died. However, the sub-apical region proliferated into a large tissue mass on which new primordia continuously arose.

Horgan and Aitken (1981) induced radiata pine buds on both an agar and a liquid SH medium containing 5 mg l^{-1} BAP. Campbell and Durzan (1976) cultured white spruce hypocotyl segments aseptically on a semi-solid medium containing 2.25 mg l^{-1} BAP. The explants produced many scale-like outgrowths and buds. Mudge (1986) found that Pinus mugo shoot development on intact embryos was greatest on a medium containing 10.0 mg l^{-1} BAP and 9.31 x 10^{-3} mg l^{-1} NAA.
Von Arnold (1987a) cultured Picea abies embryos on half-strength SH basal medium. The number of embryos forming adventitious buds was higher on media containing 10 g l^{-1} sucrose than on media containing 20 g l^{-1} or 30 g l^{-1} sucrose. The yield of adventitious buds decreased when the embryos were initially cultured on medium lacking sucrose.

2.1.3 Adventitious Shoot Elongation and Growth

As for shoot induction, elongation of the adventitious buds formed in vitro appear to be affected by a number of culture conditions. Various strategies that have been reported are: a) the transfer of the embryo explant to a hormone-free medium; b) the addition of activated charcoal; c) altering the concentration of the gelling agent; and d) cutting the explant into small pieces.

There appears to be a beneficial effect when growth regulators are removed from the culture media for a period of time subsequent to shoot induction. Aitken-Christie and Thorpe (1984) plated Pinus radiata cotyledons with meristematic tissue on a LP medium without BAP for four to six weeks to promote the elongation of adventitious shoots. During elongation it was necessary for clumps of shoots to be cut into smaller clumps so as to provide continuous growth. These researchers stated that three different adventitious shoots could be produced during the shoot elongation stage: "waxy" shoots, "wet" shoots, and translucent ("vitrified") shoots. Translucent shoots were discarded when they appeared because they did not survive hardening-off. The wet shoots often had needles which were stuck together and lacked epicuticular waxes, whereas the waxy shoots looked like shoots from a normal seedling. Both the waxy and wet shoots appeared to be normal and could be rooted, although the wet type was more prone to rot during the rooting step.

Mott and Amerson (1982) reported that by the end of the

initiation steps for Pinus taeda adventitious budding on half-strength GD medium, developing shoots had become crowded on the cotyledons and many of the shoots had reached the stage (2 to 5 mm leaf height) judged suitable for excision from the original cotyledon explant. These shoots and developing shoot clusters were placed individually on half-strength GD medium. The well developed shoots (2 to 5 mm) elongated steadily and exceeded 5 mm in height within four weeks. Similarly, Reilly and Washer (1977) found that when portions of the cotyledons bearing the bud masses and smooth meristematic swellings were dissected from the embryos and transferred to SH medium without cytokinin, the leaves elongated and after three weeks individual shoots could be recognised. Aitken-Christie and Thorpe (1984b) cultured radiata pine explants, after a BAP treatment, on a LP medium the same as the initiation medium but without BAP. Several transfers (two to six) were necessary to obtain fully elongated shoots of about 1.5 cm to 2.0 cm. Von Arnold and Eriksson (1981) found that once Pinus contorta adventitious buds were formed it was necessary to transfer the explants to basal medium either lacking cytokinin completely, or with a very reduced level (0.23 mg l^{-1} BAP), otherwise the buds became overgrown with callus. Development of adventitious buds was stimulated by using quarter-strength basal medium. Adventitious buds were isolated from embryo tissue when stem elongation had started, that is, after about two months on BAP-free medium.

Von Arnold (1982a) found that no differences were observed in the effectiveness of various cytokinins before the Picea

abies adventitious buds started to elongate into shoots. However, at the elongation stage there existed apparent differences. Kinetin gave the highest yield of adventitious shoots, while 2iP was the least effective cytokinin. Embryos cultured during the whole in vitro procedure on half-strength medium and under a 16 hour photoperiod gave the highest yield of adventitious shoots. Transfer of Picea glauca explants to a medium lacking cytokinin induced the development of the scale-like outgrowths into needles and the development of the buds into elongated shoots bearing normal needles (Campbell and Durzan, 1976). In experiments with whole embryos, no great differences in shoot number, size, and appearance were observed between the different nutrient media tested, except on SH with gibberellic acid (GA_3) where shoots turned yellow (Horgan and Aitken, 1981). The transfer of shoots from a SH medium to a GD medium was found to be an important pre-treatment which increased the survival of the shoots when they were placed in rooting medium.

The presence of activated charcoal in the medium has been shown to have a beneficial effect in many culture systems (Weatherhead et al., 1978). Charcoal adsorbs metabolites from the medium which might be inhibitory to morphogenesis (Fridborg et al., 1978). Mehra-Palta and Smeltzer (1978) reported that activated charcoal (0.5 g l^{-1}) enhanced loblolly pine shoot growth. Patel and Thorpe (1984) described a method where after three weeks of culture on BAP-containing medium, Pinus contorta embryos were transferred to a hormone-free medium (Patel and Thorpe, 1984). Bud growth was enhanced by diluting the basal medium

to quarter strength, sucrose to 20 g l^{-1} and addition of 5 g l^{-1} activated charcoal. After about three months in culture, the shoots were separated from the mother explants which further stimulated the process of elongation. Von Arnold and Eriksson (1981) reported that the growth of isolated shoots was stimulated both by a quarter dilution of the basal medium and by the addition of 5 g l^{-1} activated charcoal.

Von Arnold and Hakman (1988b) stated that the development of isolated adventitious shoots was affected by the agar concentration in the culture medium. Increasing the agar concentration from 5 g l^{-1} to 20 g l^{-1} decreased vitrification, but at the same time reduced shoot growth. The best results obtained were with 10 g l^{-1} agar. Vitrification problems were eliminated by transferring the shoots to the elongation medium solidified with Gelrite rather than Difco-Bacto agar or Phytagar.

Rumary and Thorpe (1984) made attempts to increase secondary shoot formation in black and white spruce explants. The shoot-forming clumps were cut into two or four longitudinal sections and placed on fresh medium with the cut surface in contact with the medium. Shoot proliferation occurred but the tissue turned brown at the cut surface, ultimately leading to the death of most of the shoots. Attempts to reduce the browning, which probably resulted from phenolic oxidation of the tissue, were made by using antioxidants. The final treatment selected after experimentation included 18 mg l^{-1} ascorbate in the medium for black spruce and 38 mg l^{-1} glycine for white spruce at

the time of sectioning the explants. These treatments reduced browning and death, and no callus was formed. Removal of elongated shoots enhanced the rate of elongation of the remaining shoots in a clump. Von Arnold (1982b) transferred Picea abies embryos, covered with adventitious bud primordia after four weeks on cytokinin containing media, to basal medium lacking growth regulators. When bud elongation had started the embryos were cut into smaller pieces in order to get a better supply of nutrients. After about five months, when the stem of adventitious shoots had reached a length of about 5 mm, the shoots were isolated and cultured individually .

Aitken-Christie and Jones (1987) developed a novel system for in vitro production whereby Pinus radiata shoot hedges were maintained in one vessel. Shoot clumps grown for one month on LP agar medium were replenished once a week for eighteen months with liquid LP nutrients. The liquid medium was poured onto the agar medium and four to six hours later nutrients were removed. Liquid-LP-nutrient replenishment of shoots twice weekly without transfers resulted in better shoot growth and health than monthly transfers to fresh agar medium. The production of shoots from hedges maintained in tissue culture by nutrient replenishment has several advantages over traditional methods. There is no need for regular (four to six weekly) transfers to fresh medium, large numbers of containers of fresh agar medium are not needed, and shoots or clumps do not have to be removed from their containers to be divided for further multiplication or for rooting.

2.1.4 Rooting of established shoots and Hardening-off of Regenerated Plantlets

Once the shoots have grown in vitro, they need to be rooted. The whole plantlet then requires a "hardening-off" stage which conditions them to the greenhouse environment. Rooting of conifers is often difficult (especially under in vitro conditions), mainly because it takes a long time for the roots to develop (Von Arnold and Hakman, 1988b). Various workers have employed a number of different strategies to produce a conifer plantlet that could survive in field conditions. Amerson et al. (1984) found that rooting occurred best in response to auxin pulses.
Continuous exposure to low level auxin for 42 days yielded only 17% rooting, whereas pulse exposure to the same 0.09 mg l^{-1} NAA concentration for 12 days yielded 50% rooting. Mott and Amerson (1982) stated that in the rooting of adventitious shoots the treatment which stimulates cell division at the basal end of the stem is antagonistic to subsequent root development. These researchers used two distinct substeps for the rooting process. The first involved the stimulation of cell division. This was achieved by making a fresh cut near the base of the existing stem and then placing the shoots on half-strength GD containing NAA (0.5 mg l^{-1}) and BAP (0.1 mg l^{-1}). When cell division progressed to a stage where the stem base was swollen, the epidermis was split and some callus could be seen extruding from the split epidermis (± twelve days), the shoot was transferred to half-strength GD lacking growth regulators. Shoots remained on this medium for approximately nine weeks or until roots were at least 3 mm

long. Plantlets at this stage could be transferred to soil.

Toivonen and Kartha (1988) induced rooting of _Picea glauca_ adventitious buds at four months, when shoots had elongated sufficiently to handle. The rooting medium consisted of half-strength GMD media containing 0.1 mg l^{-1} IBA. After four weeks on this root induction medium, the shoots were transferred to the same medium but without hormones. After transfer to soil, rooted shoots were acclimatised for four weeks under glass jars. After four weeks the glass jars were removed and the plantlets were exposed to ambient greenhouse conditions. For root initiation, Aitken-Christie (1984) gave her radiata pine shoots a five day auxin treatment in water agar, plus 1 mg l^{-1} IBA and 0.5 mg l^{-1} NAA. After this they were washed and planted in a non-sterile peat/perlite pumice (50% /25% /25%) mix. The trays of shoots were kept in a high humidity chamber, which was misted and aerated daily until roots were formed. Induced _Picea abies_ adventitious shoots were elongated with the aid of far-red light and rooted _in vivo_ after a long-duration (12 hour), high concentration (127 mg l^{-1}) application of IBA. IBA, when applied either as a pulse or by infusion, resulted in rooting one or two weeks earlier than when IBA (2 mg l^{-1}) was included in the medium. However, there was no difference in the mean number of rooted needles/seedling when comparing the conventional media-containing auxin response with those of the pulse and infusion treatments (Bornman, 1983). Von Arnold (1982b) treated _Picea abies_ shoots in vermiculite saturated with a sterile water solution containing 18 mg l^{-1} IAA and 20 mg l^{-1} IBA for 24 hours at 20 °C in darkness and afterwards washed in sterile

water. After the root-inducing treatment the shoots were cultured on half-strength MS basal medium at 20 °C under 16 hours light. Shoots, with 2-5 mm long roots, were rinsed under tap water to remove agar and then transferred to unsterile conditions in a greenhouse. Mehra-Palta and Smeltzer (1978) observed that the selection of adventitious shoots for rooting experiments was important. The percentage of adventitious shoots rooting on a medium containing low concentrations of BAP (0.01-0.1 mg l^{-1}) in addition to NAA (0.1 mg l^{-1}) was higher than if only auxin was used. Further growth of the roots was inhibited if maintained on cytokinin/auxin media. Rooted propagules were transferred, therefore, on to half-strength GD medium lacking growth factors, where growth of the roots continued. For rooting of adventitious shoots of Pinus carariensis, the best results were obtained by pulsing in 20 mg l^{-1} IBA, and the best substrate was found to be peat-vermiculite (1:1) moistened with quarter-strength MCM. Webb and Street (1977) found that Pinus sitchensis and Pinus contorta shoots should ideally root whilst in sterile culture. Shoots initiated on 2iP and kinetin had a higher frequency of spontaneous root initiation in culture than those produced on BAP-containing medium.

In another approach to rooting, small Pinus radiata shoots, which were dissected from embryos four to six weeks after first being initiated, formed only green friable callus at their bases when placed on SH medium containing 1 to 25 mg l^{-1} IBA (Reilly and Washer, 1977). When shoots were allowed to elongate on SH medium (without cytokinin) for three months and then placed on SH (1 to 25 mg l^{-1} IBA) little

callus formed, nor were roots initiated. However, some shoots which grew vigorously on SH (without cytokinin) for six months formed roots without the addition of auxin. Generally, when NAA was included in the medium the shoots became greener, but once roots were formed all the shoots greened considerably. After the roots had extended 10-40 mm into the medium, the plantlets were placed in test tubes containing non-sterile water which allowed further growth and elongation of the roots to occur. Two to three weeks later they were planted into soil in pots (Reilly and Washer, 1977).

Aitken-Christie and Thorpe (1984) placed single shoots (10-15 mm) in an 8 g l^{-1} Difco-Bacto water agar medium containing 1 mg l^{-1} IBA and 0.5 mg l^{-1} NAA for five days. The shoots were then planted in a non-sterile peat-pumice mix. In some of the P. radiata clones shoots formed three to six roots in four weeks, whereas in others, one or two roots were formed in eight weeks. Horgan and Aitken (1981) found that an IBA/NAA auxin treatment promoted the rooting of Pinus radiata shoots. Seven weeks after planting, 86% of the shoots, which were given an IBA/NAA treatment, had formed roots. Only 36% of the shoots placed directly in the potting mix had formed roots in the same time. Minocha (1980) suggested that the lack of rooting in the adventitious shoots of gymnosperms may be due to the presence of supra-optimal endogenous levels of plant hormones. This argument is supported by the common observation that externally supplied hormones are generally ineffective in inducing the formation of roots in these shoots and may even suppress the rooting response in many

cases (Cheng, 1975; Campbell and Durzan, 1975 and 1976; Reilly and Washer, 1977). Von Arnold and Hakman (1988b) found that the rooting ability of auxin-treated adventitious shoots (twenty four hours with 18 mg l^{-1} IAA and 20 mg l^{-1} IBA) varied depending on the agar concentration in the culture medium. About 50% of shoots raised on 5 g l^{-1} agar formed roots while only 10% did so on 10 g l^{-1} agar. The yield of rooted shoots increased when the photoperiod was shortened from 16 hours to eight hours one month before the auxin treatment. Von Arnold and Eriksson (1984) found that the rooting ability of Picea abies shoots varied depending on the agar concentration used. Increasing the agar concentration from 5 g l^{-1} to 20 g l^{-1} decreased vitrification, but at the same time reduced shoot growth and rooting potential. Two-month-old shoots were given a root-inducing treatment for 24 hours at 20 °C in the dark in sterile vermiculite saturated with a water solution containing 18 mg l^{-1} IAA and 20 mg l^{-1} IBA. After the root-inducing treatment the shoots were washed once in sterile water and transferred to the same culture conditions as before the treatment. After one or two months the roots were 2 to 5 mm long. The plantlets were then given various acclimatization treatments before they were transplanted to pots in the greenhouse.

Rooting studies on black and white spruce adventitious shoots were performed by Rumary and Thorpe (1984). Shoots, at least 10 mm in length, were dipped in a sterile 0.2 g l^{-1} solution of IBA. The best rooting response was obtained with fine vermiculite saturated with the medium, but the IBA dip caused much callus proliferation at the base of the

shoots. Lower IBA concentrations caused a reduction in callus formation without stimulating rooting. The use of a commercial rooting powder (containing 1 g l^{-1} IBA) significantly enhanced rooting. The bottom 1-2 mm of the selected shoots were dipped in sterile water and then into the rooting powder which was also sterilised. This treatment, coupled with the inclusion of charcoal (2 g l^{-1}), produced very high levels of rooting. Patel and Thorpe (1984) found that lodgepole pine shoots, which were given a 1 g l^{-1} IBA rooting powder treatment, became slightly swollen at the base of the stem. These shoots produced roots three to four weeks after they were washed and transferred to fresh agar medium. The inclusion of 1 g l^{-1} activated charcoal in the medium enhanced rooting significantly. Mudge (1986) had successful rooting of mugo pine shoots when he treated the adventitious shoots as microcuttings. A thin sliver of stem tissue was removed from the base and the cut base was dipped in 8 g l^{-1} IBA in talc. Shoot microcuttings were then placed upright in vermiculite moistened with distilled water in a covered transparent plastic box.

2.1.5 Aim of this Investigation

For the local South African requirements the unique advantage of organogenesis, via adventitious bud production, is the higher multiplication rate compared with propagation by cuttings. No specific research on _Pinus patula_ _in vitro_ propagation has been reported to date. This study, therefore, investigates the possibilities and

constraints that *P. patula* organogenesis could provide for eventual commercial production of patula pine plantlets. The important requirements for establishing an *in vitro* system are examined.

2.2 MATERIALS and METHODS

2.2.1 Seed Germination

The Pinus patula seeds (Seed Lot No. M4562) were obtained from the Mondi Forests seed orchards at Sabie, Eastern Transvaal, R.S.A. The seeds, collected from Orchard No. 3, was an "A mix" harvest. The seed-bearing trees in Orchard 3 are all half-sibling. These trees were produced from crosses between different P. patula families, with only one parent being definitely identified. After collection, the seed were stored in an air-tight plastic container at 4 °C (Stubsgaard, 1992). Before being placed in the seed germinator for pre-treatment, the seeds were surface sterilised in a 1% calcium hypochlorite solution (30 minutes, constant stirring) and then rinsed with tap water.

The seed germinator consisted of four 600 ml plastic beakers placed in a water-bath (Plate 2.1) at 25 °C. Each beaker contained 500 ml of a pre-treatment nutrient solution [KNO_3 (100 mg l^{-1}), $CaNO_3$ (10 mg l^{-1}), NaH_2PO_4 (5 mg l^{-1}) and H_3BO_3 (20 mg l^{-1})], pH 6.0. Each beaker was aerated using an airstone from an aquarium pump. Approximately two grams of seed were added to each container and the seed remained in the germinator for 72 hours. The seeds were then removed from the germinator and 50 seed lots were each plated in a 90 mm Petri dish lined with moist filter paper. To prevent drying-out of the seed, the Petri dishes were covered with lids. The seeds were then allowed to germinate on the filter paper, which was always kept moist with

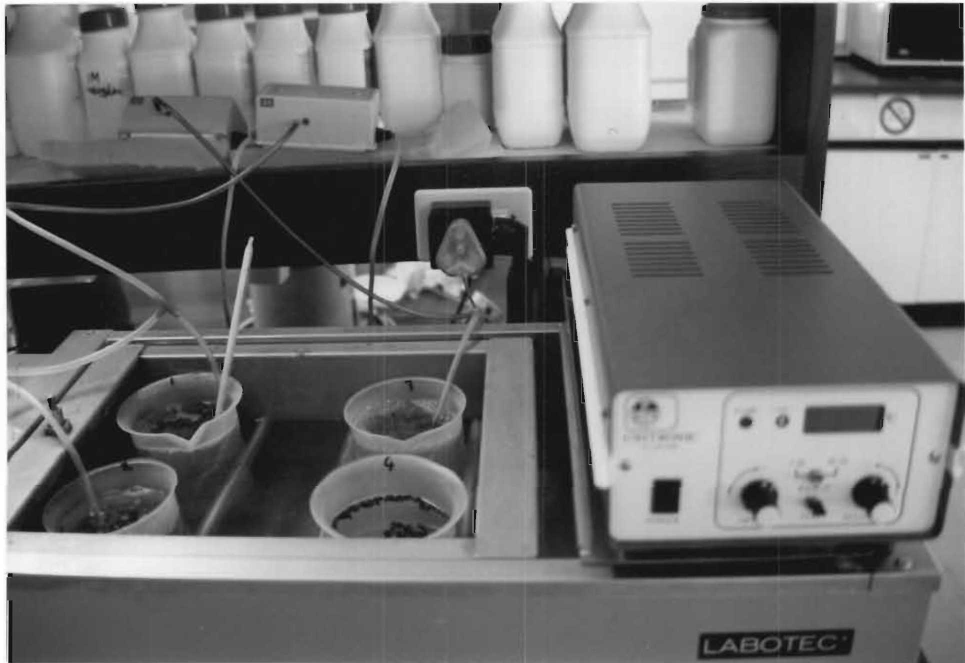

Plate 2.1 **The germinator used for the pre-treatment of the <u>P. patula</u> seed.**

Plate 2.2 **Germinating <u>P. patula</u> seed. a= 7-8 day-old germinated seed and b= 12-14 day-old germinated seed.**

reverse osmosis (RO) water, at 24 °C with a 16 hour photoperiod of 740 mols m^{-2} s^{-1}. The successful use of lighting in seed germination has been reported by Bartok (1992). The RO water was obtained by passing mains tap water, under mains pressure, through an Elgastat® membrane cartridge (Type SC30). The percentage of seed that germinated and the mean length of the radicle emerging from the seed were recorded after the seed had been plated for 10 days. A seed was considered to have germinated when at least 2 mm of the radicle had emerged from the seed coat (Plate 2.2). The "age" of the germinating seed, mentioned in this work, was calculated from the time the seed was placed in the germinator. For example, a eight day-old seed was one that had been placed in the germinator for three days and plated on moist filter paper for another five days.

2.2.2 Sterilisation of the Explant Material

All manipulations were performed aseptically, and all glassware and media were autoclaved at 1.06 kg cm^{-2} (121 °C) for 20 minutes. Sterilisation procedures for the seed coat and embryo explants were investigated. After a number of preliminary experiments (section 2.3.2), the protocol developed for the surface sterilisation of the seeds was a 10% H_2O_2 soak for 20-30 minutes followed by a thorough sterile reverse osmosis (RO) water rinse. The effectiveness of the surface sterilisation treatments tested was determined by aseptically plating the sterilised seed on a

nutrient agar medium. Seeds (25/Petri dish) were kept at 24 °C (±2 °C). The nutrient agar medium consisted of yeast extract (3 g l^{-1}), peptone (5 g l^{-1}), nutrient agar (Biolab Chemicals) (15 g l^{-1}), pH 7.0 (adapted from Seeley and Van Demark, 1981). The percentage of seeds having bacterial colonies growing on the nutrient medium surrounding each seed was recorded after 72 hours.

Once the seeds had been surface sterilised, the embryos were then excised from the seed while the whole seed was submerged in a 1.3% sodium hypochlorite (Domestos®:RO water 1:4) solution. This was done as a precautionary measure against endogenous and/or exogenous contamination. The removal of the embryo from the seed involved an incision at the micropylar end of the seed coat using sterile forceps and scalpel. Application of pressure at the opposite end with the forceps helped to force the embryo out through the incision opening. The excised embryos were then immediately rinsed in sterile RO water before being placed in the culture media. All these manipulations were performed under sterile conditions.

2.2.3 Culture Conditions for the Initiation and Elongation of Adventitious Buds

Media was dispensed (20 ml/culture vessel) and autoclaved in 100 ml glass jars sealed with clear polycarbonate lids

(Kirstenbosh Botanical Gardens, R.S.A.). Five embryos were placed in each culture vessel and these were kept in the culture room at a 16 hour photoperiod (740 mols m^{-2} s^{-1}). The culture room was maintained at a constant 24 °C (±2 °C). After approximately 10 weeks on the induction medium, the embryos were assessed for adventitious bud primordia formation. When the newly formed adventitious buds were ±3 mm tall, the whole embryo, containing the buds, was aseptically transferred to the shoot elongation medium.

The elongation medium comprised of half-strength LM salts. The LM formulation, used for adventitious bud initiation, was diluted for the elongation medium as several workers have reported on the beneficial effect of using half-strength salts (Campbell and Durzan, 1976; Von Arnold and Eriksson, 1981; Mott and Amerson, 1982). The other constituents, included in the elongation medium, were 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, 10 g l^{-1} activated charcoal, pH 5.8. The sucrose and activated charcoal were autoclaved separately to prevent sucrose hydrolysis (Druart and De Wulf, 1990). The adventitious bud-bearing explants were maintained, on this medium, under the light regime described for bud initiation. New shoots, formed from the adventitious buds, elongated under these conditions. Only the rate of shoot elongation was investigated in the shoot elongation study.

2.2.4 Data Analysis

For the adventitious bud studies, each experiment was repeated at least three times. The number of explants used for each treatment was between 30 and 40. Results were assessed using Duncan's Multiple Range test (SAS, 1982). This statistical procedure assigns alphabetical values to the means obtained for each treatment, and means can be recognised as being significantly different if they do not share the same letter. Only one variable at a time was analysed using this multiple range test. Standard deviations from the mean are recorded alongside the alphabetical values assigned for significant differences. For all analysis the level of significance was set at 0.05.

2.3 RESULTS and DISCUSSION

2.3.1 The effect of Seed Pre-treatment on Embryo Germination

For successful P. patula adventitious bud initiation it is important that the embryo explant used is in a healthy condition. Embryo development is dependent on germination. Germination is the process of reactivation of the metabolic machinery of the seed and the emergence of the radicle (root) and plumule (shoot), leading to the production of a seedling. Physiologically, germination begins with the initial stages of biochemical reactivation and ends with the emergence of the radicle (Jann and Amen, 1977). For both the adventitious bud initiation and somatic embryogenesis work, the seed embryo needed to have distinguishable radicle, hypocotyl and cotyledon components. Ungerminated (small, white and undeveloped) embryos were not suitable at all for culture initiation. Hence a seed pre-treatment method was devised to increase the number of germinated embryos acceptable for use in the adventitious bud initiation and somatic embryogenesis work.

The initiation of germination requires that three conditions must be fulfilled (Ching, 1972; Jann and Amen, 1977). First, the seed must be viable; that is, the embryo must be alive and capable of germination. Second, the seed must be non-dormant and the embryo quiescent. There must be no dormancy-inducing physiological, physical, or chemical barrier to germination. Quiescence describes the condition in which the seed can germinate immediately when water is

available and in the absence of any internal germination barriers (Brown, 1972).

A pre-incubation of Pinus caribaea seeds in half-strength Schenk and Hildebrandt (SH) salts (no hormones) has been shown to accelerate bud formation and also increase the number of buds formed subsequently on a medium containing BA (Webb and Santiago, 1983). Certain compounds have been known to stimulate seed germination but their role is not clear. For example, the use of potassium nitrate has been an important seed treatment in seed-testing laboratories for many years without a good explanation for its action (Hartman and Kester, 1983). In other techniques, the seeds are soaked in water or treated with organic or inorganic chemicals at various concentrations and time intervals (Gottfried and Heidmann, 1992). It has been suggested that chemical treatments could break seed dormancy as opposed to being stimulants of germination (Baldwin, 1942).

A simple nutrient solution was formulated to see if similar results could be obtained with the patula pine seed. The choice of the nutrient salts and their concentrations was based roughly on the ratios used in the Murashige and Skoog (1962) formulation. Only the nitrate, phosphate, potassium, calcium and boron elements were considered for the seed pre-treatment nutrient solution as these are the some of the most important elements for plant development (Whitcomb, 1988). Hoff (1987) reported that chemicals must penetrate both the inner layer of the seed coat, which is the major site of seed dormancy in some pines, and the hard outer-seed coat. The imbibing period, in the nutrient

solution, is thus very important. P. patula seed was imbibed in this pre-treatment solution to observe their germination response (Figure 2.1). Imbibition is a physical process whereby water is absorbed by the dry seed and the moisture content increases rapidly at first and then levels off (Bewley and Black, 1978). Water is necessary for the hydrolysis of reserve foods and for initiating cell division in the rest of the embryo (Baldwin, 1942). Two variables were recorded: a) the germination percentage and b) the mean length of the radicle tissue emerging from the micropylar end of the seed. The germination percentage expresses the viability of the seed, which indicates the number of seedlings produced by a given number of seeds. In this work, an embryo was considered to be germinated once at least 2 mm of the radicle had emerged from the seed coat. The results shown in Figure 2.1 indicate that it is necessary for the seeds to be pre-germinated in the pre-treatment solution for at least 72 hours before being plated on moist filter paper for continued growth. The percentage germination between the 72 hour and the 240 hour treatments was not significantly different. However, the lengths of the emerging radicles were inhibited by the 240 hour treatment. The reason for this could be that root development was not so critical, at this stage, for the embryos were germinating in the presence of excess nutrients. There was, therefore, little need for a sudden increase in root mass while the seeds were subjected to "luxury" nutrient availability. As the lighting regime, during the 240 h duration, was the natural day and night period, this factor would not have been responsible for inhibiting root elongation.

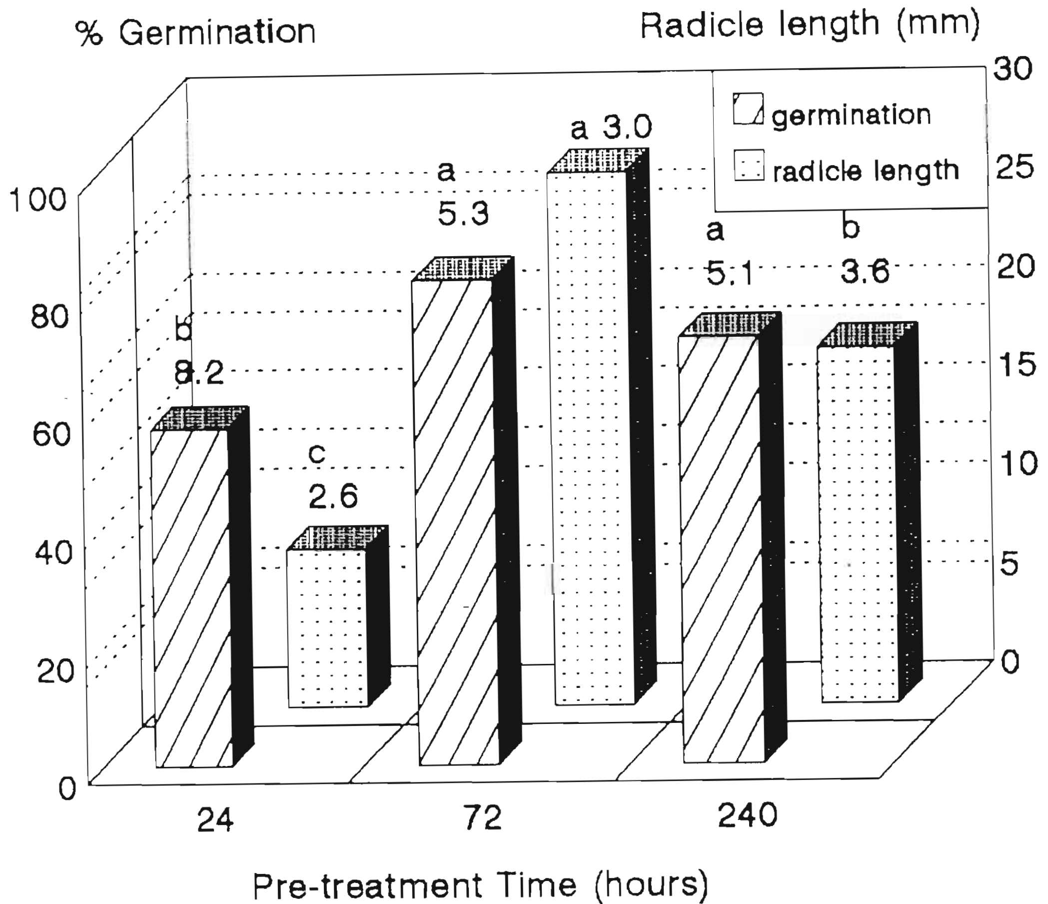

Figure 2.1 The effect of the duration of imbibing period on seed germination.

The seed were pre-treated in the full-strength nutrient solution at pH 6.0 and 25 °C with one airstone, and then placed in Petri dishes containing moist filter paper. Germination values and the lengths of the emerging radicles were recorded after 10 days. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

As the nutrient pre-treatment had a significant effect on the seed germination, further studies were performed in order to increase the percentage germination of the P. patula seed. The next consideration was to investigate the effect of altering the concentration of the nutrient formulation on percentage germination. Both higher concentrations and dilutions of the original formulation were examined (Figure 2.2). The percentage germination value was significantly higher when the seeds were pre-treated in the three quarter strength and full-strength nutrient solutions. Nutrient concentrations at higher strengths than the original formulation did not increase the germination percentage of the patula seed. The extent of radicle emergence was influenced very slightly by the various strengths of the nutrient solution. Consequently, the original (full-strength) formulation was used in all subsequent seed pre-treated work.

Among the more obscure factors influencing germination of seeds and early growth of seedlings is the pH of the soil or surroundings. It is inevitable that free ions influence germination directly or indirectly by their action on seed enzymes; for instance, it has long been known that acids stimulate germination (Baldwin, 1942). Figure 2.3 shows the results of an investigation to determine whether a more acidic pre-treatment solution would increase the germination of the P. patula seed. Seed pre-treated in the solution buffered at pH 6.0 had a significantly higher percentage germination value than those seeds germinated in the more acidic and alkaline solutions.

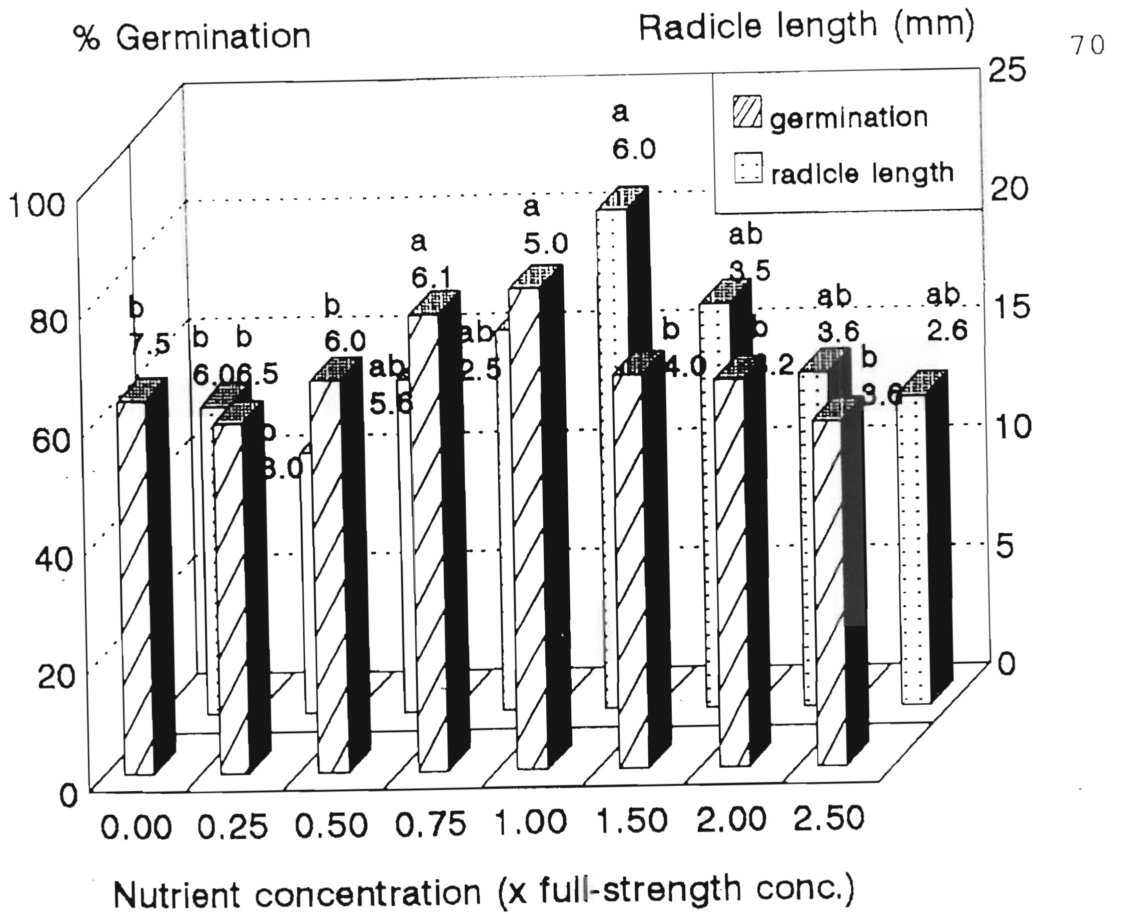

Figure 2.2 The effect of various nutrient concentrations on seed germination.

Germination conditions are as described in Figure 2.1, except for the nutrient concentrations. The imbibition period was 72 h. The nutrient concentrations depicted are both multiples and fractions of the original salt formulation shown in Table 2.2. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

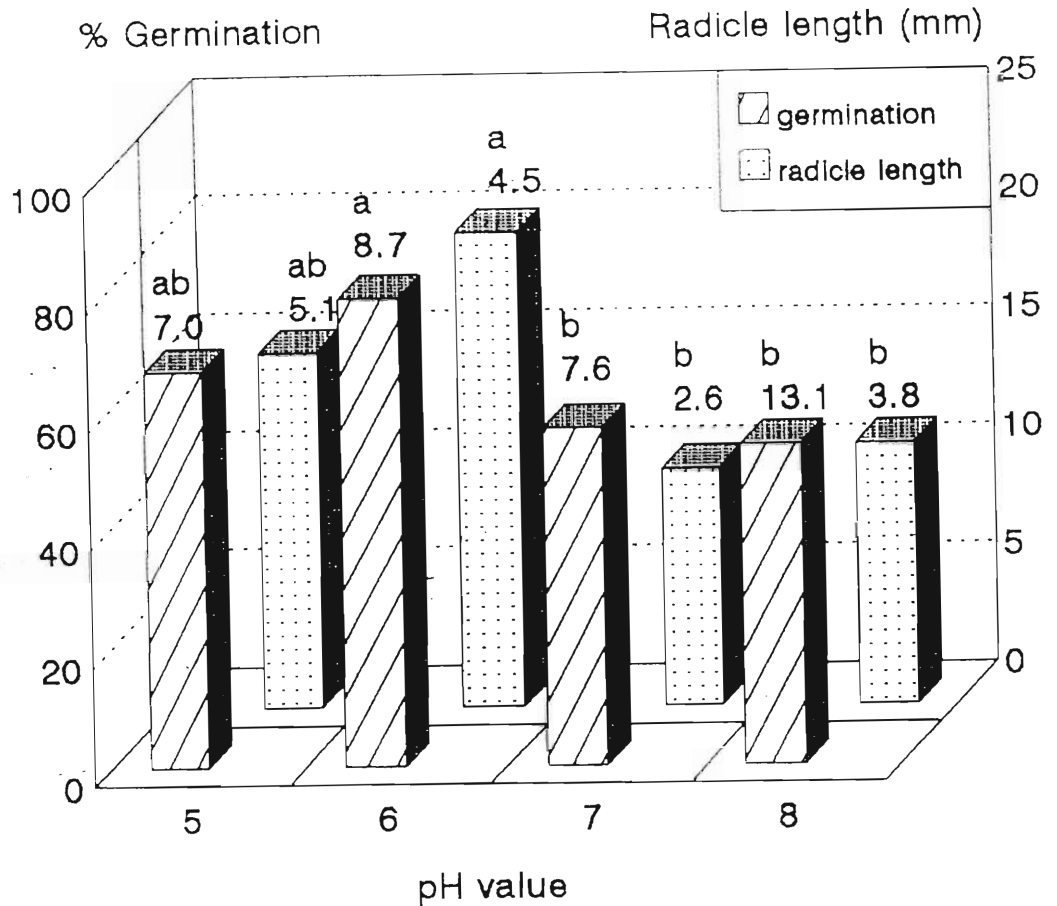

Figure 2.3 The effect of the pre-treatment solution pH on seed germination.

The seed was pre-treated for 72 h in a range of buffer solutions under the same germination conditions as described in Figure 2.1. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

Temperature is, perhaps, the most important environmental factor that regulates germination and controls subsequent seedling growth (Hartman and Kester, 1983). Optimum temperatures for seed germination fall within the range at which the largest percentage of seedlings are produced at the highest rate. The optimum for non-dormant seeds of most plants is between 25 °C and 30 °C (Edwards, 1932). Figure 2.4 shows the effect that the temperature of the pre-treatment solution has on the germination of P. patula seed. From these results a constant temperature of 25 °C was selected for the pre-treatment solution.

A good exchange of gases between the germination medium and the embryo is essential for rapid and uniform germination. Oxygen is vital for the respiratory process in the germinating seed (Hartman and Kester, 1983), but the content of oxygen in the germination medium is affected by its low solubility in water (Hanks and Thorp, 1956). Aeration of the pre-treatment solution was investigated for its effect on the P. patula germination (Figure 2.5), using an air bubbling system from a fish-tank airstone. When required, increased aeration was obtained by adding more airstones to the pre-treatment solution. Results recorded in Figure 2.5 indicate that aeration of the pre-treatment solution was necessary for higher seed germination. However, by increasing the aeration of the pre-treatment solution (2 airstones) the percentage germination of the seed tested did not differ significantly. Thus, one airstone provided all the oxygen needed for the germination process.

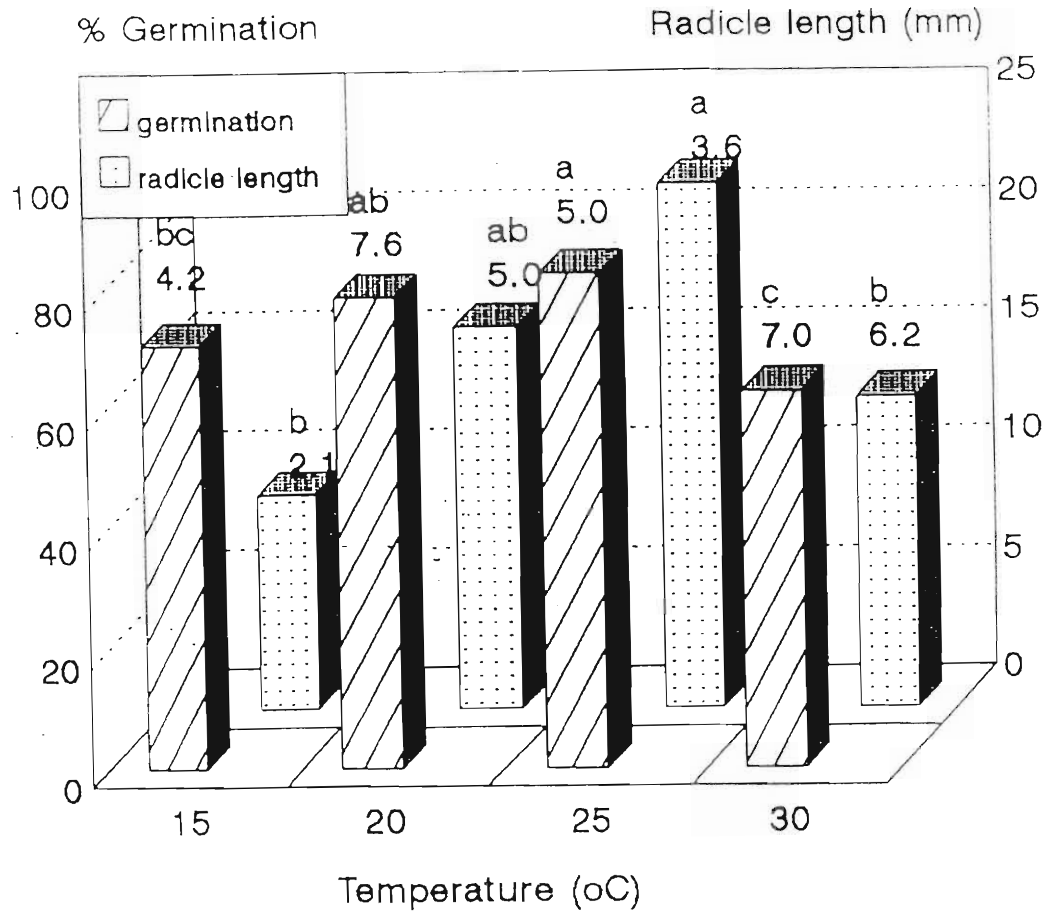

Figure 2.4 The effect of the pre-treatment solution temperature on seed germination.
The seeds were pre-treated for 72 h at various temperatures, under the same germination conditions as described in Figure 2.1. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's multiple Range test) are given (a-c).

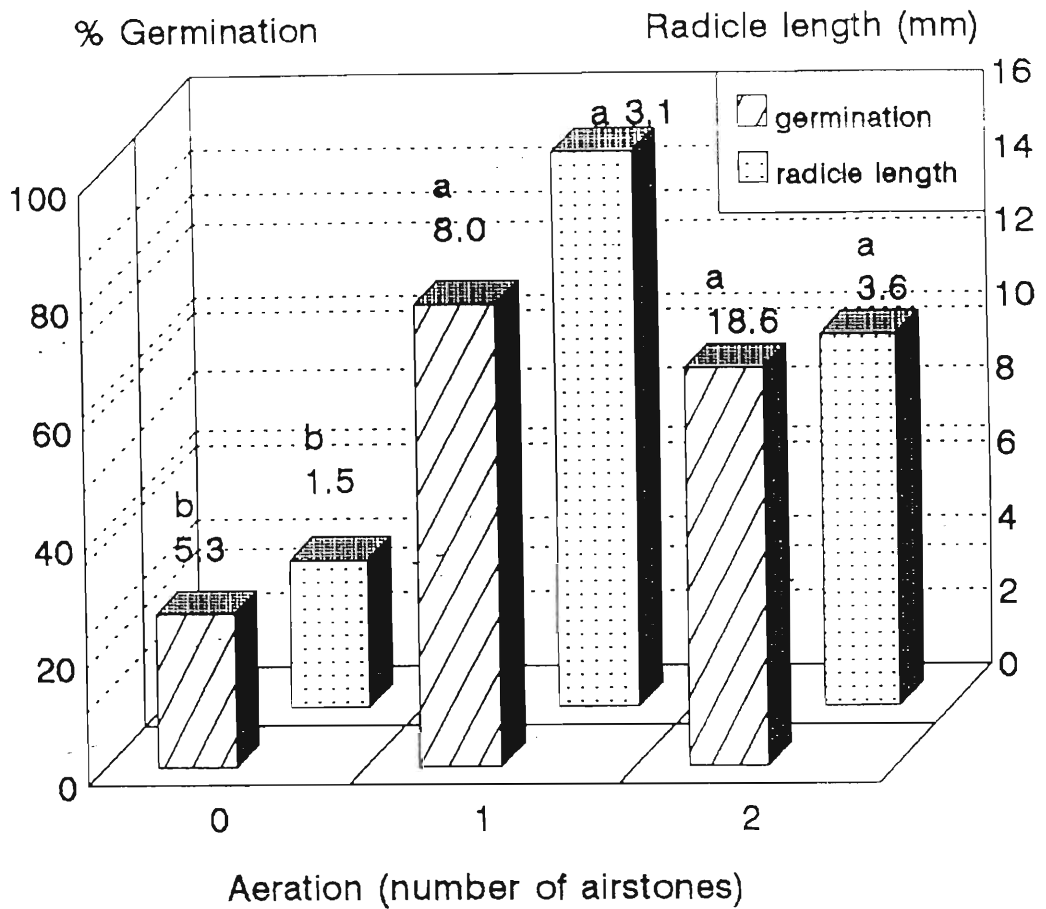

Figure 2.5 **The effect of aeration on seed germination.** The seeds were pre-treated for 72 h with the addition of airstones to the pre-treatment solution. Germination conditions are as described in Figure 2.1. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

One other parameter tested in this work was the effect of hydrogen peroxide (H_2O_2) on seed germination. The earliest references to the use of hydrogen peroxide on seed are by Miége (1908) and by Babcock (1911). Miége found that dilute solutions of hydrogen peroxide facilitated germination and stimulated the seedlings to more active development. Babcock used hydrogen peroxide as a seed sterilant in germination studies and noticed a stimulating effect on the seeds of barley, wheat, rye, corn, buckwheat, peas and beans. Babcock also recognised that the oxygen needed for respiration by the submerged seed was obtained from the reagent. In a later study (Scholer and Stubsgaard, 1989) hydrogen peroxide was found to enhance the early phases of germination by increasing the ambient oxygen level and thus stimulating respiration. In this study H_2O_2 was added to the pre-treatment solution for the 72 hour imbibition period. Results, depicted in Figure 2.6, indicate that the presence of H_2O_2 in the germination solution does not significantly increase the percentage germination nor the rate of radicle emergence. Judging from the data in Figure 2.5, the seed oxygen requirements must have been met from the aeration received from the airstone. From these results, hydrogen peroxide was not included in the standard seed pre-treatment protocol.

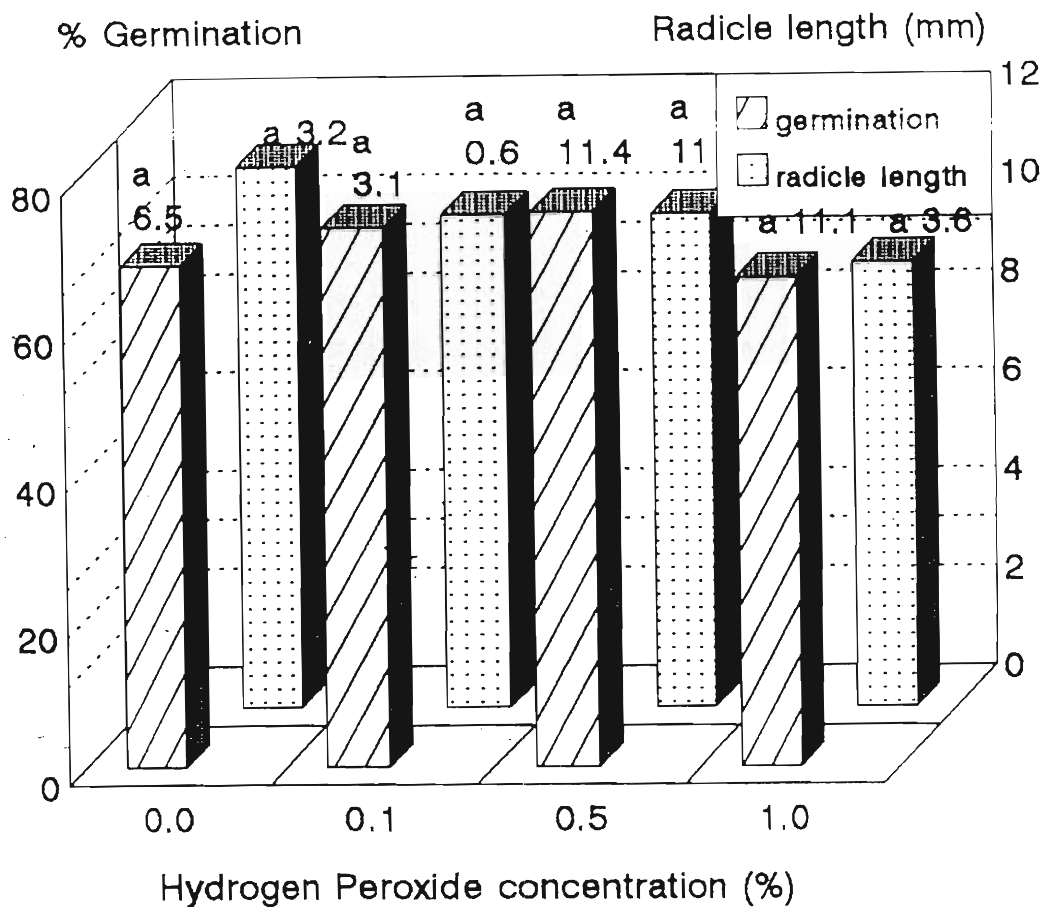

Figure 2.6 The effect of hydrogen peroxide on seed germination.

The seeds were pre-treated for 72 h in a range of hydrogen peroxide concentrations. Germination conditions are as described in Figure 2.1. n= 100 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a).

2.3.2 Surface Sterilisation of the Seed

The surface sterilisation of the seed coat is essential before the embryo can be removed from the seed. Aseptic techniques must be used to transfer the embryo, which is encased within the sterile environment of the female gametophyte tissue, from the seed to the culture medium. Any contamination transferred from the seed coat to the embryo explant would result in the proliferation of the bacterial or fungal contaminant on the nutrient medium. This, in turn, would lead to the eventual death of the embryo explant.

Surface sterilisation procedures, used by different researchers working with similar conifer embryo cultures, were used to ascertain which would be the most suited for the P. patula seed. Figure 2.7 shows that the most effective procedure was the one used by Martinez Pulido et al. (1990), who worked with mature seed of the Canary Island pine, as it decreased the contamination of the patula seeds from 100% to 16%. It was also found that only bacterial colonies grew on the nutrient agar around the contaminated seed. It is unclear why no fungal contamination appeared, even from the control seeds, within the three day incubation period; possibly, results were taken before fungal contamination could be detected or the presence of the bacteria suppressed fungal growth. The next most effective procedure was the one used by Bornman (1983). The common factor in these two treatments, which is absent in the other treatments, was the use of hydrogen peroxide (H_2O_2). Martinez Pulido et al. (1990) used a higher

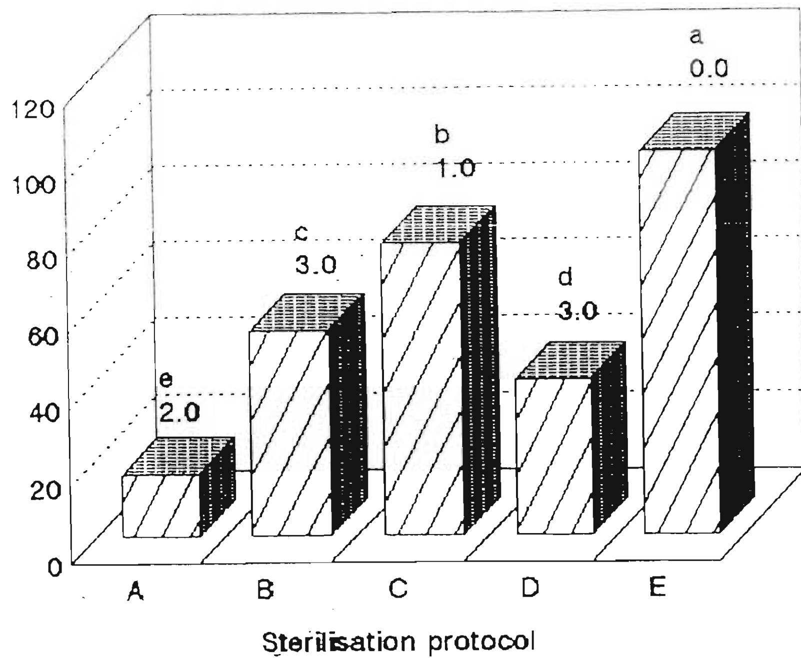

Figure 2.7 The effect of reported seed sterilisation protocols on the elimination of surface contaminants.

Seeds, removed from the germinator after 3 days, were plated on nutrient agar after having a thorough rinse in sterile distilled water after each treatment. Treatments were: A= 6% NaOCl (30 min), 10% H_2O_2 (10 min) (Martinez Pulido <u>et al.</u>, 1990); B= 5.25% NaOCl (20 min) (Ellis and Bilderback, 1984); C= 6% H_2O_2 (12 min), 5.25% NaOCl (5 min)(Bornman, 1983); D= 70% ethanol dip, 3% NaOCl (15 min), 0.01N HCl dip (Perez-Burmudez and Sommer, 1987); E= Control (no surface sterilisation).Seeds, plated on nutrient agar, were kept in sealed Petri dishes at 24 °C (±2 °C). The results were recorded as the percentage of seed having bacterial contamination after 3 days. n= 50 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-e).

concentration of H_2O_2 (10%) than did Bornman (6%) and so it was deduced that a high hydrogen peroxide concentration (10%) would be the most effective treatment to use in this work.

In order to reduce the contamination totally, another experiment was designed to further examine the effectiveness of the hydrogen peroxide. Results, shown in Table 2.2, indicate that a twenty minute soak in 10% hydrogen peroxide was sufficient to eradicate any contamination from the seed coat. Thus all seed used in this study were sterilised in a 10% hydrogen peroxide solution for at least twenty minutes.

Table 2.2 The effect of the hydrogen peroxide concentration and duration of the treatment on the eradication of contamination from the seed coat.

Seed, removed from the germinator after 3 days, were sterilised and then rinsed thoroughly in sterile RO water. Seed, plated on nutrient agar, were kept in sealed Petri dishes at 24 °C (±2 °C). Results were recorded as the percentage of seed having bacterial contamination after 3 days. No fungal contamination was found in any of the treatments. n= 50 for each treatment which was replicated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-g).

	Time (min)				
H_2O_2 (%)	**1**	**5**	**10**	**15**	**20**
1	100 ±0.0 a	100 ±0.0 a	100 ±0.0 a	100 ±0.0 a	100 ±0.0 a
5	94 ±4.0 b	95 ±2.5 b	42 ±4.0 de	39 ±2.1 e	9 ±2.3 f
10	82 ±3.2 c	80 ±3.2 c	44 ±3.2 d	11 ±1.5 f	0 ±0.0 g

2.3.3 Adventitious Bud Initiation

In the formation of adventitious buds, there is an interplay between the inoculum, the medium, and the culture conditions (Thorpe and Patel, 1984). For optimum organogenesis, each of these components has to be critically assessed.

One of the most important factors to consider when trying to induce adventitious bud formation is the cytokinin treatment (section 2.1.2). A concentration range of the cytokinin N^6-benzyladenine (BA) was tested (Figure 2.8) in order to establish which had the most positive effect on adventitious bud induction. The number of explants forming adventitious bud primordia increased with increasing supply of BA up to the 5 mg l^{-1} level. Concentrations higher than 5 mg l^{-1} BA had an inhibitory effect on the number of embryo explants producing buds. Although bud formation on the explants was clearly observed after eight to 10 weeks, the bud primordia that formed were very tightly packed together. As a result, an exact count of each individual bud that formed on each explant was not done. However, between five and twelve individual buds were initiated on those explants that did bear bud primordia. Those embryo explants that did not form bud primordia at all usually turned brown within four to six weeks of being cultured.

Having an indication of what BA concentration to use for adventitious bud induction, the next consideration was to determine the type of medium formulation that would be best suited for bud initiation from the P. patula embryos. The

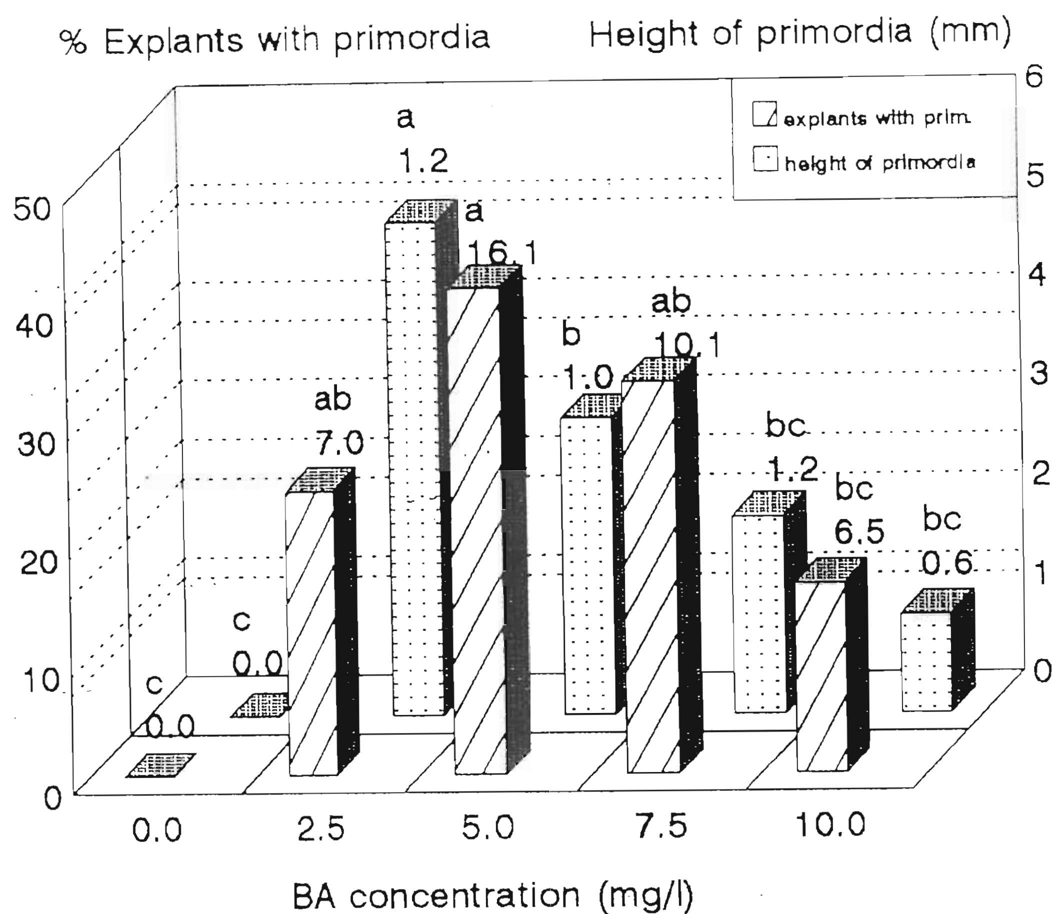

Figure 2.8 **The effect of BA concentrations on the initiation of adventitious buds on embryo explants.**

Explants (8-10 day-old) were plated on MS media containing 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Cultures were maintained at 24 °C (±2 °C). Results were recorded as the percentage of explants having adventitious bud formation and the height of those bud primordia formed, after 6 weeks. n= 30 for each treatment which was replicated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

most common medium formulations used by researchers on conifer organogenesis were investigated (Figure 2.9). The LM formulation, described by Litvay et al. (1981), proved to be the most suited for the patula cultures. The results obtained from the MCM (no explants forming bud primordia) and MS (only 7% explants forming primordia) formulations were rather surprising. Work done by Bornman (1983) indicated that the MCM medium, which was the product of intensive research on morphogenesis in Picea abies and Pinus sylvestris, resulted in the initiation of large numbers of Picea abies adventitious shoots as compared to the LM, MS, and SH media. According to Bornman (1983), reasons for the better response of his Picea abies cultures to MCM appeared to lie in the wider range of vitamins and other organic constituents, lower levels than normal of Mn^{2+} and I^- and, possibly, the addition of reduced nitrogen. In this study, however, none of the P. patula embryos cultured responded to the MCM formulation. Furthermore, the MS formulation had very little effect on patula bud initiation. It is interesting to note that the LM formulation is very similar to the MS formulation, differing only in having higher levels of KH_2PO_4, $MgSO_4.7H_2O$, H_3O_3, and $ZnSO_4.7H_2O$ (Appendix 1). The GD and LP formulations have high $MgSO_4.7H_2O$ concentrations (1000 mg l^{-1} and 1800 mg l^{-1} respectively) relative to the MCM, MS and SH concentrations, but these are similar to the LM concentration. The Mg^{2+} ion and/or the SO_4^{2-} ion present could, perhaps, be the important factor in the medium for the formation of adventitious buds from patula embryo explants. The particular salt or salt ratio in the LM formulation responsible for adventitious bud initiation in

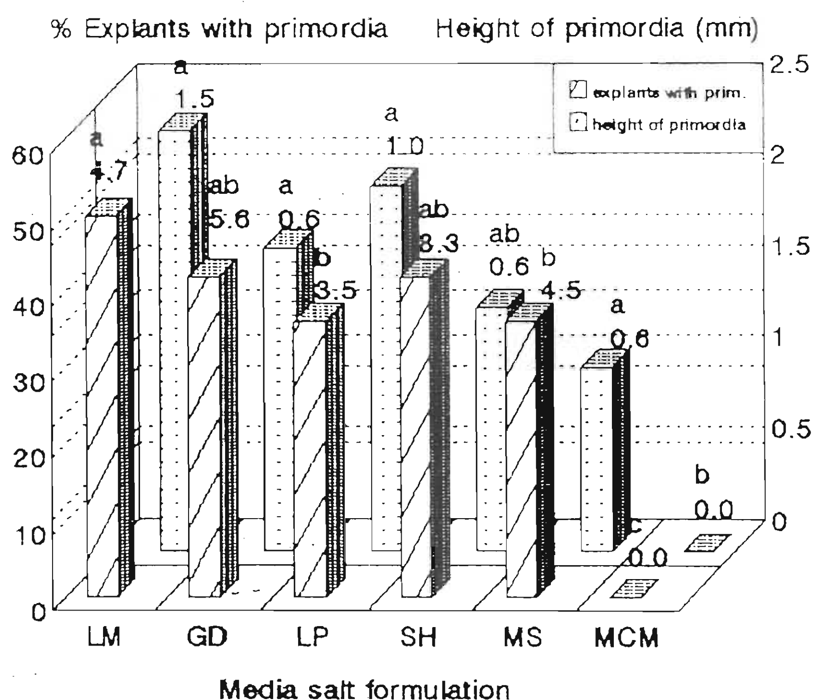

Figure 2.9 The effect of various salt formulations on the formation of adventitious buds from embryo explants.

Embryos (8-10 day-old) were plated on full-strength media containing 5 mg l^{-1} BA, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Salt formulations are: LM (Litvay et al., 1981), GD (Mehra-Palta and Smeltzer, 1978), LP (Aitken-Christie, 1984), MCM (Bornman, 1981), SH (Schenk and Hildebrant, 1972) and MS (Murashige and Skoog, 1962). Culture conditions were as described in Figure 2.8. Results were recorded, after a 10 week culture period, as the percentage of explants having adventitious buds and the height of the bud primordia. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

P. patula is not known. Further in-depth studies could be done to investigate salt combinations and concentrations for optimising patula adventitious bud initiation.

For the initiation of adventitious buds, the explant is typically placed on a medium in the presence of a cytokinin (Thorpe and Biondi, 1984). However, in some cases, a low level of auxin may be useful for increasing the yield of adventitious buds (Cheng, 1977; Jansson and Bornman, 1980). Table 2.3 gives the results of an experiment designed to determine the effect, on adventitious bud initiation, of the auxin NAA in combination with BA. The percentage of explants forming adventitious bud primordia decreased with an increasing NAA concentration. A similar trend was observed with the height of the bud primordia formed. Plate 2.3 shows clearly that with increasing NAA concentrations, more callus formation is obtained. These results are similar to those of Biondi and Thorpe (1982) who found, with radiata pine, that auxin enhanced callus formation rather than primordium initiation.

Von Arnold (1987a) reported that the number of Picea abies embryos forming adventitious buds was higher on media containing 10 g l^{-1} sucrose than on higher levels. Figure 2.10 gives the results of an investigation into the effect of sucrose concentration, in the initiation medium, on the yield of patula adventitious buds. Although no significant difference was obtained between the 20 g l^{-1} and the 30 g l^{-1} treatments with regard to the number of explants forming buds, the 20 g l^{-1} concentration, with its higher mean value, was selected as being the most suited for the

Table 2.3 The effect of BA and NAA combinations in the initiation medium on adventitious bud formation. Embryo explants (8-10 day-old) were cultured on LM media, containing 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Culture conditions were as described in Figure 2.8. Results show (A) the percentage of explants that formed adventitious bud primordia and (B) the mean height (mm) of the adventitious buds formed after 10 weeks in culture. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-e).

(A)

	BA (mg l^{-1})		
NAA (mgl^{-1})	**0.0**	**2.5**	**5.0**
0.00	0 ±0.0 e	22 ±3.5 c	50 ±8.9 a
0.06	0 ±0.0 e	32 ±4.2 b	18 ±6.5 c
0.10	0 ±0.0 e	8 ±4.6 d	10 ±3.5 d
0.15	0 ±0.0 e	0 ±0.0 e	8 ±3.5 d

(B)

	BA (mg l^{-1})		
NAA (mg l^{-1})	**0.0**	**2.5**	**5.0**
0.00	0 ±0.0 d	3.3 ±0.58 a	2.7 ±0.58 b
0.06	0 ±0.0 d	1.7 ±0.58 c	1.3 ± 0.58 c
0.10	0 ±0.0 d	1.0 ±0.0 c	1.7 ±0.58 c
0.15	0 ±0.0 d	0 ±0.0 d	1.0 ±0.0 c

Plate 2.3 The effect of combining BA with NAA, in the induction medium, on the formation of adventitious bud primordia. Plant growth regulator concentrations (mg l^{-1}) are depicted with the BA concentration written above the NAA concentration. Scale bar= 2 mm.

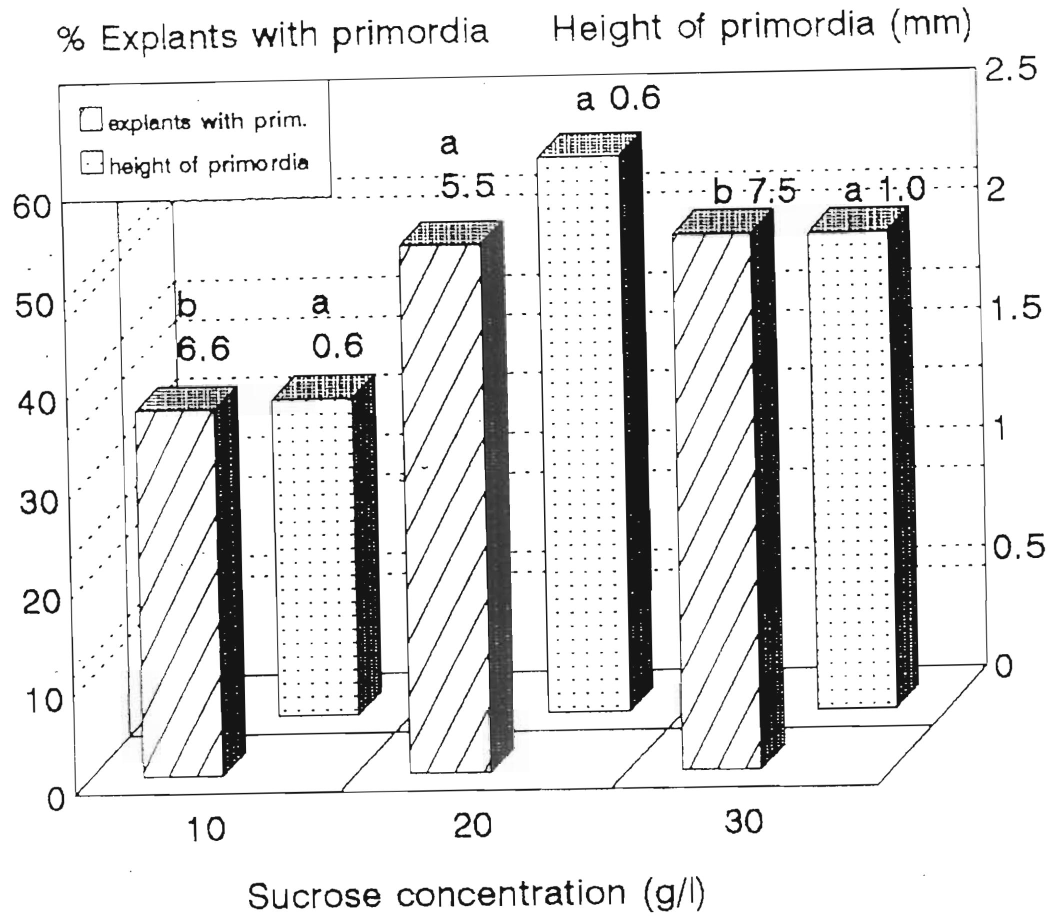

Figure 2.10 **The effect of sucrose concentration on the formation of adventitious buds from embryo explants.**

Explants (8-10 day-old) were plated on LM, 8g l^{-1} agar-agar, 5 mg l^{-1} BA, pH 5.7. Culture conditions were as described in Figure 2.8. The percentage of explants forming adventitious bud primordia and the height of those bud primordia formed after 10 weeks was recorded. n= 30 for each treatment which was replicated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

initiation medium.

For all these investigations on the yield of adventitious buds, the embryo explant used had been allowed to germinate for seven to ten days. A study was undertaken to test the effect of the age of the embryo explant as regards to the formation of adventitious buds. Figure 2.11 gives the results of this study. The embryo germination age was taken from the time when the seed was first pre-treated to when the embryo was excised from the seed. The 14-day germination period proved to be significantly more suitable as regards to the number of embryo explants that formed adventitious bud primordia. A longer germination period of 20 days did not produce a larger yield of bud primordia and so it can be concluded that there is a certain "window" period where the embryo explant tissue is developed enough and yet retains enough juvenile morphology for initiation of adventitious buds. In all further work only 13 to 15 day-old embryo explants were used.

A further study was designed to determine whether the concentration of the medium salt formulation and/or the type of gelling agent used in the initiation medium would affect the yield of adventitious buds. As regards the number of embryo explants producing adventitious buds there was no significant difference between the full-strength LM salt formulation agar-agar versus Gelrite treatments and the half-strength LM agar-agar treatment (Figure 2.12). The quarter strength LM agar-agar treatment proved to be too dilute for meaningful bud initiation. A similar trend was obtained with the heights of the buds initiated.

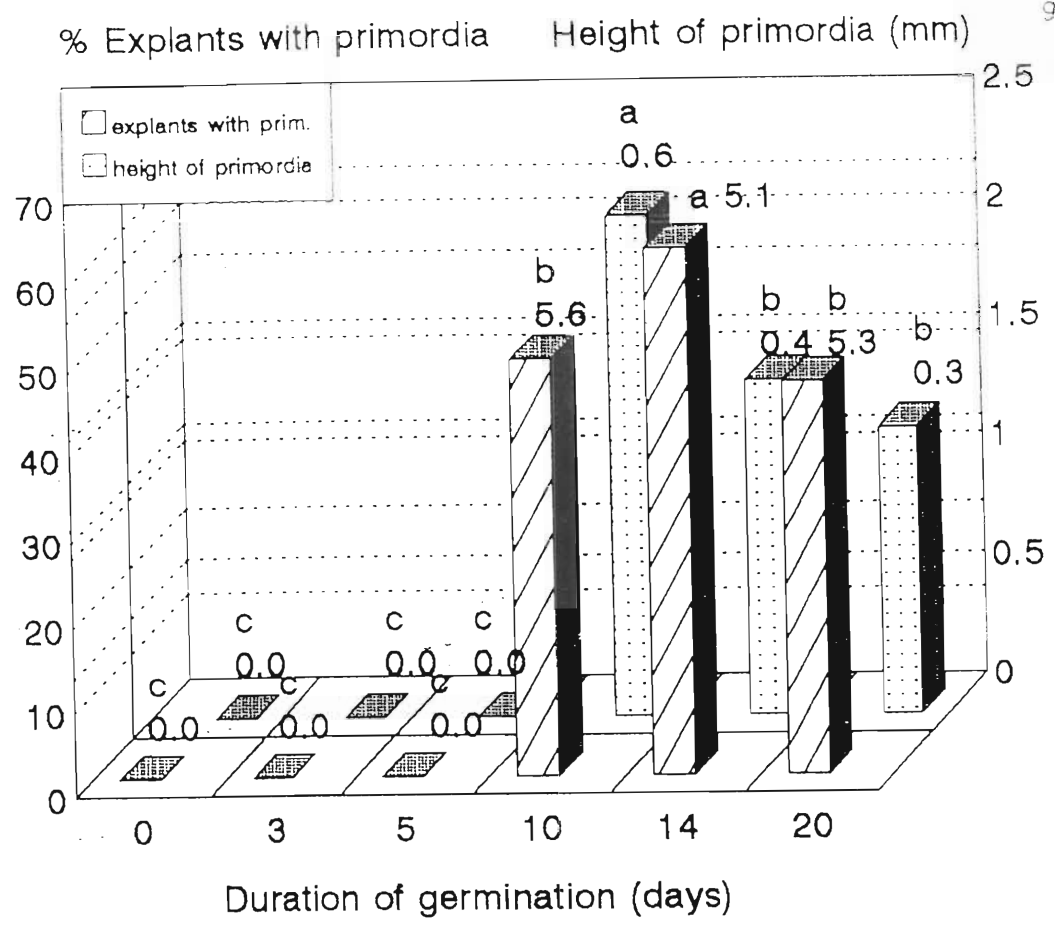

Figure 2.11 **The effect of explant age (duration of germination) on the development of adventitious bud primordia.**
Seeds were pre-treated for the standard 72 h and then plated on moist filter paper and germinated for 0-20 days. Then the embryos were excised and plated on full-strength media containing 5 mg l^{-1} BA, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Culture conditions were as described in Figure 2.8. Results were recorded, after a 10 week culture period, as the percentage of explants having adventitious bud formation and the height of those buds formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

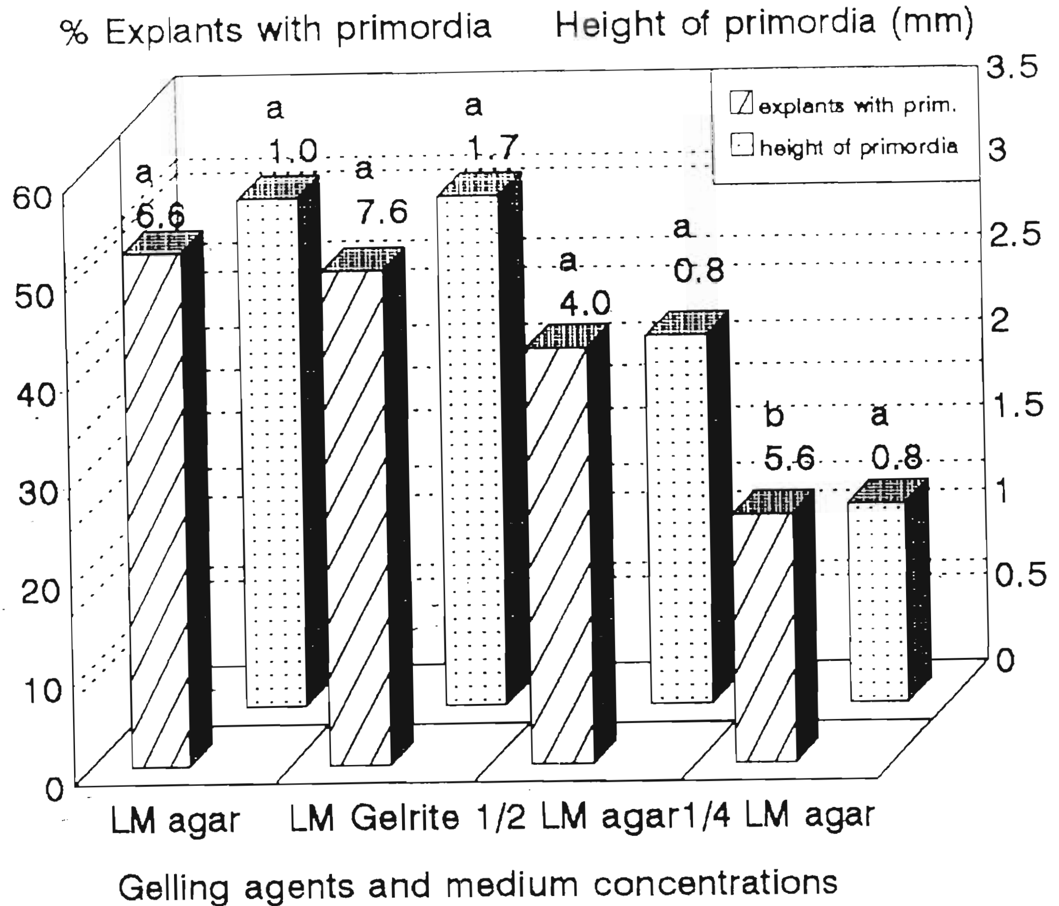

Figure 2.12 A comparison of two types of gelling agents and the effect of reducing their concentration in the initiation medium on the formation of adventitious buds from embryo explants. Embryos (14 day-old) were plated on full-strength media containing 5 mg l^{-1} BA, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Culture conditions were as described in Figure 2.8. Results were recorded, after a 9-19 week culture period, as the percentage of explants having adventitious bud formation and the height of those bud primordia formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

Several researchers have reported successes, using high-concentrated cytokinin pulses on their embryo explants, as regards adventitious bud formation (Bornman, 1983; Vogelmann et al., 1984; Von Arnold and Eriksson, 1985; Von Arnold and Hakman, 1988). Figure 2.13 shows the results of an investigation using two reported pulse treatments on the patula embryos. The two hour treatment, used by Von Arnold and Eriksson (1985), produced a higher number of bud-bearing explants than the three hour, lower BA concentration treatment used by Bornman (1983). However, the two hour, 56.3 mg l^{-1} BA pulse treatment did not produce more adventitious bud-bearing explants than the standard method of culturing the explants on medium containing a low BA concentration.

Perez-Bermudez and Sommer (1987) found that the nitrogen content in the Murashige and Skoog (MS) formulation was too high for successful culture of Pinus elliottii adventitious buds. Those researchers reported that the frequency of the Pinus elliottii embryos, forming shoots, was inversely correlated with the NH_4NO_3 concentration, a significantly higher number of P. elliottii shoots were induced when ammonium was omitted. Consequently, in this study the role of the nitrogen compounds, in the LM initiation medium, on adventitious bud development was investigated (Figure 2.14). This included KNO_3 1.90 g l^{-1}, NH_4NO_3 1.65 g l^{-1} (standard LM formulation)(Treatment A); KNO_3 1.90 g l^{-1}, NH_4NO_3 1.65 g l^{-1}, K_2SO_4 3.60 g l^{-1} (Treatment B); KNO_3 6.00 g l^{-1} (Treatment C); $(NH_4)_2SO_4$ 3.95 g l^{-1}, K_2SO_4 5.25 g l^{-1} (Treatment D); KNO_3 3.00 g l^{-1}, $(NH_4)_2SO_4$ 1.98 g l^{-1}, K_2SO_4 2.63 g l^{-1} (Treatment E). The treatment containing the

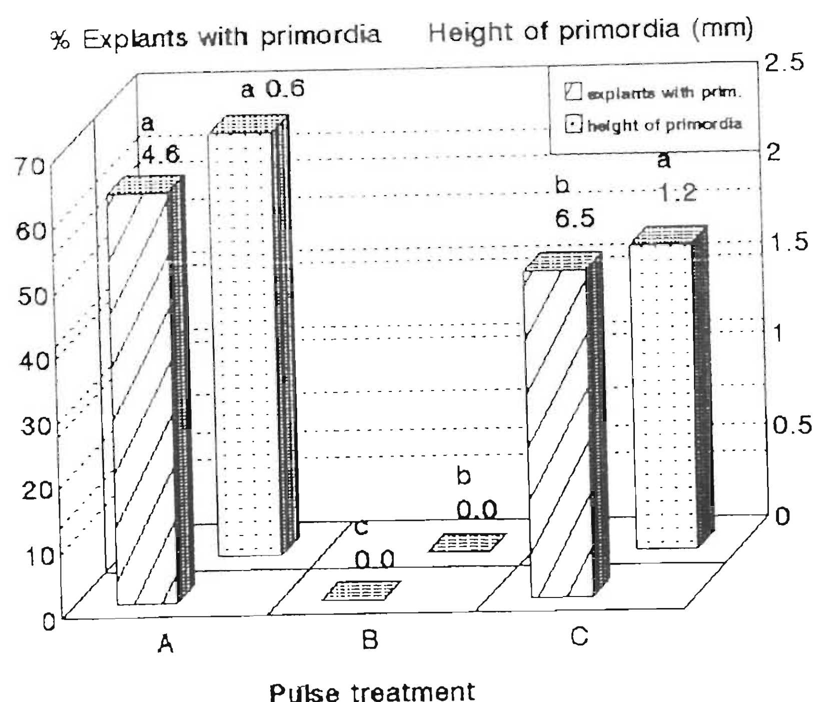

Figure 2.13 The effect of cytokinin pulse treatments on the induction of adventitious bud primordia.

Embryo explants (14 day-old) were subjected to two pulse treatments. The control (A) was the normal procedure of plating the explants on to LM media, containing 5 mg l^{-1} BA, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. The pulse treatments were: B) chilled ¼LM, pH 5.0, containing 28.1 mg l^{-1} BA (Bornman, 1983) and C) chilled ¼LM, pH 5.0, containing 56.3 mg l^{-1} (Von Arnold and Erikson, 1985). The cultures were then shaken gently for 3 h and 2 h respectively. The explants were then dried on sterile filter paper before being plated on to LM media, containing 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8 with no PGRs. Culture conditions were as described in Figure 2.8. The percentage of explants which formed adventitious bud primordia and the height of those primordia formed after 10 weeks was recorded. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

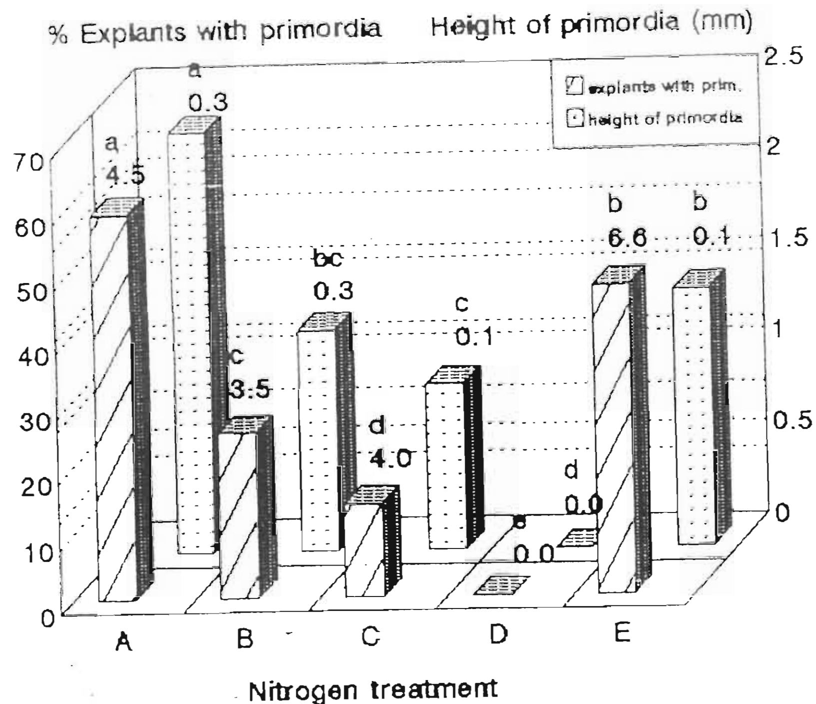

Figure 2.14 The effect of the form and concentration of nitrogen, in initiation medium, on adventitious bud formation.

The initiation media consisted of full-strength LM salts apart from the KNO_3 and NH_4NO_3 salts. The adjusted formulations were: A) KNO_3 1.90 g l^{-1}, NH_4NO_3 1.65 g l^{-1} (standard LM formulation); B) KNO_3 1.90 g l^{-1}, NH_4NO_3 1.65 g l^{-1}, K_2SO_4 3.60 g l^{-1}; C) KNO_3 6.00 g l^{-1}; D) $(NH_4)_2SO_4$ 3.95 g l^{-1}, K_2SO_4 5.25 g l^{-1}; E) KNO_3 3.00 g l^{-1}, $(NH_4)_2SO_4$ 1.98 g l^{-1}, K_2SO_4 2.63 g l^{-1}. Embryos (14 day-old) were cultured on each of these treatments which also contained 5 mg l^{-1} BA, 20 g l^{-1} sucrose, 8 g l^{-1}, pH 5.8. Culture conditions were as described in Figure 2.8. Results were recorded, after a 10 week culture period, as the percentage of explants having adventitious bud formation and the height of those bud primordia formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

standard KNO_3 and NH_4NO_3 concentration (Treatment A) had a more significant effect than the other treatments as regards adventitious bud development. The treatment with the NO_3^-/NH_4^+ ratio being equal to one (Treatment E) resulted in significantly more explants forming adventitious buds than treatments B, C and D. This result, however, does not correspond with the work of David et al. (1982) which reported a positive effect on Pinus pinaster adventitious budding when the NH_4^+/K^+ ratio was one. Treatment B was included to observe the effect of adding the extra K^+ in treatment C. Extra K^+ was present in treatment C due to higher concentration of KNO_3 being used to provide all the nitrogen available in the form of NO_3. As the percentage of explants (possessing adventitious bud primordia) cultured on treatment B was very low, it can be concluded that high concentrations of potassium ions (K^+) are detrimental to adventitious bud development. Therefore, the poor results obtained for treatment C could be a result of the high level of K^+ ions and not necessarily the high concentration of nitrate (NO_3^-) ions present. Treatment D shows the effect of using ammonium ions (NH_4^+) as the sole nitrogen source. In this case all the embryo explants turned brown and died within two to three weeks of culturing. This result thus agrees with the findings of Perez-Bermudez and Sommer (1987) as regards to the detrimental effect of high levels of NH_4^+ ions in the adventitious bud initiation media. This is not surprising as nitrate (NO_3^-), rather than ammonium (NH_4^+), seems to be the form of nitrogen preferentially utilised by higher plants (Sutcliffe and Baker, 1976).

2.3.4 Adventitious Bud Elongation

When adventitious bud primordia had developed on the original embryo explant to approximately 5 mm in height, the explants, bearing bud primordia, were aseptically transferred to the elongation medium. This comprised half-strength LM salts, 10 g l^{-1} activated charcoal, 8 g l^{-1} agar-agar, 20 g l^{-1} sucrose, pH 5.8. No growth regulators were included in the elongation media as this is recommended in the literature (Reilly and Washer, 1977; Von Arnold and Eriksson, 1981; Aitken Christie and Thorpe, 1984). Several reports have emphasised the importance of having activated charcoal present in the elongation medium to ensure the removal of endogenous growth regulators (Mehra-Palta and Smeltzer, 1978; Von Arnold and Eriksson, 1981 and Patel and Thorpe, 1984). As with adventitious bud development (Plate 2.4), the elongation of the adventitious buds was a very slow process (Plate 2.5). If individual buds were removed entirely from the embryo explants they would inevitably turn brown and die. Only those buds that were still attached to the original explant tissue survived. Figure 2.15 shows the time taken for adventitious bud elongation. Due to the long time required for the adventitious shoots to develop, no rooting studies were done on these shoots. However, spontaneous rooting did occur from a few shoots while they were being cultured on the elongation medium.

2.3.5 Concluding Remarks

In conclusion, a protocol was devised to obtain adventitious shoots from mature P. patula zygotic embryos.

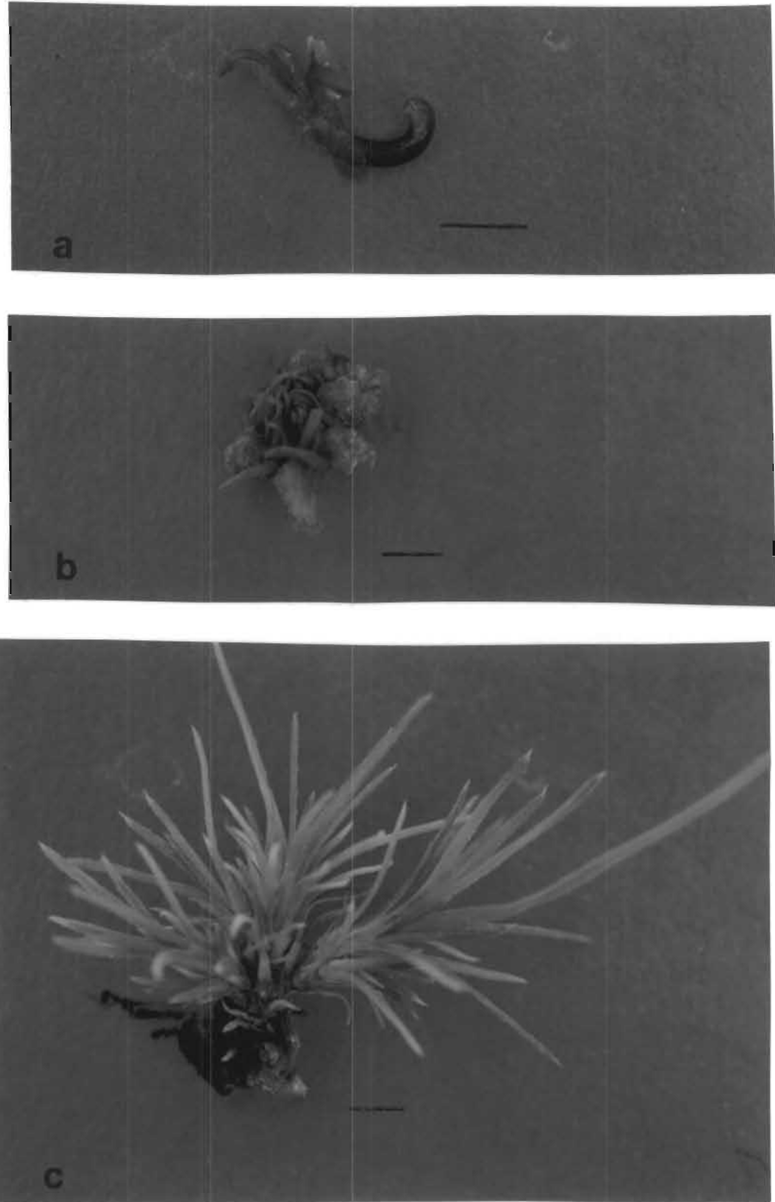

Plate 2.4 The development of adventitious bud primordia from the embryo explant when cultured on the LM initiation medium containing 5 mg l^{-1} BA. Scale bar= 2 mm.

a) 2 week culture period

b) 8 week culture period

c) 20 week culture period

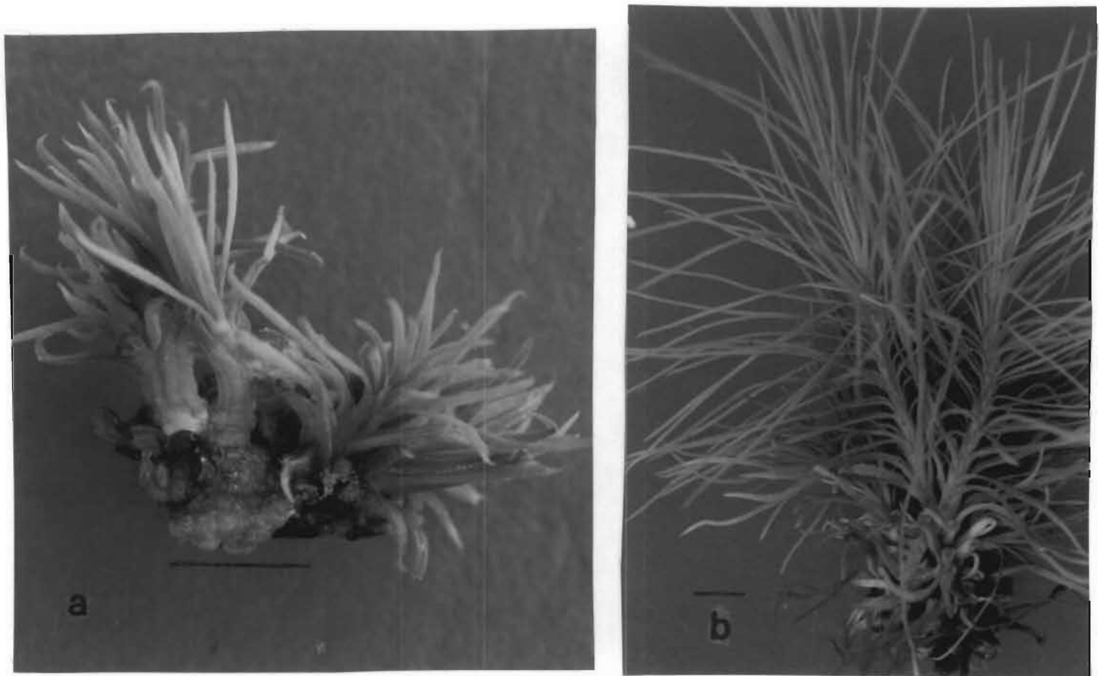

Plate 2.5 The elongation of adventitious buds. 13-15 week-old cultured explants were transferred to the LM elongation medium containing 10 g l^{-1} activated charcoal.

a) 6 weeks on the elongation medium.

b) 16 weeks on the elongation medium.

Scale bar= 4 mm.

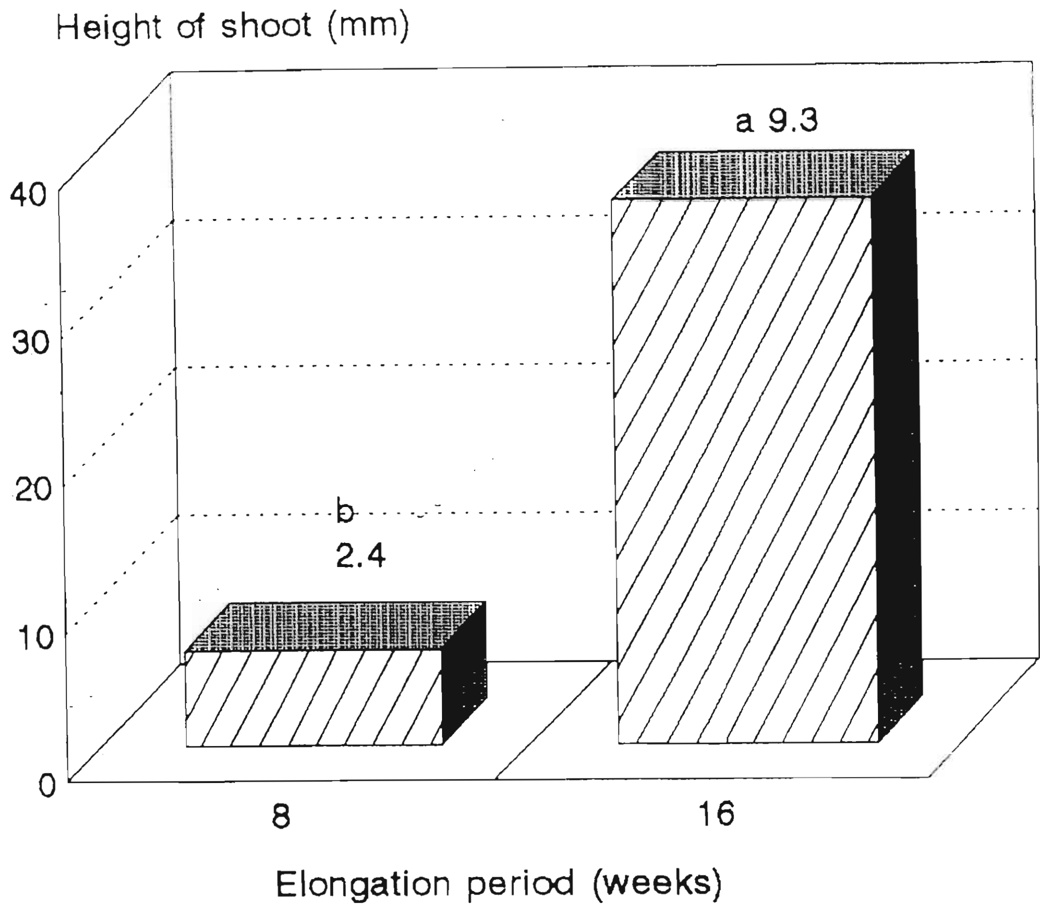

Figure 2.15 **The rate of adventitious bud elongation.** Intact embryo explants were aseptically transferred to the elongation medium after the embryo explant had been cultured on initiation medium for 15 weeks. The elongation medium comprised of half-strength LM, 10 g l^{-1} activated charcoal, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. Culture conditions were as described in Figure 2.8. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

Seeds are first pre-treated for three days in a nutrient solution and then plated for a further 10 to 12 days for further germination. After the seeds have been surface sterilised in 10% hydrogen peroxide for thirty minutes, the embryo is removed and aseptically placed in culture. The embryo explants are cultured on initiation medium until the adventitious buds formed have developed into shoots, approximately 5 mm in height. About three to five shoots normally develop from each explant. The intact embryo explants, containing adventitious shoots, are then aseptically transferred to the elongation medium where the shoots grow until they are ready for rooting. Adventitious shoots need to be approximately 50 mm in height before they can be rooted.

CHAPTER 3

MICROPROPAGATION OF *Pinus patula* VIA THE ORGANOGENIC ROUTE OF AXILLARY BUD INDUCTION

3.1 INTRODUCTION AND LITERATURE REVIEW

In the formation of *in vitro* axillary shoots, lateral growing points on the shoot explant at the nodes below the apical meristem are stimulated to grow (Hartman and Kester, 1983). Pines normally bear buds (axillary buds) in the axils of all their reduced scale-like leaves on a shoot axis. Most of these buds immediately give rise to the needle-bearing short shoots (Foster and Gifford, 1959; Fink, 1984). Each short shoot consists of the needles and a diminutive shoot apex (Cohen, 1977). In all the pine species there are branches of two types, long shoots and short shoots, the foliage leaves being confined to the latter, except in young seedlings. According to the species, there are two, three or five needle-shaped leaves in a cluster at the apex of each short shoot. There are also scale-leaves which occur on the long shoot branches and it is in their axils that both long and short shoots arise (Foster and Gifford, 1959; Sporne, 1965). In literature written on pine morphology, the terminology used to describe the shoots formed from axillary buds has not been consistent. These shoots have been referred to as dwarf shoots (Sacher, 1955; Cohen, 1977), short shoots

(Sporne, 1965; Fink, 1984), spur shoots (Foster and Gifford, 1959), needle fascicles (Stone and Stone, 1943; Kummerow, 1966; Mehra-Palta and Smeltzer, 1978; Stiff et al., 1985 and 1989) and brachyblast (Cohen and Shanks, 1975). In this study the shoots formed from axillary buds will be referred to as dwarf shoots. Sacher (1955) showed that the development of dwarf shoots was fundamentally similar to that of vegetative axillary buds of other taxa.

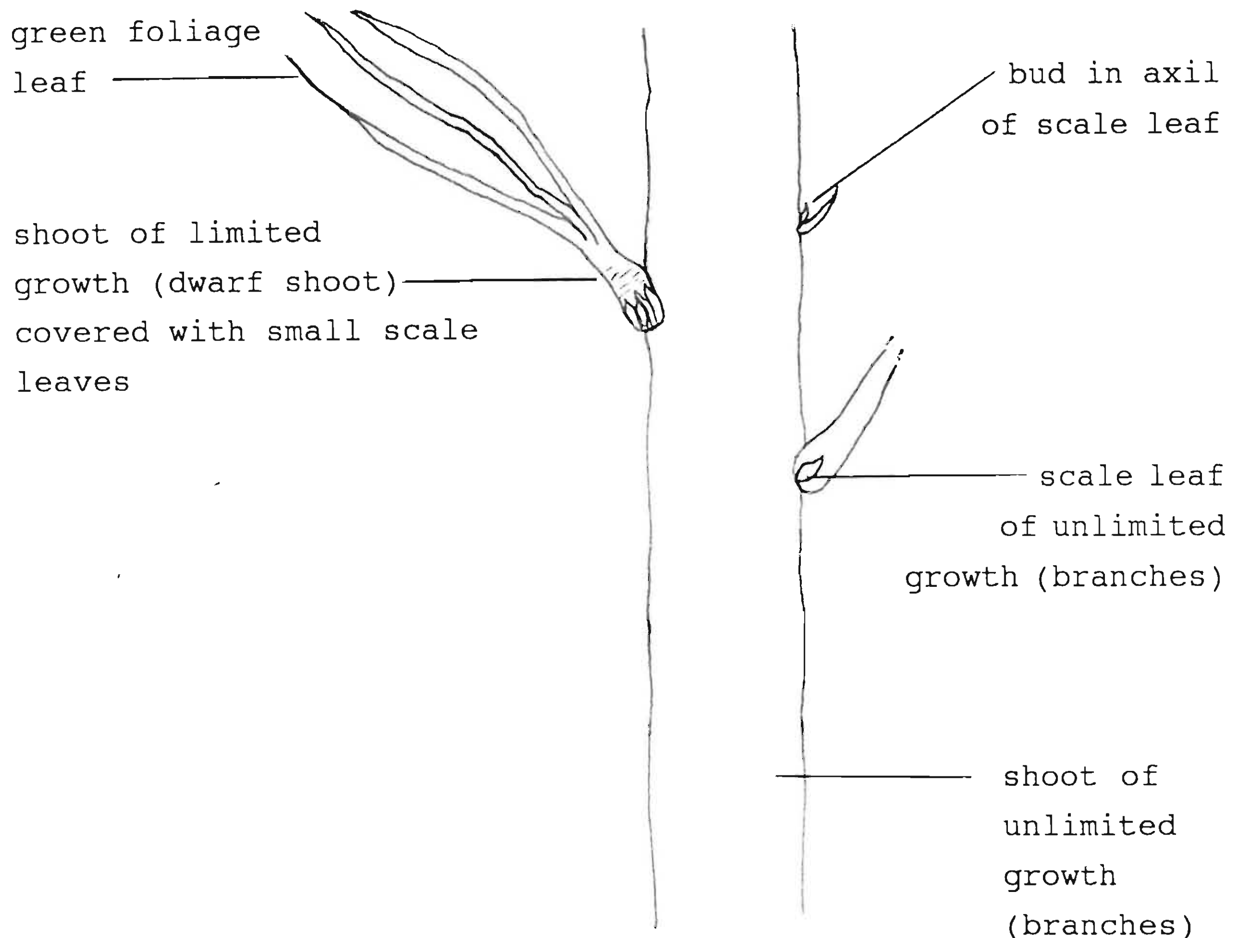

Figure 3.1 A simplified diagram of pine vegetative morphology. (Adapted from Sporne, 1965)

The foliage leaves of all members of the Coniferales are simple, and most commonly are scale-like or needle-like in form. In genera such as Pinus, a large proportion of the needle-like leaves produced during a growing season are devoid of axillary buds; in these plants branching proceeds from a few axillary buds located in a pseudowhorl just below the terminal bud. Under conditions of unusual stimulation, fully mature dwarf shoots may proliferate into long shoots through the reactivation of their shoot apices (Foster and Gifford, 1959). Stone and Stone (1943) stated that with Pinus rigida, following severe injury, both branches of limited development and dormant buds respond by profuse and vigorous growth. This effect is particularly noticeable in P. rigida when it has been damaged by fire. McLintock (1940) reported that the number and length of the sprouts in P. rigida increase with greater intensity of pruning.

Axillary bud induction is the formation of buds from axillary meristems produced by immature shoots (Lin et al., 1991). The axillary and/or fascicular shoot formation in vitro is often referred to as micropropagation (Amerson et al., 1988). Numerous investigations of the histogenesis of vegetative axillary buds have shown that the organisation of their meristems commonly duplicates that found in the terminal buds of the parent axes (Philipson, 1949; Gifford, 1951; Esau, 1954). In vitro induction of axillary buds capable of forming shoots is a widely used method for the propagation of flowering plants and trees (Lin et al.,

1991). Axillary bud induction for micropropagation can be considered as an extension of the more traditional methods of propagation. Buds from a desired plant are placed on an appropriate culture medium under specific growth conditions to enhance production of axillary shoots. Subculture of the buds and shoots is repeated until many plants are produced, all having the genetic characteristics of the original plant (Hussey, 1978 and 1983). Clones, derived from axillary buds, maintain genetic stability with less risk of mutation that may arise during organogenesis from callus or suspension culture (Vasil and Vasil, 1980; Pierik, 1987).

3.1.1 Explants used for the Induction of Axillary Buds

Shoot explants for axillary bud initiation have been taken from mature trees as well as from very juvenile parent stock. Gupta and Durzan (1985) obtained shoot multiplication from mature trees of Douglas fir and sugar pine. Lateral branches (100-200 mm length), containing preformed apical and axillary buds, were collected from the lower branches. In vitro shoot formation among seventeen year-old Douglas fir provenances was investigated by Dunstan et al. (1986). Portions of first and second order branches bearing vegetative buds were harvested from the lower two-thirds of each tree. Bud scales were excised and the meristematic dome was removed from the exposed shoot axis, giving rise to a "bud-base" explant. Amerson et al. (1988) developed a micropropagation procedure for the

propagation of loblolly pine from fully mature eleven year-old explants maintained as grafts in a greenhouse. Those researchers severely hedged the parent tree so as to stimulate the production of shoots exhibiting juvenile morphology with elongated primary needles.

Work with explants from young (five to seven year-old) trees included that of Mehra-Palta and Smeltzer (1978)(loblolly pine), Stiff et al. (1985)(mugo pine) and Stiff et al. (1989)(white pine). With respect to juvenile material, axillary shoot production was achieved also by Abdullah et al. (1986) using excised shoot explants from Pinus brutia seedlings. Baxter et al. (1989) described a method for the production of clonal plantlets of Pinus caribaea, P. oocarpa and P. tecunumanii. All shoots used were taken from clones of shoots derived from epicotyls or were inter-cotyledonary axillary shoots of seedlings freshly germinated under sterile conditions. Skidmore et al. (1988) took shoot explants, ranging in height from 8 to 30 mm, from a clone of P. caribaea. All the explants had the same history of subculturing, being derived originally from a single embryo excised six months previously and subsequently cultured in vitro on LP medium.

3.1.2 Axillary Bud Induction

A number of very different strategies have been employed by other workers for the induction of axillary budding. In

most of the reported work, the presence of the cytokinin 6-benzyladenine (BA) is essential for bud break. To induce the growth of the primary axillary buds, Abdullah et al. (1986) placed Calabrian pine explants on a modified SH medium. This medium was supplemented with BA alone at a concentration of 0.7 mg l^{-1} or 1.1 mg l^{-1} or together with kinetin, each at 2 mg l^{-1}. After six weeks, the largest number of induced primary shoots was produced from explants placed on SH medium containing 2 mg l^{-1} BA and 2 mg l^{-1} kinetin. Those explants that were cultured with their apical buds removed produced more primary axillary buds than those that were cultured as entire shoot explants with apical buds. During culture, many secondary buds were also observed at the base of the newly formed primary shoots. Mehra-Palta and Smeltzer (1978) placed loblolly pine needle fascicles on GD medium with different cytokinin and auxin combinations to induce multiple adventive buds. Multiple adventitious buds were cultured on media containing higher cytokinin concentrations. Disinfected mugo pine fascicles were dipped for five seconds in a 2 mg l^{-1} NAA solution, and then cultured on a hormone-free medium. Fascicular buds were also placed in media containing BA and NAA. Those fascicles dipped aseptically in NAA exhibited bud elongation, axillary bud development and basal callus, but not as extensively as seen in the BA/NAA treatments (Stiff et al., 1985). Amerson et al. (1988) dipped sterilised juvenile loblolly pine shoots intact for one to two minutes in 0.1 g l^{-1} BA dissolved in 70% ethanol. The ethanol was allowed to evaporate from the shoots and, after receiving

a fresh basal cut, they were placed into culture on GD medium containing 5 to 10 g l^{-1} charcoal and 20 g l^{-1} sucrose. Within two to four weeks of the BA dip, responsive plants produced swollen buds in many of their leaf axils.

However, there have been reports on axillary bud induction without the use of plant growth regulators. Baxter et al. (1989) cultured the initial shoot explant of three pine species on half-strength MS medium without growth regulators. The shoot was allowed to elongate for six weeks and then was divided into a tip and one or more nodal segments. Subsequent growth allowed the excised tip to elongate and axillary shoots to form on, and grow out from, the nodal segments. Gupta and Durzan (1985) found that, within four to five weeks, buds were opened on 70-80% of the shoots on DCR basal medium without any growth regulators both from Douglas fir and sugar pine.

Other approaches to axillary bud induction have been attempted by a number of researchers. Dunstan et al. (1986) cultured the Douglas fir explants on medium containing 1 mg l^{-1} BA and 400 mg l^{-1} NH_4NO_3 for the first six weeks. Stiff et al. (1989) cultured western white pine fascicle buds in vitro. Bud elongation and secondary needle growth was obtained on GD medium containing 1 mg l^{-1} BA with an increased concentration of KNO_3. Skidmore et al. (1988) described a novel method whereby shoot apices of a clone of Pinus caribaea were cultured and multiplied in vitro by

supporting them with their basal cut ends immersed in a liquid nutrient medium. Shoot explants were first cultured on a bud induction medium comprising liquid LP medium supplemented with 2 μg ml^{-1} BA. Lin et al. (1991) reported the first in vitro induction of axillary buds from mature ponderosa pine. After disinfection the shoot apex was removed and each immature shoot explant was cut into three transverse segments and the scales removed. Explant elongation occurred and basal needle primordia swelled on MS media containing 0.5 mg l^{-1} BA and 1 mg l^{-1} NAA. When transferred to a MS medium containing 1.0 mg l^{-1} BA, 59% of explants formed axillary buds (Lin et al., 1991).

3.1.3 Elongation and Growth of the Axillary Buds

Once the axillary buds have formed on the shoot explant, the buds need to be subcultured on to a medium that will allow the establishment of a well-formed shoot. It appears that the trigger for the elongation of the dwarf shoot is the removal of growth regulators either by changing the cultures to a hormone-free medium and/or the addition of activated charcoal. For example, Calabrian pine axillary shoot elongation was achieved on both half-strength SH medium without cytokinin and on the same medium containing 10 g l^{-1} activated charcoal (Abdullah et al., 1986). The presence of activated charcoal decreased the incubation time by two weeks. Other workers found that when newly flushed buds were excised and transferred to half-strength

DCR basal medium with 3 g l^{-1} activated charcoal (DCR-1), all the flushed shoots elongated and formed complete shoots within five to six weeks for Douglas fir and seven to eight weeks for sugar pine (Gupta and Durzan, 1985).

Prior to Douglas fir shoot remultiplication, Dunstan et al. (1986) made a fresh cut to the base of any shoots that were being recycled onto fresh nutrient medium. This medium contained neither BA or NH_4NO_3. Skidmore et al. (1988) reported that after 21 days in culture, on bud induction medium, Pinus caribaea explants were placed in new containers and incubated for a further four weeks on the shoot elongation medium to allow elongation of the induced axillary buds. In a comparison between the growth of the P. caribaea shoot explants on liquid and solid media, shoots incubated on the liquid medium showed significantly greater increases in length in a four-week period than those cultured on solid medium.

3.1.4 Rooting and Hardening-off of Shoots

The final step in obtaining a plantlet from axillary buds is the rooting of the newly formed shoot. Once a root system has been established, the plantlet needs to be acclimatised to greenhouse conditions (hardening-off) before being planted in the field. Rancillac et al. (1982) studied the rooting of Pinus pinaster shoots derived from axillary budding in vitro. Those workers reported that an

agar medium was effective for root initiation but a permeable substrate such as peat-perlite mixture seemed more suitable for the continuing development of the root system. For hormonal treatment, explants submitted to a twelve day period with NAA (0.2 mg l^{-1}) and then subcultured in an auxin-free medium, gave rooting percentages ranging from 80 to 100%. In contrast, an *in vitro* dip in 2 g l^{-1} NAA promoted the formation of white glassy callus, potential root primordia, and root-growth in 33% of the western white pine explants (Stiff *et al.*, 1989). Only 10% of the explants developed elongated roots 120 days after initial culture.

Another reported approach to rooting of *in vitro* shoots is that of Baxter *et al.* (1989) who set shoots of three pine species in 2:1 peat-perlite medium. The shoots were sprayed initially with fungicide solution; no auxin treatment was used. Mean rooting percentages in the region of 50% were obtained after the shoots had remained in a sealed propagator, with a daily misting of water, for seven weeks. Once rooted, the plants continued to develop normally and could be hardened for greenhouse growth.

3.1.5 Aim of this Investigation

In this study, the potential of existing axillary buds as a source of material for the *in vitro* multiplication of *Pinus patula* was evaluated. This chapter presents a

systematic study of the induction of shoots derived from axillary budding with the purpose of implementing rooted plantlets into the patula pine vegetative production program. The objectives thus were to investigate the influence of plant growth regulators and salt formulations on the formation of shoots from axillary buds. Once shoots were formed, further work investigated the elongation of these shoots and then the rooting abilities of the shoots.

3.2 MATERIALS and METHODS

3.2.1 Preparation of the Parent Material

One year-old P. patula seedlings (Seed Lot No. M4562) were each potted in a litre of composted pine bark growing medium. All plants were kept in a nursery tunnel which was covered with Patilux® plastic and surrounded with a 55% shade-cloth windbreak. The seedlings were watered twice a day from a deep-drenching boom system. Fertiliser was applied twice weekly in the form of soluble Hortichem® (N 21.2%, P 7.1%, K 14.2%) at 600 μS conductivity. The apices of the seedlings were removed to allow for bud break just below the severed tops (Plate 3.1). Juvenile shoots that emerged below the severed apex were ready for harvesting at ten weeks from the time of topping the seedlings (Plate 3.2).

3.2.2 Surface Sterilisation of the Shoot Explant

Juvenile shoots were removed from the parent seedling by cutting the base of the shoots with clean scissors. The shoots were then placed in 1 ml l^{-1} Bravo-500® (Appendix 2) contact fungicide for one hour. The apex was removed from each shoot and the needles trimmed along the stem of each shoot. Each shoot was cut to a 55 mm length. Shoots were rinsed well under running tap water before being surface sterilised with 0.02% mercuric chloride ($HgCl_2$) for three to four minutes, with constant agitation. After a thorough rinse in sterile reverse osmosis (RO) water the shoots were

Plate 3.1 One year-old P. patula seedlings with new juvenile shoot formation just below the severed apicies. Scale bar= 50 mm.

Plate 3.2 Ten week-old juvenile shoots ready to be harvested as explants for in vitro axillary bud induction. Scale bar= 20 mm.

aseptically placed in the culture vessels.

3.2.3 Culture Conditions for Axillary Bud Induction from the Shoot Explants

Each juvenile shoot was aseptically placed in a glass tube containing 10 ml of DCR induction medium. Tubes were kept in the culture room with a 16 hour photoperiod at 740 mols m^{-2} s^{-1}. The temperature of the culture room was maintained at 24 °C (± 2 °C). The juvenile shoot explants were kept under these conditions until the dwarf shoots, formed from induced axillary buds, were large enough (30-40 mm in height) to be excised from the parent shoot. Then these dwarf shoots were transferred aseptically to the shoot elongation medium. Parameters investigated for axillary bud induction were the trimming of the explant material, the effect of cytokinin concentrations and salt formulations, the explant size and growth regulator pulses.

3.2.4 Culture Conditions for Dwarf Shoot Elongation

Under aseptic conditions, the explant shoots were removed from the induction medium and the dwarf shoots severed from the parent shoot with a cut at their base. The dwarf shoots were soaked in an antibiotic cocktail for 5-10 minutes. This cocktail consisted of four antibiotics in 100 ml of sterile RO water [Zinacef® (17 mg), Novo-Strep® (56 mg), Novocillin® (50 mg) and Claforan® (42 mg)] (Appendix 2). Each individual shoot was transferred to a glass tube

containing 10 ml of elongation media. The elongation medium comprised of half-strength DCR salts, 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar, 10 g l^{-1} activated charcoal, pH 5.7. Shoots were allowed to elongate under the same culture conditions as for shoot induction. When the dwarf shoots had elongated to 60-70 mm, they were transferred to the greenhouse for rooting. Only the rate of axillary shoot elongation was investigated on this medium.

3.2.5 Rooting Conditions for the Dwarf Shoots

On removal from the elongation medium the dwarf shoots were set directly into a pasteurised composted pine bark growing medium, which had been drenched with 1 ml l^{-1} Bravo-500 fungicide (Appendix 2). Each dwarf shoot was set in an unigro® insert, each containing approximately 66 ml of bark medium, before being placed in the greenhouse. Parameters investigated for rooting were basal wounding of the shoot and plant growth regulator applications. During hardening-off the shoots received frequent misting (10 seconds every 25 minutes, between 6.00 am and 8.00 pm) for the first two weeks, with a gradual reduction in misting (30 seconds every 30 minutes, 8.00 am - 9.00 am and 12.00 pm - 8.00 pm) over the following weeks.

3.2.6 Data Analysis

Analysis of the data, in this chapter, was as described in section 2.2.4.

3.3 RESULTS and DISCUSSION

3.3.1 Sterilisation of the juvenile Shoot Explants

The juvenile shoots, collected from the pruned stock plants, contained both endogenous and exogenous bacterial and fungal contaminants. Before such shoots could be used as explants for axillary bud initiation in vitro, they required a thorough sterilisation treatment. Gupta and Durzan (1985) and Lin et al. (1991) described extensive sterilisation treatments on their sugar pine and ponderosa pine shoots, respectively. However, a much quicker and simpler sterilisation method was implemented in this work. Various chemical sterilants were tested as decontaminants (Figure 3.2). Only the 0.02% mercuric chloride ($HgCl_2$) treatment was found to be effective for surface sterilisation of the explants. Mercuric chloride has been used successfully for sterilising Eucalyptus species (le Roux and van Staden, 1991). However, the use of $HgCl_2$ as a surface sterilant for pine shoots has not been reported in literature reviewed to date. The hazardous nature of this chemical could be the main reason for the reluctance of researchers to use mercuric chloride. The optimum soaking time required when using the $HgCl_2$ treatment was found to be between 4-5 minutes (Figure 3.3). Soaking the shoots in 0.02% $HgCl_2$ for longer periods resulted in browning of the shoots and their eventual death.

3.3.2 Induction of Axillary Buds

In all plant species the apical bud exerts an inhibitory

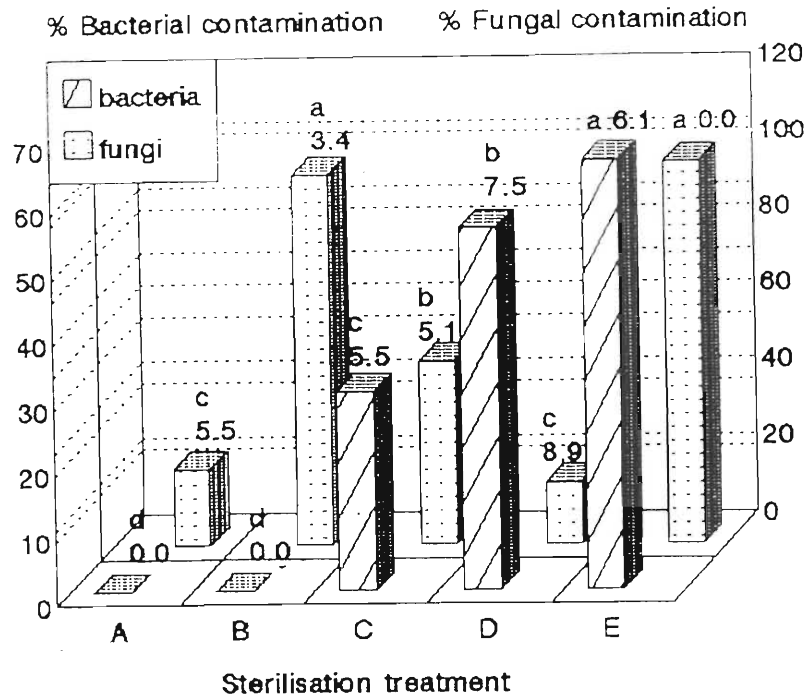

Figure 3.2 The effect of various chemical agents on the sterilisation of juvenile explant shoots. Shoots were soaked in 1 ml l^{-1} Bravo 500 contact fungicide (30 min) and then rinsed with tap water before the sterilisation treatment. The treatments tested were: A= 0.02% $HgCl_2$ (2 min); B= 1% H_2O_2 (1 min); C= 70% ethanol (2 min); D= 1.3% NaOCl (2 min); E= control (sterile water). All explants were rinsed in sterile RO water after each treatment. The shoots were placed on to ½MS, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, 0.5 mg l^{-1} BA, pH 5.7. Results were recorded after 1 week in culture and indicate the percentage of explants having bacterial and/or fungal contamination present. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

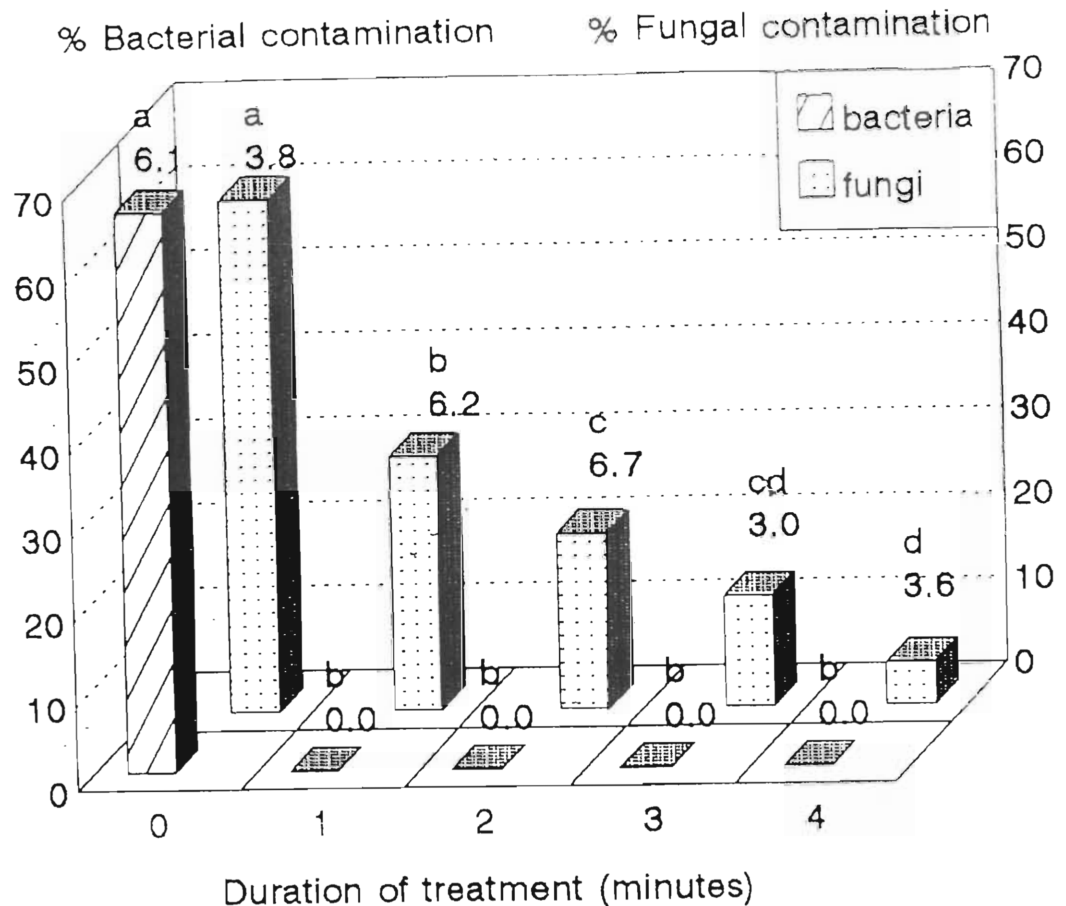

Figure 3.3 **Optimising the mercuric chloride sterilisation procedure for the juvenile shoot explant.**

Shoots explants were soaked in 1 ml l^{-1} Bravo-500 contact fungicide (1 hour), followed by a rinse in sterile RO water before the 0.02% $HgCl_2$ treatment. Then the shoots were cultured on ½MS media, containing 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, 0.5 mg l^{-1} BA, pH 5.7. The data shows the percentage of explants having bacterial and fungal contamination after 1 week in culture. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

influence (apical dominance) upon the axillary buds, preventing their development. The active substance normally produced by the apical bud is an auxin (Salisbury and Ross, 1969). By removing the terminal bud, the inhibitory effect of auxin on the development of axillary buds is thought to be reduced (Robbins et al., 1966). Abdullah et al. (1986) and Lin et al. (1991) found that the removal of the apical meristematic region of calabrian and ponderosa pine explants, significantly enhanced the yield of axillary buds. Stiff et al. (1989) found that removal of the needles from western white pine shoot explants increased the induction of axillary buds. An investigation of these effects was undertaken (Figure 3.4). There was no significant difference between the number of shoots formed on each explant in all the treatments, apart from the treatment where only the apex was removed (-a,+n). In this treatment, the number of shoots formed on each explant was much lower than the other treatments. However, the largest number of explants, possessing axillary shoots, was obtained from the treatment where both the apices and needles were removed. The number of shoots, formed on each explant, was also large with this same treatment. As a result of these findings all juvenile shoot explants, used in the axillary bud work, had their apices removed and needles trimmed before being placed in culture (Plate 3.3).

The next important factor examined was the effect of cytokinin levels in the medium on the induction of axillary buds. David (1982) noted that amongst the cytokinins that are generally used for axillary bud induction (kinetin, zeatin, and BA), BA was the most effective one if used at

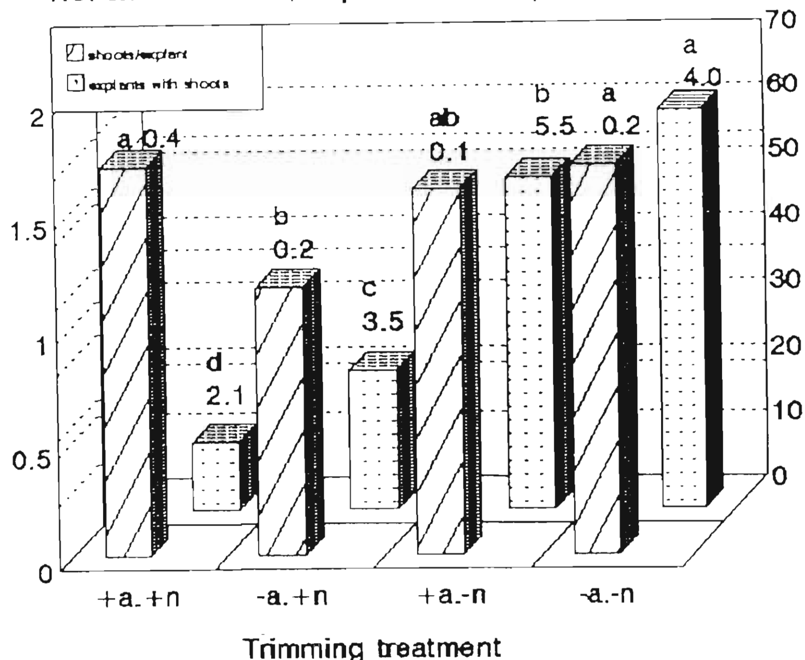

Figure 3.4 The effect of trimming parts of the juvenile shoot explant on dwarf shoot formation.

Explants received a 1 ml l^{-1} Bravo-500 contact fungicide soak for 1 hour, after which they were rinsed with tap water, and surface sterilised with 0.02% $HgCl_2$ (4 min). After a thorough rinse in sterile RO water, the explants were placed on DCR media, 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar, 0.5 mg l^{-1} BA, pH 5.7 and cultured at 24 °C (±2 °C) in the light. The trimming treatments were: +a.+n= apex and needles remaining (control), +a.-n= only needles removed, -a.+n= apex removed and -a.-n= both apex and needles removed. The mean number of dwarf shoots formed on each explant and the percentage of explants on which dwarf shoots developed from the induced axillary buds was recorded after 8 weeks. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

concentrations of 2.3 mg l^{-1} to 11 mg l^{-1}. David (1982) also stated that auxin (NAA) is often used, in combination with the cytokinin, at concentrations of 1.9 μg l^{-1}. In this study, a BA concentration of 0.75 mg l^{-1} was found to be the best suited for the induction of P. patula axillary buds with regard to the number of explants forming dwarf shoots (Figure 3.5)(Plate 3.4). Increasing the concentration of BA from 0.75 mg l^{-1} to 2.5 mg l^{-1} did not further increase the number of explants on which dwarf shoot formation occurred, but it did significantly increase the number of dwarf shoots formed on an individual explant. Nevertheless, in total, the 0.75 mg l^{-1} BA concentration resulted in a greater yield of dwarf shoots. Abdullah et al. (1986) found that the maximum number of vigorous calabrian pine shoots produced per explant occurred with 0.70 mg l^{-1} BA and a higher concentration of BA (1.1 mg l^{-1}) inhibited shoot development. In this study, no auxin was added to the patula culture media as Rancillac (1977 and 1981) found that auxin did not stimulate axillary bud development but rather stimulated callus formation instead.

As discussed previously (section 3.1.2) different media salt formulations have been used by various workers for axillary bud culture. The most popular medium formulation has been the Murashige and Skoog (1962) MS salts (Rancillac et al., 1882; Baxter et al., 1989; Lin et al., 1991). The Schenk and Hildebrandt (SH) formulation has been used successfully by Abdullah et al. (1986) on their Calabrian pine cultures, as was the DCR basal medium used by Gupta and Durzan (1985) on Douglas-fir and sugar pine. Media formulations employed for other types of conifer culture

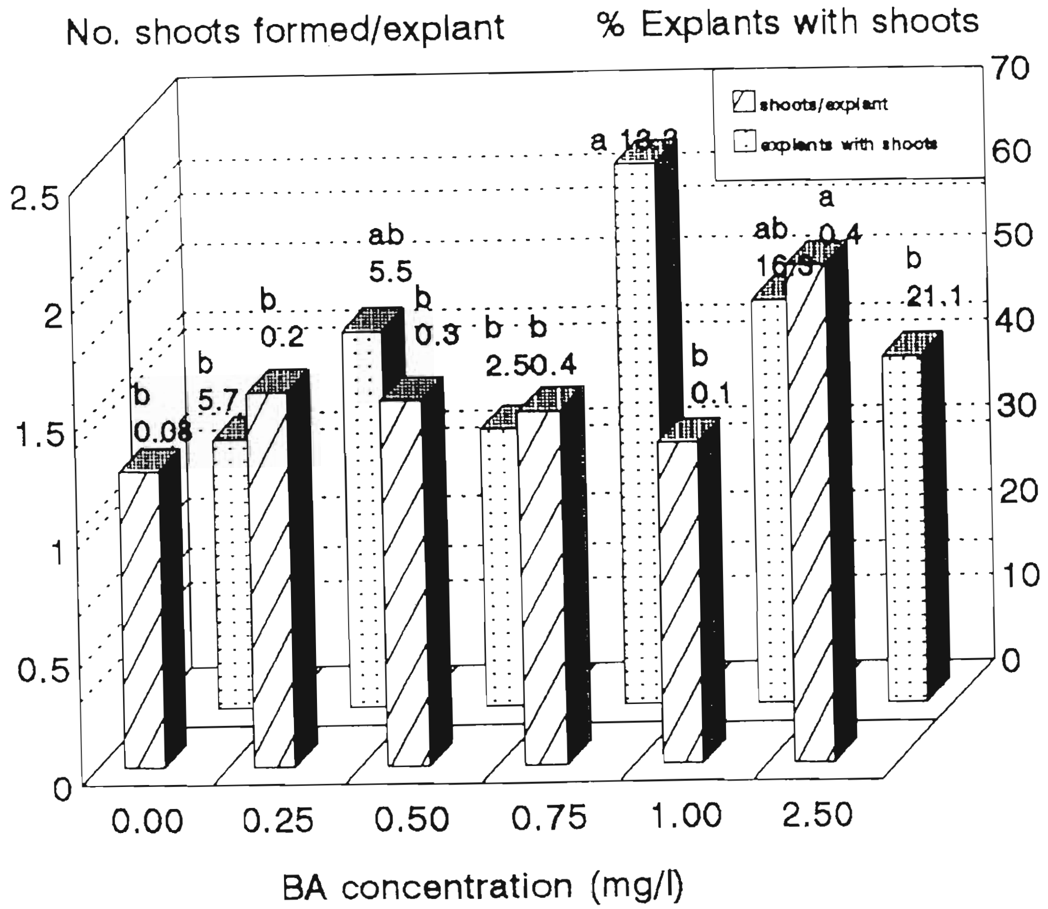

Figure 3.5 The effect of BA concentrations on the formation dwarf shoots.

Explants were collected in 1 ml l^{-1} Bravo-500 contact fungicide (30 min) and the apices and needles were trimmed off. Sterilisation and culture procedures were as described in Figure 3.4. Explants were placed on DCR medium, containing 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7. Data was recorded after 8 weeks in culture. The results shown are from two types of assessments, the percentage of explants having one or more dwarf shoots formed and the mean number of dwarf shoots formed on each explant. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

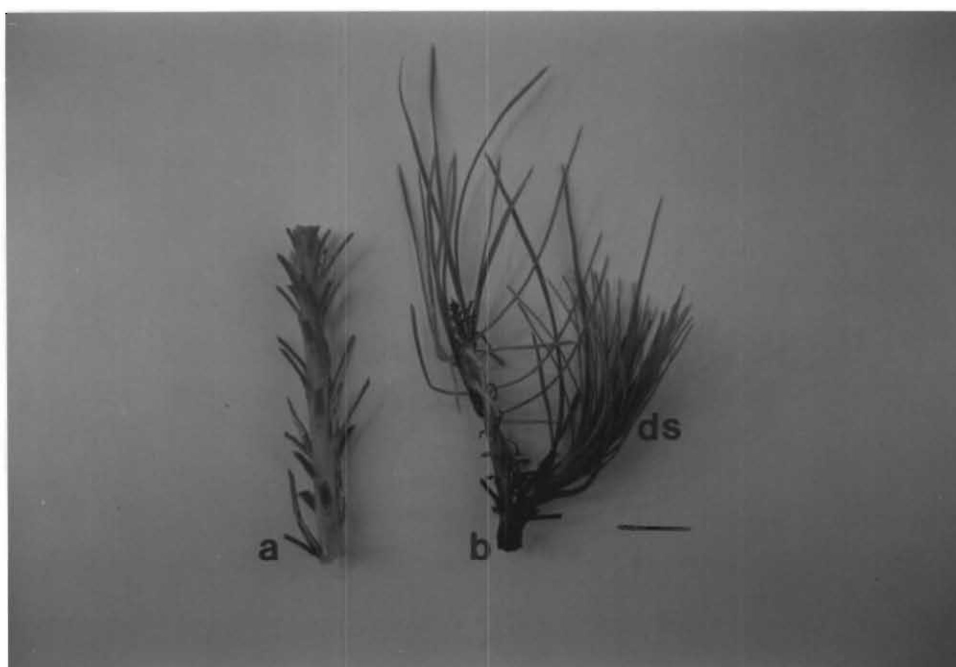

Plate 3.3 a) Juvenile shoot explant, with apex removed and needles trimmed back, before being placed in culture. b) The formation of a dwarf shoot (ds) after the explant has been in culture for 12 weeks. Scale bar= 10 mm.

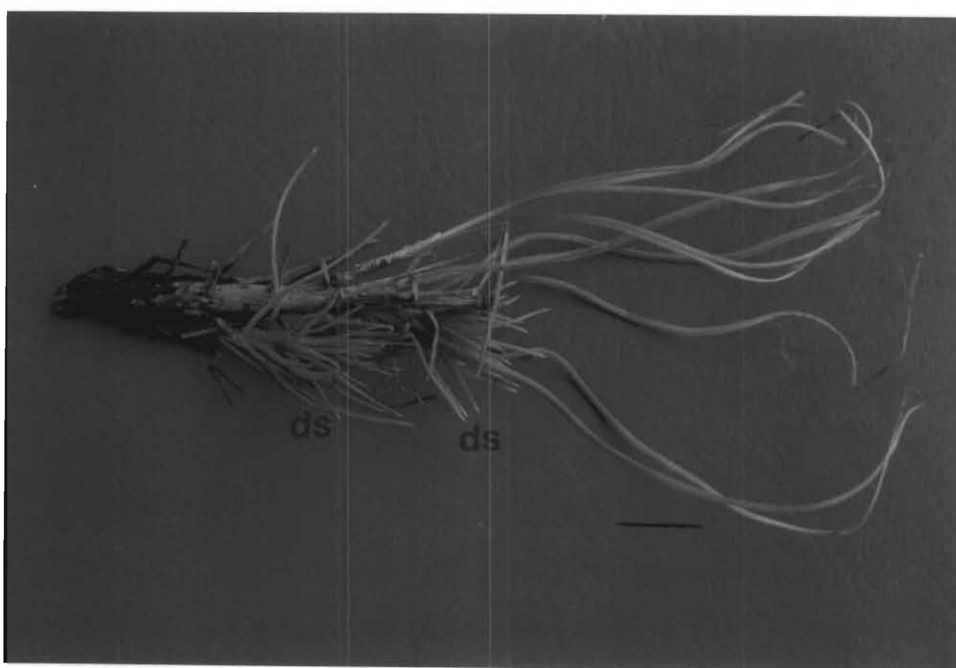

Plate 3.4 Dwarf shoot (ds) formation from axillary buds after 10 weeks in culture. Scale bar= 10 mm.

were also investigated in this study. The LM formulation was included as this was the medium of choice for both the P. patula adventitious bud (Chapter 2) and somatic embryogenesis (Chapter 4) work in this study. Results shown in Figure 3.6 indicate that while the DCR medium produced a higher percentage of explants having dwarf shoots, the LM medium had a noticeable effect on the number of shoots formed per explant. David (1982) states, in his review on axillary bud culture, that a high potassium concentration in the medium promotes morphogenesis. However, both the LM and SH formulations have a similar potassium content (Appendix 1) and yet there was a significantly higher yield of dwarf shoots from explants placed on the LM media. The greatest yield of dwarf shoots were obtained from the shoot explants placed on the DCR salt formulation. The DCR formulation was, therefore, chosen as the standard salt preparation for the axillary bud induction medium.

Successful results were reported by Lin et al. (1991) on the segmenting of Pinus ponderosa shoot explants in order to increase the yield of dwarf shoots. A similar treatment was done in this P. patula work to examine whether more dwarf shoots could be obtained, by using segments of a juvenile shoot, rather than just from one unsegmented juvenile shoot explant (Figure 3.7). There was a significantly lower total yield of dwarf shoots from the shorter explants than from the longer explants. The number of dwarf shoots formed from the 15 mm segment explants would be lower, in any case, as there were fewer nodes present for axillary bud formation to take place. In fact, if the number of shoots formed on each explant, for the

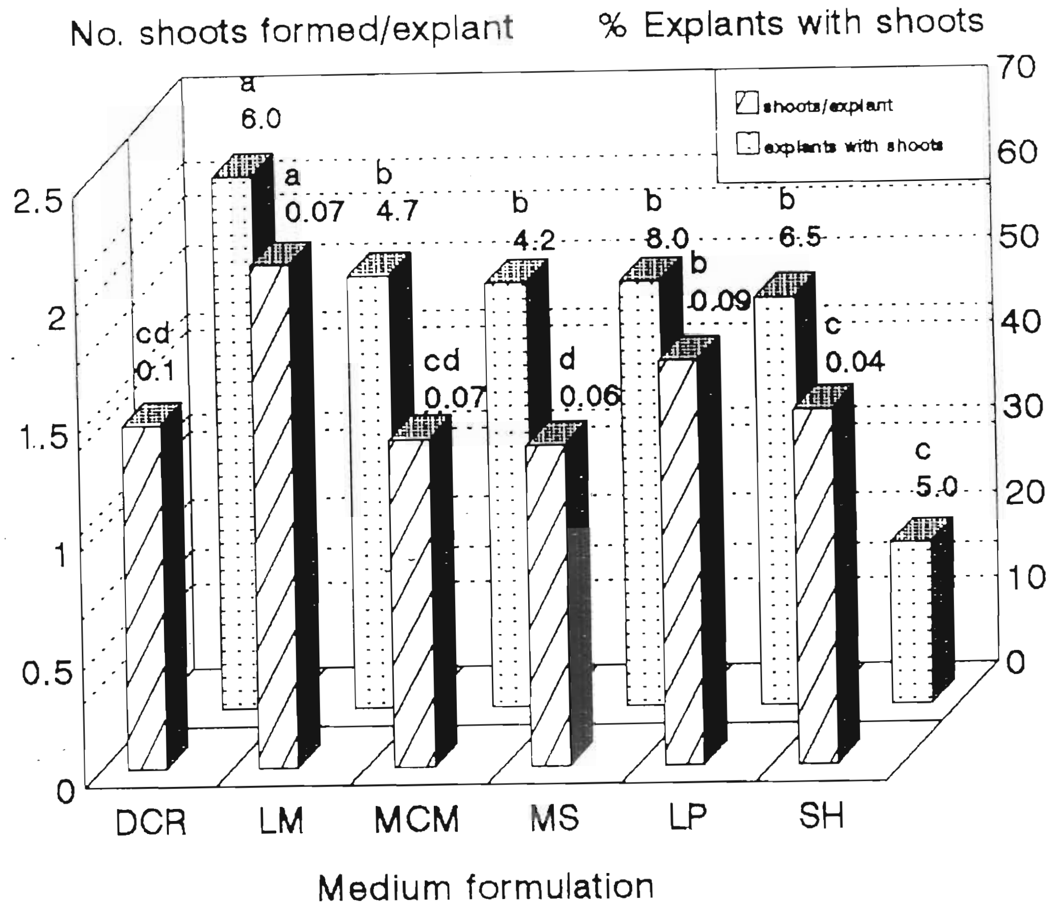

Figure 3.6 The effect of reported media salt formulations on the formation of dwarf shoots.

Sterilisation and culture procedures were as described in Figure 3.4. Salt formulations are: MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), MCM (Bornman, 1981),LM (Litvay et al., 1981), LP (Aitken-Christie, 1984), and DCR (Gupta and Durzan, 1985). The results shown include the percentage of explants having one or more dwarf shoots formed and the mean number of dwarf shoots formed on each explant. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

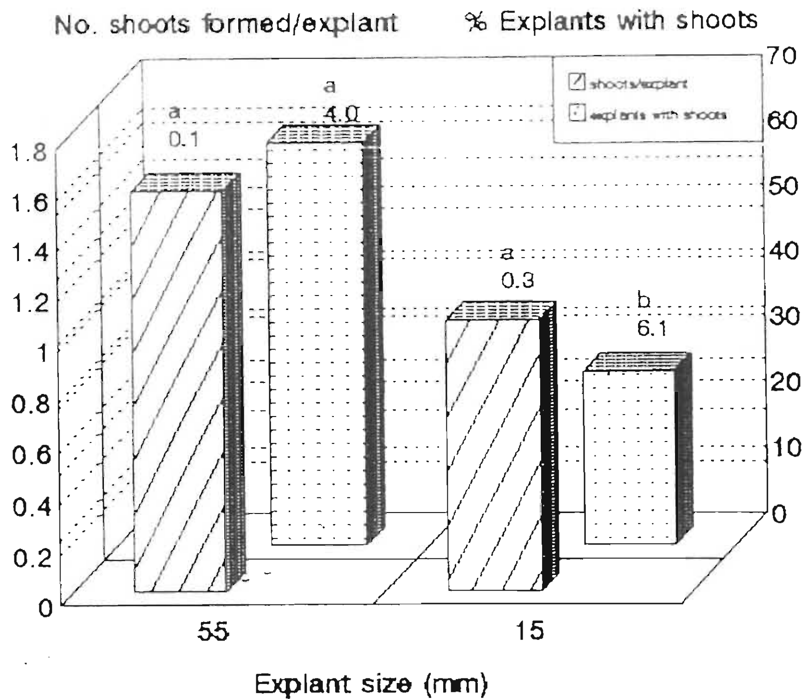

Figure 3.7 The effect of explant size on dwarf shoot formation.

Juvenile shoot explants were cut into 55 mm and 15 mm segments before being sterilised. Sterilisation and culture procedures were as described in Figure 3.4. The results shown include the percentage of explants having one or more dwarf shoots formed and the mean number of dwarf shoots formed on each explant after a 8 week culture period. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

shorter explants, were to be extrapolated by the number of short segments that would be similar to the length of a longer segment, there would be more dwarf shoots formed with the shorter segments. However, the percentage of explants, possessing dwarf shoots, for the shorter segments was significantly lower than for the longer shoot segments. Based on this result the total yield of dwarf shoots would be greater if the longer segments, used in the previous P. patula experiments, were to continue to be utilised.

Another procedure, tested in this study, was the protocol used by Amerson et al. (1988) which allowed those researchers to multiply successfully shoots of Pinus taeda obtained from mature explants (Figure 3.8). The Amerson procedure was to subject the shoot explant to a high cytokinin pulse and then place the explant on medium containing activated charcoal. These workers soaked the shoot explant in a 99 mg l^{-1} BA solution for approximately two minutes before transferring the explant to a GD medium containing 10 g l^{-1} activated charcoal. The control (Treatment A) used in Figure 3.8 was the standard procedure of placing the P. patula shoot explant on DCR medium containing 0.75 mg l^{-1} BA with no activated charcoal. Treatment B (GD medium, 10 g l^{-1} activated charcoal, no BA pulse or BA presence in the medium) was included to observe whether the BA pulse or the activated charcoal was the effective agent in the Amerson protocol. The high-concentrate BA dip, used by Amerson et al.(1988), proved to be the best treatment as regards to the formation of new P. patula dwarf shoots. In this treatment (Treatment C), the juvenile shoot explants were dipped intact for one to two

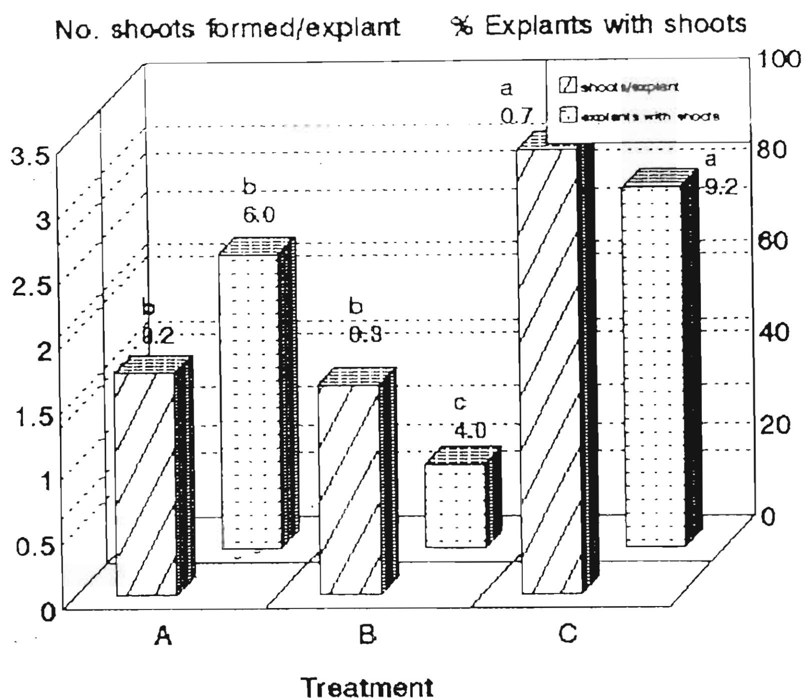

Figure 3.8 **The effect of using various protocols to induce dwarf shoot formation.**

Treatments are: A) DCR, 0.75 mg l^{-1} BA, 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7; B) GD, 10 g l^{-1} activated charcoal, 20 g l^{-1} sucrose, 4 g l^{-1} gelrite, pH 5.7; C) 99 mg l^{-1} BA soak (1-2 min), GD, 10 g l^{-1} activated charcoal, 20 g l^{-1} sucrose, 4 g l^{-1} gelrite, pH 5.7 (Amerson et al., 1988). Sterilisation and culture procedures were as described in Figure 3.4. The results shown include the percentage of explants having one or more dwarf shoots formed and the mean number of dwarf shoots formed on each explant after 7 weeks in culture. n= 30-40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

minutes in 99 mg l^{-1} BA (dissolved in 70% ethanol) and the ethanol was allowed to evaporate from the shoots before being placed in culture. Within two to four weeks of the BA dip, responsive patula explants produced swollen buds in many of their leaf axils. After seven weeks in culture the physical appearance of the micropropagated shoots (Plate 3.5) was very different to the dwarf shoots that had developed in previous studies (Plate 3.4). Shoots, that initially formed primary needles, elongated only 10 to 20 mm, then produced apical fascicles and little subsequent growth. This same effect was reported in the paper produced by Amerson et al. (1988). Treatment B was identical to treatment C except for the BA application to the explants. The number of juvenile shoot explants that produced dwarf shoots in this treatment (B) was very low. Although the number of dwarf shoots, formed under treatment A, was lower than those formed under treatment C, there was normal development of the dwarf shoots. Another interesting observation in this experiment was the fact that the number of shoots, formed per explant, was similar in both treatments A and B. Treatment A contained BA, but no activated charcoal whereas treatment B contained no BA, but activated charcoal was present in the medium. The use of activated charcoal could be an important factor for dwarf shoot formation. Amerson et al. (1988) found that 66% of explants produced new shoots when charcoal was present in the medium whereas, in contrast, only 3% of explants produced any new shoots when charcoal was absent. The other differences between treatments A and B, namely the sucrose concentration and gelling agents used, could also have been responsible for the differences detected.

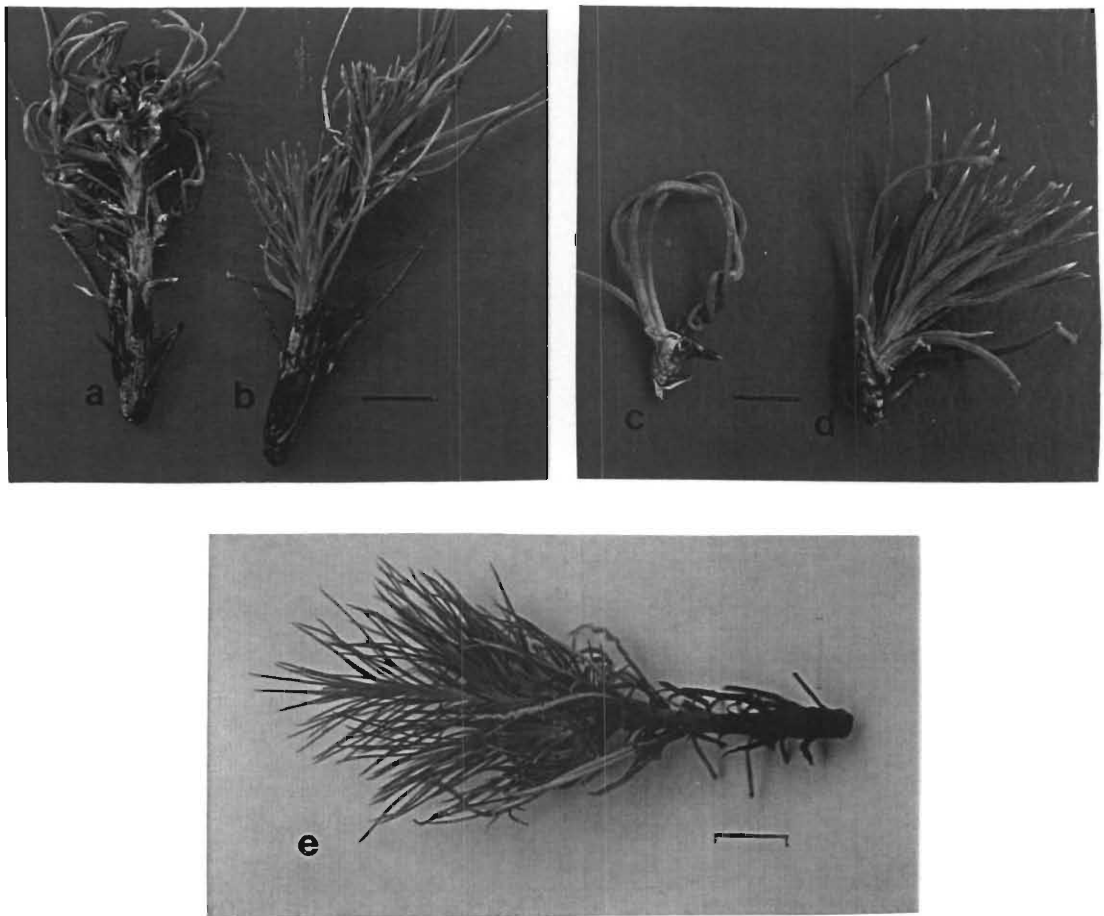

Plate 3.5 The effect of a 2 minute pulse with 99 mg l^{-1} BA on the formation of dwarf shoots.

a) Axillary bud break after 7 weeks on Amerson et al. (1988) medium showing poor dwarf shoot formation.

b) Normal dwarf shoot formation after 7 weeks on DCR 0.75 mg l^{-1} induction medium. Scale bar= 10 mm.

c) Isolated abnormal dwarf shoot from the explant shown in (a) and d) isolated normal dwarf shoot from explant shown in (b). Scale bar= 5 mm.

e) Dwarf shoot development after 16 weeks on the Amerson et al. (1988) medium. Scale bar= 10 mm.

3.3.3 Elongation and Rooting of Dwarf Shoots

Figure 3.9 and Plate 3.6 illustrate a study on the rate (±10 mm/ 4 weeks) of dwarf shoot elongation and the appearance of the elongated shoots, respectively. Due to the low numbers of elongated dwarf shoots that were ready for rooting, a study on the rooting capabilities of Pinus patula was done using the same juvenile shoots used as explant material for the axillary bud induction studies. The results obtained (Figure 3.10) give an indication of what treatment to use for rooting studies on dwarf shoots. The treatments tested were the basal wounding of the dwarf shoot and the application of a few commercial rooting agents. The shoots that received only a vertical basal wound, using scissors, rooted much better than those on the other treatments. Basal wounding is a common treatment used in horticulture as wounded tissues are stimulated into cell division and production of root primordia (Hartman and Kester, 1883). The highest percentage rooting, however, was expected from the two rooting compound treatments Seradix 3® (8 g kg^{-1} IBA) and Rootstim® (0.57 g kg^{-1} IBA and 0.33 g kg^{-1} NAA)(Appendix 2). Haissig (1986) states that applied synthetic auxins are the only chemicals that have consistently enhanced adventitious root formation in diverse species of cuttings under a wide-ranging test conditions. IBA has been the most used auxin for propagation of recalcitrant (usually woody) species of cuttings, and is present in most commercially produced formulations used to promote rooting of cuttings (Blazich, 1988). The reason why the rooting percentage was so low with treatment 4 (wounding and Seradix 3 application

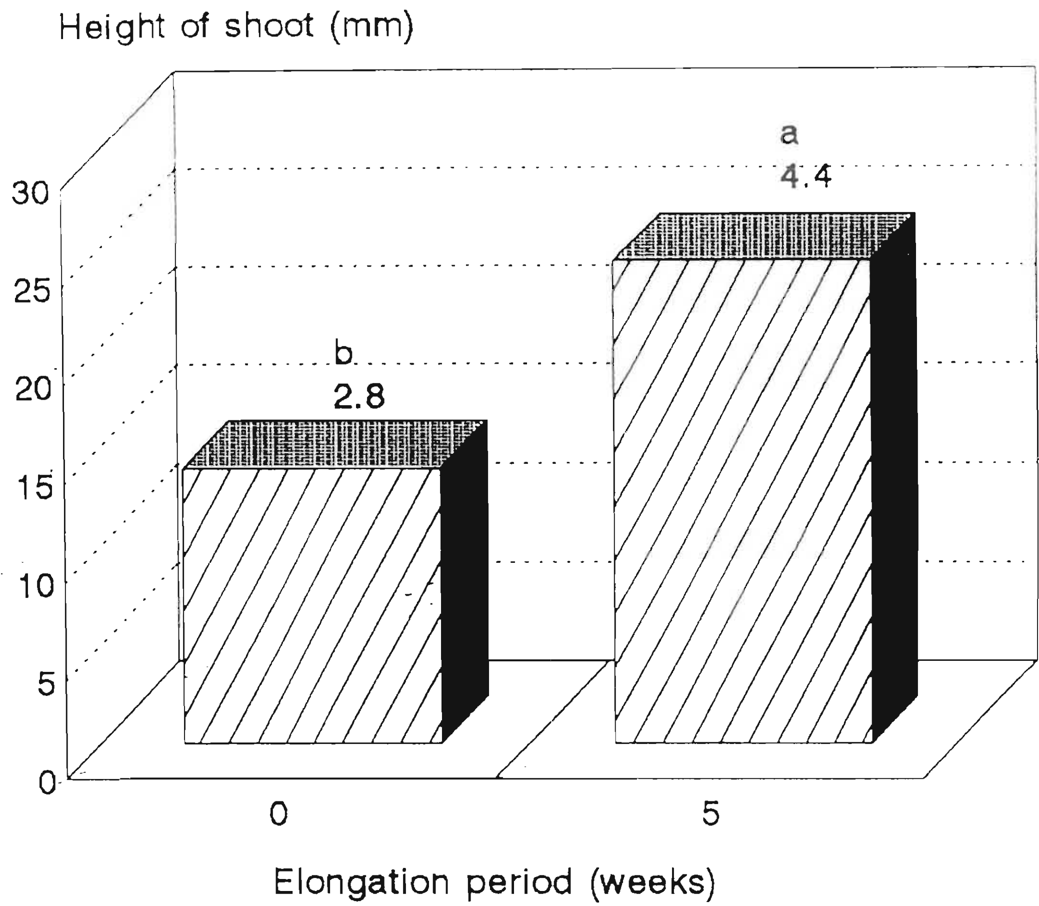

Figure 3.9 **The rate of dwarf shoot elongation.**
Dwarf shoots were removed from the juvenile shoot explants after 10 weeks in culture, soaked in an antibiotic cocktail for 5 minutes and placed on half-strength DCR, 10 g l^{-1} activated charcoal, 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.8. n= 25 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

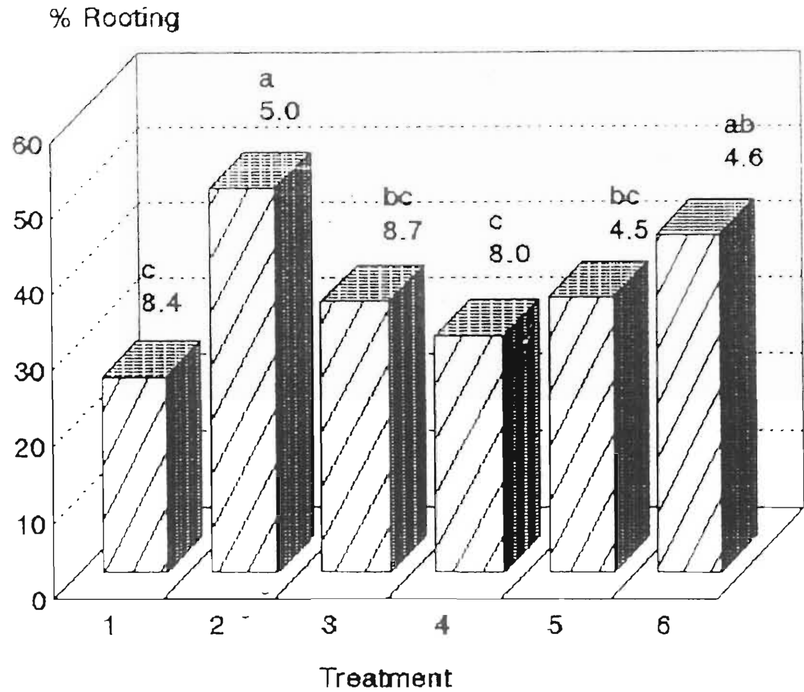

Figure 3.10 The effect of various rooting treatments on root formation from juvenile shoots.
Juvenile shoots (10 week-old) received the rooting treatments and then were set in pasteurised bark under greenhouse conditions. Rooting treatments were: 1) no basal wounding, no rooting hormone application; 2) basal wounding, no hormone application; 3) no wounding, Seradix 3 application; 4) wounding, Seradix 3; 5) no wounding, Rootstim; 6) wounding, Rootstim. Results were recorded after 5 months. n= 40 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

together) is unknown. Plate 3.7 shows the type of root formation obtained from a cultured dwarf shoot; there is a distinct tap root but very poor lateral root formation.

3.3.4 Concluding remarks

In this study axillary buds were induced on juvenile shoot segments. The juvenile shoot explants were surface sterilised with 0.02% $HgCl_2$ before being placed in DCR induction medium containing 0.75 mg l^{-1} BA. The axillary buds developed into dwarf shoots over a ten week period. Only about 60% of shoot explants produced dwarf shoots and of these approximately two dwarf shoots formed per explant. When the dwarf shoots were approximately 30 mm in length they were removed from the explant shoot and soaked in an antibiotic cocktail for 5-10 minutes to remove bacterial contaminants. The dwarf shoots were then placed in half-strength DCR elongation medium, containing 10 g l^{-1} activated charcoal, for 2-3 months when they were ready for rooting. Rooting studies were performed on juvenile shoots. The highest number of rooted cuttings (53%) was obtained from the basal wounding treatment.

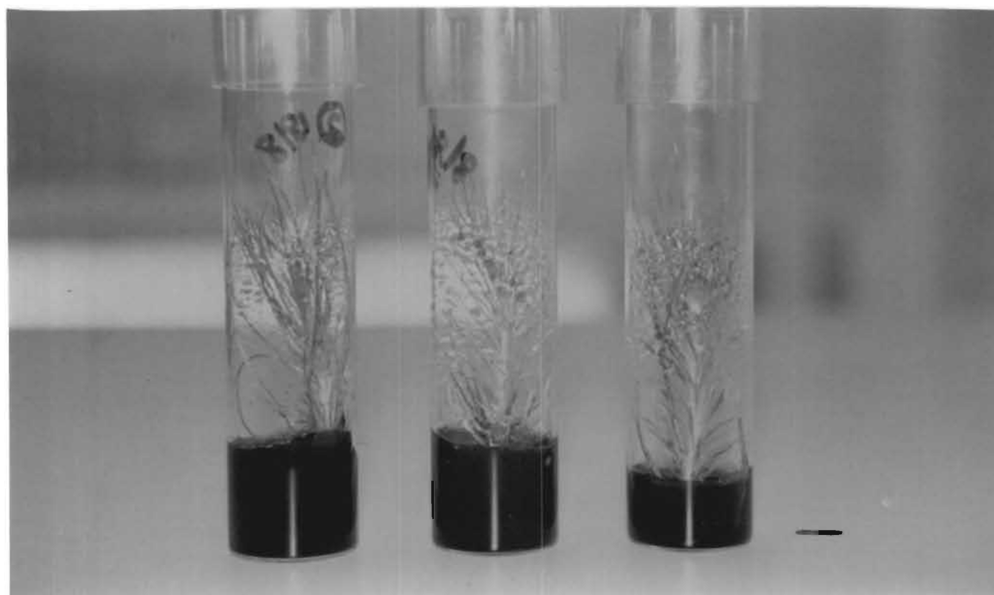

Plate 3.6 Dwarf shoot elongation on half-strength DCR medium containing 10 g l^{-1} activated charcoal. Scale bar= 10 mm.

Plate 3.7 Root formation from an elongated dwarf shoot after 16 weeks in bark medium. The dwarf shoot was cultured on half-strength DCR containing 10 g l^{-1} activated charcoal for 12 weeks before being placed in the greenhouse for rooting. Scale bar= 20 mm.

CHAPTER 4

In vitro CULTURE OF Pinus patula VIA SOMATIC EMBRYOGENESIS

4.1 INTRODUCTION AND LITERATURE REVIEW

The act of fertilisation, in the sexual reproductive cycle of plants, triggers the egg cell (known as the zygote, after fertilisation) to divide and develop into an embryo, through the process known as embryogenesis. However, fertilisation is not always essential to stimulate the egg to undergo embryogenesis (Haccius, 1978; Bhojwani and Razdan, 1983; Ammirato, 1989; Jones, 1990). The pollination stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development (Bhojwani and Razdan, 1983; Williams and Maheswaran, 1986; Komamine et al., 1990). Moreover, it is not the monopoly of the egg to form an embryo. Any cell of the female gametophyte (embryo sac), or even that of the sporophytic tissues around the embryo sac may give rise to an embryo (Bhojwani and Razdan, 1983). Somatic embryogenesis is the process by which previously unspecialised vegetative (somatic) cells develop into entire plants through a series of stages characteristic of zygotic embryo development (Ammirato, 1973; Wann, 1988).

4.1.1 Phases and Events in Conifer Somatic Embryogenesis

Very little is currently known about the origin and development of somatic embryos in conifer culture (Tautorus

et al., 1991). The origin of somatic embryos may also vary depending on the type of explant used (Nagmani et al., 1987; Finer et al., 1989). Hakman et al. (1987) suggested three different processes that could account for the origin of conifer somatic embryos: (a) somatic embryos may arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryonal apex and suspensor region; (b) somatic embryos may develop from small meristematic cells within the suspensor; and (c) somatic embryos could arise by a mechanism similar to cleavage polyembryogenesis, with the initial separation occurring in the embryonic region.

Zygotic embryo development in Pinus is separated into two phases: a brief pro-embryo phase, that occurs within the archegonium and an embryo phase that occurs after the pro-embryo elongates into the nutritive female gametophytic tissue (Owens and Molder, 1984a and 1984b). A difficulty in attempting to compare zygotic versus somatic embryogenesis is the inconsistent terminology used by different researchers to classify the various stages in somatic embryogenesis (Tautorus et al.. 1991). Von Arnold and Hakman (1988a) distinguished four stages during the development of plants from somatic embryos (Figure 4.1). Stage 1 embryos consist of small cells, containing a dense cytoplasm, subtended by a suspensor comprised of long highly vacuolated cells. The suspensor is an embryonic organ which connects the developing embryos to the maternal tissues and is the nutrient uptake site during early stages of embryo development (Evans et al., 1986). The Stage 1 embryos arise in proliferating calli grown on medium

containing auxin and cytokinin (Hakman and Von Arnold, 1985). Stage 1 embryogenic cells can be recognised by their conspicuous starch contents and their close morphological and cytological resemblance to the apical meristem or zygotic embryo cells, that is, they are relatively isodiametric, have prominent nuclei, are thin-walled, and minimally vacuolated (Sharp et al., 1980). The Stage 2 embryos have a more prominent and dense meristematic region and are still attached to the callus by long suspensor cells. Stage 2 embryos are transferred to a medium containing abscisic acid (ABA). After four weeks on ABA medium the somatic embryos closely resemble mature zygotic embryos as they possess cotyledons. These are referred to as Stage 3 embryos. The fourth stage of development involves isolating the embryos and culturing them individually to form green plantlets (Figure 4.1).

As for organogenesis (section 2.1), two general patterns of embryogenic development of in vitro embryogenesis are discernible: direct and indirect embryogenesis. The former has been found in some plants which have a natural tendency to form asexual embryos from ovular tissues without the fusion of cells or nuclei (George and Sherrington, 1984). In indirect embryogenesis, callus proliferation is a prerequisite to embryo development (Sharp et al., 1980). Because the cells within the explant are differentiated and are incorporated into specific meristems or mature tissues, an induction treatment is needed to foster dedifferentiation and cell division and then subsequently redetermination of the differentiated cells into the embryogenically determined cells.

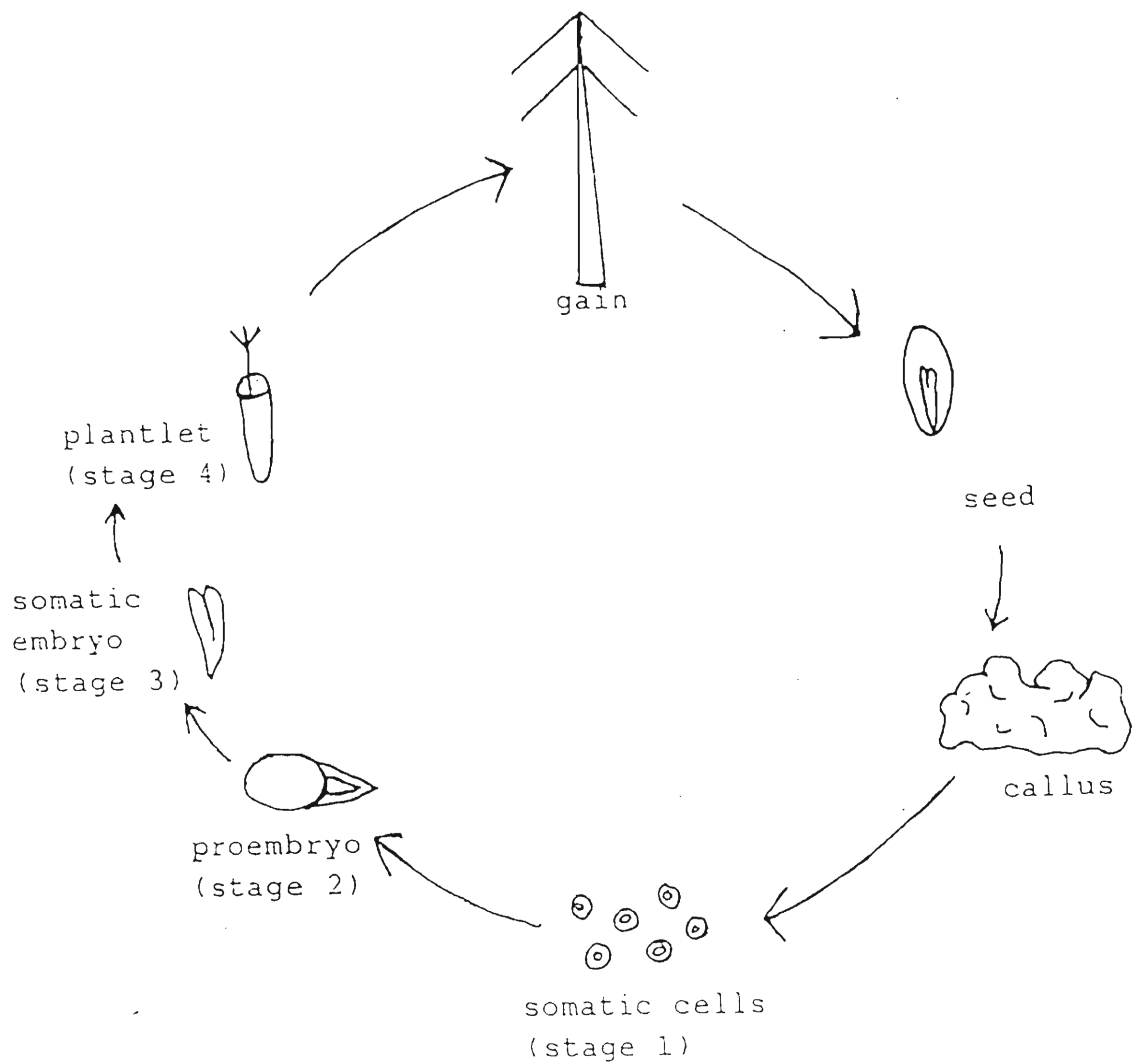

Figure 4.1 The stages of somatic embryogenesis.
(Adapted from Durzan and Gupta, 1988)

Direct somatic embryogenesis is primarily, but not exclusively, a phenomenon of nucellar tissue in temperate and tropical fruit trees. While direct embryogenesis can produce somatic embryos in only a few dicotyledoneous species, indirect embryogenesis has found a wide range of applicability in monocots and dicots (Wann, 1988). In the indirect system, differentiated cells must become

redetermined by a successive series of mitotic divisions. Ultimately, all cells, regardless of their initial state of differentiation, must arrive at a similar meristematic ground state. In this review of the literature on conifer somatic embryogenesis, only the indirect embryogenesis system is addressed as the indirect route was investigated in the Pinus patula study.

Indirect embryogenesis follows the development of induced embryogenically determined cells (IEDCs) (Ammirato, 1987). Despite the fact that the cells undergoing IEDC differentiation require an induction treatment with growth regulators, the explant and certain of its associated physiological qualities are the most significant determinants of embryo initiation (Sharp et al., 1980). The in vitro environment acts primarily to enhance or repress the embryogenic process, that is, the cells that undergo embryo initiation are predetermined, and their subsequent exposure to exogenous growth regulators simply allows embryogenesis to occur (Sharp et al., 1980). In the majority of examples of IEDC, the appropriate stimulus is auxin, the predominant auxin used being 2,4-dichlorophenoxyacetic acid (2,4-D) (Wann, 1988). Other auxins that have been employed are indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) (Wann, 1988).

Systems involving IEDCs are noted for the presence of a callus phase (Wann, 1988). Although the term callus has been used by many authors (Becwar et al., 1987; Mohan Jain et al., 1989; Webb et al., 1989; Laine and David, 1990;

Tremblay, 1990), this cellular mass is composed of organized structures and thus the term embryogenic mass is more appropriate (Tautorus et al., 1991). While variations exist between species as to the level of development that somatic embryos exhibit while in the embryogenic callus matrix, in all the species researched, embryogenic callus masses exhibit some aspect of organisation. In general, they tend to be friable parenchymatous matrices containing cream-coloured nodules or growth centres characterised by the presence of a thickened mother cell wall (Wann, 1988). The general morphology of young somatic embryos and their development into plantlets appear to be similar in all coniferous species (Von Arnold and Hakman, 1988b). Unlike most angiosperm somatic embryos, those of conifers have an extensive suspensor consisting of very long, highly vacuolated cells extending from an embryonic region which is composed of small meristematic cells.

4.1.2 The Importance of Explant Type in Indirect Somatic Embryogenesis

There has been a large variety of explants used in producing somatic embryos from many plant species. For the cereals and grasses, the use of immature and mature embryos (Vasil, 1988) to establish embryogenic cultures has been accompanied by successes with non-embryonic material, primarily immature leaf tissue, as in sugarcane (Guiderdoni and Demarly, 1988), root tips (rice)(Zimny and Lorz, 1986), immature inflorescences (Lolium perenne and L. multiflorum) (Creemers-Molenaar et al., 1988) and shoot tips (Sorghum

bicolor)(Bhaskaran et al., 1988). In conifers, proper explant selection has been critical in order to achieve successful induction of somatic embryogenesis. This is important because various tissues of the same plant or tissues at various stages of development can differ in their response when cultured in vitro (Roberts et al., 1989). In general, in Pinus species, precotyledonary zygotic embryos have been found to be best for induction of embryogenic tissue, whereas in Picea species, cotyledonary zygotic embryos have been proven to be the most successful type of explant (Becwar et al., 1988).

Somatic embryogenesis of gymnosperm forest trees was first claimed by U.S. Patent 4217730 in 1980, where a method is described for plantlet production from cotyledonary callus of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] (Von Arnold and Hakman, 1986). Most coniferous somatic embryogenesis research has been done on Norway spruce (Picea abies (L.) Karst.) and one of the first reports of embryogenic callus in coniferous species referred to the use of immature zygotic embryos as the best explants (Hakman et al., 1985). Those workers collected Picea abies cones during the maturation period of the embryos. The immature embryos with suspensors were dissected out aseptically from the surrounding female gametophyte and the two tissues were cultured separately. Hakmann et al. (1985) found that, in contrast to immature embryos, most of the mature embryos proliferated. The cultured megagametophytes could not be induced to form callus. Nagmani et al. (1987) cultured immature embryos of Picea abies and Picea glauca. Callus that proliferated from the hypocotyl region was

embryogenic whereas callus originating from the radicle was non-embryogenic. In another study, immature embryos from the female gametophyte of Picea abies seeds were used by Becwar et al. (1987). Both Roberts et al. (1990a and 1990b) and Webster et al. (1990) worked with immature embryos of interior spruce. Tautorus et al. (1990) compared the frequency of embryonic calli initiated from immature zygotic embryos of black spruce (Picea mariana Mill.), collected during the maturation period, with mature zygotic embryos dissected from seed which had been stored for 13 years. Those researchers found that a 65% frequency of embryogenic calli was obtained from the immature explants compared to only a 10% frequency from the mature explants. Immature and mature zygotic Picea abies embryos cultured on a modified MS medium produced a whitish, translucent tissue at a frequency of up to 38% (immature) and 8% (mature) of explants (Chalupa, 1985).

Excised zygotic embryos, particularly if immature, have permitted the development of embryogenic cultures in many hitherto recalcitrant species (Ammirato, 1989). However, the process of obtaining immature zygotic embryos is tedious and sometimes impossible. Rapidly dividing, undifferentiated embryonic cells grow easily in culture and readily generate embryos. Also, they may reduce the potential for genetic variability in the cultured tissue and recovered plants (Ammirato, 1989). There have, however, been reports on producing somatic embryos from mature seed (Krogstrup, 1986; Von Arnold and Hakman, 1986 and 1988a). One of the main advantages of using mature zygotic embryos from stored seed is that plant material can be provided

throughout the year. Von Arnold (1987b) and Von Arnold and Hakman (1986 and 1988a) obtained embryonic callus from mature zygotic Picea abies embryos dissected from the surrounding gametophyte tissue; embryogenic callus was first seen to arise from the region just beneath the cotyledons of the cultured embryos (Von Arnold, 1988b). Embryos from imbibed ripe seeds and cotyledon explants of seven day-old Picea abies seedlings were used by Krogstrup (1986) as explants for somatic embryogenesis. Mo and Von Arnold (1991) used zygotic embryos and in vitro grown seedlings of Picea abies, and found that embryogenic structures differentiated from the epicotyl, hypocotyl and cotyledons. Mature zygotic embryos have been used also in studies with Picea glauca and P. engelmanii (Webb et al. , 1989) and P. mariana (Attree et al., 1990). Nollet and Deberg (1989) reported that damaged embryos, from mature Picea abies seed, produced more callus than intact ones.

Picea species appear to be more amenable to somatic embryogenesis than Pinus (Finer et al., 1989; Tautorus et al., 1990). It is not known if this recalcitrance is partially due to Pinus species being more primitive in terms of evolution (Coulter and Chamberlain, 1910) or simply to the fact that more research has been focused on Picea species, as Picea abies was the first species to have successfully been induced for somatic embryogenesis. In addition, soft pines (that is, five-needled pines) such as Pinus lambertiana (Hosie, 1979) may be more responsive to somatic embryogenesis protocols than hard pines (that is, two-to-three-needles pines)(Tautorus et al., 1991). Table 4.1 lists those few Pinus species that have been

investigated for somatic embryogenesis. With respect to explants used to initiate somatic embryogenesis in Pinus species, mature zygotic embryos have been used successfully onlt for induction of somatic embryogenesis with P. lambertiana (Gupta and Durzan, 1986). Immature embryos of Pinus caribaea Morelet var. hondurensis were used by Laine and David (1990). Those researchers found that the presence of the endosperm aided pine somatic embryogenesis. This observation was also reported by Gupta and Durzan (1986). Mohan Jain et al. (1989) worked with immature embryo explants of four different genotypes of slash pine (Pinus elliottii). Pinus taeda gametophytes with attached suspensors and proembryos were used by Gupta and Durzan (1987), whereas Finer et al. (1989) found that whole gametophyte from immature seeds of eastern white pine (Pinus strobus) was far superior than the excised zygotic embryo as an explant source for the initiation of embryogenic callus.

4.1.3 Nutritional and Environmental Requirements for the Induction of Embryogenesis

Von Arnold (1988b) stressed that when using mature zygotic embryos, some factors that have to be considered are: (a) plant growth regulators; (b) concentration of the basal medium, sucrose and NH_4NO_3; (c) pH of the medium, medium consistency; and (d) light regimes.

In vitro development of somatic embryos is essentially a two-step process, each requiring a different medium

(Bhojwani and Razdan, 1983). The embryogenic mass is first initiated and multiplied in an auxin-rich medium (induction or proliferation medium) where embryogenic tissue differentiation occurs in localised groups of meristematic cells called "embryonic clumps". In repeated subcultures on the induction medium the embryogenic clumps continue to multiply without the appearance of mature embryos (Bhojwani and Razdan, 1983). It is thought that the primary mode of action of the known natural growth regulating substances is probably their action on membrane systems, particularly their control of ion fluxes (Trewavas, 1976). This, in turn, could lead to many of the biochemical changes which they are known to induce (Sharp et al., 1980).

Although the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is the most commonly used growth regulator for somatic embryogenesis, in some plant species cytokinins have been found to have a promotive effect on embryogenesis. For example, leaf explant cultures of Coffea arabica require a high kinetin to auxin ratio for high frequency embryo induction (Sharp et al., 1980). There are numerous reports on the use of 6-benzyladenine (BA), combined with 2,4-D, for the induction of embryogenesis in Picea abies (Hakman et al., 1985; Krogstrup, 1986; Becwar et al., 1987; Mo and Von Arnold, 1991). Lelu and Bornman (1990) found that the exposure of Picea glauca and P. mariana cotyledons to a high ratio (90:1) of BA to NAA for one week before subculturing on media with ratios 2:1 (2,4-D:BA) and 50:1 (NAA:BA) improved embryogenic response. Recently somatic embryogenesis was induced from immature (precotyledonary) embryos of Abies nordmanniana with cytokinins as the sole

growth regulator (Nørgaard and Krogstrup, 1991). There are reported instances where IAA, ABA and gibberellic acid (GA_3) have suppressed embryogenesis in carrot (Fujimura and Komamine, 1975) and Citrus (Tisserat and Murashige, 1977).

Various medium salt formulations have been used for the induction of conifer somatic embryogenesis. Durzan and Gupta (1987) stipulated that the LP medium (Litvay et al., 1981) was not beneficial for Douglas-fir embryogenic cultures. However, a modified half-strength MS and half-strength DCR basal medium each supplemented with myo-inositol, casein hydrolysate and L-glutamine proved adequate for the proliferating embryonal-suspensor mass. Mohan Jain et al. (1989) tested several basal culture media for the initiation of somatic embryogenesis from immature Pinus elliottii embryos and found that MNC1 medium was the most suitable. Excised sugar pine embryos, plated by Gupta and Durzan (1986), developed callus within four to five weeks. By ten to twelve weeks (five year-old seeds) an unusually white, mucilaginous callus was obtained from explants around the radicle on DCR basal medium containing 2,4-D (3 mg l^{-1}), L-glutamine (50 mg l^{-1}) and casein hydrolysate (500 mg l^{-1}). Microscopic examination of the white callus revealed globular embryos at various stages of development with large suspensors protruding from the callus. Tremblay (1990) initiated white spruce embryogenesis on half-strength Litvay salts with 2,4-D (2.2 mg l^{-1}) and BAP (1.1 mg l^{-1}). Embryogenic callus was typically composed of long vacuolated cells organised as suspensor-like structures and highly active meristematic centres that formed an early embryo mass. Interior spruce

embryogenic calli were initiated for two weeks on VE basal medium (Von Arnold, 1987b) containing 1.1 mg l^{-1} 2,4-D and 4.5 mg l^{-1} BA (Roberts et al., 1990a). Finer et al. (1989) plated whole female Pinus strobus gametophytes on to a DCR basal medium containing 2 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. Embryogenic calli could be seen as early as five days following culture. Pinus taeda tissues were inoculated on to a half-strength MS basal medium containing 2,4-D (1.1 g l^{-1}), kinetin (4.3 mg l^{-1}) and BAP (4.5 mg l^{-1}) by Gupta and Durzan (1987).

Of all the nutrients in the medium, the form of nitrogen appears to be the one that is most important in the process of in vitro embryogenesis. Halperin and Wetherell (1965) reported that in the cultures of wild carrot raised from petiolar segments, embryo development occurred only if the medium contained some amount of reduced nitrogen. The calli initiated on medium with KNO_3 as the sole nitrogen source failed to form embryos upon removal of auxin. However, the addition of a small amount of NH_4^+ - ammonium nitrogen (0.27 g l^{-1}), in the form of NH_4Cl, in the presence of KNO_3 (5.62 g l^{-1}) allowed embryo development. Halperin and Wetherell (1965) also demonstrated that the presence of reduced nitrogen was critical in the induction medium. Furthermore, the 2,4-D concentration in the induction medium appears to have an influence on the effects of nitrogen on embryogenesis. Evidence in support of this is found in the fact that Halperin and Wetherell (1965), working with the wild carrot cultures, did not find an ammonium requirement in the medium in which the embryos were developing (0.1 mg l^{-1} 2,4-D), but only in the induction medium (10 mg l^{-1}

2,4-D).

Von Arnold (1987a) did extensive research on nitrogen requirements during initiation of embryogenesis in black spruce. Her results showed that the NH_4NO_3 concentration could be varied at high sucrose concentrations, and organic nitrogen, in the form of L-proline, alanine, glutamine and asparagine, could not supplement too low NH_4NO_3 concentrations. Addition of alanine, asparagine and glutamine to the culture medium reduced the yield of embryonic callus substantially, but the addition of an amino-acid supplement, as casein hydrolysate, was found not to be beneficial. Casein hydrolysate, although frequently included as a medium component for the initiation of embryogenesis of other conifer species (larch: Nagmani and Bonga, 1985 and white spruce: Lu and Thorpe, 1987), it has not been proven to be beneficial. In contrast, Hakman _et al._ (1985) suggested that casein hydrolysate improved the frequency of somatic embryos from immature zygotic embryos of Norway spruce, especially for the most immature stages.

The sucrose concentration in the basal medium has been found to be important also for the initiation and development of somatic embryos. Von Arnold and Hakman (1986) showed that the highest yield of embryogenic types of callus of _Picea abies_ was obtained on medium containing 10 g l^{-1} sucrose. This same concentration of sucrose has been successfully used with immature interior spruce embryos (Webster _et al._, 1990) and _Picea glauca_ and _Picea engelmanii_ embryos (Webb _et al._, 1989). Laine and David (1990) tested four initiation media on _Pinus caribaea_

embryos and found that the LP media containing 30 g l^{-1} sucrose was the most satisfactory. Nagmani et al. (1987) used basal LP medium with four different types of sugars (sucrose, arabinose, glucose and xylose) for initiating callus on Norway and white spruce embryos.

Other factors that have had an effect on conifer somatic embryogenesis are the type of gelling agent used and the light regime of the culture environment. Tremblay and Tremblay (1991) found that the number of somatic embryos formed from immature zygotic embryos of black spruce was significantly higher on medium solidified with Gelrite gellan gum than with Difco Bacto-agar. Corn, potato, wheat, or rice starches as gelling agents either did not influence or drastically reduce the development of somatic embryos. The presence or absence of light is recognisably important in loblolly pine embryogenesis (Amerson et al., 1988). White light inhibits initial culture establishment but is stimulatory to late-stage embryo development (Gupta and Durzan, 1987b).

4.1.4 Maturation and Germination of Somatic Embryos

At present, the recovery of plantlets from somatic embryos is very poor and remains a limitation to the implementation of this technology. Somatic embryos occur in a repressed state of development while on their induction media and, in successful studies, have required passage on a "differentiation" medium to obtain further maturation (Dunstan et al., 1991; Tautorus et al., 1991). In some cases this has involved the removal of phytohormones from

Table 4.1 Explant type and Induction media reported for somatic embryogenesis of Pinus species.

Induction media are: MS (Murashige and Skoog, 1962), WPMG (Lloyd and McCown, 1981), DCR (Gupta and Durzan, 1985), LP (Hakman and Von Arnold, 1985), MSG (Becwar et al., 1988).

Species	Explant	Media	Reference
P.caribaea	Immature embryos	LP	Laine & David, 1990
P.elliottii	Immature embryos	WPMG	Mohan Jain et al., 1989
P.lambertiana	Immature & Mature embryos	DCR	Gupta & Durzan, 1986
P.serotina	Gametophyte	MSG	Becwar et al., 1988
P.strobus	Immature embryos Gametophyte	DCR	Finer et al., 1989
P.taeda	Gametophyte	MS	Gupta & Durzan, 1987

the induction medium (Kartha et al., 1988), while in others abscisic acid (ABA) has been used. The addition of exogenous ABA (usually supplied in the racemic (±) form) to conifer somatic embryo cultures can result in somatic embryo maturation (Dunstan et al., 1991). Racemic ABA [(±)-ABA] is a synthetic mixture having a 1:1 ratio of the natural compound and the unnatural R(−)- enantiomer (Dunstan et al., 1991). The racemic form was first used because of the implications of the involvement of natural ABA in the maturation of the zygotic embryo and because of prior use with somatic embryos of angiosperms (Ammirato, 1973; Ackerson, 1984). Several authors have suggested that an effect of exogenous (±)- ABA is the inhibition of cleavage polyembryony and the consequent development of individual somatic embryos (Durzan and Gupta, 1987; Boulay et al., 1988; Krogstrup et al., 1988). Von Arnold and Hakman (1988a) observed that the embryonic region of their Picea abies embryos became firmer and assumed a glossy surface. Lipids were observed to accumulate in the embryos during ABA treatment in a way similar to what is found in mature seed embryos. The addition of auxin and/or cytokinin to the ABA-containing medium only inhibited the effect of the ABA. After transferring the cultures to medium lacking ABA, the meristematic region of the embryos became more organised and cotyledons appeared after about one month in culture.

There have been a wide range of ABA concentrations, exposure times on ABA, and culture conditions, reported for maturation and germination of conifer somatic embryos. However, many reports are unclear as to what methods were

used and what results were actually obtained. For example, although the solvent (acetone, ethanol, DMSO, NaOH)[2] used to dissolve the ABA has been shown to have an effect on somatic embryo maturation (Dunstan et al., 1991), few authors have reported their method of preparation of ABA (Tautorus et al., 1991).

There have been numerous reports on the use of ABA for the maturation of conifer somatic embryos. Von Arnold (1988a) grew Stage 2 embryonic calli (that is, embryos with a more prominent and dense meristematic region) on half-strength LP without PGR or with 1 mg l^{-1} ABA, 1 mg l^{-1} IBA, or 1.5 mg l^{-1} zeatin alone or in combination. Somatic embryos grown on ABA alone became firmer and had a smooth surface compared to those grown on the other growth regulators. The ABA-treated somatic embryos resembled mature zygotic embryos in appearance, growth habit and in vitro culturing requirements. Roberts et al. (1990b) found, with interior spruce explants, that little or no organised somatic embryo development occurred in the absence of ABA. As the level of ABA was increased, a range of embryo types was produced. Roberts et al. (1990b) reported that "shooty embryo" structures predominated in many callus lines at low levels of ABA (0.3-3.0 mg l^{-1}), while 3-6 mg l^{-1} ABA promoted the formation of bipolar embryos that germinated precociously. When ABA was increased to 9-12 mg l^{-1}, precocious germination was inhibited and opaque cotyledonary embryos, characteristic of their zygotic counterparts, were formed which entered a period of quiescence. IBA included with ABA

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[2] Sigma Chemical Company catalog, 1991. p. 1426.

increased the number of mature embryos. Similarly, Mo and Von Arnold (1991) stimulated Picea abies somatic embryos to mature by transferring them to half-strength LP medium containing 2 mg l^{-1} ABA. After one month the mature somatic embryos were then stimulated to develop into plantlets by transferring them to medium lacking plant growth regulators. Attree et al. (1990) conducted a study on two spruce species in order to improve maturation efficiencies. Suspension-cultured somatic embryos, when transferred to a maturation medium containing ABA, developed a globular opaque embryonal region after two weeks and small emerging cotyledons after three weeks. Abscisic acid at 4.2 mg l^{-1}, over a twenty eight day culture period, consistently yielded the most mature embryos. Only normal-looking embryos, as judged by comparing their appearance with mature zygotic embryos, germinated. Mature embryos that were individually separated from the calli and transferred to low light germinated immediately. Embryos first turned green, then hypocotyl elongation and radicle emergence followed, yielding young plants after approximately fourteen days. In another study, various proliferation media were tested (Laine and David, 1990) for Pinus caribaea embryogenic calli but only two, BMS-B and DCR-MM, supported sustained growth. For pro-embryo formation and subsequent embryo differentiation those calli were transferred to various media, of which LPS, containing 2 mg l^{-1} ABA as the sole growth regulator, was most suited for pro-embryo formation. Maturation of these pro-embryos into somatic embryos bearing well differentiated cotyledons was achieved by a doubling of macronutrients and ABA concentration.

The use of activated charcoal in the maturation process has been reported by several authors. Webster et al. (1990) divided interior spruce embryogenic tissue into approximately 150 mg pieces, following a two-week subculture on initiation medium. The pieces were weighed and placed onto a hormone-free VE basal medium containing 10 g l^{-1} activated charcoal and 34 g l^{-1} sucrose. After seven days on charcoal, the cultures were transferred to a VE basal medium with 34 g l^{-1} sucrose and supplemented with 0.2 mg l^{-1} IBA and ABA levels between 10 mg l^{-1} and 15 mg l^{-1}. Mature embryos, that is, those remaining opaque with well-developed cotyledons, were subjected to a high relative humidity treatment for 16 days before being placed on half-strength VE basal medium with 20 g l^{-1} sucrose and no hormones. In another investigation, Roberts et al. (1990) transferred interior spruce calli onto VE medium containing 10 g l^{-1} charcoal for one week and then to VE basal medium containing 10 mg l^{-1} ABA and 0.2 mg l^{-1} IBA for four weeks. Mature embryos were removed from the calli and germinated on half-strength VE containing 20 g l^{-1} sucrose. Becwar et al. (1987) subcultured Picea abies callus, after two weeks on initiation medium, onto the same LP basal medium containing 10 g l^{-1} activated charcoal with no growth regulators. After another week, the embryogenic callus was transferred to a basal medium containing 0.2 mg l^{-1} of IBA and 0.3 mg l^{-1} ABA. Somatic embryos developed either directly from the subcultured callus or after being dispersed in a thin layer of agarose medium. The transfer of embryos to a filter paper support in liquid medium lacking growth regulators promoted embryo elongation and the greening of cotyledons over thirty days. At this stage

transfer of embryos to a basal medium without supplements and with 2.5 g l^{-1} activated charcoal under continuous light produced complete plantlets within 40 days. When cultured on a medium containing activated charcoal the immature Picea abies embryos, used by Hakman et al. (1985), developed visible cotyledons and roots. However, this was only possible if the embryos had reached a certain maturity.

Numerous other procedures for embryo maturation have been described in literature, none of these being similar. For example, to complete early embryony, Gupta and Durzan (1987)_subcultured the proliferating Pinus taeda embryonal-suspensor mass onto the same half-strength MS initiation medium except for lower concentrations of 2,4-D (1 mg l^{-1}), kinetin (0.4 mg l^{-1}) and BAP (0.45 mg l^{-1}). After three or four subcultures, the globular stage of embryogenesis was fully evident. Embryos elongated and developed cotyledons eight to ten weeks when transferred to a sterile-filtered liquid medium with filter paper support without growth regulators. Complete plants were developed in a half-strength MS basal medium containing 2.5 g l^{-1} activated charcoal, myo-inositol (100 mg l^{-1}) and sucrose (20 g l^{-1}). Gupta and Durzan (1986) found that the development of the globular somatic embryos of sugar pine did not proceed beyond twelve weeks unless they were transferred to a medium lacking 2,4-D and containing 0.1 mg l^{-1} BAP. Transfer of embryos encouraged elongation of the embryonic axis and the true-to-type development of six to eight cotyledons in all cases. Mohan Jain et al. (1989) subcultured Pinus elliottii embryogenic callus on medium containing 0.3 mg l^{-1}

ABA, 1 mg l^{-1} 2,4-D and 0.22 mg l^{-1} BAP. However, after three weeks, embryogenic callus did not show the formation of an embryonal mass of cells in any of the media tested. Somatic embryos of Picea glauca were matured after transfer to an embryo development medium consisting of half-strength Litvay salts, 60 g l^{-1} sucrose, 2,4-D (0.22 mg l^{-1}) and kinetin (1 mg l^{-1}). Although the increased sucrose concentration gave better and more reliable embryo development, not all embryos produced were normal (Tremblay, 1990).

It has been proposed that osmotic treatments may also stimulate germination of somatic embryos (Gingas and Lineburger, 1989). Consequently, Roberts (1991) investigated the role of osmoticum during somatic embryogenesis and germination of interior spruce. Low levels of mannitol (20-60 g l^{-1}) were found to promote the formation of globular embryos in the embryonic cultures. However, those concentrations of mannitol were inhibitory to the formation of cotyledonary embryos. A short (one week) pulse of mannitol in combination with abscisic acid doubled the production of late cotyledonary somatic embryos compared with the standard abscisic acid treatment.

The light regime during culturing appears not to have an effect as maintenance of black spruce embryogenic tissue under light or in darkness and use of different fluorescent lamps during the maturation stage did not influence the total number of somatic embryos produced (Tremblay and Tremblay, 1991). However, in that study, a maximum number of germinating embryos was produced when the embryogenic

tissue was maintained in darkness, followed by a maturation stage in light.

There is little information concerning soil establishment of conifer plants derived from somatic embryos. In _Picea abies_ 56% of matured somatic embryos shooted and rooted, and 29% were successfully established in soil (Becwar _et al._, 1989). In _Picea mariana_, 61% of plants survived when transferred to soil (Attree _et al._, 1990). Dunstan _et al._ (1991) obtained 52% survival of _Picea glauca_ plants in soil using a self-watering propagation unit for acclimatisation.

4.1.5 Aims of this Study

There have been no in depth studies on _Pinus patula_ somatic embryogenesis reported, consequently this study explores the feasiblity of producing somatic embryos from mature seed of the most economically important pine species in South Africa. The system could provide a means of mass propagation of selected _P. patula_ genotypes. The main focus of this work was on the induction phase of the embryogenic cycle. A number of parameters required examination in order to optimise the production of the embryos. These included investigating the state of the explant as well as the induction conditions such as growth regulators and media formulations. Maturation conditions, for obtaining pro-embryos from the embryogenic cells, were also examined.

4.2 MATERIALS and METHODS

4.2.1 Seed Germination and Sterilisation of the Explant Material

As for 2.2.1 and 2.2.2.

4.2.2 Culture Conditions for the Induction of Embryogenic Calli

The explants were embryos aseptically excised from mature P. patula seed (Seed Lot No. M4562). Cultures were incubated in the dark at 24 °C (±2 °C) for 28 days on induction media, contained in sterile 100ml glass jars. The cultures were then placed under a 16 hour photoperiod at 740 mols m^{-2} s^{-1} for another 14-28 days, after which they were assessed for the number of embryos producing callus and for the fresh weight of the calli produced. A number of variables were examined for their effect on calli induction. These were the 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations and the combination effect with cytokinins, media salts formulations, concentrations of media components and age of the embryo explant.

4.2.3 Culture Conditions for the formation of Pro-embryos

Calli were aseptically transferred from the induction medium to the maturation medium, containing 12 mg l^{-1} abscisic acid (ABA). The maturation medium was identical to the induction medium except that the 2,4-D, found in the induction medium, was replaced by ABA. Calli were cultured

for another two months under 16 hour photoperiod and 740 mols m^{-2} sec^{-1}.

4.2.4 Microscopy and Photography

Squashes of calli were prepared and stained with 1% Safranin Red stain (Harrigan and McCance, 1966). An Euromex light microscope was used in the examination of the callus squashes. Stages of embryo development were photographically recorded using a Wild Photoautomat MPS 55 system and a Nikon FX 35A and UFX photorecording system. Gross morphological characteristics of the calli were recorded with a Wild Photoautomat MPS 55 system and a Nikon FX 35A and UFX photorecording system.

After the two month culture period each callus mass was examined under a Kyowa Optical (model SD-2R) stereo microscope while carefully being teased open using two 18-gauge syringe needles to expose the suspensors and pro-embryo material. Suspensor and pro-embryo tissue was photographically recorded using a Wild Photoautomat MPS 55 system and a Nikon FX 35A and UFX photorecording system.

4.2.5 Data Analysis

Analysis of the data, in this chapter, was as described in section 2.2.4.

4.3 RESULTS and DISCUSSION

4.3.1 Optimisation of the Induction Process

This study investigated the possibilities of producing somatic embryos from the tissue of mature Pinus patula embryos using the indirect route which involves the formation of a callus stage. The preliminary investigations involved examining some of the most important factors of somatic embryo induction. Seed pre-treatment and sterilisation procedures, for obtaining contaminant-free embryo explants, have already been described in sections 2.2.1 and 2.2.2.

The importance of using 2,4-dichlorophenoxyacetic acid (2,4-D) as the auxin growth regulator for the induction of embryogenic development has been emphasised by several authors (Sharp et al., 1980; Bhojwani and Razdan, 1983; Gupta and Durzan, 1986; Von Arnold, 1987b; Komamine et al., 1990). A low 2,4-D concentration range (0-2 mg l^{-1}) was used initially as such concentrations were found to be successful with the conifer cultures of Von Arnold (1987b), Finer et al. (1989) and Webster et al. (1990). The sucrose content in the induction medium has been shown to have an effect on embryogenesis (Von Arnold and Hakman, 1986; Von Arnold, 1987b). Webb et al. (1989) included sucrose at concentrations ranging from 5 to 40 g l^{-1}. Table 4.2 and Plate 4.3 illustrate the results obtained from a preliminary experiment designed to investigate the auxin/sucrose combination that would be best suited for embryogenic induction. Parameters examined for providing an

Table 4.2 The percentage of embryo explants forming callus at various sucrose and 2,4-D concentrations.

Embryos (8 day-old) were plated on ½MS, 8 g l^{-1} agar-agar, pH 5.7, and cultured in the dark. Results were recorded after 4 weeks in culture. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-h).

	Sucrose (g l^{-1})			
2.4-D (mg l^{-1})	**0**	**10**	**20**	**30**
0	0 ±0.0 h	0 ±0.0 h	0 ±0.0 h	0 ±0.0 h
0.1	0 ±0.0 h	31 ±3.1 f	33 ±2.7 f	51 ±2.7 d
0.5	0 ±0.0 h	41 ±3.6 e	42 ±3.2 e	77 ±4.4 b
1.0	0 ±0.0 h	29 ±4.2 f	40 ±3.0 e	79 ±4.2 b
2.0	0 ±0.0 h	24 ±4.0 g	71 ±3.6 c	96 ±4.0 a

indication of potential for embryogenesis were the number of embryo explants that developed into a callus mass and microscopic examination of those callus masses for the presence of embryogenic cells. The basal salt medium used in this preliminary study was half-strength Murashige and Skoog (1962) (½MS), as the MS medium formulation has been the most commonly used for tissues of various woody species (Watt et al., 1991). As the MS medium is the highest of all the formulations with respect to salt content (Gupta and Durzan, 1987b), a half-strength concentration was used initially to prevent any adverse effect that might arise from the full-strength MS salt concentration. Cultures were kept in the dark for four weeks.

The results shown in Table 4.2 indicate that more explants formed callus at the higher 2,4-D and sucrose concentrations. On examination of the squashes of calli formed, the 2 mg l^{-1} 2,4-D/ 20 g l^{-1} sucrose treatment had the greatest number of embryogenic cells present out of all the treatments examined (Plate 4.1). Embryogenic cells were distinguishable from non-embryogenic cells (Plate 4.2) in that they were aggregated into clumps, highly nucleated, relatively small and isodiametric, rich in cytoplasm and minimally vacuolated (Sharp et al., 1980). However, the occurrence of the embryogenic clumps was very low. These embryogenic clumps will be referred to as stage 1 embryos as classified by Von Arnold and Hakman (1988a).

A further investigation into the optimum 2,4-D concentration to be used in the induction medium was undertaken. This was done as the results in Table 4.2

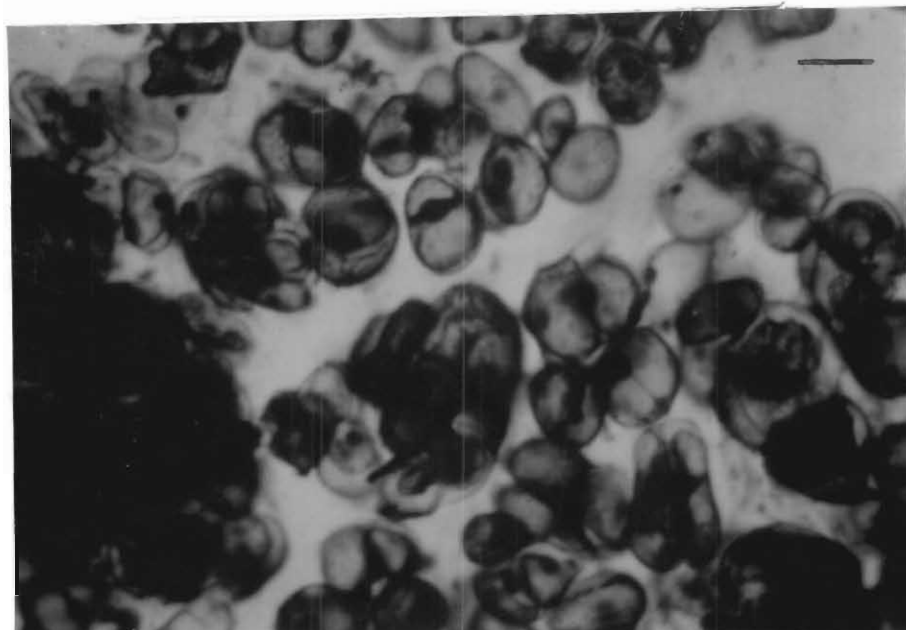

Plate 4.1 Embryogenic cells (stage 1) present in the callus after a 6 week culture period on half-strength MS induction medium containing 2 mg l^{-1} 2,4-D and 20 g l^{-1} sucrose. Scale bar= 0.26 mm.

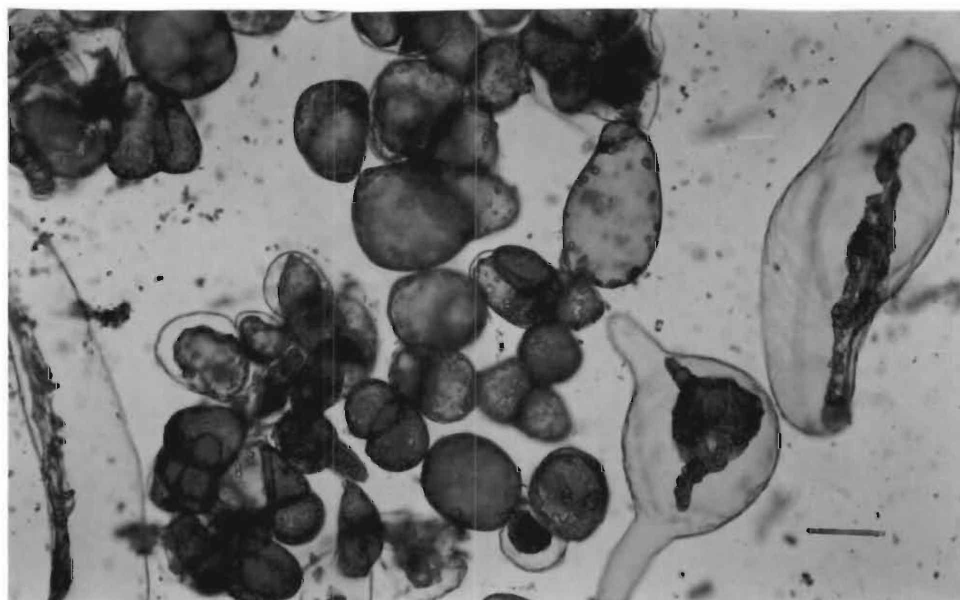

Plate 4.2 Non-embryogenic cells present in the callus after a 6 week culture period on half-strength MS induction medium. Scale bar= 0.26 mm.

indicated that the 2 mg l^{-1} 2,4-D concentration provided the best yield of embryogenic cells and it was unknown whether a higher 2,4-D concentration would, in fact, increase this yield. Results of this study (Figure 4.2) indicate that higher 2,4-D concentrations did not increase the calli yield. Microscopic examination of the calli formed in each treatment showed that there was no change with respect to the number of stage 1 embryogenic cells in the higher 2,4-D treatments.

The next important consideration for optimising the induction medium was the investigation of the effect of various media salt formulations successfully used by other researchers on their conifer cultures. Figures 4.3 shows that both the percentage of explants forming callus and the fresh weight of the calli formed had the highest value when cultured on the LM medium described by Litvay et al. (1981). The LM medium was developed at the International Institute of Paper Chemistry, Wisconsin, U.S.A. (Bornman, 1983). This medium appears to be based on MS, although there are striking differences in the concentration of a number of macro- and micronutrient components. The most significant of these is that LM has a much higher $MgSO_4.7H_2O$, H_3O_3 and $ZnSO_4.7H_2O$ concentration than the MS formulation (see Appendix 1). The magnesium sulphate in LM is the only source of magnesium and the only significant source of sulphate in the LM formulation. Magnesium serves as a cation capable of balancing and neutralising inorganic cations and organic acids whereas the sulphate component of $MgSO_4.7H_2O$ provides sulphur for lipid synthesis and governing the structure of proteins through the formation

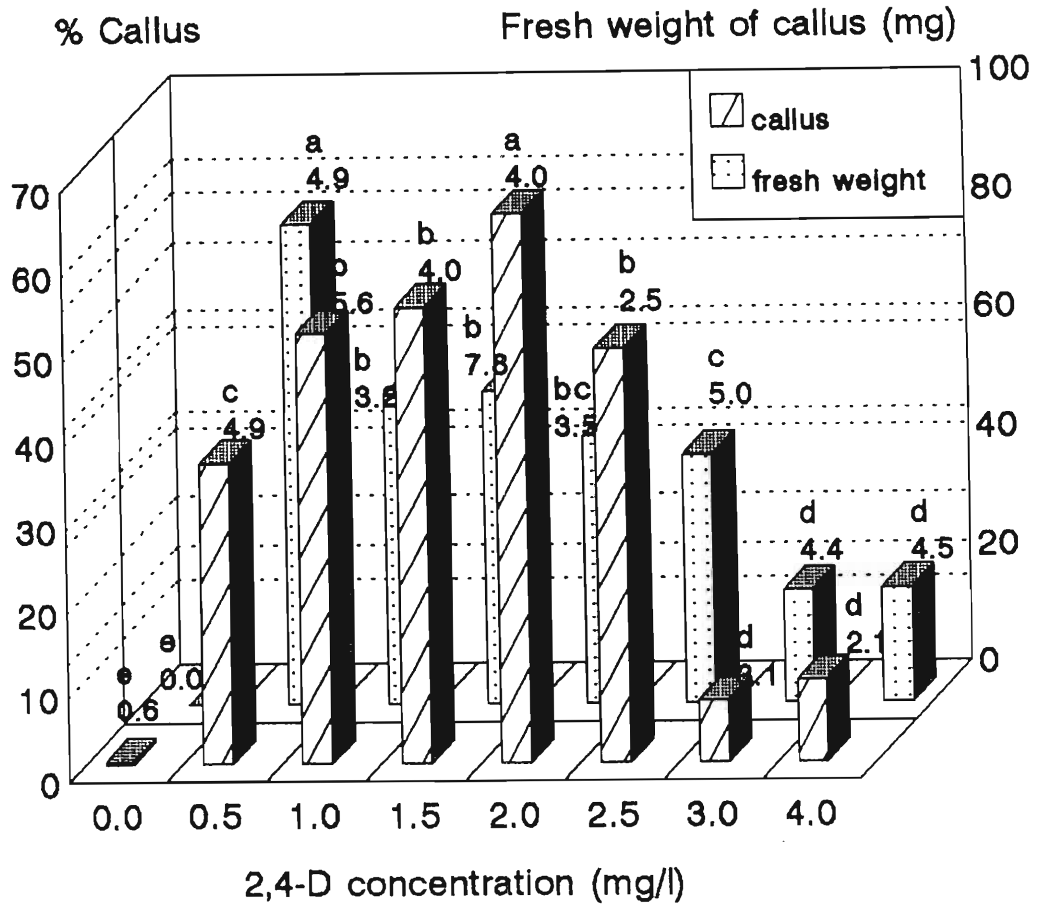

Figure 4.2 Optimising the 2,4-D concentration for callus production from 8-10 day-old embryo explants. Explants were plated on ½MS, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7 and cultured in the dark at 24 °C (±2 °C). Results were recorded, after 6 weeks in culture, as the percentage of explants forming callus and the fresh weight of the calli formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-e).

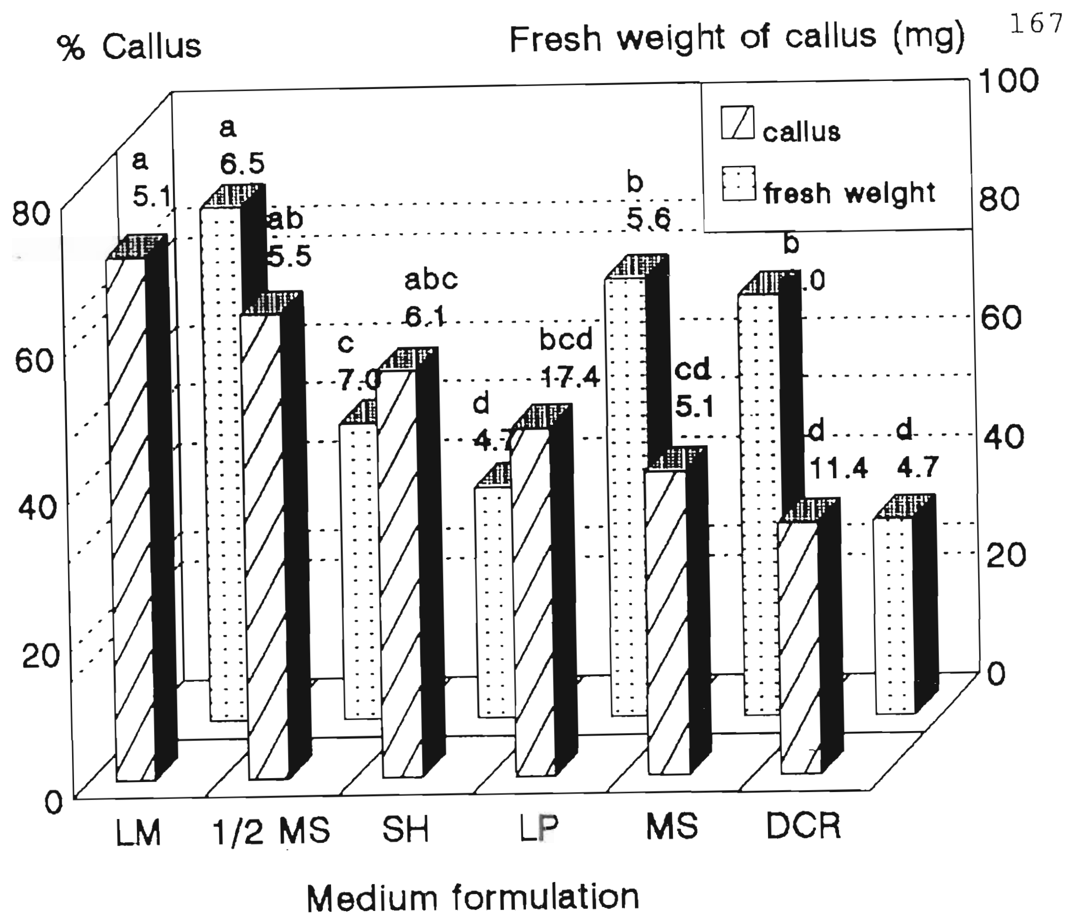

Figure 4.3 Callus formation from 8-10 day-old embryo explants when cultured on various medium salt formulations.

All media were at full-strength and contained 2 mg l^{-1} 2,4-D, 8 g l^{-1} agar-agar, 20 g l^{-1} sucrose, pH 5.7. The medium salts are: MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrant, 1972), GD (Mehra-Palta, 1978), LM (Litvay <u>et al</u>, 1981), LP (Aitken-Christie, 1984) and DCR (Gupta and Durzan, 1985). Results are recorded as the percentage of explants forming callus and the fresh weight of the calli formed after 6 weeks in culture. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

of S-S bridges (George et al., 1988b). It is unknown as to what extent these particular salt concentrations might have on the process of embryogenic induction of the cultures used in this study. It is repeatedly mentioned in literature that the form of nitrogen in the induction medium significantly effects in vitro embryogenesis (Halperin and Wetherell, 1965; Bhojwani and Razdan, 1983; Von Arnold, 1987a)(section 4.1.3). However, the nitrogen content in both the LM and MS formulations are identical so the nitrogen factor had no influence on the differing results obtained from the LM and MS formulations.
Embryogenic cells were present in squashes done on the LM calli, although present in no greater amounts than embryogenic cells found in the other salt formulations. Due to the high yield of calli produced on LM nutrients, this medium was selected as the basal salt formulation for subsequent studies on somatic embryogenesis induction of P. patula. The gross morphology of a six week-old callus mass is shown in Plate (4.4).

Most conifer somatic embryogenesis work has been done with immature zygotic embryos. However, the process of obtaining immature embryos is very tedious as much time is required to monitor the development of the cones on the tree. Only at a particular "window" stage can the cones be collected and the immature embryos removed (Jones and van Staden, 1992). However, by using mature zygotic embryo explants from stored seed, not only is material available at all times but also less work is required to obtain the embryo from the seed. One of the main objectives of this study was to induce somatic embryogenesis from mature embryos. It was

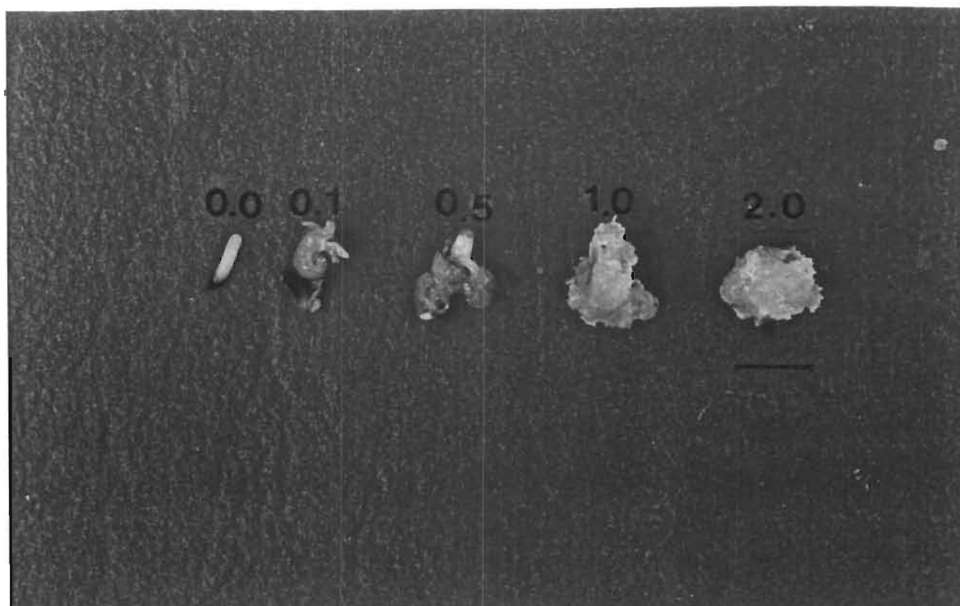

Plate 4.3 The effect of increasing the 2,4-D concentration ($mg\ l^{-1}$) on callus formation. Embryo explants were cultured for 6 weeks on half-strength MS induction medium containing 20 $g\ l^{-1}$ sucrose. Scale bar= 5 mm.

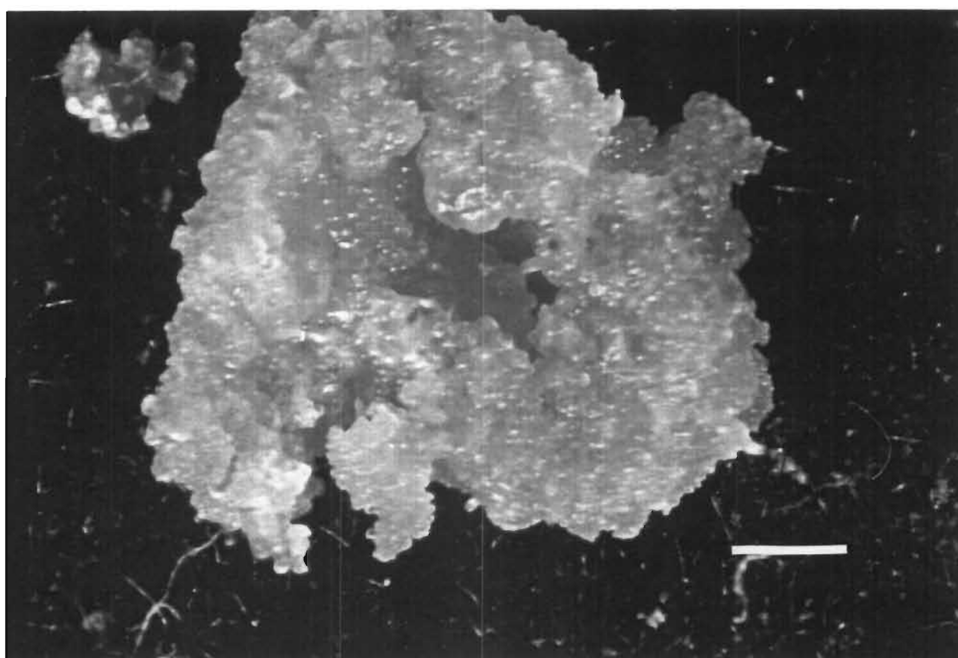

Plate 4.4 Gross morphology of callus tissue formed after 6 weeks on LM induction medium containing 2 $mg\ l^{-1}$ 2,4-D and 20 $g\ l^{-1}$ sucrose. Scale bar= 2 mm.

felt that investigating somatic embryogenesis induction using immature embryos would be a futile exercise especially if ever somatic embryogenesis were to be used as a commercial system. Figure 4.4 shows the results of an experiment performed to find the most suitable stage of embryo development required for the production of callus. Embryo explants, derived from seed that had been pre-treated for 72 hours and allowed to germinate for a further two days, did not respond to the culture conditions and no change was observed. Only those embryos, that had been allowed at least ten days of germination, responded to the culture conditions and produced callus. The largest number of embryo explants that produced callus were those that had germinated for ten days. However, the fresh weight of the callus formed, from the 10 day-old germinated embryos, was lower than that produced from the 14 day-old explants. Consequently, in all further work the embryos were allowed to germinate for at 10-14 before being used as explants.

Some researchers have reported that the inclusion of the cytokinins 6-benzyladenine (BA) and kinetin (FAP) along with the 2,4-dichlorophenoxyacetic acid (2,4-D) in the induction medium have had a stimulatory effect on conifer somatic embryogenesis (Krogstrup, 1986; Gupta and Durzan, 1987a; Mohan Jain et al., 1989). The results depicted in Table 4.3 clearly reveal that a no greater number of explants forming callus was obtained when these cytokinins were included, with the 2,4-D, in the induction medium. By increasing the FAP concentration to 1 mg l^{-1} the number of explants forming callus decreased by 67%. When BA was added to the FAP/2,4-D combination, the percentage of callus-

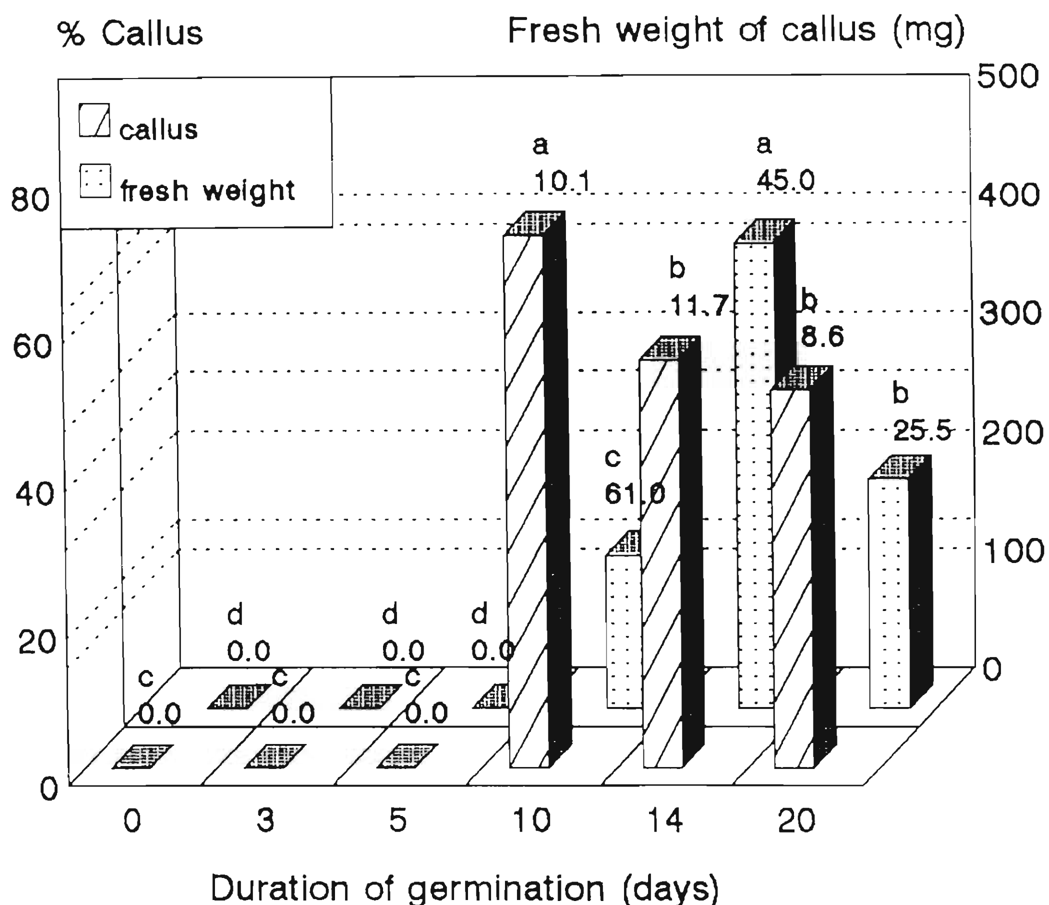

Figure 4.4 **The effect of the duration of germination on callus formation.**

The explant age was taken as the duration between the beginning of the seed pre-treatment process and the excision of the embryo from the seed. Explants were plated on LM, 2 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7 and cultured in the dark at 24 °C (±2 °C). Results were recorded, after 6 weeks in culture, as the percentage explants forming callus and the fresh weight of the calli formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-d).

Table 4.3 The effect of combining BA and FAP with 2,4-D on callus formation.

Varying concentrations of BA and FAP were combined in the LM induction medium containing 2 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7. Results were recorded as A) the percentage of explants forming callus and B) the fresh weight of the callus formed. Culture conditions were as described in Figure 4.4. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-f).

A)

	FAP (mg l^{-1})		
BA (mg l^{-1})	**0.0**	**0.3**	**1.0**
0.0	75 (±8.0) a	38 (±5.3) c	8 (±4.5) e
0.3	35 (±5.6) c	20 (±3.8) d	30 (±7.4) c
1.0	51 (±8.0) b	32 (±5.6) c	31 (±4.5) c

B)

	FAP (mg l^{-1})		
BA (mg l^{-1})	**0.0**	**0.3**	**1.0**
0.0	94 (±12.2)c	132 (±14.0)b	68 (±7.0)de
0.3	81 (±16)cde	78 (±3.6)cde	48 (±8.3)f
1.0	62 (±5.0)ef	152 (±12)a	86 (±9.6)cd

forming explants, although low (±30%), did not decrease further as the FAP concentration increased. The increased BA concentration did not result in an increase in the percentage of explants forming callus. There was no clear trend observed with the addition of BA and FAP as regards to the weight of the callus formed. Microscopic examination of the callus tissue showed no greater presence of embryogenic cells in the treatments containing the cytokinins. Consequently, all further callus induction work used 2,4-D as the sole growth regulator.

A further experiment was designed to examine the effect of using a gelling agent other than the agar-agar used thus far, and diluting the basal salt concentration, on callus formation from the embryo explants. Figure 4.5 shows that a reduction in the mineral salt content of the induction medium had a deleterious effect on the number of explants forming callus. There was no significant difference between the two types of gelling agents as regards to the number of explants forming callus. However, more callus was produced, per explant, when agar-agar was used as the gelling agent. Thus agar-agar was retained as the standard gelling agent for the somatic embryogenesis work.

Gupta and Durzan (1986) reported successful induction of globular somatic embryos from callus of mature pine embryos. To date this is the only report on somatic embryogenesis of a pine species from mature embryos. Because the present study involved working with mature pine embryos, the protocol used by those workers was tested. Figure 4.6 shows the results of this trial. The Gupta and

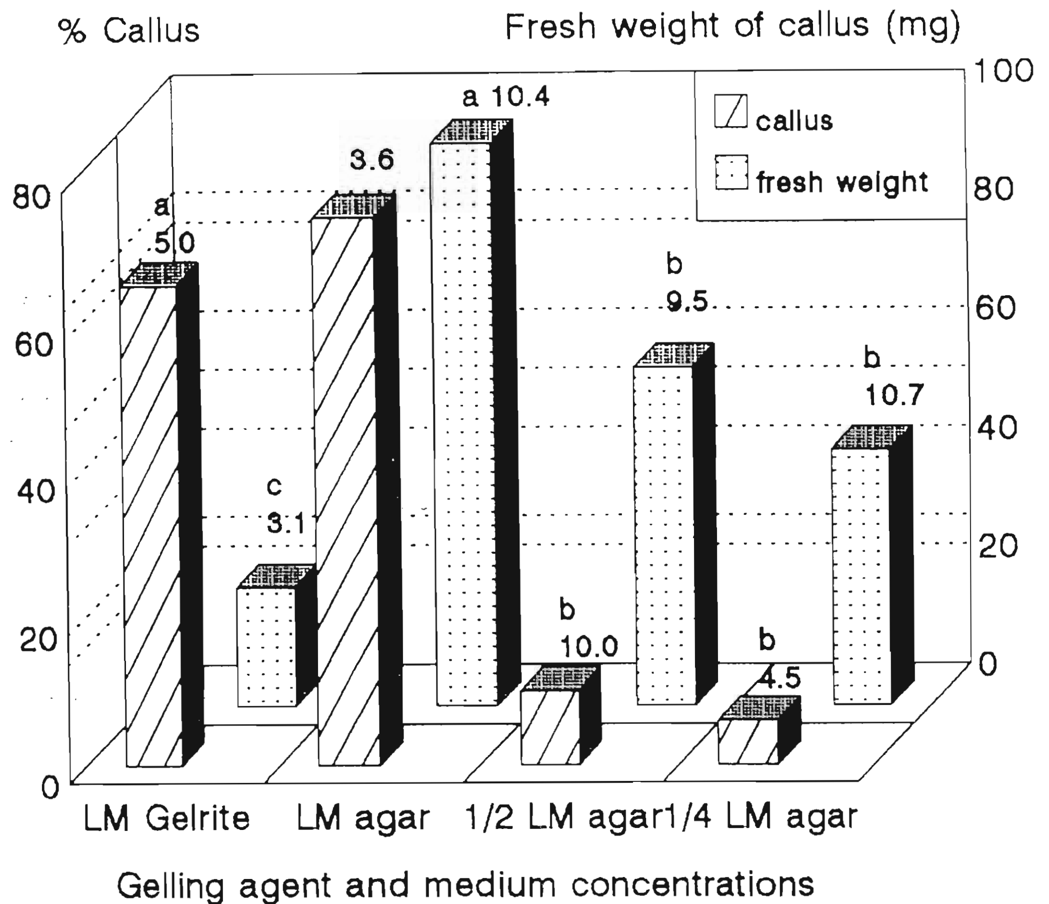

Figure 4.5 **The effect of the gelling agent and levels of nutrients in the medium on callus formation.** Explants were plated on various strengths of LM media, 8 g l^{-1} agar-agar or 4 g l^{-1} gelrite, 20 g l^{-1} sucrose, pH 5.7 and cultured in the dark at 24 °c (±2 °C). Results were recorded, after 6 weeks in culture, as the percentage of explants forming callus and the fresh weight of the calli formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple range test) are given (a-c).

Durzan (1986) report indicated that the sugar pine embryos were excised directly from stored seed without a germination pre-treatment stage. In this work, however, the patula seed received a 72 hour pre-treatment before the embryos were excised and cultured. The reason for this was that the removal of the patula embryos directly from the untreated seed, without damaging them, was very difficult. In order to have an acceptable number of explants for statistical analysis, the seed was imbibed for 72 hours so that the embryos could be easily removed. The patula embryos were cultured under the same conditions as described by Gupta and Durzan (1986). The LM medium salt formulation was included in the trial as a comparison to the DCR formulation used by Gupta and Durzan (1986). The results obtained were unexpected for two reasons. Firstly, few explants, cultured on the LM, 3 mg l^{-1} 2,4-D medium, formed callus whereas, as shown in Figure 4.4, explants, at the 3 day, LM 2 mg l^{-1} 2,4-D treatment, did not form callus at all. The only difference between these two treatments was an increase of 1 mg l^{-1} 2,4-D in the induction medium used in the trial on the applicability of the Gupta and Durzan (1986) protocol. The other observation was the fact that more explants formed callus on the DCR, 3 mg l^{-1} 2,4-D medium than on the LM, 3 mg l^{-1} 2,4-D medium. Results, in Figure 4.3, show that the explants cultured on the LM medium produced more callus than those on the DCR medium. However, in the Gupta and Durzan method, casein hydrolysate and L-glutamine were added to the DCR medium whereas these components were not added to the LM medium used in this experiment. Hence these substances could be responsible for the increase in callus formation in this work. Bhojwani and

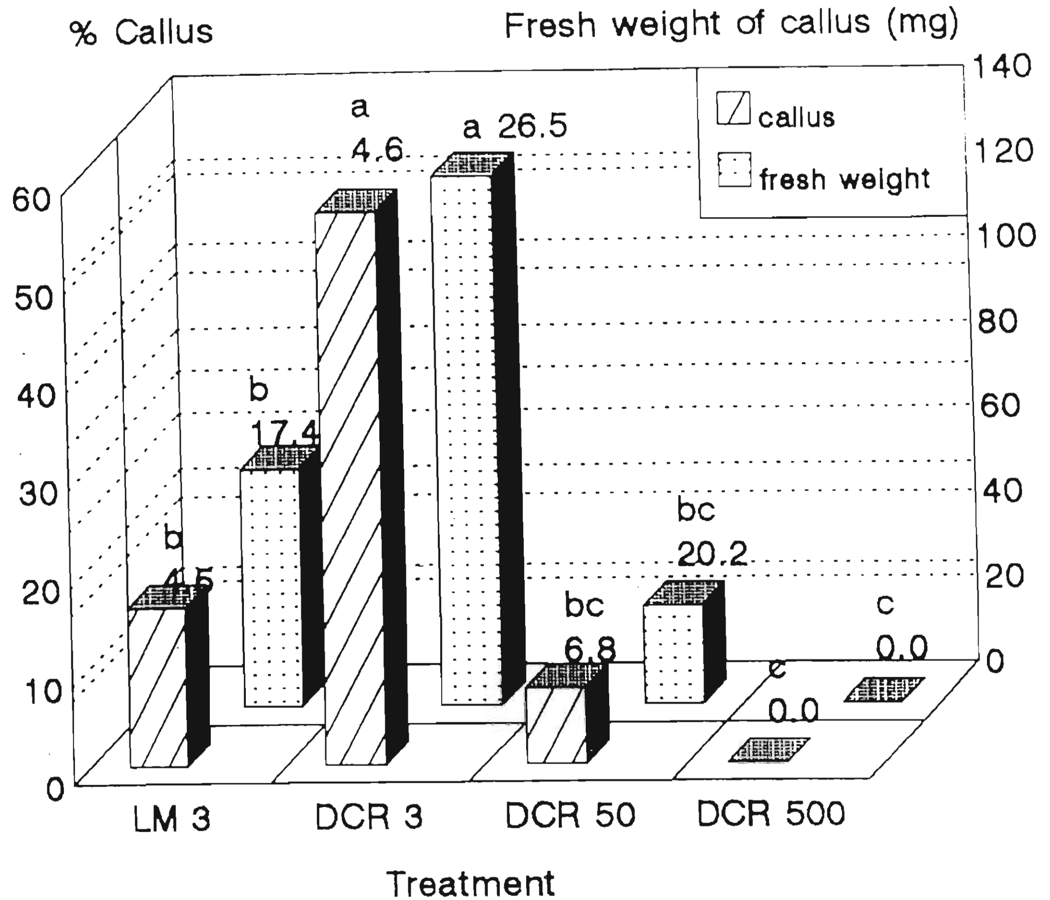

Figure 4.6 Applicability of using the Gupta and Durzan (1986) protocol for callus formation.

Embryo explants (3 day-old) were cultured on the induction medium, in the dark, for 10 weeks. The induction media were: LM, 3 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7 and DCR, 3, 50, 500 mg l^{-1} 2,4-D, casein hydrolysate 500 mg l^{-1}, L-glutamine 200 mg l^{-1}, 30 g l^{-1} sucrose, 6 g l^{-1} agar-agar, pH 5.7. Results were recorded as the percentage of explants forming callus and the fresh weight of the callus formed. n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-c).

Razdan (1983) reported on the promotive effect of casein hydrolysate on the growth of calli. The main reason why many researchers avoid the use of this substance is that different samples of casein hydrolysate may affect the reproducibility of results. The increase in 2,4-D concentration in the DCR medium (3-500 mg l^{-1}) inhibited callus formation.

4.3.2 Pro-embryo Formation

Once the Pinus patula somatic embryogenesis induction medium had been tested and a suitable medium was formulated, the next consideration was the period of time required for complete induction and consequently the culture duration needed on the maturation medium for pro-embryo development. Table 4.4 shows the results of an experiment examining these parameters. The formation of Stage 1 embryogenic cells in the callus required at least six weeks of culturing on the induction medium. Calli from each of the induction periods were aseptically transferred to the maturation medium for the development of Stage 2 pro-embryos from the Stage 1 embryogenic cells. A maturation period of at least six weeks was necessary for the formation of pro-embryo structures (Plates 4.5 and 4.6). A few pro-embryo structures were detected and these were all embedded in the centre of the callus mass and could not be observed without teasing the callus mass open with fine dissecting needles (Plate 4.7 and 4.8).

Table 4.4 The effect of the duration of culture, on both the induction and maturation media, on callus and pro-embryo formation respectively.

Explants were cultured on the induction medium as described in Figure 4.4 and then plated on LM maturation medium containing 12mg l^{-1} ABA, 20 g l^{-1} sucrose, 8 g l^{-1} agar-agar, pH 5.7. Results were recorded as the percentage of explants forming callus and the presence of pro-embryo structures in the callus mass (- =no structures, + =±2 structures, ++ =±5 structures). n= 30 for each treatment which was repeated three times. Standard deviations and levels of significant difference (Duncan's Multiple Range test) are given (a-b).

		Period on ABA (weeks)		
Period on induction medium (weeks)	**% explants forming callus**	**4**	**6**	**8**
4	26 ±5.3 b	-	+	-
6	66 ±6.1 a	-	-	++
8	64 ±12.5 a	-	+	-

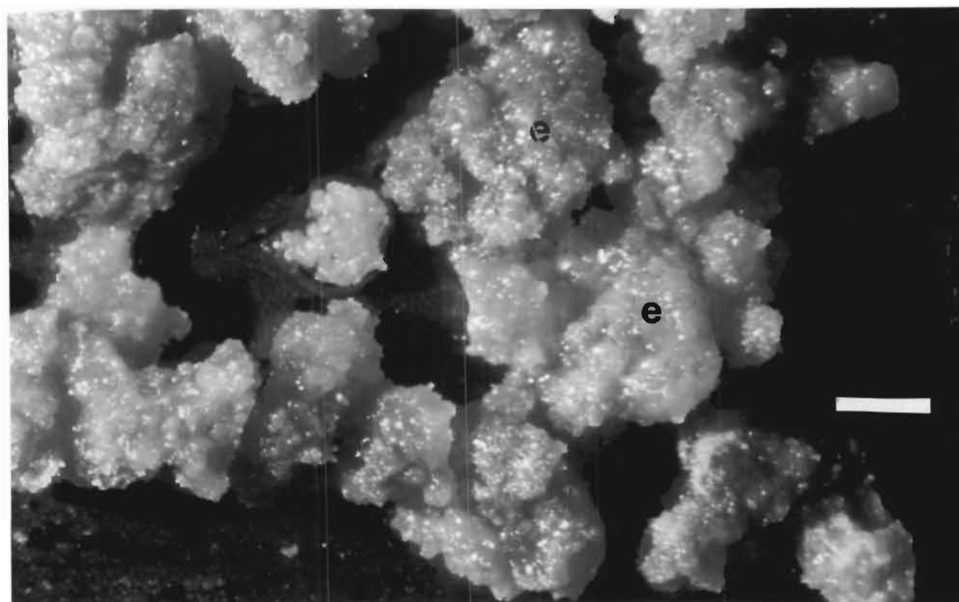

Plate 4.5 Clumps of embryogenic cells (e) grouped together in callus tissue. No distinct pro-embryo structures are present. Callus tissue was cultured om LM induction medium for 6 weeks before being transferred to the maturation medium (containing 12 mg l^{-1} ABA) for a further 9 weeks. Scale bar= 2 mm.

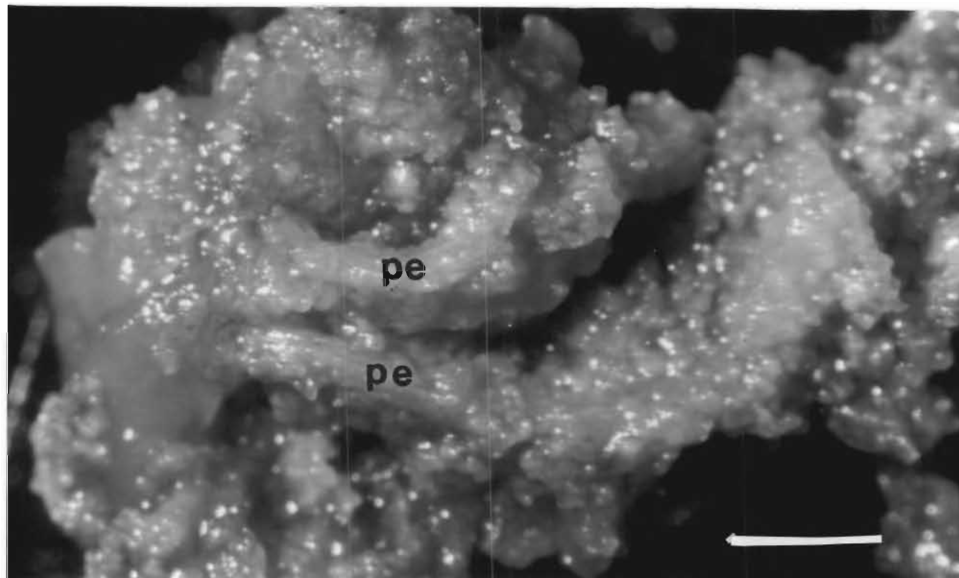

Plate 4.6 Callus tissue containing embedded stage 2 pro-embryos (pe). Callus tissue was cultured on LM induction medium for 6 weeks before being transferred to the maturation medium for a further 11 weeks. Scale bar= 0.5 mm.

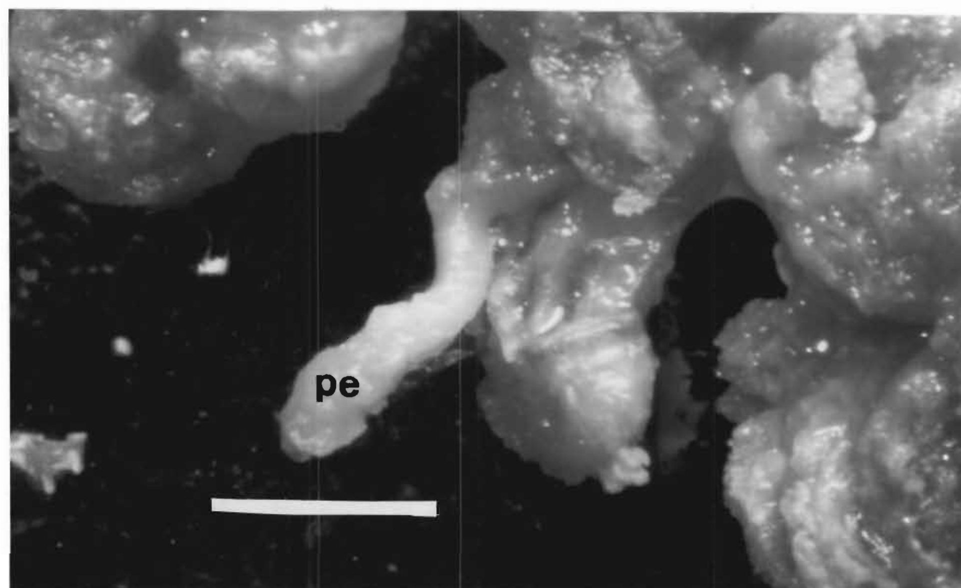

Plate 4.7 Stage 2 pro-embryo structure (pe) formed after an induction culture period of 6 weeks and a maturation culture period of 12 weeks. A stage 2 pro-embryo is one that is prominent, smooth in outline, opaque, cream to pale yellow and subtended by a suspensor (Tautorus et al., 1991). Scale bar= 0.83 mm.

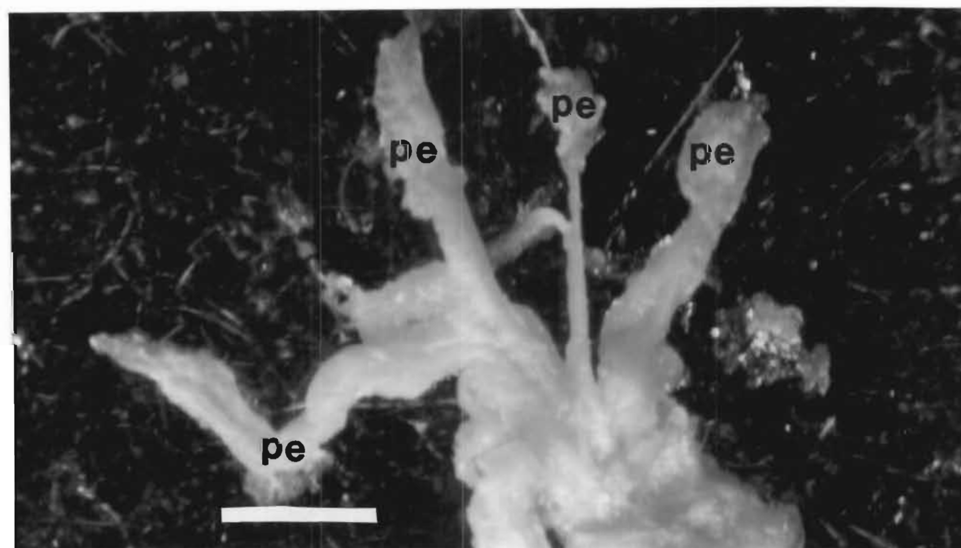

Plate 4.8 Stage 2 pro-embryo structures (pe) teased out from the surrounding callus tissue. Callus was cultured on induction medium for 6 weeks and maturation medium for 12 weeks. Scale bar= 1.0 mm.

4.3.3 Conclusion

A protocol was devised for the formation of the embryogenic cell and pro-embryo stages of P. patula somatic embryogenesis. Germinated embryos (10-14 day-old) were cultured on a LM induction medium containing 2 mg l^{-1} 2,4-D for six weeks. Embryogenic cells in the callus were clearly distinguishable from the non-embryogenic cells. Callus masses were then placed on a LM maturation medium containing 12 mg l^{-1} ABA. Within 6-8 weeks pro-embryos could be detected embedded in the calli masses. The yield of pro-embryos was low (3-5 pro-embryos/callus).

OVERALL DISCUSSION AND CONCLUSIONS

The main objective for investigating Pinus patula in vitro propagation techniques was to determine whether a successful mass propagation system could be found for producing large numbers of selected genotypes.By propagating trees, using micropropagation techniques, the immediate gains can be expected to be greater than by the production of seed in seed orchards (Nel, 1985). Seeds from orchards are at best open pollinated with only one parent known (John, 1983). Techniques exist to make controlled genetic crosses, but they are expensive. Vegetative propagation can be seen as a method of multiplying selected genotypes on a large scale. Throughout agriculture and forestry, in vitro methods of vegetative propagation are being studied as potential propagation systems for selected plants. Such strategies are being applied to a number of agricultural crops such as citrus (Kochba and Spiegel-Roy, 1977), tobacco and rice (Yoshida and Kohno, 1982). With respect to forest species, in vitro culture has been studied as a technique for shortening the generation time of the breeding programme and producing large numbers of selected plants (Thorpe and Biondi, 1984; Nel, 1985). The selected clones could be plus trees with superior timber quality, trees which have been selected for a particular pulp requirement or for specific site types. In view of these potential benefits, this study investigated three in vitro systems for the mass propagation of Pinus patula: (1) the production of adventitious buds from mature zygotic embryos (Chapter 2); (2) micropropagation via axillary bud induction on juvenile shoots and subsequent dwarf shoot

development (Chapter 3); and (3) pro-embryo formation from mature zygotic embryos through the process of somatic embryogenesis (Chapter 4).

An adventitious bud system was obtained for Pinus patula using germinated zygotic embryos as the explant. Germination techniques were developed for conditioning the embryo explant material (Figs 2.1, 2.2, 2.3, 2.4, 2.5, 2.6) and sterilisation procedures for producing contaminant-free cultures were implemented (Figure 2.7 and Table 2.2). Culture conditions for adventitious bud initiation were investigated (Figure 2.16) and suitable hormone and media formulations were established (Figs 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14). The main drawback with adventitious buds is the low recovery rate of utilisable shoots (approximately 20%). The yield (50-70% embryo explants with adventitious bud formation) obtained in this study was not as high as that (80-90%) reported by Mott et al. (1977) who worked on Pinus taeda. Similarly, the actual yield of P. patula shoots, obtained from each explant in this work was extremely low (between three to five shoots) compared to yields reported by other researchers. For example, Aitken et al. (1981) observed that nine rootable shoots were produced per seed for excised Pinus radiata embryos. Although 50-70% of the embryo explants produced bud primordia, only a few dominant shoots elongated from the clump of adventitious buds (Plate 2.5). The shoots, which did elongate, developed very slowly (Fig 2.15). This problem has been experienced by other researchers. Martinez-Pulido et al. (1990) reported that after nine weeks only 16% of buds, initiated on 3 day-old Pinus

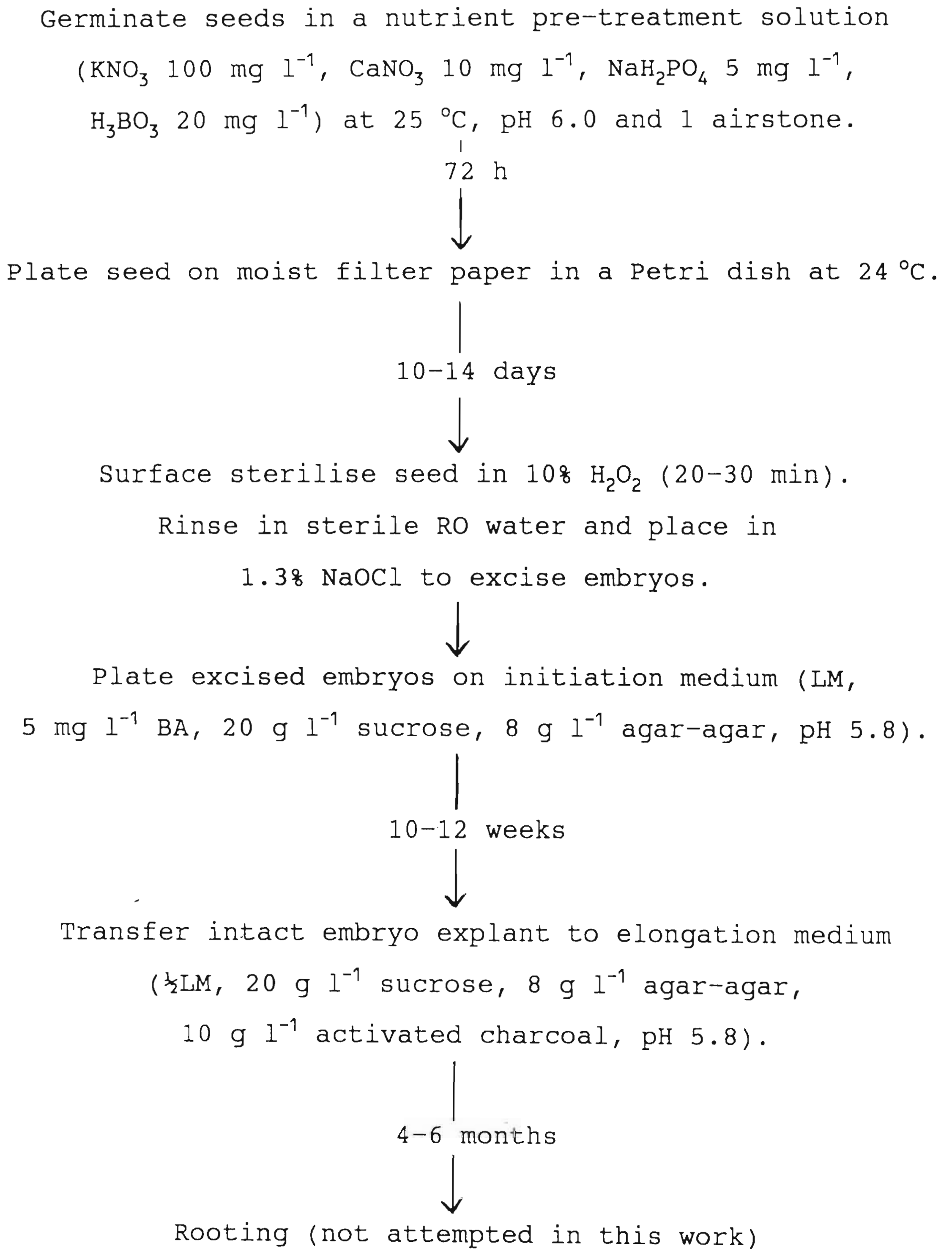

Figure 2.16 Summary of developed protocol for in vitro production of Pinus patula adventitious shoots from mature embryos. Cultures were kept in a 16 h photoperiod (740 mols m^{-2} s^{-1}).

canariensis explants, were over 5 mm in height. The time taken, from embryo explant placement to the stage when the shoots were ready for rooting, was approximately seven months.

In contrast to adventitious bud culture, axillary bud propagation (Figure 3.11) proved to be more successful as a shorter period (approximately five months) was necessary for shoots to develop to the rooting stage. Juvenile shoots were harvested from one year-old P. patula seedlings and cut into 55 mm segments (Plate 3.3) prior to surface sterilisation. An effective sterilisation treatment controlling both bacterial and fungal contamination without damaging explant material, was developed (Fig 3.2 and Fig 3.3). Culture conditions, with respect to explant preparation, cytokinin concentrations and media formulations, were examined for dwarf shoot production (Figs 3.4, 3.5, 3.6, 3.7, 3.8). Approximately 60% of the juvenile shoot explants produced one or more dwarf shoots per explant. Similar results were obtained by Gupta and Durzan (1985) with Douglas fir (two to three axillary buds/shoot) and Lin et al. (1991) with ponderosa pine (two to four axillary buds/shoot). The P. patula dwarf shoots were successfully elongated (Fig 3.9) to a size suitable for rooting. As insufficient numbers of dwarf shoots were produced for rooting studies to be conducted on the dwarf shoots, juvenile shoots were used in the rooting study in order to ascertain possible rooting requirements for dwarf shoots (Fig 3.10). The rooting investigation was carried out under greenhouse conditions as most reports on in vitro-produced conifers suggest treating the shoots as

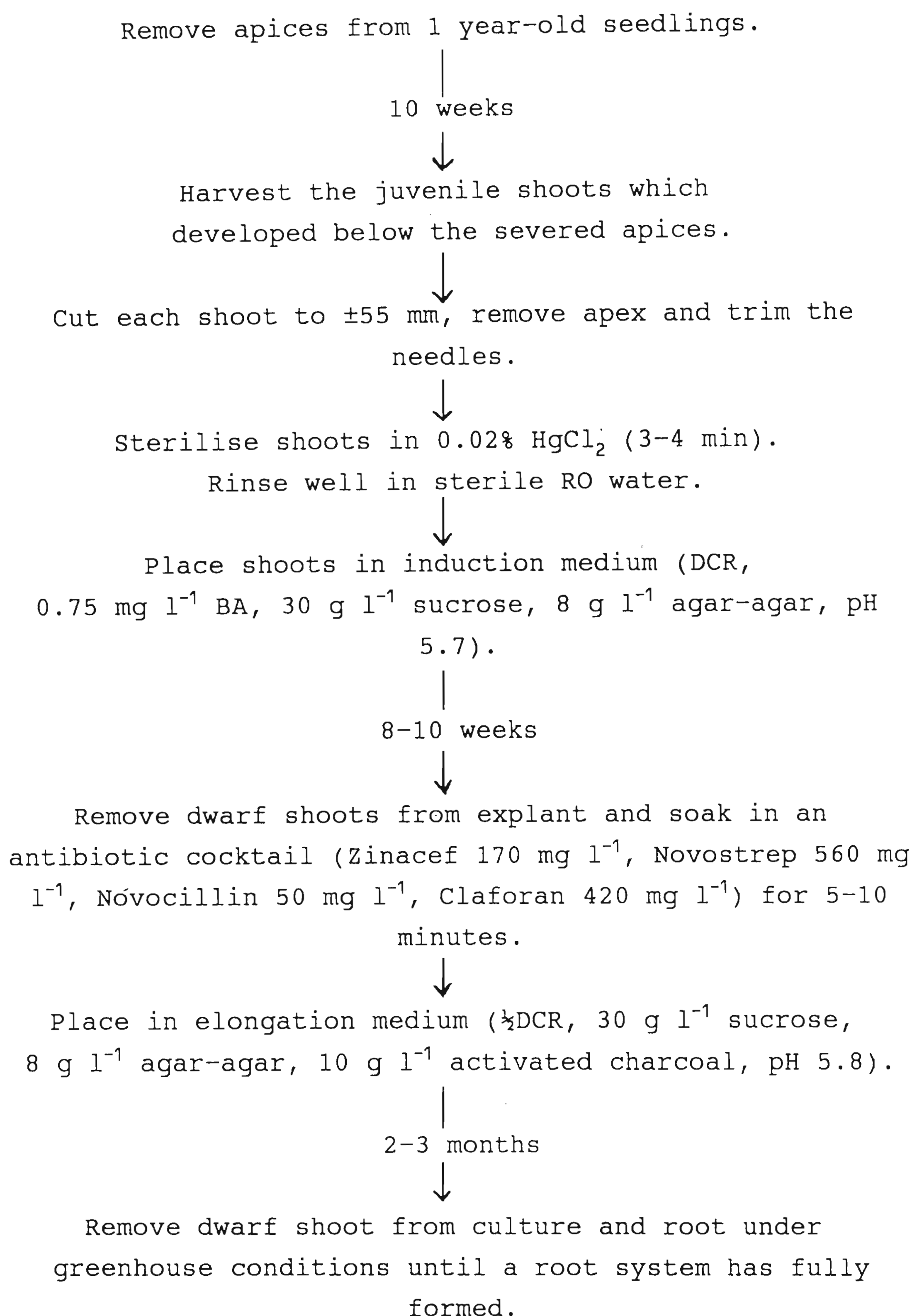

Figure 3.11 Summary of developed protocol for in vitro production of axillary dwarf shoots from juvenile shoots. Cultures were maintained in a 16 h photoperiod (740 mols m^{-2} s^{-1}).

cuttings (Rancillac et al., 1982 and Baxter et al., 1989). The rooting percentages, from the best rooting treatment, were low (50 to 60%). This was expected as Pinus patula is known to be a poor rooter (Osborne, 1992). Rooting studies, similar to those undertaken in this study, were carried out on Pinus tecunumanii, a closely related species to P. patula, by Baxter et al. (1989). Those workers found that P. tecunumanii had the lowest axillary shoot rooting percentage (44%) out of the three pine species (P. caribaea, P. oocarpa, P. tecunumanii) examined.

Smith and Drew (1990) stated several advantages of recovery of plants from cells via somatic embryogenesis compared with other in vitro systems. Somatic embryos can be produced from cells growing in suspension, thereby making possible batch culture techniques which can be scaled-up with minimum increase in handling costs (Smith and Drew, 1990). Cell suspensions have the advantage over callus cultures in that cells develop more or less individually, have more nutritional access to the culture medium, and some of the physiological variations of callus cultures are avoided (Hartmann and Kester, 1983). In view of this and the fact that the Pinus patula somatic embryogenesis system has not been reported in any detail, this in vitro system was examined in the present study. The seed germination and sterilisation protocols, used for the embryogenesis work, were the same used in the adventitious work (sections 2.2.1 and 2.2.2). Culture conditions for the induction of embryogenic cells, namely auxin concentrations and medium formulations (Figs 4.2, 4.3, 4.4, 4.5, 4.6 and Table 4.2) were developed (Figure 4.7). Although 60 to 70% of the

embryo explants formed a callus mass, the yield of embryogenic cells within each callus, after a six week culture period, was very low judging from microscopic examination of calli squashes. The embryogenic cells developed further to form pro-embryo structures (Table 4.4, Plates 4.7 and 4.8) when cultured on maturation medium for a further six weeks. Due to the lengthy development of pine embryogenesis _in vitro_, subsequent maturation and germination studies on the pro-embryos were not undertaken in this study. Further developmental stages of embryogenesis that are required to produce a plant are maturation (where the embryo possesses cotyledons) and then the isolation of the embryos and culturing them to form plantlets.

From the work summarised above, the common constraint with each _in vitro_ system examined was the duration it took to produce plants. This was true even with the somatic embryogenesis work where, although no plants were obtained, the culture period to the pro-embryo stage was in excess of three months. Reports on pine embryogenesis (Nagmani and Bonga, 1985 and Tremblay, 1990) suggest that a further six months of culture could be necessary for obtaining germinated plantlets ready for field planting. This duration is similar to the period required for producing pine seedlings in South African nurseries. Similarly, to develop hardened-off _P. patula_ plants from _in vitro_ shoots could take as long as seven to eight months. In _Eucalyptus_ species, production of rooted organogenic plants can take as little as four months (Blakeway, 1992), thus making _in vitro_ culture of this species much more commercially viable

Seed germination and sterilisation procedures.
(refer to Figure 2.16).

Plate excised embryos on induction medium
(LM, 2 mg l^{-1} 2,4-D, 20 g l^{-1} sucrose,
8 g l^{-1} agar-agar, pH 5.7).

4 weeks in dark

2 weeks in light
(16 h photoperiod, 740 mols m^{-2} s^{-2}).

Transfer calli to maturation medium
(LM, 12 mg l^{-1} ABA, 20 g l^{-1} sucrose,
8 g l^{-1} agar-agar, pH 5.7).

6-10 weeks
(16 h photoperiod, 740 mols m^{-2} s^{-1})

Pro-embryos embedded in callus mass.

Figure 4.7 Summary of developed protocol for obtaining Pinus patula pro-embryos from mature zygotic embryos.

than *Pinus* species.

Of the three *in vitro* systems investigated for *P. patula*, the axillary bud system was the one with the most potential (approximately six months to obtain a rooted shoot). However, the yield of dwarf shoots per explant (three to five shoots) was low. In this regard somatic embryogenesis could offer the most potential in terms of the theoretical yield of plants. For example, in *Pinus radiata* there are at least 10 000 embryo initials per gram fresh weight of embryogenic tissue (Jones, 1990). This technique could substantially reduce the high labour input that would be required for the multiplication and transfer of shoots and plantlets produced via organogenesis.

There have been conflicting reports with regard to the cost of producing plants in culture. Rediske (1978) stated that the cost of *in vitro* plants should be significantly lower than that of rooted stem cuttings which, in 1974, were estimated to cost at least 30% more than seedlings (Kleinschmidt, 1974). In contrast, Sommer and Caldas (1981) estimated that rooted plantlets produced by *in vitro* methods might cost 3-30 times as much as seedlings produced using normal nursery techniques. Therefore, due to the low yields and time taken for *in vitro* propagated *P. patula* plants to be ready for field planting, it is essential that the protocols must be optimised further. It is also important that the genotype, selected as the explant source for *in vitro* propagation, must be superior since the increased growth of the genotype and its subsequent increased yield of products will have to pay for the

increased cost of the method of production of the planting stock. The high expenses of in vitro propagation are found in the capital cost of laboratory facilities, the need for trained staff and the cost of chemicals required for the culture media. Hartney and Svensson (1992), however, described simple micropropagation techniques that do not demand expensive facilities or large amounts of capital equipment. These include growing cultures in stacks of polypropylene food containers under natural light in shaded polythene greenhouses, using pressure cookers instead of autoclaves, replacing laminar flow benches with glove boxes and rooting shoots ex vitro. Expenses could be further reduced if automated production systems were implemented. For example, Commonwealth Industrial Gases (CIG) in Australia have developed an automated system which performs visual assessment of shoots, automatically cuts and transfers shoots from one container to another (Johnson, 1989).

Although the axillary bud route is the one that seems to take the shortest time, and therefore is the most cost effective route for commercial P. patula in vitro propagation, another factor that needs to be considered is the poor rooting ability of this species, both in vivo and in vitro. Osborne (1992) discussed methods for trying to improve the rooting of P. patula cuttings. However, only about 50% of the cuttings set rooted. The same limitation is placed on producing plantlets from adventitious or axillary dwarf shoots as these are rooted under similar conditions to cuttings. Rooting of in vitro conifer shoots have presented problems to a number of workers (Campbell

and Durzan, 1975; Cheng, 1975; Sommer et al., 1975; Coleman and Thorpe, 1977; Winton and Verhagen, 1977). Despite this constraint, successful field plantings of Pinus species have been reported by Smith (1986)(P. radiata), Amerson et al. (1988)(P. taeda) and Horgan and Holland (1990)(P. radiata).

Significant progress has been made in biotechnology aimed at conifer tree propagation and tree improvement (Dunstan, 1988). There is, however, a vast amount of work that still remains to be done in tree improvement biotechnology. For example, investigating the possibility that more cutting material and higher rooting percentages could be obtained from in vitro produced patula pine. The capacity to propagate trees vegetatively is associated with juvenility, generally, the more juvenile the parent plant, the easier it is to propagate vegetatively (Bonga, 1982). Although there have been no reports on the use of in vitro-produced hedges for pines, a Vitis clone, which for centuries has been propagated only in its mature form, reverted to the juvenile form after several generations of in vitro repropagation of propagules (Mullins et al., 1979). Another aspect of tree breeding research is somatic hybridisation. Somatic hybridisation, through protoplast fusion, permits the synthesis of crosses that would not normally be possible, for example, between different genera or species that produce infertile offspring (Burley, 1989). As an alternative to sexual reproduction, protoplast culture combines the entire nuclear and non-nuclear genomes of the parents (Caplan et al., 1983). This is of importance to tree improvement as new hybrids, between sexually

incompatible species, can be produced (Jones and Lindsey, 1988). Also, the techniques of somaclonal variation and selection, in vitro selection, and haploid culture could have very valid roles to play in forest biotechnology (Dunstan, 1988). The potential of this variation as a source of useful mutants for crop improvements is vast, as discussed in reviews by Mitra (1985) and Larkin (1987). For example, in vitro selection of somaclonal variants can be used for the production of plants which are resistant/tolerant to stresses produced by phytotoxins from pathogens, herbicides, cold stress, and aluminium, manganese and salt toxicity (Tomes and Swanson, 1982; Chaleff, 1983). Another technique of value is the culture of gametophytic tissue, that is, the microspores (pollen) and the megagametophyte of conifers. These cells are the product of meiosis and therefore have half the genetic complement of the parent. Haploid culture is of interest to tree breeders who want to use homozygous diploid (double haploid) plants in their study of inheritance (Bonga et al., 1988). There have been no reports of these techniques being used for P. patula, hence, the tree breeding research possibilities for this species are enormous.

In conclusion, the low yields and time taken for plants to be produced may result in the in vitro systems, described in this work, not being used for commercial production of Pinus patula. If such systems were to be considered for mass propagation, further work would be needed to greatly improve the yields as reducing the culture period would be very difficult for this species. One important priority would be investigating the somatic embryogenesis route

further in order to produce plantlets. The advantages of this system are (1) the potential large numbers of plants that can be produced and (2) the difficult rooting stage would not be encountered as germinated somatic embryos possess both shoot and root axes as do germinated zygotic embryos. Once a protocol is established for producing complete somatic embryogenic plants from mature seed, further work could examine the possibilities of producing somatic embryos in suspension culture in order to increase the yield of plants even more.

APPENDIX 1

Table 1.3 Composition of the media used in this study on micropropagation of P. patula. The media are: MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), GD (Mehra-Palta and Smeltzer, 1978), MCM (Bornman, 1981), LM (Litvay et al., 1981), LP (Aitken-Christie, 1984) and DCR (Gupta and Durzan, 1985).

Compound	Media Mass (mg l^{-1})						
	MS	SH	GD	MCM	LM	LP	DCR
Macronutrients							
CH_4N_2O	-	-	-	150	-	-	
KNO_3	1900	2500	1000	2000	1900	1800	340
$Ca(NO_3)_2.4H_2O$	-	-	-	500	-	1200	556
NH_4NO_3	1650	-	-	-	1650	400	400
$(NH_4)_2SO_4$	-	-	200	400	-	-	-
$NH_4H_2PO_4$	-	300	-	-	-	-	-
KH_2PO_4	170	-	-	270	340	270	170
$CaCl_2.2H_2O$	440	200	150	-	22	-	85
KCl	-	-	300	150	-	-	-
$MgSO_4.7H_2O$	370	400	250	250	1850	360	370
$NaH_2PO_4.H_2O$	-	-	90	-	-	-	-
Na_2HPO_4	-	-	30	-	-	-	-
Micronutrients							
$FeSO_4.7H_2O$	27.8	15	27.8	-	27.8	30	27.8
$Na_2.EDTA.H_2O$	37.3	20	37.3	-	37.3	40	37.3
$NaFe.EDTA.2H_2O$	-	-	-	37.5	-	-	-
H_3BO_3	6.2	5	3	1.5	31	6.2	6.2
$ZnSO_4.7H_2O$	8.6	1	3	3	43	8.6	8.6
$MnSO_4.H_2O$	16.9	10	10	0.17	21	1	22.3
$Na_2MoO_4.2H_2O$	0.25	0.1	0.25	0.25	1.25	0.25	0.25
KI	0.83	1	0.75	0.25	4.15	0.08	0.83
$CuSO_4.5H_2O$	0.025	0.2	0.25	0.025	0.025	0.025	0.25
$CoCl_2.6H_2O$	0.025	0.1	0.25	0.025	0.13	0.025	0.025
Vitamins & other organic constituents							
Myoinositol	100	1000	10	90	100	1000	200
Glycine	2	-	-	2	-	-	2.0
Thamine.HCl	0.1	5	1	1.7	0.1	0.4	1.0
Nicotinic acid	0.5	5	0.1	0.6	0.5	-	0.5
Pantothenate	-	-	-	0.5	-	-	-
Pyridoxine.HCl	0.5	0.5	0.01	1.2	0.1	-	0.5
Folic acid	-	-	-	1.1	-	-	-
Biotin	-	-	-	0.125	-	-	-

APPENDIX 2

Bravo-500®	Chlorothalonil; manufactured by SDS Biotech Corporation.
Claforan®	Cefotaxime base; manufactured by Roussel.
Novocillin®	Procaine Penicillin; manufactured by Novo Industries.
Novo-Strep®	Streptomycin Sulphate; manufactured by Novo Industries.
Rootstim®	1-Naphthylacetamide 0.67 g kg^{-1} 2-Methyl-1-Naphthylacetic Acid 0.33 g kg^{-1} 2- Methyl-1-Naphthacetamide 0.13 g kg^{-1} indolyl Butyric Acid 0.57 g kg^{-1} Manufactured by Applied Agricultural Products (Pty) Ltd.
Seradix 3®	4-(indol-3-yl)-butyric acid 8 g kg^{-1} Manufactured by Maybaker.
Zinacef®	Cefuroxime; manufactured by Glaxo S.A. (Pty) Ltd.

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