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*Micropropagation of
Pinus Species*

By

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*Submitted in fulfilment of
the requirements for the degree
Magister Scientiae*

*in the Faculty of Science
(Department of Botany and Genetics)
University of the Orange Free State
Bloemfontein
1999*

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Universiteit van die
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BLOEMFONTEIN

8 - SEP 2000

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Preface

Investigations leading to the results presented in this study were carried out in the Department Botany and Genetics, at the University of the Orange Free State, Bloemfontein, under the supervision of Professors A.J. van der Westhuizen and A.M. Botha-Oberholster (currently at UP).

The studies have not been submitted in any form to another University. The work of others is acknowledged in the text, and results given are of my own investigation.

Angeline Jacoby

April 1999

Acknowledgements

A great thank you to the Lord for wisdom and perseverance.

I am grateful to Professors A.J. van der Westhuizen and A-M Botha-Oberholster, for the opportunity to conduct my studies. I would like to thank them for their time, valuable input and assistance.

I also would like to thank the University of the Orange Free State and the Department of Botany and Genetics for allowing me to use their facilities to complete my studies.

I would like to acknowledge the financial assistance of the FRD and the support of SAFCOL towards this research. A sincere thank you to SAFCOL for providing the plant material and to M. Wilding, A. van Zyl and K. Maasdorp my co-workers on the SAFCOL project, for their contributions towards some of the results obtained in this study.

I wish to thank Dr. Groenewald and Dr. Meyer for their contribution and interest in my work and the numerous people not mentioned by name who in some way have contributed to this study.

I would like to thank my husband Shane for all his valuable support and constant encouragement.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
4-chloroIAA	4-chloroindoleacetic acid
ABA	abscisic acid
BA	N ⁶ -benzyladenine
BAP	benzylaminopurine
cm ³	cubic centimeter
DCR	Douglas-Fir Cotyledon Revised Medium (Gupta & Durzan, 1985)
<i>et al.</i>	and others (et alii)
EDTA	ethyl diamine tetra acetic acid
EIA	enzyme immunoassays
ESM	embryonal suspensor mass
g l ⁻¹	gram per litre
GC-MS	gas-chromatography-mass spectrometry
GD	Gresshof and Doy (1972) medium
h	hour(s)
IAA	indoleacetic acid
IBA	indolebutyric acid
IEDC	induced embryogenic-determined cells
IPA	isopentenyl adenine
Kin	kinetin
LM	Litvay medium (Litvay <i>et al.</i> 1981)
LP	Quoirin and Lepoivre (1977) medium
M	molar
MCPA	2-methyl-4chlorophenoxyacetic acid
mg	milligram(s)
mg l ⁻¹	milligram(s) per litre

min.	minute(s)
ml	millilitre(s)
mm	millimeter
mM	millimolar
MS	Murashige and Skoog (1962) medium.
MSG	Modified MS medium Becwar <i>et al.</i> (1990)
m/v	mass per volume
N	Normal
NAA	naphthaleneacetic acid
nm	nanometer
NSB	non-specific bindings
OD	optical densities
PAA	phenylacetic acid
PEDC	pro-embryonic-determined cells
PEG	polyethylene glycol
PGR	plant growth regulators
psi	pound per square inch
RIA	radio-immunoassays
rpm	revolutions per minute
SAFCOL	South African Forestry Company Ltd.
sec.	seconds
SH	Schenk and Hildebrandt (1972) medium
sp.	species
TLC	Thin Layer Chromatography
Tris	tris(hydroxymethyl)methylamine buffer
Tween 20	polyoxyethylene-sorbitan-monooleate
t-ZR	trans-zeatin riboside
v/v	volume per volume
wt	weight
&	and

%	percentage
°C	degree Celsius
$\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$	micro Einstein per square meter per second.
μl	microlitre
μM	micromolar
μm	micrometer

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Chapter 1

Introduction

Pines belong to the Division Coniferophyta, Family Pinaceae. This family is one of the most dominant, conspicuous and best known gymnosperms among the floras of the modern world.

The genus *Pinus* is of immense economic importance to man. These plants are a major source of paper pulp and timber. There is a great variety of other uses including the supply of building material, firewood and material to the furniture industry. Wood products like tar, oils and turpentine are also obtained by distillation. Certain Asian species are also tapped on a large scale for resin (Poynton, 1977).

Naturally these trees are very widely distributed in the Northern hemisphere, and almost entirely absent south of the equator. Because of their economic importance, pine trees have been introduced and cultivated, in the Southern hemisphere (Poynton, 1977). According to Mc Kellar *et al.* (1994), one of the most commercially important softwood species grown in South Africa is *P. patula*. Species like *P. radiata* have been planted on a very large scale and *P. kesiya*, *P. elliottii*, *P. taeda* and *P. pinaster* have also been planted and grown for their timber (Poynton, 1977).

Forestry in South Africa has been established for over a 100 years and is a very vibrant and growing industry. From a humble beginning the forestry industry has become one of the fastest growing sectors and a major force in the South African economy (Delaney, 1994). The industry is dominated, by large forest owners, such as Sappi, Mondi and SAFCOL and numerous other smaller ventures exist. They provide employment for a vast number of people.

In 1980, R275 million worth of forest products were exported and in 1991, the products exported were estimated at R2 billion (Cellier, 1993). Growing pines have become more profitable over the period 1991 to 1995, mainly as a result of increasing timber prices. Growers benefited by an average of 142% improvement in profits (Oliver & Rusk, 1997).

The most common means of propagation of *Pinus* sp. is by seed. The seeds are collected from natural stands and seed orchards. Problems with seed dormancy, embryo and seed coat dormancy makes the propagation of some species difficult (Hartmann *et al.* 1990a). The rooting of cuttings or needle fascicles is also a well-known traditional method of vegetative propagation. However, for the majority of trees this method is often characterized by an inverse relationship between rooting capacity of the cutting and the age of the parent plant (Thorpe & Biondi, 1984).

Grafting methods such as tip cleft, side veneer and budding were among the first techniques used to propagate forest trees vegetatively (Barnes & Burley, 1987). Considering the expense and limited yield of these methods, they can hardly be considered economically viable.

It was generally believed that vegetative propagation offered the best solution to meet the high yield uniformity and quality requirements of the industry. The superiority of vegetative propagation, over sexual regeneration, lies in the ability to transfer the entire genetic potential of a selected tree. Thus vegetative propagation allows one to take advantage of genetic progress as soon as it is visible (Poynton, 1977). A disadvantage, however is the long period required for these trees to grow and reach maturity. Forests are normally managed on a 20-25 year rotation. The conventional methods of tree improvement and selection are impractical and have their problems (Reilly & Washer, 1977). They therefore offer a very limited probability of meeting the growing demands (Minocha & Minocha, 1995).

Another prominent feature of the *Pinus* reproduction cycle is the relatively long interval between pollination and fertilization. In most cases it is about twelve months (Weier *et al.* 1982).

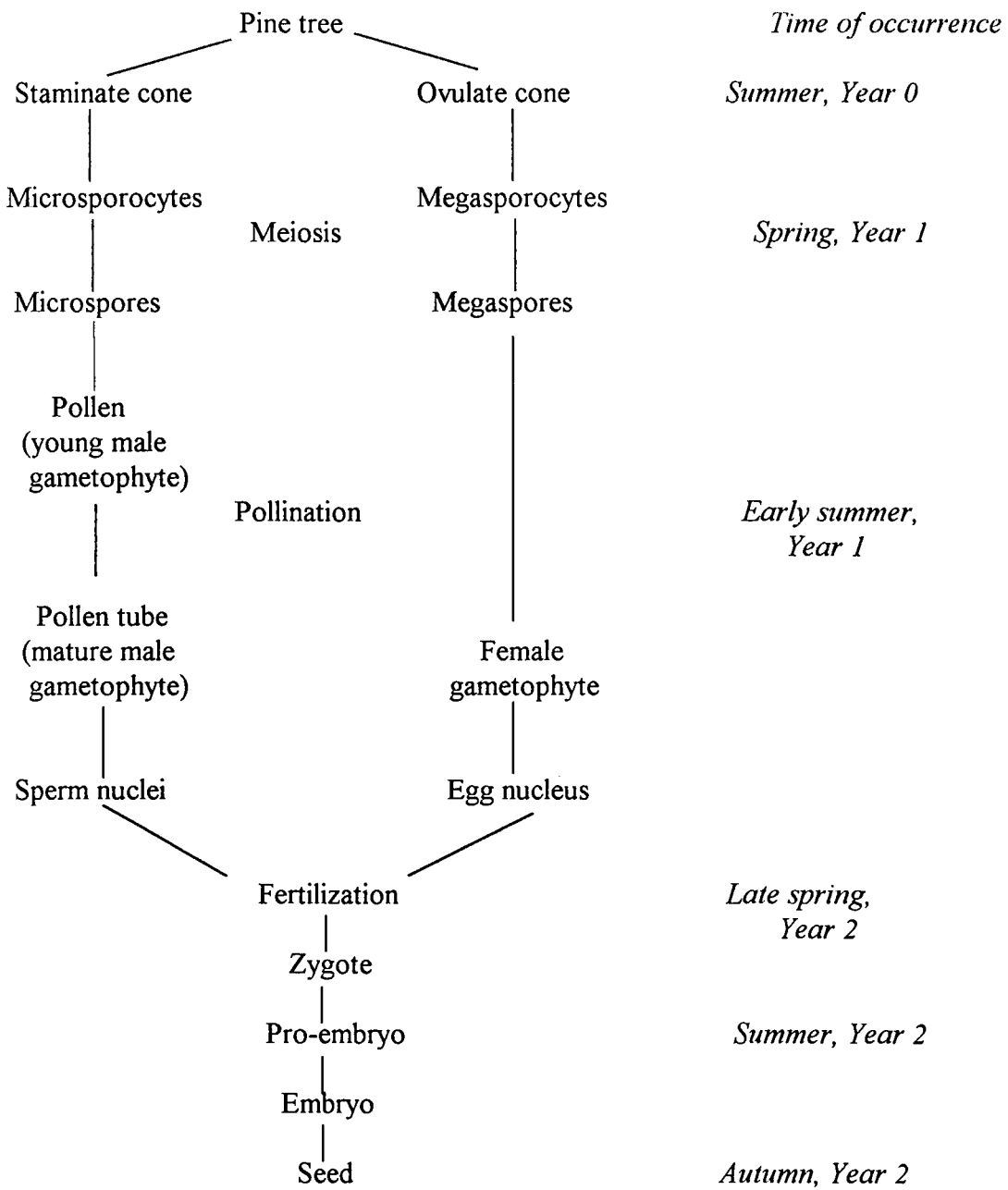


Fig. 1. A generalized life history of a pine.

The world's demand for wood and wood products is expected to rise very sharply in the future. To meet this growing demand, there will be an increasing need for mass production of improved quality stock. Forest trees, however present a challenge to the geneticist due to their long reproductive cycle, which requires many years of research to achieve significant improvement. The option used in the improvement of forest trees, is therefore a programme of selection, cloning progeny testing and controlled breeding (Kriebel, 1995).

The criteria for tree selection are determined by particular commercial requirements: fast growth rate, disease resistance, wood density, growth form, bio-mass and bio-energy production, and environmental tolerance (Dunstan & Thorpe, 1986). Outstanding individuals could be specifically selected for these requirements and used as the basis for breeding programmes.

Clonal forestry caught the imagination of prominent South African companies in 1982/83 as a result of wide publicity of the "Aracruz Florestal Success Story" which coincided with the rapid expansion of the pulp, paper and mining industry. These developments resulted in major forestry expansion programmes to meet the future demand (Denison & Kietzka, 1993).

The modern approach to propagation is by cell and tissue culture. Methods already exist including the use of buds, and the production of plantlets from stem callus (organogenesis) and the production of embryos from embryonic callus mass (somatic embryogenesis), for a wide variety of plant species. The main advantage of using cell culture as a tool in breeding programs and mass clonal propagation is its potential for virtually unlimited multiplication rates (Thorpe & Biondi, 1984) with the reproduction of large quantities of uniform plants of selected qualities. It is, however only during the past two decades that concerted efforts have been made to adapt these methods for the propagation of commercially important tree species. The information at hand does not, however allow for the routine propagation of all

conifer species. It is obvious from published literature that the results can seldom be generalized and extrapolated from one species to another with regard to the nutritional requirements for growth and differentiation *in vitro*. Therefore, detailed steps for successful propagation of each particular species must be worked out individually (Minocha & Minocha, 1995).

The aim of this study.

The economic importance of pine species such as *P. radiata* and *P. patula* is the underlying motivation to the following:

- To initiate embryonic callus formation and development thereof on solid medium.
- To find the most effective embryonic cell suspension culture medium.
- To develop the most effective medium for organogenesis from explant sources like, embryos, cotyledons, and the distal portion of the hypocotyl of seedlings.
- To investigate a probable relationship between phenolic, auxin and cytokinin contents and the tendency of hybrid cuttings to root.

Chapter 2

Literature Review

2.1. Micropropagation.

The term micropropagation is used specifically to refer to the application of tissue culture techniques to the propagation of plants, starting with a very small plant part grown aseptically in a container. The propagule used to start the process is known as an explant (Hartmann *et al.* 1990b). Most often micropropagation is also associated with mass propagation at a competitive price.

Up to 1975 micropropagation involved the production of callus, and the subsequent regeneration of plantlets from these callus cultures (Thorpe *et al.* 1991). Since the first protocol for micropropagation was proposed in the 70's much has changed and there are now only five stages critical to successful micropropagation:

- Stage 1 is the preparative stage. It is just as important as any of the other stages for development of a reliable and repeatable micropropagation scheme.
- Stage 2 is to establish a viable culture.
- Stage 3 aims towards proliferation.
- Stage 4 is the production of plantlets.
- Stage 5 is the re-establishment in a greenhouse.

Each of these stages however has it's own inherent difficulties.

It is very important that the right starting material (explant) for each micropropagation principle must be used. The developmental stage of an explant can be of tremendous importance. The age of the mother plant, the physiological age of the explant and its developmental stage, as well as its size can determine the success (Debergh & Read, 1991).

Explants for *in vitro* cultures have been derived from almost every tissue present during the life cycle of conifers. Male and female gametophytic tissue, immature and mature embryos, hypocotyl, cotyledons and shoot apex have been used. However newly germinated seedlings, apical meristems, whole shoots, juvenile and mature needles, needle fascicles (brachyblasts) and various cambia of more mature shoots have also been used (John, 1983). Various authors have shown that immature seeds or intact female gametophytes are the most suitable explants from which to initiate embryonic cultures in pines (Kriebel & Finer, 1990; Becwar *et al.* 1991; Gupta & Grob, 1995).

The duration and conditions of storage of these explants are also critical. Extended storage can result in severe reduction in embryonic induction, possibly as a result of excessive desiccation or further maturation of the embryos (Tautorus *et al.* 1991).

Great success was achieved in tissue culture following the discoveries of auxin and cytokinin. Skoog and Miller (1957) proposed the concept of hormonal control over organ formation. High auxin concentrations promoted rooting, whereas proportionally more cytokinin initiated bud or shoot formation. The cytokinin/auxin interaction provides the basis upon which all micropropagation procedures depend (Hartmann *et al.* 1990b). Growth regulators are often the most manipulated parameters in micropropagation (Debergh & Read, 1991).

Micropropagation can be expressed by two principles in tissue cultures namely: 1) somatic embryogenesis and 2) organogenesis (Dunstan & Thorpe, 1986). *In vitro* micropropagation of *gymnosperms* has only been successfully achieved within the last \pm 15 years (Jang & Tainter, 1991).

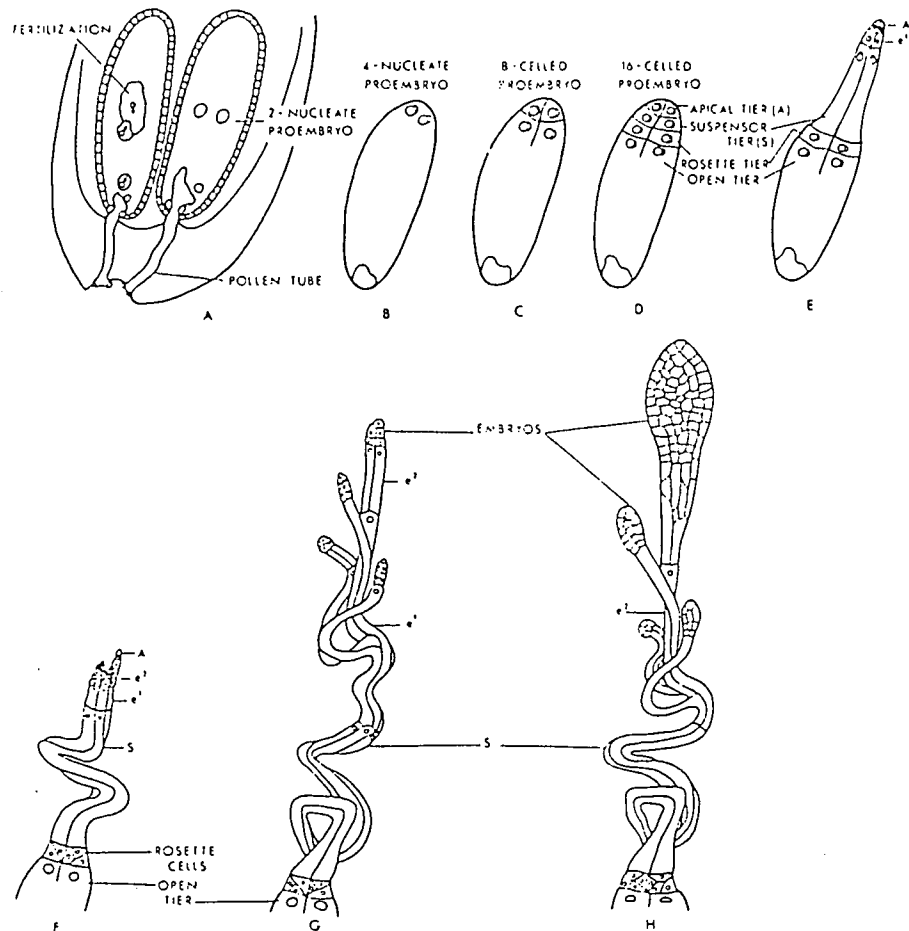


Fig. 2.1. Fertilization, pro-embryo and early embryo development in Pine sp.

A, B - Zygote nucleus divides forming two free nuclei which in turn divide to form free nuclei. These nuclei migrate to the distal end of the archegonium.

C, D - Division and cell wall formation occur forming 16-celled pro-embryo.

E - The suspensor tier (S) elongates. The apical tier divides to form apical cells (A) and embryonal tubes.

F - Cleavage polyembryony occurs when the apical cells and embryonal tubes separate to form four files of cells.

G,H - Apical cells divide forming multicellular embryos.

(After Sharma & Thorpe, 1995).

2.1.1. Somatic embryogenesis.

The process of embryogenesis *in vivo* is similar in most conifers but differs significantly from the embryo development occurring in angiosperms. The mature seeds of conifers generally contain only one embryo while only a very small percentage (1-3%) show polyembryony. In a young seed however, the presence of more than one embryo during late embryogenesis is very common. This is mostly due to the fertilization of more than one egg and the development of multiple zygotes. This is known as "simple polyembryony". Frequently, a single zygote may also form multiple embryos by the cleavage or splitting of the cells of the embryonal tier into several embryonal units, this results in "cleavage polyembryony" (Owens & Molder, 1984; Sharma & Thorpe, 1995) in which multiple embryos arise from a single zygote (Fig. 2.1). These resulting embryos are genetically identical. One of the embryos normally become dominant, while the others abort.

The term somatic embryogenesis refers to the *in vitro* development of a complete embryo from vegetative (somatic) cells derived from various sources of explants (Hartmann *et al.* 1990b; Gupta & Grob, 1995). This process was first reported in 1958 for *Daucus carota* (Steward *et al.* 1958).

The somatic embryogenesis (*in vitro*) follows a similar development pattern to the zygotic embryos for most conifers and certain generalizations can be made. These stages are:

- Stage 1. Selection and culture of appropriate explant material. The first step is to produce embryonal suspensor mass (ESM). This is initiated from immature or mature zygotic embryos. The developmental stage of the zygotic embryo plays a crucial role in the production of ESM.

- Stage 2. Initiation or induction of somatic embryos from primary explants. Induction is achieved through the transfer of cells to a basal medium with a high auxin concentration.
- Stage 3. Proliferation or multiplication of somatic embryonic cultures. The proembryonic masses are shifted to an auxin-free basal medium. By adding abscisic acid (ABA) to the medium, uniformity and normal development is promoted.
- Stage 4. Development and maturation of somatic embryos. Normal size embryos are planted onto a solidified medium.
- Stage 5. Transplanting. After leaves and roots have formed it can be handled as any seedling plant (Hartmann *et al.* 1990b).

Somatic embryogenesis thus has the ability to recapitulate the events of zygotic embryogeny *in vitro*, producing bipolar structures with the capacity for both root and shoot production (Tautorus *et al.* 1991). However, the final size of the cotyledons is usually reduced and there is no development of endosperm or a seed coat (Gray & Purohit, 1991). Many researchers in forest biotechnology regard somatic embryogenesis as the *in vitro* system of choice for mass propagation (Gupta *et al.* 1991).

There are some limitations to the application of somatic embryogenesis of forest trees. The first major limitation is the low numbers of field-plantable clonal plantlets produced per embryonic culture. Although reports of somatic embryogenesis are plentiful, data on plantlet production is scarce. The second major limitation is the inability to initiate embryonic cultures from mature trees. Most of the systems have relied on explanting material of seeds or seedlings. The material propagated is thus of unproven genetic value (Merkle, 1995). According to Minocha & Minocha (1995) confusion also existed regarding the regeneration of somatic embryos from cell/tissue cultures of woody plants. Part of this confusion arose from the lack of a clear definition of a somatic embryo. Alternative terms such as *embryoids*, *embryo-*

like structures, adventitious embryos and others have been used to describe the regenerants. An additional problem relates to the origin of these structures.

Very little is currently known about the origin and development of conifer somatic embryos in cultures. The origin of somatic embryos may also vary depending on the type and origin of explant used (Nagmani *et al.* 1987; Finer *et al.* 1989). In 1980 Sharp *et al.* distinguished two different patterns of origin of somatic embryos from explants grown *in vitro*. The first category provides for the direct production of somatic embryos from the explant cells, called the pro-embryonic-determined cells (PEDC). The second includes somatic embryo production indirectly from an unorganized callus/tissue mass called the induced embryonic-determined cells (IEDC). In the PEDC pattern somatic embryos are presumed to originate from explant cells that only require an *in vitro* environment to be released from some suppressive condition imposed by the organization of the explant. The IEDC pattern is more difficult to induce, since the starting material consists of vegetative cells that must undergo major epigenetic changes to initiate somatic embryo production (Merkle, 1995). The first pattern requires no growth regulators, while the latter pattern depends on a sequence of growth regulator treatments, first to form ESM from which the somatic embryos are regenerated (Minocha & Minocha, 1995). Gupta and Grob (1995) also state that somatic embryogenesis proceeds directly or indirectly after exposure of responsive explants to critical concentrations of plant growth regulators during the initial culture phase.

Hakman *et al.* (1987); Hartmann *et al.* (1990b) and Tautorus *et al.* (1991) suggested the following three different processes that could account for the origin of somatic embryogenesis *in vitro*. These processes are known to occur in angiosperms and to a lesser extent in gymnosperms (Tautorus *et al.* 1991).

2.1.1.1. Somatic adventitious embryogenesis.

Somatic embryos develop directly from cells or callus, which are associated with the explant. These cells have been embryonically predetermined prior to their excision as explant and can be referred to as embryonic. This type can originate from three fundamentally different kinds of explants:

Type 1. Includes nucellus tissue or integuments of young ovule tissue of poly- or monoembryonic species like grasses (Litz, 1987), grapes (Mullins, 1987) and coffee (Sondahl & Monaco, 1981). This type reproduces the genotype of the mother plant. This method is thus useful in clonal propagation.

Type 2. Includes the ESM at the very earliest stage that precedes embryo development (Durzan & Gupta, 1988). This tissue is prominent in conifers.

Type 3. Includes the developing zygotic embryo at various stages of development or seedling tissue as the explant source. The embryo may thus arise from a single cell or small cell cluster on the surface of the immature zygotic embryos in culture by an initial asymmetric division which delimits the embryonal apex and suspensor region (Hakman *et al.* 1987; Nagmani *et al.* 1987; Jain *et al.* 1989). This is a common pattern of somatic embryogenesis in a wide variety of plants.

2.1.1.2. Somatic polyembryogenesis.

Somatic embryos could arise by a mechanism similar to cleavage polyembryogenesis, with the initial separation occurring in the embryonic region (Tautorus *et al.* 1991). The proliferation of these cells within very immature gymnosperm ovules gives rise to the phenomenon of cleavage polyembryogenesis. Based on the similarity of this tissue with the *in vivo* cleavage polyembryony in conifers, Gupta and Durzan (1986a,b; 1987) suggested the term "somatic polyembryogenesis" for this tissue. This involves the transplanting of highly embryonic ESM, which precedes formation of the embryo (Durzan & Gupta, 1988).

The ESM consists anatomically of early stage embryos, which have an embryonal head and a suspensor system (Gupta & Grob, 1995). This ESM tissue is unique and can be distinguished from non-embryonic tissue in culture by its white translucent mucilaginous appearance and ability to stain red with acetocarmine (0.10%, m/v) (Gupta & Durzan, 1986b). This embryogenesis does not involve callus but results from "cleavage" and "budding" of the original ESM (Hartmann *et al.* 1990b). This has been described in cultures of *Abies alba* (Schuller *et al.* 1989), *Larix decidua* (Nagmani & Bonga, 1985; Von Aderkas & Bonga, 1988), *Pinus* and *Picea* species (Becwar *et al.* 1988; Von Arnold & Woodward, 1988) and *Pseudotsuga menziesii* (Durzan & Gupta, 1987).

2.1.1.3. Induced somatic embryogenesis.

This type of embryogenesis results from somatic callus and cell suspensions only after the tissue is subjected to specific treatments, which result in the induction of embryonic competence. The phenomenon requires a systematic analysis of the embryonic potential of the different explant sources and the required culture conditions. The two potential uses of this procedure are:

- a) Mass propagation of "synthetic" somatic seeds and
- b) Genetic improvement programmes of plant cultivars (Hartmann *et al.* 1990b).

This process has been successful with a number of crop species, including carrot (Lutz *et al.* 1985), alfalfa (Stuart *et al.* 1987), grasses (Vasil, 1985), coffee (Sondahl & Monaco, 1981), palm species (Tisserat *et al.* 1979) and soybean (Christianson, 1985).

2.1.1.4. Classification of somatic embryo development.

The description by Von Arnold and Hakman (1988) of somatic embryos is a very useful means of classifying somatic embryo development and to categorize the maturation of somatic embryos into four stages. The advantage of this system is that it avoids any confusion that may arise when terms used specifically for zygotic embryogeny are applied to somatic embryogenesis.

- Stage 1. The somatic embryo is characterized by a dense embryonal apex, subtended by a more elongated, translucent suspensor cell.
- Stage 2. The embryos are more prominent, appear smooth in outline and are opaque, usually cream to pale yellow in color. The apex is still subtended by the suspensor cell.
- Stage 3. Small cotyledons appear clustered around a central meristem. The suspensor may or may not be present. The embryo resembles a zygotic embryo and may be cream to pale green in color. This stage is usually derived from embryonic tissue cultured on ABA-containing medium.
- Stage 4. This stage is divided into an early (a) and late (b) phase. In the early stage, the plantlet structure is visible, with distinct, partly elongated cotyledons, clustered around the central meristem. The hypocotyl may show some elongation, and the structure is green. The late stage 4 is usually evident, after plantlets have been cultured for approximately three weeks on a hormone-free medium. Elongating cotyledons and hypocotyl are evident, and rudimentary radicle development is visible.

2.1.1.5. General.

The importance of somatic embryogenesis cannot be overemphasized. In 1987 Boulay identified a number of advantages over other micropropagation techniques. The most commonly cited is the tremendous potential for large scale propagation, of superior genotypes, even in liquid cultures. Due to the lower labour costs per unit,

this technique is significantly more economical (Thorpe *et al.* 1991; Gupta & Grob, 1995; Merkle, 1995).

Somatic embryogenesis not only represents a method to obtain true rejuvenation from mature trees but its aim is to obtain as many embryos as possible from a single immature embryo. It is believed that this method holds such large potential because it is labour-saving, needs less space than other propagation methods and its propagation rate is enormous if successfully conducted (Ishii, 1991). Cells can also be cryopreserved for future use (Thorpe *et al.* 1991).

Over the past few years, much progress has been made in the area of somatic embryogenesis in conifers. The first report of somatic embryogenesis was that of Norway spruce (*Picea abies* (L) Karst) in 1985 (Hakman *et al.*). Within a year, the genus *Pinus* also appeared on the list, with species like *Pinus lambertiana* Lamb. and *Pinus taeda* L. (Gupta & Durzan, 1986b and 1987).

Other species reported have been Douglas-fir (*Pseudotsuga menziesii*) (Durzan & Gupta, 1987), black spruce (*Picea mariana* (Mill.) BSP.), and white spruce (*Picea glauca* (Moench) Voss.) (Hakman & Fowke, 1987a). In 1990, Laine and David obtained somatic embryogenesis of *Pinus caribaea*. Since then numerous reports of somatic embryogenesis in tissue culture of conifer species have appeared.

It is interesting that the same general principles and strategies that have been used successfully during the early 1980s for the initiation of somatic embryogenesis in recalcitrant cereals and grasses, can also be applied to conifers (Vasil, 1988). A key factor to many of the successes has been the choice of explant.

Various explants such as megagametophytes, immature and mature zygotic embryos, young seedlings and re-induced cotyledonary somatic embryos have been used to initiate somatic embryogenesis (Tautorus *et al.* 1991), but success has been achieved mainly with immature and mature embryos (Thorpe *et al.* 1991).

Those working with conifer somatic embryogenesis have found that there is a striking difference between *Picea* and *Pinus* conifers, with regard to the ease with which somatic embryogenesis can be induced and plantlets regenerated. The literature indicates that greater success has been achieved with *Picea* than with *Pinus*. Initiation frequencies of about 1% to 5% are routinely cited by those working with *Pinus* species (Gupta & Durzan, 1987; Becwar *et al.* 1988; Jain *et al.* 1989; Becwar *et al.* 1990).

The single report claiming a 54% initiation rate from immature zygotic embryos of *P. strobus* (Finer *et al.* 1989) has yet to be repeated or duplicated by others working with this species (Becwar *et al.* 1995). Subsequent production of plantlets has been extremely limited in *Pinus* species. Tautorus *et al.* (1991) cited only 3 of 7 reports, which indicated plantlets were obtained via somatic embryogenesis in *Pinus*. In contrast 30 of 43 reports relating to *Picea* species reported obtaining plantlets via somatic embryogenesis.

The key medium constituents important to somatic embryogenesis, are auxins and nitrogen. The hormone auxin facilitates the induction of meristematic cells to form embryonic clumps or pro-embryos. Nitrogen assists the continued expression of embryogenesis, leading to the formation of embryos (Dunstan & Thorpe, 1986). The media requirements for the initiation of embryonic cultures do not appear to be very specific. There are several basal media (e.g. modified MS, LP, DCR) which have been used for the initiation of embryonic cultures of *Picea* and *Pinus* sp. (Gupta & Grob, 1995).

It is also important to remember that some growth factors such as ABA, zeatin, indolebutyric acid (IBA) and certain vitamins are heat-labile (they cannot be autoclaved with the rest of the nutrient medium). These solutions are then filter-sterilized (0.22 μ m filter) and added to the rest of the medium, which has been previously autoclaved. The filter-sterilized solution is added just before the medium sets at about 40 - 60°C.

According to Von Arnold (1987) the nutrient media and the growth regulator additives used, play an important role in determining the embryonic potential of the explant. The culture medium is thus one of the most important components of an efficient and effective somatic embryogenesis regeneration system.

It has been established that auxin and a low level of cytokinin are required to stimulate the production of the ESM. The auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) has generally been the preferred auxin used for the initiation of ESM of most conifer species (Tautorus *et al.* 1991). Initiation of ESM for conifers has been achieved with different concentrations of growth regulators, such as 10 to 110 mg l⁻¹ 2,4-D (Hakman *et al.* 1985; Gupta & Durzan, 1986b) up to 5 mg l⁻¹ naphthalene acetic acid (NAA) (Lelu *et al.* 1987; Von Arnold, 1987) and up to 5 mg l⁻¹ each, of benzyladenine (BA) and kinetin (Kin) (Becwar *et al.* 1988). Induction of ESM has mostly been done in the dark (Gupta & Grob, 1995).

The addition of ABA to conifer somatic embryo cultures can result in the improvement of somatic embryo maturation (Tautorus *et al.* 1991) and the stimulation of this process. Concentrations of 0.1-25 mg l⁻¹ have been used. Several authors have suggested that the inhibition of cleavage polyembryony and the consequent development of individual somatic embryos is an effect of ABA (Durzan & Gupta, 1987; Boulay *et al.* 1988; Krogstrup *et al.* 1988). It has also been shown that transferring the somatic embryos onto media containing ABA, followed

by further development on medium with reduced growth regulator concentrations or no growth regulators, results in embryo maturation and plantlet development.

Although agar has most commonly been used as gelling agent, four reports have commented on the effect of gelrite on conifer somatic embryogenesis (Von Arnold, 1987; Klimaszewska, 1989; Harry & Thorpe, 1991 and Tremblay & Tremblay, 1991). In their studies on *Picea* sp. Tremblay & Tremblay (1991) found that gelrite was superior to agar, in that 3 to 5 times more somatic embryos developed on medium containing gelrite than on medium containing agar.

2.1.2. Cell suspension cultures.

Cell suspension cultures can be created and established from ESM derived from immature seeds by placing a piece of ESM in a liquid medium so that the cells dissociate from each other (Hartmann *et al.* 1990b). The ESM is first subcultured on a solid medium to build up a mass of tissue and to improve the friability of the mass, which is a prerequisite for raising a fine cell suspension in the liquid medium (Razdan, 1993). This is a type of somatic embryogenesis, because of the explant used. The main advantage of using cell cultures as a tool in breeding programmes and mass clonal production is its potential for enormous (potentially unlimited) multiplication rates (Thorpe & Biondi, 1984).

The culture medium normally used for suspension cultures includes the complete range of ingredients: inorganic salts, sucrose, vitamins and a proper balance of growth regulators, and is usually based upon that medium which maintains good growth of the somatic embryonal tissue. It may, however, be necessary to modify it slightly. The suspension culture normally grows in a moving liquid medium. The movement of the liquid medium serves to maintain the even distribution of the cells and promotes adequate gaseous exchange between the culture medium and the air (Street, 1977). Platform (orbital) shakers are used for the initiation and propagation

of the cell suspension cultures. The platform is fitted with clips to carry the chosen culture vessels.

During incubation, batch cultures in a fixed volume of culture medium increase in bio-mass by cell division and cell growth until a factor in the culture environment (nutrients or oxygen availability) becomes limiting. When the cell number in suspension cultures is plotted against time of incubation, a growth curve is obtained. The growth curve depicts that initially the culture passes through a lag phase (where cells divide very slowly), followed by a brief exponential growth phase (the most fertile period for active cell division) and increasing to a steady state (linear). The growth declines after three to four cell generations, signaling that the culture has entered the stationary phase (Fig.2.2).

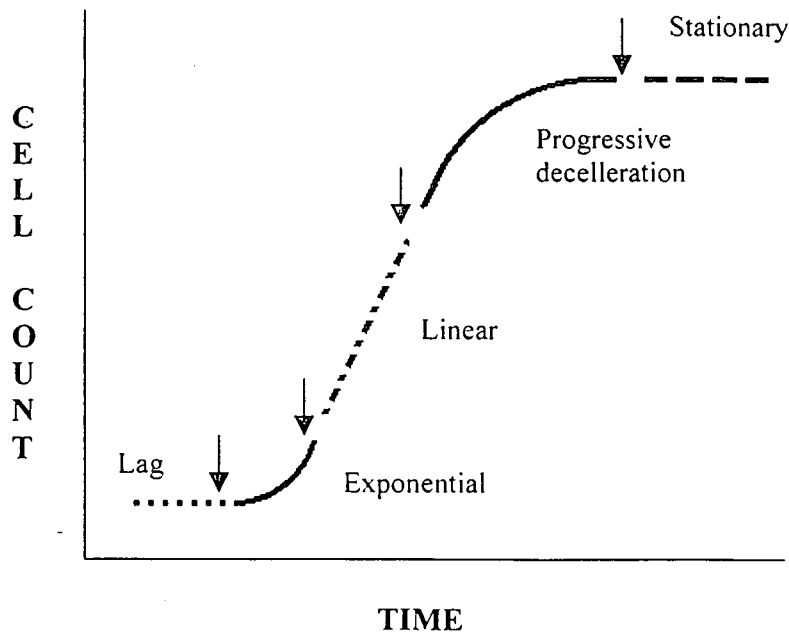


Fig. 2.2. Model curve relating cell number per unit volume of culture to time in a batch grown cell suspension culture. Growth phase of the cycle are labeled. (After Street, 1977).

These cultures are maintained continuously by propagating the inoculum in the moving liquid medium and transferring a small aliquot of it to a fresh medium (subculture) at regular intervals (Razdan, 1993). A similar pattern of growth and yield will occur every time. The general technique of cloning cell suspensions is to subculture them in the early stationary phase (Torres, 1989). This means subculture at a time when cell aggregation is maximal and leads to progressively increased aggregation (Street, 1977). Beyond this point, the viability of the cell suspension may decrease (Torres, 1989).

When cells of a stationary phase cell suspension are subcultured, the cells in succession pass through a lag phase, a short-lived period of exponential growth, and then again enter the stationary phase. Traditionally such subcultures are initiated from a relatively high cell density and the cell therefore accomplishes only a very limited number of divisions before again entering the stationary phase. Cultures can, however, be initiated successfully from lower initial densities. The use of these low cell densities will however prolong the lag and exponential phases of growth. For any combination of cell culture strain and culture medium there is a minimum inoculation density, below which the culture will fail to grow on subculture. At the densities approaching this point, the lag phase will be prolonged and variable, as between separate cultures (Street, 1977). Krogstrup (1990) reported that culture density is critical and often determined the quality of early stage embryos in suspension cultures. For example, ESM densities of 5 ml packed cells in a volume of 40 ml fresh medium were required for maximum embryo quality in Douglas-fir (Durzan & Gupta, 1987). Lower densities led to insufficient growth while higher densities caused browning of cells (Gupta *et al.* 1991).

The period from culture initiation to stationary phase is determined primarily by initial cell density, duration of lag phase and growth rate. Where subculture is attempted with cells in a very active state of division the passage length is likely to be between 6 and 9 days. The normal incubation time of stock cultures is 21-28 days (Razdan, 1993).

The ideal cell suspension culture is characterized by morphological and biochemical homogeneity (Torres, 1989) and it is important to ensure that sufficient cells are transferred at each subculture to develop a medium for stock culture maintenance (Street, 1977). Growth in plant cell suspension cultures is normally measured by cell counting, determination of the total cell volume and by determinations of cell fresh weight, cell dry weight and content of cellular protein.

As early as 1968, Durzan and Steward obtained single cell suspension cultures of *Picea glauca* and *Pinus banksiana*, which underwent cell division and growth. Chalupa and Durzan (1973) obtained suspension cultures of *Picea abies* and found that after repeated transfers to a fresh medium, some small embryoids of 5-10 cells had formed. Winton and Verhagen (1977) initiated embryoids of 3-5 cells in suspension cultures derived from callus of *Pseudotsuga menziesii* and *Pinus taeda*. In 1979, Durzan reported the establishment of suspension cultures from shoot tips of 3-4 year old *Pseudotsuga menziesii*.

The first cell suspension culture reported for *Pinus radiata* was in 1986, by Teasdale. The only medium reported to sustain a cell culture of any pine species over many subcultures was the Litvay medium (LM) (Litvay *et al.* 1981). In 1989 Finer *et al.* obtained embryonic callus and suspension cultures of *Pinus strobus*. The induction of embryonic tissue from precotyledonary zygotic embryos and the subsequent maintenance of the tissue of *Pinus strobus* by using suspension cultures was also reported by Kriebel and Finer (1990). Although maintenance of the cultures was easy, maturation and the development of plantlets were not obtained in liquid media.

Laine and David (1990) also initiated cell suspension cultures, from immature seeds for *Pinus caribaea* Mor. var. *hondurensis*. Work done on *Pseudotsuga menziesii* revealed that replacement of sucrose with maltose in combination with ABA promoted development of somatic embryos to the cotyledonary stage (Nagmani & Dinus, 1991). For *Pinus taeda* very similar results were obtained (Uddin *et al.* 1990).

2.1.3. Organogenesis.

Organogenesis is the major method by which *in vitro* plant regeneration is obtained specially in softwood species. This form of micropropagation involves various development stages, including:

- Culture establishment and bud induction
- Shoot development and multiplication
- Rooting of developed shoots and
- Hardening-off of the plantlets (Thorpe *et al.* 1991).

The *in vitro* development is affected by many factors, such as: the explant genotype, source and age, as well as the season in which the explant is obtained. The size of the explant, the nutrients used, the quality and concentration of the gelling agent also plays a great role. The duration, type and mode of application of plant growth regulators are also important factors (Dunstan & Thorpe, 1986; Thorpe *et al.* 1991). Another problem associated with micropropagation is clone to clone variation in both rooting frequency and the ability to initiate shoots in the medium (Chandler & Young, 1995).

Organogenesis can be obtained via a direct or indirect pathway. The direct pathways are either the continued development of shoot meristematic activity from lateral or axillary buds in shoot cultures or the *de novo* induction of adventitious buds from loci that would not otherwise have been shoot forming. The indirect pathway relies on a callus intermediary step. In conifers efforts were made to achieve *de novo* organogenesis directly on the explant (Dunstan & Thorpe, 1986).

2.1.3.1. Axillary bud multiplication.

In conifer cultures the original explant is a terminal or lateral vegetative bud, or actively growing shoot tip. By adding growth substances to the medium, axillary bud initiation can be promoted. Generally benzylaminopurine (BAP) at between 10^{-5} and 5×10^{-5} M with an auxin (NAA or IBA) at 10^{-8} M have been suggested as the most successful concentrations (David, 1982). Mapes *et al.* (1981) found in their study with *Pseudotsuga menziesii* that cytokinin and auxin were a prerequisite for development. However extension of the axillary buds was only achieved when the bud clusters were transferred to a medium free of growth regulators. Boulay (1979) stated that the presence of cytokinins in the medium of some conifer species is not necessary for axillary bud development (John, 1983).

Axillary bud production is described as conservative and also of relatively low multiplication potential in comparison to the adventitious bud or asexual embryogenesis routes. It is conservative because it produces the smallest number of plants since the number of axillary buds placed on medium limits the number of shoots produced. Although the initial multiplication rates are low, increases occur during the first few subcultures and eventually reach a steady rate, which may be maintained through numerous subcultures. This method is often the preferred technique used by commercial woody plant laboratories, because of its relative ability to produce true-to-type plants without genetic change (Dunstan & Thorpe, 1986; Thorpe *et al.* 1991).

2.1.3.2. Adventitious shoot production.

This approach offers a greater potential for shoot production than the above route, since shoot induction occurs at sites other than bud meristems. The method involves the induction of localized meristematic activity by hormone or growth regulator treatments, leading to primordium differentiation and eventually to shoot development, often under growth regulator-free conditions. Buds are generally

induced directly on the explant. In general the more juvenile the tissue, the better it will respond to *in vitro* treatments leading to organogenesis (Thorpe *et al.* 1991). The most frequently used explants, which have led to successful regeneration have been seed or seedling parts including the cotyledons, hypocotyl, epicotyl and embryonic axis. BA has been the preferred cytokinin for bud induction. (Dunstan & Thorpe, 1986). The concentration of BA needed for formation of the adventitious buds increased with the physiological age of the explant (Zel, 1993). To date only very juvenile explants (i.e. seedlings less than 10-12 days post germination) have been shown to be capable of adventitious shoot formation (Chandler & Young, 1995). The addition of auxin or growth regulators other than cytokinin tends to enhance callus formation and reduce organogenesis (Biondi & Thorpe, 1982).

2.1.3.3. Indirect Organogenesis via Callus.

Indirect adventitious budding can also occur via callus formation, but difficulty is often experienced in the regeneration of plantlets from callus of the conifer tissue. In the late 1970's many researchers were led away from callus culture when organ cultures began to be increasingly successful (Dunstan & Thorpe, 1986; Thorpe *et al.* 1991).

2.1.3.4. Development and multiplication of buds.

The main objective of organogenesis is to produce the maximum number of rootable shoots. Generally, the formation of true shoot apices with juvenile leaf primordia in conifers requires transfer unto a medium with altered nutritional and/or growth regulator levels, often with the inclusion of activated charcoal (Biondi & Thorpe, 1982; Thorpe & Biondi, 1984). A change in medium is also needed for stem elongation. For most conifers, no growth regulators are needed at these stages, but several cycles of culture may be required to produce rootable shoots (Thorpe *et al.* 1991). Re-multiplication of shoots can be carried out by a topping method, in which the shoot apex and apical shaft of the primary needles are severed from each shoot.

New shoots arise from the axillary meristems in the basal portions (Aitken-Christie & Thorpe, 1984; Smith, 1986).

2.1.3.5. Rooting and hardening of plantlets.

When rooting is done under *in vitro* conditions, basically two types of substrates and several auxins are used. Substrates like solidified medium and soilless mixes like peat, vermiculite and perlite moistened with nutrient medium are used.

In general, rootable shoots are pulsed for a short time in solutions of auxins, IBA being the most commonly used auxin in this case (Thorpe *et al.* 1991). Smith & Thorpe (1977) showed that exogenously applied aromatic amino acids and simple phenolics can enhance root primordium formation in *Pinus radiata*. When a solidified medium is used, the nutrients can be reduced to $\frac{1}{2}$ or $\frac{1}{4}$ of the original compositions and in general, sucrose is reduced to 1% or 2%. Occasionally, the concentration of the gelling agent used is increased (Thorpe *et al.* 1991).

The rooting process can be separated into two stages: root initiation and root growth. These stages have been found to occur in response to different media. Sommer *et al.* (1975) showed that adventitious shoots of *Pinus palustris* rooted spontaneously on a hormone-free medium, but four weeks pretreatment with an auxin increased rooting. For *Pinus taeda*, roots were initiated on shoots after five weeks in a medium containing 0.05 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. Root growth was then achieved, by transferring to a hormone-free medium (Mehra-Palta *et al.* 1977).

2.1.3.6. General.

Softwood species were among the first to be used for *in vitro* studies. As early as the 1930's Gautheret worked extensively on the culture of cambial tissue from a variety of woody species especially *Pinus pinaster*. It was not until 40 years later that an entire plant could be regenerated for *Populus tremuloides* (Winton, 1970)

and for *Pinus palustris* (Sommer *et al.* 1975) by organogenesis from callus and by adventitious bud development on cotyledons, respectively (Dunstan & Thorpe, 1986).

Since these first reports many problems have been overcome and much progress has been made. Many recent reviews, cover the progress of a wide variety of species and explants used, the number of species in which plantlets have been produced, and different methods used to produce them (Mott, 1978, 1981; Sommer & Brown, 1979; Biondi & Thorpe, 1982; Karnosky, 1981; David, 1982; Thorpe & Biondi, 1984).

The first report of regeneration by organogenesis (adventitious shoot formation) in *Pinus radiata* was in 1976 (Reilly & Brown). In this first report, the mature embryo was shown to have a high regeneration capacity. In 1977 Reilly and Washer reported viable plantlets, regenerated from adventitious meristematic tissue induced by the action of BAP on the cotyledons of mature *P. radiata* zygotic embryos. Smith *et al.* reported in 1982 that *P. radiata* micropropagated shoots could be stored at 2°C for up to 8 months, with shoot growth resuming upon transfer to fresh medium and normal culture conditions. By excising the terminal portion of individual shoots and by placing both, the shoot and base back into the growth medium multiplication is achieved. The terminal portion grew on to form a normal shoot, while the basal portion formed axillary shoots which when excised and when transferred to fresh medium developed into normal shoots. The *in vitro* multiplication by axillary meristems has become the standard method for commercial micropropagation of *P. radiata* trees in New Zealand.

2.2. Total phenolic contents and phenolic acids.

It is also well known that phenolic compounds play an important role in the plant defense response (Ingham, 1973) against herbivores and pathogens. Phenols and phenolic acids have been attributed with important roles in the inhibition of the

germination of seeds and of plant growth generally (Van Sumere, 1989). They also play a variety of other roles like attracting pollinators or reducing the growth of nearby competing plants, or fulfill a function in mechanical support (Taiz & Zeiger, 1991). Plant hormones, such as auxins, cytokinins, gibberellins and ethylene; as well as inhibitors, such as ABA and phenolic compounds, influence root initiation (Hartmann *et al.* 1990c).

Phenolic synthesis is one of the most extensively studied pathways of secondary metabolism in forest tree species. The use of plant cell cultures as a tool to study the vast array of biosynthetic pathways culminating in secondary metabolites has been extended in the recent years. Plant cell cultures nevertheless possess certain desirable characteristics for the study of the secondary metabolism, these features are:

- a) independence of cells from intricate inter-relations in the parent plant.
- b) rapid uptake and metabolism of precursor substances and
- c) control of nutrition and environmental conditions with the possibility of short term alterations in selected parameters (Grey *et al.* 1987).

Tissues placed in culture sometimes produce phenolic compounds, turn brown and appear non-viable. However these brown reaction products are not necessarily inhibitory to the process of somatic embryogenesis. The use of antioxidants such as ascorbic acid or cystine has been suggested to reduce phenolic oxidation. Incubation of tissue in the dark and the use of activated charcoal have also been recommended. The biosynthesis and productivity of phenolic compounds are regulated by a variety of external and biological factors. Westcott and Henshaw (1976) reported that by raising the sucrose concentration in the medium from 2% to 4% a three-fold stimulation of phenolic production occurred in sycamore cells.

Hess (1962) remarked on the presence of phenolic type co-factors in *Hedera* and *Hibiscus*, by analysing cultivars which were easy- or difficult-to-root. Findings in a variety of other plants also seem to credit the hypothesis of the intervention of phenolic compounds in the induction process of adventitious roots (Moncousin,

1991). Bachelard and Stowe (1962) have shown a clear relation between the formation of phenolic substances and adventitious rooting in *Acer rubrum*. Bora *et al.* (1991) found in their study on mung bean seedlings, a decrease in phenolic compounds in the cuttings, which were treated to form an increased number of adventitious roots. Rajasekar and Sharma (1989) found that the phenolic compounds did not have a favourable affect on rooting, regardless of whether they were applied alone or in combination with IBA and micronutrients.

All phenolic compounds have an aromatic ring structure, which contains various attached substituent groups, such as hydroxyl, carboxyl and methoxyl (-O-CH₃) groups and often other non-aromatic ring structures. Phenolics are more soluble in water and less soluble in non-polar organic solvents. Some phenolics are rather soluble in ether, especially when the pH is low enough to prevent ionization of any carboxyl and hydroxyl groups present. These properties greatly aid separation of phenolics from other compounds (Salisbury & Ross, 1992).

Many of the basic techniques of phenolic analysis were developed during the first half of the present century when there was already considerable interest in some classes of natural phenols, because of their biological and medicinal properties. The natural phenols arise biogenetically from mainly two basic pathways: the shikimate pathway which directly provides phenylpropanoids such as the hydroxycinnamic acids (Harborne, 1989), and the malonic acid pathway (Taiz & Zeiger, 1991).

At one time it was common practice to determine the total phenols in a plant, either in relationship to phenolic biosynthesis or to biological function. For analysis we need a method which is not only very sensitive but also highly specific. Various methods are described for the extraction of total phenols.

One of the first methods regularly used was that of Swain and Hillis (1959). This is a quantitative analytical method. The total phenolic compounds in the plant tissue were extracted with boiling methanol (Hillis & Swain, 1959). Then the absorptivity was

determined in 1-cm³ cells at 725nm using water and the reagents as a blank (Swain & Hillis, 1959). This method extracts monosaccharides along with the phenolic compounds. Singh *et al.* (1978) observed interference due to the presence of these natural sugars.

In 1964 Swain and Goldstein finally recommended the Folin reagent for total phenols and the vanillin reaction. In 1977 Price and Butler developed a spectrophotometric method which detects low concentrations of tannin and other polyphenolics by the formation of the Prussian blue complex. This test is based on the reduction of ferric ions to ferrous ions by the presence of phenols, followed by the formation of a ferricyanide-ferrous ion complex. This coloured product commonly known as Prussian blue absorbs maximally at 720nm.

In 1978 Singh and his co-workers developed a colorimetric method using ferric chloride and potassium ferricyanide. This method is more sensitive than the method used by Swain and Hillis (1959) and no interference by natural free sugars occurs. According to Budini *et al.* (1980) the most applicable method is the Prussian Blue method which is also very easy to perform. Budini and co-workers (1980) modified the method of Price and Butler (1977) to improve the sensitivity thereof.

Recently investigators have proposed the titanium chloride and Prussian blue methods as being somewhat superior to the others. The titanium method gives comparable values to Folin. For example the measurement of chlorogenic acid in sunflower meal gives values of 3.36 ± 0.023 g 100g⁻¹ dry wt by Folin and 3.32 ± 0.063 g 100g⁻¹ dry wt by titanium. The Prussian blue method is reported to be three times more sensitive than the titanium procedure and twenty times as sensitive as the vanillin reaction (Harborne, 1989; Budini *et al.* 1980).

Phenolic compounds are determined for some *Pinus* sp. for different reasons. Total phenolic contents were determined for *P. banksiana* in methanol soluble extracts of the pine needles, with gallic acid as a standard by the method of Swain and Hillis (1959)

(Nozzolillo *et al.* 1990). Karolewski and Giertych (1995) studied the changes in the level of phenolic compounds in the needles of *P. sylvestris*. This study was done in an environment polluted by sulfur dioxide and fluorine compounds. The method described by Johnson and Schaal (1957) was used and an increase in the total phenol content was found in the needles that developed in the polluted environment. Northup *et al.* (1995) used *P. contorta* var. *bolanderi* and *P. muricata* mature foliage in a study where the aqueous methanol extracts were analyzed for the concentrations of total phenols by the Prussian blue method. Vazquez *et al.* (1995) studied the phenolic composition of *P. pinaster* foliage. The amount of extracted polyphenolic material was determined by the Stiasny gravimetric procedure.

2.3. Plant hormones.

Salisbury and Ross (1992) define a plant hormone as an organic compound synthesized in one part of a plant and translocated to another part, where in very low concentrations it causes a physiological response. Hormones are often effective at internal concentrations near 1 μ M.

While the traditional concept of growth regulation invokes changes in concentration of growth substances, it is the sensitivity of tissue to these growth substances that is the key factor in plant development (Pattabhi, 1990). In the early 1980's Trewavas repeatedly emphasized this concept. Although many researchers have argued convincingly against Trewavas's overall conclusion, his papers forced researchers to consider and measure tissue sensitivity to hormones (Salisbury & Ross, 1992).

There are currently only five generally acknowledged groups of hormones, viz. auxins, gibberellins, cytokinins, abscisic acid and ethylene. According to Biondi and Thorpe (1981) the two groups of hormones which are of very special importance in plant tissue culture are the cytokinins and auxins.

2.3.1. Auxin.

The term auxin (Greek auxein, "to increase") was first used by Frits Went in 1926. What he then called auxin is now known as indoleacetic acid (IAA). Plants contain three other compounds which are structurally similar to IAA and which cause the same response. They are 4-chloroindoleacetic acid (4-chloroIAA), phenylacetic acid (PAA) and IBA. Little is known about the transport characteristics of these three compounds and whether they in fact normally function as plant hormones, but it seems likely (Salisbury & Ross, 1992).

The term auxin has become much more encompassing since the discovery of IAA, because certain synthesized compounds are structurally similar to IAA and cause similar responses. Compounds such as NAA, 2,4-D and 2-methyl-4-chloro phenoxyacetic acid (MCPA) are the best known. Because they are not synthesized by the plant, but through a chemical process, they are not hormones but are classified as plant growth regulators (PGR). PGR are defined as a chemical substance known to be involved in the control of normal development in plants by delicately balancing growth-coordinating factors (Pattabhi, 1990).

The most widely used function of auxin in plant propagation is in the induction of adventitious roots on cuttings and the control of morphogenesis in micropropagation. The determination of the amount of endogenous auxin sets well the relation existing between auxins and adventitious rooting. It has been noticed that endogenous auxinic compounds accumulate in areas such as nodes, where root development is frequent. The exogenous application of IAA also confirms the importance of the auxin activity for adventitious rooting (Salisbury & Ross, 1992).

Some studies show that the concentration of auxin-like substances increases, in the basal zone of the cutting before the appearance of roots. The number of roots formed per cutting is related to the total amount of these substances present. It has been

found that there is a clear positive correlation between rooting percentage and richness in internal auxin-like substances in *Prunus* cuttings (Moncousin, 1991).

2.3.2. Cytokinins.

In 1913 Gottlieb Haberlandt discovered a compound present in vascular tissue of various plants which stimulated cell division. In the 1940's Van Overbeek also found compounds in the milky endosperm of immature coconut which promotes cell division. In the early 1950's research by Skoog verified Haberlandt's results. It was not until 1964 that the identity of such a substance was determined in kernels of corn. These various stimulants of cell division came to be known as cytokinins (Salisbury & Ross, 1992).

The several cytokinins now known differ somewhat in their molecular structure and possibly also in origin, but they all have an adenine moiety. Cytokinins participate in the enlarging of cells, the differentiation of tissue, the development of chloroplasts, the stimulation of cotyledon growth, the delay of ageing in leaves and in many of the growth phenomena also brought about by auxins and gibberellins (Stern, 1991).

The most common and abundant cytokinin in plants seems to be zeatin, first isolated from immature seeds of *Zea mays*. Two other commonly detected and most physiologically active cytokinins in various plants are dihydrozeatin and isopentenyl adenine (IPA) (Starr & Taggart, 1989). Auer (1997) clearly stated that in *Pinus* sp. the cytokinin that occurs commonly is of the zeatin type.

The effects of a PGR are determined by the kind of growth regulator, its concentration, the presence or absence of other PGRs and by the genetic make up and the physiological status of the target tissue. The same physiological response in different tissue even of the same plant may require different growth regulators or different combinations of growth regulators (Minocha, 1987).

The interaction between auxin and cytokinin is one of the primary relationships in plant propagation. A high auxin/cytokinin ratio favours rooting and a high cytokinin/auxin ratio favours shoot formation (Salisbury & Ross, 1992). Okoro and Grace (1978) state that cuttings of species with high natural cytokinin levels have been more difficult to root than those with a low cytokinin level. To analyse these hormones we must have a method, which not only is very sensitive, but also highly specific.

2.3.3. Determination of the hormone concentrations.

The hormone concentrations can be determined by bioassays. Bioassays, however have their limitations in that they do not differentiate between the various kinds of auxins present in the tissue. Superior to bioassays are a number of chemical or physiochemical assays; these include gas-chromatography-mass spectrometry (GC-MS), radio immunoassays (RIA) or colorimetric and fluorometric assays (Brock & Kaufman, 1991). In general, these physiochemical methods provide greater specificity than a bioassay and permit identification of a specific compound. They are more sensitive, thus permitting detection of considerably smaller amounts of hormones, yield less variation of the results, and therefore permit resolution of smaller differences between samples. Shorter analysis times also permit the analysis of more samples (Brenner, 1981).

Immunoassays are based on the ability of animals to produce proteins (antibodies) which recognize and bind to specific compounds (antigens) foreign to the animal. Such antibodies can be raised against plant hormones and used for hormone quantitation. The technique was first applied to plant hormones in an assay measuring the inactivation of hormone-labeled bacteriophage by antibodies raised against hormones (Fuchs *et al.* 1971). It has been RIA and enzyme immunoassays (EIA) which have been most widely used in this field. The specificity and sensitivity of antibodies make immunoassays attractive for the quantitation of hormones from small amounts of plant tissue. Large numbers of samples can be processed simultaneously, therefore the expectation has been that immunoassays should allow rapid quantitation

of hormone levels in relatively crude extracts, in contrast to the more extensive clean-up needed by most physicochemical methods (Pence & Caruso, 1987).

The first step is to extract the hormone with an organic solvent, which will neither extract numerous contaminating compounds nor destroy the hormone. It is sometimes necessary to purify the hormone by using various chromatographic procedures. A variety of techniques can now be used to determine the hormone concentration in plant extracts (Salisbury & Ross, 1992).

Chapter 3

Plant Material and Methods

3.1. Plant material.

Cones of *P. patula* and *P. radiata* were collected by the South African Forestry Company Ltd. (SAFCOL). The collection period was during the summer months from 16 November 1995 to 29 March 1996 and from 24 October 1996 to 30 March 1997. Twelve *P. patula* trees from the Sabie region were represented (only five of which were received cones in the first year). Five trees of *P. radiata*, from the George/Knysna area were represented throughout.

Cones were collected from the individual trees on a 2 weekly basis. During the expected window period, cones were collected weekly. The collections for the somatic embryogenesis culture began just after the stage when fertilization was thought to occur and continued until seeds were nearing maturity approximately 12 months after pollination. The collected cones were transported in brown paper bags and surface sterilized on arrival with 70% (v/v) ethanol and dried on paper and then stored at 4°C until further use.

The cold stored cones were washed for 20 min. in running tap water before being immersed into 70% (v/v) ethanol (containing a few drops of Tween 20) for 5 min. That was followed by a surface sterilization using 2% (v/v) NaOCl for 20 min. and then rinsed three times with sterile distilled water. Older cones were submerged in boiling water for 30 sec. The cones were cracked open in a cone breaker.

The intact gametophytes with immature embryos were excised and separated from the wing under sterile conditions in a laminar flow cabinet. After the removal of the "seed coat" they were sterilized for 30 sec. in 70% (v/v) ethanol followed by 0.5%

(v/v) NaOCl for 1 min. and rinsed in a 0.5% (v/v) anti-oxidant mixture for 10-20 sec. The rinsing in an antioxidant was done to prevent browning of the explant. The explants were then cultured on the different media (Table 3.1, Appendix A). Some media were supplemented with antioxidants or activated charcoal or a combination of the two. This was also done to combat browning of the explant and the calli that formed. Where the cones were heavily contaminated, the sterilizing time of the immature seeds were extended to a rinsing of 1 min. in 70% (v/v) ethanol and 3 min. in 0.5% (v/v) NaOCl and then rinsed in the 0.5% (v/v) antioxidant for 10-20 sec.

For the initiation of multiple budding, mature seeds from open pollinated cones of *P. patula* lines; AP150, AP736 and AP737 and of *P. radiata* lines; AR106, AR678 and AR836 were supplied by SAFCOL. Explants from older cones, which were used in the somatic embryogenesis studies, but had germinated rather than produce ESM, were used to initiate multiple buds. Germination of both explants occurred in the dark at 23 ± 2 °C. Embryos of AP737 and AR 836 were also excised and transferred to solidified media for cultivation under low light ($5\mu\text{E.m}^{-2}.\text{s}^{-1}$) conditions or in the dark.

ESM developed by somatic embryogenesis of *P. radiata* (line AR366) collected on 3 January 1997 was used as inoculant to initiate suspension cultures.

For the total phenolic contents, phenolic acid, and the hormonal studies, softwood cuttings of different *P. elliotii* hybrids (Table 4.12) were used. This material was chosen because the rooting percentage of these cuttings was known and supplied by SAFCOL. Cuttings of young tip growth from a clonal hedge were weighed and frozen in liquid nitrogen within 12 hours of collection and stored at -20°C until used.

3.2. Methods.

3.2.1. Medium and Culture Conditions.

3.2.1.1. Somatic embryogenesis.

3.2.1.1.1. Initiation.

Initially different variations (Table 3.1, Appendix A) of the Douglas-Fir Cotyledon Revised medium (DCR) (Gupta & Durzan, 1985); a MSG medium (Becwar *et al.* 1990) and a Schenk and Hildebrandt medium (SH)(1972) were used. The following year three different variations of the DCR medium (Gupta & Durzan, 1985), and the mediums of Gresshoff and Doy (GD)(1972); Quoirin and Lepoivre (LP)(1977) modified by Aitken-Christie *et al.* (1987); MSG (Becwar *et al.* 1990) and Schenk and Hildebrandt (SH)(1972) were used to initiate ESM formation. The different media were supplemented with vitamins and growth regulators as seen in Table 3.1 (Appendix A). The pH of all media was adjusted to 5.8 with 1N NaOH and 1N HCl prior to autoclaving. Where activated charcoal was used it was added to the medium before the pH of the medium was adjusted. Aqueous stock solutions of L-glutamine were filter sterilized (0.22 μ m filter) and added to the warm medium under sterile conditions. Where an antioxidant was used it was also filter sterilized and then added to the medium after autoclaving. The medium was sterilized by autoclaving it at 121°C and 20 psi. The time required for sterilization depends upon the volume of the media in the vessel (250-500 ml for 25 min, 1000 ml for 30 min.) Ten ml of medium was poured into 65mm sterile plastic petri dishes. Six explants were cultured in each petri dish. The petri dishes were sealed with parafilm and incubated at 23 \pm 2°C in the dark. After 2 weeks in culture the explants were shifted to a new position in the same petri dish or subcultured onto fresh media every fourth week. Media were initially also supplemented with 3% (m/v) polyethyleneglycol (PEG)-4000 to stimulate embryogenesis.

3.2.1.1.2. Maintenance of ESM.

Initially the ESM obtained in 3.2.1.1.1 were maintained on SH and DCR2 solidified [0.25% (m/v) gelrite] basal medium in 65mm petri dishes as given in Table 3.1 (Appendix A), but containing 2,4-D (0,3 mg l^{-1}) and BA (0.05 mg l^{-1}) or no growth regulators at all. Maintenance of the ESM was conducted in the latter part of the study on a ½ strength LP medium (½ the concentration of the macro and micro-elements given in Table 3.1, Appendix A) with no growth regulators and 3% (m/v) maltose as carbohydrate source. Not more than eight small pieces (5mm in diameter each) of ESM were cultured in each petri dish. The culture conditions were the same as in 3.2.1.1.1.

3.2.1.1.3. Embryo maturation and development.

The ESM previously obtained were matured on a DCR2 medium containing glucose (30 gl $^{-1}$), ABA (1.3 mg l^{-1}) and 1% (m/v) activated charcoal to form somatic embryos. The ABA was filter sterilized with a 0.22 μ m filter. Gelrite (0.25%, m/v) was used to solidify the medium. Cultures were incubated in the dark at 23 \pm 2°C. Culture conditions were the same as in 3.2.1.1.1.

3.2.1.1.4. Root initiation.

Embryos with well developed cotyledons were transferred to a modified GD medium containing NAA (0.5 mg l^{-1}) and IBA (2 mg l^{-1}) for root initiation, as described by Reilly and Washer (1977). Gelrite (0.3%, m/v) was used as the gelling agent. The IBA was filter sterilized (0.22 μ m filter) into the medium. The cultures were incubated at a light intensity of 5 μ E.m $^{-2}$.s $^{-1}$. Five replicates were used and the experiment was repeated three times.

3.2.1.2. Cell suspension Cultures.

The basal medium used for initiation of the suspension cultures was very similar to that used for the solidified cultures (Table 3.1, Appendix A), except that the gelrite was omitted. The ESM was initiated on a solidified medium and maintained on ½ LP medium (modified by Aitken-Christie *et al.* 1987) without any growth regulators (3.2.1.1.2). The stock cell suspension cultures were initiated by the inoculation of a small piece (10mm in diameter) of ESM into 35ml fresh ½ LP liquid medium without any growth regulators in a culture vessel.

Five ml of the ESM cultures was used to inoculate 25ml of one of four different liquid media: GD (Gresshoff and Doy, 1972); ½ LP (as modified by Aitken-Christie *et al.* 1987); DCR (Gupta and Durzan, 1985) and SH (Schenk and Hildebrandt, 1972).

In this study Erlenmeyer flasks were used as culture vessels. They were modified with side tubes so that cell growth (turbidity) could be measured with a Klett-Summerson photoelectric colorimeter. The flasks were normally plugged with cotton wool, which were then covered with aluminum foil. The cultures were agitated at 100rpm on an orbital shaker in the dark at $23 \pm 2^\circ\text{C}$. As the cultures developed, subculturing was initiated.

The effect of different treatments on cell growth was determined against a growth regulator-free control. The different treatments were:

- 0.5, 1 or 2 mg l^{-1} 2,4-D.
- 3% maltose.
- 2 and 5 μM BAP.

For embryo maturation the liquid cultures were re-established onto solid ½ LP medium with no growth regulators. The medium containing the cells was filtered

through sterile filter paper under sterile conditions. The filter paper was then placed on top of the medium containing 0.4% (m/v) gelrite, in sterile plastic petri dishes (65mm). The petri dishes were sealed with parafilm and incubated in the dark at $23 \pm 2^\circ\text{C}$. After a week the filter papers were transferred to fresh $\frac{1}{2}$ LP medium containing 5 mg l^{-1} ABA and 0.4% (m/v) gelrite. The ABA and gelrite concentrations were increased at weekly intervals from 5, 10, 15, 20 and 25 mg l^{-1} ABA and 0.4, 0.5, 0.6, 0.7 and 0.75% (m/v) gelrite. After eight weeks the cultures that showed no development were transferred to maintenance medium.

3.2.1.2.1. Processing of results.

Each time period represents five replicates. All experiments were repeated three times. The mean turbidity values (Klett units) of each five replicates were plotted on a log scale and standard deviations were calculated.

3.2.1.3. Organogenesis.

3.2.1.3.1. Sterilization.

Different sterilization procedures (Table 3.2, Appendix A) were investigated for the two *Pinus* sp. After each treatment the seeds were rinsed three times in sterile distilled water. Thereafter 10 seeds were placed on sterile filter paper under aseptic conditions into each sterile 90 mm glass petri dish to germinate. Distilled water (2 ml) was added and the petri dishes were closed but not sealed. Incubation was at $23 \pm 2^\circ\text{C}$ in the dark.

In another study, embryos were excised under sterile conditions and used as explants, to obtain organogenesis. First the seeds were sterilized by soaking in concentrated H_2SO_4 for 5 min, and washed overnight in running tap water. They were then treated, first with 1.75% (v/v) NaOCl for 30 min, rinsed in sterile water and then treated with 10% (v/v) H_2O_2 for 10 min. Finally they were rinsed three

times in sterile distilled water. The embryos were then excised and cultured in batches of ten embryos each and each batch placed under different conditions to develop (3.2.1.3.4.).

3.2.1.3.2. Germination.

For this study germination was considered to have taken place when the radicle emerged from the seed coat. Germination of the seeds on filter paper was compared to germination on a solidified DCR medium (Table 3.3, Appendix A) without growth regulators. The seeds were incubated at $23 \pm 2^\circ\text{C}$ in the dark to germinate. Progress was monitored daily with the aid of a safe light. Seeds which germinated in this study were subsequently used in further organogenesis studies.

3.2.1.3.3. Initiation of axillary and adventitious buds.

Five basal media were first tested for multiple axillary budding of the two *Pinus sp.* (Table 3.3, Appendix A).

- A Douglas-fir Cotyledon Revised medium, DCR (Gupta and Durzan, 1985).
- A GD medium based on Gresshoff and Doy (1972) and modified by Horgan and Aitken (1981).
- LP medium based on Quoirin and Lepoivre (1977) and modified by Aitken-Christie *et al.* (1987).
- An MS medium based on Murashige and Skoog (1962). The macro and micro elements (salts) were bought pre-packed from Sigma-Aldrich.
- A SH medium based on Schenk and Hildebrandt (1972) and modified by Horgan and Aitken (1981).

The three media that resulted in the highest percentage bud formation were used in a study where the formation of adventitious buds on cotyledons and the further development of these buds were recorded (4.3.4).

The different media in all cases were supplemented with vitamins and growth regulators as specified in Table 3.3 (Appendix A). The pH of the DCR medium was adjusted to 6.0 and the pH of all the other to 5.8. The nutrient media were all gelled with gelrite (0.25%, m/v) and autoclaved for 20 min. at 121°C and 20 psi. The cultures were maintained in plastic jars (65 x 65 x 70 mm) and sealed with parafilm at $25 \pm 2^\circ\text{C}$ at a light intensity of $85\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Twenty five ml of medium was poured into each jar. The explants were transferred every two weeks to a new position on the medium or to fresh medium. Cultures were maintained for a period of eight weeks, before being used in elongation trails.

3.2.1.3.4. Development of excised embryos.

The seeds were sterilized as in 3.2.1.3.1 and the embryos were excised under sterile conditions. The embryos were then cultured on three different media (DCR, LP and SH) (Table 3.3, Appendix A) to determine the best medium for seedling development.

The media were supplemented with vitamins and growth regulators as in Table 3.3 (Appendix A). The pH and sterilization of the media was conducted as described in 3.2.1.3.3. The embryos were incubated at a low light intensity of $5\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or in the dark to determine the best culture conditions.

3.2.1.3.5 Elongation medium.

The elongation of the adventitious and axillary buds formed was obtained on one of three different media (DCR, LP and SH) (Table 3.3, Appendix A) containing no growth regulators. The pH of the medium was adjusted and the culture conditions are described in 3.2.1.3.3.

3.2.1.3.6. Rooting.

The elongated shoots were transferred to a GD medium (Table 3.3, Appendix A) containing 2.0 mg l⁻¹ IBA and 0.5 mg l⁻¹ NAA and 2% sucrose (m/v) for 5 days. The gelrite concentration was also altered to 0.35% (m/v). The aqueous stock solutions of IBA were filter sterilized with a 0.22µm filter and added to the warm autoclaved media. The pH of the medium was adjusted to 5.8, and it was autoclaved for 20 min. at 121°C and 20 psi. Twenty five ml of medium was poured into culture vessels. The next transfer was onto LP medium (Table 3.3, Appendix A) with no growth regulators. The explants were transferred to a new position in the culture vessel, or to fresh medium every second week. The cultures were maintained at 25 ± 2°C and a light intensity of 70µE.m⁻².s⁻¹.

3.2.2. Anatomical studies for the identification of ESM.

To study and identify the ESM microscopically the acetocarmine squash method was used (Gupta & Durzan, 1987). Small samples (± 1mm) of the ESM were placed onto a glass slide and submerged in a few drops of 1%(m/v) acetocarmine. It was then heated for a few seconds over an open flame and then washed with distilled H₂O and stained for a few seconds with 0.05% (m/v) Evan's blue and washed again. These prepared slides were observed and photographed under a Nikon microphoto-FXA microscope to follow the stage of zygotic and somatic embryo development.

3.2.3. Determination of possible chemical markers for rooting ability of Pinus cuttings.

3.2.3.1. Determination of the phenolic contents.

The total phenolic content was determined according the Prussian blue method of Budini *et al.* (1980). A weighed sample of the *P.elliottii* shoot tip cuttings, consisting of the shoot and leaves, was ground in liquid nitrogen to a powder. This was incubated in 2M HCl in a 1:10 (m/v) ratio and heated in a water bath for 30 min. at

95°C in screw cap test tubes to minimize air contact. The mixture was cooled on ice and then filtered. The residue was rinsed with 2x distilled water to a volume of 15 ml.

Fifty μl 0.008M $\text{K}_3\text{Fe}(\text{CN})_6$ and 50 μl of 0.1M FeCl_3 in 0.1M HCl was added to 750 μl diluted (1:1) extract. This was transferred to a 2 ml cuvette. The optical density was read after 5 min. at 700nm with a Hitachi U-2000 spectrophotometer, against a blank of identical compositions in which the pine extract was replaced with distilled water.

Fresh gallic acid standards were prepared for each trial. The phenolic content is expressed as mM gallic acid equivalents g^{-1} fresh material.

3.2.3.2. Extraction of the phenolic acids and TLC separation.

The aqueous plant extracts were prepared as described in 3.2.3.1 and extracted with diethylether (3 times) in a separating funnel. The pooled ether extract was dried under vacuum in a Buchi rotavapor. The residue was dissolved in 1 ml 95% (v/v) ethanol. An aliquot (10 μl) of the ethanolic phenolic acid solution was applied on cellulose thin layer plates (1mm) (TLC) (Merck) for separation according to Ibrahim and Towers (1960). The top layer of a benzene: acetic acid: water (6:7:3 v/v/v), mixture was used to develop the plate in the first direction. The solvent for development in the second direction was a mixture of sodium formate: formic acid: water (10:1:200 m/v/v).

The plates were then sprayed with Pauly's reagent (Jatzkewitz & Hoppe-Seylers, 1953) to diazotise the acids. The reagent was prepared as follows: 4.5 g sulfanilic acid in 45 ml warm 10N HCl made up to 500 ml with distilled water. Ten ml of this solution (0°C) was added to 10 ml of a cool 4.5% (m/v) NaNO_2 solution and cooled

down on ice for 15 min. This mixture is stable for 1 to 3 days at 0°C. Just before use 20 ml of a 10% (m/v) Na₂CO₃ solution was added.

3.2.3.3. Immunoassays for determination of auxin and cytokinin concentrations.

3.2.3.3.1. Method.

Immunoassays kits were purchased from Sigma-Aldrich for the quantitative determination of the auxin (IAA) and cytokinin (zeatin) in the pine hybrid cuttings. The IAA and the *trans*-zeatin riboside (t-ZR) tests both utilized a monoclonal antibody to IAA and to t-ZR respectively. The test is sensitive in the range of 5-1000 pmol l⁻¹ for IAA and 0.2-50 pmol l⁻¹ for t-ZR. The immunoassays were carried out according to the instructions of the manufacturer and methyl indole-3-acetate and *trans*-zeatin riboside were used as standards. The assay utilizes the competitive antibody binding principle to measure concentrations of the hormones in the plant extract. The hormones are labeled with alkaline phosphatase (tracer) and then added along with the plant extract to the antibody coated microwells. A competitive binding reaction is set up between a constant amount of tracer, a limited amount of the antibody and the unknown sample containing the hormones. The samples used for the IAA assay was methylated via diazomethane, before testing. The extract used for the immunoassays for IAA and t-ZR was prepared according to the method described by Weiler and Spanier (1981). The soft pine cuttings were ground in liquid nitrogen to a powder which was incubated in 80% (v/v) ice cold aqueous methanol (100 mg ml⁻¹) for three days at -20°C. The suspension was centrifuged at 1500 rpm at 0°C for 10 min. The supernatants were stored at -20°C until used.

For the determination of IAA 0.1 ml aliquots of the supernatants were dried under nitrogen in polytops, redissolved in methanol and treated for 5 min. at 0°C with an excess of ethereal diazomethane. After destruction of excessive diazomethane with

diluted methanolic acetic acid, the samples were dried again and redissolved to contain not more than 10% (v/v) organic solvent in 25 mM Tris buffer.

For the cytokinin determination 0.1 ml aliquots of supernatants were dried under nitrogen and redissolved with 10 μ l methanol and 90 μ l 25 mM Tris buffer, to contain no more than 10% (v/v) organic solvent.

According to the instructions of the manufacturer 100 μ l tracer and 100 μ l extract were added to the wells. Plates were incubated at 0 - 8°C for 3 hours. After incubation the wells were washed three times with the wash solution supplied. Substrate solution (200 μ l) was added to the wells and incubated for 60 min. at 37°C. After the incubation period a stop reagent (50 μ l) was added and after 5 min. the absorbancy was read at 405nm with a Bio-Rad Model 3550 Microplate reader.

3.2.3.3.2. Calculations.

First the means of the optical densities (OD) of the duplicate standard or samples must be calculated. The % binding for each standard point or sample is then calculated as instructed by the manufacturer:

$$\% \text{ Binding} = \frac{\text{Standard or Sample OD} - \text{Non-Specific Binding(NSB) OD}}{B_0 \text{ OD} - \text{NSB OD}} \times 100$$

B_0 = 100 μ l Tracer + 100 μ l Tris Buffer = 100% Binding

NSB = 500 pmol 0.1 ml⁻¹ IAA or 100 pmol 0.1 ml⁻¹ t-ZR + 100 μ l Tracer = 0% Binding.

3.2.3.3.3. Processing of results.

Determinations were done in three fold and all experiments were repeated three times. Regression analysis was performed and the resulting regression line was plotted.

Chapter 4

Results

4.1. Somatic embryogenesis.

4.1.1. Initiation.

Two easily distinguishable types of callus tissue developed from different parts of the explant. Development of the ESM at the micropylar end of the immature embryo was translucent to white, mucilaginous and probably developed from suspensor cells of the immature embryo (Fig. 4.1). The other type of cell mass consisted of creamy coloured, small compact spherical cells, originating from the surface of the female gametophytes (Fig. 4.2). This creamy coloured cell mass developed before the ESM and at a faster rate, but was prone to turn brown as seen in Fig. 4.2.

The results of the ESM formed and the embryo formation on the ESM for *P. radiata* during the collecting period 28 November 1995 to 25 March 1996 are given in Table 4.1. This was tested on four basic media, SH, DCR1-DCR4 (Table 3.1, Appendix A). The initiation was only obtained for the period 28 November 1995 until 27 December 1995. After these dates germination of the explant occurred (Fig. 4.3). Approximately 75% of the female gametophytes were initiated to form ESM. Only a small percentage of these ESM (4-17%) survived after a three-month period, despite regular transfers to new media.

The level of ESM initiation increased in all media as maturation of the gametophyte proceeded, from sample date 28.11.95 to 27.12.95, as seen in Table 4.1. On average the best ESM initiation occurred on the SH medium (82%) followed by DCR2 medium (77%).

Table 4.1. Effect of different media on ESM formed and embryo formation for the explants of five *P. radiata* families.

Family Number	Medium	Collection date of cones					
		28.11.95		11.12.95		27.12.95	
		% Explants forming ESM and % embryos that form on the ESM.					
		ESM	Embryo	ESM	Embryo	ESM	Embryo
Tree 1	SH	50.00	0.00	84.00	0.00	95.65	0.00
	DCR1	76.47	0.00	88.00	0.00	84.00	0.00
	DCR2	57.89	0.00	52.94	0.00	100.00	10.00
	DCR3	90.48	0.00	78.95	0.00	82.61	5.26
	DCR4	89.47	0.00	20.00	0.00	91.67	4.55
Tree 2	SH	75.00	0.00	92.00	0.00	100.00	0.00
	DCR1	45.00	0.00	80.00	0.00	88.00	0.00
	DCR2	60.00	0.00	76.00	0.00	91.30	9.52
	DCR3	52.00	0.00	84.00	0.00	92.00	0.00
	DCR4	72.00	0.00	88.00	0.00	96.00	0.00
Tree 3	SH	40.00	0.00	--	--	100.00	0.00
	DCR1	80.00	0.00	--	--	70.83	0.00
	DCR2	57.14	0.00	--	--	80.00	5.00
	DCR3	29.41	0.00	--	--	72.00	0.00
	DCR4	64.00	0.00	--	--	70.83	5.88
Tree 4	SH	--	--	81.82	0.00	95.45	0.00
	DCR1	42.86	0.00	50.00	0.00	85.00	0.00
	DCR2	80.00	0.00	75.00	0.00	81.82	0.00
	DCR3	46.15	0.00	50.00	0.00	84.00	0.00
	DCR4	42.86	0.00	52.38	0.00	71.43	0.00
Tree 5	SH	--	--	80.00	0.00	86.96	0.00
	DCR1	--	--	100.00	0.00	80.95	0.00
	DCR2	--	--	78.26	0.00	100.00	0.00
	DCR3	--	--	77.78	0.00	91.30	0.00
	DCR4	--	--	85.71	0.00	84.00	0.00

Observations were recorded at 4 and 8 weeks after culture initiation. ESM formation was observed at 4 weeks and embryo formation on the ESM at 8 weeks after culture initiation.

0 = Denotes no response.

-- = Denotes no response due to fungal infection on the cones.

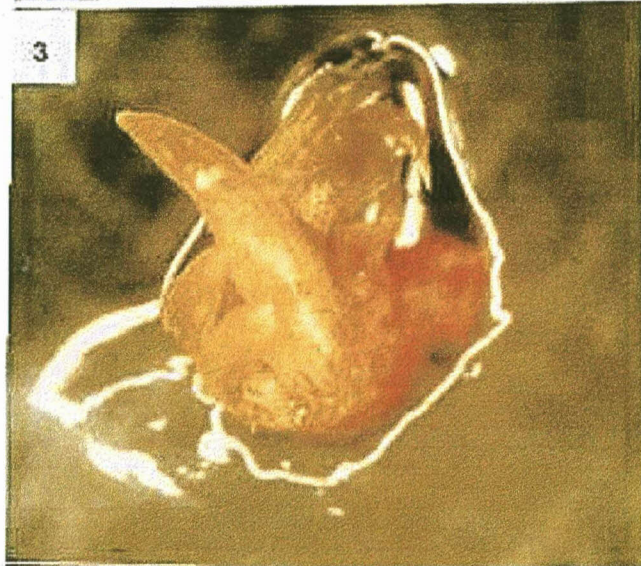
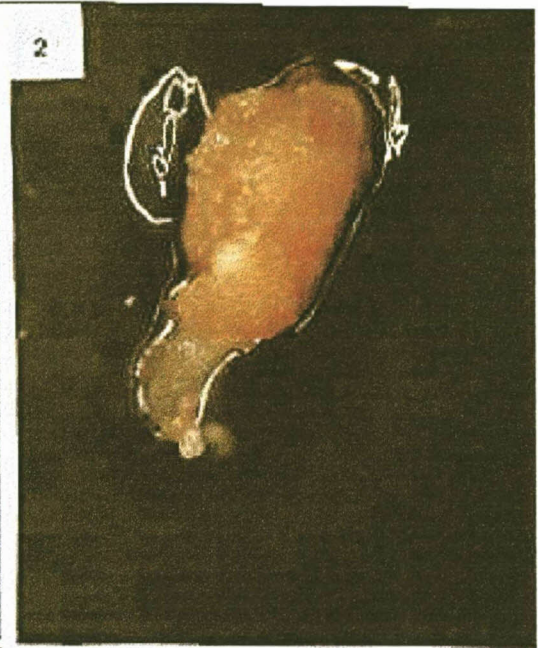
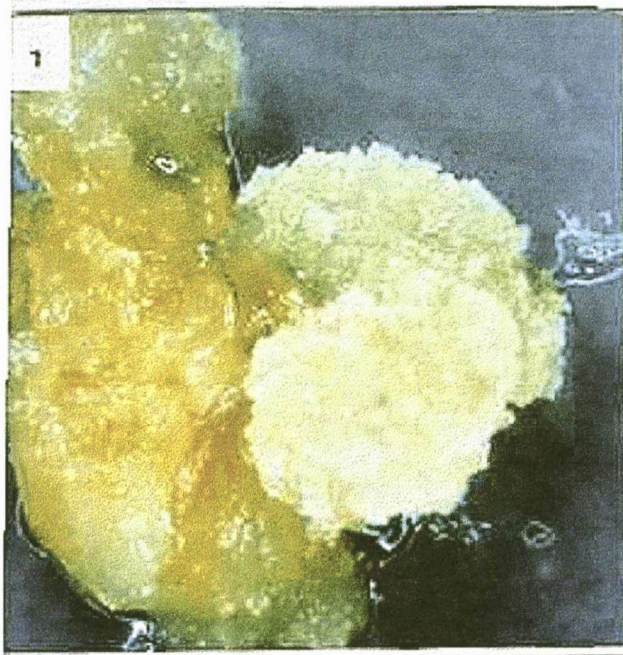


Fig. 4.1. Formation of the translucent white ESM from the micropylar end of the immature embryo after 4 weeks in culture.

Fig. 4.2. Formation of creamy coloured (non-embryonic) cell mass originated from the surface of the immature embryo after 4 weeks in culture.

Fig. 4.3. Germinated explant.

The ESM formation and embryos formed on the ESM for *P. patula* was obtained for the collection period 16 November 1995 to 18 January 1996. The results for the six basic media (Table 3.1, Appendix A) are given in Table 4.2. After this period, the germination of the embryo commenced as seen in Fig. 4.3 and no ESM formed. The best ESM formation for *P. patula* was obtained on the DCR5 medium.

Table 4.2. Effect of different media on ESM formed and embryo formation for the explants of five *P. patula* families.

Family number	Media	Collection period 16.11.95 to 18.01.96.	
		% Explants forming ESM and % embryos that form on the ESM.	
		ESM	Embryo
AP 297	MSG	1.54	--
	DCR1	12.35	--
	DCR2	20.00	--
	DCR3	11.11	--
	DCR4	10.00	3.33
	DCR5	15.00	5.00
AP 263	MSG	--	--
	DCR1	--	--
	DCR2	--	--
	DCR3	--	--
	DCR4	--	--
	DCR5	4.76	2.38
AP 289	MSG	16.00	--
	DCR1	18.18	--
	DCR2	25.53	--
	DCR3	53.33	--
	DCR4	33.33	4.76
	DCR5	39.29	8.93
AP 287	MSG	--	--
	DCR1	--	--
	DCR2	--	--
	DCR3	20.00	--
	DCR4	--	--
	DCR5	8.89	--
AP 261	MSG	8.57	--
	DCR1	--	--
	DCR2	25.00	--
	DCR3	35.00	--
	DCR4	32.31	4.62
	DCR5	37.50	2.50

= Denotes no response due to fungal infections on cones after 3 weeks in culture.

Cones of both species collected in the period 24 October 1996 to 30 March 1997 were highly infected. The excised immature seeds with intact female gametophytes were sterilized as described in 3.1. Sterilization by rinsing for 1 min. in 70% (v/v) ethanol and 3 min. in 0.5% (v/v) NaOCl was not done the previous year. A lot of the explants cultured turned brown and an antioxidant was added to the medium to combat browning, but without success. The explants were cultured on media selected for best growth the previous year. For *P. patula*; DCR1, DCR2 and DCR5 (Table 3.1, Appendix A) and for *P. radiata*; DCR2 and SH (Table 3.1, Appendix A) were used. In some cases the media were supplemented with 3% (m/v) PEG-4000 to stimulate embryogenesis for a period, but this was later omitted due to poor growth of the ESM.

The best embryonic cell mass development, for both species occurred for cones harvested during the period 20.12.96 to 02.01.97. ESM establishment varied between different families, being worst for AP128 and AR346. The success rate of ESM which developed pro-embryos was very variable (20-100%).

The solidified media finally selected for initiation of ESM were DCR2 for *P. radiata* and DCR1 and DCR5 for *P. patula*.

4.1.2. Maintenance.

After six months, in spite of regular transplanting to fresh media, the survival level of the ESM of the earlier harvest dates in the first year surpassed that of the latter harvest dates, where ESM initiation was higher. Maintenance of the ESM of one specific *P. radiata* line (AR366) which formed in the second year was very successful. This ESM was maintained on a solidified ½ LP medium (Table 3.1, Appendix A) without any growth regulators and cultured as in 3.2.1.1.2. By subculturing every four weeks ESM multiplication was obtained.

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4.1.3. Embryo maturation and development.

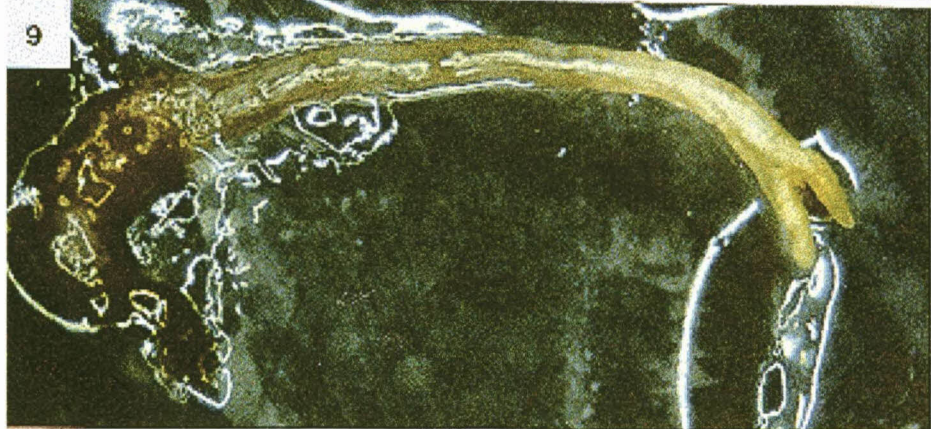
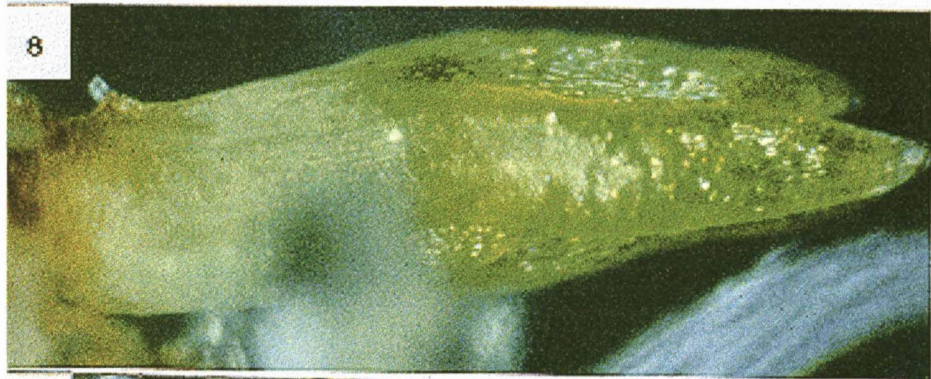
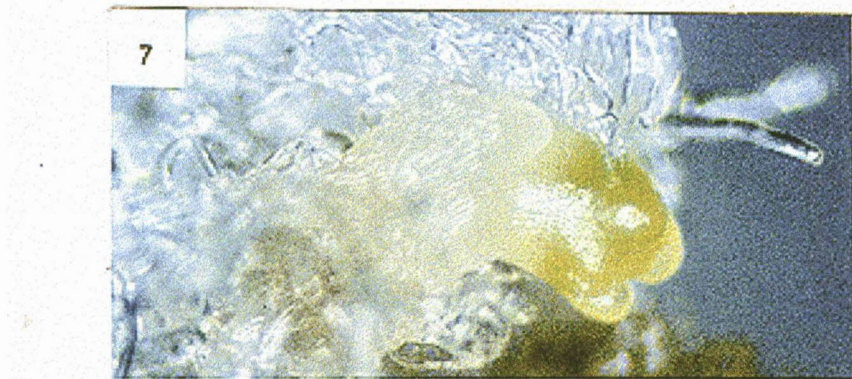
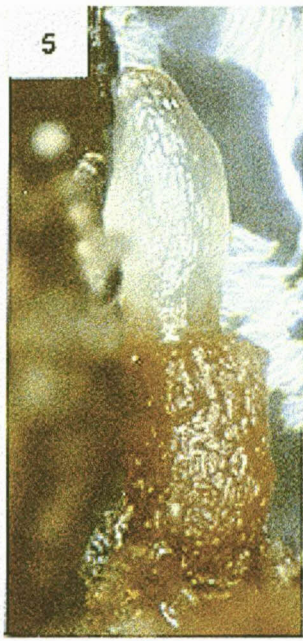
For *P. radiata* somatic embryos, characterized by a dense embryonal apex, subtended by a more elongated, translucent suspensor cell (Fig. 4.4), formed only on ESM derived from the immature embryos of cones harvested on the 25 January 1996. These embryos were placed on a DCR2 medium (Table 3.1, Appendix A). Although the SH medium resulted in a 82% initiation of the callus, no embryo development took place on this medium. Taking into account only those conditions, which gave positive embryo formation an average of only 6.7% explants formed embryos on the DCR2 medium. Stage 2 embryos as seen in Fig. 4.5 were more prominent and appear smooth in outline and are opaque. The apex is still subtended by the suspensor. The exposure of the ESM to ABA (1.3 mg l^{-1}) and 1% (m/v) activated charcoal resulted in maturation, or the development of stage 3 somatic embryos in a process which closely resembles zygotic embryo development (Fig. 4.6). The cotyledons elongate and start to turn green (Fig. 4.7). The incidence of production of these pro-embryos however, remained low or it did not occur at all. Although ESM initiation occurred for *P. patula* on DCR1 and DCR5 media (Table 3.1, Appendix A), no embryo development occurred.

4.1.4. Rooting.

The resultant stage 3 embryos for *P. radiata* were transplanted on a growth regulator-free GD medium and a plantlet structure, with partly elongated cotyledons developed (Fig. 4.8). The hypocotyl showed elongation and is green in colour. A rudimentary radicle developed (Fig. 4.9).

The GD rooting medium tested (3.2.1.1.4), only induced further cell mass formation. This cell mass that formed, showed no differentiation and no roots were produced.

- Fig. 4.4. Stage 1. Somatic embryo characterized by a dense embryonal apex, subtended by a more elongated, translucent suspensor cell.
- Fig. 4.5. Stage 2. Embryo appears smooth in outline and is opaque, apex still subtended by the suspensor cell.
- Fig. 4.6. Stage 3. Small developing cotyledons clustered around a central meristem.
- Fig. 4.7. The cotyledons elongate and start to turn green.
- Fig. 4.8. Stage 4 (early) Plantlet with partly elongated cotyledons, clustered around the central meristem. The hypocotyl shows some elongation.
- Fig. 4.9. Stage 4 (late) elongated hypocotyl and cotyledons are evident, and rudimentary radicle development visible.



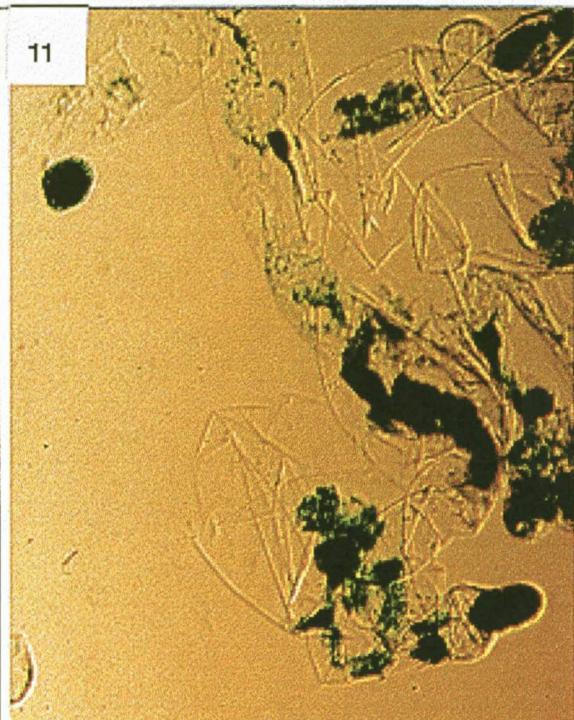
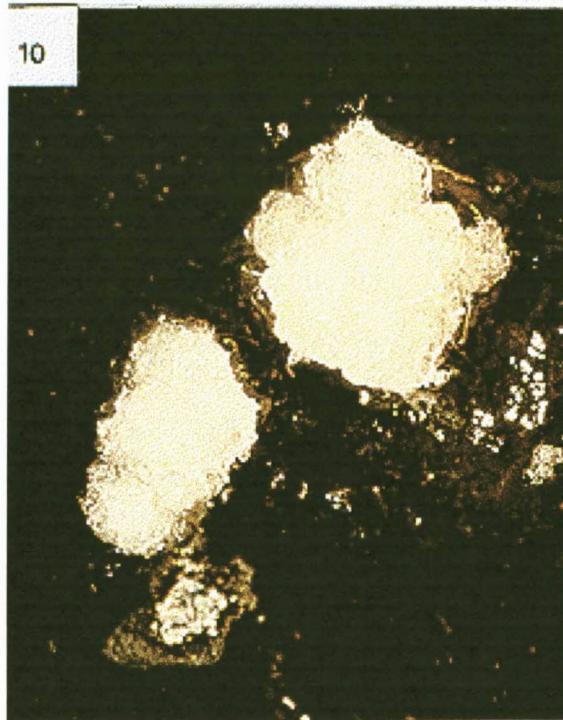


Fig. 4.10. White mucilaginous, translucent ESM used to start the inoculum for the cell suspension cultures.

Fig. 4.11. Cells stained with acetocarmine and Evan's blue. Nuclei visible in apical region.

Fig. 4.12. Modified Erlenmeyer flasks used during the cell suspension cultures.

4.2. Cell suspension cultures.

Embryonic cell suspension cultures were easily initiated from the white mucilaginous, translucent ESM (Fig. 4.10) obtained after culturing of the explants as described in 3.2.1.1.1. Staining with acetocarmine and Evan's blue (as described in 3.2.2) revealed the nuclei in the apical region (Fig. 4.11). These cultures were grown in modified Erlenmeyer flasks (Fig. 4.12), with side tubes allowing the use of a Klett-meter to determine the turbidity of the culture as a measure of growth.

The first experiment was done with the four liquid media selected in 3.2.1.2 and supplemented with 3% (m/v) sucrose. In addition, the effect of auxin supplementation (0.5 mg l^{-1} 2,4-D) was investigated for each medium. GD medium with and without the auxin supplementation had a long lag phase of between 8 and 12 days, respectively (Fig. 4.13,A and B). After that, cell doubling occurred at a rate of 2.6 days in GD medium containing auxin, and 3 days in the medium without auxin supplementation.

The DCR medium without any auxin (Fig. 4.13,A) achieved cell doubling after 3 days and the stationary phase after 5.5 days. Auxin supplementation increased the growth rate to a doubling time of 2.6 days and retarded the onset of the stationary phase to 10 days.

The auxin supplementation increased the growth rate of the SH medium from a cell doubling time of 3 days to 2 days and retarded the onset of the stationary phase to 6.8 days. The onset of the stationary phase for the SH medium without auxin was 6 days.

In the absence of auxin the $\frac{1}{2}$ LP medium yielded a cell doubling time of 2.5 days. Auxin supplementation decreased this period to 2 days. This was the

best growth rate obtained (Fig. 4.13,A and B and Table 4.3).

Table 4.3. Effect of different sucrose containing media and auxin (2,4-D) supplementation on the growth of *P. radiata* embryonic cells.

Liquid media	Cell doubling time during growth phase (Days)	Onset of stationary phase (Days)
½ LP	2.5	10.3
½ LP + 0.5 mg l ⁻¹ 2,4-D	2.0	6.4
SH	3.0	6.0
SH + 0.5 mg l ⁻¹ 2,4-D	2.0	6.8
DCR	3.0	5.5
DCR + 0.5 mg l ⁻¹ 2,4-D	2.6	10.0
GD	3.0	17.5
GD + 0.5 mg l ⁻¹ 2,4-D	2.6	16.5

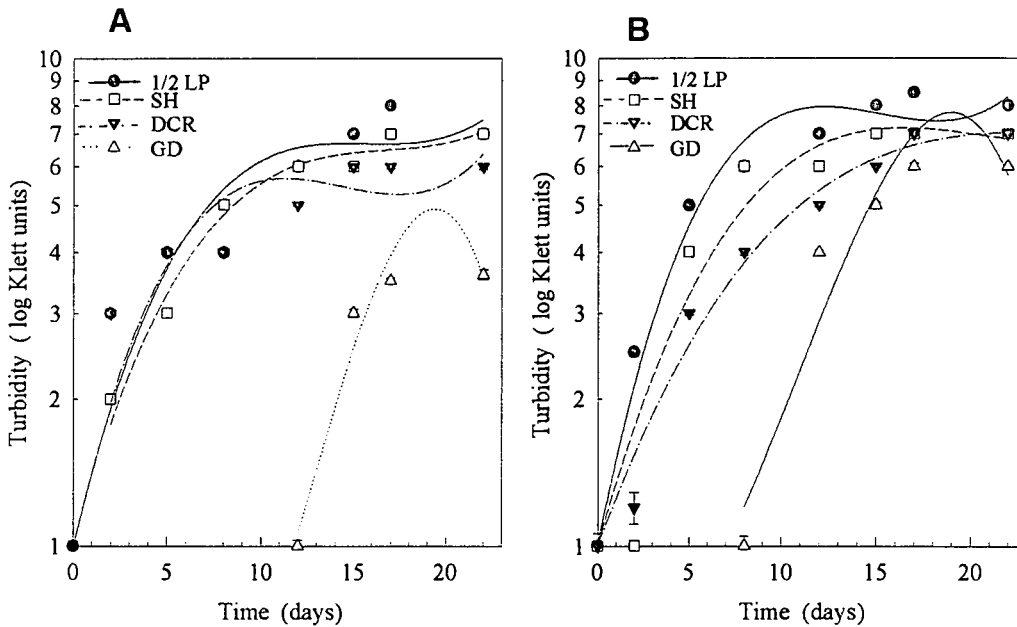


Fig. 4.13. The effect of auxin on the growth of *P. radiata* embryonic cells in four different liquid media. Turbidity was measured with a Klett meter.

A: Media containing 3% (m/v) sucrose without 2,4-D

B: Media containing 3% (m/v) sucrose with 2,4-D

According to Gupta¹ (personal communication) sucrose as carbon source results in the development of B-type embryos. B-type embryos do not mature and will not develop past the bullet stage. Changing the carbon source to maltose however resulted in A-type embryos that develop further.

In the study comparing sucrose (3%, m/v) and maltose (3%, m/v) as carbon sources for the four media tested, it was found that the GD and DCR media, had a lag phase before the cells started to multiply as seen in Fig. 4.14,A and B. It seems that maltose also increased the growth rate by decreasing the cell doubling time from 2 days in the ½ LP liquid medium containing sucrose to 1 day in the same medium containing maltose (Table 4.4).

The cell doubling time decreased from 2.5 days in the SH medium containing sucrose to 2 days in the same medium containing maltose as carbon source (Table 4.4) and a more prominent and longer linear growth phase occurred (Fig. 4.14,A and B).

Supplementation of sucrose or maltose to the DCR medium resulted in cell doubling times of 2.6 days. The stationary phase increased from 6 days in the medium containing sucrose to 6.5 days in the medium supplemented with maltose. The same result is seen in the GD medium (Table 4.4). The different carbon sources thus had a very small effect on the onset of the stationary phase in the DCR and GD media.

¹ Gupta: Weyerhaeuser, Tacoma, Washington, 98477.

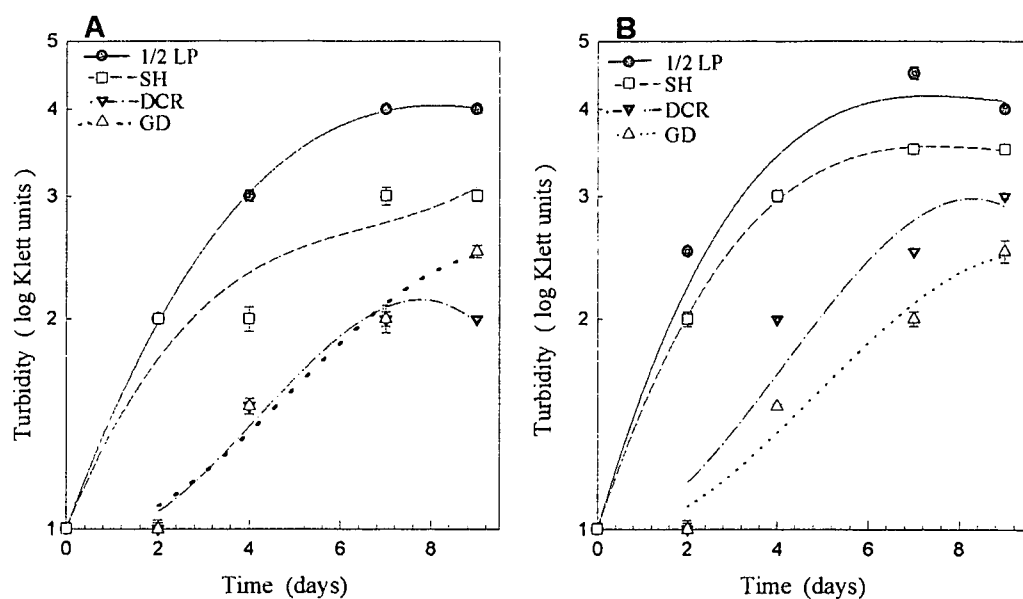


Fig. 4.14. The effect of carbohydrate source on the growth of *P. radiata* embryonic cells in four different liquid media. Turbidity was measured with a Klett meter.

A: Sucrose (3%, m/v) containing media.

B: Maltose (3%, m/v) containing media.

Table 4.4. Effect of different carbon sources (maltose and sucrose) on the growth of *P. radiata* embryonic cells in different liquid media, all containing 0,5 mg l⁻¹ 2,4-D.

Liquid media	Cell doubling time during growth phase (Days)	Onset of stationary phase (Days)
½ LP (maltose)	1.0	2.5
½ LP (sucrose)	2.0	3.0
SH (maltose)	2.0	3.0
SH (sucrose)	2.5	2.5
DCR (maltose)	2.6	6.5
DCR (sucrose)	2.6	6.0
GD (maltose)	3.0	7.5
GD (sucrose)	3.0	7.0

In a follow up study with maltose as carbon source another two 2,4-D concentrations (1 mg l⁻¹ and 2 mg l⁻¹) were used to determine the 2,4-D concentration for optimal growth.

It appears that an auxin concentration of 1 mg l⁻¹ 2,4-D stimulated ESM growth in ½ LP medium more than the 2 mg l⁻¹ 2,4-D (Table 4.5 and Fig. 4.15,A). The cell doubling time in ½ LP medium containing 1 mg l⁻¹ 2,4-D was 2 days compared to 3 days in ½ LP medium containing 2 mg l⁻¹ 2,4-D. According to Table 4.4 0.5 mg l⁻¹ 2,4-D in ½ LP medium supplemented with maltose resulted in the best growth rate. Cell doubling time was recorded as 1 day.

In SH medium a doubling time of 3 days prevailed for both 2,4-D concentrations (Table 4.5 and Fig. 4.15,B).

Table 4.5. Effect of auxin (2,4-D) concentration on the growth of *P. radiata* embryonic cells in ½ LP and SH liquid media containing maltose.

Liquid media	Cell doubling time during growth phase (Days)	Onset of stationary phase (Days)
½ LP	2.0	9.0
½ LP + 1 mg l ⁻¹ 2,4-D	2.0	9.0
½ LP + 2 mg l ⁻¹ 2,4-D	3.0	10.0
SH	4.0	7.0
SH + 1 mg l ⁻¹ 2,4-D	3.0	9.0
SH + 2 mg l ⁻¹ 2,4-D	3.0	13.0

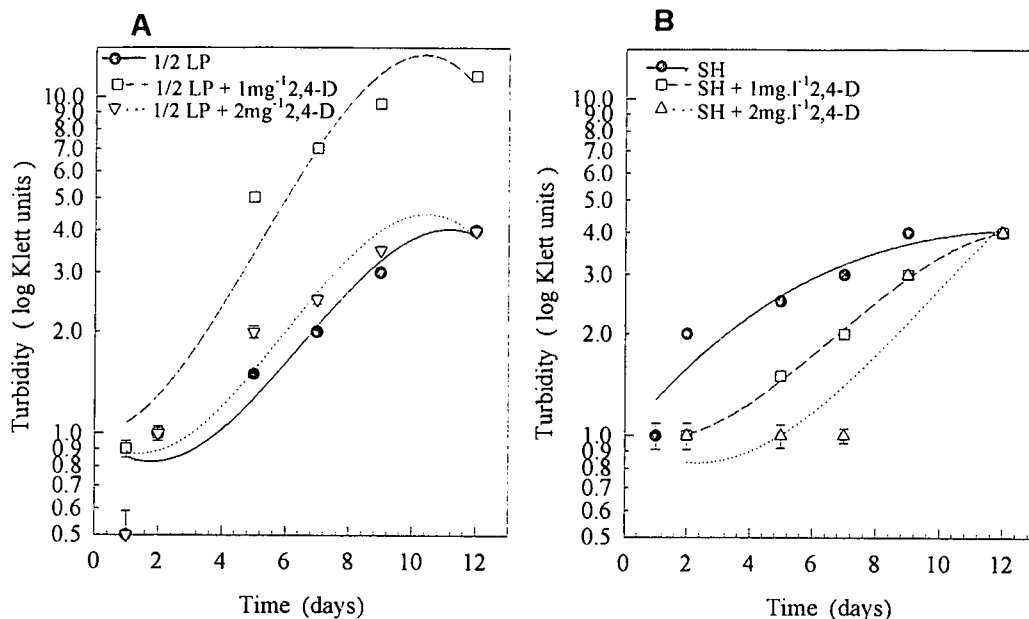


Fig. 4. 15. The effect of different 2,4 -D concentrations on the growth of *P. radiata* embryonic cells in ½ LP and SH liquid media.

Turbidity was measured with a Klett meter.

A: ½ LP medium containing 1 and 2mg.l⁻¹ 2,4 -D.

B: SH medium containing 1 and 2mg.l⁻¹ 2,4 - D.

The effect of cytokinin (2µM and 5µM BAP) on the growth of *P. radiata* embryonic cells in suspension cultures was also determined. Both media tested (½ LP and SH) showed a lag phase for all the treatments as seen in Fig. 4.16,A and B. According to Table 4.6 and Fig. 4.16,B addition of cytokinin tended to retard the growth in the SH medium.

For the ½ LP medium the 5µM BAP tended to retard the growth of the embryonic cells by increasing cell doubling time from 1 (Table 4.4) to 2 days (Table 4.6). This growth rate corresponded to that of ½ LP containing 1 mg.l⁻¹ 2,4-D (Table 4.5 and 4.6).

Table 4.6. Effect of different concentrations cytokinin (BAP) on the growth of *P. radiata* embryonic cells in different liquid media.

Liquid media	Cell doubling time during growth phase (Days)	Onset of stationary phase (Days)
½ LP	2.0	8.0
½ LP + 1 mg.l ⁻¹ 2,4-D	2.0	9.0
½ LP + 1 mg.l ⁻¹ 2,4-D + 2 µM BAP	3.0	6.0
½ LP + 1 mg.l ⁻¹ 2,4-D + 5 µM BAP	2.0	3.0
SH	3.0	7.0
SH + 1 mg.l ⁻¹ 2,4-D	2.0	9.0
SH + 1 mg.l ⁻¹ 2,4-D + 2 µM BAP	3.0	6.0
SH + 1 mg.l ⁻¹ 2,4-D + 5 µM BAP	3.0	5.0

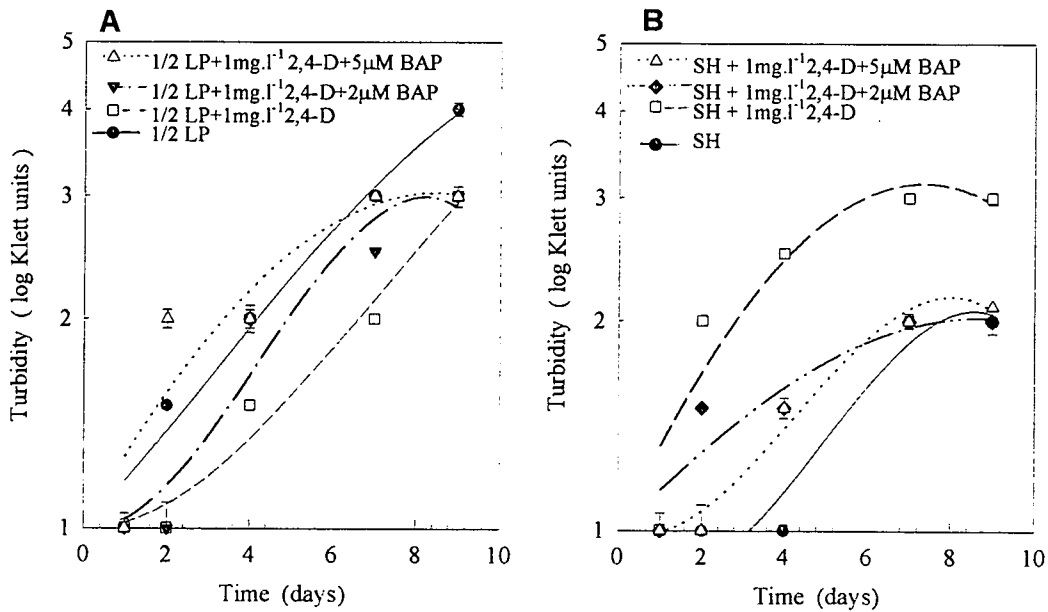


Fig. 4.16. The effect of cytokinin (BAP) on the growth of *P. radiata* embryonic cells in ½ LP and SH liquid medium. Turbidity was measured with a Klett meter.

A: ½ LP medium.

B: SH medium.

The liquid cultures were re-established onto solid media for embryo maturation as described in 3.2.1.2. Establishment of translucent, mucilaginous, embryonic tissue was easily achieved and it rapidly covered the filter paper within a week of transferring it. Development of pro-embryos and further development was very slow. After six weeks on the media containing 25 mg l^{-1} ABA and 0.75% gelrite only 4.5% of the cell clusters transferred showed development of stage 1 and 2 embryos.

4.3. Organogenesis.

4.3.1. Sterilization and germination of mature seeds.

The 21 different sterilization treatments and the control (Treatment no. 1) (Table 3.2, Appendix A) described in 3.2.1.3.1 resulted in a range of effects regarding contamination and germination. The % seeds germinating successfully, and the % seeds showing contamination following each treatment, are depicted in Table 4.7.

Table 4.7. Percentage *P. patula* and *P. radiata* seeds germinated and contaminated 21 days after different sterilization treatments.

Treatment number	<i>P. patula</i> (%)		<i>P. radiata</i> (%)	
	Germination	Contamination	Germination	Contamination
1	70	100	20	100
2	80	10	0	0
3	40	0	0	0
4	70	50	50	20
5	70	50	50	20
6	80	10	100	0
7	80	0	50	10
8	80	0	40	0
9	100	0	60	30
10	0	0	0	0
11	0	0	0	0
12	90	60	40	20
13	70	70	60	50
14	0	0	0	0
15	0	0	0	0
16	0	10	0	20
17	0	0	0	0
18	80	70	50	40
19	0	0	0	0
20	60	30	30	30
21	90	30	50	20
22	0	0	0	0

The germination response was greatly affected by the different sterilization procedures as depicted in Table 4.7. The two species also reacted very differently to these treatments. The germination percentage of *P. patula* was in most cases higher than that of *P. radiata*. Treatments with CaOCl_2 (Treatment 10 and 11) as well as 1.5% and 2% NaOCl (Treatment 14,15 and 17,19) and 3.5% NaOCl (Treatment 22) caused total inhibition of germination and no contamination occurred for either species. For treatment 16 (2% NaOCl for 5 min.) contamination (10-20%) still occurred. For *P. radiata* the treatments with HgCl_2 also resulted in no germination.

It is clear that for *P. patula* the highest germination rate was obtained by sterilizing for 10 min. with 30% H_2O_2 (Treatment 9). For *P. radiata* 5 min. with a 10% H_2O_2 solution produced the best germination rate (Treatment 6). For these two treatments zero contamination occurred and the germination rate was improved by the treatment as compared to the control (Treatment 1) (Table 4.7). These sterilizing treatments constantly resulted in more than 80% germination. These two treatments were subsequently used to sterilize the mother material.

Initially seeds were germinated in the dark on wet sterile filter paper, at $\pm 23^\circ\text{C}$ (Fig. 4. 17). Germinated seeds were then transferred, under sterile conditions to a solidified DCR medium, containing no growth regulators. Within 4-7 weeks the germlings developed to contain fully developed cotyledons. These explants were then sterilized again by using 0.5% NaOCl for 1 min. and transplanted. This secondary sterilization resulted in an unacceptable loss. Therefore seeds were sterilized with the best method of those tried in Table 4.7, and placed directly onto solidified DCR medium (Table 3.3, Appendix A) containing no growth regulators. By germinating the seeds directly on the solidified medium (Fig. 4.18), contamination could easily be detected. This process also proved to be labour saving.

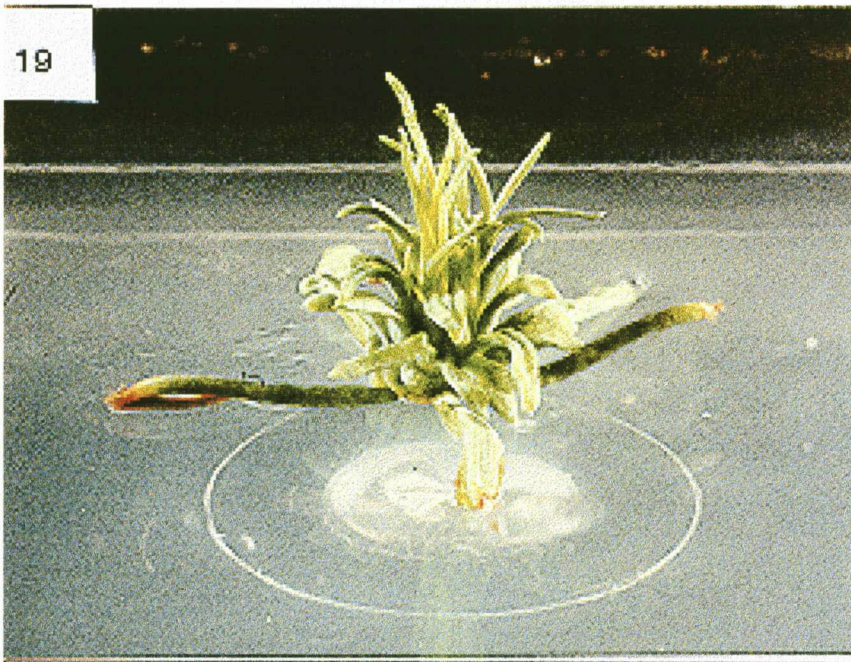
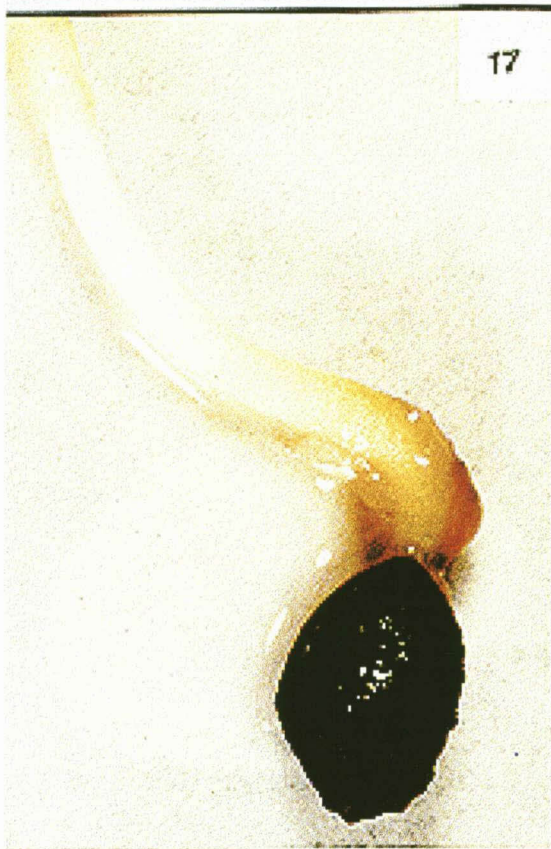


Fig. 4.17. Seed germinated on sterile filter paper.

Fig. 4.18. Seeds germinated on solidified medium.

Fig. 4.19. Explant with distal portion of hypocotyl and cotyledons still attached.

When the pine seed germinates, the entire embryo emerges from the ruptured seed coat, which may adhere for a short time to the tips of the cotyledons. The primary shoot is formed by the terminal bud of the seedling, and at first bears only a spiral series of needle like leaves. Later the first spur shoots arise in the axils of some of the primary leaves.

4.3.2. Axillary bud formation and multiplication.

For the study, 4-7 week old germlings were used. The explant consisted of the distal portion of the hypocotyl with the cotyledons still attached (Fig. 4.19). In the latter part of the study the cotyledons were removed and 2 - 4 week old germlings were used. It was found that the development of the apical buds improved. The excised cotyledons were cultured individually (see 4.3.4). The explants which consisted of the distal portion of the hypocotyl, were cultured for axillary bud formation on five different media as shown in Table 3.3 (Appendix A). The percentage explants which formed axillary buds after 8 weeks in culture are shown in Table 4.8.

Table 4.8. Percentage explants which formed axillary buds on the different media used.

Medium	% Explants which formed axillary buds for <i>P. radiata</i>	% Explants which formed axillary buds for <i>P. patula</i>
DCR	52	64
LP	50	60
SH	40	60
GD	32	36
MS	5	16

The explant development on the GD medium was good but a relatively low percentage (32 - 36%) formed axillary buds. The growth and development on the MS medium resulted in a very low percentage (5 -16%) formation of axillary buds (Table 4.8 and Fig. 4.20 D & E).

Of the five media tested three (DCR, LP and SH) were selected for further studies as they adequately supported sustained growth (Fig. 4.20 A -C). The percentage explants which formed axillary buds on these media varied between 64% and 40%. *P. patula* performed on average 10% better than *P. radiata* (Table 4.8).

Developed axillary buds were excised and transplanted unto the same medium. The axillary buds that survived three transplants unto fresh media were then transferred to the different media for elongation trials (4.3.6).

4.3.3. Development of axillary buds from immature seeds.

Explants (embryos) from the older cones received from SAFCOL, germinated spontaneously on media (Fig. 4.3) and were used in this investigation. Some did not develop cotyledons, but produced multiple buds (Fig. 4.21). The buds were separated and placed on different media supplemented with growth regulators as in Table 3.3 (Appendix A) for further development.

The explants on the MS and SH media turned brown or yellow and died after the third transfer to fresh media. Multiple buds formed on the GD medium, but better overall growth and development was however obtained on the LP and DCR media. The development and growth of the buds obtained on these two media was very similar.

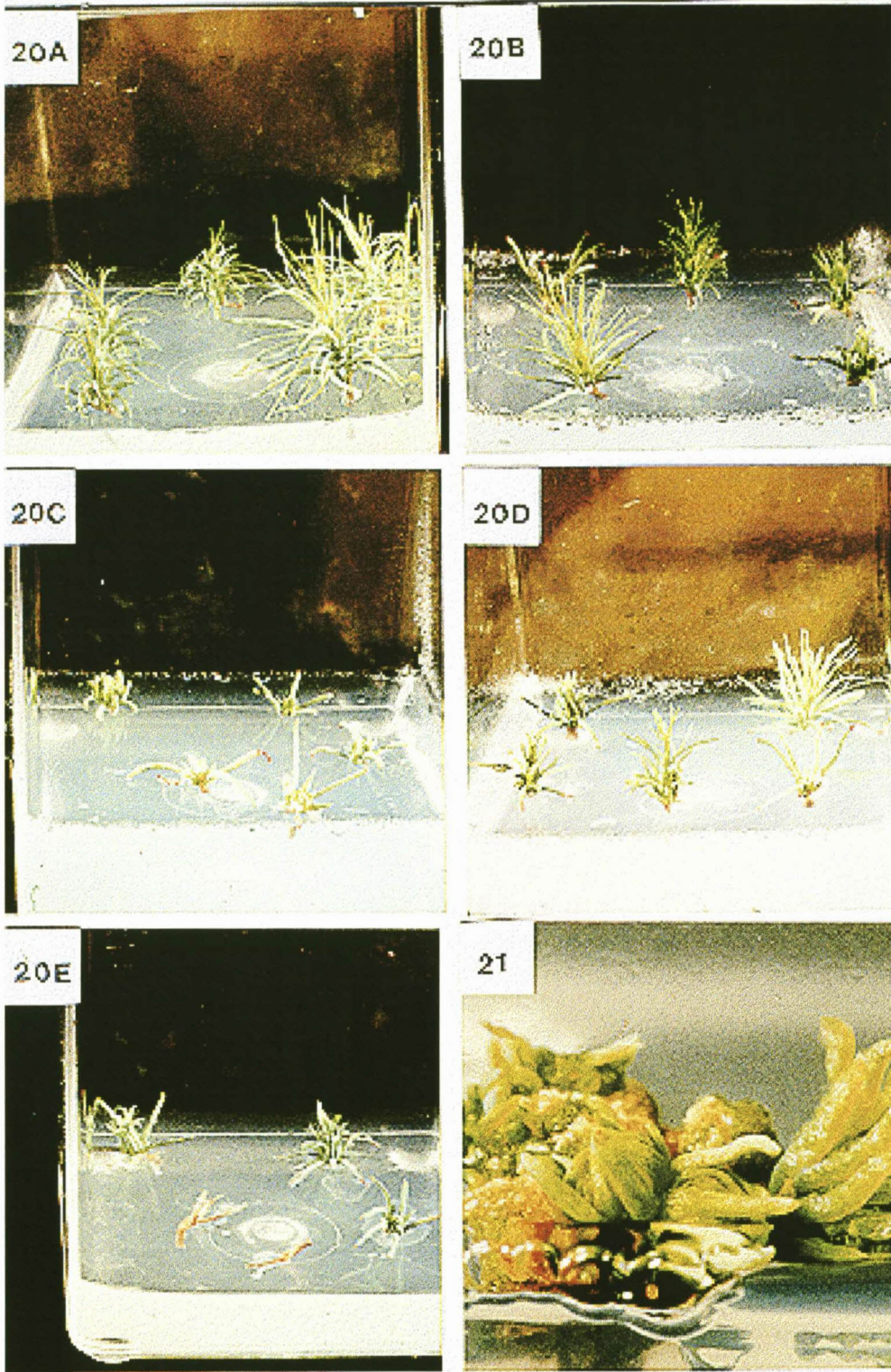


Fig. 4.20. Development and growth on the different media for the formation of axillary buds. A: DCR medium; B: LP medium; C: SH medium; D: GD medium and E: MS medium.

Fig. 4.21. Multiple buds formed after germination of an explant from an older cone.

4.3.4. Adventitious bud formation.

In only one case (Fig. 4.22) adventitious buds were induced on cotyledons still attached to the hypocotyl of *P. radiata*. This development occurred on SH medium (Table 3.1, Appendix A).

Development of the adventitious buds on the cotyledons was improved by removal of the cotyledons from the explant. The cotyledons of 14 day old seedlings were excised from the seedlings and cultured lengthwise on top of the media (LP, SH and DCR media supplemented as stated in Table 3.3 (Appendix A). After 3 weeks yellow meristematic callus tissue developed on the cut edges of the cotyledons (Fig. 4.23). After about 6-8 weeks scale-like structures and adventitious buds formed on some of the cotyledons (Fig. 4.24 A & B). A very low percentage cotyledons (6%) formed callus on the SH medium, but no further development took place (Table 4.9).

Table 4.9. The percentage *P. patula* cotyledons which formed callus, scale-like structures and buds on different media.

Medium	% Cotyledons which formed callus	% Cotyledons which formed buds and scale-like structures.
DCR	46%	14%
LP	31%	4%
SH	6%	0%

For *P. radiata* 25% cotyledons formed callus and only 11% cotyledons formed buds and scale-like structures. This development only occurred on DCR medium (Table 3.3, Appendix A) and cotyledons with a minimum length of 2 cm. No development occurred on the other media tested.

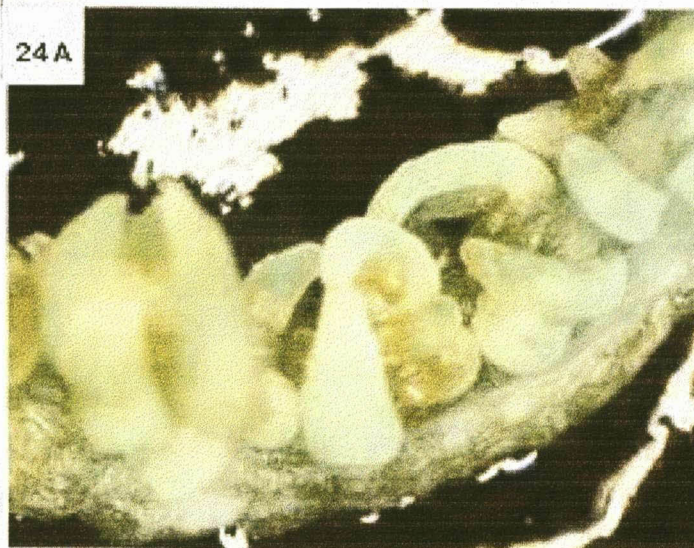
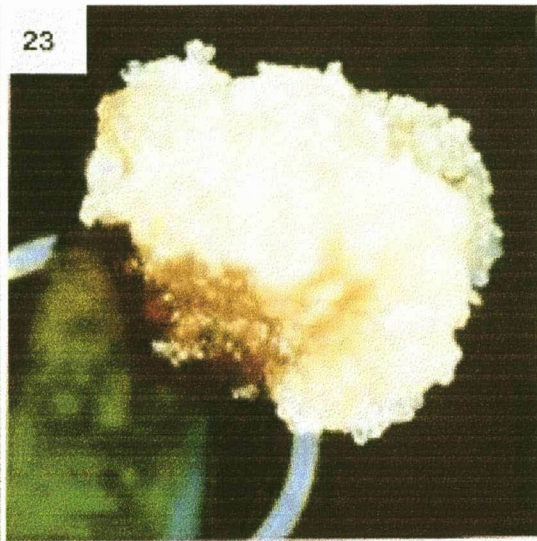


Fig. 4.22. Adventitious buds formed on explant with intact cotyledons.

Fig. 4.23. Yellow meristematic callus developed on the cut edge of individual cotyledons.

Fig. 4.24. A: Scale-like structures formed on individual cotyledons.
B: Adventitious buds formed on individual cotyledons.

The remaining cotyledons showed no other visual development after 12 weeks in culture, but remained green and alive. The developed buds were transferred to an elongation medium, but even after a period of 12 weeks no further development occurred.

The only development on cotyledons of 4 week old seedlings was yellow meristematic callus that formed on the cut edges of the cotyledons. (Table 4.10).

Table 4.10. The percentage older cotyledons which formed callus on the different media.

Medium	% <i>P. patula</i> cotyledons which formed callus.	% <i>P. radiata</i> cotyledons which formed callus.
DCR	100%	100%
LP	50%	75%
SH	18%	36%

4.3.5. *Development of diverse structures from embryos.*

The excised embryos were placed on the three different solidified media (DCR, LP and SH) as described in Table 3.3 (Appendix A), and cultured under low light conditions or in the dark.

Some embryos were then cultured in an inverted position on the media with cotyledons submerged, but this treatment did not result in further development. Other embryos were put in a horizontal position on the media. These embryos became noticeably swollen in the hypocotyl and cotyledon regions. Callus and unidentified diverse structures subsequently developed as seen in Fig. 4.25. Embryos with excised radicle tips, showed the same development than intact embryos. The embryos cultured in the dark on the DCR medium became noticeably swollen, but no further development took place. This phenomenon occurred in both species. On the LP medium 25% of the *P. patula* embryos showed development

and for *P. radiata* 33% embryos developed diverse structures. On the SH medium only *P. radiata* showed development of structures as seen in Fig. 4.25. Only 25% embryos developed these structures which became visible after 6 weeks. The embryos cultured under low light conditions turned green and then browned after 4 weeks and no further development occurred.

4.3.6. Elongation of axillary and adventitious buds.

The developed axillary buds (Fig. 4.26) were excised and transplanted on LP, SH, and DCR medium (Table 3.3, Appendix A) without any growth regulators for elongation (Fig. 4.27). The best success rate (68%) for elongation of *P. patula* buds was obtained on DCR medium (Table 4.11).

The LP medium yielded only 59% buds which elongated, but 37% of the explants on the same medium developed secondary axillary buds. These shoots which formed secondary axillary buds during the elongation study were excised for re-multiplication.

Table 4.11. The percentage buds which elongated and the percentage which formed secondary axillary buds on the different elongation media.

Medium	% Axillary buds that elongated	% Buds which formed secondary axillary buds
DCR	68%	27%
LP	59%	37%
SH	45%	27%

The adventitious buds which formed on the cotyledons were transferred to the elongation media (DCR, LP and SH , Table 3.3, Appendix A) containing no growth regulators. Only 20 % of the buds transplanted on the DCR medium elongated, but no further development took place. The adventitious buds which were transplanted onto the SH and LP media turned brown and failed to elongate even after regular transfers to fresh media.

4.3.7. Rooting of the elongated shoots.

Different media were used to investigate the rooting of elongated shoots. Initially 20 shoots were put on solidified LP medium with a high cytokinin concentration (10 mg l^{-1} BAP). No roots formed over a period of 12 weeks. Consequently a GD medium supplemented with 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} IBA was used as root-initiation medium. During the first 2 weeks all 50 shoots formed meristematic callus. The shoots were then transferred to LP medium without any growth regulators. The rooting percentage was established at 10% (Fig. 4.28).

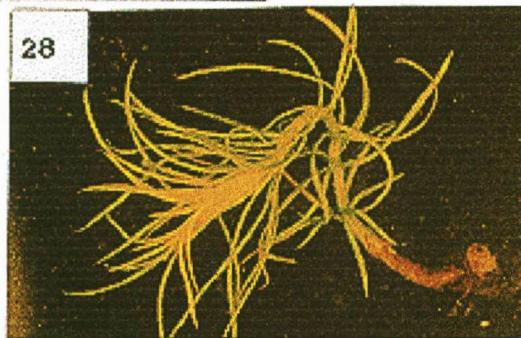
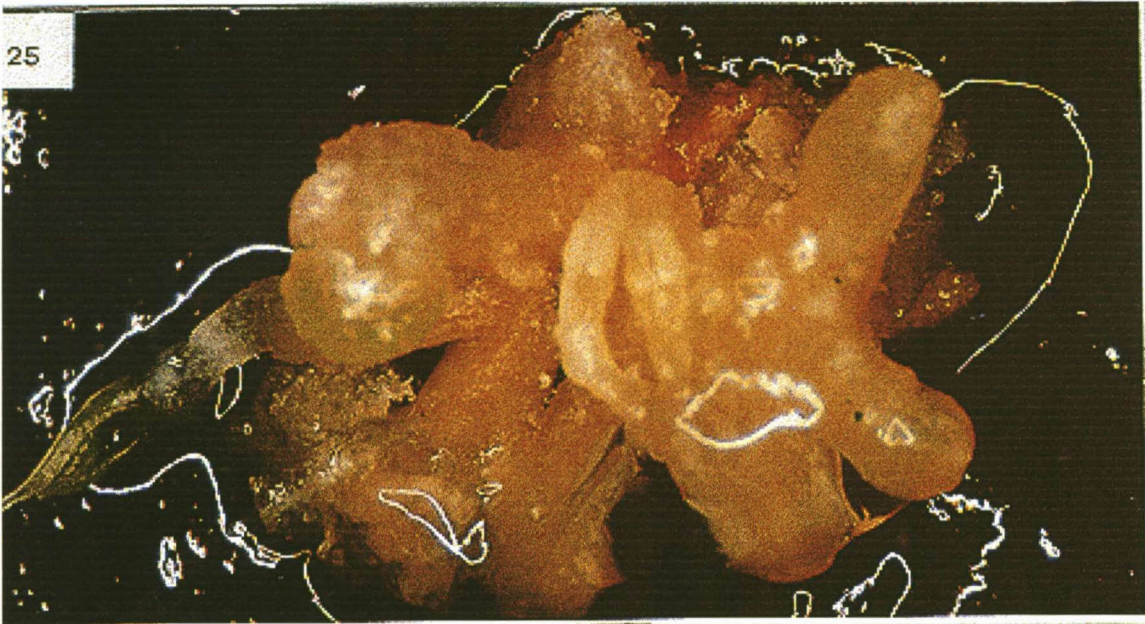


Fig. 4.25. Callus and diverse structures formed on embryos in the dark.

Fig. 4.26. Developed axillary buds.

Fig. 4.27. Shoots placed out for elongation.

Fig. 4.28. Root formation.

4.4 Chemical markers of the rooting ability of *Pinus* cuttings.

4.4.1. Total phenolic contents.

There was a vast difference in the average rooting percentage for the hybrids used in the studies, as given by the supplier (Table 4.12).

Table 4.12. The rooting percentage of different hybrid cuttings supplied by SAFCOL.

Number	Clone number	Rooting %
1	E26 x PCH	88
2	E17 x PCH	69
3	E833 x PCH	67
4	E38 X C6.AE218	61
5	E768 X PCH	73
6	AE219 x C167	73
7	E511 x PCH	64
8	E591 x PCHM	62
9	E23 x PCH	59
10	E30 x C5	40
11	E820 x PCH	46
12	AE218 x PCH	40
13	E587 x PCH	56
14	AE200 x C157	57
15	E661 x PCH	43
16	E551 x PCH	53
17	AE218 x C162	45
18	E862 x PCH	50
19	E38 x C6.C157	33
20	E1 x C5.C5	24

AE/E = *P. elliottii*

PCH = *P. caribaea* var. *hondurensis*

PCHM = *P. caribaea* var. *hondurensis* mix.

Samples of these cuttings were prepared as in 3.2.3.1 and their total phenolic content determined. The total phenolic content was expressed in gallic acid equivalents g^{-1} fresh material. The absorption values at 700 nm used in the method (3.2.3.1) to determine the phenolic content, increased linearly over the gallic acid concentration range of 0.50 to 2.00 mM ($r^2 = 0.9$) (Fig. 4.29).

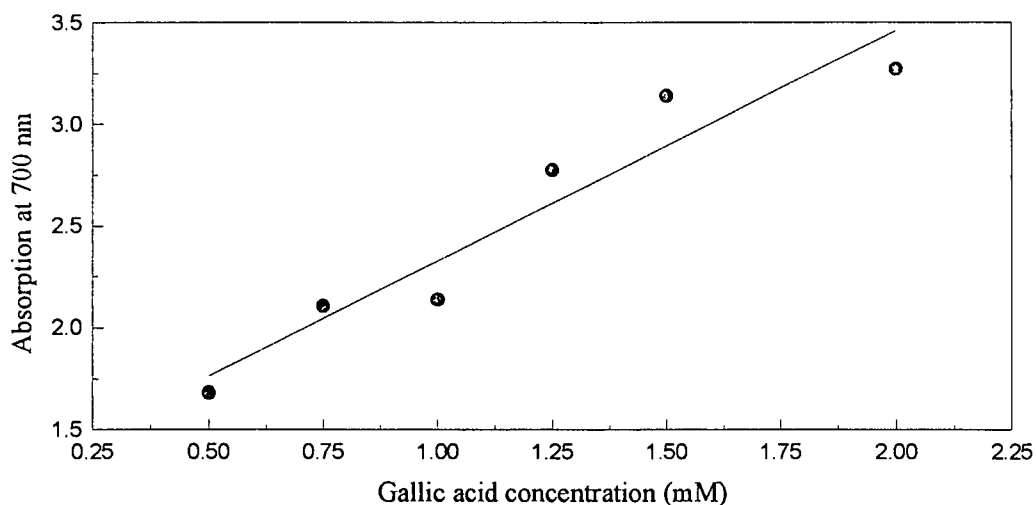


Fig. 4.29. Relation between the absorption at 700 nm and gallic acid concentration ($r^2=0.9$).

The phenolic content decreased with increasing rooting percentages ($r^2 = 0.7$) for the cuttings tested (Fig. 4.30). A relationship of low phenolic content and high rooting percentage was therefore established.

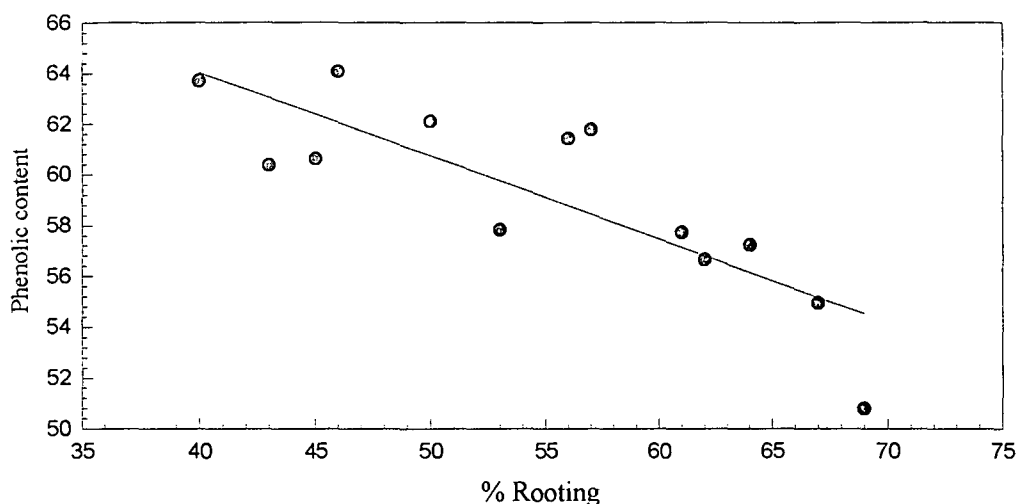


Fig. 4.30. Relation between the total phenolic content and rooting percentage ($r^2=0.7$)

4.4.2. Phenolic acid composition of cuttings.

Six different cutting samples with different rooting % were chosen and prepared as in 3.2.3.2. Low molecular weight phenolic acid extracts were separated on cellulose thin layer plates. The cellulose TLC plates produced six spots after spraying with a reagent and are depicted in Fig. 4.31 A-F).

Good separation of the phenolic acids was observed and the chromatograms of two replicates of each sample were essentially similar. Chromatograms in Fig. 4.31 A & B and Fig. 4.31 C-D were developed separately which did give rise to slightly different patterns i.e. disappearance of spot 2 and appearance of spot 7 in Fig. 4.31 C-D. Otherwise these two sets of chromatograms were essentially similar, indicating no difference between phenolic acid composition of cuttings with poor rooting potential and cuttings with better rooting potential. Quantitative differences indicated by the size and colour intensities of the spots were inconsistent and could not be correlated with rooting potential.

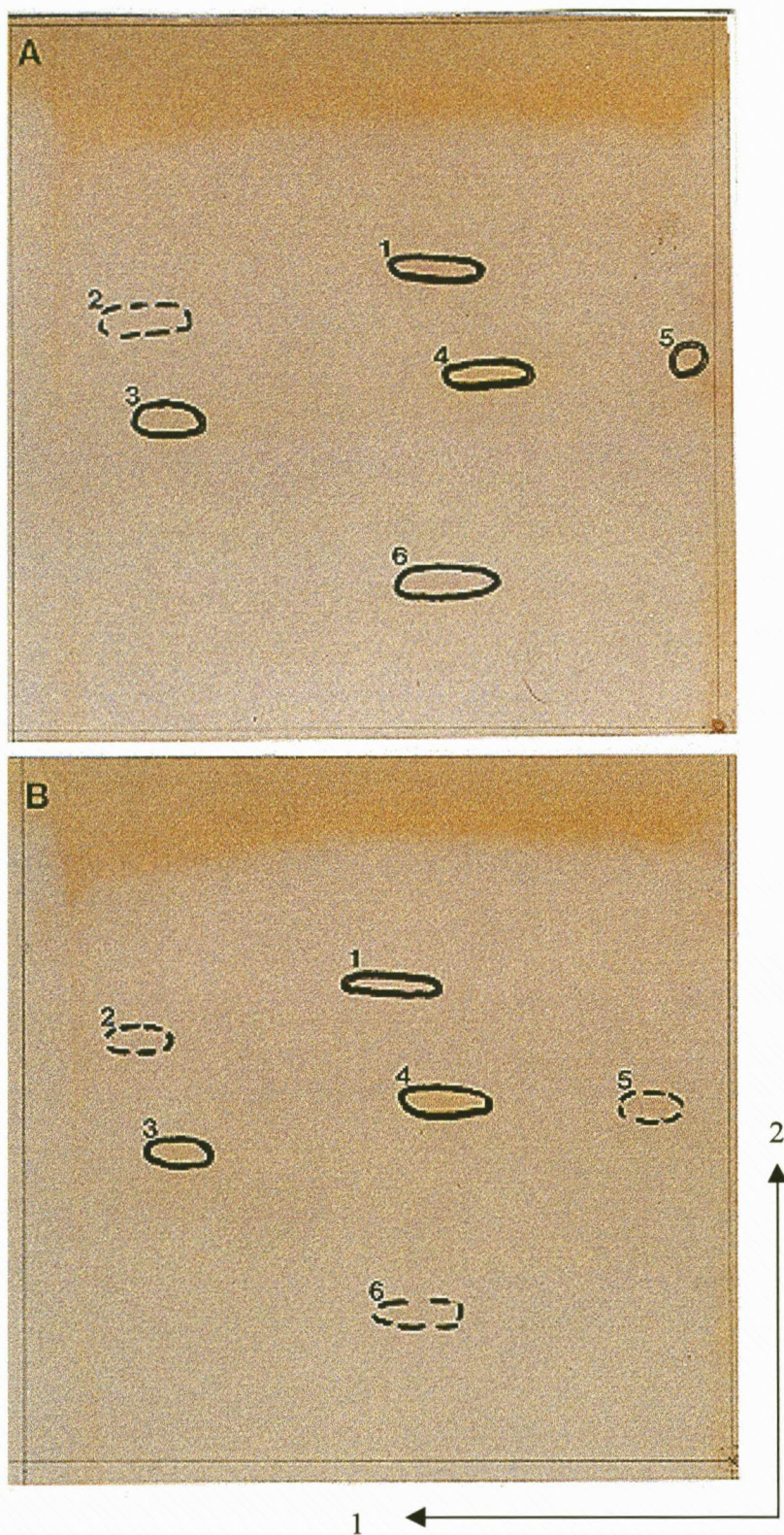


Fig. 4.31. A: TLC plate with separated phenolic acids of a 88% rooting extract.
 B: TLC plate with separated phenolic acids of a 24% rooting extract.
 1. Benzene: acetic: water (6:7:3 v/v/v).
 2. Sodium formate: formic acid: water (10:1:200 m/v/v).

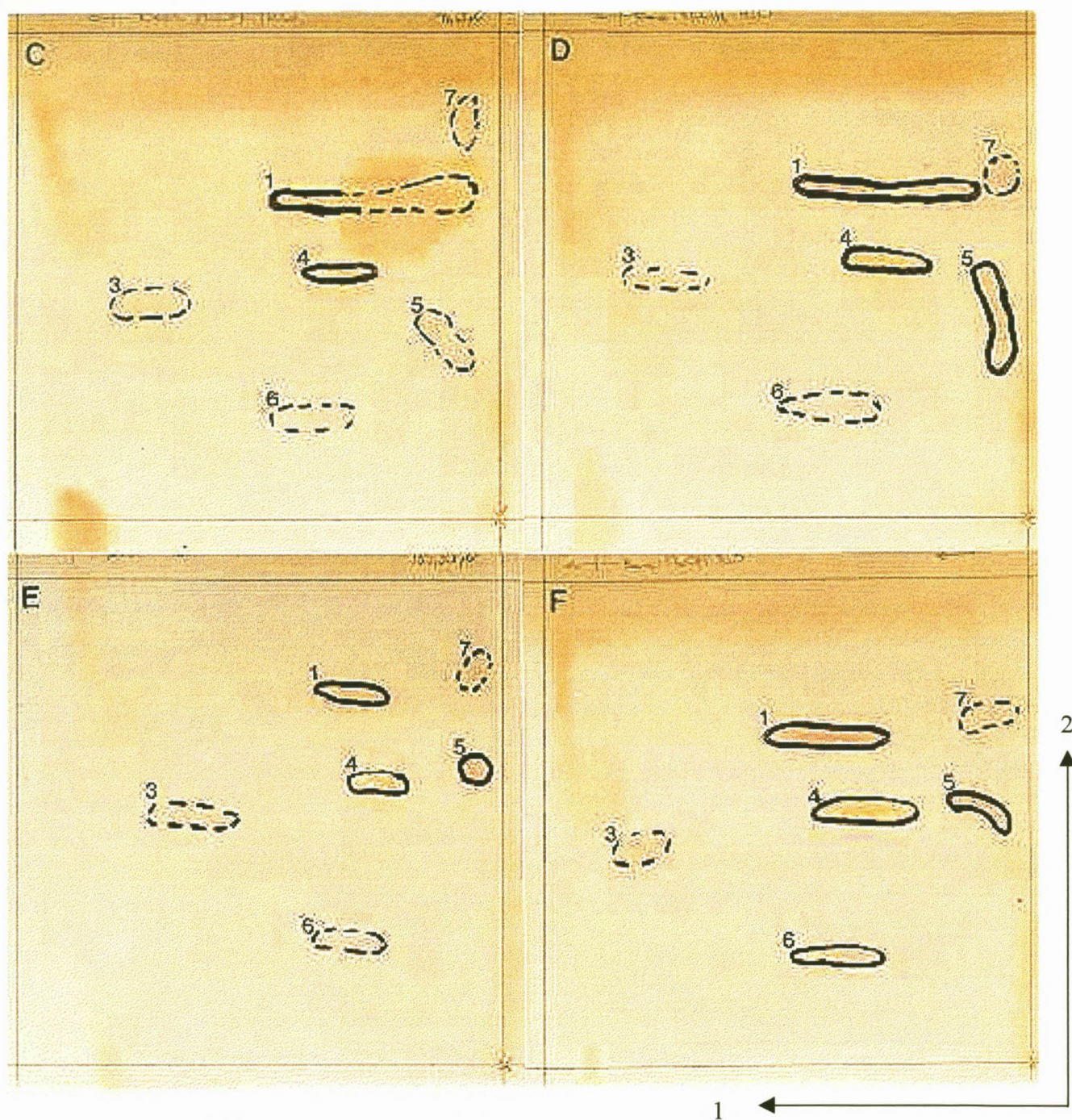


Fig. 4.31. C: TLC plate with separated phenolic acids of a 67% rooting extract.

D: TLC plate with separated phenolic acids of a 57% rooting extract.

E: TLC plate with separated phenolic acids of a 45% rooting extract.

F: TLC plate with separated phenolic acids of a 33% rooting extract.

1. Benzene: acetic: water (6:7:3 v/v/v).

2. Sodium formate: formic acid: water (10:1:200 m/v/v).

4.4.3. Auxin and cytokinin concentrations of *Pinus* cuttings.

Immunoassays for the quantitative measurements of trans-zeatin riboside and IAA were performed on *P. elliotii* hybrid cuttings as described in 3.2.3.3.1. The % binding versus the concentration (pmol 0.1ml⁻¹ methyl indole-3-acetate or trans-zeatin riboside) is calculated as described in 3.2.3.3.2 and represented in the standard curves [Fig. 4.32 ($r^2 = 0.99$) and Fig. 4.33 ($r^2 = 0.98$)] used to calculate the concentrations.

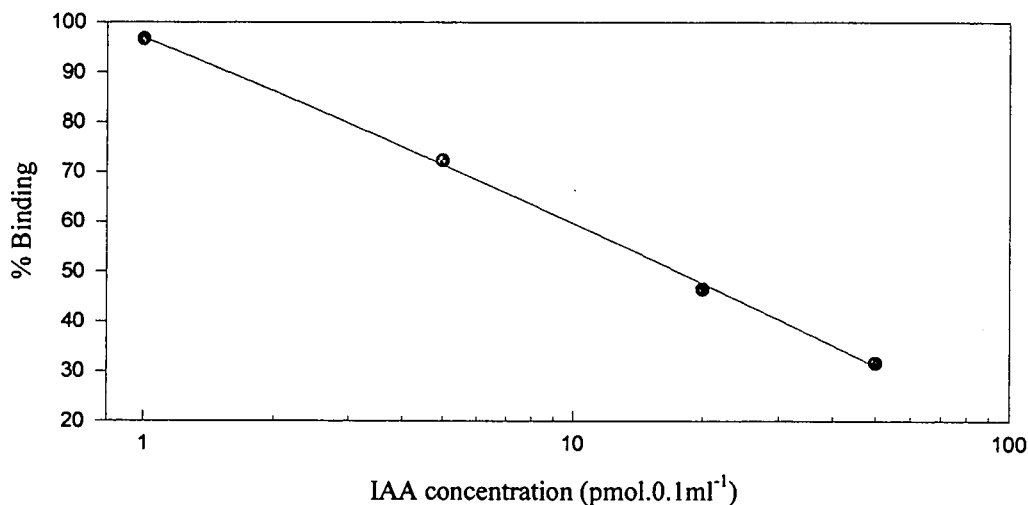


Fig. 4.32. Relation between the % binding of the hormone to the antibody and the IAA concentration. ($r^2 = 0.99$)

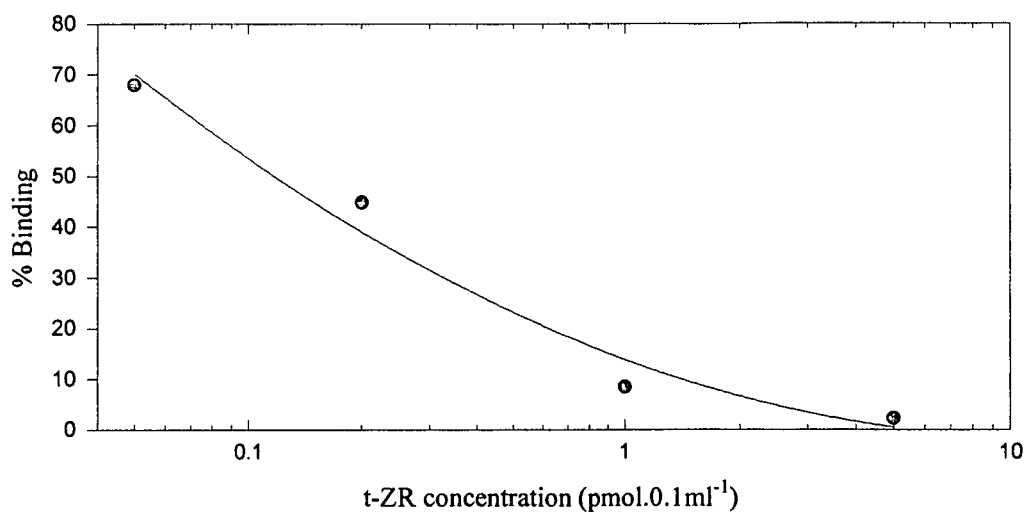


Fig. 4.33. Relation between the % binding of the hormone to the antibody and the t-ZR concentration ($r^2 = 0.98$)

The different hormone concentrations were determined by interpolation of the sample's percent binding from the different standard curves. The rooting percentage correlated positively with the auxin concentration (Fig. 4.34). High auxin concentrations were indicative of high rooting percentages in the cuttings tested.

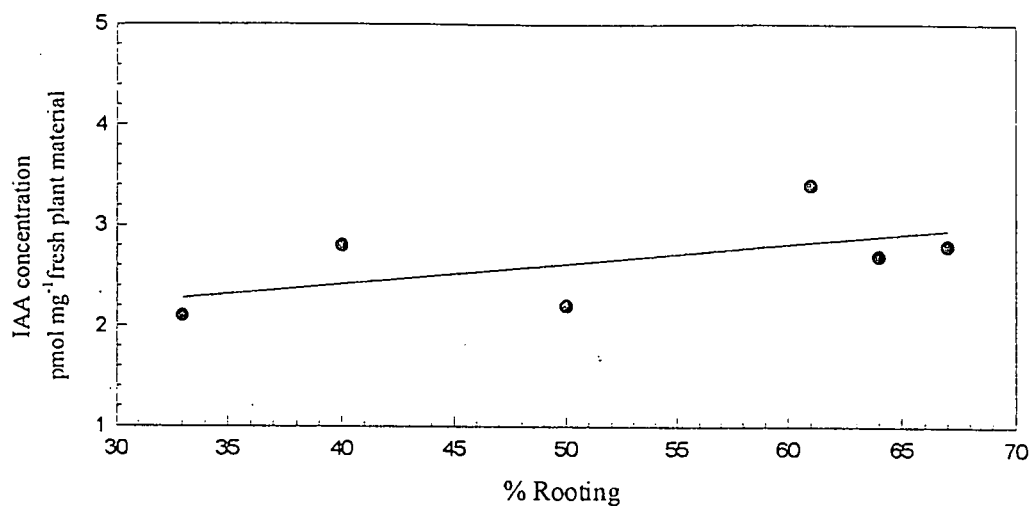


Fig. 4.34. Relation between the IAA concentration and the rooting percentage ($r^2=0.7$)

The rooting percentage correlated negatively with the cytokinin concentration (Fig.4.35)

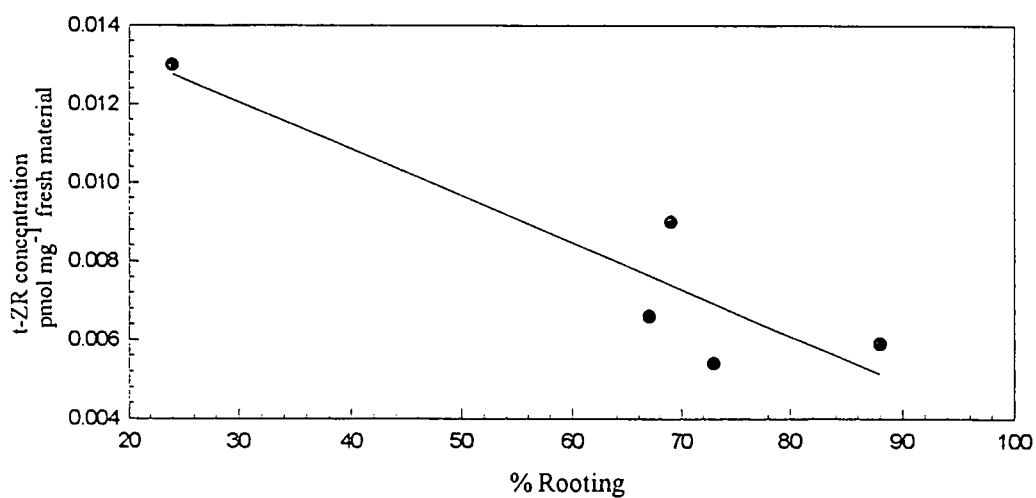


Fig. 4. 35. Relation between the t-ZR concentration and the rooting percentage ($r^2=0.8$).

Increasing auxin/cytokinin ratios correlated with increasing rooting percentage. (Fig. 4.36).

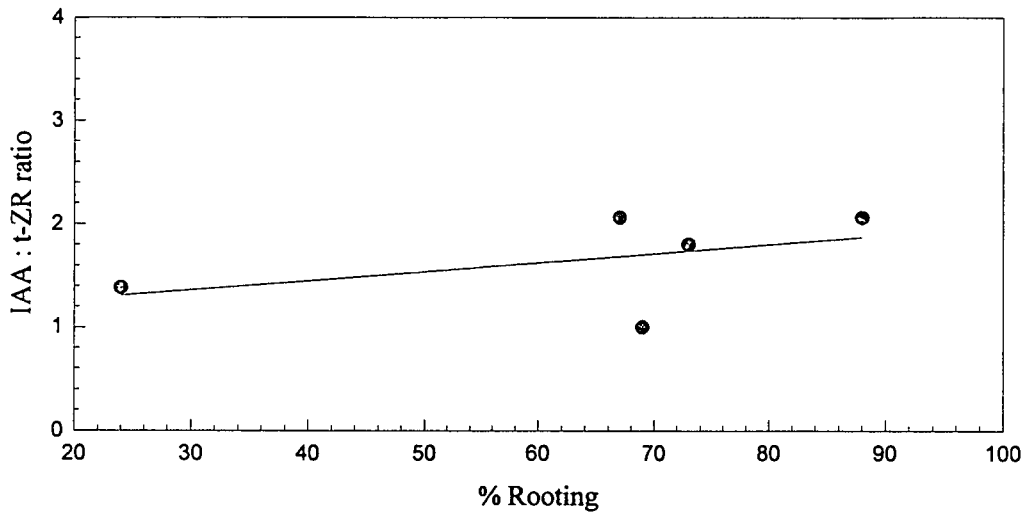


Fig. 4. 36. Relation between the auxin/cytokinin ratios and the rooting percentages ($r^2=0.7$).

The auxin/phenolic ratio increases with increased rooting percentages as seen in Fig. 4.37.

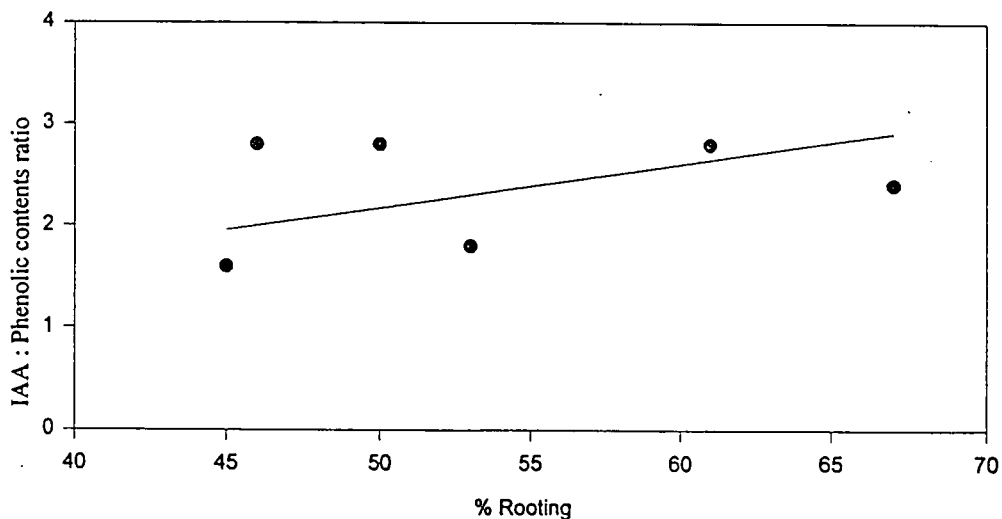


Fig. 4. 37. Relation between the auxin/phenolic ratios and the rooting percentages ($r^2=0.7$).

Chapter 5

Discussion

5.1. Somatic embryogenesis.

In this study embryonic cultures were initiated from immature female gametophytes containing zygotic embryos, on solidified medium. Different variations of the DCR medium with altered concentrations of the key components were used. The SH and MSG media were also tested as described in Table 3.1 (Appendix A).

There were differences in the initiation percentages for both species among female gametophytes from the same trees (Table 4.1 & 4.2) on the different media and among female gametophytes from different trees on the same medium. The differences may be due to genetic variation in the embryonic potential of the trees tested, or it may simply reflect variation in the medium requirements among the explants of the different trees (Becwar *et al.* 1990).

The performance of the different trees, regarding ESM formation also varied according to sample dates, probably due to the developmental stage of the explant. The stage of development of the explants was thus found to be critical for the establishment of ESM. This is in agreement to findings of Lu and Thorpe (1987), for *Picea*, Vasil (1987) for cereal and grass crops and Debergh and Read (1991) for conifers. According to Tautorius *et al.* (1991) proper explant selection is critical in order to achieve successful induction of somatic embryogenesis in conifers. The optimum stage of development for establishment of pine embryonic cultures is approximately three to five weeks post-fertilisation (Becwar *et al.* 1991).

Embryo development is not always found to be synchronous in a sample of cones collected at a particular time and the size of the explant is not a good indication of the morphological stage of development. It is clear that at the harvest date important variables exist, which influence optimal somatic embryo formation. The harvesting of the cones must fall into the so called "window period" before somatic embryogenesis will occur (Becwar *et al.* 1988; Nagmani *et al.* 1995).

The "window period" in this study differed for the two species. In the first season a longer development period was experienced for *P. patula*, ranging from 16.11.95 to 18.01.96. For *P. radiata* ESM formation only occurred on explants collected for the period 28.11.95 to 27.12.95. During the second season the collecting dates were between 24.10.96 and 30.03.97. The explants most responsive to ESM formation were collected from the 20.12.96 to the 06.02.97 for *P. patula* and 27.12.96 to 06.02.97 for *P. radiata*.

Explant identification using the time of collection is useful, but it is important to keep in mind that the developmental stage of the explant varies, depending on the climate and location of the donor tree. This was experienced with the two collection periods of plant material used in this study. During the second season a wet climate was experienced which might also been the reason for the increased contamination rate of the mother material. Moisture stress may also affect seed development and therefore indirectly influence the embryonic induction potential of the explants (Jones & Van Staden, 1995). Our results thus indicate that a combination of factors play an important role in the initiation of the embryonic tissue.

In all conifer species reported to date, the whitish, mucilaginous ESM is very characteristic and can easily be distinguished from the non-embryogenic tissue (Tautorus *et al.* 1991). The ESM were sustained on different media to identify

the best medium for sustainable growth, development and hardening off. The embryonic tissue (Fig. 4.1) was selectively cultured further while the non-embryonic calli (Fig. 4.2) were discarded.

Different processes have been suggested for the possible origin of somatic embryogenesis as described in 2.1.1.1 to 2.1.1.3. One such proposal is that the somatic embryos could arise by a mechanism similar to cleavage polyembryogenesis. This process is known to occur naturally in *Pinus* zygotic embryogeny (Owens & Blake, 1985). In this study cleavage polyembryogenesis occurred for the most responsive explants. This coincided specifically with the initiation of ESM.

Initiation percentages were based on data collected after 3 to 4 weeks for cultures which proliferated rapidly. It was found that not all embryonic cultures initiated were maintained in long term culture. This was also true for work done on *Picea* (Webb *et al.* 1989) and Von Arnold and Woodward (1988) reported a 60% loss for cultures of *P.stichensis*.

Although Gupta and Grob (1995) suggested that several basal media could be used for the initiation of embryonic cultures, Von Arnold (1987) is of the opinion that the composition of the culture medium is one of the most important factors determining the effective and efficient establishment of cultures.

For this reason, various variations of the basal media were evaluated in this study. For *P.radiata*, four variations of the DCR media were tested, and compared with SH medium with regard to the initiation of ESM. DCR media 1 to 4 all contained 3 mg l⁻¹ 2,4-D; compared to 0.1 mg l⁻¹ 2,4-D in the SH medium.

DCR media 1 to 3 contained 0.5 mg l⁻¹ BAP, and the DCR4 medium contained 1 mg l⁻¹ BAP. The SH medium, in contrast had a concentration of 2 mg l⁻¹ BAP (Table 3.1, Appendix A).

Gupta & Durzan (1986a) found that low auxin concentrations and darkness were essential for the initiation of somatic embryos. In this study, results confirmed that the low auxin concentration of the SH medium offered the best initiation of ESM (82%), but no further embryo development occurred on this medium. The DCR2 medium yielded ESM initiation in 77% of explants and subsequently lead to further development of embryos in 6.7% of these explants. For this reason DCR2 was selected as the preferred medium for somatic embryonic initiation.

The initiation frequency on DCR medium, also confirms results obtained by other workers with loblolly pine (Becwar *et al.* 1988; Becwar *et al.* 1990) and sugar pine (Gupta & Durzan, 1986b). Jones *et al.* (1993) reported on the somatic embryogenesis in *P. patula*, where results indicated that the interaction between BA and 2,4-D played a stimulating role in the induction of the embryonic responses.

Studies with many plant species, have indicated that nitrogen also plays a critical role in cell growth (Street, 1966; Kirby, 1982). Dunstan and Thorpe (1986) also stated that auxin and nitrogen are important to somatic embryogenesis. Kirby *et al.* (1987) stated that nitrogen is of singular importance for the growth and development of plant cells in culture.

Glutamine is often the choice of organic nitrogen source in the culture medium (Feirer, 1995). Laine and David (1990) found in their studies on *P. caribaea* that an organic nitrogen source (glutamine with or without casein hydrolysate) and 2,4-D in the initiation media produced beneficial results. Casein hydrolysate is seen to provide growth benefit when the inorganic nitrogen concentration is low.

The higher levels of casein hydrolysate are seen to be slightly inhibitory when not overshadowed by nitrogen limitations (Teasdale, 1986).

The different DCR media tested in this study on *P. radiata* had the same glutamine concentration. The SH medium contained no glutamine and the MSG medium contained double the concentration of glutamine of the DCR media. The DCR media differed with respect to the other nitrogen components in the macro elements of the basal medium. The DCR2 medium used for the initiation of *P. radiata* contained a higher concentration inorganic nitrogen than the other media used (NH_4NO_3 500 mg l^{-1} and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 600 mg l^{-1} Table 3.1, Appendix A). These higher concentrations of nitrogen seem to have had a positive effect on the initiation of the ESM, when compared to the other media tested, which have lower nitrogen concentrations.

The use of ABA for maturation of gymnosperm embryos is well-known (Gupta & Durzan 1986a; Hakman & Fowke, 1987a). Von Arnold and Hakman (1988) and Boulay *et al.* (1988) also reported that full concentration macro-nutrients yielded better results than diluted mineral media for embryo maturation and that ABA addition is beneficial. They found that by transferring somatic embryos onto media containing ABA, followed by further development on medium with reduced levels or no PGR, resulted in embryo maturation and plantlet development. Cleavage polyembryony is inhibited by ABA, allowing singulation and continued growth of individual embryos. ABA has also been found to stimulate the accumulation of storage proteins in somatic embryos of interior spruce (Roberts *et al.* 1990) and to enhance accumulation of storage lipid in somatic embryos of Norway spruce (Feirer *et al.* 1989). Kriebel and Finer (1990) found that 10 mg l^{-1} ABA promoted the development of stage 2 somatic embryos of white pines. Complex carbohydrates have been combined with ABA for improved embryo development and maturation. Finer *et al.* (1989) and Becwar and Feirer (1989) showed that 6% sucrose and ABA were useful for loblolly and white pines.

Maltose, lactose and starch were useful for white fir embryo development (Schuller *et al.* 1989).

Maturation of the somatic embryos in this study proved to be problematic. Only 6.7% of the explants formed matured embryos (4.1.3). This development occurred only on DCR2 medium (Table 3.1, Appendix A) containing 30 g l^{-1} glucose, 1.3 mg l^{-1} ABA and 1%(w/v) activated charcoal. The presence of the ABA in the medium was beneficial in furthering the development of the somatic embryos, possibly as a result of the synthesis and mobilisation of the storage reserves. The slight variation in macro elements as well as the different carbon source (glucose) of the DCR2 medium, possibly played a role in this regard. This was also proposed by Jones and Van Staden (1995). The other media tested resulted in browning and dying-off of the somatic embryos. The most advanced stage of development observed can be seen in Fig. 4.9 where the root started to form.

According to Smith *et al.* (1994) roots appeared 10 days after transfer to an embryo germination medium. As the cells age, roots took longer to emerge but usually appeared within 12 weeks. Chandler and Young (1995) achieved limited germination in the absence of growth regulators on a modified SH medium. The use of a high concentration sucrose also enhanced the results for *P. radiata*. Menzies (1992) stated that the "New Zealand group" obtained full maturation of somatic embryos which germinated normally, leading to establishment of fast growing plants in the field in 1991-92.

There is little information concerning soil establishment of conifer plants derived from somatic embryos (Tautorius *et al.* 1991). Von Arnold *et al.* (1995) observed strikingly uniform growth for Norway spruce within a clone as compared to less uniform growth from conventional seedlings.

Clonal propagation via somatic embryogenesis offers a number of previously unavailable benefits. This includes production of larger numbers of propagules, potentially greater genetic gains and capture of genetic gain much sooner than is possible from traditional programmes (Tautorus *et al.* 1991). Considerable advances have been made in conifers since 1985 when the first somatic embryogenesis was achieved (Hakman & Von Arnold, 1985). Despite all the progress, certain limitations still exist. A major limitation is the low numbers of field plantable clonal plantlets and the inability to initiate embryonic cultures from mature trees. The zygotic embryo used as explant arises from the sexual fusion of gametes and its clonal derivatives are genetically dissimilar from the parental tree. Therefore, it is not the preferred explant for true-to-type cloning of phenotypically superior trees (Attree *et al.* 1991).

Our results indicate that initiation of somatic polyembryogenesis of intact gametophytes with immature embryos is possible for both *Pinus* sp. at a low yield. The solidified media finally selected for initiation of the ESM were DCR2 (Table 3.1, Appendix A) for *P. radiata* and DCR1 and DCR5 (Table 3.1 Appendix A) for *P. patula*. Maintenance of the ESM was done on a ½ LP medium (Table 3.1, Appendix A) containing no growth regulators.

5.2. Cell suspension cultures.

Suspension cultures present several advantages over other micropropagation techniques. This method of culture offers good gaseous exchange. The maintenance of embryonic tissue via suspension cultures is less time consuming and could be a useful tool for the bulking up of the tissue. According to Ammirato (1986) 60 000 embryos per liter of medium is achievable. This method can be applied in order to increase the production of somatic embryos. It also has the potential for higher growth rates and reduced cell doubling times (Becwar *et al.* 1988). An interesting feature is that it contains numerous somatic

embryos of different developmental stages at any given time (Hakman & Fowke, 1987b). Another advantage of using cell cultures as a tool in plant breeding programmes and mass clonal production is its potential to deliver practically unlimited multiplication rates.

The suspension culture cells form terminal embryonic regions, comprising meristematic cells, and a suspensor region (Fig. 4.11). The suspensor region consists of large elongated highly vacuolated cells (Hakman & Fowke, 1987b).

One of the major benefits of this part of the study was the ease with which the cultures were initiated and the favourable growth response observed. A typical sigmoid growth curve is exhibited in the cell suspension culture. These curves are characterised by having a lag phase followed by exponential and linear phases, a period of progressive deceleration, a stationary growth phase and a subsequent culture decline (Street, 1977; Dodds & Roberts, 1985). The growth curves in the study (Fig. 4.13; 4.14; 4.15; 4.16) showed a similar pattern. Similar growth patterns have been reported in embryogenic suspension cultures of *Picea sitchensis* (Krogstrup, 1990) and *Picea glauca* (Dong & Dunstan, 1994).

The growth curves indicate the relatively rapid rate at which cultures can multiply, far exceeding that of cultures incubated on solid media. The curves also indicate when subculturing should occur, in order to maximise growth and use of the medium.

A reduced concentration of nutrient elements ($\frac{1}{2}$ LP medium) was tested and resulted in the best growth rate. At first 3% (m/v) sucrose was used as carbon source, which led to the development of B-type embryos. This type of embryo does not mature and will not develop past the bullet stage. Maltose as carbon source was responsible for transforming advanced somatic embryos of Douglas-Fir to the mature cotyledonary stage embryos (Nagmani & Dinus, 1991).

Maltose also proved in this study to be a good stimulator for growth (Fig. 4.14 and Table 4.4).

The effect of the auxin (2,4-D) was also investigated (Fig. 4.14, Table 4.4 and Fig. 4.15, Table 4.5). Results indicate that ½ LP liquid medium supplemented with 3% maltose and a 2,4-D concentration of 0.5 mg l⁻¹ gave the optimal growth for *P. radiata* embryonic cells. Gupta and Durzan (1986a) stated that a sequential reduction of 2,4-D levels improves the growth, morphogenesis and quality of the somatic embryos.

Some researchers have reported that the inclusion of BA and kinetin in the 2,4-D containing medium resulted in a stimulation of somatic embryogenesis in conifers. This was however, not observed for *P. patula* (Mc Keller *et al.* 1994). The effect of the cytokinin tested in this study (Fig. 4.16 and Table 4.6) also indicated no significant effect on the growth.

To mature the somatic embryos, the cells in suspension were re-established onto solidified medium with great ease. In our study stage 1 and 2 embryos were formed at a low percentage yield (4.5%). Boulay *et al.* (1988) found that by transferring the liquid media to solid supports was essential for maturation to occur. Gupta *et al.* (1991) stated that usually the pro-embryos require plating onto a semisolid medium or artificial support system after development in liquid medium. Twenty to thirty cotyledonary embryos of norway spruce have been reported to form from 1ml of suspension culture when placed on cheesecloth or filter paper supports wetted with liquid medium (Boulay *et al.* 1988). For Douglas-fir or norway spruce 50-100 cotyledonary embryos developed from 1ml of suspension culture plated onto semisolid medium (Gupta *et al.* 1991).

Cryopreservation is very important for the large-scale commercial use of somatic embryogenesis. Clonal field testing in forestry often takes 5-10 years before

superior genotypes are identified. ESM and liquid cultures provide an excellent cell type for storage of juvenile tissue during this field testing period. Cryopreservation also provides an opportunity for creation of a bank of superior genotypes which can be retrieved when needed (Gupta *et al.* 1991).

Successful storage and plant regeneration have been demonstrated for Norway spruce, white spruce and loblolly pine (Gupta *et al.* 1987; Kartha *et al.* 1988). *Picea abies* (Bercetche *et al.* 1990), Caribbean pine (*Pinus caribaea*) (Laine *et al.* 1992), larch and black spruce (*Picea mariana*) (Klimaszewaska *et al.* 1992) were also recovered from cryopreserved conditions. Hargreaves and Smith (1993) described a complete protocol for the cryopreservation of *Pinus radiata* embryonic cells. After 6 months 50% of the embryonic cultures in their study were recovered. Cryopreservation can become an extremely valuable storage system during tree improvement and while the clones are tested in field trials.

Despite the obvious advantage of using liquid cultures, very little information exists on suspension growth characteristics and somatic embryo yield.

5.3. Organogenesis.

Seeds are inevitably contaminated with a wide range of micro-organisms. Sterilisation of the seeds is a vital pre-requisite to the success of micropropagation. There is a large variety of chemical agents in use for the surface sterilisation of the plant material. The choice of the agent and the time of treatment depends on the sensitivity of the material. Hutzell and Durzan (1993) reported that the transplanting of surface sterilised seeds onto sterile filter paper in petri dishes did not always render optimal conditions as expected. Sterile seeds germinated and grown on solidified medium have the advantage that contaminating organisms which were not killed during sterilisation of the seeds are easily detected during germination.

In the literature various methods are used to surface sterilise seeds. According to Narayanaswamy (1977) a 10% (m/v) filtered solution of calcium hypochlorite can be used for a period ranging from 20 min. to several hours, depending on the hardness of the seedcoat. A solution of HgCl_2 (0.1%, m/v) or bromine water (1-2%, v/v) is also used in difficult cases, but needs thorough washing in sterile water. H_2O_2 (10-12%, v/v) is another widely used sterilising agent. Mc Keller *et al.* (1994) treated germinated seeds with 10% H_2O_2 for 30 min., rinsed them with sterile water and then submerged them in 1,3% (v/v) NaOCl during the excision of the seedlings.

The sterilisation trials in this study resulted in the selection of a different treatment for each species. For *P. patula* the best sterilisation, with the highest germination rate and lowest contamination percentages, was obtained by sterilising for 10 min. with 30% H_2O_2 (Treatment No.9, Table 4.7). For *P. radiata* 5 min. with a 10% H_2O_2 solution resulted in the best germination and lowest contamination rate (Treatment No.6, Table 4.7).

Mc Keller *et al.* (1994) stated that the most promising protocol for the micropropagation of *P. patula* appeared to be that which involves the route of axillary bud proliferation. Axillary buds in this study were induced by placing the distal portions of the hypocotyls (4.3.2) of both species out on a nutrient medium containing cytokinins (Table 3.3, Appendix A).

Cytokinins are essential for bud development as stated by various authors (Bronson & Dixon, 1991). The cytokinin BA or BAP used at various concentrations, is usually the selected growth regulator for shoot induction (Rumary & Thorpe, 1984). Amerson *et al.* (1985); Ellis and Bilderback (1989) and Martinez-Pulido *et al.* (1990) reported that shoot initiation requires exposure to cytokinin.

Nairn (1993) found that the LP medium forms the base of the growth media in his studies on *P. radiata*. Though he also found that satisfactory growth was maintained on GD medium, the MS medium gave undesirable results. The MS medium yielded shorter yellow shoots in our study which correlates with the results obtained by Nairn (1993).

Although this is described as conservative and of relatively low multiplication compared to the adventitious bud formation route, our results offered the highest percentage yield buds for all the routes tested. Results showed that the DCR medium (Table 3.3, Appendix A) offered the highest percentage yield. For *P. patula* 64% explants developed buds and for *P. radiata* 52% explant development occurred on the DCR medium (Table 4.8). Consecutive explanting of newly formed buds can potentially produce large numbers of progeny with similar genetic make-up.

A persistent problem in culturing the pine buds was the sudden appearance of a white bacterial infection around the base of the explant. This resulted in the loss of a significant number of buds. James and Thurbon (1979) reported that 25% of tissue sections from *in vitro* cultured apples, were screened monthly with negative results for five consecutive screenings, but contained bacterial contaminants when screened a sixth time. This bacterial infection is also mentioned by Horgan (1987) for *P. radiata*. The sudden appearance of bacteria were experienced in our studies.

Results showed that adventitious buds can be induced by placing young cotyledons of both pine species on nutrient medium (4.3.4). For the older cotyledons only yellow meristematic callus formation occurred (Table 4.9 and 4.10). The medium tested contained cytokinins (Table 3.3 Appendix A). BA was the cytokinin used. According to Biondi and Thorpe (1982) auxin and other growth regulators tend to enhance callus formation. Biondi and Thorpe (1982)

and Yeung *et al.* (1981) showed that the presence or absence of BA in the culture medium determines the subsequent development of radiata pine cotyledons. In the presence of cytokinin, the cotyledons elongated only slightly and became swollen as a result of cell divisions which ultimately led to the formation of *de novo* bud formation. In the absence of cytokinin the cotyledons elongated significantly without producing bud primordia. The younger the tissue the better it responded (Thorpe *et al.* 1991). Yeung *et al.* (1981) reported that of key importance is the time at which selection of cotyledons took place, i.e. when the cotyledon explants are still in the meristematic state of growth. Aitken *et al.* (1981) also found that cotyledons selected from older germinated seeds produced shoots only on the tip of the cotyledons, if at all, and often formed callus. Chandler and Young (1995) showed that 10-12 day post germination cotyledons are more capable of adventitious bud formation. Elongation and proliferation of the adventitious buds in this study are still problematic.

The excision of embryos from the seeds is a delicate and time consuming process and therefore impractical in a commercial micropropagation endeavour (Mc Keller *et al.* 1994). Reilly and Washer (1977) observed 1-200 buds per embryo with embryo cultures of *P. radiata*. To date it has not been possible to increase the mean number of shoots per shoot-forming embryo above 15 (Aitken *et al.* 1981). Cultured embryos of slash pine yield approximately 14 shoots (Perez-Bermudez & Sommer, 1987).

The embryos in this study which were transplanted onto different media (4.3.5) only developed in the dark. The best results were obtained on LP medium (Table 3.3, Appendix A). For *P. patula* 25% of the embryos showed development of morphogenetic structures and for *P. radiata* 33% of the embryos placed out developed morphogenetic structures.

The elongation of the axillary buds was very successful. Extension of the axillary buds was only achieved when the bud clusters were transferred to a growth regulator-free medium. Previous investigations demonstrated that transfer of buds to a growth regulator-free medium after an induction period, promoted shoot differentiation and growth in several conifers (Mehra-Palta *et al.* 1978; Mapes *et al.* 1981; Mott & Amerson, 1982; Patel *et al.* 1986; Kaul, 1987).

In this study 68% of the bud clusters cultivated on the DCR medium (Table 3.3, Appendix A) elongated successfully. Shoot tips were placed back into culture where shoot elongation was stimulated and the process was repeated. Aitken-Christie and Thorpe (1984) found that shoots of *P. radiata* elongated at different rates depending on the amount of shoots produced per clone. David (1982) found the addition of activated charcoal to the growth medium had a stimulatory effect on elongation. DCR proved to be the medium of choice (Table 4.11) for elongation for both species in this study.

Root formation *in vitro* is the first step towards plantlet establishment *ex vitro*. *In vitro* rooting of conifers remains a limiting factor in achieving whole plant regeneration (Bronson & Dixon, 1991 and Zel, 1993). This problem is aggravated by large clonal differences in rooting potential (Jang & Tainter, 1991). Each stage of the *in vitro* development may also influence the rooting performance. The state of growth of the stock plant is also an important factor in determining rooting capacity (Horgan & Aitken, 1981). Mohammed and Vidaver (1988) stated that the effects of the growth regulators used during the previous stages may influence rooting. Auxins have been used either alone or in combination to stimulate rooting. Combinations of NAA, IBA or IAA have been effective with *P. radiata* (Aitken-Christie & Thorpe, 1984), Norway spruce (Von Arnold, 1982), Douglas-fir (Boulay, 1979) and pitch pine (Patel *et al.* 1986).

Sommer and Caldas (1981) stated that often a combination of two auxins (IBA and NAA) improved *in vitro* rooting than one for conifer cultures, and a sucrose concentration of 1% (m/v) is generally better than 2 or 3 % (m/v). It is also important to reduce the callusing at the base of the shoots, to prevent callus growing faster than the root primordia and suppressing the outgrowth of the roots. Bronson and Dixon (1991) reported root induction of slash pine after 14 days in ½ GD medium containing NAA. Root formation and elongation followed after a further 14-21 days on growth regulator-free ½ GD medium. Horgan and Aitken (1981) reported that the rooting percentage of *Pinus radiata* shoots is generally below 50%.

Roots formed in this study on a GD medium (Table 3.3, Appendix A) containing 0.5 mg l⁻¹ NAA, 2 mg l⁻¹ IBA and Gelrite (0.35%, m/v) (4.3.7). The concentration of GD nutrients may possibly allow the shoots to shift their carbohydrate/nitrogen balance in favour of nitrogen, which has been shown to favour rooting (Horgan & Aitken, 1981).

Major influences on root production include shoot quality, donor age, temperature and substrate. One of the most important factors is the type and concentration of auxin (Mohammed & Vidaver, 1988). It appears that a major drawback with organogenesis is the long period of time required for completion of the entire process from germination to root generation.

5.4. Chemical markers for the rooting ability of the Pinus cuttings.

Smith and Thorpe (1977) in contrast, showed that exogenously applied simple phenolic compounds can enhance root primordium formation in *P. radiata*. Gaspar and Coumans (1987) stated that there is substantial evidence that applied phenolics may influence rooting. Rajasekar and Sharma (1989) in

contrast thereto, showed that the addition of phenolic compounds had no favourable affect on rooting. They proposed that phenolics affect auxin metabolism, and thereby indirectly influence the rooting response of cuttings. According to Mohammed and Vidaver (1988) the effect of auxin is sometimes enhanced by the presence of rooting cofactors or auxin synergists, some of which probably are phenolics. Moncousin (1991) stated that an intervention of phenolic compounds in the induction process of adventitious roots occurred. Bora *et al.* (1991) found a decrease of phenolic compounds in mung bean cuttings that form an increased number of adventitious roots. Our results corresponded (Fig. 4.30) with this finding. The phenolic acid composition of cuttings most probably plays no role in the rooting potential of cuttings, since no differences could be found between phenolic acid compositions of poor and good rooting cuttings (Fig. 4.31 A-F).

The subtle interactions among the different groups of plant hormones and specifically the auxin/cytokinin ratio play an important role in plant development as a whole (Salisbury & Ross, 1992). Since the hormone balance is so important, the concentration of auxin and cytokinin were determined by immunoassay.

The technique of plant hormone immunoassay is a relatively recent development. Immunoassays, recently introduced into the analysis of a variety of plant hormones, allow a rapid and reliable quantitative determination of several important hormones simultaneously (Weiler *et al.* 1981). Due to their high selectivity, minimal sample purification is required. The assays can thus be applied to the analysis of crude extracts, no purification losses occurs and recoveries of hormones is relatively high (Weiler, 1980,1982). When properly executed, immunological assays offer a rapid, sensitive method for the analysis of hormones (Brenner, 1981).

In plants relatively high concentrations of auxin induce the regeneration of roots (Minocha, 1987). The rooting response in tissue culture varied according to the type and concentration of auxin applied (Abdullah *et al.* 1989). It is well known that the interaction between auxin and cytokinin is a primary relationship in plant propagation. It is also generally accepted that auxins play a central role in the initiation of adventitious roots (Gaspar & Coumans, 1987; Abdullah *et al.* 1989). It is well known that a high auxin/cytokinin ratio favours rooting (Salisbury & Ross, 1992). In our study we found a clear positive correlation between rooting percentage and the internal auxin concentrations of cuttings (Fig. 4.34).

Adventitious root formation is generally inhibited by apical or basal application of cytokinin to cuttings. Some cytokinins however, may be necessary for root formation since addition of small amounts of cytokinin is necessary in tissue culture and since root initiation in cuttings is inhibited by a cytokinin antagonist (Skoog *et al.* 1973). Root initiation is inhibited by increased endogenous cytokinin levels (Fig. 4.35) and promoted by a favourable auxin/cytokinin ratio (Fig. 4.36). A favourable auxin/phenolic ratio was also required for root formation (Fig. 4.37).

5.5. Concluding remarks.

To meet the growing demand of the forestry industry for mass production of plantlets bearing the effects of genetic improvement, an alternative to traditional generative production of seedlings and the rooting of cuttings from hedges is sought. The ideal would be the cloning of genetically proven material. The modern approach to mass production is micropropagation by cell and tissue culture. The main advantage of these techniques is the potential for virtually unlimited multiplication of a given material (Thorpe & Biondi, 1984), resulting in the reproduction of large quantities of uniform plants bearing selected qualities.

A disadvantage however is that it is usually the new generation and not the mother tree that is propagated this way, because explant material which will allow real cloning is normally recalcitrant to development in tissue culture. Unfortunately, spontaneous mutations may occur occasionally in tissue cultures, which may result in genetic variation (Libby & Ahuja, 1993). According to Leach (1979) plantlets generated by somatic cell cultures *in vitro* showed a higher mortality, less shoot growth and more curved stems than seedlings. Adequate quality checks and field testing are therefore necessary (Dunstan & Thorpe, 1986).

The research conducted in this study resulted in the successful induction and maintenance of ESM, as well as the establishment of cell suspension cultures. This was however achieved only for some of the tree lines tested due to genetic variation between the different trees. The induction, maintenance and rooting of multiple buds were also achieved. The success of these methods illustrates the viability of using a biotechnical approach to tree breeding, and specifically identifies protocols which can be applied to species of economic importance to this country. We have succeeded in cloning genetically unproven zygotic material by means of embryogenesis and organogenesis. However, the embryos generated by our method can be cryopreserved, while samples of the plantlets generated undergo field trials. Should the genetic properties prove to be desirable, stocks of cryopreserved cultures can be matured, rooted and planted out to satisfy the demand.

Literature indicates a limitation, in that the number of field plantable clones produced per embryonic culture is less than optimal. It is therefore felt that further attention needs to be afforded to the hardening off of plantlets. In spite of this limitation, we believe that our findings could contribute to the successful establishment of micropropagation techniques to meet this country's need for mass propagation within the forestry industry.

Woody plant species have often been viewed as difficult subjects to work with, and until there is the input of research effort into these systems that philosophy may well remain.

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1986

Chapter 6

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Appendix A

Table 3.2. Different sterilizing treatments.

Treatment number	Treatment
1	Control
2	0.1% HgCl ₂ for 5 min.
3	0.1% HgCl ₂ for 10 min.
4	0.5% Panazide for 5 min.
5	0.5% Panazide for 10 min.
6	10% H ₂ O ₂ for 5 min.
7	10% H ₂ O ₂ for 10 min.
8	30% H ₂ O ₂ for 5 min.
9	30% H ₂ O ₂ for 10 min.
10	7% CaOCl ₂ + 0.1% Tween 20 for 15 min. 70% Ethanol for 2 min.
11	7% CaOCl ₂ + 0.1% Tween 20 for 25 min. 70% Ethanol for 2 min.
12	0.5% NaOCl for 10 min.
13	0.5% NaOCl for 20 min.
14	1.5% NaOCl for 5 min.
15	1.5% NaOCl for 10 min.
16	2% NaOCl for 5 min. + Tween 20
17	2% NaOCl for 20 min. + Tween 20
18	Soak in distilled water for 30 min. 30% H ₂ O ₂ for 10 min.
19	Soak overnight in distilled water. 2% NaOCl + 1% Tween 20 for 20 min.
20	Soak 24 h in distilled water. 70% ethanol for 10 sec. 30% H ₂ O ₂ for 10 min.
21	Soak in a 0.5% NaOCl for 30 min. 70% ethanol for 2 min.
22	Rinse in 70% ethanol 3.5% NaOCl for 10 min. Rinse in distilled water for 15 min.

Table 3.3. Formulation of the basal culture media used for organogenesis.

Compounds	DCR	GD	LP	MS #	SH
Macro elements	(mg l ⁻¹)	(mg l ⁻¹)	(mg l ⁻¹)	(mg l ⁻¹)	(mg l ⁻¹)
KNO ₃	340	1000	1800		2500
MgSO ₄ .7H ₂ O	370	250	360		400
CaCl ₂ .2 H ₂ O	64	150	-		200
NH ₄ H ₂ PO ₄	-	-	-		300
(NH ₄) ₂ SO ₄	-	200	-		-
KCl	-	300	-		-
NaH ₂ PO ₄ .2 H ₂ O	-	100	-		-
Na ₂ HPO ₄ .12 H ₂ O	-	75	-		-
NH ₄ NO ₃	400	-	400		-
KH ₂ PO ₄	170	-	270		-
Ca(NO ₃) ₂ .4 H ₂ O	556	-	1200		-
Micro elements					
FeSO ₄ .7H ₂ O	27.8	30	30		15
Na ₂ EDTA.2 H ₂ O	37.3	40	40		20
MnSO ₄ .4H ₂ O	22.3	20	20		20
H ₃ BO ₃	6.20	5	6.2		5
ZnSO ₄ .7H ₂ O	8.60	1	8.6		1
KI	0.83	1	0.08		1
CuSO ₄ .5H ₂ O	0.25	0.2	0.25		0.2
Na ₂ MoO ₄ .2H ₂ O	0.25	0.2	0.25		0.2
CoCl ₂ .6H ₂ O	0.025	0.2	0.025		0.2
NiCl ₂	0.025	-	-		-
Vitamins					
Thiamine HCl	1.0	5.0	0.4	0.1	5.0
Nicotinic acid	0.5	5.0	-	0.5	5.0
Pyridoxine HCl	0.5	0.5	-	0.1	0.5
Glycine	2	-	-	-	-
Inositol (myo)	100	1000	1000	100	100
Growth regulators					
BAP	2.0	5.0	5.0	5.0	5.0
Kin	0.5	-	-	-	-
NAA	-	*0.5	-	-	-
IBA	-	*2.0	-	-	-
Other					
Sucrose	2%	2%	3%	3%	3%
Gelrite	0.25%	0.25%	0.25%	0.25%	0.25%
pH	6.0	5.8	5.8	5.8	5.8

Macro and micro elements purchased pre-packed from Sigma-Aldrich.

* These growth regulators and 0.35% gelrite were used for rooting.

Summary

The aim of this study was to develop an effective protocol for the micropropagation of *Pinus patula* and *P. radiata*. Micropropagation procedures by means of somatic embryogenesis on solidified medium and in cell suspension cultures as well as organogenesis were investigated. In addition the possible relationship between phenolic, auxin and cytokinin content within cuttings and the tendency of these cuttings to root, were to be investigated.

The cones of *P. patula* and *P. radiata* were collected for the somatic embryogenesis study, on a two weekly basis during the summer months of 1995 to 1997. Somatic embryonic cultures were initiated from the immature female gametophytes containing zygotic embryos. The embryonal suspensor mass (ESM) formed, was used as starting material for cell suspension cultures.

Organogenesis included axillary and adventitious budding on hypocotyls and cotyledons respectively of young germlings deriving from seeds of open pollinated cones. Various techniques to sterilize the seeds were evaluated and it was found that 30% H₂O₂ (10 min.) proved most effective for *P. patula*, and 10% H₂O₂ (5 min.) was most effective for *P. radiata*.

The initiation of somatic embryonic cultures was attempted on solidified modified Murashige and Skoog medium (MSG), Schenk and Hildebrandt (SH), Gresshof and Doy (GD), Quoirin and Lepoivre (LP) and variations of the Douglas-fir Cotyledon Revised (DCR) media, each differing with regard to nitrogen sources and growth regulator composition. It was concluded that the most effective initiation media for *P. patula* were DCR1 and DCR5, and that DCR2 was most effective for *P. radiata*. Maintenance of the embryonic cultures was most successfully achieved for both species on ½ LP medium containing 3% maltose and no growth regulators.

Maturation of *P. radiata* somatic embryos was achieved on solidified DCR2 medium supplemented with 1.3 mg l^{-1} ABA, 30 g l^{-1} glucose and 1% (m/v) activated charcoal. Attempts to mature *P. patula* embryos were unsuccessful.

Embryonic cell suspension cultures were established in liquid GD, DCR, SH and $\frac{1}{2}$ LP media. The best culture growth was achieved on $\frac{1}{2}$ LP medium supplemented with 0.5 mg l^{-1} 2,4-D and maltose as carbon source. Re-establishment of these cultures onto solidified $\frac{1}{2}$ LP medium, supplemented with ABA, for further development and maturation was successful.

Adventitious buds were induced on young (14 day old) cotyledons on nutrient (DCR) medium containing cytokinins (2 mg l^{-1} BAP and 0.5 mg l^{-1} Kin). In addition axillary buds were initiated on hypocotyls. A better success rate was obtained by axillary budding on hypocotyls than adventitious budding on cotyledons. Best elongation of the axillary buds was recorded on DCR medium containing no growth regulators. Rooting of these elongated shoots was subsequently successfully conducted on a GD medium supplemented with 0.5 mg l^{-1} NAA and 2 mg l^{-1} IBA.

An investigation on possible chemical markers of the rooting potential of cuttings was conducted on softwood cuttings of *P. elliotii* hybrids. The rooting percentage correlated inversely with the total phenolic content of the cuttings. According to TLC chromatograms for the separation of phenolic acids no special phenolic acid could be related to high or low rooting potential. Immunoassays were used to determine the endogenous auxin and cytokinin levels of cuttings. The rooting percentage correlated positively with the auxin concentration and negatively with the cytokinin concentration as expected.

Results obtained in this study showed that the micropropagation of *P. patula* and *P. radiata* is feasible. These results contribute to a better understanding of micropropagation of *Pinus* species which has great potential for mass propagation demanded by forestry.

Opsomming

Die doel van hierdie studie was om 'n effektiewe protokol vir die mikropropagering van *Pinus patula* en *P. radiata* te ontwikkel. Mikropropagering by wyse van somatiese embriogenese op vaste medium en in selsuspensiekulture sowel as organogenese is ondersoek. Hierbenewens is die verband tussen fenol-, ouksien- en sitokiniënhoud van steggies en die bewortelingstendens van steggies ondersoek.

Die keëls van *P. patula* en *P. radiata* benodig vir die somatiese embriogenesestudie, is tweewekliks gedurende die somermaande van 1995 tot 1997 versamel. Somatiese embriokulture is vanaf onvolwasse vroulike gametofiete wat sigotiese embrios bevat, geïnisieer. Die embrionale suspensie massa (ESM) wat gevorm het, is daarna in die selsuspensiekulture as moedermateriaal gebruik.

Organogenese het oksel- en byknopontwikkeling op onderskeidelik hipokotiele en saadlobbe van jong kiemplantjies afkomstig van volwasse sade uit natuurlik bestuifde keëls behels. Verskeie tegnieke om saad te steriliseer, is beproef. Daar is bevind dat 30% H₂O₂ (10 min.) en 10% H₂O₂ (5 min.) die beste resultate vir *P. patula* en *P. radiata* onderskeidelik gelewer het.

Daar is gepoog om somatiese-embriokulture op verskeie vaste media soos 'n veranderde Murashige en Skoog medium (MSG), Schenk en Hildebrandt (SH), Gresshof en Doy (GD), Quoirin en Lepoivre (LP) en variasies van die Douglas-fir Cotyledon Revised (DCR) media te inisieer. Die media verskil van mekaar t.o.v. stikstofbronne en die groeireguleerders. DCR1 en DCR5 was die effektiëste inisiëringsmedia vir *P. patula* en DCR2 was die effektiëste vir *P. radiata*. Instandhouding van hierdie kulture is op ½ LP medium met 3% maltose en sonder enige groeireguleerders bereik.

Veroudering van *P. radiata* somatiese embryo's tot volwassenheid was suksesvol op 'n vaste DCR2 medium wat met 1.3 mg l^{-1} ABA, 30 g l^{-1} glukose en 1% (m/v) geaktiveerde koolstof verryk is. Pogings om *P. patula* embryos te verouder was onsuksesvol.

Embriogenetiese selsuspensiekulture is in vloeibare GD, DCR, SH en $\frac{1}{2}$ LP-media gevestig. Die beste kultuurgroei is op $\frac{1}{2}$ LP medium verryk met 0.5 mg l^{-1} 2,4-D en maltose as koolstofbron behaal. Hervestiging van hierdie kulture is suksesvol op vaste $\frac{1}{2}$ LP medium met ABA uitgevoer, vir verouderingsdoeleindes.

Byknoppe is op jong (14 dae oue) saadlobbe op 'n sitokinienbevattende (2 mg l^{-1} BAP and 0.5 mg l^{-1} Kin) DCR-medium geïnduseer. Hierbenewens is okselknoppe op hipokotiele geïnisieer. 'n Hoër suksestempo is met die ontwikkeling van okselknoppe op hipokotiele as met die ontwikkeling van byknoppe op saadlobbe verkry. Die beste verlenging van die okselknoppe is op 'n DCR-medium met geen groeireguleerder waargeneem. Beworteling van die verlengde lote is suksesvol op 'n GD medium met $0,5 \text{ mg l}^{-1}$ NAA en 2 mg l^{-1} IBA uitgevoer.

Ondersoeke om moontlike chemiese aanduidings van bewortelingspotensiaal van steggies te identifiseer is op sagtehoutsteggies van *P. elliottii* hibriede uitgevoer. Die totale fenoliese inhoud was omgekeerd eweredig aan die bewortelingspersentasie van die steggies. Volgens TLC-chromatogramme vir die skeiding van fenoliese sure is daar geen spesiale fenoliese sure wat met hoë of lae bewortelingspotensiaal geassosieer kan word nie. Die endogene ouksien- en sitokinienvlakke van die steggies is met behulp van immunoessaïeringsmetodes bepaal. Die bewortelingspersentasie het positief ooreengestem met die ouksienkonsentrasie en negatief met die sitokinienkonsentrasie gekorreleer.

Resultate van hierdie studie het bewys dat mikropropagering van *P. patula* en *P. radiata* moontlik is. Hierdie resultate het tot 'n beter begrip van die mikropropagering van *Pinus* spesies bygedra. Mikropropagering het groot potensiaal vir massapropageering wat in aanvraag by die bosboubedryf is.

Keywords

Pinus radiata

Pinus patula

Micropropagation

Somatic embryogenesis

Embryonic cell suspension cultures

Tissue culture - Organogenesis

Axillary & Adventitious budding

Phenolic

Immunoassays

Auxin & Cytokinin

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