SOMATIC EMBRYOGENESIS OF *PINUS PATULA* SCHEIDE ET DEPPE

BY

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Not here for centuries the winds shall sweep

Freely again, for here my tree shall rise

To print leaf-patterns on the empty skies

And fret the sunlight. Here where grasses creep

Great roots shall thrust and life run slow and deep:

Perhaps strange children, with my children's eyes

Shall love it, listening as the daylight dies

To hear its branches singing them to sleep.

MARGARET ANDERSON (1950)

PREFACE

The experimental work described in this thesis was carried out in the Botany Department, University of Natal, Pietermaritzburg, from January 1991 to July 1994 under the supervision of Professor J van Staden.

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

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Vorrei ringraziarvi mamma e papá, per avermi datto l'opportunitá di studiare. Senza i vostri sacrifici, non potrei esser' arrivata fino a questo punto.

I wish to dedicate this thesis to my husband, Wayne, for his valuable input, his insight, assistance and constant encouragement to persevere.

ABSTRACT

Immature, green female cones of *Pinus patula* Scheide et Deppe. were collected on a weekly basis during the South African summer months from December 1991 to February 1992 and from December 1992 to March 1993. Embryogenic tissue was initiated from excised megagametophyte explants containing immature zygotic embryos. Embryogenic induction was achieved using both MSG (BECWAR, NAGMANI & WANN 1990) and DCR (GUPTA & DURZAN 1985) media. The highest induction frequency was obtained on DCR1 (Douglas-fir Cotyledon Revised) medium supplemented with 0.5 mg l^{-1} BA and 3.0 mg l^{-1} 2,4-D, using L-glutamine as the major nitrogen source. Embryogenic tissue was translucent-to-white and mucilaginous in nature, composed of elongated, suspensor-like cells. The tissue was extruded from the micropylar end of the female gametophyte. In comparison, non-embryogenic tissue was produced from the gametophytic tissue itself and consisted of small, compact, spherical cells, crystalline in nature. Anatomical studies of developing patula seed demonstrated that the production of embryogenic tissue from the immature explants co-incided with the period, approximately two weeks after fertilization and with the occurrence of cleavage polyembryony in the developing zygotic embryos. Embryogenic tissue was maintained in culture by a recapitulation of the cleavage process.

Transfer of the embryogenic tissue to DCR2 medium containing 1.3 mg l^{-1} ABA resulted in tissue maturation and in the subsequent development of somatic embryos. Presence of ABA in the culture medium stimulated the development of cotyledonary initials in the apical region of the embryos. Elongated embryos, possessing small cotyledons, were rooted (50 to 60 %) on MSG6 medium containing no plant growth regulators. Somatic plantlets were successfully hardened-off under greenhouse conditions.

Liquid culture methods were found to be a useful means of rapidly increasing the volume of embryogenic suspensor masses. Maturation, in terms of somatic embryo development and the production of cotyledonary initials, though, was not obtained in suspension. Re-establishment onto agar-solidified medium (DCR2) was required before maturation could

occur. ABA is also responsible for stimulating reserve deposition and mobilization. In this regard, lipid accumulation in the developing somatic embryos was quantified and found to be significantly lower than in developing zygotic embryos. Similarly, non-matured embryogenic tissue contained less lipid deposits than matured (ABA-treated) tissue, indicating the requirement for ABA during maturation. Quantification of the lipids deposits is useful in determining the potential for somatic embryos to acclimatize to *ex vitro* conditions since their further growth and development is based on their ability to accumulate storage reserves.

Somatic embryogenesis was found to be a useful method of propagation, producing plantlets with seedling-like qualities. This development has important consequences for the production of clonal plantlets in the Forestry Industry.

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COMMONLY USED ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
BA	Benzyladenine
CM	Coconut milk
d	day
DCR	Douglas-fir Cotyledon Revised Medium
2,4-D	2,4-Dichlorophenoxyacetic acid
ESM	Embryonal suspensor masses
FAA	Formalin-acetic acid-alcohol
FDA	Fluorescein diacetate
GA	Gibberellin
GC	Gas liquid chromatography
IAA	Indoleacetic acid
IBA	Indolebutyric acid
Kin	Kinetin
m	month
min	minute
MS	MURASHIGE & SKOOG (1962) Medium
MSG	Modified MURASHIGE & SKOOG (1962) Medium
NAA	Naphthaleneacetic acid
PEG	Polyethyleneglycol
SCV	Settled cell volume
SEM	Scanning electron microscopy
TAG	Triacylglycerol
TEM	Transmission electron microscopy
TMAH	Tetramethylammonium
w	week

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and initiation of embryo growth towards the surrounding nucellus tissue (N). **E** Development of the zygotic embryo (ZE) in the corrosion cavity (CC), with attached suspensor (S) and developing cleavage embryo (arrowhead).

CHAPTER 1

GENERAL INTRODUCTION

1.1 The South African Forestry Industry

The South African forestry industry is a vibrant and growing endeavour which has been well established for over 100 years. For a country with limited natural forest resources, South Africa has emerged among the world leaders in the industry, providing employment for 120 000 people and earning a place as one of the largest exporters of manufactured goods (including kraft and fine papers, rayon and timber products). Despite the ravages of the recession, most forestry industries are confident of an imminent economic upswing, even though the demands of the Reconstruction and Development Programme (RDP) are likely to place serious challenges on the country's limited timber resources.

The industry is dominated by large forest owners including Sappi, Mondi, Safcol (the recently commercialized State-owned enterprise), HL&H Timber and numerous other smaller ventures which collectively own or lease 75 % of the plantations. The small private timber owners and wattle growers account for about 20 % of the land under afforestation. Today, plantations cover over 1.3-million hectares or 1.2 % of the country's total surface area (DELANEY 1994). Softwood species occupy the largest proportion (51.3 %) of the plantation area, bringing economic activity and rural prosperity to a broad belt concentrated mainly in the Eastern and South Eastern Transvaal, extending through the Natal Midlands and down to the Southern Cape (FOREST OWNERS ASSOCIATION 1994).

From the humble beginnings of a single plantation established in the Western Cape

in the late 1870s, the forestry and forestry products industry has become one of the fastest growing sectors of the South African economy. Sales of pulp and paper products are proving the most lucrative in the industry as demonstrated by the 1992/1993 pulp figures which were in excess of R 3 570-million, followed by those of sawn timber, panel products and mining timber (DELANEY 1994).

1.2 Early Propagation Methods

The ever-increasing demand for timber and wood products has resulted in the necessity for optimum land usage. This includes the use of marginal areas where growth conditions are not ideal. These circumstances have forced researchers to develop new and more reliable methods of tree selection and propagation. Commercial methods, used by foresters to retain desirable qualities in forest trees, have been mainly by sexual reproduction. Traditionally, forest planting stock production was dependent on the use of seed collection from natural stands, a process which to some extent still occurs today. Outstanding individuals, in terms of tree form, yield and volume, have been selected and used as the basis for breeding programmes. Progeny trials are used to test the best genetic combinations and seed orchards are then established to realize the potential gains (POWLEDGE 1984).

Besides using seeds as planting stock, attempts have been made to multiply selected genotypes vegetatively. Retaining superior clones is especially important in selection for pulp, paper, lumber and ornamental value. Grafting methods such as tip cleft, side veneer and budding were among the first techniques used to propagate tropical forest trees vegetatively (BARNES & BURLEY 1987). The aim of these techniques was to preserve selected phenotypes and to multiply them to create clonal orchards to produce seed for progeny tests and commercial use. Since then, grafting has played a major role in tree breeding programmes for many years (SLEE 1967; DORMAN 1976; ZOBEL & TALBERT 1984). The use of physiologically older material in the graft scion has also significantly reduced the

period required before orchards can produce seed (DONALD 1987). Despite the potential benefits of grafting, these propagation methods cannot be considered economically viable. The expense and the limited numbers which can be produced also provided the first incentive for tree breeders to attempt producing ramets with their own root systems.

The use of cuttings for the development of orchards and hence the establishment of superior plantations, received much attention in the sixties and seventies (THULIN & FAULDS 1968; SHELLBOURNE & THULIN 1974). The only pine species that has enjoyed success using vegetative propagation of cuttings in large numbers, has been *Pinus radiata* (KUMMEROW 1969). A possible reason for the limited use of this practice has been the difficulty of rooting cuttings of commercial species, successfully and consistently.

1.3 Modern Approaches to Propagation

The current activity of developing appropriate methods of clone propagation by means of cell and tissue culture hold promise for reliable, practical propagation techniques in the future. Already, tissue culture methods exist for the production of clones of ten or more individuals from single embryos or young seedlings. Clones of this size can substantially increase the efficiency of breeding programmes. Experiments are beginning to suggest the possibility of mass propagation as well as an ability to propagate from mature trees, through tissue culture. Better selection of desirable genotypes can be made when conifers are mature although the capacity for propagation by conventional methods (rooted cuttings) is usually lost at tree maturity.

Tissue culture propagation of forest trees is a new and developing area which is making rapid progress. Greenhouse and field testing of plantlets produced from tissue culture has also just begun. It is hampered by the long time required for conifers to grow and reach maturity (from years to decades), suggesting that

meaningful evaluation of these clones will require some time. The *in vitro* methods for elite tree production include use of buds (terminal, axillary, fascicle) and plantlet production from stem callus cultures (organogenesis) as well as artificial embryo production from such callus cultures (somatic embryogenesis).

Somatic embryogenesis, although generally considered "high risk" research, is aimed at manipulating the genetic gains associated with elite trees in tree breeding programmes, which usually also involve the rooting of cuttings, micropropagation and seed orchard management (TIMMIS, ABO EL-NIL & STONECYPHER 1987). This approach has not solved the problem of rooting, but provides an alternative to the rooting of cuttings and of micropropagated shoots. The limitation however, remains that it is usually the new generation and not the mother tree that is propagated by somatic polyembryogenesis (DURZAN 1988). BECWAR (1993) reaffirmed the concern that embryogenic lines derived from a single seed containing numerous embryos (or sub dominant embryos) could result in multiple genotypes and that clonal integrity of cell lines from each seed could not be guaranteed. This problem could be solved by culturing excised immature embryos, or through the use of more mature seed explants. Alternatively, multiple genotype production could be encouraged as part of the selection process in the breeding programme.

Somatic embryogenesis has numerous promising applications (AMMIRATO & STYER 1985). The most important of which is the mass clonal propagation of many normally seed-propagated crops. Other advantages include: the production of both root and shoot apices on the same structure; the relative ease of bulking up tissue, especially if suspension cultures are utilized; induction of dormancy, as well as the possibility of long term storage; and the production of encapsulated seed or direct production of young plantlets (LUTZ, WONG, ROWE, TRICOLI & LAWRENCE 1985). The production of artificial seed is a process still in its infancy, but one with numerous potential benefits. Precision planting by machine; synchronous germination and yield uniformity as well as the gains derived from using superior germplasm are some of the potentials envisaged through the use of this approach (DURZAN 1988).

1.4 Introduction of *Pinus patula* to South Africa

Pinus patula Scheide et Deppe, also known as the Mexican weeping pine, Mexican red pine or "patula" pine is a woody conifer which is considered a difficult-to-root species. It was first introduced to South Africa from Mexico in 1907 (POYNTON 1961), marking the start of the large scale use of this softwood species for forestry purposes. Desirable characteristics such as fast growth, good silvicultural features and easy availability of seed from South Africa, made *P. patula* the obvious plantation species for many east, central and southern African countries (WORMALD 1975). Since its early introduction *P. patula* has become one of the most important softwood timber species in South Africa.

P. patula was previously placed in the large group *Insignes* (SHAW 1914), but was later reclassified as *Pinus* subsection *Oocarpae* (LITTLE & CRITCHFIELD 1969). The subsection *Oocarpae* consists of six other New World pine species besides *P. patula*. *P. radiata*, *P. attenuata* and *P. muricata* occur in North America; *P. greggi* and *P. pringlei* are confined to Mexico while *P. oocarpa* is distributed from Mexico throughout Central America to Northern Nicaragua.

Species belonging to the genus *Pinus* are evergreen, although deciduous since they gradually shed their needles in autumn. Pines can reach 20 to 30 m in height. The bark on the lower part of the trunk is broken into irregularly shaped plates while higher up, it becomes thin, papery and reddish brown in appearance (DALLIMORE & JACKSON 1966) a feature from which the common name is derived. Pine needles are the principle photosynthesising structures which develop in fascicles containing three to five needles. The needles develop from meristematic cells emanating from the tiny short shoots of the branches (MIROV & HASBROUCK 1976). The pollen-producing microsporangia are borne on the tips of the lower branches while the megasporangia or ovulate cones develop on the higher branches (RAVEN, EVERT & CURTIS 1981).

1.5 Aims of the Study

The economic importance of this softwood species, as well as the implications of introducing a viable and practical system of improving existing methods of propagation are the underlying motivations for this research. The major objectives were therefore, to attempt the vegetative propagation of *Pinus patula* by generating somatic embryos. The approach involved:

i) Conducting a survey of immature zygotic explants of *P. patula* in order to determine the optimum stage of embryo development and the culture conditions required for the initiation and maintenance of embryogenic tissue.

ii) Attempts at maturing the embryogenic tissue for the production of somatic embryos; and

iii) the hardening-off and establishment of somatic plantlets in the soil. This was done with the aim of developing a practical and reliable method of clonal propagation with possible commercial application.

CHAPTER 2

MACROPROPAGATION OF *Pinus* SPECIES

2.1 Introduction

The potential benefits of using clonally propagated material in forestry have long been recognized and a substantial increase in gain can be expected by planting selected clonal material rather than selected seed-derived families (KLEINSCHMIDT 1974). In order to realise genetic benefits, both sexual reproduction and vegetative propagation need to be included in the tree breeding programme. Sexual reproduction ensures that genetic variety is maintained and clonal propagation enables multiplication of the selected families.

Cloning ensures that desirable characteristics such as disease or frost resistance, environmental adaptability, high yield and vigour and superior wood quality and tree form are maintained. In addition, this method of propagation shortens the breeding cycle, thus saving time. It now takes at least 16 years after selection, for either a Douglas-fir or loblolly pine seed orchard to attain full production and thus for the gains of the improvement effort to be realized. Although this period can be reduced by gibberellin treatments it has been anticipated that in some cases, this 16 year period could be reduced to four years or less with vegetative propagation. The added advantage of growth uniformity is also a desirable response for forestry management.

2.2 Methods of Clonal Propagation

Clonal propagation of forest tree species has been achieved by rooting of cuttings

(PATTON & RIKER 1958), or fascicular buds (PHILLION, WHITTAKER & BUNTING 1983) and using various grafting methods (HEAMAN & OWENS 1972). However, for many woody species, the realization of clonal benefits is impeded by the generally recalcitrant nature of coniferous species to vegetative propagation. One of the major problems is the effect of maturation on rootability. Maturation is a developmental process relating mainly to woody species, during which growth rate and rooting of cuttings is reduced by the onset of morphological changes (HAFFNER, ENJALRIC, LARDET & CARRON 1991). It has been shown that by the time superior growth characteristics have been identified, ramets (cuttings) have usually lost their rooting capacity, as a result of the increasing age of the ortet (parent plant) (THORPE & BIONDI 1984). The rooting ability, number and length of roots as well as the speed of rooting and subsequent survival and development of the cuttings, all decline in proportion to the age of the ortet (GIROUARD 1974).

In order to overcome these difficulties, once a superior tree has been selected, hedging (repeated pruning back of shoots to a specific height) can be used to retard maturation and hence maintain the ortet in a juvenile state (DOOLEY 1986). Using this method buds less mature than the tissue being removed are stimulated to develop. Maturation though, is only temporarily delayed since repeated trimming activates the meristems and hence ontogenetic ageing (genetically controlled change from the juvenile to adult state). Serial propagation is another method of retaining juvenile characteristics. This method involves taking cuttings from a selected tree. The cuttings are then rooted and established. These then become the secondary ortet and source of further cutting material. Both these methods ensure that rootability of cuttings is maintained.

An alternative method of ensuring sufficient rootable cuttings from selected material, involves the rejuvenation of already mature material, rather than the maintenance of juvenility. This method involves the serial or repetitive grafting of mature material onto juvenile rootstocks (DOOLEY 1986). Although delayed graft incompatibility may cause problems in established clone banks (DUFFIELD & WHEAT 1964; SLEE & SPIDY 1970), the advantage of this method is that the initially selected material is mature enough for the identification of desirable characteristics. The methods

described earlier, in which juvenility is merely maintained, prevent adequate selection based on morphological and physiological characteristics. Root formation in pine cuttings is therefore strongly dependent on the growth condition of the parent plant (STRÖMQUIST & HANSEN 1980).

Other factors, besides the state of the mother plant, that need to be considered for the successful rooting of cuttings include: the size, type and position of the cutting on the tree; exogenous factors such as the concentration, and type of rooting agents and treatments; mode of application; suitable rooting environment and rooting media as well as the most suitable time to collect cuttings (DOOLEY 1986).

Numerous rooting agents have been employed to promote rooting. Auxins are known to improve the root formation of numerous species (WENT & THIMANN 1937) and mixtures of synthetic substances with auxin activity are used for propagation of ornamental plants by cuttings. Auxins are responsible for increasing the number of cuttings that root, and the number of roots produced per cutting (HARTMAN & KESTER 1983). The hormonal effects are dependent on both the concentration and duration of the treatment.

Coniferous species are difficult to root from cuttings resulting in low rooting frequencies or slow unsynchronized rooting (FLYGH, GRÖNROOS, GULIN & VON ARNOLD 1993). Other difficulties associated with propagation of plantlets from branch cuttings are the often horizontal orientation and bilateral symmetry (plagiotropy) both of which are displayed during growth. Cuttings tend to continue growing horizontally in branch-like fashion before the terminal meristem changes to radial symmetry and vertical growth (orthotropy) is resumed. The resumption of normal growth may take some time to occur (THORPE, HARRY & KUMAR 1991).

Forestry as well as agricultural and horticultural practices have for a long time relied on traditional methods of propagation. Much has been learnt from the experience with vegetative propagation by using branch (KLEINSCHMIDT & SCHMIDT 1977) and brachyblast (KUMMEROW 1966; INGLIS 1984) cuttings. However, these

techniques have only been successfully applied to a limited number of species, for example *Pinus radiata*. In view of the limitations associated with traditional clonal propagation, and the fact that *Pinus patula* is considered a "difficult-to-root" species, not easily established by vegetative cuttings, alternative methods of propagation have been sought. Cell and tissue culture techniques offer the possibility for large scale production of selected families or clones from control-pollinated crosses (AITKEN-CHRISTIE & THORPE 1984). This would enable faster establishment of improved genotypes and result in economic gains from tree breeding by making the technology commercially viable (SMITH, AITKEN & SWEET 1981; SMITH, HORGAN & AITKEN-CHRISTIE 1982).

CHAPTER 3

MICROPROPAGATION OF *Pinus* SPECIES

3.1 Introduction

Tree species were among the first plants to be cultured *in vitro*. As early as 1934, GAUTHERET cultured cambial tissue of woody species including *Pinus pinaster* and *Abies alba*. In spite of this, *in vitro* micropropagation of gymnosperms has only been successfully achieved within the last 15 years (JANG & TAINTER 1991a). Improvements through organogenesis have been made but the critical component necessary for success remains the rooting of micropropagated shoots (MOHAMMED & VIDAVER 1988). Tissue culture of gymnosperms has not only become important from a propagation point of view, but has also been studied in terms of physiological, morphological and anatomical characteristics. Morphogenesis of primary organized explants *in vitro*, is affected by various factors including: the explant genotype, source and age; the nutrients used; agar quality; and the concentration, duration, type and mode of application of plant growth regulators (DEBERGH, HARBAOUI & LEMEUR 1981; DAVID, DAVID & MATEILLE 1982b; BORNMAN 1983; DEBERGH 1983). A review of the recent *Pinus* species utilized and protocols employed to establish various explants in culture is presented in Table 3.1.

3.2 Micropropagation via Organogenesis

Organogenesis is the major method by which *in vitro* plantlet regeneration is obtained in softwood species. It is a process involving various developmental stages including: culture establishment and/or bud induction; shoot development and multiplication; rooting of developed shoots; and hardening-off of plantlets. Organogenesis has been achieved in various pine species (*P. palustris*, *P. pinaster*, *P. radiata*, *P. strobus*, *P.*

sylvestris and *P. taeda*) by culturing whole embryo or embryonic explants (Table 3.1). During *in vitro* organogenesis, competent cells within an explant respond to an organogenic stimulus (such as phytohormones) and after an appropriate induction period become determined towards a specific pattern of differentiation (CHRISTIANSON & WARNICK 1983). Other cultural factors such as basal medium components and age and source of explant also play a role in determining the differentiation pattern.

3.2.1 Shoot Initiation

Embryonic and juvenile tissues have been commonly employed explants from which to induce shoot development (KAUL 1987; WEBB, FLINN & GEORGIS 1988; SCHWARZ, SCHLARBAUM & BEATY 1988). Shoot initiation requires exposure to cytokinin which may be combined with a low concentration of auxin (AMERSON, FRAMPTON, McKEAND, MOTT & WEIR 1985). Exposure time is critical in obtaining shoots capable of subsequent growth (BIONDI & THORPE 1982; MOTT & AMERSON 1981a; VON ARNOLD & ERIKSSON 1981). JANG & TAINTER (1991a) found that for *Pinus echinata*, *P. taeda*, *P. virginiana* and *P. taeda* x *P. echinata* hybrids reduced medium strength was beneficial for shoot formation and inclusion of activated charcoal in the medium stimulated shoot development.

3.2.2 Shoot Multiplication and Elongation

The strategy for inducing shoot multiplication in conifers involves carefully severing shoot apices and the apical tuft of primary needles to allow new shoots to develop from the axillary meristems located at the base of the needles of the nodal segment. The shoot tips are then placed back into culture where shoot elongation is stimulated and the process is repeated (AITKEN-CHRISTIE & THORPE 1984; BAXTER, BROWN, ENGLAND, LUDLOW, TAYLOR & WOMACK 1989).

AITKEN-CHRISTIE & THORPE (1984) found that shoots of *Pinus radiata* elongated at different rates depending on the amount of shoots produced per clone. Nutrients were also found to be limiting in clones producing large numbers of shoots due to competition effects. The addition of activated charcoal to the growth medium has been found to have a stimulatory effect on elongation (DAVID 1982).

3.2.3 *In vitro* Rooting

In vitro rooting of woody species and especially of conifers remains the limiting factor in achieving whole plant regeneration (FAYE, OURRY, SAIDALI-SAVI, DARGENT, BOUCARD & DAVID 1989). The problem is aggravated by large clonal differences in rooting potential (JANG & TAINTER 1991a). Spontaneous rooting is rare and treatment is therefore required for root initiation. Owing to uniform distribution of nutrients and hormones and good contact between the shoot and the medium there is a possibility of attaining more synchronous rooting *in vitro*, however root quality is often poor (MOHAMMED & VIDAVER 1988).

Each stage of the *in vitro* development process may influence the rooting performance. Root formation is affected by both the form and concentration of nitrogen (HASSIG 1974), as well as the presence of endogenous and exogenous amino acids (WELANDER 1978). Carry-over effects of the phytohormones used during the multiplication stage may also influence rooting. Furthermore, the agar itself may impede gaseous exchange and appropriate root development (MOHAMMED & VIDAVER 1988).

Auxins have been used either alone or in combination, to stimulate rooting. Levels of NAA used, vary from 0.05 to 0.5 mg l^{-1} (KAUL 1987; MOTT & AMERSON 1981a; 1981b; PATEL, KIM & THORPE 1986; RANCILLAC, FAYE & DAVID 1982), while those of IBA range from 1 to 10 mg l^{-1} (ABDULLAH, GRACE & YEOMAN 1989; LOPEZ-PERALTA & SANCHEZ-CABRERA 1991). Auxins are usually omitted from the medium during root elongation.

Ectomycorrhizal fungi have also been used to induce short, dichotomously branched roots in pine shoots (SLANKIS 1973). These fungi release IAA (GAY & DEBAUD 1987) which is responsible for enhancing the rhizogenic potential of the host plant (GAY 1990). Ineffective rooting and hardening-off of adventitious shoots derived *in vitro* has been the major limitation for the use of micropropagated conifer plantlets (COLEMAN & THORPE 1977; AMERSON, FRAMPTON, McKEAND, MOTT & WEIR 1985).

3.3 Adventitious Budding

The first complete conifer plantlet obtained *in vitro* was one of *Pinus palustris* (longleaf pine) produced in 1974 (SOMMER, BROWN & KORMANIK 1975). Plantlets were produced from adventitious buds initiated from cotyledons of embryos in culture. Since then numerous reports have followed on plantlet regeneration in culture, among them being: *Sequoia sempervirens* (BOULAY 1979); *Pinus radiata* (REILLY & WASHER 1977; YEUNG, AITKEN, BIONDI & THORPE 1981; VILLALOBOS, LEUNG & THORPE 1984; VILLALOBOS, YEUNG & THORPE 1985); *P. taeda* (MEHRA-PALTA, SMELTZER, & MOTT 1978); *P. pinaster* (DAVID, DAVID & MATEILLE 1982b) and *P. brutia* (ABDULLAH, YEOMAN & GRACE 1985).

Adventitious bud and shoot formation is the most effective *in vitro* method of propagating forest tree species, resulting in direct production of whole plants or adventitious shoots from embryos, shoot tips or stem segments (MOTT & AMERSON 1981a; JONES 1983; AITKEN-CHRISTIE, SINGH, HORGAN & THORPE 1985). This process has been well documented through numerous anatomical studies (VILLALOBOS, YEUNG & THORPE 1985; RUMARY, PATEL & THORPE 1986; VON ARNOLD & GRÖNROOS 1986) which show that adventitious organs arise from groups of small, isodiametric, densely cytoplasmic cells, termed meristemoids (FLINN, WEBB & NEWCOMB 1989).

Cytokinins are essential for adventitious bud development. The cytokinin, BA used at various concentrations, is usually the phytohormone selected for shoot induction. Mixed cytokinins have also been beneficial for some conifers (RUMARY & THORPE 1984). Auxin may occasionally also be used (CHALUPA 1985a).

Indirect adventitious budding can also occur via callus formation, a process which occurs predominantly in angiosperms (AHUJA 1983; SOMMER & WETZSTEIN 1984) and tends to be extremely rare in conifer regeneration (NOH, MINOCHA & RIEMENSCHNEIDER 1988).

3.4 Axillary Bud Stimulation

This method involves the use of shoot tips, lateral buds and small nodal cuttings. The process is especially useful for hardwood species, relying on the presence of axillary buds on the explants used. Development is therefore direct, usually by-passing a callus stage. Using juvenile explants BAXTER, BROWN, ENGLAND, LUDLOW, TAYLOR & WOMACK (1989) initially stimulated bud production and then the formation of intercotyledonary axillary buds from nodal stem segments of *Pinus caribaea*, *P. oocarpa* and *P. patula*. Stimulating the axillary buds at the base of mature needle fascicles, to produce shoots has proved very difficult (BOULAY 1987). Little success has been obtained using this method, since rooting of the shoot also poses difficulty.

3.5 Protoplast Culture

Protoplasts have been defined as that "part of the plant cell which lies within the cell wall and can be plasmolysed and isolated by removing the cell wall either mechanically or enzymatically. The protoplast is therefore only a naked cell - surrounded by the plasma membrane - which is potentially capable of cell wall regeneration, growth and division" (VASIL 1976). In view of this, protoplast culture

is becoming one of the more sophisticated tools enabling exploration of various aspects of fundamental and applied biology.

Despite the fact that conifer protoplasts are considered recalcitrant to culture (ATTREE, DUNSTAN & FOWKE 1989a) and some *in vitro* responses appear genotype-dependent, the genetic benefits of protoplast culture techniques are becoming more significant (KIRBY 1988). Protoplasts have been isolated from cotyledons of *Pinus coulteri* (PATEL, SHEKHAWAT, BERLYN & THORPE 1984); *P. pinaster* (DAVID & DAVID 1979; DAVID, DAVID & MATEILLE 1982a); as well as suspension cultures of *P. contorta* (HAKMAN & VON ARNOLD 1983); *P. taeda* (TEASDALE & RUGINI 1983) and *P. lambertiana* (GUPTA & DURZAN 1986c) in order to generate colonies.

Problems associated with the recalcitrant nature of conifer protoplast cultures were overcome when embryogenic material was used as the source material. In this regard, protoplasts have been isolated from embryonal suspensor masses of *Pinus taeda* resulting in the production of somatic embryos (GUPTA & DURZAN 1987a). Furthermore, protoplasts have also been derived from *Picea glauca* (ATTREE, BEKKAOUI, DUNSTAN & FOWKE 1987; ATTREE, DUNSTAN & FOWKE 1989b) and *Pseudotsuga menziesii* (GUPTA, DANDEKAR & DURZAN 1988) although no plantlet regeneration was recorded.

Although promising, this application awaits the development of reliable protocols for the regeneration of trees from protoplast-derived cell cultures. The recent development of embryogenic tissue may lead to more efficient production of trees from protoplasts (KIRBY 1988). This type of system also lends itself to genetic manipulation involving *Agrobacterium*-mediated gene transfers, or the use of technologies such as microprojectile bombardment of protoplasts or cells with DNA-coated tungsten pellets (KARNOSKY, DINER & BARNES 1988).

Table 3.1 Summary of the *in vitro* establishment of *Pinus* species. The initial explant and the subsequently derived explants are listed in Column 2. The resulting morphogenesis is described in Column 3, achieved using various media formulations (Column 4), pH values (Column 5), sucrose contents (Column 6), and solidifying agents (Column 7). The relevant plant growth regulators (mg l^{-1}) are tabulated in Column 8. Cited references appear in Column 9.

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
Pinus spp.	Cambium, resin duct cells, pith, phloem parenchyma and leaf trace cells	Callus induction	MS				NAA 2,4-D	BYCHENK-OVA (1977)
P. banksiana	Cotyledons (8-10-d-old seedlings) Cell suspensions initiated from seedlings Protoplasts	Protoplast isolation and cell division Green callus Microcalli	LAINÉ medium C ½ LV (modified NAGMANI & BONGA 1985) Diluted LAINÉ medium C	5.6 5.6	81 (G) 20.5	 8	(1.9)NAA (1.5)BA (2)2,4-D (1)BA	TAUTORUS *et al.* (1990b)
P. brutia	Shoot explants from 10-w-old seedlings	Axillary shoot production	SH (modified)				Cyt [6 w]	ABDULLAH *et al.* (1986)
P. brutia	Apical buds (8-w-old seedlings) Shoots	Multiple axillary shoots Shoots rooted, plantlets produced	 SH (modified)[5]		 20	 6	 (1)NAA (2)IBA (0.05)BA	ABDULLAH *et al.* (1989)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. brutia	Dormant fascicular meristems (10-y-old trees)	Shoot bud initiation	SH (modified)[5]	5.8	30	6	(5)BA (5)Kin	ABDULLAH *et al.* (1987)
	Shoot buds	Shoots elongation	½ SH (modified)		20	6	(1)BA (1)Kin	
	Elongated shoots (10-15; 20-28 mm)	Shoot multiplication	SH (modified)		30	6	(2.5)BA (2.5)Kin	
	Elongated shoots (> 20 mm)	Rooting pre-treatment	SH (modified)				(1)NAA (2)IBA (0.05)BA [12-18 d]	
	Shoots	Shoots rooted	½ SH (modified)				None	
P. canariensis	Excised embryos	Germination			10	8		MARTINEZ-PULIDO *et al.* (1990)
	Excised 3-d-old cotyledons	Adventitious bud induction	MCM		30	8	(2.3)BA [2-3 w]	
	Adventitious buds	Bud development	½ MCM				(2.3)BA [15 w]	
	Buds	Bud elongation	½ SH		20	5(Ge)		
	Shoots	Shoots rooted (80-100 %)	Peat:Vermiculite 1:1 + ¼ MCM				(203)IBA [liquid pulse]	
P. canariensis	Cotyledons (3-d-old)	Bud development	MCM				Cyt	MARTINEZ-PULIDO *et al.* (1991)
	Buds	Shoot production	MCM				None	
	Shoots	Shoots rooted	Peat/Vermiculite				IBA pulse	
P. caribaea	Excised embryos	Bud induction	½ CD	5.6	20	10	(3-5)BA	BERLYN *et al.* (1987)
	Multiple adventitious buds	Root induction	½ GD	5.6	20		(18)IBA	
	Rooted shoots	Hardened-off	Sterile peatmoss : commercial potting soil : vermiculite : perlite (2:2:1:1) + ¼ Knop's nutrient solution					
P. caribaea	Cotyledons (attached and detached from excised embryo	Adventitious buds					BA	SKIDMORE (1988)
	Buds	Shoots					IAA, BA	

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. caribaea	Epicotyl explants or inter cotyledonary axillary shoots	Bud development and shoot elongation	½ MS	5.6	20	8	(2.2)BA (4.7)NAA [2 w]	BAXTER *et al.* (1989)
P. contorta	Hypocotyls from 3-w-old explants	Direct root production	Hoagland nutrient solution (ELIASSON 1978)	5.8		-	(0.2-0.3)IBA [4-6 d]	GRÖNROOS & VON ARNOLD (1987)
P. contorta	Stock plant Fascicular buds	Fascicular bud induction Shoots rooted					Cyt spray treatments (100)IBA or (25)IBA + (25)NAA [24 h]	SALONEN (1991)
P. contorta var. *latifolia*	Callus from leaf explants	Somatic polar structures resembling embryos, no further development obtained					(0.2)2,4-D (0.9)BA	MACDOUGALL *et al.* (1988)
P. coulteri	Excised embryos	Adventitious buds	D Medium (modified BERLYN & BECK 1980)		30		(2.25)BA	PATEL & BERLYN (1982, 1983)
P. densiflora	Cotyledons from 9-d-old seedlings Protoplasts	Protoplast isolation Protoplast budding (3-4 cell stage)	B5 medium				BA, 2,4-D	LEE *et al.* (1985)
P. durangensis	Cotyledons Shoots Elongated shoots	Shoot induction Shoot multiplication/elongation Shoots rooted	SH (modified) ½ SH (modified) ½ SH (modified)	5.7	30 20	8 8	(3)BA (1.0-4.0)BA (2.0-7.0)IBA	LOPEZ-PERALTA & SANCHEZ-CABRERA (1991)
P. echinata	Cotyledonary explants Meristematic tissue Shoots	Meristematic tissue induction Shoots Root induction	GD (modified) ½ GD[6] GD (modified)[7]		 30 20	7 7	(10)BA (0.01)NAA None (0.5)NAA (0.1)IBA	JANG & TAINTER (1991a)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. echinata	Hypocotyl and radicle	Callus induction	MS & LV (both modified)		30	7	(11)2,4-D (0.5)BA	JANG & TAINTER (1991b)
P. elliottii	Hypocotyls from 1-w-old seedlings	Callus induction	MS (modified)				(20%)CM	LAU *et al.* (1980)
P. elliottii	Excised cotyledons Adventitious shoots Elongated shoots Rooted shoots	Adventitious shoots Shoot elongation Shoots rooted Root elongation	GD (modified) GD (modified) ½ GD ½ GD				(10)BA (0.01)NAA None (0.5)NAA None	BRONSON & DIXON (1988)
P. elliottii	Stem segments from 2-m-old seedlings Callus	Callus induction Callus maintenance	MS LV (suspension culture)				(2)2,4-D	LESNEY (1989)
P. elliottii	Cotyledons Shoots Shoots Elongated shoots	Adventitious shoots Shoot differentiation & elongation Root induction Root formation and elongation	GD (modified) ½ GD[7] ½ GD ½ GD	5.7 5.7 5.7 5.7	20 20 20 20	8 8 8 8	(14.9)BA None [28 d] (0.5)NAA [14 d] None [14-21 d]	BRONSON & DIXON (1991)
P. elliottii	Hypocotyl explants from 30-40-d-old seedlings Axillary shoots	Multiple buds Shoot elongation	 GD			 3	BA (25)BA [45 s dip]	BURNS *et al.* (1991)
P. greggii	Cotyledons Shoots Elongated shoots	Shoot induction Shoot multiplication/elongation Shoots rooted	SH (modified) ½ SH (modified) ½ SH (modified)	5.7	30 20	8 8	(3)BA (1-4)BA (2-7)IBA [72 h]	LOPEZ-PERALTA & SANCHEZ-CABRERA (1991)
P. halpensis	Excised hypocotyls inoculated with *Hebeloma hiemale* (ectomycorrhizal fungus)	Hypocotyls rooted (96.6 %)	P_2[11]	6.5	4.8	7.5	(20.4) Tryptophan	GAY (1990)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. halpensis	Excised embryos Shoot buds Shoots Elongated shoots	Adventitious shoot bud induction Shoot development Shoot elongation Shoots rooted (100 %)	AE AE ½ AE[10] Peat:Vermiculite 1:1		30	8	(1.1)BA [3 w] None [4 w] None [8-12 w] (203)IBA [6 h pulse]	LAMBARDI *et al.* (1991)
P. koraiensis	Excised mature embryos	Successful organogenesis					(0.1)2,4-D (0.1)BA or (0.1)Kin	KIM & PARK (1986)
P. monticola	Seedlings (4-y-old) Fascicular buds Shoots	Fascicular bud induction Bud elongation, secondary needles Root induction (10 %)	Containerized GD ½ GD[7]				(450)BA [spray, 8 w] None (2 500)NAA [dip]	STIFF *et al.* (1988)
P. monticola	Induced fascicle buds Buds Rooted plantlets	Bud development Buds rooted Hardened-off	GD ½ GD[7] Peat/perlite	5.8 5.8		7 7	(1.1)BA, then none (2 000)NAA [dip]	STIFF *et al.* (1989)
P. monticola	Intact embryos Cotyledons Buds (75 mm) Shoots	Bud initiation Bud initiation Rooting pre-treatment Shoots rooted (30 %)	GD GD ½ GD ½ GD		 40 40		(10)BA (0.01)NAA [8 w] (1)BA (0.01) NAA [8 w] (0.1)BA (0.1)NAA [5 d] None	MOTT & AMERSON (1981a)
P. mugo	Root organ cultures	Dichotomous branching of lateral roots, inhibition of root hair formation					(7.2 or 14.5) Ethephon	RUPP & MUDGE (1985)
P. mugo var. *mugo*	Excised embryos and explants from 2-w-old and 4-m-old seedlings Shoots Shoots	Shoot development via adventitious buds Shoot elongation Shoots rooted	 Non-sterile medium				(10)BA (0.01)NAA None (0.008)IBA	MUDGE (1986)
P. mugo var. *mughus*, *rostrata* and *pumilio*	Excised embryos	Callus induction	MS	5.8	30	8	(1.5)Kin (0.5)2,4-D	PEI (1989)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. nigra var. *maritima*	Excised embryos	Callus induction	MS	5.8	30	8	(1.5)Kin (0.5)2,4-D	PEI (1989)
P. oocarpa	Cotyledonary explants from 7-10-d-old seedlings Adventitious buds Elongated shoots Plantlets	Adventitious buds Bud development and elongation Shoots rooted Hardened-off	 Soil in greenhouse conditions				Cyt [21 d] None Auxin	FRANCO & SCHWARZ (1985)
P. oocarpa	Cotyledons from 11-d-old seedlings Fragmented colonies	Protoplast isolation Microcalli	Suspension culture[8] Medium C (DAVID *et al.* 1986)				(0.9)NAA (2.2)Zeatin	LAINÉ *et al.* (1988)
P. oocarpa	Shoots	Shoot elongation	½ MS[8]	5.6	20	8	None	BAXTER *et al.* (1989)
P. palustris	Mature embryos Differentiated cotyledons.	Cellular differentiation Plantlet production	GD (modified) RW (modified)	5.8	20 20	7 7	(1)BA (2)NAA None	SOMMER *et al.* (1975)
P. patula subsp. *tecunumanii*	Cotyledons from 11-d-old seedlings Fragmented colonies	Protoplast isolation Microcalli	Suspension culture[8] Medium C (DAVID *et al.* 1986)				(0.9)NAA (2.2)Zeatin	LAINÉ *et al.* (1988)
P. pinaster	Seedling explants Shoots	Axillary budding Shoots rooted (92 %)	 Basal nutrient medium[9]			7	(0.2)NAA [16 d]	FAYE *et al.* (1989)
P. pinaster	Whole cotyledons (2.5 mm) from aseptically excised embryos Whole cotyledons	Adventitious budding Cotyledon elongation	Solution I (DAVID *et al.* 1982b) Solution I (DAVID *et al.* 1982b)			8 8	(0.1)BA (0.01)IBA [28 d] None [28 d]	TRANVAN *et al.* (1988)
P. pinaster	Vegetative floral buds from 8-10-y-old trees Basal section, male and female sexual organs	Brachyblast and callus production Heterogenous callus	HW (modified) HW (modified)				(1.1)BA (0.2)NAA (1.1)BA (0.2)NAA	HUGUES-JARLET & NITSCH (1988)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. pinaster	Primary explants Axillary buds Shoots Elongated shoots	Axillary budding Shoots Shoot elongation Shoots rooted	MG (macro) MS (micro-elements) MG (macro) MS (micro-elements)[6] Basal nutrient medium + peat:perlite, 1:3		30 20	8 8 8	(4.5)BA (0.002)NAA (0.2)NAA [2-19 d] or (5.6)NAA (30.5)IBA [7 °C, 24 h]	RANCILLAC *et al.* (1982)
P. pinaster	Apical meristems	Organogenesis	MS (modified)	6.0	low			WALKER *et al.* (1985)
P. pinaster	Cotyledonary explants Needles/short shoots Adventitious buds	Adventitious budding Adventitious buds Shoot elongation	CH (macro) MS (micro-elements) ½ MS	5.7	 40 20	10 10 8	(0.2)BA (0.01)NAA BA (0.005)NAA (4.5)BA (0.01)NAA	DAVID *et al.* (1982b)
P. pinaster	Apical and *in vitro*-induced axillary buds from seedlings	Leaf protoplast isolation	P_2[11]					DAVID *et al.* (1986)
P. ponderosa	Cotyledons	Multiple buds					(5)BA	ELLIS (1987)
P. radiata	Sterile seed Cotyledons from 4-5-d-old seedlings	Stratification 4 °C, germination Bud induction	Moist vermiculite SH (modified REILLY & WASHER 1977)			8	(5.6)BA [3 w]	BIONDI *et al* (1986); KUMAR *et al.* (1987); KUMAR & THORPE (1989)
P. radiata	Sterile seed Cotyledons (3-5 mm)	Germination Shoot formation	Moist vermiculite SH (modified REILLY & WASHER 1977)				[6-7 d, dark] (11.3)BA [3 w]; None [3 w]	BIONDI & THORPE (1982)
P. radiata	Sterile seed Cotyledons from 5-7-d-old seedlings	Germination Shoot initiation	SH (modified REILLY & WASHER 1977)				(5.6)BA	PATEL & THORPE (1984)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. radiata	Excised embryos Adventitious shoots Rooted plantlets	Shoot initiation & elongation Root initiation Hardened-off	SH GD (modified) Soil:vermiculite:pumice:peat 4:1:2:1	5.8	30 30	3.7	(0.1-25)BA (0.5)NAA (2)IBA	REILLY & WASHER (1977)
P. radiata	Excised embryos	Cell suspension	SH (modified)[9]				(2)NAA	TEASDALE (1986)
P. radiata	Aseptic seed Cotyledons from 4-5-d-old seedlings	Germination	SH (modified)				(5.6)BA	KUMAR *et al.* (1988)
P. radiata	Mature enclosed buds Shoots Plantlets	Shoot multiplication Shoots rooted, plantlets Hardened-off	LP (modified)[3] GD (modified)[4] Peat:pumice 1:1		20	6	(0.5)NAA (1)IBA [1-2 w]	HORGAN & HOLLAND (1989)
P. radiata	Aseptically excised embryos Shoots	Shoot initiation Shoot elongation	QL (modified AITKEN-CHRISTIE *et al.* 1987) QL (modified AITKEN-CHRISTIE *et al.* 1987)		30 30	8 97:3[10]	(5)BA [16 h photoperiod] None	NAIRN (1988)
P. radiata	Excised embryos Primary needles from germinated seedlings Swollen embryos and primary needles Elongated shoots Shoots	Shoot induction Shoot induction Shoot & needle elongation (2 transfers before rooting) Rooting pre-treatment Shoots rooted (86 %)	SH (no micro-elements) SH (liquid) SH SH Peat:Pumice 1:1 (non-sterile)	5.8	30 20 20	4.5	(5)BA [2 w] (5)BA [2 w] None (2)IBA (0.5)NAA [5 d]	HORGAN & AITKEN (1981)
P. radiata	Sterile seed Cotyledons	Germination Shoot induction	Moist vermiculite SH (modified AITKEN *et al.* 1981)		30		[6 d, dark] (5.6)BA	DOUGLAS *et al.* (1982)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. resinosa	Aseptically excised embryos	Adventitious shoot induction	½ LP (AITKEN-CHRISTIE 1984)				(5.6)BA [10 d]	NOH *et al.* (1988)
	Shoots	Shoot multiplication	½ LP[7] (AITKEN-CHRISTIE 1984)				None	
	Shoots (20-30 mm)	Root induction	½ LP (AITKEN-CHRISTIE 1984)				(0.004)IBA [1 m]	
P. resinosa	Female gametophyte containing embryos	Embryo development	¼ BLG		30		None	GATES & GREEN-WOOD (1991)
P. rigida	Excised embryos and young seedling parts	Multiple shoots					Cyt	PATEL *et al.* (1986)
	Shoots	Shoot elongation					None	
	Shoots	Shoots rooted	Soil				IBA, NAA [5 d, then none]	
P. rigida	Embryos	Shoot production	MS, LM or SH (all modified)				(2 or 5)BA (0.1)NAA	YI (1989)
P. roxburghii	Sterilized seed	Seedling germination	MS		20			MEHRA & ANAND (1983)
	Cotyledons, shoot tips, hypocotyls and roots from 3-5-w-old seedlings	Callus induction	MS	5.8		10	(4)NAA or 2,4-D (1)Kin (15%)CM	
	Excised embryos	Callus induction	GD				(4)NAA (1)Kin (15%)CM	
P. roxburghii	Excised cotyledons	Bud induction	½ MCM	5.8	30	8	(2.3)BA [7 d]	MURIITHI *et al.* (1993)
	Cotyledons (5-w-old)	Bud development	½ MCM[13]		30		(2.2)Zeatin [7 d]	
	Buds	Shoot elongation	½ SH					
	Shoots (24-w-old)	Shoots rooted (80 %)	¼ MCM		10	5(Ge)	(0.1)NAA [4 w]	
	Plantlets	Plantlets hardened-off (85 %)	Peat:vermiculite 1:1 and moistened with ¼ MCM					
P. strobus	2-4-w-old vegetative shoots from 15-18-y-old trees	Callus induction	MS (modified)				BA NAA	KAUL (1986)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. strobus	Embryo explants	Caulogenesis	SH				BA [1 w]	FLINN *et al.* (1986)
P. strobus	Aseptically excised embryos	Caulogenesis (shoot production)	SH (modified REILLY & WASHER 1977)	5.7	30	8	(1)BA	FLINN *et al.* (1989)
P. strobus	Excised embryos	Caulogenesis (shoot production)	SH (modified REILLY & WASHER 1977)	5.7	30	8	(1)BA	FLINN *et al.* (1988)
P. strobus	Cotyledons	Shoot induction	SH (modified REILLY & WASHER 1977)	5.7		8	(1)BA [2 w]	WEBB *et al.* (1988)
	Epicotyls	Primary shoots and callusing	SH (modified REILLY & WASHER 1977)	5.7		8	(0.1-10)BA	
	Hypocotyls	Shoot induction	SH (modified REILLY & WASHER 1977)	5.7		8	(1-25)BA	
	Shoots	Shoot elongation	SH (modified REILLY & WASHER 1977)	5.7		8	None	
	Microshoots	Shoots rooted	¼ SH (modified REILLY & WASHER 1977)	5.7		8	(1)IBA (0.5)NAA [5 d pulse, then none]	
P. strobus	Whole embryo Buds Elongated shoots	Bud induction Bud development, shoot elongation Shoots rooted	MS (modified) MS (modified) Artificial mix (non-sterile)				(2.3)BA [21 d] None (0.003)NAA	SCHWARZ *et al.* (1988)
P. strobus	 Shoots	Shoot growth Shoots rooted (80 % at 17 °C)	LITVAY *et al.* (1981) ½ GD				None (0.5)NAA [2 w]	KAUL (1990)
P. strobus	Embryos Adventitious shoots Shoots Shoots	Adventitious shoot induction Shoot elongation Rooted initiation Shoots rooted (50 %)	LP (modified) ½ LP[1] LP	5.5 5.5 5.5	30 30 30 30	6 10 6	(4.5)BA None (10.2)IBA [8 d] None [light]	CHESICK *et al.* (1991)
P. strobus	Shoots Shoots	Rooting pre-treatment Shoots rooted (43 %) and hardened-off in greenhouse	½ MCM ½ MCM		35		(1.3)Ancymidol (0.1)NAA [pulse]	BURKHART & MEYER (1991)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. sylvestris	Stem segments Stem seg. with buds Elongated shoots Elongated shoots Rooted shoots	Axillary bud break Bud elongation New axillary buds Shoots rooted Root elongation	WPM and QL WPM[2] WPM or QL WPM WPM		20-30	6	(0.4-1)BA (0.1)IBA	CHALUPA (1985a)
P. sylvestris	Cotyledonary explants	Protoplast isolation and fusion: *P. sylvestris* x *P. sylvestris* *P. sylvestris* x *Picea abies*						KIRSTEN *et al.* (1986)
P. sylvestris	Hypocotyls from 20-d-old seedlings	Root development	INGESTAD (1979) medium[12]			7	None	GRÖNROOS & VON ARNOLD (1985)
P. sylvestris	Hypocotyls from 3-5-w-old seedlings Rooted hypocotyls	Direct rooting (77 % in 42 d) Root elongation	¼ Hoagland nutrient solution (ELIASSON 1978) Hydroponic culture				(250)IBA [24 h pulse]	GRÖNROOS & VON ARNOLD (1988)
P. sylvestris		Callus tissue	MS				2,4-D	CHUPKA *et al.* (1990)
P. sylvestris	Shoot tips excised from mature buds of 10-40-y-old trees Purified protoplasts	Protoplast production Microcalli					(0.2)BA (0.2)2,4-D	HOHTOLA & KVIST (1991)
P. sylvestris	Mature apical buds from 8-30-y-old trees (buds subjected to cryopreservation prior to culture initiation)	Callus induction	MS (modified HOHTOLA 1988)		25		(0.2)BA (0.2)2,4-D	KUOKSA & HOHTOLA (1991)
P. sylvestris	Stock plant Fascicular buds	Fascicular bud induction Shoots rooted					Cyt spray treatments (100)IBA or (25)IBA + (25)NAA [24 h]	SALONEN (1991)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. sylvestris	Excised embryos	Callus induction	MS	5.8	30	8	(1.5)Kin (0.5)2,4-D	PEI (1989)
P. taeda	Intact embryos	Bud initiation	GD				(10)BA (0.1 or 0.01)NAA [8 w]	McKEAND & ALLEN (1984)
	Cotyledons	Bud initiation	GD				(1)BA (0.01)NAA [8 w]	
	Buds (7.5 mm)	Buds rooted (30 %)	½ GD		40		(0.1)BA (0.1)NAA [5 d], then none	
P. taeda	Hypocotyls	Bud induction	½ GD (modified MOTT & AMERSON 1981b)				(0.5)NAA (0.1)BA [10-12 d]	FRAMPTON & ISIK (1987)
	Shoots	Root induction	½ GD (mod MOTT & AMERSON 1981b)					
	Excised cotyledons	Cell division activity	BLG				(10)BA (0.01)NAA [4-6 w]	
	Cotyledons	Shoot differentiation and development	½ GD (modified MOTT & AMERSON 1981b)[2]				None [4 w], then no activated charcoal	
	Shoots	Root induction	½ GD (modified MOTT & AMERSON 1981b)					
P. taeda	Cotyledons	Callus induction	MS (liquid, using HELLER (1949) supports & No. 2 Filter paper)		30	-	(3)2,4-D	NEWTON *et al.* (1986); NEWTON *et al.* (1989a); NEWTON *et al.* (1989b); NEWTON *et al.* (1990)
P. taeda	Cotyledonary explants	Shoot induction	GD				BA, NAA, (0.03)ABA	SEN *et al.* (1989)
P. taeda	Embryos	Shoot production	MS, LM or SH (all modified)				(5)BA (0.1 or 0.01)NAA	YI (1989)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. taeda	Hypocotyl and radicle	Callus induction	MS & LV (both modified)		30	7	(11)2,4-d; (0.5)BA	JANG & TAINTER (1991b)
P. taeda	Cotyledonary explants	Meristematic tissue induction	GD (modified)				(20)BA (0.01)NAA	JANG & TAINTER (1991a)
P. taeda	Cotyledons Shoot buds	Shoot bud induction Shoot elongation	GD ½ GD			 20	(0.01)NAA (10)BA (0.026)ABA [1-4 w] None	NEUMAN *et al.* (1992)
P. taeda x *P. echinata*	Seed Meristematic tissue Shoots Elongated shoots	Meristematic tissue induction Shoot formation Shoot elongation Root induction and growth	GD (modified) ½ GD[2] GD GD (modified)[7]		 30 20	7 7 8 	(20)BA (0.01)NAA None None (0.5)NAA (1)IBA	JANG & TAINTER (1991a)
P. taeda x *P. rigida*	Embryos	Shoot production	MS or LM (both modified)				(1.2 or 5)BA (0.1)NAA	YI (1989)
P. tecunumanii	Elongated shoots	Shoots rooted (50 %)	Peat:perlite 2:1					BAXTER *et al.* (1989)
P. thunbergii	Hypocotyls	Callus induction					(2.2)2,4-D	KONDO & KUSHIMA (1987)
P. thunbergii	Mature embryos Shoots	Bud induction Shoot elongation					BA	ISHII (1988)
P. thunbergii	Excised embryos Adventitious buds Shoots Rooted plantlets	Adventitious buds Shoot elongation Root induction (<40 %) Hardened-off in greenhouse	⅔ GD Soil				BA, NAA None NAA, IBA, Riboflavin	FUKUDA *et al.* (1989)

Species	Explant	Morphogenesis	Media	pH	Sucrose (g l^{-1})	Agar (g l^{-1})	Hormones (mg l^{-1})	References
P. virginiana	Cotyledonary explants	Meristematic tissue induction	GD (modified)			7	(10)BA (0.01)NAA	JANG & TAINTER (1991a)
	Meristematic tissue	Shoot formation	½ GD[2]		30	7		
	Shoots	Shoot elongation	GD		30	8	None	
	Elongated shoots	Root induction and growth	GD (modified)[7]		20	8	(0.5)NAA (1)IBA	
P. virginiana	Cotyledonary explants	Adventitious shoots	BLG (modified AMERSON *et al.* 1985)	5.5	30	8	(10)BA	SARAVITZ *et al.* (1991)
	Excised shoots	Shoot elongation	GD[2] (modified MOTT & AMERSON 1981b)	5.5	20	8	None	
	Elongated shoots	Shoots rooted (50 %)	½ GD	5.5	10	8	(5-40)IBA	
	Rooted shoots	Hardened-off (± 100 %)	Peat/vermiculite					
P. virginiana	Hypocotyl and radicle	Callus induction	MS & LV (both modified)		30	7	(11)2,4-D (0.5)BA	JANG & TAINTER (1991b)
P. virginiana	Cotyledonary explants	Shoot induction	GD				(5)BA (0.01)NAA	CHANG *et al.* (1991)
	Shoots	Enhanced shoot formation by 65 %	GD				7.6 ABA (5)BA (0.01)NAA	
	Shoots	Root initiation	½ GD				(0.2)NAA (0.2)IBA (0.1)BA	

Table 3.2 Abbreviations used in the text and in Table 3.1.

AE	VON ARNOLD & ERIKSSON (1981) Medium
BLG	BROWN & LAWRENCE (1968) Medium
CD	CAMPBELL & DURZAN (1975) Medium
CH	CHONG (1977) Medium (in DAVID, DAVID & MATEILLE 1982b)
DCR	Douglas-fir Cotyledon Revised Medium (GUPTA & DURZAN 1985)
D medium	CAMPBELL & DURZAN (1975)
G	Glucose
Ge	Gelrite
GD	GRESHOFF & DOY (1972) Medium
HW	HALPERIN & WETHERELL Medium (in HUGUES-JARLET & NITSCH 1988)
LAINÉ medium C	LAINÉ, DAVID & DAVID (1988)
LP	LEPOIVRE (1977) Medium (in QUOIRIN & LEPOIVRE 1977)
LV	LITVAY Medium (LITVAY, JOHNSON, VERMA, EINSPAHR & WEYRAUCH 1981)
LM	LLOYD McCROWN (1981) Medium
MCM	Medium for Conifer Morphogenesis (BORNMAN 1981)
MG	MARGARA (1977) Medium
MS	MURASHIGE & SKOOG (1962) Medium
QL	QUOIRIN & LEPOIVRE (1977) Medium
RW	RISSER & WHITE (1964) Medium
SH	SCHENK & HILDERBRANDT (1972) Medium
WPM	Woody Plant Medium (LLOYD & McCROWN 1981)

Table 3.3 Annotations with regard to media supplements in Table 3.1.

1	Medium contained 0.2 % activated charcoal.
2	Medium contained 1 % activated charcoal.
3	Medium modified as per AITKEN-CHRISTIE, SINGH & DAVIES (1987) containing 0.5 % activated charcoal.
4	Medium modified as per SOMMER, BROWN & KORMANIK (1975).
5	Medium modified as per ABDULLAH, YEOMAN & GRACE (1985)
6	Medium contained 2 % activated charcoal
7	Medium contained 0.5 % activated charcoal.
8	Medium contained 0.1 % activated charcoal.
9	Medium was modified to included 2.6 mM ammonium phosphate and 10 mM argenine hydrochloride. Nitrogen supply was a mixture of nitrate (3.3 mM as N) and glutamine (2 mM as N). Referred to as SHR4 medium.
10	97:3 - Gelrite:Difco Bacto Agar
11	Macro-elements from BJÖRKMAN (1942) and micro-elements from HELLER (1953).
12	The medium contained 0.4 mg l^{-1} thiamine-HCl and 100 mg l^{-1} myo-inositol. Nitrogen concentration in the nutrient solution was 0.7 $mmol^{-1}$ and 0.1-20 g l^{-1} activated charcoal was added.
13	Medium contained 0.05 % activated charcoal.

CHAPTER 4

SOMATIC EMBRYOGENESIS

4.1 Introduction

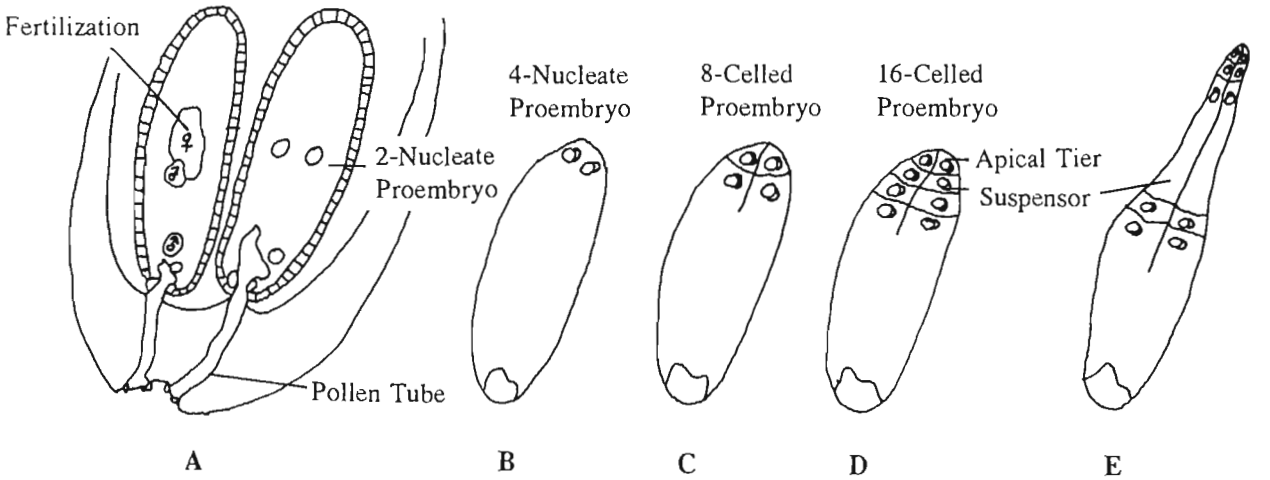
Somatic embryogenesis (asexual or adventive embryogenesis) is a process analogous to zygotic embryogenesis, in which a single cell or group of vegetative (somatic) cells become the precursors for the development of embryos (AMMIRATO 1983). Early in the study of cell suspension systems, STEWARD, MAPES & MEARS (1958) discovered that when treated with coconut milk, carrot cells did not continue to multiply but differentiated into miniature embryo-like structures called embryoids. A similar effect was simultaneously produced using a high auxin concentration (REINERT 1959). Since then many species have been found to have the capacity for somatic embryogenesis either through the selection of embryogenically competent cells or tissues or by their induction from the culture treatment. The process of embryogenesis is similar in most conifers, but differs significantly from the processes that usually occur in angiosperms. Conifer somatic embryogenesis is also still a relatively new area of research, in comparison to the progress made with the study of embryogenesis in angiosperms.

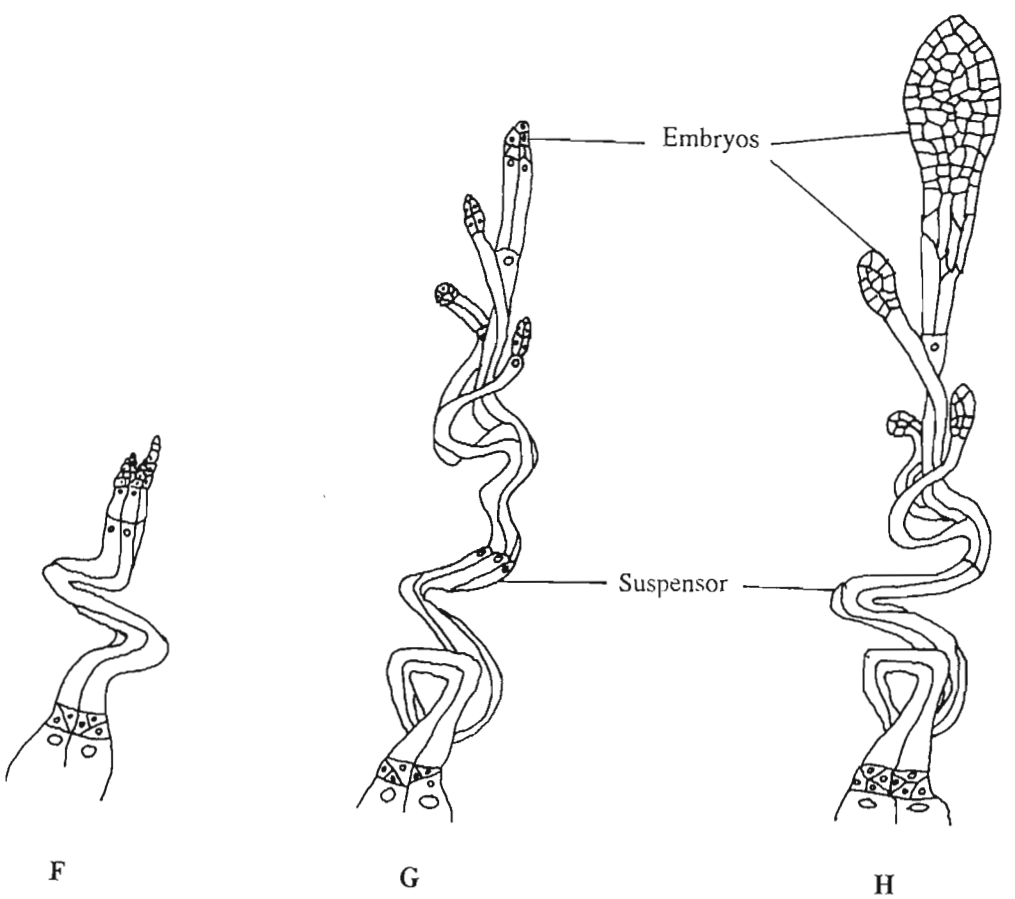
Studies into the growth, metabolism and developmental patterns that characterize callus and cell suspensions of conifers have been conducted from as early as 1968 (DURZAN & STEWARD 1968; CHALUPA & DURZAN 1973; DURZAN & CHALUPA 1976a; 1976b; 1976c; 1976d; DURZAN 1980). During the course of these early investigations, polarized, organized structures, resembling embryos (embryoids) were noticed, although further differentiation into plantlets was not observed (DURZAN & GUPTA 1987). These structures later became known

as sphaeroblasts to distinguish them from somatic embryos (DURZAN & GUPTA 1988). Similar cell aggregates are still observed today in non-embryogenic cultures (TAUTORUS 1990).

Within the Pinaceae, embryogenic cultures are translucent to white, and when cultured on solid media, appear glossy and mucilaginous in nature. Cultures consist primarily of somatic embryos, which are polarized structures possessing a densely meristematic embryonic region subtended by elongated, vacuolated suspensor cells (HAKMAN, FOWKE, VON ARNOLD & ERIKSSON 1985; ATTREE & FOWKE 1993). Many authors have termed these cultures calli. However, due to the presence of highly organized structures, the terms "embryogenic tissue" or "embryonal suspensor masses" are more appropriate (TAUTORUS, FOWKE & DUNSTAN 1991; ATTREE & FOWKE 1993). Furthermore, the inappropriate use of terminology reserved specifically for zygotic embryogenesis, to describe processes that occur during somatic embryogenesis, has led to some confusion. It has therefore become acceptable to use the terms (such as stage 1 to describe the proembryo stage) introduced by VON ARNOLD & HAKMAN (1988) to characterize somatic embryo development.

Unlike organogenesis, somatic embryogenesis has the ability to recapitulate the events of zygotic embryogeny *in vitro*, thus producing bipolar structures with the capacity for both root and shoot production. Two natural processes may occur in conifer embryogeny: simple or cleavage polyembryony. Simple or archegonial polyembryony occurs in both *Picea* and *Pinus* species. During this process, different pollen grains may fertilize more than one egg per ovule, resulting in the development of genetically different proembyros (SINGH 1978). Usually only one of the proembryo completes development while the others degenerate.


Fertilization
2-Nucleate
Proembryo
Pollen Tube
4-Nucleate
Proembryo
8-Celled
Proembryo
16-Celled
Proembryo
Apical Tier
Suspensor
A
B
C
D
E


Embryos
Suspensor
F
G
H

In addition *Pinus* species undergo cleavage polyembryony (BUCHHLOZ 1926; OWENS & MOLDER 1984) in which multiple embryos arise from a single zygote (Fig. 4.1). The apical tier cells of the proembryo divide vertically into four files of cells, each of which has the potential to develop into a separate embryo. These resulting embryos are therefore genetically identical. One of the embryos becomes dominant, while the others abort. Polyembryony is thought to be a primitive gymnosperm character, useful for the elimination of unfit embryos (BUCHHOLZ 1918). The selective pressures involved in determining which embryo becomes dominant and which degenerate, are unknown, although mechanical, nutritional and growth suppressing influences may be involved (BUCHHOLZ 1918; DOGRA 1967; OWENS & BLAKE 1985).

Depending on the original explants used, three possible modes of *in vitro* embryogenesis have been identified. These processes are known to occur in angiosperms and to a lesser extent in gymnosperms, since very little is currently known about the origin of somatic embryogenesis in conifers (TAUTORUS, FOWKE & DUNSTAN 1991).

4.1.1 Somatic Adventitious Embryogenesis

Somatic embryos may develop from cells or callus associated with the reproductive apparatus. These structures have been referred to as pre-embryonically determined cells (PEDC). Embryogenic tissue can arise from nucellus tissue or very young ovule tissue of polyembryonic or monoembryonic species eg. grasses (LITZ 1987), grape (MULLINS 1987) and coffee (SONDAHL & MONACO 1981). This method is useful in clonal propagation, since the cultured explants have the same genotype as the stock plant, resulting in genetically identical plantlets.

Adventitious embryos may also arise from single cells or small cell clusters on the surface of immature zygotic embryos in culture, by an initial asymmetric

division that delimits the embryonal apex and suspensor region (HAKMAN, RENNIE & FOWKE 1987; NAGMANI, BECWAR & WANN 1987; JAIN, DONG & NEWTON 1989). Somatic embryos may also arise indirectly from the callus produced from cell clusters (SHARP, SONDAHL, CALDS & MARAFFA 1980). Although this proliferation involves somatic tissue, the original explant is zygotic in origin and would not duplicate the genotype of the source plant. This type of culture would therefore not be suitable for clonal propagation, but rather for genetic improvement programmes, where the culture treatment would function to "rescue" embryos that would naturally be aborted.

4.1.2 Somatic Polyembryogenesis

This type of embryogenesis involves the culture of the highly embryogenic, embryonal-suspensor masses (ESM) (small meristematic cells within the suspensor) obtained from very immature gymnosperm ovules approximately two to four weeks after fertilization. Under suitable culture conditions, proliferation of the ESM can result in direct embryo formation. The type of tissue produced is unique and can easily be distinguished from non-embryogenic callus, by its translucent to white mucilaginous nature. Somatic polyembryogenesis is particularly relevant in gymnosperms, but can also occur in angiosperms.

Somatic embryos can arise via a method similar to cleavage polyembryony. This has been observed to occur in cultures of *Abies alba* (SCHULLER, REUTHER & GEIER 1989), *Larix decidua* (NAGMANI & BONGA 1985; VON ADERKAS & BONGA 1988) and *Pinus* species (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988; VON ARNOLD & WOODWARD 1988). The term somatic polyembryogenesis has therefore been used to describe the origin of adventive embryos from the zygotic suspensor cells of *Pinus lambertiana*. GUPTA & DURZAN (1986b) state that *Pinus* somatic embryogenesis is a true reflection of zygotic polyembryony.

4.1.3 Induced Somatic Embryogenesis

Specific treatments are used to induce embryogenic competence in the explants being cultured. This concept is based on a general biological phenomenon (KRIKORIAN 1982; WILLIAMS & MAHESWARAN 1986; PIERIK 1987) and usually requires prior callus proliferation before embryos can originate from the induced embryogenic cells. Although individual carrot cells are totipotent, isolated single cells do not readily become transformed into embryos by repeated divisions and, therefore require a callus induction stage before embryoids can be produced (DODDS & ROBERTS 1985). This process has been successful with a number of crop species, including carrot (LUTZ, WONG, ROWE, TRICOLI & LAWRENCE 1985), alfalfa (STUART, STRICKLAND & WALKER 1987), grasses (VASIL 1985), coffee (SONDAHL & MONACO 1981), palm species (TISSERAT, ESAN & MURASHIGE 1979) and soybean (CHRISTIANSON 1985).

4.2 Historical Background

Embryogenic culture initiation from conifers was first established from immature zygotic embryos of *Picea abies* L. (HAKMAN, FOWKE, VON ARNOLD & ERIKSSON 1985). CHALUPA (1985b), simultaneously described embryogenic induction from the same species and NAGMANI & BONGA (1985) reported on the induction of haploid European larch (*Larix decidua* Mill.) embryos from megagametophyte tissue. Since then, somatic embryogenesis has been achieved in gymnosperms such as *Picea glauca* (HAKMAN & FOWKE 1987b; LU & THORPE 1987); *P. mariana* (HAKMAN & FOWKE 1987b); *Pinus taeda* (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988); *P. caribaea* (LAINÉ & DAVID 1990) and *Larix decidua* (NAGMANI & BONGA 1985), with plantlet regeneration occurring in *Pinus lambertiana* (GUPTA & DURZAN 1986a) and *P. taeda* (GUPTA & DURZAN 1987b). Induction of embryogenic tissue in other *Picea* and *Pinus* species is detailed in Table 4.1.

Current research appears to be focused on methods of optimising somatic embryo maturation and improving the hardening-off potential of the "emblings". This term is used to describe plantlets derived from somatic embryos (LIBBY 1986).

Embryogenic cell suspension cultures have also been initiated and serve as a useful source from which protoplasts can be obtained. Using protoplasts, regeneration and gene transfers can be conducted. Somatic embryos have been regenerated from protoplasts of *Pinus taeda* (GUPTA & DURZAN 1987b) and *Picea glauca* (ATTREE, BEKKAOUI, DUNSTAN & FOWKE 1987). Studies have also been initiated on the analysis of molecular and biochemical events that occur during induction and maturation. Culturing zygotic embryos and gametophytes *in vitro* have also been useful in the study of nutritional requirements of developing embryos and their morphogenesis (NORSTOG 1982). It was found that during the *in vitro* culture of *Larix decidua* proembryos, cleavage polyembryony was mainly related to nutrient availability, and was not only an invariable attribute of the embryo (STERLING 1949). Similar observations were made for *Pinus* (WOODS 1953), for which polyembryony appeared to be a result of physiological conditions with no phylogenetic relevance. Furthermore, since somatic embryos may arise from a single cell (NAGMANI, BECWAR & WANN 1987), regenerated plantlets are uniform, showing less chromosomal variation (HANNA, LU & VASIL 1984). Plantlets derived in such a manner would be ideal for clonal propagation of forest trees.

Table 4.1 Induction of somatic embryogenesis and production of plantlets in conifer species.

Species and explant	Basal media[a]	Embryogenic tissue (%)	Plantlet production	References
Picea abies				
Female gametophyte[b]	N6	Not reported	Unsuccessful	SIMOLA & SANTANEN (1990)
Immature embryos	MS	38	Plantlets produced and established in soil	CHALUPA (1985b)
Immature embryos	LP	50	Unsuccessful	HAKMAN *et al.* (1985)
Immature embryos	LP	95	Plantlets produced	HAKMAN & VON ARNOLD (1985)
Immature embryos	LP	78	Plantlets produced	BECWAR *et al.* (1987a)
Immature embryos	LP	Not reported	Plantlets produced and established in soil	VON ARNOLD & HAKMAN (1988); BECWAR *et al.* (1989)
Immature embryos	½LP	Not reported	Plantlets produced	HAKMAN *et al.* (1990)
Immature embryos	LP	72	Not reported	BECWAR *et al.* (1988)
Immature embryos	HFAE	Not reported	Not reported	NAGMANI *et al.* (1987)
Immature embryos	HFAE	>75	Plantlets produced and established in soil	BECWAR *et al.* (1987a)
Immature embryos	LP	>75	Plantlets produced and established in soil	BECWAR *et al.* (1987b)

Species and explant	Basal media	Embryogenic tissue (%)	Plantlet production	References
Picea abies				
Mature embryos	MS	8	Plantlets produced	CHALUPA (1985b)
Mature embryos	½MS	6	Plantlets produced	GUPTA & DURZAN (1986a)
Mature embryos	LP	11	Not reported	VON ARNOLD & HAKMAN (1986)
Mature embryos	½LP	50	Plantlets produced	VON ARNOLD (1987)
Mature embryos	½MS	Not reported	Plantlets produced	BOULAY *et al.* (1988)
Mature embryos	LP	55	Plantlets produced	JAIN *et al.* (1988)
Mature embryos	½LP	Not reported	Plantlets produced and established in soil	VON ARNOLD & HAKMAN (1988)
Mature embryos	MSG	32	Plantlets produced	VERHAGEN & WANN (1989)
Mature embryos	½LP	45	Plantlets produced	MO *et al.* (1989)
Mature embryos	½BLG	25	Not reported	BECWAR *et al.* (1988)
Mature embryos	½LP		Unsuccessful	NOLLET & DEBERGH (1989)
Cotyledons	½MS	5	Not reported	KROGSTRUP (1986)
Cotyledons	MS	23	Not reported	LELU *et al.* (1987, 1990)
Picea abies				
Somatic embryos	½LP	81	Plantlets produced	MO *et al.* (1989)
Somatic embryos	MS	80	Not reported	RUAUD *et al.* (1992)
Zygotic embryos	MS	10	Not reported	RUAUD *et al.* (1992)
Somatic needles of 14-m-old plantlet	MS	3	Not reported	RUAUD *et al.* (1992)
Somatic cotyledons	GD	12-50	Plantlets produced from all explant sources	RUAUD (1993)
Zygotic cotyledons	GD	0-21		
Somatic needles	GD	20.8		
Picea engelmanii				
Mature embryos	LP	11.3	Plantlets produced	WEBB *et al.* (1989)

Species and explant	Basal media	Embryogenic tissue (%)	Plantlet production	References
Picea glauca				
Not reported	½LP	Not reported	Plantlets produced	ATTREE *et al.* (1992)
	LP	Not reported	Plantlets produced	ATTREE *et al.* (1989a)
Immature embryos	LP	22	Plantlets produced	HAKMAN & FOWKE (1987a)
Immature embryos	LP	67	Plantlets produced	LU & THORPE (1987)
Immature embryos	Not reported	Not reported	Plantlets produced	ATTREE *et al.* (1990b)
Mature embryos	½LM	51	Plantlets produced and established in soil	TREMBLAY (1990)
Mature embryos	LP	11.3	Plantlets produced	WEBB *et al.* (1989)
Cotyledons	LP	38	Not reported	ATTREE *et al.* (1990a)
Cotyledons	MS	6	Plantlets produced	LELU & BORNMAN (1990)
Picea glauca - engelmannii complex				
Immature embryos	LP	60	Plantlets produced	WEBB *et al.* (1989)
Immature embryos	LP	60	Not reported	ROBERTS *et al.* (1989)
Immature embryos	LP	Not reported	Plantlets produced, established in the soil and used in field trial	ROBERTS *et al.* (1990a); WEBSTER *et al.* (1990)
Picea glehnii				
Mature embryos			Plantlets produced and established in the soil	ISHII (1991)
Picea jezoensis				
Mature embryos			Plantlets produced and established in the soil	ISHII (1991)

Species and explant	Basal media	Embryogenic tissue (%)	Plantlet production	References
Picea jezoensis var. *hondoensis*				
Mature embryos			Plantlets produced and established in the soil	ISHII (1991)
Picea mariana				
Immature embryos	LP	5	Plantlets produced	HAKMAN & FOWKE (1987b)
Immature embryos	LP	Not reported	Plantlets produced and established in soil	ATTREE *et al.* (1990b)
Mature embryos		8	Plantlets produced and established in soil	CHELIAK & KLIMASZEWSKA (1991)
Mature embryos	LP	8	Protoplasts produced in all cases, with somatic embryo development on LV, but no plantlets produced	TAUTORUS *et al.* (1990)
Mature embryos	½ LV	10		
Immature embryos	LP	65		
Cotyledons	MS	26	Plantlets produced	LELU & BORNMAN (1990)
Picea omorika				
Shoot explants	LP	6	Plantlets produced and established in the soil	BUDIMIR & VUJIČIĆ (1992)
Picea rubens				
Mature embryos	LP	17	Plantlets produced and established in soil	HARRY & THORPE (1991)

Species and explant	Basal media	Embryogenic tissue (%)	Plantlet production	References
Picea sitchensis				
Immature embryos	MS	5	Plantlets produced and established in soil	KROGSTRUP *et al.* (1988)
Immature embryos	LP	Not reported	Plantlets produced and established in the soil	ROBERTS *et al.* (1991a)
Mature embryos	½LP	20	No plantlets produced	VON ARNOLD & WOODWARD (1988)
Picea wilsonii				
Immature embryos	LP	Not reported	Plantlets produced	LI & GUO (1990)
Immature embryos			Plantlets produced	YING-HONG & ZHONG-SHEN (1990)
Pinus caribaea				
Female gametophyte[b]	½LP	2	Plantlets produced	LAINÉ & DAVID (1990)
Pinus contorta var. *latifolia*				
Mature leaf explants			Unsuccessful	MACDOUGALL *et al.* (1988)
Pinus elliottii				
Immature embryos	WPMG	6	Unsuccessful	JAIN *et al.* (1989)
Pinus lambertiana				
Immature embryos	DCR	Not reported	Plantlets produced[c]	GUPTA & DURZAN (1986b)
Mature embryos	DCR	5		
Pinus nigra				
Immature embryos	DCR	2	Unsuccessful	SALAJOVÁ & SALAJ (1992)

Species and explant	Basal media[a]	Embryogenic tissue (%)	Plantlet production	References
Pinus serotina				
Female gametophyte[b]	MSG	12	Not reported	BECWAR *et al.* (1988)
Pinus strobus				
Female gametophyte[b]	DCR	54	Unsuccessful	FINER *et al.* (1989)
Female gametophyte[b]	DCR	64	Unsuccessful	KRIEBEL & FINER (1990)
Immature embryos	DCR	3	Unsuccessful	FINER *et al.* (1989)
Immature embryos	MSG/DCR	2.7	Not reported	BECWAR *et al.* (1988)
Pinus taeda				
Female gametophyte[b]	½MS	10	Plantlets produced and established in soil	GUPTA & DUZAN (1987b)
Female gametophyte[b]	MSG	3	Unsuccessful	BECWAR *et al.* (1990)
Immature embryos	DCR	5	Unsuccessful	BECWAR *et al.* (1990)
Immature embryos	DCR	9	Not reported	BECWAR *et al.* (1991)
Immature embryos	DCR	2.3	Not reported	BECWAR *et al.* (1988)

Table 4.2 Notes pertaining to Table 4.1

a Basal media used for induction of somatic embryogenesis; some basal media have been adjusted, consult references for modifications:

BLG	BROWN & LAWRENCE (1968) Medium
DCR	Douglas-fir Cotyledon Revised Medium (GUPTA & DURZAN 1985)
GD	GUPTA & DURZAN (1986a) Medium
HFAE	HAKMAN, FOWKE, VON ARNOLD & ERIKSSON (1985) Medium
LP	VON ARNOLD & ERIKSSON (1981) Medium
LV	LITVAY Medium (LITVAY, JOHNSON, VERMA, EINSPAHR & WEYRAUCH 1981)
MS	MURASHIGE & SKOOG (1962) Medium
MSG	MS modified as per BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI (1988)
N6	CHU, WANG, SUN, HSU, YIN, CHU & BI (1975)
WPMG	LLOYD & McCROWN (1981) Medium

b Containing zygotic embryo

c Authors did not indicate which original explant was used for plantlet production

CHAPTER 5

PLANT MATERIALS

5.1 Introduction

In order to initiate studies on the somatic embryogenesis of pine, it was essential to have an appropriate explant source. This involved selecting suitable donor trees. Due to the initial uncertainty of the period during which explants would be most responsive to embryogenic induction in culture, a pilot study was conducted from December 1991 to February 1992, prior to the actual investigation. The main study, based on the results obtained from the initial analysis was conducted from December 1992 to March 1993. Results of both the trial run (1991/92) and the subsequent study (1992/93) will be presented. Numerous authors have shown that in pine, immature seeds are the most suitable explants from which to initiate embryogenic cultures (KRIEBEL & FINER 1990; BECWAR, BLUSH, BROWN & CHESICK 1991; SALAJOVÁ & SALAJ 1992). On this basis, trees bearing large numbers of immature cones were sought.

5.2 Study Site Selection and Description

The pilot study site was located on De Rust Plantation near Greytown in the Natal Midlands region and consisted of twenty-five-year-old, open-pollinated, commercial *P. patula* stands, provided by SAPPI Forests Research. This region contains flat crest areas with moderate slopes ranging from 1 400 to 1 600 m. The underlying geology is composed mainly of dolerite (96 %), with deep, yellow-brown or red subsoils and frequently humic top soils (PALLETT & MITCHELL 1993).

For the subsequent study (1992/93), a closer location was sought and again SAPPI Forests provided the material. The site was located at Pinewoods Plantation also in the Natal Midlands (29° 39' S 30° 4' E). The topography of this area is flat to undulating with slopes between 1 400 and 1 600 m above sea level. The underlying geology is mudstone and sandstone of the Adelaide (47 %) and Tarkastad (24 %) Formations and dolerite (29 %). The major soil group (52 %) is deep (600 to 1 200 mm) with apedal subsoils and frequently humic topsoils. The minor soil group (32 %) is shallow (100 to 600 mm) and contains lithocutanic subsoils (Pallett & Mitchell 1993).

The climate of both areas is similar, described as fairly cool, with a mean annual temperature of 15.2 °C. The mean monthly maximum temperature for January is approximately 24.1 °C, while the monthly minimum for July has been recorded as 3.9 °C. The estimated mean annual rainfall for both regions is 900 mm. The softwood species particularly suited for growth on the sites is *P. patula*.

5.3 Experimental Design

The aim of the pilot study on the De Rust site was to determine whether embryogenesis could, firstly, be induced from *P. Patula* and, secondly, when the optimum collection period was, in order to stimulate an embryogenic response in culture. On this basis, trees which bore large numbers of easily accessible, immature cones were primarily selected. A pine tree was felled on a weekly basis and all the immature female cones collected for culture initiation. This method ensured that half-sib material from various pollen origins was tested every week. It also proved to be a costly and time-consuming operation.

In the second study, five experimental trees were again selected on their cone bearing potential, in order to ensure that sufficient cones could be collected from each tree repeatedly for an extended period of time. Trees were marked and numbered and used throughout the study period.

5.4 Cone Collection

Green, ovulate cones were collected from each individual on a weekly basis. Initially immature cones were collected on a fortnightly basis for embedding purposes (described further in Chapter 10) and, later, on a weekly basis for placement in culture. The collections for culture purposes began just after the stage when fertilization was thought to occur and continued until seeds were nearing maturity i.e. the stage when cotyledon development was first observed. The collection period was during the summer months from 3 December 1991 to 10 February 1992 in the initial study, and from 1 December 1992 to 1 March 1993 in the second investigation. Ladders of various heights and extended pruning shears were used to reach cones in the upper branches. The collected cones were transported in brown paper bags and maintained at 4 °C. All cones were placed into culture within 1 or 2 days of collection. Very young, newly developed cones were also collected for anatomical purposes.

Two-year-old cones containing mature seed were also collected from each tree in the latter study for use as an embryogenic explant source and for histological studies. Seeds were extracted by placing the cones in boiling water for 30 to 45 s and then into a drying oven, to enable the ovuliferous scales to open. Once the scales had opened, the seeds were easily removed and separated from the wing. Seeds were stored in a sealed container at 4 °C until required.

Embryogenic induction and very limited maturation was obtained from the tissue produced in the pilot study. The embryogenic tissue induced in the second study was subjected to maturation procedures, suspension culture initiation and lipid analyses.

CHAPTER 6

INDUCTION OF SOMATIC EMBRYOGENESIS

6.1 Introduction

Various embryonic explants have been used to initiate somatic embryogenesis. These include the megagametophytes, immature and mature zygotic embryos and reinduced cotyledonary somatic embryos. The most mature explant tissue reported has been young seedling material (TAUTORUS, FOWKE & DUNSTAN 1991). The review in the previous Chapter, shows that the most common explants for embryogenic induction from conifer species are both mature and immature seed in the case of *Picea*. It appears that immature embryos are more responsive for induction in *Pinus* species. In pine studies both excised immature zygotic embryos (BECWAR, NAGMANI & WANN 1990) and the female gametophyte containing the immature embryo (GUPTA & DURZAN 1987b; BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988; BECWAR, NAGMANI & WANN 1990) have been used as an explant source. An initiation frequency of 6 % was obtained using isolated zygotic embryos of *Pinus elliottii* (JAIN, DONG & NEWTON 1989), while initiation as high as 54 % has been reported for *Pinus strobus*, using gametophytic explants (FINER, KRIEBEL & BECWAR 1989).

Both, explant selection and developmental stage of the explant are critical factors in the establishment of embryogenic tissue (VASIL 1987). In *Picea abies*, the most appropriate developmental stage for the induction of embryogenic tissue is at the time of cotyledon primordia formation (HAKMAN & FOWKE 1987b; LU & THORPE 1987; BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988). The most responsive explant

stage for *Pinus* species has yet to be adequately determined, but has been described by BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI (1988), as the precotyledonary immature embryo stage. In *Pinus* species especially, it has been observed that the embryogenic induction frequencies are greatly reduced, with increasing maturity of the zygotic explant (CHANDLER, BATEMAN, BLOMSTEDT, WILLYAMS & YOUNG 1989; JAIN, DONG & NEWTON 1989; BECWAR, NAGMANI & WANN 1990; LAINÉ & DAVID 1990). The sensitivity of the explant to forming embryogenic tissue, has also been observed with Douglas-fir (DURZAN & GUPTA 1987) and diploid larch (KLIMASZEWSKA 1989). These observations suggest that in *Pinus*, precotyledonary explants are most suitable for the induction of embryogenic tissue. These considerations will be discussed further in Chapter 10.

In order to prolong the period during which seeds are available for experimentation, some researchers have successfully attempted to refrigerate seed cones prior to embryo dissection. In some cases, this procedure was found to increase induction frequency of embryogenesis (ATTREE, DUNSTAN & FOWKE 1991). The length and conditions of storage are critical, though. Storage for two months at 4 °C was optimal for *Picea glauca* seeds (HAKMAN & FOWKE 1987b). The storage container is also of importance, sealed paper bags being superior to plastic holders. Extended storage can result in severe reduction in embryogenic induction, possibly as a result of excessive desiccation or further maturation of embryos (TAUTORUS, FOWKE & DUNSTAN 1991). Induction of embryogenic tissue from older embryonic material, such as seedling explants, has only been achieved in *Picea* species. The implications of this are that the process of tissue culture is less labour-intensive and seedlings themselves may eventually become incorporated in a process of selecting superior trees, thereby making somatic embryogenesis a more powerful tool to work with. Despite the various explants used to induce embryogenic tissue, it has been shown that factors such as the age of seeds, period of storage, imbibition time of seeds, as well as geographical provenance, play a role in

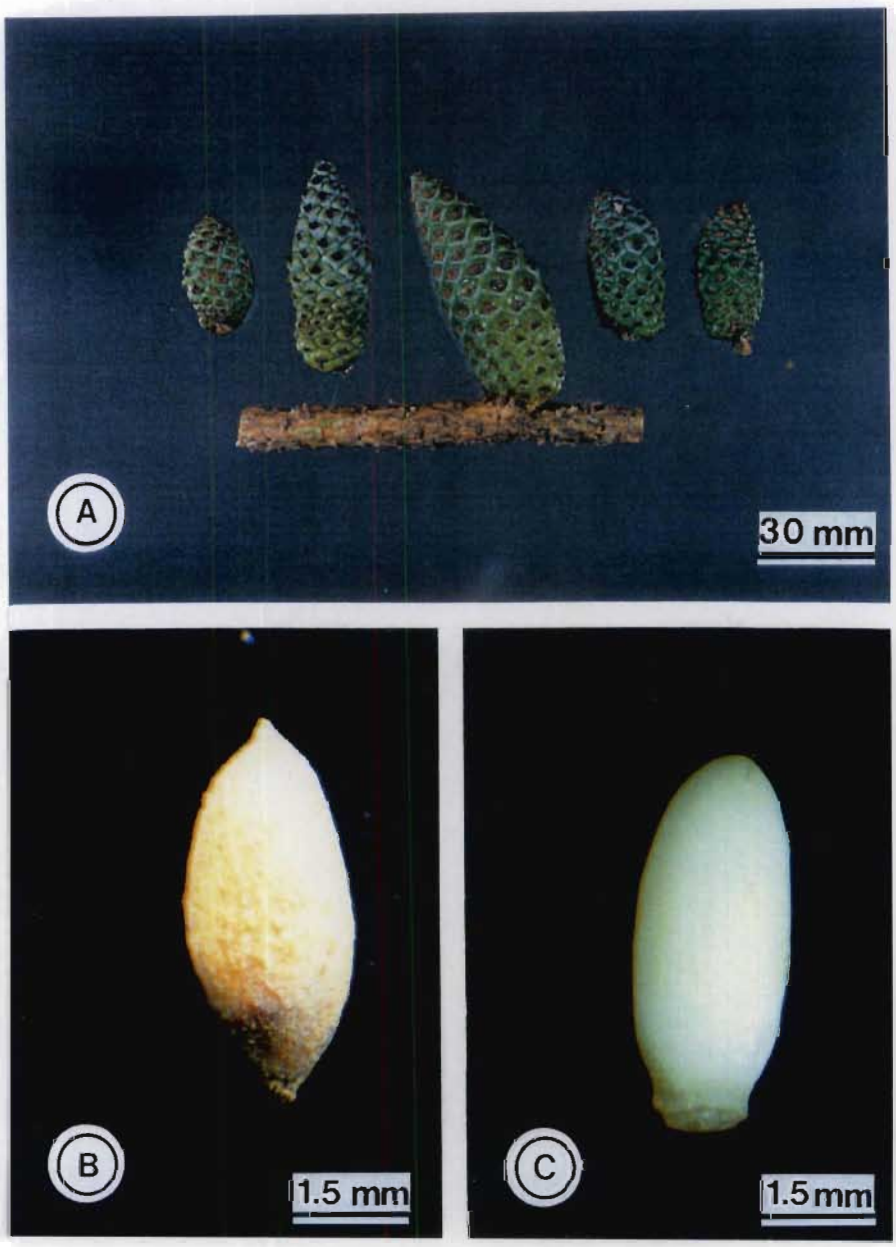
determining the embryogenic potential of the explant (TREMBLAY 1990).

The nutrient media and hormonal additives used, also play a great role in determining the embryogenic potential of explants and the ability to initiate embryogenic tissue from mature explants (VON ARNOLD 1987; JAIN, NEWTON & SOLTES 1988). The factors to consider in attempting to initiate embryogenic cultures are: light regimes; concentration of the basal medium, especially that of sucrose; the composition and level of nitrogen, mineral elements, agar, plant growth regulators and pH. Culturing immature and mature zygotic embryos on a modified Murashige and Skoog (MS) medium (MURASHIGE & SKOOG 1962) containing 2.2 to 4.4 mg l^{-1} 2,4-D and 1.1 mg l^{-1} BA, resulted in the production of a translucent to white mucilaginous tissue (TAUTORUS, FOWKE & DUNSTAN 1991). Through the use of light microscopy, distinct polarized structures resembling immature zygotic embryos could be seen emerging from the tissue.

6.2 Materials and Methods

6.2.1 Sterilization Procedures

The harvested cones (Fig. 6.1A) were washed briefly in running tap water before being immersed into 75 % ethanol (containing a few drops of Tween® 20 per l) for 4 min. They were then surface sterilized using 1.3 % NaOCl (containing Tween® 20) for 20 min, before being rinsed several times with sterile distilled water. The immature seed (Fig. 6.1B) were extracted aseptically and the intact female gametophyte excised microscopically. The gametophyte, containing the immature zygotic embryo served as the explant (Fig. 6.1C). Two-year-old mature seeds were used as an alternative source from which to induce embryogenic tissue. One hundred and twenty five explants were placed on five different media on a weekly basis in order to determine the optimal period of explant responsiveness.


A
30 mm
B
1.5 mm
C
1.5 mm

6.2.2 Media and Culture Conditions

Two basal media were used for embryogenic induction, the first consisted of MSG (BECWAR, NAGMANI & WANN 1990) a modification of MS (MURASHIGE & SKOOG 1962) in which NH_4NO_3 was replaced by L-glutamine; the KNO_3 level was reduced and KCl was added. The second was Douglas-fir Cotyledon Revised medium, DCR (GUPTA & DURZAN 1985), which also contained elevated levels of L-glutamine, although less concentrated than the MSG media. The complete formulation of the media employed to initiate and maintain somatic embryo development was based on that used by BECWAR, NAGMANI & WANN (1990) (Table 6.1). Variations in the media supplements and growth regulators to create five induction media (MSG1, MSG2, MSG3, MSG4 and DCR1) are listed in Table 6.2. The pH of the media was adjusted to 5.8 with 1 N NaOH and 1 N HCl prior to autoclaving. In the case of MSG1 medium, the pH was adjusted after addition of the activated charcoal. Aqueous stock solutions of L-glutamine were filter sterilized and added to the warm media. Ten ml of medium was poured into 65 mm sterile plastic petri dishes (Labotec). Five explants were cultured in each plate. The perimeter of each plate was sealed with Parafilm®. Approximately 25 explants were cultured per medium. Cultures were maintained in the dark at 23 to 25 °C. After approximately 4 weeks in culture, explants were moved onto new positions in the same petri dish, or subcultured onto fresh media.

6.2.3 Anatomical Studies

For light microscopy, samples of tissue were placed onto glass slides and stained with 0.5 % toluidine blue in glycerol and pressed gently with a cover slip. Acetocarmine (0.5 %) diluted five to ten times with water was also used (BECWAR, NAGMANI & WANN 1990). Slide preparations were observed and photographed under bright field microscopy to follow the stages of zygotic and somatic embryo development.

Table 6.1 Formulation of MSG and DCR basal media according to BECWAR, NAGMANI & WANN (1990).

Inorganic Compounds	MSG (mg l^{-1})	DCR (mg l^{-1})
NH_4NO_3	-	400.0
KNO_3	100.0	340.0
$Ca(NO_3)_2.4H_2O$	-	556.0
$MgSO_4.7H_2O$	370.0	370.0
KH_2PO_4	170.0	170.0
$CaCl_2.2H_2O$	440.0	85.0
KCl	745.0	-
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4.H_2O$	16.9	22.3
$ZnSO_4.7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25
$CoCl_2.6H_2O$	0.025	0.025
$NiCl_2.6H_2O$	-	0.025
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
Vitamins and Amino Acids (mg l^{-1})		
Nicotinic acid	0.5	0.5
Pyridoxine-HCl	0.1	0.5
Thiamine-HCl	0.1	1.0
Glycine	-	2.0
Carbohydrate and Gelling Agent (g l^{-1})		
Sucrose	30.0	30.0
Agar	8.0	8.0

Table 6.2 Variations in media used to initiate and maintain embryogenic cultures of *P. patula* (Adapted from BECWAR, NAGMANI & WANN 1990).

	Embryogenic culture initiation media				
Component	MSG1	MSG2	MSG3	MSG4	DCR1
Basal medium	MSG	MSG	MSG	MSG	DCR
	Supplements (g l^{-1})				
Inositol	0.1	0.1	0.1	0.1	0.2
Casein hydrolysate	-	1.0	-	1.0	0.5
L-glutamine	1.5	0.5	1.5	0.5	0.25
Activated charcoal	10.0	-	-	-	-
	Plant growth regulators (mg l^{-1})				
2,4-D	-	5.0	2.0	5.0	3.0
BA	-	-	1.0	2.5	0.5

Embryogenic and non-embryogenic material was prepared for scanning and transmission electron microscopy by fixing in 3 % glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.1). Material was post-fixed with cacodylate buffered osmium tetroxide (2 %) and dehydrated through a graded ethanol series, including uranyl acetate at the 70 % ethanol stage for TEM samples. Scanning electron microscopy samples were critical point dried (Hitachi HCP2) and sputter coated (Polaron Equipment Limited E5100) before viewing in the scanning electron microscope (Hitachi S570). The TEM samples were embedded in low viscosity resin (SPURR 1969). Ultrathin sections (approximately 90 nm) obtained for electron microscopy were post-stained with lead citrate (REYNOLDS 1963).

6.2.4 Data collection

A representative sample of approximately 25 embryos (depending on the number of

seed per cone remaining after placement in culture) from each tree sample at various collection dates was used to determine mean zygotic embryo length as well as the percentage of cleavage and cotyledonary embryos present in seed. These parameters were used as possible indicators of the most suitable stage of embryo development for the collection of responsive explants. Observations were carried out using a photomicroscope (Olympus BH-2) equipped with a graduated eye piece. All cultures were monitored on a weekly basis for induction of embryogenic tissue and the developmental stages in the embryogenic process were recorded.

6.2.5 Data Analysis

Due to the different sampling procedures used in the two studies, separate analytical techniques were employed to interpret the data. In both cases, statistical analyses were carried out using the Statgraphics statistical programme.

In the first study (1991/92), the total induction of embryogenic tissue across all media was analysed using Chi-square two by two Contingency Tables. The embryogenic induction frequency, from the first three collection dates was grouped (in order to obtain values greater than five) and compared with values obtained for the rest of the collection period. Two by four Contingency Tables were used to analyse the effects of the different media on the embryogenic induction frequency.

In the second study (1992/93), where necessary, data were normalized by an arcsine conversion prior to a one-way ANOVA. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

6.3 Results

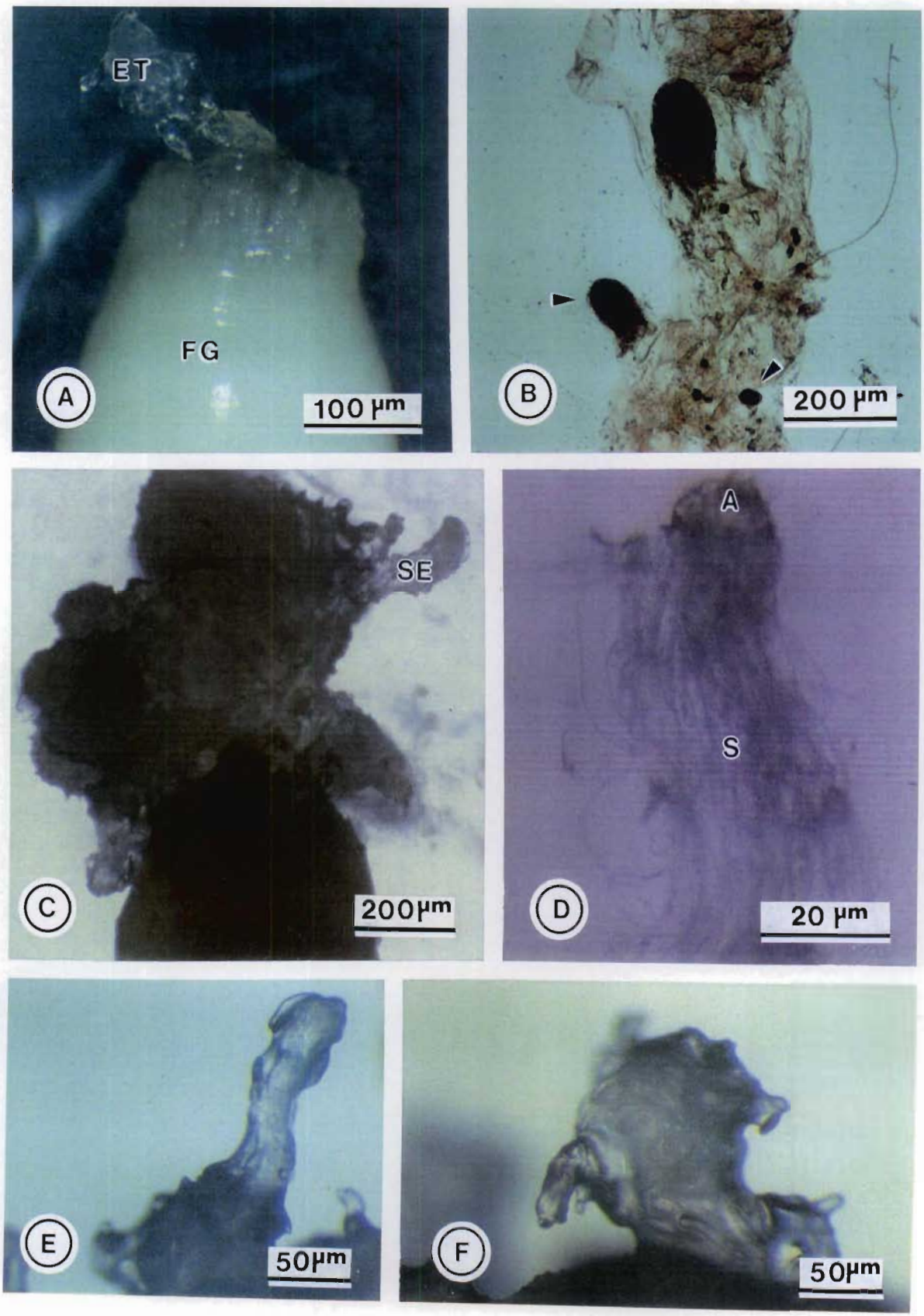
6.3.1 Preliminary Study

In the initial study (1991/92), embryogenic callus was initiated from intact female gametophytes containing precotyledonary zygotic embryos. Thirty three explants out of a total of 1 252 (2.6 %) gametophytes cultured, produced a mucilaginous tissue. Although up to 3.35 % of the explants originally extruded this tissue from the archegonial end of the ovules, only 2.6 % of these lines could be maintained (Table 6.3).

Embryogenic tissue was translucent to white, mucilaginous and prolific, once initiated. The tissue was extruded from the micropylar end of the female gametophyte (Fig. 6.2A), originating from the apical tier cells of the zygotic embryo as a result of cleavage polyembryony (Fig. 6.2B). The proliferative capacity of the tissue led to the rapid development of the early stages of somatic embryogenesis, consisting of globular masses of highly cytoplasmic cells scattered among loose suspensor-like cells. Similar observations were made by BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER and NAGMANI (1988) in *Pinus serotina*. Subculture of this tissue, initially onto a new position in the same petri dish, and later onto fresh medium, resulted in further differentiation of the tissue into somatic embryos (Fig. 6.2C). The translucent embryos consisted of a distinct embryonal apex, subtending a suspensor composed of elongated cells (Fig. 6.2D). Both stage 1 (translucent suspensor) and stage 2 (opaque head) embryos could be identified (Figs 6.2E & 6.2F).

Besides the induction of embryogenic tissue, histological examination indicated the presence of two other distinct tissue types: non-embryogenic and a mixture of the two tissue types. The most common tissue to occur was non-embryogenic and was initially predominantly produced, declining towards the end of the collection period. A mean of 12.4 % of the cultured explants produced this tissue type. Non-embryogenic tissue originated from the surface of the female gametophyte and was

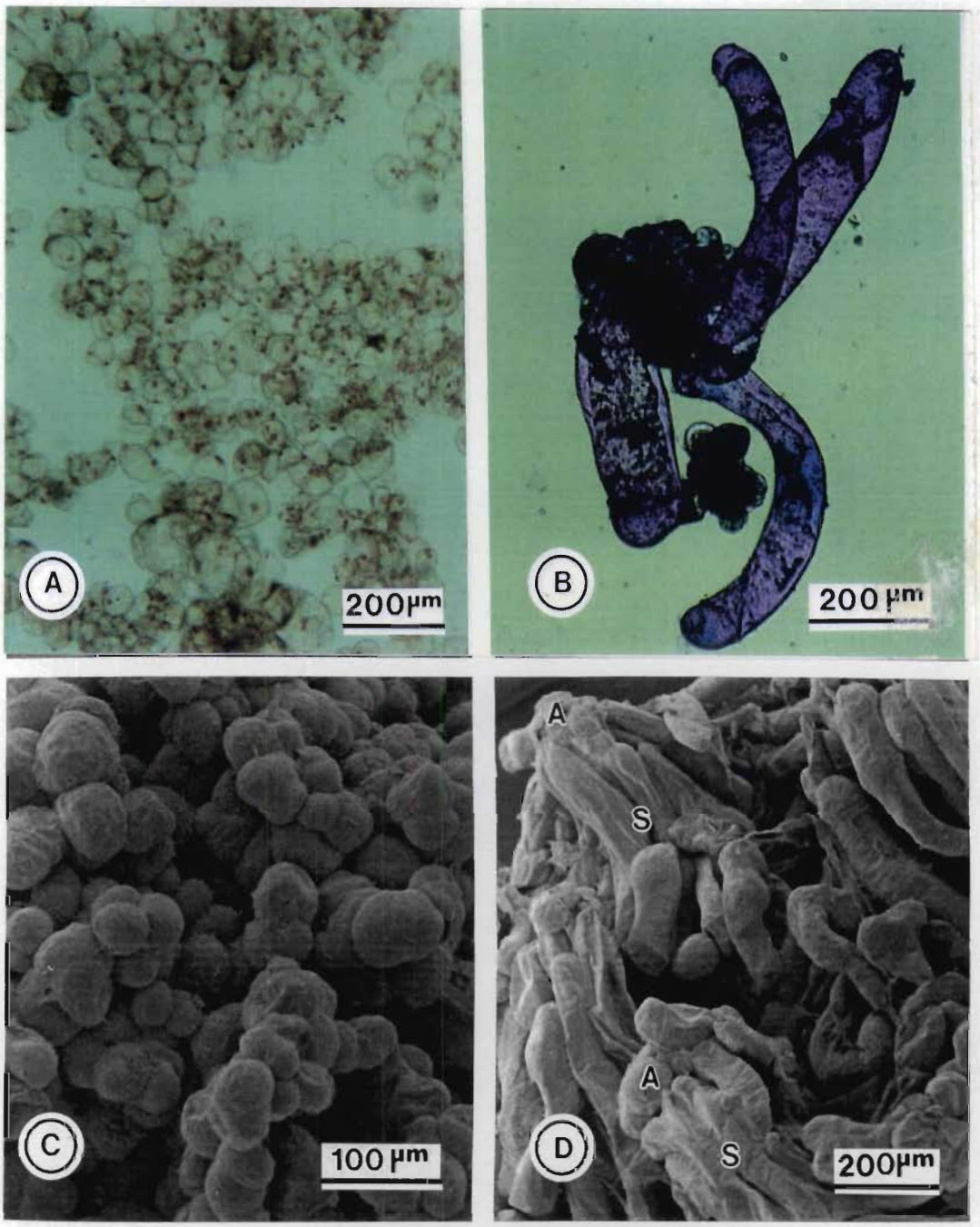
Fig. 6.2 A-F Somatic embryogenesis from the culture of female gametophytes with intact zygotic embryos of *Pinus patula*. **A** Embryogenic tissue (ET) extruded from the micropylar end of the female gametophyte (FG). **B** Staining with 0.5 % acetocarmine shows cleavage polyembryony in the suspensor region of a zygotic embryo (collected 20-01-1992). Developing cleavage embryos are indicated by arrowheads. **C** The translucent to white mucilaginous tissue was prolific and led to the development of somatic embryos (SE). **D** Somatic embryo consisting of densely cytoplasmic cells at the apex (A) subtended by elongated cells constituting the suspensor region (S). **E** Development of stage 1 somatic embryo, showing translucent suspensor. **F** Early stage 2 embryos were larger, the apex being more opaque.


ET
FG
A
100 µm
B
200 µm
SE
C
200µm
A
S
D
20 µm
E
50µm
F
50µm

very distinct from the embryogenic type. Staining with 0.5 % acetocarmine indicated that the tissue was characterized by small, compact spherical cells (Fig. 6.3A) and never gave rise to embryogenic tissue. The mixture of embryogenic and non-embryogenic tissue was also characterized by loose, suspensor-like cells, interspersed among small spherical, compact cells, closely resembling the pre-embryonal masses found in the embryogenic tissue. Although difficult to distinguish from the early stages of somatic embryogenesis, these structures were identified as sphaeroblasts (Fig. 6.3B) since attempts to subculture them onto fresh media resulted in a lack of further development. The embryogenic tissue was short-lived and rapidly became smothered by the non-embryogenic type. The mixture tissue type was observed in 0.8 % of cultured explants.

The morphological studies revealed that the non-embryogenic tissue was highly compact and crystalline in appearance, with distinctly spherical cells (Fig. 6.3C), as opposed to the more elongated cells of the embryogenic tissue. Distinct somatic embryos, consisted of characteristic apical and suspensor cells (Fig. 6.3D). While the morphology of the tissue types was vastly different, ultrastructural differences between embryogenic and non-embryogenic tissues were not as apparent. Slight differences were observed, though, between the embryonal apex cells and those of the suspensor region in that the former were much smaller, more rounded and had smaller scattered vacuoles (Fig. 6.3E). The small meristematic cells constituting the embryonal apex, had a very dense cytoplasm scattered with mitochondria, rough endoplasmic reticulum (rER) and starch grains. The elongated suspensor-cells were characteristically more vacuolate, containing fewer amyloplasts. Organelles such as rough endoplasmic reticulum, Golgi bodies and mitochondria were restricted to a thin peripheral layer of cytoplasm, surrounding large vacuoles (Fig. 6.3F). Chloroplasts were not observed in the embryonal ultrastructure, perhaps since cultures were maintained permanently in the dark.

Using the mean zygotic embryo length as a possible indicator of the optimum developmental stage to select for, in attempting to induce embryogenesis in culture, it was found that embryos measuring between 0.25 mm (collected on 30 December


A
200μm
B
200 μm
C
100 μm
A
S
A
S
D
200μm
V
ER
A
M
E
1 μm
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ER
M
V
A
F
1 μm

1991) and 1.35 mm (collected on 28 January 1992) in length, were the most suitable (Table 6.3). The embryos within this range exhibited cleavage polyembryony, resulting in the production of numerous smaller embryos. The precotyledonary explants from the initial three collections contained embryos smaller than 0.01 mm and resulted mainly in the production of the non-embryogenic tissue and sphaeroblasts. Both of these tissue types failed to differentiate further. No embryogenic tissue or cleavage polyembryony was observed. Despite the lower

Table 6.3 Induction frequency of embryogenic tissue *in vitro* from embryonic explants of *P. patula* cultured on five media from December, 3, 1991 to February 11, 1992. Treatments followed by different letters are significantly different at (*) the 0.001 % and (**) the 0.05 % level.

	Percent of explants embryogenic						
Collection Date	MSG1	MSG2	MSG3	MSG4	DCR1	Totals*	Zygotic Embryo Length (mm)
04 Dec 91	0	0	0	0	0	0^{a}	-
11 Dec 91	0	0	0	0	0	0^{a}	0.010
18 Dec 91	0	0	0	0	0	0^{a}	0.015
30 Dec 91	0	0	4	0	0	0.8^{b}	0.250
07 Jan 92	4	4	12	0	12	6.4^{b}	0.575
14 Jan 92	8	0	4	0	4	3.2^{b}	0.775
21 Jan 92	0	0	0	12	8	4.0^{b}	1.010
28 Jan 92	0	0	4	12	12	5.6^{b}	1.350
03 Feb 92	0	8	4	4	4	4.0^{b}	2.005
11 Feb 92	0	0	0	5	10	3.1^{b}	2.895
Totals**	1.2^{c}	1.2^{c}	2.8^{d}	3.2^{d}	4.8^{d}	2.6	-

occurrence of cleavage and the observed presence of cotyledon primordia, embryos larger than 1.35 mm, (3 to 11 February 1992 - Table 6.3) were still capable of producing embryogenic tissue.

Embryogenic callus was initiated from the fourth collection (30 December 1991) at a very low frequency (0.8 %), and was observed to occur in various degrees in all following collections, spanning a period of seven weeks (Table 6.3). The fifth collection (7 January 1992) produced an initiation frequency of 6.4 % while seed from the following collections varied in frequency from 3.1 to 5.6 % (Table 6.3). Explants from collections four to ten produced significantly ($P < 0.001$) more embryogenic tissue than collections one to three.

Explants responded on all media. Use of Chi-square two by four Contingency Tables, indicated that explants placed on MSG3, MSG4 and DCR1 media, all containing both BA and 2,4-D, produced significantly ($P < 0.05$) higher induction frequencies than explants cultured on MSG1 and MSG2 media. The former medium contained no plant growth regulators and the latter medium contained 2,4-D alone (Table 6.3).

6.3.2 Principal Investigation

Embryogenic tissue was successfully initiated in 0.99 % of explants (69 out of 6 996) cultured over a period of three months. Despite this very low induction frequency, the embryogenic tissue was successfully maintained and manipulated. The induction frequency from each tree sample showed wide variation (Fig. 6.4), not only in the amount of tissue induced, but also in the time of initiation, possibly as a result of open pollination and hence genetic differences. The non-embryogenic callus which developed was discarded, and no sphaeroblast production was observed, as noted in the previous study.

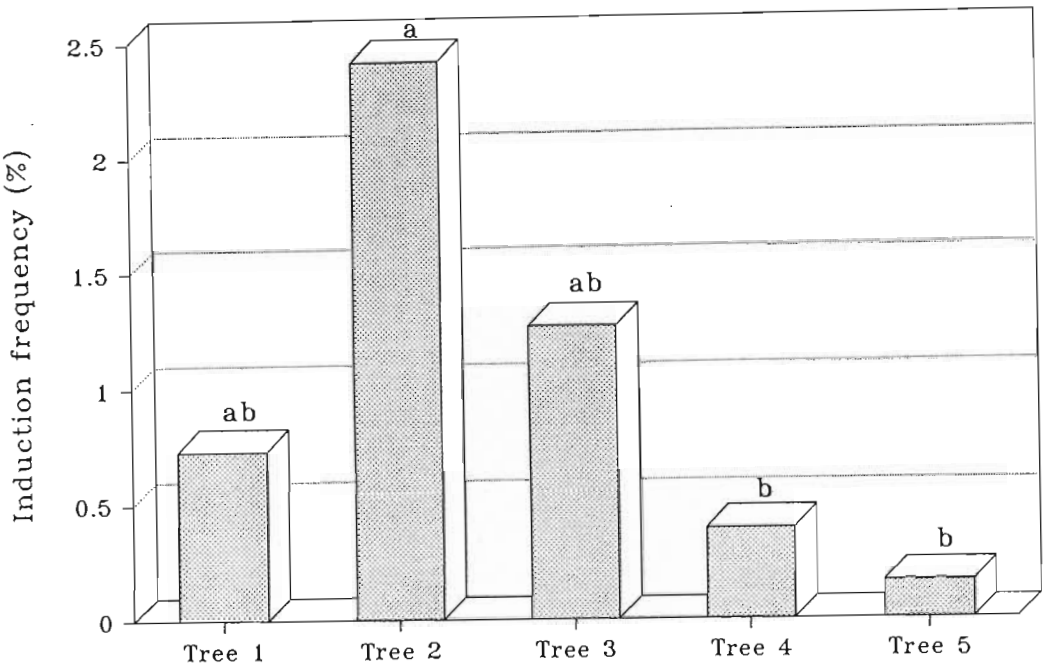


Fig. 6.4 Mean embryogenic induction frequency from source material.

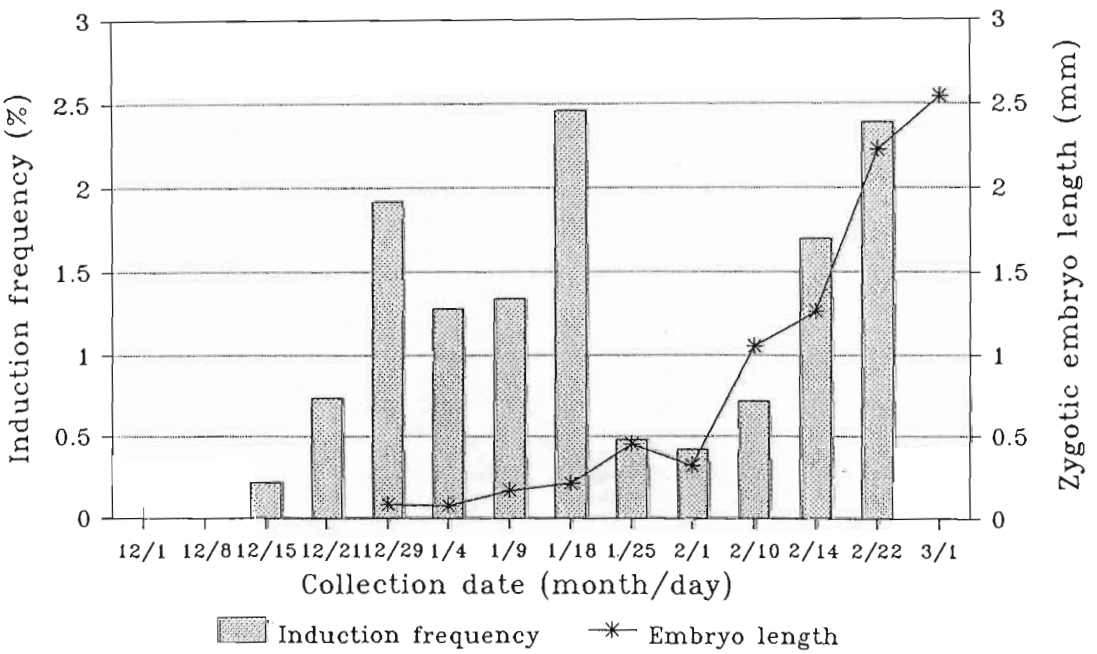


Fig. 6.5 Mean embryogenic tissue induction (%) and coinciding zygotic embryo length (mm) for the collection period.

The mean embryogenic induction for the entire 14 week collection period, showed an inconsistent induction frequency (Fig. 6.5). Immediately apparent, was the reduced induction from explants collected on 25 January and 1 February 1993. This phenomenon was prevalent in all five samples, despite genetic variability.

The corresponding mean zygotic embryo length was also determined for this period (Fig. 6.5). Embryogenesis was evident at all collection dates, except during the first two weeks of December when explants were possibly too immature. No embryogenesis was evident on the final collection date (1 March 1993) when the zygotic embryo length had exceeded 2.5 mm (Fig. 6.5). On closer observation of the percentage of zygotic embryos undergoing cleavage polyembryony, and those possessing cotyledonary primordia (Fig. 6.6), it was noticed that initially all the immature embryos exhibited cleavage. The number of embryos undergoing cleavage began to decline as the presence of cotyledonary primordia became apparent. On the final collection date (1 March 1993) in which embryogenesis was not observed, cleavage embryos were absent and explants were all in possession of cotyledonary initials. This resulted in the germination of some explants. This suggests that the culmination of embryogenic induction may coincide with the termination of cleavage embryo production in the zygotic embryo.

The effect of the initiation media on embryogenic frequency showed a significantly ($P < 0.05$) greater induction on MSG3, MSG4 and DRC1 in comparison to MSG1 and MSG2 media (Fig. 6.7). All callus lines were subsequently subcultured onto DCR1 medium and successfully maintained. Subsequent exposure of the tissue to ABA, resulted in maturation or the development of somatic embryos in a process which closely resembles zygotic embryogeny. No embryogenic induction was obtained using mature seeds as an explant source.

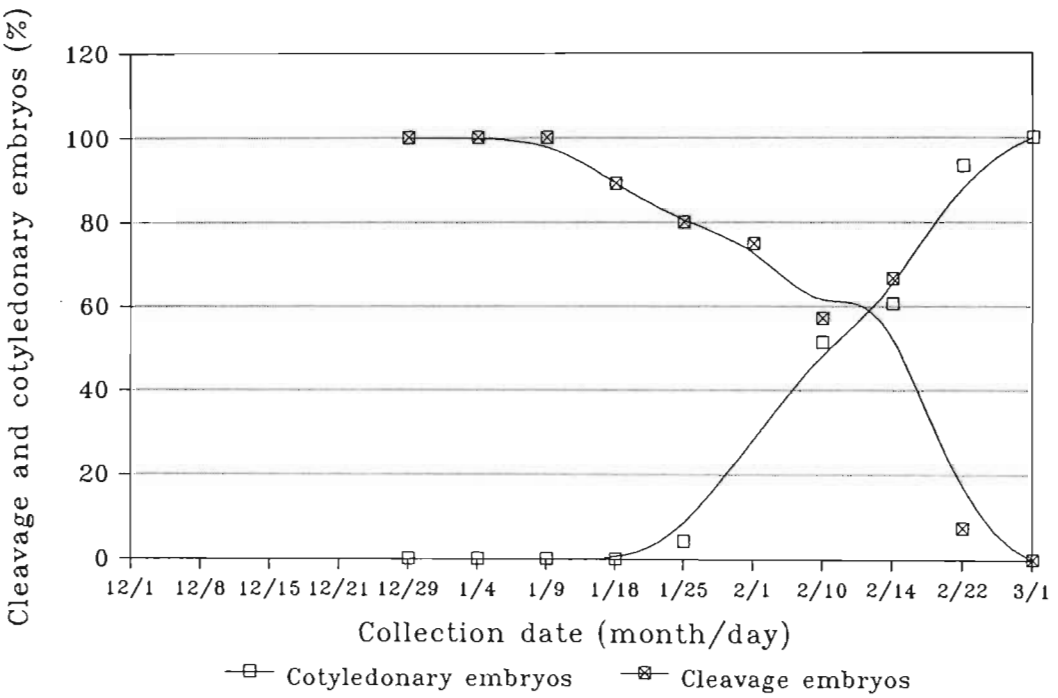


Fig. 6.6 The percentage of zygotic embryos undergoing cleavage and/or demonstrating the presence of cotyledonary initials during the cone collection period.

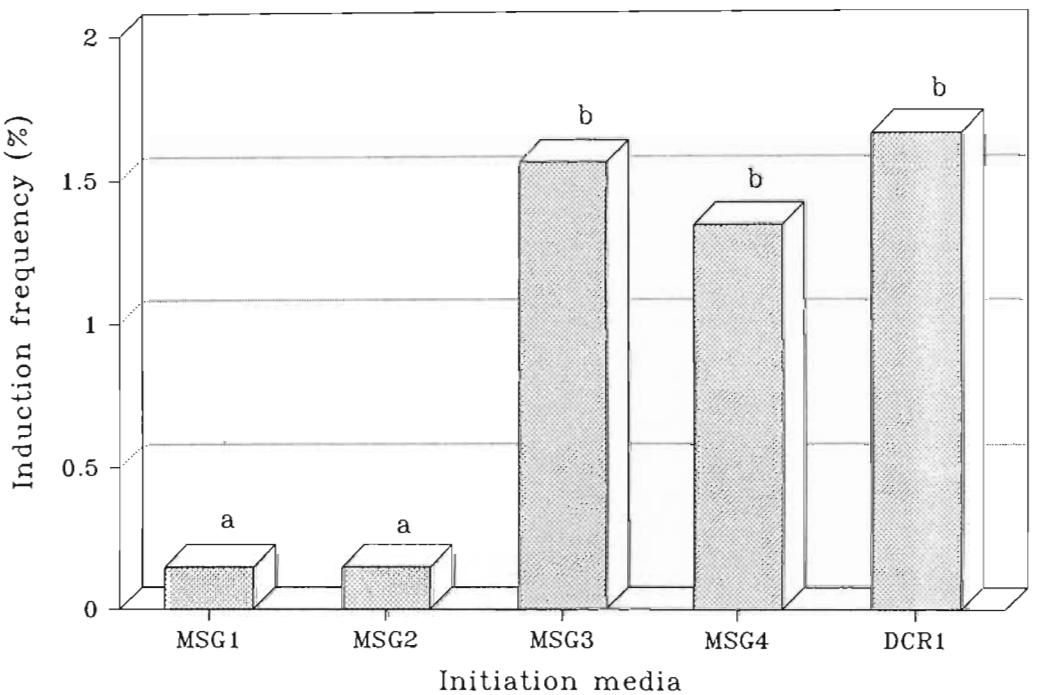


Fig. 6.7 Mean induction frequency (%) of embryogenic tissue on initiation media. (Treatments denoted by the same letter are not significantly different at the 0.05 % level).

6.4 Discussion

This report is the first to describe somatic embryogenesis in *Pinus patula*, and demonstrates that low frequencies of embryogenic tissue can be obtained from immature ovules, provided that explants are obtained at the correct developmental stage, and that the nutrient requirements of the developing zygotic embryos are met. The optimum stage of zygotic embryo development for establishment of pine embryogenic cultures is approximately three to five weeks post-fertilization (BECWAR, BLUSH, BROWN & CHESWICK 1991). At this stage, the ovule contains more than one viable embryo, including the dominant one, as a result of cleavage polyembryony (OWENS & MOLDER 1984). Culture of embryos at this stage leads to an "embryo rescue" situation, which may result in somatic embryogenesis. This process has been confirmed by BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER and NAGMANI (1988), who observed multiple embryos attached to a common suspensor, suggesting that in *Pinus* somatic embryogenesis may be a re-initiation of the cleavage process. In the initial study, these results were re-affirmed by the origin of multiple embryos from explants that later produced embryogenic callus (Figs 6.2B and 6.2C), while zygotic embryos not undergoing cleavage (from the first three collections, Table 6.3), failed to produce embryogenic tissue. The second study appears to indicate that embryogenic tissue was induced throughout the collection period, almost irrespective of the zygotic embryo length, suggesting perhaps that other factors may also be involved in determining the responsiveness of explants.

In this respect, it appears that the occurrence of cleavage embryos, associated with the developing zygotic embryo, play a large role in determining whether explants become responsive. This was demonstrated by determining the number of developing zygotic embryos exhibiting cleavage, and those possessing cotyledonary primordia. In the presence of cleavage embryos, embryogenic induction was observed at various degrees, irrespective of embryo size. Even in the penultimate collection, when embryos were almost 95 % cotyledonary (Fig. 6.6), a high induction (Fig. 6.5) was observed. Furthermore simultaneous cleavage and

cotyledonary development was observed in some zygotic embryos. On the final collection date (1 March 1993), when all explants manifested cotyledonary presence alone, embryogenesis was conspicuously absent. Instead, germination of the zygotic explants was observed to occur, especially on the hormone-free, MSG1 medium.

The observed variation in performance of the weekly cone collections in the pilot study, indicated that the initial explants were at a premature developmental stage. There was a noticeable lack of cleavage polyembryony in these early zygotic explants, resulting in the production of sphaeroblasts rather than embryogenic tissue. The most responsive explants were collected from December 30, 1991 to February 3, 1992 (Table 6.3) where the occurrence of cleavage polyembryony was observed to coincide specifically with the initiation of embryogenic tissue in culture.

The results of the second study (Fig. 6.5), show the typical "window" effect, in which receptive explants were responsive to embryogenic induction for a short period of time during zygotic embryo development. The total embryogenic induction frequency, although low, was successfully maintained and proliferated, despite genetic differences among the open pollinated patula families tested.

Tentative attempts at linking reduced induction frequency to environmental parameters such as rainfall and temperature suggested the possibility of a corresponding response (only with regard to rainfall) providing circumstantial evidence for the effect of environmental factors on induction frequency. Numerous authors have shown that ABA and moisture stress are of consequence for the maturation of seeds of many angiosperm species (KERMODE 1990; HETHERINGTON & QUATRANO 1991). Moisture stress may affect seed development and therefore indirectly influence the embryogenic induction potential of embryonic explants. These considerations are relevant with respect to the drought conditions experienced in southern Africa. The direct physiological effects of drought stress on embryogenic induction, though, are unknown.

The induction of the three types of tissue under identical culture conditions

(1991\92), can be attributed to the various cell populations which constitute the gametophytic explants (BHASKARAN & SMITH 1988), as well as the genotypic responses to the exogenous growth regulators in the various media. This can result in large changes in embryogenic potential. A further, more critical factor, contributing to callus differentiation, is the developmental stage of the explant (LU & THORPE 1987). The present results show that it is a combination of these factors that play an important role in the induction of embryogenic tissue. Sphaeroblast production was not observed in the 1992/93 study, at any stage during the collection period. Embryogenic tissue was selectively cultured further while non-embryogenic callus was discarded.

Although the morphological differences between embryogenic and non-embryogenic tissue were striking, ultrastructural differences were less obvious only becoming more apparent when explants have been cultured in the light (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988), allowing chloroplast morphology to be used as a diagnostic feature. The ultrastructure of somatic embryos has only been studied in a limited number of conifers including, *Picea glauca* (HAKMAN, RENNIE, & FOWKE 1987; JOY, YEUNG, KONG, & THORPE 1991) and *Larix decidua* (ROHR, VON ADERKAS & BONGA 1989). The ultrastructure of *P. patula* somatic embryos showed development similar to that observed in *Picea glauca* (TAUTORUS, FOWKE & DUNSTAN 1991). Ultrastructural differences occur between the apical and suspensor cells. The numerous starch granules located in the suspensor region are indicative of energy reserves required for growth, while the abundant presence of mitochondria, Golgi bodies and rough endoplasmic reticulum are indicators of rapid cell growth (TAUTORUS, FOWKE & DUNSTAN 1991). BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER and NAGMANI (1988) suggested that attempts at discerning ultrastructural differences between embryogenic and non-embryogenic tissue, would aid both the verification and prediction of embryogenic potential.

The embryogenic induction-media interactions for the two studies were similar. The results (Table 6.3, Fig. 6.7) indicate that all media were capable of initiating

embryogenic responses, and that the hormonal interaction between BA and 2,4-D play a role in stimulating embryogenic induction, since explants on media containing both plant growth regulators produced significantly more embryogenic tissue than other media tested. The high initiation frequency on DCR1 medium, also confirms results obtained by other workers with loblolly pine (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988; BECWAR, NAGMANI & WANN 1990) and sugar pine (GUPTA & DURZAN 1986b). Initiation of embryogenic tissue on MSG1 medium suggests that, if explants are obtained at the precise developmental stage, exogenous growth regulators are not essential, yet their presence may significantly enhance frequency of initiation as was observed on DCR1 medium. This phenomenon has been observed previously, TAUTORUS, FOWKE and DUNSTAN (1991) reported on the induction of embryogenic callus from *Pinus radiata* on media containing activated charcoal and no hormonal constituents. *Picea*, in contrast has an obligatory requirement for exogenous auxin and cytokinin (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988).

Collectively these results indicate that initiation of somatic embryogenesis in immature explants of *P. patula* is possible, and coincides well with the process of cleavage polyembryony. Further research is required to determine the precise time of fertilization, since some authors (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988) have reported that explants are most responsive approximately three weeks after fertilization. Research is also required to determine the optimum time for induction of embryogenic tissue as well as modifications of the medium formulations in order to maximize the embryogenic potential of these explants. These aspects are discussed in later Chapters.

CHAPTER 7

SOMATIC EMBRYO MATURATION AND PLANTLET DEVELOPMENT

7.1 Introduction

Although the concept of somatic embryogenesis is an important one, this process has not yet been successfully established on a scale that would be commercially profitable. The *Pinus* embryogenic induction process is well documented (GUPTA & DURZAN 1987b; LAINÉ & DAVID 1990, TAUTORUS, FOWKE & DUNSTAN 1991). It has been established that auxin and a low level of cytokinin are required to stimulate the production of a translucent, mucilaginous tissue. The most successfully used plant growth regulator to stimulate the maturation process is abscisic acid (ABA). The requirement of ABA in the control of embryo development and maturation in both monocotyledonous and dicotyledonous plants has been demonstrated in *Triticum aestivum* (KING 1976; WILLIAMSON & QUATRANO 1988); *Carum carvi*; *Daucus carota* (AMMIRATO 1974; 1983) and *Glycine max* (ACKERSON 1984). In conifers, researchers have shown that transferring somatic embryos onto media containing ABA, followed by further development on medium with reduced levels or no phytohormones, resulted in embryo maturation and plantlet development (DURZAN & GUPTA 1987; HAKMAN & VON ARNOLD 1988; DUNSTAN, BEKKAOUI, PILON, FOWKE & ABRAMS 1988; DUNSTAN, BETHUNE & ABRAMS 1991; ATTREE, TAUTORUS, DUNSTAN & FOWKE 1990b; ROBERTS, FLINN, WEBB, WEBSTER & SUTTON 1990a; ROBERTS, SUTTON & FLINN 1990b). Maturation of *Pinus* embryogenic tissue, though, has proved to be far more difficult than that of other conifer species such as *Picea*.

7.1.1 ABA Effects

ABA was initially incorporated into conifer maturation media because of the implication of the presence of natural (+)-ABA in the maturing zygotic embryo. Workers had also successfully used ABA in the maturation of angiosperm somatic embryos (AMMIRATO 1973; 1974). It is believed that the natural enantiomer of ABA supplied in culture behaves as its endogenous counterpart would. A common suggestion is that the presence of (±)-ABA (composed of both the natural (+) and unnatural (-) enantiomers) may inhibit cleavage polyembryony, resulting in the maturation of individual somatic embryos (DURZAN & GUPTA 1987; KROGSTRUP, ERIKSEN, MÖLLER & ROULUND 1988). This may also provide an explanation for the lack of induction of embryogenic tissue from mature explants of *Pinus* species.

ABA has been shown to accumulate naturally in developing seed with the onset of embryo ripening (FELLENBERG 1982) and to inhibit precocious germination of the zygotic embryo (ROBERTS, FLINN, WEBB, WEBSTER & SUTTON 1990a). Abscisic acid also promotes the deposition of storage reserves (ROBERTS, FLINN, WEBB, WEBSTER & SUTTON 1990a). The reduction or absence of auxin in the medium during maturation may retard the cleavage process, thus yielding somatic embryos that may be more receptive to ABA (ATTREE & FOWKE 1993).

A wide range of ABA concentrations as well as exposure times have been reported for the maturation and germination of conifer somatic embryos. Initially, ABA concentrations below 2.1 to 3.1 mg l^{-1} were considered optimal (BECWAR, NOLAND & WANN 1987a; BECWAR, NOLAND & WYCKOFF 1989; HAKMAN & VON ARNOLD 1988). Levels as high as 10.6 to 28.1 mg l^{-1} have subsequently been used to mature spruce (ROBERTS 1991). The reason for this trend has been an increase in exposure times and the fact that somatic embryos appear to decrease in sensitivity to ABA with time. This has been shown by the precocious germination of some embryos. Higher ABA

concentrations thus ensure more uniform germination (ATTREE & FOWKE 1993). For some *Picea* (VON ARNOLD & HAKMAN 1988; ATTREE, TAUTORUS, DUNSTAN & FOWKE 1990b) and *Pinus* (FINER, KRIEBEL & BECWAR 1989) species it was found that ABA concentrations of 2.0 to 4.2 mg l^{-1}, for four to five weeks followed by a period (up to four months) on hormone free medium were successful in establishing plantlets.

Even the method of preparing ABA for inclusion in the medium, has been shown to have an effect on embryo maturation (DUNSTAN, BETHUNE & ABRAMS 1991), yet authors do not usually describe the precise procedures followed. DUNSTAN, BETHUNE & ABRAMS (1991) found that acetone was the only water-miscible organic solvent which did not appear to affect the yield of stage 3 embryos. Use of ethanol/water or dimethyl sulphoxide (DMSO)/water (1:9 v/v in both cases) as ABA-dissolving agents resulted in a lack of maturation or reduced yield of somatic embryos.

7.1.2 Osmoticum Effects

Another factor significantly affecting the quality of conifer somatic embryos, is the level and type of osmoticum employed simultaneously with ABA. While a low sucrose concentration seems effective in inducing embryogenic tissue, it appears that maturation requires elevated osmotica levels. It has been shown that simple salts and sugars may not be as effective as the use of high molecular weight compounds such as polyethylene glycols (PEG) or dextrans (ATTREE, MOORE, SAWHNEY & FOWKE 1991). The reason for this is that permeating (low molecular weight) compounds, such as sucrose, initially result in plasmolysis. After prolonged culture, deplasmolysis or osmotic recovery occurs where both water and sucrose move into the cells via osmosis, thereby preventing a water deficit. The use of high molecular weight or non-permeating compounds activate a non-osmotic moisture stress at the cellular level which is comparable to drought-induced stress. The large size of the molecules inhibits their passage

through the cell wall and, although water is lost from the cells creating a negative pressure, no plasmolysis occurs. The turgor pressure may only be restored by the cells actively increasing sucrose levels. This may then stimulate the deposition of other reserves and hence enhance maturation. Optimal L-glutamine levels also need to be determined to promote successful maturation.

7.1.3 Classification of the Maturation Process

VON ARNOLD & HAKMAN (1988) developed a useful classification system, to categorise somatic embryo maturation into four distinct stages. The added 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. The apex can appear slightly irregular in outline.

Stage 2 At this stage, embryos are more prominent, appear smooth in outline and are opaque, usually cream to pale yellow in colour. The apex is still subtended by the suspensor.

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 colour. This stage is usually derived from embryogenic tissue cultured on ABA-containing medium.

Stage 4a Stage 4 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.

Stage 4b This stage is usually evident, after plantlets have been cultured for approximately three weeks on hormone-free medium. Elongating

cotyledons and hypocotyl are evident, and rudimentary radicle development is visible.

LIBBY (1986) used the term "embling" to describe plantlets derived from somatic embryos. VON ARNOLD & HAKMAN (1988) working on *Picea abies* found that germination was best when stage 3 embryos were separated and cultured individually. Similar results were obtained for other *Picea* species (ATTREE, TAUTORUS, DUNSTAN & FOWKE 1990b; TAUTORUS 1990; DUNSTAN, BETHUNE & ABRAMS 1991).

7.1.4 Acclimatization

Recent studies have been successful in ameliorating the hardening-off process by including regimes of partial drying at high humidity following exposure to ABA (ROBERTS, SUTTON & FLINN 1990b). The success of this treatment may lie in that exogenous ABA increases the tolerance of plant tissues to water deficit (MIZRAHI, SCHERINGS, ARAD & RICHMOND 1974) by stimulating lipid reserve deposition (KIM & JANICK 1990). This approach was effective in improving germination of *Picea glauca-engelmanii* complex and *Picea sitchensis* somatic embryos (ROBERTS, SUTTON & FLINN 1990b; ROBERTS, LAZAROFF & WEBSTER 1991a). Other methods have involved using cold stratification to diminish endogenous ABA levels, hence allowing further plant development to occur (ATTREE & FOWKE 1993). Researchers have also produced cultures up until the precotyledonary stage on ABA-containing media solidified with agar (BECWAR, NAGMANI & WANN 1990). The use of liquid media and a solid support (such as filter paper or cheesecloth) have also been found to be beneficial to the maturation process. To date maturation of *Pinus* species has not been achieved using suspension cultures.

Little information exists on the hardening-off of somatic embryo-derived plantlets.

In *Picea abies,* 56 % of matured somatic embryos rooted and 9 out of 31 plantlets (29 %) were successfully established in the soil (BECWAR, NOLAND & WYCKOFF 1989). *Pinus* species that have successfully been cultured from somatic embryos to produce plantlets include: *P. lambertiana* (GUPTA & DURZAN 1986b), *P. caribaea* (LAINÉ & DAVID 1990) and *P. taeda* (GUPTA & DURZAN 1987b), although figures relating to the establishment of the plantlets in the soil are not provided. This chapter reports on the successful maturation and establishment of *P. patula* somatic embryos in *ex vitro* conditions.

7.2 Materials and Methods

7.2.1 Media and Culture Conditions

By varying the constituents of the basal media used for embryogenic induction (BECWAR, NAGMANI & WANN 1990) two maturation media (MSG5 and DCR2) and two rooting media (MSG6 and DCR3) were created (Table 7.1). Modifications to the rooting media included the addition of i) activated charcoal (1 %) and ii) the addition of 0.05 mg l^{-1} IBA to MSG6. The pH of the media was adjusted to 5.8 with 1 N NaOH and 1 N HCl prior to autoclaving. Aqueous stock solutions of L-glutamine and abscisic acid (ABA - dissolved in acetone) were filter sterilized before being added to the media. Associated Chemical Enterprises agar (8 g l^{-1}) was used to solidify media. Fifteen ml of medium was poured into 65 mm plastic petri dishes (Labotec). Five explants were cultured in each dish. The perimeter of each petri dish was sealed with Parafilm®. Approximately 25 explants were cultured per medium. Cultures were initiated, maintained (Chapter 6) and matured to the cotyledonary stage, in the dark at 25 °C. Rooting was achieved in the light, using cool white fluorescent light (19.7 μmol m^{-2} s^{-1}) at a similar temperature.

7.2.2 Maturation

Somatic embryo maturation was accomplished by a replicated quantitative test using three levels of sucrose and L-glutamine in DCR2 medium containing 1.3 mg l^{-1} ABA. Sucrose at 1, 3 and 6 % was tested in factorial combination with L-glutamine at 0.025, 0.25 and 2.5 g l^{-1}. This experiment was repeated using the same sucrose levels (1, 3 and 6 %), but increasing the concentration range of L-glutamine (0, 0.25, 2.5, 5.0 and 10 g l^{-1}). Embryogenic tissue samples of approximately 50 mg were used per replicate and the number of maturing (stage 3) somatic embryos recorded after three weeks in culture. Cultures were incubated in the dark at 25 °C since all attempts to mature embryogenic tissue in the light resulted in necrosis.

Table 7.1 Variations in media used to mature and root somatic embryos of *P. patula*.

	Maturation and rooting media			
Component	MSG5	DCR2	MSG6	DCR3
Basal medium	MSG	DCR	MSG	DCR
	Supplements (g l^{-1})			
Sucrose	60.0	30.0	60.0	30.0
Inositol	0.1	0.2	0.1	0.2
Casein hydrolysate	-	0.5	-	0.5
L-glutamine	1.5	0.25	1.5	0.25
	Plant growth regulators (mg l^{-1})			
ABA	0	0	-	-
	1.3	1.3		
	2.6	2.6		
	3.9	3.9		
	5.0	5.0		

7.2.3 Hardening-off

Approximately 230 rooted and non-rooted emblings were hardened-off either under misthouse conditions at 28 °C, where watering was controlled by an electronic leaf and bed heating was provided (25 °C) or placed directly into the greenhouse and covered by a clear perspex lid where watering was done manually on a daily basis. Emblings were potted in trays containing an autoclaved (1 h at 1 KPa, 120 °C) seedling mix composed of 60 % pine bark and 40 % sand, to which lime and superphosphates was added. In order to ensure high humidity, but prevent over-watering, the plantlets in the misthouse were kept under raised K-cloth*. Once a day, the cloth was removed and misting was allowed to fall directly onto the plantlets. Emblings were kept in the misthouse for a period of about two months, or until active shoot growth was observed, before transferal to the greenhouse, where the K-cloth* was kept in place until the plantlets had acclimatized to the new conditions.

7.2.4 Chromosome Number Determination

Chromosome number determination was conducted using both embryogenic tissue and the root tips of somatically-derived plantlets. The protocol included an initial pre-treatment with 0.2 % colchicine (SALAJOVÁ & SALAJ 1992) for 4 h at 10 °C in the dark (to halt spindle formation). The tissues were then fixed in a mixture of glacial acetic acid and ethanol (1:2) for 1 h, and soaked in 45 % acetic acid for 15 min. The samples were stained in a 2 % solution of acetic orcein:HCl (9:1) and heated slightly. Cytological observations were made by placing the tissues on a glass slide in a 1 % acetic orcein solution and pressing gently with a cover slip, to release the chromosomes. Counting of the chromosomes was facilitated through the use of a photomicroscope (Olympus BH-2).

7.2.5 Data Collection

All cultures were monitored on a weekly basis for signs of tissue maturation and the developmental stages in the embryogenic process were recorded.

7.2.6 Data Analysis

Statistical analysis was carried out using the Statgraphics statistical programme. Where necessary, data were normalized by an arcsine conversion prior to a one-way ANOVA. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

7.3 Results

Exposure of embryogenic tissue to ABA resulted in maturation or the development of somatic embryos in a process which closely resembles zygotic embryo development. Although the presence of ABA produced a larger number of somatic embryos, there was no significant difference between the various hormone levels tested in MSG5 medium and the hormone-free control medium (Fig. 7.1). DCR2 medium, containing 1.3 mg l^{-1} ABA, produced significantly more somatic embryos than the DCR2 control medium three weeks after inoculation. Despite the initial signs of maturation on ABA-free medium, the embryogenic tissue rapidly senesced and embryo development did not exceed stage 1 (HAKMAN & VON ARNOLD 1985). Despite the lack of difference in the number of somatic embryos matured on MSG5 and DCR2 media, development on the latter medium was more pronounced and more successful.

Somatic embryo maturation was further manipulated by altering the levels of sucrose and L-glutamine in DCR2 medium containing 1.3 mg l^{-1} ABA. It was observed that irrespective of the L-glutamine level used, a low sucrose

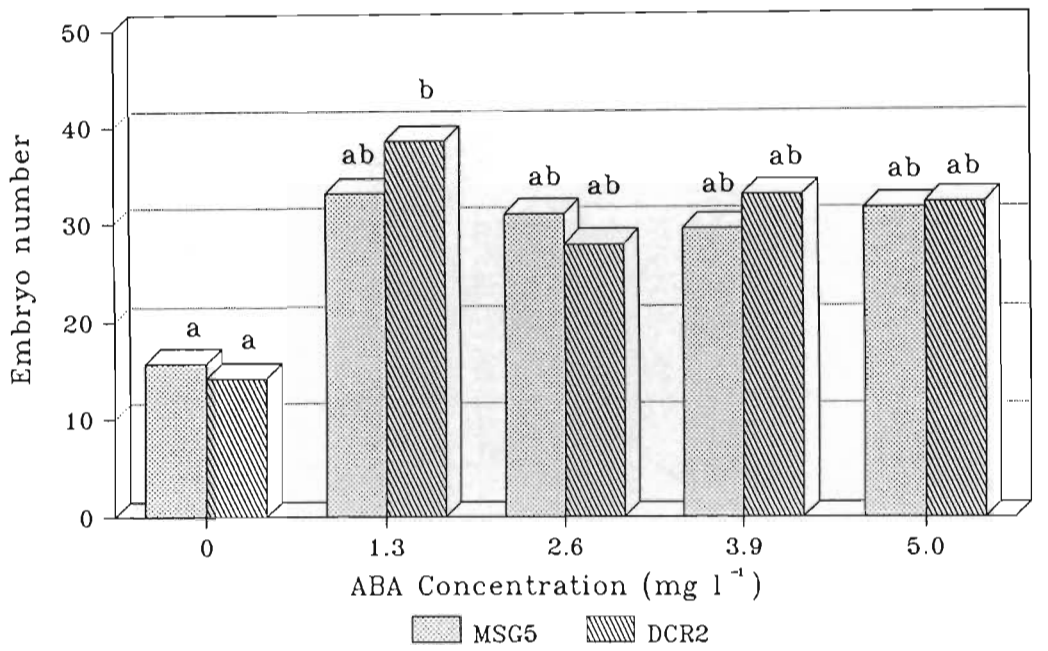


Fig. 7.1 The effect of various ABA concentrations on somatic embryo maturation (Treatments denoted by the same letters are not significantly different at the 0.05 % level).

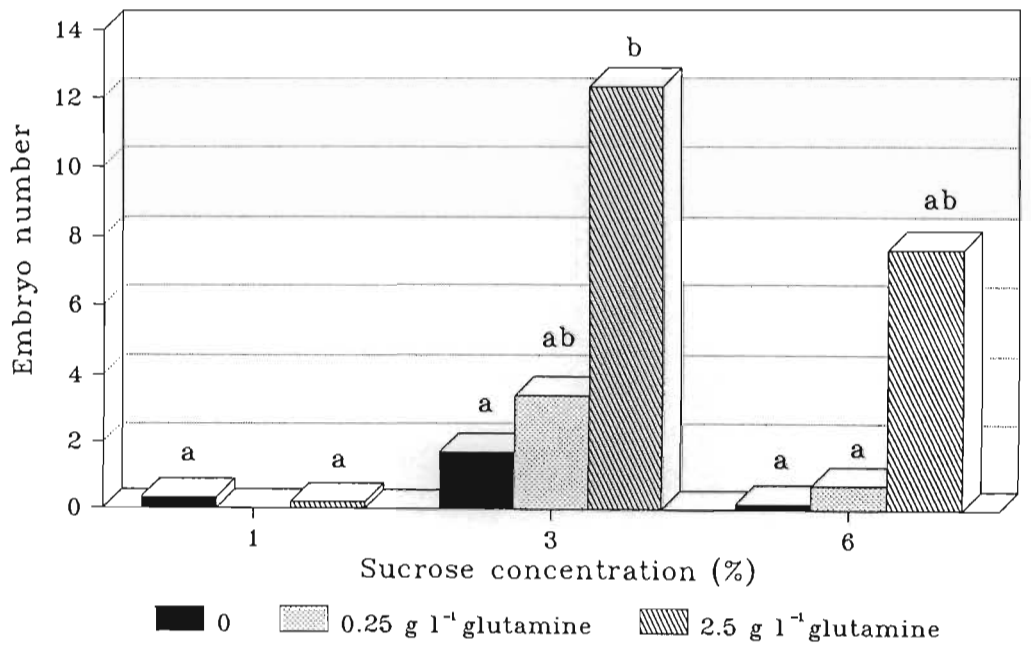


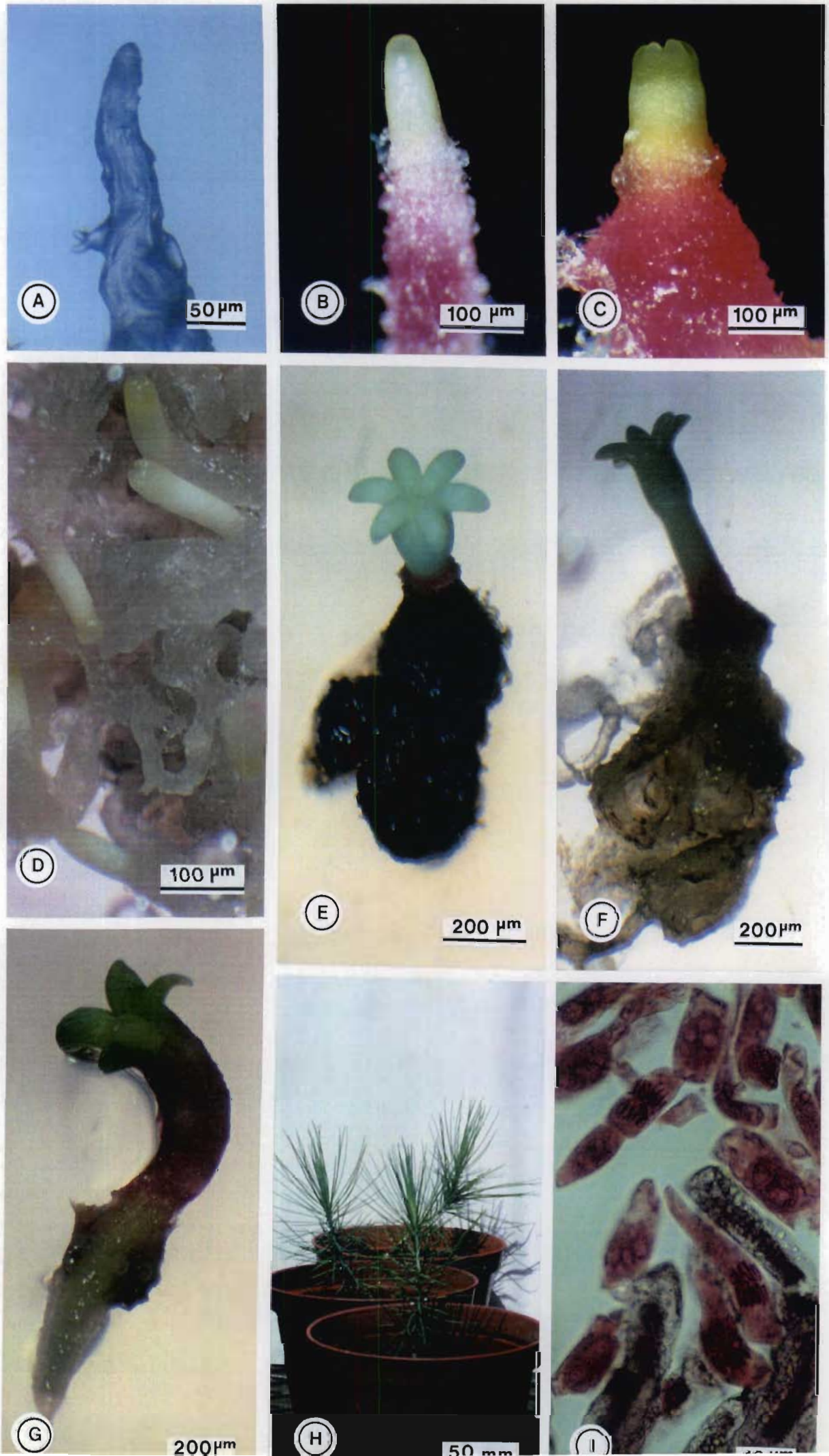
Fig. 7.2 The effect of altering L-glutamine levels on the number of developing somatic embryos (Treatments denoted by the same letters are not significantly different at the 0.05 % level).

concentration (1 %) resulted in very poor maturation (Fig. 7.2). At an intermediate (3 %) and high (6 %) sucrose level, the largest L-glutamine concentration produced significantly more stage 3 somatic embryos than media containing less L-glutamine. Repetition of this experiment, using the same sucrose levels while increasing the concentration range of L-glutamine, resulted in a fairly similar response, with the lower L-glutamine levels (0, 0.25 and 2.5 g l^{-1}), at the 3 % sucrose level producing a larger number of stage 3 embryos, while the higher L-glutamine levels (5.0 and 10 g l^{-1}) were inhibitory to somatic embryo production, except at the lowest sucrose concentration (1 %).

In the presence of ABA the translucent stage 1 embryos, produced on the initiation media (Fig. 7.3A) , developed into opaque structures, much smoother in outline (Fig. 7.3B). Early stage 3 somatic embryos were characterized by the development of cotyledonary primordia around the central apical meristem (Fig. 7.3C). Stage 2 and 3 somatic embryos developed profusely from the embryogenic tissue (Fig. 7.3D) and once the cotyledons had expanded further (Fig. 7.3E), stage 3 embryos were isolated from the embryogenic mass. After four to six weeks on ABA-containing medium, stage 4a embryos exhibited elongation of the hypocotyl and of the cotyledonary needles (Fig. 7.3F).

Rooting was unsuccessful using DCR3 medium (Table 7.1). Presence of activated charcoal in the medium resulted in necrosis and poor survival of explants while very poor rooting was stimulated on IBA-containing MSG6 medium. Transferral of isolated developing embryos onto hormone-free MSG6 medium (Table 7.1) resulted in the development of a rudimentary radicle, indicative of stage 4b embryos (Fig. 7.3G). The *in vitro* rooting of *P. patula* somatic embryos was achieved in 50 to 60 % of explants after three to four weeks of culture (Fig. 7.4). Rooting of embryos was also more successful with the embryogenic lines which were initially the most responsive to induction in culture (described in Chapter 6, Fig. 6.4). Most commonly a single root developed from each embryo, but in some cases secondary root development was also observed.

Fig. 7.3 A-I Morphological development of *Pinus patula* somatic embryos during maturation. **A** The characteristic stage 1 somatic embryo showing the translucent suspensor region. **B** Stage 2 embryo, regular in outline with an opaque embryonal apex. **C** An early stage 3 embryo, showing the cotyledonary initials just beginning to develop at the apex. **D** A mass of stage 2 and 3 embryos developing from embryogenic tissue after exposure to ABA (1.3 mg l^{-1}). **E** Further growth and development of the cotyledons around the central meristem occurs (stage 3). **F** Hypocotyl elongation and enlargement of the cotyledons, denoting stage 4a. **G** A stage 4b embryo showing development of the radicle. **H** Somatic embryos hardened-off under greenhouse conditions. **I** Somatic embryo root tip chromosomes stained with acetic orcein.


A
50 µm
B
100 µm
C
100 µm
D
100 µm
E
200 µm
F
200µm
G
200µm
H
50 mm
I

Maintenance of rooted plantlets in culture for extended time periods led to a loss of viability, manifested by chlorosis of needles, swelling of the hypocotyl and a simultaneous accumulation of anthocyanin. Callusing of the thickened hypocotyl and root tip was also observed. These observations suggest that once root formation has occurred, plantlets should be transferred to *ex vitro* conditions.

Hardening-off of rooted emblings under misthouse conditions (Fig. 7.3H), before they were placed into the greenhouse, resulted in low survival success (8 %). Problems were mainly ones of over-watering which resulted in rotting of plantlets. When emblings were placed directly in the greenhouse and covered with a perspex lid, survival was greatly improved (40 %).

Determination of chromosome number, proved unsuccessful using the embryogenic tissue as a source. Using root tip material, chromosomes could be determined, but not easily counted. The high magnification required to observe the chromosomes resulted in great loss of resolution. The indications are, though, that the samples analysed contained more than 12 chromosomes, which is the haploid chromosome number for pine (Fig. 7.3I).

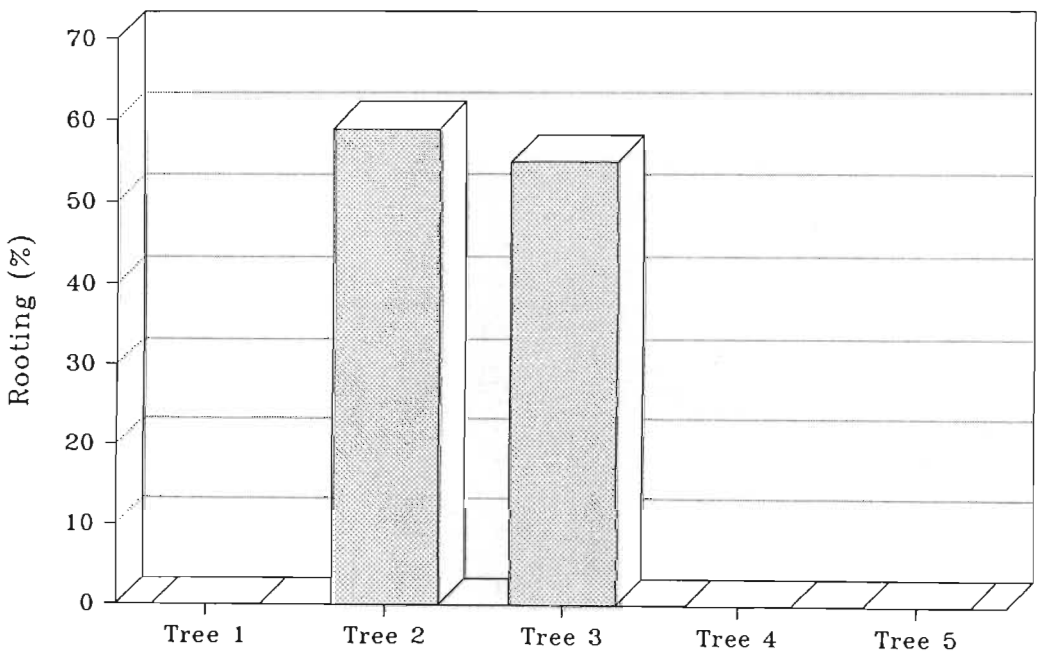


Fig. 7.4 *In vitro* rooting of somatic embryo lines maintained in culture using MSG6 hormone-free medium.

7.4 Discussion

The use of ABA in the maturation of both angiosperm (AMMIRATO 1974; CROUCH & SUSSEX 1981) and gymnosperm (GUPTA & DURZAN 1986a; HAKMAN & FOWKE 1987a) somatic embryos is well known. ABA has been shown to inhibit precocious germination and to promote maturation and the accumulation of storage products (ACKERSON 1984; FINKLESTEIN, TENBARGE, SHUMWAY & CROUCH 1985; BARRATT 1986; KUHLEMEIR, GREEN & CHUA 1987). This plant growth regulator has also been found to stimulate the accumulation of storage proteins in somatic embryos of interior spruce (ROBERTS, FLINN, WEBB, WEBSTER & SUTTON 1990a) and to enhance accumulation of storage lipid in somatic embryos of Norway spruce (FIERER, CONKEY & VERHAGEN 1989).

The presence of ABA in the medium (DCR2) was beneficial in furthering the development of the somatic embryos, possibly as a result of the synthesis and mobilization of storage reserves. Cotyledon development from the embryonal apices of the somatic embryos was observed, especially at the lower ABA concentrations. The various ABA levels tested did not appear to significantly affect the number of somatic embryos produced. Similarly, NAGMANI, BECWAR & WANN (unpublished results) were unable to determine the optimal ABA concentration after using levels ranging from 1.3 to 10 mg l^{-1}. Despite the indication that somatic embryo maturation was equally successful on either MSG5 or DCR2 media, the latter medium was more conducive to the further survival of the maturing embryos. The slight variation in macro- and micro-elements, as well as the lower sucrose and L-glutamine levels of DCR2 medium, possibly played a role in this regard. VON ARNOLD & HAKMAN (1988) found that the frequency of stage 3 embryos also depended on the size of the calli explants. Yield was also found to be higher when embryos were left intact within the callus and not excised and isolated.

KRIEBEL & FINER (1990) found that high levels of L-glutamine (7.31 g l^{-1}) were

successful in stimulating maturation of *Pinus strobus* embryogenic tissue to stage 2 somatic embryos, while much higher levels (21.92 g l^{-1}) retarded growth. These authors also found that 10 mg l^{-1} ABA promoted the development of stage 2 somatic embryos of white pine, and that this process was enhanced by increasing the sucrose level to 6 %. Although increasing sucrose content may be advantageous for some species, it has been found that sucrose requirements among clones alone may differ (NAGMANI, BECWAR & WANN unpublished results). In *P. patula* it appears that an intermediate to high sucrose content (3 to 6 %) coupled with L-glutamine levels of 0.25 to 2.5 g l^{-1} are the most beneficial in terms of the number of embryos produced. The difference in the total number of developing somatic embryos in Figs 7.1 and 7.2 may be due to competition effects, as a result of nutrient depletion and possibly the formation of substances produced by the suspensor which may inhibit the development of other somatic embryos (GUPTA & DURZAN 1987b).

Transferal of suitably developed somatic embryos (stage 4a) onto MSG6 medium resulted in senescence of the remaining suspensor region, and the development of a rudimentary radicle from the base of the somatic embryo. Rooting percentages varied from 50 to 60 %. The enhanced rooting on MSG6 medium may have been the result of the increased sucrose levels (double that in the DCR3 medium). Some workers have found that increasing sucrose levels to 60 g l^{-1} promoted starch accumulation and improved plantlet establishment in the soil (CONNER, XINRUN & WOODING 1993). In some cases, red pigmentation of the hypocotyl was noted, a feature which has previously been observed (KRIEBEL & FINER 1990) and has been associated with final necrosis, yet in this study anthocyanin deposition did not appear to be detrimental or to affect rooting if plantlets were transferred to *ex vitro* conditions within a few weeks of rooting. Although all rooting experiments were conducted in the light, germination has also been obtained in the dark (VON ARNOLD & HAKMAN 1988). Relocation into the light, though, is essential for root elongation.

Numerous authors have succeeded in germinating conifer somatic embryos

(HAKMAN & VON ARNOLD 1985; NAGMANI & BONGA 1985; GUPTA & DURZAN 1986a; 1986b; BECWAR, NOLAND & WANN 1987a; HAKMAN & FOWKE 1987a; VON ARNOLD 1987; GUPTA & DURZAN 1987b), but only the last report describes *Pinus* plantlet establishment in the soil. Hardening-off of the emblings is the most important step of the entire process, but also the most difficult to achieve. Despite the fact that somatic embryos have the potential to produce both roots and shoots, very poor survival was obtained from embryos that had not produced roots *in vitro*.

Hardening-off under misthouse conditions has been achieved on a small scale. The problem associated with this phase of development appears to be one of over-watering which leads to rotting of plantlets. Hardening-off directly in the greenhouse under a clear perspex lid, offered more promising results for the further establishment of plantlets. The advantages of this system were that evaporation was prevented by the solid lid, although high humidity was successfully maintained. Watering plantlets only once a day also prevented over-watering.

Consideration of the available literature, regarding the possible origins of embryogenic tissue (TAUTORUS, FOWKE & DUNSTAN 1991), would suggest that the tissue produced from the embryonic explants was in fact diploid in nature ($2n = 24$) (SALAZAR 1983). The chromosome number determination studies tentatively suggest that this is the case. The difficulty experienced with these karyological studies has also been described by other authors. VON ADERKAS, BONGA & NAGMANI (1987) working on *Larix decidua* were unable to obtain adequate samples containing metaphases with chromosomes sufficiently contracted and separated in order to facilitate reliable counting.

Maturation of the *P. patula* somatic embryos is still problematic. Research so far has yielded low numbers of emblings and there is a need for further study before any conclusions can be drawn with regard to their viability and establishment in the field.

CHAPTER 8

EMBRYOGENIC SUSPENSION CULTURE

8.1 Introduction

Cell suspension culture offers an alternative method of culturing plant tissue. This system can be created by transferring fragments of undifferentiated callus into a liquid medium, or by inoculating the medium with differentiated tissue, which is then agitated during the culture period (DODDS & ROBERTS 1985). The "friability" (the separation of cells following cell division) of cultures is an important aspect to consider in establishing suspension cultures. In this regard, the mucilaginous nature of embryogenic tissue is ideal for liquid culture initiation.

Suspension cultures may present several advantages over conventional solid culture techniques. Maintenance of these cultures is less time-consuming since sub-cultures can be handled in bulk, making the process more efficient, economical and commercially viable. Liquid cultures also have potential for higher growth rates and reduced cell doubling times (BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI 1988). One of the most important benefits of liquid cultures, is that they are more amenable to automation of both embryo proliferation and mass production through the use of large bioreactors (AMMIRATO & STYER 1985).

Despite the obvious advantages of using liquid cultures, very little information exists on suspension growth characteristics and somatic embryo yield. KRIEBEL & FINER (1990) reported on the induction of embryogenic tissue from precotyledonary zygotic embryos of *Pinus strobus* and subsequent maintenance of the tissue using suspension cultures. Although these cultures were easily

maintained, maturation and plantlet development in liquid medium was not obtained. NAGMANI & DINUS (1991), working with *Pseudotsuga menziesii*, found that replacement of sucrose with maltose in combination with ABA promoted somatic embryo development to the cotyledonary stage. Similar results were obtained by UDDIN, DINUS & WEBB (1990) for *Pinus taeda*.

One of the few studies to report complete plantlet development from liquid culture includes that of DURZAN & GUPTA (1987), who repeatedly produced 20 to 25 embryos of *Pinus taeda* with both shoot and root apices from 15 ml of suspension culture within 140 days. Three complete plantlets were successfully established in the soil from 65 such embryos, suggesting that embryo maturation, using this technique is possible, although further refinement of the technique is required before commercial gains can be realized.

In the present study suspension cultures were initiated in an attempt to increase the multiplication rates of the embryogenic tissue on maintenance medium and to attempt maturation under liquid conditions.

8.2 Materials and Methods

8.2.1 Plant Materials

Several of the embryogenic cell lines induced on agar-solidified media (Chapter 6) were introduced to liquid culture. One line in particular (designated K) was successfully initiated and used for maintenance and maturation experiments. Cell line K was initiated in culture from immature seed collected on 21 December 1992, from Tree 3 and maintained on MSG3 medium (Table 6.2). After a maintenance period of 14 months, during which cultures had been transferred on to DCR1 medium (the medium found to be the most suitable for maintenance purposes, Table 6.2), suspension cultures were initiated.

8.2.2 Initiation Media and Culture Conditions

The medium used for initiation and maintenance of cultures was similar to that used for solid culture, except that the agar component was omitted (DCR1 medium - Table 6.2). The medium was autoclaved and allowed to cool, before the addition of filter-sterilized L-glutamine. Embryogenic suspension cultures were initiated by inoculating 10 to 100 mg of tissue into 35 ml of liquid medium in 100 ml Erlenmeyer flasks, which were sealed with a cotton wool bung and covered with a tinfoil cap. The flasks were agitated at 150 rpm and maintained in the dark at 25 °C. As the cultures developed, subculturing took place in progressively larger vessels, until 500 ml flasks containing 100 ml of cellular suspension were used. Flasks of this nature were used for all subsequent experimentation. Sub-culturing involved taking a 10 to 20 ml packed volume of cells and diluting with 480 to 490 ml fresh medium. The resulting 500 ml suspension culture was sub-divided into 100 ml aliquots and placed into 500 ml side-arm flasks for experimentation purposes. Sub-culturing took place approximately every two weeks.

8.2.3 Maturation in Suspension

Unlike solid cultures, the use of liquid media ensures that all cells are constantly in contact with the agitated medium. In view of this, the ABA concentration, found to be optimal for maturation of embryogenic tissues on solid medium, and dilutions thereof were tested. A hormone-free control was tested against ABA concentrations of 0.5 and 1.3 mg l^{-1}. This experiment was re-run using pre-conditioned cultures. Maturation was also manipulated by regulating the osmolarity of the culture medium. The effect of two levels of maltose (1 and 3 %) were tested on the further development of Stage 1 somatic embryos. DCR2 medium (Table 7.1), containing 1.3 mg l^{-1} ABA, in which sucrose was replaced with maltose, was used for maturation.

8.2.4 Anatomical Studies

Light microscopy (Olympus BH-2 photomicroscope) was used to conduct anatomical studies on the somatic embryo developmental process in liquid culture. The studies were facilitated by the cytochemical staining of cells in suspension using acetocarmine (0.5 %) and Evans blue for diagnostic purposes.

8.2.5 Viability Determination

To determine the viability of cultures, representative samples were stained with a few drops of fluorescein diacetate (FDA). One ml of FDA was mixed with 10 ml of medium containing cellular aggregates and observed under an inverted microscope (Zeiss IM 35), equipped with an ultra violet lighting facility.

8.2.6 Data Collection

Cell growth was determined with the aid of 500 ml side-arm flasks, using the settled cell volume technique. Flasks were suspended so that each angle of the arm was perpendicular for a period of 5 min to allow final settling of cells at the base of the arm after a period of 10 min. Cell growth was recorded at the same time on a daily basis. The recorded growth indices were converted to percentage values.

8.2.7 Data Analysis

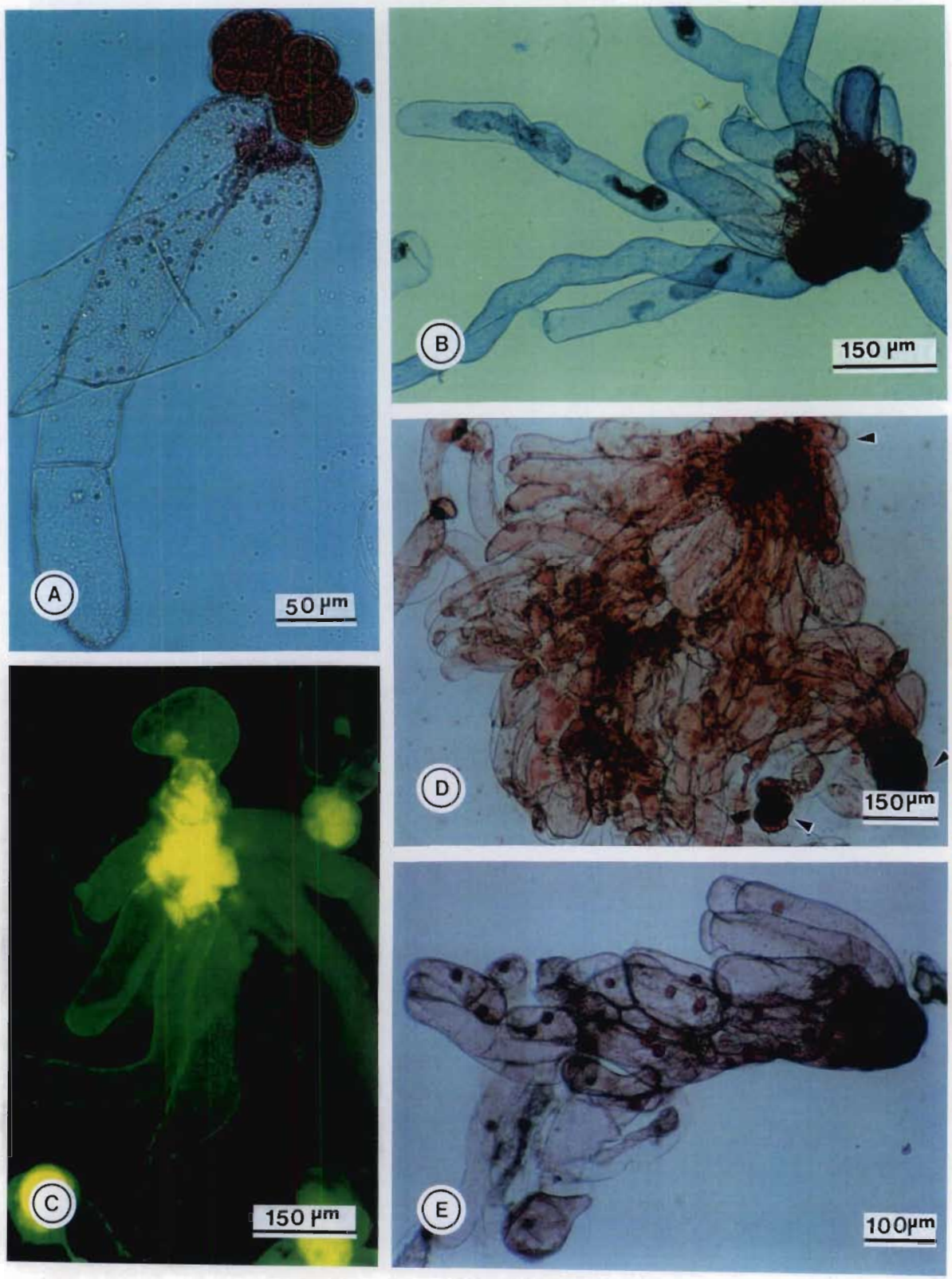
Each experiment consisted of five replicates (flasks) per treatment and experiments were repeated three to four times each.

8.2.8 Re-establishment of Suspension Cultures onto Solid Media

A 100 ml aliquot of maintenance suspension-culture (DCR1 medium) was allowed to settle in a 100 ml measuring cylinder for 20 min. The excess "old medium" was poured off and about 20 ml fresh DCR1 medium was added and the cellular aggregates resuspended. Volumes of between 3 and 5 ml of the suspension culture were then poured onto agar-solidified maturation media (DCR2) containing ABA (Table 7.1). The liquid film was allowed to cover the surface of the medium. Petri dishes were sealed with Parafilm® and incubated in the dark at 25 °C.

8.3 Results

Suspension cultures were easily initiated from embryogenic tissue previously cultured on solid medium. The mucilaginous, friable nature of the embryogenic tissue, facilitated fragmentation and dispersion of cellular aggregates throughout the liquid medium. Microscopic examination of the cellular aggregates developing in suspension demonstrated the presence of pre-embryonal-suspensor masses (PEM), characteristic of embryogenic tissue maintained on solid DCR1 medium. Staining with acetocarmine (a chromatin stain) revealed the nuclei in the apical region of the proembryonic tissue. The neocytoplasm (new cytoplasm which develops around the nucleus in the zygote after fertilization) gives an intense red-staining product which can be observed in the developing pro-embryonal cells and suspensor cells undergoing cleavage (DURZAN 1991). The early apical division pattern of the pro-embryo and the initial elongation of the suspensor was observed in embryos developing on maintenance medium (Fig. 8.1A). Cell divisions gradually proliferated to produce a recognizable somatic embryo (Fig. 8.1B) consisting of a red-staining embryonal apex subtended by elongated, blue-staining suspensor cells. Evans blue has a greater affinity for the suspensor cells and nuclei and may be used to determine the viability of cells (GUPTA & DURZAN 1987b) since less viable cells and nuclei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permit more dye to enter (GAHAN 1984). Embryo viability was also tested using FDA. Fluorescent staining demonstrated high viability especially in the meristematic apical regions (Fig. 8.1C). Somatic embryo proliferation in liquid culture appears to occur via a process of cleavage (Fig. 8.1D). Once cleavage embryos have developed, they begin to separate as a result of the agitation of the medium to form discrete entities (Fig. 8.1E).

With regard to induction and maintenance of cultures in suspension, replicated growth analyses indicated that when a high volume (> 20 %) of initial inoculum was used, growth in terms of settled cell volume (time dependent measurement) showed a typically linear growth response (Fig. 8.2). Settled cell volume (%) increased linearly to the point of saturation where possibly the nutrients became the limiting factor, inhibiting further growth. By lowering the initial inoculum volume to approximately 6 % (settled cell volume), a sigmoidal growth curve was obtained, representative of most suspension cultures (Fig. 8.2).

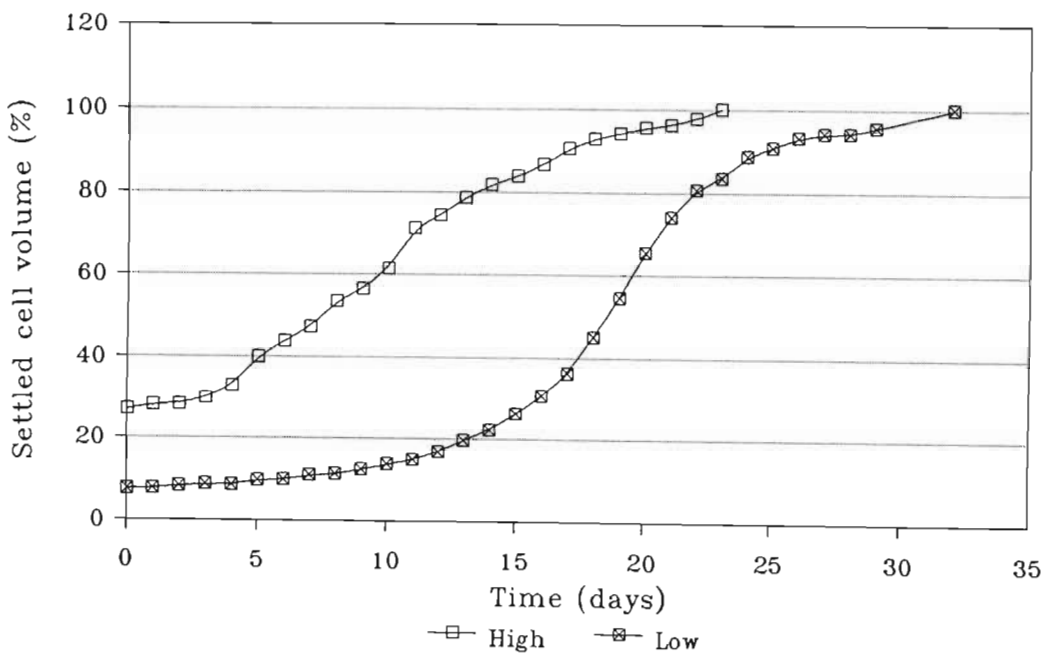


Fig. 8.2 Growth of an embryogenic suspension culture on DCR1 maintenance medium, using a high and low initial inoculum.

Maturation, in terms of the development of cotyledonary initials from the central apical meristem of the developing somatic embryo was not obtained in suspension. Placing cultures directly from initiation medium into media containing various levels of ABA, initially showed no treatment effects since equal growth was obtained irrespective of the hormone treatment applied (Fig 8.3). Using an inoculum of less than 20 % (SCV) exceptional growth but poor maturation was obtained (Fig. 8.3). Irrespective of the ABA treatment, cultures reached saturation about two weeks after their induction. There was also little difference between treatments with respect to somatic embryo development, although those exposed to ABA appeared slightly enlarged and more opaque while those on hormone-free medium remained undifferentiated.

Using pre-conditioned cultures, large differences were observed in culture growth between the ABA treatments, especially in the time required to reach saturation. By day 20, differences in the growth rate between treatments were already discernable, and these were linked to similar developmental patterns. The treatment containing no hormone showed a great reduction in both growth and development (Fig. 8.4), emphasised by the very fine consistency of the cultures, reflecting a lack of differentiation, coupled with a necrotic appearance of the cellular aggregates. The two treatments containing ABA showed a slower growth rate than that observed in the initial experiment (Fig. 8.4). Cultures growing in the lower ABA level, appeared very fine, consisting of small cellular aggregates, lacking in differentiation and appearing highly translucent. Cultures exposed to 1.3 mg l^{-1} ABA were characterized by the presence of larger, creamy embryonal apices, still attached to semi-translucent suspensor regions which had thickened and elongated slightly. No cell necrosis was evident and a very high embryo density was observed. The most advanced stage of development achieved was barely past stage 1 as described for solid cultures (Chapter 7). Despite the potential for embryogenic tissue to grow on the ABA-containing DCR2 medium, maturation in suspension was not pronounced.

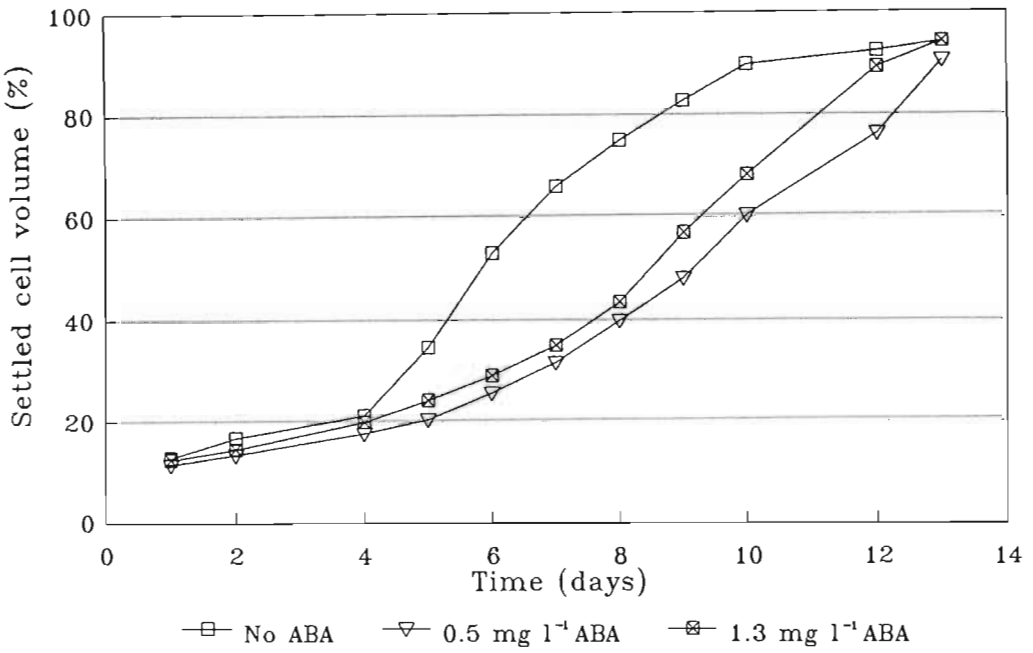


Fig. 8.3 Growth of embryogenic suspension cultures on DCR2 maturation medium containing various concentrations of ABA.

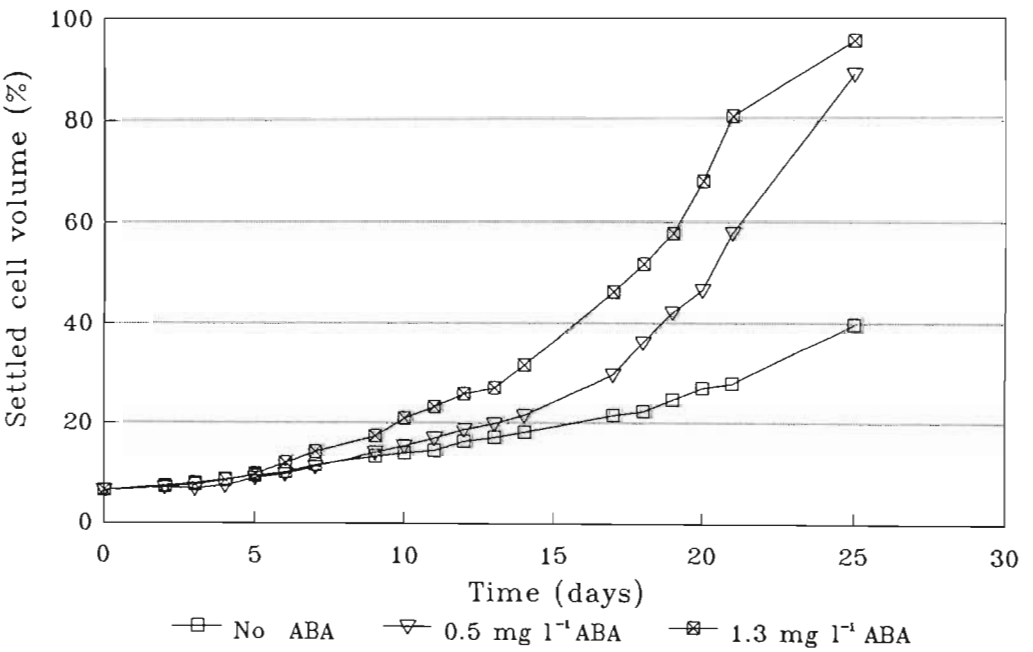


Fig. 8.4 Growth of liquid cultures on DCR2 maturation medium containing various levels of ABA, after pre-conditioning on the same medium for a period of 10 days.

Replacement of sucrose with maltose resulted in no growth at the 3 % level, although some growth was observed at the lower concentration (1 %). In both cases, maturation as described on solid media was not observed. Lack of growth coupled with necrosis of tissue was observed at the higher maltose level. No embryo differentiation was observed.

Liquid cultures were easily re-established onto solid media by inoculating agar-solidified medium with a few ml of the suspension culture. The initially semi-liquid appearance of the cultures soon developed into firmly established, semi-translucent, mucilaginous, embryogenic tissue which rapidly covered the agar surface within a week of inoculation. Plantlet maturation and hardening-off of emblings was achieved by transferring liquid maintenance cultures back onto solid media using the protocol described in Chapter 7.

8.4 Discussion

The most beneficial responses obtained from the initiation of embryogenic suspension cultures were the relative ease with which cultures could be initiated, and the favourable growth response observed when a very low volume of initial inoculum was used. This suggests that even if the initial embryogenic induction frequency from cultured explants is low, tissue can easily be bulked using a suspension system. The sigmoidal growth pattern (Fig. 8.2) obtained using a low inoculum was characterised by an initial lag phase during which cell division was not apparent, followed by an exponential rise in cell number, and a linear increase in the cell population (DODDS & ROBERTS 1985). This phase was followed by a levelling off of the curve, indicative of the stationary or non-dividing phase. Subculturing has been recommended at this stage or when maximum cell density is reached. These plots therefore indicate when subculturing should occur, in order to maximise growth and use of the medium. The data presented (Fig. 8.2) shows that maximum cell density was reached within approximately 23 days. Suspension cultures have been found to reach

their maximum cell density within 8 to 25 days depending on the species being cultured (DODDS & ROBERTS 1985). The suspension culture growth curves indicate the relatively rapid rate at which cultures can multiply, far exceeding that of cultures incubated on solid media.

Although various attempts were made to mature somatic embryos using liquid conditions, no successful development was obtained, either by manipulating the hormonal additives, or the osmotic potential of the medium. No reports are presently available on the successful maturation of *Pinus* species using liquid culture media, although numerous groups are working on this particular aspect of propagation. A number of factors may be responsible for the poor performance of embryos subjected to liquid conditions. These include the osmolarity of the culture medium, and the inability of the maturing embryos to undergo desiccation while submerged in the liquid as well as problems related to gaseous exchange.

When conifer somatic embryos were submerged in liquid or agar, maturation was inhibited (BOULAY, GUPTA, KROGSTRUP & DURZAN 1988; HAKMAN & VON ARNOLD 1988). Preliminary studies demonstrated that the use of individual culture flasks were ineffectual in promoting maturation processes in conifer embryogenic cultures (ROBERTS personal communication). The use of a number of culture vessels linked together by a series of tubes to enable a through-flow of medium in a non-agitated system have proved more beneficial. The cultivation of embryos in this manner, using suitable support mechanisms, enable the required desiccation process to occur in the maturing embryos (ROBERTS personal communication). Similar observations were made by BOULAY, GUPTA, KROGSTRUP & DURZAN (1988) who found that transfer to solid supports suspended above liquid media was essential for maturation to occur. This alternative method of culture offers good gaseous exchange and also provides a moisture-stressing environment. Although the addition of PEG to liquid media is successful in producing a moisture stress, the accompanying reduced oxygen tension is not desirable (ATTREE & FOWKE 1993).

The osmolarity of culture media, as described earlier (Chapter 7) plays an important role in the ability to mature somatic embryos. The addition of sugars, hexitols or cyclitols capable of raising the osmotic potential of media, has benefitted the maturation of numerous conifer species (NAGMANI & DINUS 1991). These authors found that maltose in particular was responsible for transforming advanced somatic embryos of Douglas-fir to the mature cotyledonary stages.

Because so many difficulties are encountered when attempting to mature embryogenic tissue using liquid means, it seems evident that future research should concentrate on this area. If liquid suspension culture could be refined, it might provide a viable option for commercial purposes, as the system does have great advantages. The maintenance of embryogenic tissue suspension cultures could be useful as a tool for bulking up the tissue in order to increase somatic embryo production as much as possible.

CHAPTER 9

LIPID DEPOSITION IN SOMATIC AND ZYGOTIC EMBRYOS OF *Pinus patula*

9.1 Introduction

Storage lipids (triacylglycerols - TAG) occur in seed as oil droplets commonly referred to as lipid bodies (GURR 1980; APPELQVIST 1982). Numerous ultrastructural studies have been conducted on lipid bodies of oil seed at various stages of development (ADAMS, NORBY & RINNE 1983; MURPHY, CUMMINS & KANG 1989). An unresolved issue concerns the ontogeny of lipid bodies. This involves the association of organelles (such as plastids) and membrane systems (as the endoplasmic reticulum) in the synthesis and organisation of lipid bodies and their surrounding membrane (DUTTA, APPELQVIST, GUNNARSSON & VON HOFSTEN 1991). Despite the uncertain ontological development of lipid bodies, it is generally accepted that these organelles are surrounded by an osmiophilic (unit or half unit) membrane (GARCIA, QUINTERO & MANCHA 1988; MURPHY, CUMMINS & KANG 1989).

It is well-established that the accumulation of storage products is part of the normal process of orthodox seed and embryo differentiation (BEWLEY & BLACK 1986). It is also known that exposure of embryogenic tissue to ABA results in mobilization of reserves (ROBERTS, LAZAROFF & WEBSTER 1991a; KIM & JANICK 1991). Qualitative and quantitative studies have been conducted on the development and accumulation of lipid bodies in somatic and zygotic embryos of carrot (DUTTA, APPELQVIST, GUNNARSSON & VON HOFSTEN 1991). Similar patterns of accumulation have been found to occur

in conifers, where lipids tend to increase with the onset of maturation, in a process closely resembling the zygotic developmental pattern.

Some authors found that a combination of a high ABA concentration together with a non-plasmolysing osmoticum (such as PEG 4 000) induced a nine-fold increase in the amount of TAG per embryo, and imposed an associated desiccation tolerance (ATTREE, POMEROY & FOWKE 1992). During subsequent desiccation there was a further graded accumulation of lipids.

These observations prompted a histochemical investigation of lipid localization in maturing embryogenic tissue of *P. patula*. This study had three objectives: (a) to determine the presence and pattern of lipid accumulation in maturing embryogenic tissue; (b) to compare quantitatively, total levels of lipid in the somatic and zygotic embryos and to examine fatty acid levels in the different embryos by Gas Liquid Chromatography (GC) and (c) to compare ultrastructural aspects of lipid body accumulation during somatic embryo development with that of mature zygotic embryos of pine.

9.2 Materials and Methods

Embryogenic tissue maintained on BA and 2,4-D containing-medium (non-matured) and tissue that had been exposed to ABA (matured) as well as portions of zygotic embryos were investigated for lipid content (Details regarding induction and maturation of the tissues are provided in Chapters 6 and 7).

9.2.1 Sudan Black B Staining for Lipids

Embryogenic tissues were fixed in formal calcium (40 % formaldehyde (10 ml); distilled water (90 ml) and 10 % calcium chloride (10 ml) as described by BANCROFT & STEVENS (1982)) for approximately 1 h before being rinsed in

70 % ethanol. Tissues were then stained for 15 to 30 min in saturated Sudan Black B (0.5 g) dissolved in 70 % ethanol (100 ml). Prior to staining, the Sudan Black B solution was boiled for 10 min, allowed to cool and filtered (GURR 1956). Samples were differentiated with 70 % ethanol. Acetocarmine was used as a counterstain. Tissues were rinsed and mounted in glycerol. Lettuce seeds which contain high quantities of lipid (PAULSON & SRIVASTAVA 1968) were used as a control. Observations were conducted using bright field light microscopy and results recorded using a photomicroscope (Olympus BH-2).

9.2.2 Lipid Analysis

A total lipid analysis was conducted on matured and non-matured embryogenic tissue as well as gametophytic tissue (from mature seed). Samples weighing 1 to 1.5 g fresh mass were used for each extraction. Three replicates were used for each treatment, and the experiments were repeated six times. Initially, embryogenic tissue from various tree lines was randomly selected for extraction purposes, but was found to yield inconsistent results. When lipids were extracted from tissue samples of the same tree, trends were more consistent.

After recording the fresh mass of the tissues, the samples were extracted twice in a volume (60 ml g^{-1} fresh weight) of chloroform:methanol (2:1 v/v) and homogenized with an Ultra-Turrax. The solvent solution was separated from the cellular debris by filtration using a Buchner funnel. A double layer of pre-weighed Whatman No. 1 and No. 42 filter paper, was used to trap the cellular debris. The clear filtrate was then placed into a separating funnel and given a Folsch wash (1 % aqueous NaCl equal to ⅓ the volume of the solvent). Once the emulsion had cleared, and two separate layers were evident, the lower chloroform:methanol layer was drained off and dried with anhydrous Na_2SO_4. The solvent was then filtered through a 0.45 μm DynaGard$^{\bullet}$ column and taken almost to dryness on a rotary evaporator at 30 °C (Buchi). The lipid extracts were then transferred into pre-weighed vials and evaporated under a stream of

nitrogen. After a 24 h period of drying over silica gel, the mass of each sample was determined. Lipid content was calculated on a fresh and dry mass basis.

9.2.3 Esterification of Fatty Acids

The fatty acid component of mature and immature embryogenic tissue was assessed. Methyl esters of the fatty acids were obtained using a modification of the organic base-catalysed technique of METCALFE & WANG (1981). An aliquot of lipid (approximately 100 μl) was dissolved in 400 μl of diethyl ether. Four hundred μl of tetramethylamonium (TMAH) were added and the mixture shaken for 1 min. Six hundred μl of water were added, the methyl esters partitioning into the upper ether phase. An aliquot of this upper phase was analysed using Gas Liqiuid Chromatography. Fatty acid methyl esters were identified by comparison with the retention times of authentic standards (Rapeseed, Sigma).

9.2.4 Gas Liquid Chromatography

A Varian model 3300 Gas Chromatograph, fitted with a flame ionization detector (FID) and a 25 m x 0.53 mm I.D. fused silica column coated with polyethylene glycol (SGE, BP2D), was used. Helium was used as the carrier gas at 25 ml min^{-1}. For fatty acid analysis, the injection temperature was 260 °C, the detector temperature was 200 °C and the column temperature was programmed from 100 to 200 °C, at 3 °C min^{-1}. Peak areas were integrated electronically and expressed as area percentages.

9.2.5 Ultrastructural Studies

Samples of embryogenic tissue, maturing somatic embryos (exposed to ABA), as

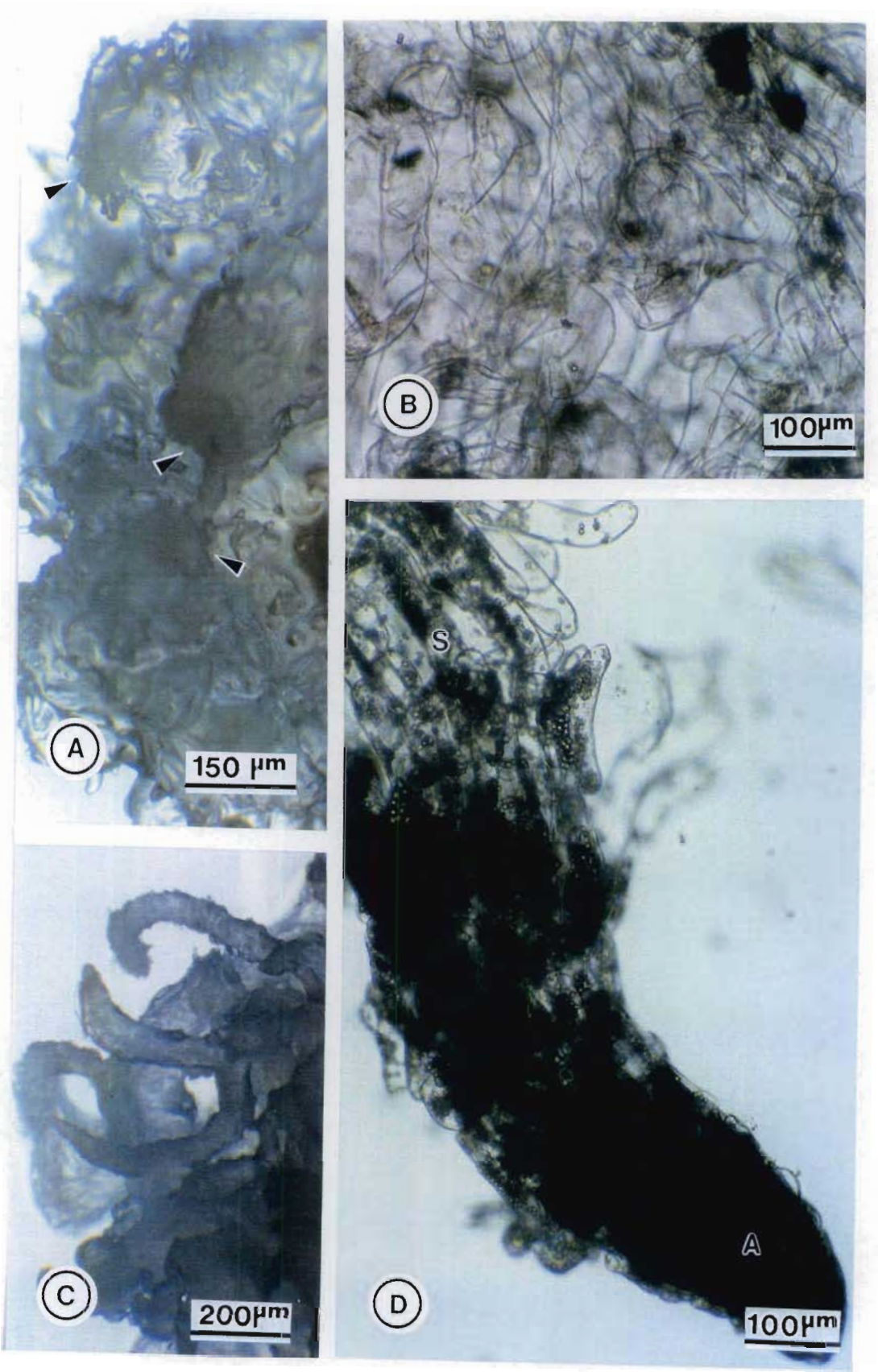
well as matured zygotic embryos were prepared for transmission electron microscopy by fixing in 3 % glutaraldehye in a 0.05 M sodium cacodylate buffer (pH 7.1) for 24 h. After two buffer washes, tissues were post-fixed with cacodylate buffered osmium tetroxide (2 %) and dehydrated through a graded ethanol series. Embedding was achieved using a low viscosity resin (SPURR 1969). Ultrathin sections (approximately 90 nm) were post-stained with lead citrate (REYNOLDS 1963) and uranyl acetate, prior to viewing.

9.2.6. Data Analysis

Data were analysed by analysis of variance using the Statgraphics Statistical Programme. An arcsine conversion was conducted to normalize data and Tukey's multiple comparison test was used to indicate significant difference between means of treatments.

9.3 Results

All lettuce seed tissues showed numerous discrete bodies which stained black with Sudan Stain, demonstrating a highly positive response to the presence of lipid. Staining of immature embryogenic tissue (Fig 9.1A) indicated little or no presence of lipid bodies in the cells (Fig. 9.1B), even though counterstaining with acetocarmine revealed the presence of numerous embryonal apices (indicative of developing somatic embryos) within the tissue. Staining of tissue that had been exposed to ABA (Fig. 9.1C) revealed an abundance of lipid droplets in the embryonal apex, while in the suspensor region they were less common (Fig. 9.1D). This suggests that lipid accumulation occurred predominantly in the apex. Zygotic and gametophytic tissues from mature seed stained positively for the presence of lipids.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The percentage lipid content of the various tissues examined, was significantly different ($P < 0.05$) at all levels tested (Fig. 9.2). The gametophytic component of the zygotic seed contained significantly higher levels of lipid than the embryogenic tissue. Similarly, the matured tissue (ABA-treated) contained larger lipid deposits than embryogenic tissue which had not been exposed to ABA (Fig. 9.2). This trend was reflected on both a fresh and dry weight basis. The results, as presented on a dry weight basis, indicated that the lipid content of the maturing tissue was exceptionally high, and largely responsible for the mass increase of tissues. These levels were approximately half of those found in the seed (Fig. 9.2). On a fresh weight basis, the lipid content of the embryogenic tissue was a fraction of that occurring in the seed. This suggests that the embryogenic tissue was considerably more vacuolated than the seed, thus reflecting the desiccated state of the latter.

The fatty acid composition of tissues examined, revealed the predominant presence of 16:0 (stearic), 18:1 (oleic) and 18:2 (linoleic) fatty acids, although no significant quantitative and qualitative differences were observed between the mature and immature tissues (Fig. 9.3).

Ultrastructural observations of non-matured embryogenic tissue revealed cells which were meristematic in appearance. They were characterized by a noticeable lack of storage reserves, which included lipids, protein bodies and starch granules. Despite the general lack of storage products in the tissue, an abundance of mitochondria, Golgi bodies, rough ER (endoplasmic reticulum) and small scattered vacuoles was observed (Fig. 9.4A).

The maturing embryonal apices consisted of tightly fitting isodiametric cells with densely packed cellular contents. Mitochondria and amyloplasts were abundant as were the numerous small vacuoles scattered throughout the cytoplasm. The nucleus often occupied a large portion of the cell. Lipid reserves were scattered

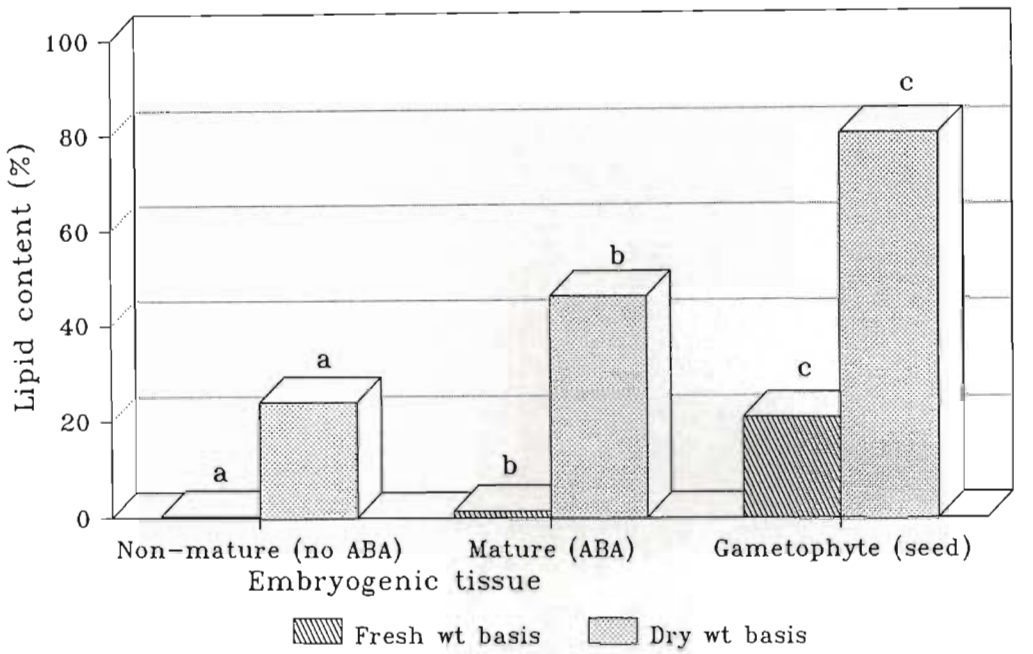


Fig. 9.2 Lipid content (%) of mature and immature embryogenic tissue in comparison to that of mature pine seed, presented on a fresh and dry weight basis (Treatments denoted by the same letters are not significantly different at the 0.5 % level).

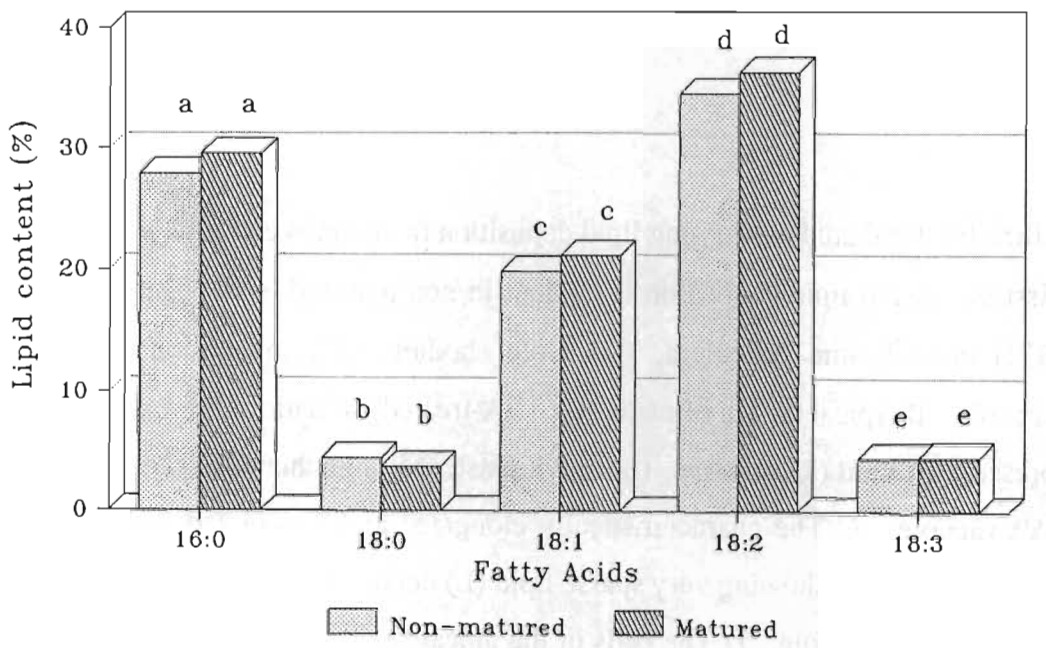
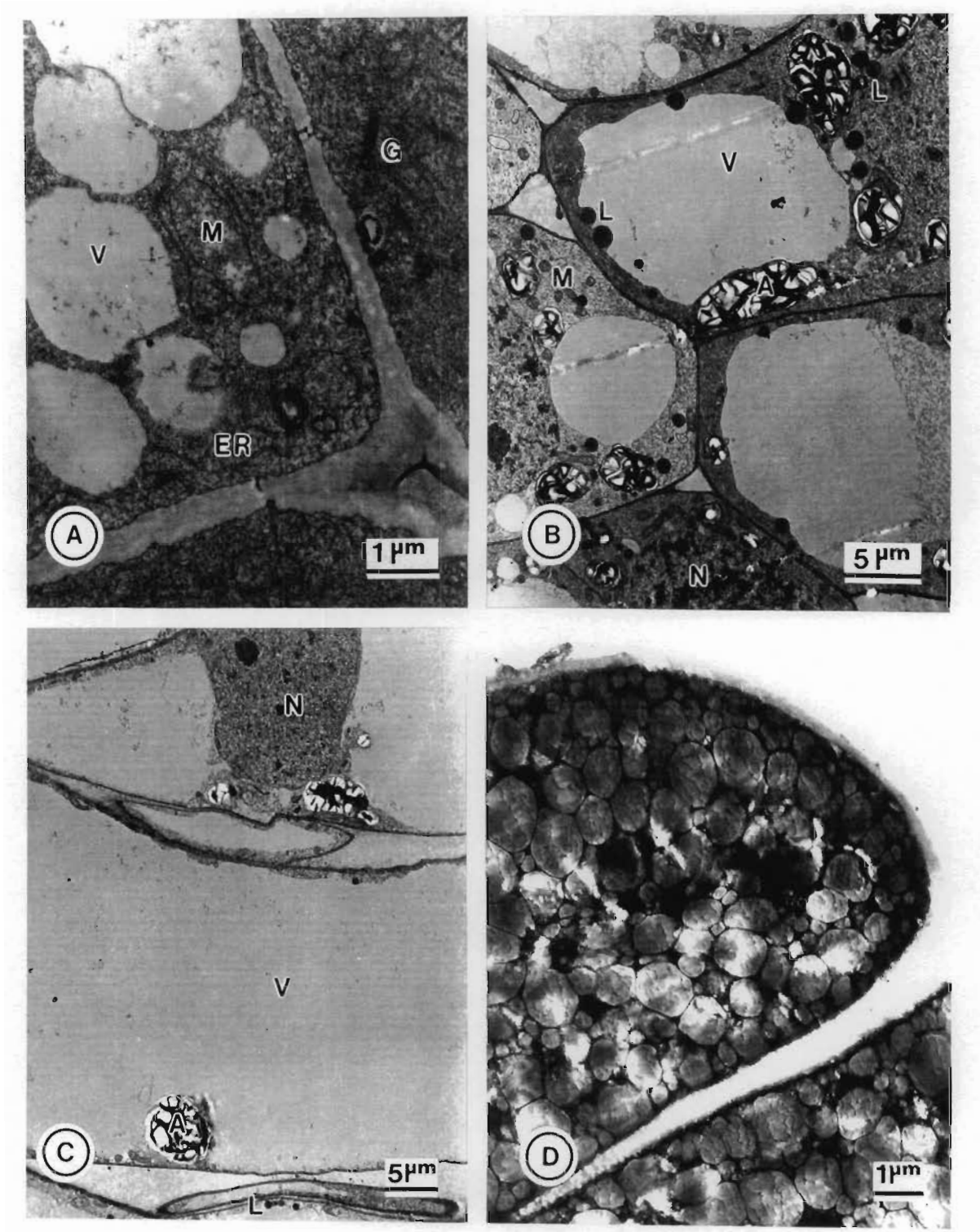


Fig. 9.3 Fatty acid component of mature and immature embryogenic tissue (Treatments denoted by the same letters are not significantly different at the 0.5 % level).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throughout the cytoplasm of cells and occasionally were associated with the vacuolar membrane. Lipid was sequestered in single membrane-bound organelles, appearing more abundant in the apical region of the somatic embryo (Fig. 9.4B), than in the suspensor region (Fig. 9.4C). This is in keeping with the observations using Sudan Black B. Very few lipid deposits were observed in the suspensor region, and were restricted to the peripheral cytoplasm surrounding the characteristically large vacuoles (Fig. 9.4C). The appearance of the lipid deposits in the apical and suspensor regions was similar. Relatively few starch grains were observed in the suspensor region (Fig. 9.4C). When the zygotic embryo apical region was studied, cells were found to consist almost entirely of lipid deposits while much of the other cell detail was obscured (Fig. 9.4D).

9.4 Discussion

The lipid analyses confirmed that ABA is essential for lipid deposition and the maturation process since non-matured tissue demonstrated very low lipid accumulation (Fig. 9.2) and somatic embryo differentiation was limited in the absence of ABA (Fig. 9.1A). The accumulation of storage reserves in conifer somatic embryos is known to be influenced by ABA and water stress (ATTREE & FOWKE 1993). Reserve deposition can therefore be regarded as a characteristic event indicative of the maturational phase. The ability of somatic embryos to accumulate reserves, is significant with respect to the ability of emblings to acclimatize to *ex vitro* conditions. This consideration is of greater consequence to somatic embryos than their zygotic counterparts, in that somatic embryos lack gametophytic tissue which provides both nutrition and protection for the zygotic embryo during germination (ATTREE, POMEROY & FOWKE 1992).

Significant differences in lipid contents of conifer zygotic and somatic embryos have also been observed in other species (KIM & JANICK 1991). Some reports show total lipids to comprise 30 to 50 % of the dry weight of conifer seed

(KONAR 1958; CHING 1963; 1966). Others describe the substantially lower amounts of triacylglycerols in mature somatic embryos of Norway and white spruce when compared to zygotic embryos (FEIRER, CONKEY & VERHAGEN 1989; JOY, YEUNG, KONG & THORPE 1991). These differences may suggest that somatic embryos which are capable of germination (approximately 6 to 8 weeks old) are still at an immature stage compared with zygotic embryos which require a much longer developmental period before they are ready for germination.

The use of non-plasmolysing agents (such as PEG 4 000) can increase somatic embryo TAG content by as much as four times that of the zygotic embryo (ATTREE, POMEROY & FOWKE 1992). It appears therefore that, in determining optimal maturation conditions, a compromise may need to be reached in terms of the optimum ABA concentration and the most appropriate type and concentration of osmoticum to use in order to yield the maximum number of superior quality embryos in terms of lipid accumulation and reserve deposition.

ATTREE, POMEROY & FOWKE (1992) found that inclusion of PEG-4000 together with ABA in culture increased the quantity of TAG in somatic embryos of *Picea glauca*. These fatty acid levels (predominantly 18:1 (oleic) and 18:2 (linoleic) acids) were not as high as those reported for zygotic embryos. The authors also observed that during an eight week period the 18:2 fatty acid composition in mature embryos increased, while all other fatty acids showed a proportional decline. The fatty acid levels of the somatic embryos treated with PEG closely resembled those of the zygotic embryos. The use of a non-permeating osmoticum in the medium appears to be effective in encouraging fatty acid synthesis and hence enhancing the storage deposition. The similar fatty acid levels in both the mature and immature embryogenic tissue of *P. patula* suggest the need for the addition of an osmoticum to the medium to enhance lipid accumulation in the maturing tissue. Alternatively, exposure to ABA may need to be increased to promote lipid biosynthesis.

Sudan Black B is a bisazo dye lacking ionic substituents. It dissolves easily in hydrophilic lipids, but is far less soluble in hydrated substances, which explains its effectiveness as a lipid stain. The standard Sudan Black procedure is non-selective and causes the staining of unsaturated cholesterol esters, triglycerides and some phospholipids (HOROBIN 1982). The results show an abundance of lipid deposits in the maturing tissue in comparison to immature embryogenic tissue, with reserves being located predominantly in the apical regions of the developing somatic embryos.

The ultrastructural studies support the results obtained from the lipid analyses, indicating that immature somatic embryos contain few lipid droplets or other storage reserves. The presence of lipids and starch in maturing embryogenic cells, and the noticeable absence of storage protein bodies, recalls the situation observed in the early stages of zygotic embryo development (DUTTA, APPELQVIST, GUNNARSSON & VON HOFSTEN 1991). These authors suggest further that the absence of protein bodies may be an indication that somatic embryos are at an early stage of reserve accumulation. It is possible that storage protein is synthesized later than storage lipid. This is a phenomenon also observed in developing rapeseed embryos (MURPHY & CUMMINS 1989).

The value of studying storage reserve deposition is that culture conditions may then be used in order to maximize the somatic embryo developmental potential. The ultimate objective is to achieve a system which produces embryos with a physiological resemblance to their zygotic counterparts. For white spruce, this approach appears to entail incubation of embryos for 8 weeks on media containing 4.2 to 6.3 mg l^{-1} ABA and 7.5 % PEG, followed by a desiccation treatment (ATTREE, POMEROY & FOWKE 1992). For pine the protocol has yet to be defined, but similarities with spruce suggest that this may be achieved with the appropriate tissue culture manipulations.

CHAPTER 10

POLLINATION, FERTILIZATION AND ANATOMICAL DEVELOPMENT OF ZYGOTIC EMBRYOS

10.1 Introduction

Somatic embryogenesis of conifers and that of pines in particular, closely resembles zygotic embryogenesis (TAUTORUS, FOWKE & DUNSTAN 1991). Understanding the zygotic developmental processes may therefore aid the interpretation of somatic events. Although studies of zygotic embryo development in conifers are numerous and well documented (CHAMBERLAIN 1935; NORSTOG 1982; SEDGLEY & GRIFFIN 1989), knowledge of the precise time at which certain events occur is critical for the induction of embryogenic tissue *in vitro*. The period during which fertilization takes place in *P. patula* is unknown. The exact time of fertilization is of importance as, in pines, embryogenesis has been found to occur approximately three weeks after fertilization (BECWAR, BLUSH, BROWN & CHESWICK 1991). Knowledge of when fertilization occurs may therefore give an indication as to when cones should be collected and when embryogenic induction could be expected to occur in culture. This information would help to determine the "window" period or the phase during which explants are most responsive. An anatomical investigation was conducted on the zygotic embryogeny of *P. patula*, in order to relate the developmental events to embryogenic induction in culture.

10.1.1 Ovulate Cones

Ovulate or female cones are borne on the tips of young branches in early spring (September/October). The cones consist of a central axis which bears several woody scales in a spiral. Two ovules develop on the upper surface of each ovuliferous scale. Each ovule is enveloped in a protective layer called the integument and a small aperture develops at the end proximal to the axis. This opening, known as the micropyle, permits pollen to enter the ovule. In pine, the megasporocyte lies in the centre of each ovule and is surrounded by the nutritive nucellus tissue.

10.1.2 Female Gametophyte

The diploid megasporocyte divides meiotically to produce four haploid megaspores. Only one of the megaspores will develop into the female gametophyte, while the rest will gradually degenerate. Growth of the megaspore and gametophyte is a very slow process, requiring up to 13 months for development to occur in pine species (RAVEN, EVERT & CURTIS 1981). After a number of mitotic divisions, cell walls begin to develop within the megaspore, which is surrounded by a diploid layer of cells. Two archegonia begin to develop within the female gametophyte, close to the micropylar end of the ovule (WEIER, STOCKING, BARBOUR & ROST 1982).

10.1.3 Pollination

Pollination in most conifers occurs as soon as the ovulate cones begin to develop in early spring when cones are soft and purplish in colour, and when the scales are opened. Wind is the major pollinating agent. Pollen adheres to a sticky substance produced by the ovule and, as the material dries, pollen grains are drawn through the micropyle into the micropylar chamber where they lodge close to the

developing female gametophyte. The pollen grains slowly germinate into a tubular male gametophyte which grows through the nucellus tissue. This tissue has a nutritive and protective function for both the developing male and female gametophytes. Numerous nuclear divisions occur within the tube, but no cross walls develop. Two of the last formed nuclei, the sperm nuclei, are responsible for fertilization and, together with several vegetative nuclei, comprise the male gametophyte.

10.1.4 Fertilization and Embryo Development

The development of the male and female gametophytes is a co-ordinated process. The egg is fully developed for fertilization by the time the pollen tube has reached the archegonium. At the time of fertilization, in comparison with pollination, the cones are larger, green, tightly closed and a year older. The contents of the pollen tube are discharged directly into the egg. One of the sperm nuclei comes into contact with the egg nucleus and unites, while the non-functional sperm nuclei and other protoplasmic material discharged into the egg cell disintegrate. Usually the eggs of both archegonia are fertilized and embryo development, a process referred to as polyembryony, commences. Despite this multiple embryo development, only one embryo will develop completely.

10.2 Materials and Methods

10.2.1 Cone Collections

Cone collections for embedding purposes were initiated in August 1992, and continued for over a year until September 1993. Both newly-formed cones and first year cones were collected from all five experimental trees in order to determine when pollination and fertilization take place in *P. patula*.

10.2.2 Wax Embedding Procedure

Initially, young cones were halved for embedding purposes. From 5 November 1992 and subsequent collection dates, large cones were quartered before embedding. When cones became too large for efficient embedding (15 December 1992), the seeds were extracted and embedded. At each collection date, cone samples were fixed in formalin-acetic acid-alcohol (FAA), dehydrated and infiltrated as described in Table 10.1. Cones were left in each solution for approximately two weeks to facilitate thorough infiltration of the woody tissue.

10.2.3 Staining Protocol

Once embedded and blocked, sections were cut, using a microtome (R Jung, AG Heidelberg). Cone sections of approximately 20 μM and seed sections of about 7 μM were obtained. The sections were fixed onto glass slides, using a few drops of Haupt's Adhesive and 3 % formalin, before staining (Table 10.2). The stained sections were then mounted with glass cover slips using "Entellan" (E. Merck) to obtain permanent slides.

10.2.4 Data Collection

Sections were cut from cones and seed of each of the five experimental trees in order to obtain representative samples of the pollination and fertilization stages. The sections were viewed and photographed using a photomicroscope (Olympus BH-2). The collection dates of samples manifesting the relevant stage of development were recorded. The determined time of fertilization was then assessed in conjunction with the *in vitro* induction of embryogenic tissue (discussed in Chapter 6) in order to determine at which zygotic developmental stage embryogenesis was initially induced.

Table 10.1 Procedure for fixation, dehydration and wax infiltration of *P. patula* female cones and seed.

TREATMENT	TEMP (°C)
FIXATION	
FAA (formalin-acetic acid-alcohol)	20
DEHYDRATION	
Water:Ethanol:Tertiary-butanol	
45:45:10	20
30:50:20	20
15:50:35	20
15:40:55	20
0:25:75	20
0:0:100	40
0:0:100	40
INFILTRATION	
Tertiary butanol:liquid paraffin 50:50	40
Liquid paraffin	40
Liquid paraffin and a few wax pellets	40
Liquid paraffin and a few wax pellets in an open vial	60
Pure molten wax	60

Table 10.2 Dewaxing and staining procedure for wax embedded sections.

STAIN/SOLVENT	DURATION (min)
Xylene	3
Xylene	3
Xylene : ethanol	1
70 % ethanol	30 sec
90 % ethanol	30 sec
Safrannin in 90 % ethanol	20
95 % ethanol	30 sec
100 % ethanol	1
100 % ethanol	1
Fast green in 90 % ethanol	a few seconds
Xylene : ethanol	30 sec
Xylene	1

10.3 Results

When first year cones were initially collected (25 August 1992) they were brownish in colour, although after a month, greening was evident between the ovuliferous scales. Expansion or swelling was also increasingly evident in the middle-to-lower portion of the cone. With each collection, cones became progressively larger. The new season's cones were first observed in Tree 1 on 8 October 1992 and only two weeks later on the other tree samples. These young cones were very small (approximately 12 x 7 mm), soft and purple in colour. By the beginning of December 1992, most of the newly-formed cones had turned green.

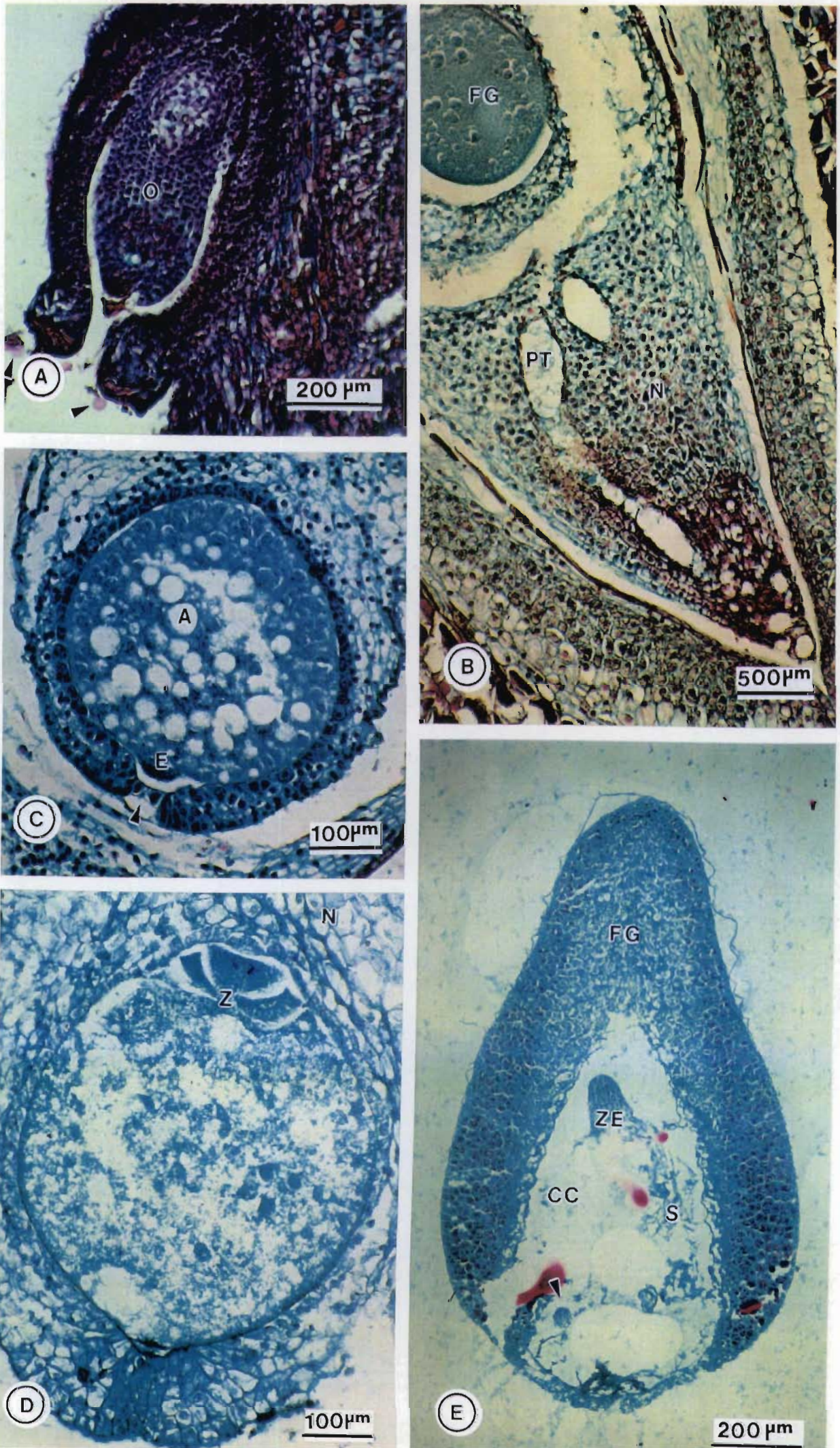
Anatomical observation of the young developing cones indicated that pollination of *P. patula* occurred in mid-November (19 November 1992), only a few weeks

after the appearance of the new female cones. This observation was supported by the presence of pollen grains situated between the ovuliferous scales, close to the micropylar end of the ovule (Fig. 10.1A). Cones that were a year older exhibited the presence of a well developed female gametophyte surrounded by the nutritive nucellus tissue (Fig. 10.1B). Growth of the pollen tube was also evident, initiating at the micropylar end and developing through the nucellus towards the female gametophyte (Fig. 10.1B). These observations were made on the ovules of cones collected on 1 December 1992.

By the time the pollen tube had reached the female gametophyte (a process which takes approximately a year), both the egg and sperm nuclei were sufficiently developed for fertilization to occur. This phase is characterized by a narrowing of the archegonial neck cells, and a migration of the egg towards the narrower end. This development facilitates the transfer of the sperm nuclei to the female gametophyte (Fig. 10.1C). The fertilization stage was identified in all five tree samples tested and found to occur in cones collected on 1 December 1992.

Two weeks following putative fertilization (15 December 1992), the archegonia of the *Pinus* ovule appeared to be filled by a transparent viscous gel (Fig. 10.1D). These structures were in fact composed of many thin-walled cells (GATES & GREENWOOD 1991). The zygote began to develop in each archegonium. On closer examination of the archegonium the first zygotic divisions were noticeable and the development of the zygote into the surrounding nucellus tissue was observed (Fig. 10.1D).

Approximately a month later (9 January 1993), substantial development of the zygotic embryo was observed. The cells in the centre of the gametophyte disintegrated to form the cylindrical corrosion cavity which surrounds the developing embryo (GATES & GREENWOOD 1991). The suspensor which is responsible for forcing the embryo deeply into the gametophyte tissue was visible, as was the accompanying presence of developing cleavage embryos (Fig. 10.1E).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Zygotic embryogeny was interpreted in relation to the embryogenic tissue induction obtained in culture (Fig. 6.5, Chapter 6). The observations suggested that the lack of response, obtained from the explants cultured on the first two collection dates, might have been owed to the fact that ovules had either not been fertilized, or were too immature to respond to the culture conditions provided. The anatomical observations also suggest that *in vitro* induction initially occurs approximately two to three weeks after fertilization has taken place, while the embryo is still very immature: a stage when embryo length is difficult to determine accurately.

10.4 Discussion

The onset of fertilization may be associated with a number of developmental processes as well as morphological changes in the cone. The fertilization stage was isolated in all the tree samples tested and was found predominantly in cones collected at the beginning of December, indicating the fairly synchronous nature of the process. The onset of embryogenic induction *in vitro* was initially reported in explants that had been collected in mid-December (15 December 1992) (Fig. 6.5). These observations show that, when using immature seed as the source explant, a period of two to three weeks after fertilization marks the first instance in which embryogenic initiation can be expected to occur. The cones collected during this period, though, will only produce embryogenic tissue four to six weeks after inoculation *in vitro*. BECWAR, BLUSH, BROWN & CHESWICK (1991) found that the optimum stage of zygotic embryo development for the establishment of pine embryogenic cultures was approximately three to five weeks after fertilization.

BECWAR, WANN, JOHNSON, VERHAGEN, FEIRER & NAGMANI (1988) reported that the most responsive explant stage for *Pinus* species has yet to be adequately determined, but has been considered to be the precotyledonary immature embryo stage. The anatomical observations conducted in this study (Fig. 10.1D) confirm this observation and are enhanced by the fact that embryogenesis

was not obtained through the culture of mature seed (Chapter 6) suggesting that in *P. patula* (as for most other *Pinus* species) precotyledonary explants are the most suitable with which to initiate embryogenic cultures. Selection, of immature explants at a suitable developmental stage, is crucial in order to obtain successful embryogenic induction *in vitro*. The point of fertilization in *P. patula* as determined by anatomical studies determines the embryogenic-inductive "window" period with greater accuracy and could also ensure an improved response from cultured explants. Results obtained (Chapter 6, Fig. 6.6) further confirm that the presence of developing cleavage embryos enhance the embryogenic process by recapitulating zygotic embryogeny.

CHAPTER 11

CONCLUSIONS AND FUTURE PROSPECTS

11.1 Conclusions

The research conducted in this study has illustrated the benefits and possibilities of using a biotechnological approach to tree breeding. The initial objectives of this study were essentially satisfied and, furthermore, new avenues of research and future challenges have been uncovered.

Embryogenic tissue was successfully induced, and matured to produce somatic plantlets. Despite the development of numerous plantlets *in vitro*, one of the greatest drawbacks was the low hardening-off efficiency, suggesting that an alternative or more controlled acclimatization process is required. Furthermore, media manipulations to increase the deposition of storage reserves may need to be considered in order to improve the hardening-off ability of the somatic plantlets.

Clonal propagation via somatic embryogenesis offers prospects for mass cloning of selected material at low cost. Somatic embryogenesis, in terms of producing structures which are bi-polar and hence have the natural ability to produce both root and shoot apices, is favoured over traditional clonal propagation methods which result in the induction of adventitious roots from stem cuttings.

Considerable advances have been made in conifer somatic embryogenesis in the last nine years since the process was first achieved with *Picea abies* in 1985 (HAKMAN & VON ARNOLD 1985). Despite this progress, certain limitations still exist with regard to the utilization of this technology for clonal forestry

purposes. A pre-requisite for commercial scale clonal propagation would include high frequency somatic embryogenesis, genetic fidelity and normal growth and development of somatic seedlings and plants (AHUJA 1991). In this regard, recent evidence in favour of somatic embryo quiescence (seed drying) has shown that desiccation plays an integral role in the maturation of somatic embryos (ROBERTS, SUTTON & FLINN 1990b).

Although genetic variability as a result of somaclonal variation is undesirable in the clonal forestry programme, such variations can offer prospects for recovery of desirable regenerants. Studies investigating the field performance of somatic plantlets have begun to show phenotypic stability within clones, indicating low somaclonal variation. Comparison of embling and seedling performance of interior spruce after one growing season, showed no visual differences between the somatic and zygotic plantlets (ROBERTS, WEBSTER, FLINN, LAZAROFF, McINNIS & SUTTON 1991b). Other researchers have found that somatic plantlets of loblolly pine were smaller than seedlings after six months growth in the field (RUTTER 1994), despite the presence of well-structured roots and root:shoot ratios similar to those of seedlings.

11.2 Future Prospects

Future research appears to be focused on large scale production of plantlets through the use of somatic embryogenesis and the production of artificial seed for commercial purposes. Numerous methods to scale-up and automate tissue culture technology and to reduce handling costs have been developed. These include the use of liquid media in bioreactors (DEBERGH 1994); image analysis; various forms of semi-automation and robotics for cutting and transplanting tissues and plantlets and encapsulating somatic embryos. Although these facilities are available, very few developments in automation are being used commercially. Reasons for this include problems associated with contamination, hyperhydricity, repeatability, cost effectiveness, choice of materials, synchronization of growth,

removal of undesirable tissue, plant performance and genetic stability and selection methods (AITKEN-CHRISTIE 1994).

The technology for artificial encapsulation is of particular interest, enabling a more rapid system of packaging somatic embryos (AITKEN-CHRISTIE personal communication). Research is also focusing on the improvement of the coatings used in artificial seed production in order to ameliorate germination and subsequent development of somatic embryos. The major problem associated with synthetic coatings is their fragility and nutrient permeability.

The advantages and limitations of the process of somatic embryogenesis, having been discussed, there is no doubt that use of this type of approach for commercial application is still considered "high risk". While significant progress has been made using this method for the commercial propagation of various angiosperm species (including carrot and coffee) it is clear that woody species still require some time and effort before a similar level of success can be reached, offering considerable challenges for the future.

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

TS ELLIOT
Little Gidding (1942)

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