SOMATIC EMBRYOGENESIS IN *Pinus radiata*

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Abstract

Somatic embryogenesis of *Pinus radiata* has been seen as an attractive alternative to traditional propagation methods. This is due to its potential for high multiplication rates and rejuvenation, as well as its compatibility with molecular techniques. To date no commercial production of somatic embryos has been undertaken for direct planting, but field trials are currently being conducted for a combination of propagation methods utilising somatic embryos.

The aims of this study were to provide a greater understanding of the factors that influence the initiation and maturation within the somatic embryogenesis process, in order to further optimise conditions and efficiencies. Another objective was to analyse the technique's potential under Australian conditions, such as planting locations, genotypes and collection dates.

The initiation of *Pinus radiata* embryogenic tissue was achieved in this study. Many factors influenced the frequencies of initiation. The main three factors were explant development (providing the greatest influence), genetic background and media/component type.

The maturation of *Pinus radiata* somatic embryos occurred to some degree on most maturation media used in this study. The media components that had the greatest influence on maturation frequencies were; presence of abscisic acid (ABA), different basal media (mainly the New Zealand maturation medium (NZ-EMM)), levels of Gelrite and polyethylene glycol (PEG).

A pre-germination treatment of high relative humidity (HRH) aided the germination of the mature somatic embryos. This step led to the partial drying process observed in the natural development of the zygotic embryo. This treatment resulted in a higher rate of germination success.

The quality of the mature somatic embryo was vital to the success of germination. Selection of mature somatic embryos at the appropriate stage of development

influenced the rate of germination completion, with the inappropriate stages developing abnormally (either without shoots or without roots).

Somatic embryogenesis of *Pinus radiata* was achieved throughout this study. The research highlighted the complexities of obtaining consistent and reliable initiation of embryogenic tissue and its subsequent conversion into mature somatic embryos and plants. Several findings from this study could be used to aid the incorporation of somatic embryogenesis into private forestry breeding programs in Australia. Areas identified include the success of the technique with Australian breeding clones, the capture of embryogenic tissue from explants from different areas within Australia, and the consistency and reproducibility of results using the New Zealand developed media and embryogenic tissue from Australian clones.

Statement of Authorship

This thesis is submitted in accordance with the regulations of Victoria University in fulfilment of the requirements for the degree of Doctor of Philosophy. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and no material previously published or written by another person except where duly acknowledged or referenced.

Susan Pascoe

May 2002

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List of Abbreviations

mg/l	milligrams/litre
g/l	grams/litre
ml	millilitres
mm	millimetres
cm	centimetres
μM	micromolar
mM	millimolar
ANOVA	Analysis of Variance
$HgCl_2$	Mercuric chloride
NaOH	Sodium Hydroxide
2,4-D	2,4- dichlorophenoxy acetic acid
BA	N^6- Benzyladenine
ABA	Abscisic acid
PEG	Polyethylene glycol
KCl	Potassium chloride
HRH	High Relative Humidity treatment
DCR	Douglas fir basal medium
MS	Murashige and Skoog basal medium
GD	Gresshoff and Doy basal medium
P21	Conifer tissue culture basal medium
WPM	Woody plant basal medium
LP	Hakman basal medium
NZ	New Zealand Forest Research developed medium
NZ-EMM	Embryo maturation medium (NZ developed)

CHAPTER 1

Somatic embryogenesis in *Pinus radiata* – An Overview

1.1 Introduction

It has long been recognized that native forests need to be protected for ecological and environmental reasons. The decreasing availability of native forests for wood harvesting and the forecasted increases in demand for timber products has led conservationists and the forestry industry to acknowledge that more timber needs to come from plantations (Chandler *et al.* 1989; Townsend and Mahendrarajah 1997). Current Figures from the National Plantation Inventory (NPI) March 2000 Tabular Report reveal that there are about 1.3 million hectares of plantation in Australia (planted to the end of September 1999), of which 71% is softwood (948,255 hectares) and 29% is hardwood (389,028 hectares). National figures indicate that wood flow for softwood in the local and export markets will increase from 10.5 million cubic metres a year in the 1995-1999 forecast period, to 13.2 million cubic metres a year by 2035-39. (National Forest Inventory 1997). Therefore placing more emphasis on the development of plantations for wood production will be the result of pressure in the political arena and from the conservation lobby.

Forestry, in general, contributes significantly to the Australian livelihood and economy. According to the Australian Bureau of Statistics (ABS) data the forests and wood products industries based on native and plantation forests contribute about 2% of Gross Domestic Product (GDP) and the employment of over 60,000 people. Australia's wood and paper products industries provided a turnover value in 1997-98 of $A11.5 billion with wood processing establishments contributing $A6.3 billion (McLennan 2000). In 1997-98 Australian exports of forest products were $A1.25 billion of which 52% were woodchips (24% were from softwoods) and 30% paper and paperboard products. Imports were $A2.7 billion, of which 52% were paper and paperboard products and 16% sawnwood. This indicates that Australia is a net importer of forest products (McLennan 2000). Australia produces about 83% of its sawn timber needs, with 36% from native forests and 64% from plantations. Timber

is imported from North America (Douglas fir) and New Zealand (*Pinus radiata*) (McLennan 2000).

By increasing the numbers and sizes of native and softwood plantations there would be a reduction in the dependence on imported timber and a decrease in the trade deficit in forest products as well as the chance to meet the increased local demands for timber and timber products. To aid this a Commonwealth, state and territory government initiative was established in 1996 with the target of trebling Australia's plantation area to 3 million hectares by 2020. The developed proposal is known as the '2020 vision' (Townsend and Mahendrarajah 1997; Stephens *et al.* 1998). This initiative aims to expand Australia's commercial plantations to provide an additional reliable, high quality wood resource for industry and to meet environmental and economic objectives (Stephens *et al.* 1998; McLennan 2000).

Another initiative developed by the government was the establishment of the National Plantation Inventory (NPI) in 1993. The inventory is part of the National Forest Inventory within the Bureau of Rural Sciences, Canberra and allows up-to-date reporting of Australia's plantations. The report on the plantation timber resources provides regionally and nationally collated data on where plantations occur, the age of the plantations, species of trees and estimates of regional wood flows (National Forest Inventory 1997).

1.2 The importance of *Pinus radiata*

Pinus radiata Don or Monterey pine, is indigenous to the coast and offshore islands of California. In Australia, *Pinus radiata* was introduced into Victoria and South Australia around the 1860s and the first forest plantings were established in the 1870s (Moran and Bell 1987). *Pinus radiata* has the ability to grow in a variety of environments but generally it requires a cool, dry summer and a warm, moist winter with an absence of extreme cold. The minimum winter temperature is critical. As a result it grows poorly in its native habitat but exceptionally well in temperate regions of South America (Chile), South Africa, New Zealand and Australia. In these countries the average temperatures range from 10-27^{0}C in summer and from 6-17^{0}C in

winter, with limited incidence of damp summer heat, hail storms and low incidences of low temperature extremes (Lewis and Ferguson 1993).

In Australia private plantations have been established in Tasmania, Victoria, South Australia, Western Australia and New South Wales, where climatic conditions are suitable for optimum production (refer to Figure 1.1 for distribution of softwood plantations) (National Forest Inventory 1997). From Figure 1.1, the regions in which *Pinus radiata* are grown are regions 1 to 11. Under these conditions *Pinus radiata* grows rapidly during the first years with the growth rate as much as 1.5 metres per year and the height over the full rotation reaching approximately 50 metres. The diameter of *Pinus radiata* trees can exceed 1.5 metres making this species highly suitable for the production of paper products such as newsprint, cardboard and wrapping paper. The timber is a popular source for furniture products and a wide range of structural applications (McLennan 2000). *Pinus radiata* is now the preferred commercial forestry plantation softwood species in Australia and New Zealand.

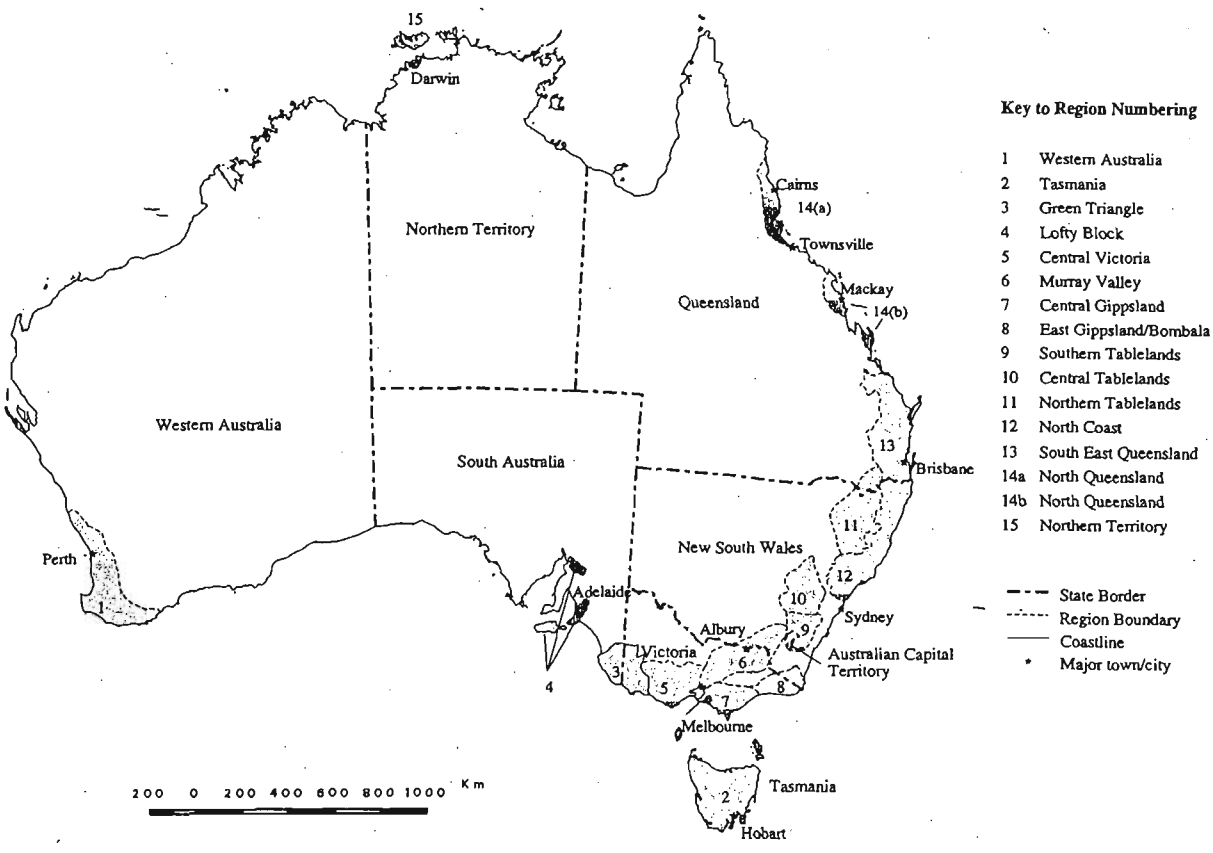


Figure 1.1 Plantation regions of Australia
(with permission from the National Forestry Inventory 1997)

1.3 Breeding of *Pinus radiata*

Breeding of *Pinus radiata* has been extensive, largely focusing on overall quality including resistance to stem deformation, high wood density and growth rate. Other factors that have been targeted through conventional breeding programs include resistance to needle blight (*Dothistroma septaspora*), and resistance to the root fungus *Phytophthora cinnamoni* (Chandler and Young 1995; Boomsma 1997). The long life cycle (25-50 years) of conifers slows the genetic gains and the returns on investment in new technology. It is for this reason that alternative methods of tree improvement and mass propagation have been considered. The application of tissue culture and biotechnology are still in the developmental stage but progressing well. However, there has been progress in the development of alternate propagation methods to advance tree breeding especially in shortening the generation interval through the selection cycle (Jones *et al.* 1993; Jones and van Staden 1995; Lelu *et al.* 1999).

Seedlings derived from open pollinated trees were originally used for *Pinus radiata* plantations, however, through breeding of genetically superior trees other methods were developed to improve and maintain the genetic qualities. Seed produced from control pollinated trees were first used commercially in 1986-87 in New Zealand and have now been adopted widely in Australia. Control pollinated seed is produced by collecting pollen from the best breeding trees and puffing it into bags enclosing receptive female cones of the second best breeders. This procedure eliminates wild pollen and self fertilization and gives higher levels of genetic improvement than selecting one parent and leaving the wind to decide the other (Clarke 1992; Horgan 1993).

Control pollinated seed is costly to produce and the demand for seed is greater than supply. To amplify the planting stock of high genetic quality, vegetative propagation techniques have therefore been implemented. Control pollinated seed from highly rated parents are planted and managed as stock plants (or stool plants) where cuttings are taken and rooted in nursery beds or in glasshouses. Cuttings produced from stem sections are routinely used for the establishment of plantations in Australia and New Zealand as they produce sturdy plants for field establishment. Fascicle cuttings have

also been used where the apical buds on the seedling have been removed to induce the fascicle shoot (Smith 1997). Over 80 cuttings can be produced from a 9-month-old seedling. A pilot plant has been established at the Forest Research (FR) (previously known as New Zealand Forest Research Institute) site in Rotorua, New Zealand, to produce large-scale numbers of fascicle cuttings (Smith 1997).

Cuttings of *Pinus radiata* can be taken from stock plants up to about four years of age. Plants older than four years have shown unproductive results in volume growth and growth rate due to the phenomenon of physiological ageing (Horgan 1993; Smith *et al.* 1994). Research has focused on maintaining the juvenile state of the stool plants, though maturation has still been observed in plants less than 0.5m tall (Smith 1997).

Other vegetative propagation techniques have been developed to overcome some of the problems of loss of juvenility and supply of tissue. The forestry industry in New Zealand utilizes *in vitro* techniques to further the availability of juvenile material (Smith *et al.* 1994; Devey *et al.* 1994). Micropropagation involves the multiplication of *Pinus radiata* plants from a small amount of plant tissue. This technique involves either the induction of shoot buds (adventitious bud formation) or stimulation of pre-existing buds (axillary bud proliferation) (Menzies and Aimers-Halliday 1997; Horgan 1993). Shoot buds are elongated and multiplied within a controlled, sterile environment. Root initiation is carried out using an auxin treatment with subsequent plants set as small cuttings in a glasshouse for further acclimation in the nursery (Menzies and Aimers-Halliday 1997; Smith 1997). High multiplication rates have been achieved, however, genotype variations have been found with only about 60% of families being initiated in culture (Smith *et al.* 1994; Menzies and Aimers-Halliday 1997). Storage of shoots during field assessment has been researched using techniques of cold storage at 2-7°C with good outcomes of survival rates of shoots upon re-culture (Smith *et al.* 1994; Menzies and Aimers-Halliday 1997).

Field trials of micropropagated *Pinus radiata* plants have shown some problems with accelerated maturation and plants showing reduction of volume growth rates and height compared to control seedlings. However, more recent trials may have resolved these problems (Menzies and Aimers-Halliday 1997; Smith 1997). As multiplication rates are more than sufficient to meet the expectations of clonal forestry

micropropagation has been developed on a larger scale. Fletcher Challenge Forests Ltd in New Zealand established a laboratory in 1984 and further expanded this in 1990 to evaluate the use of micropropagated planting stock using the techniques initially developed at Forest Research. Production of over 3 million micropropagated radiata pine plants per annum from select, control pollinated seeds has now been achieved (Smith 1997).

1.4 Somatic embryogenesis

Another vegetative propagation technique developed in the effort to reduce the problems associated with physiological ageing and selection of superior genotypes is somatic embryogenesis. Somatic embryogenesis is a process by which embryos develop from a single cell or a small group of vegetative cells. The normal developmental pathway of zygotic embryogeny is maintained whereby embryogenic tissue consisting of immature somatic embryos resemble immature zygotic embyros. The somatic embryo closely resembles the zygotic embryo with its polarized structure comprising of an embryogenic region (head) with elongated suspensor cells (Attree and Fowke 1993; Gupta 1988). Williams and Maheswaran (1986) also described somatic embryogenesis as "the process by which haploid or diploid somatic cells develop into whole plants through characteristic embryological stages without the fusion of gametes". The somatic embryogenic technique involves the *in vitro* capture of embryogenic tissue to produce a high number of somatic embryos. These can then be matured into somatic plantlets capable of growth in the field (Smith *et al.* 1994; Walter and Smith 1995).

The development of somatic embryogenesis has resulted in the embryogenic tissue being a suitable explant system for cryopreservation. Assessment of field trials of *Pinus radiata* occur at 7-9 years, therefore cryo-storing embryogenic tissue can provide the opportunity to evaluate individual clones for their field performances whilst maintaining tissue for a long time period (Smith *et al.* 1994). Cryopreservation also halts the growth of embryogenic tissue to keep it in an undifferentiated state (Horgan *et al.* 1997). This technique has been successfully used at New Zealand's Forest Research for tissue that has been cryopreserved for several years (Smith *et al.* 1994).

1.4.1 Somatic embryogenesis – Background

Since the development of somatic embryogenesis in carrot tap root cells (Steward 1958), numerous plant species have been regenerated *in vitro* by somatic embryogenesis. These include many cereals and grasses (such as *Oryza sativa*, *Panicum maximum* and *Zea mays*), ornamental plants (such as *Prunus, Ranunculus, Narcissus* and Orchid species), fruits and vegetables (such as mango, celery, avocado, mandarin and *Allium* species) and forestry trees (such as *Pinus* and *Picea* species) (Lu and Vasil 1981; Wernicke *et al.* 1981; Litz *et al.* 1982; Lu *et al.* 1982; Vasil 1985; Vasil and Vasil 1986; Mooney and van Staden 1987; Nadel *et al.* 1989; Tautorus *et al.* 1991; van der Valk *et al.* 1992; Gill *et al.* 1995; Margherita *et al.* 1996; Tang *et al.* 1999; Chen and Chang 2000; Sage *et al.* 2000). *In vitro* techniques in plant propagation were originally utilized to overcome the problems and limitations of conventional plant breeding. Problems associated with susceptibility to diseases and pests, long generation times, long juvenility periods and the lack of supply for the increased demand for some plants aided the development of *in vitro* techniques (Zimmerman 1985; Mooney and van Staden 1987; Rugini 1988; Nadel *et al.* 1989; Chen *et al.* 1990; Dineshkumar *et al.* 1995; Gill *et al.* 1995; Tang *et al.* 1999; Das *et al.* 2001; Shibli *et al.* 2001). Over the last 25 years, somatic embryogenesis has also enabled plant regeneration in some plant species which was otherwise difficult with other *in vitro* techniques such as regeneration from protoplasts and micropropagation methods (Vasil 1985; Finer 1988; Horn *et al.* 1988; Kysely and Jacobson 1990; Loiseau *et al.* 1998; Raimondi *et al.* 2001).

Experimentation with somatic embryogenesis was conducted throughout the 1960s and 70s, however the first convincing accounts of initiation of embryogenic tissue were not published until the 1980s. Renewed interest in this technology rapidly evolved from the early to mid 1980s, especially when somatic embryogenesis was successfully induced in cereals and grasses (Vasil and Vasil 1980; Lu and Vasil 1981; Wernicke *et al.* 1981.). Early studies of somatic embryogenesis in plants often showed problems with sporadic plant regeneration, genotypic variations and responses and a loss of embryogenic potential of cell lines (Vasil 1982; Lutz *et al.* 1985; Chen *et al.* 1990; van der Valk *et al.* 1992). Some of these problems still exist, such as variations in genotypic responses but the frequencies of plant regeneration have been increased and will most likely improve with advancing technologies. Additional problems of

production costs and uniformity of regenerated plants have also limited somatic embryogenesis reaching its full commercial potential (Zimmerman 1985; Margherita *et al.* 1996; Perrin *et al.* 2001; Raimondi *et al.* 2001). Vasil (1982) made a statement that can be applied to all plants, not just cereals. He suggested that the developing technologies of *in vitro* techniques including somatic embryogenesis would be used in conjunction with existing techniques and not necessarily replace them (Vasil 1982). Zimmerman (1985) also made comparisons with conventional breeding methods mentioning that further production of woody plants by somatic embryogenesis would depend on the production cost comparisons of conventional and somatic methods and the comparisons of performance characteristics.

With the advent of genetic transformation technology, somatic embryogenesis has taken on another role. Plant regeneration is necessary for the development of transgenic plants, and somatic embryogenesis has been identified as the optimal method in some species. Vasil (1995) noted that somatic embryogenesis was the predominant form of regeneration in cereals, therefore many of the cereal crops that have been transformed with genes that confer resistance to herbicide, insects or viruses have been regenerated from embryogenic cells. Many researchers have undertaken further improvements of plant systems through biotechnological methods therefore efficient regeneration system are necessary (Gill *et al.* 1995; Guis *et al.* 1997; Loiseau *et al.* 1998; Islas-Flores *et al.* 2000; Sage *et al.* 2000; Chengalrayan *et al.* 2001; Nakagawa *et al.* 2001).

The development of the technique of somatic embryogenesis has enabled research into the biochemical processes within the plant and how these can be altered. Examples using the carrot system include polyamine studies and single cell differentiation (Nomura and Komamine 1985; Khan and Minocha 1991). Polyamine studies have also been analyzed in embryogenic tissues of Indica rice (Shoeb *et al.* 2001). Somatic embryogenesis has been used to observe the effects of sugars in celery and *Medicago arborea* embryogenic tissues (Nadel *et al.* 1989; Nadel *et al.* 1990; Martin *et al.* 2000). The detection and measurement of the levels of proteins in coconut tissues has also been undertaken using somatic embryogenesis (Islas-Flores *et al.* 2000). The process has also enabled the direct observation of the plant's

embryony such as shown with *Zea mays*, coffee and peanut (Fransz and Schel 1991; Nakamura *et al.* 1992; Chengalrayan *et al.* 2001).

From its original role to aid the breeding of plant species that proved difficult to multiply *in vitro* and providing the mass propagation of plants, the process of somatic embryogenesis has evolved to provide a greater assistance with many other aspects of plant studies. These include an efficient regeneration system for transformation studies and the access for studies into the embryony and biochemistry of the plant's system.

1.4.2 Somatic embryogenesis in conifers

Several independent groups first reported somatic embryogenesis in conifers in 1985. Hakman *et al.* (1985) and Chalupa (1985) developed somatic embryos from Norway Spruce (*Picea abies*) and Nagmani and Bonga (1985) reported somatic embryos from European larch (*Larix decidua*). Since then, embryogenic tissue and consequently somatic plants in many coniferous species have been successfully produced (Finer *et al.* 1989; Jain *et al.* 1989; Jones *et al.* 1993; Huang *et al.* 1995; Salajova *et al.* 1999). A review by Tautorus *et al.* (1991) also provides a comprehensive list of embryogenic tissue initiation in conifer species.

Somatic embryogenesis has a number of advantages over micropropagation techniques. It offers a process by which unlimited numbers of identical somatic embryos and ultimately trees can be obtained. Somatic embryogenesis therefore offers the production of genetically homogenous plant material for research and breeding and for the propagation of elite trees for reforestation (Gupta *et al.* 1993; Keinonen-Mettälä *et al.* 1996). The application of somatic embryogenesis includes the development of cell lines for genetic engineering and for use in research to improve our understanding of conifer genetics and development (Tautorus 1991; Park *et al.* 1998).

A promising aspect of somatic embryogenesis is in high value clonal forestry. Highly valuable genotypes with desirable qualities, such as high timber volume, fast growth and disease resistance can be cloned and utilized in the forestry industry (Jones 1990; Park *et al.* 1998). Encapsulated somatic embryos comparable with natural seeds can

be produced for low seed yielding species such as Norway spruce as well as providing a cost efficient system with rapid multiplication (Attree and Fowke 1993). There is also the potential for scale up and delivery via bioreactors (Merkle and Dean 2000).

The clonal propagation techniques developed have enabled tissue culture systems to be used in genetic engineering to transfer genes that produce novel traits (Lelu *et al.* 1999). The aim of using transformation technology is to introduce genes into trees to make them resistant to herbicides, insects and for introducing desired traits (Walter and Smith 1995). Embryogenic cultures developed through somatic embryogenesis are seen as a suitable target tissue for genetic transformation (Merkle and Dean 2000). Direct gene transfer methods have been developed with research yielding regenerated transgenic *Pinus radiata* plants (Walter *et al.* 1998). Genetic manipulation of embryogenic tissue also offers opportunities to study the developmental regulation of genes. The ability to identify regulatory genes may influence developmental pathways to further improve wood quality and productivity gains (Smith *et al.* 1994; Walter and Smith 1995).

1.5 *Pinus radiata* – embryology

The most competent explant for embryogenic tissue initiation in *Pinus* species is the immature zygotic embryo. To fully understand the developmental system of somatic embryogenesis, a description of embryo and seed development has been included here.

The formation of the pine zygotic embryo, its origin, fertilization and development takes about two years. Conifers, including pines, produce two kinds of cones. The staminate (pollen) cone or male strobili, which produces the haploid pollen grain (microspore) and the ovulate (seed or female) cone, which produces the megaspores. Staminate cones are quite small and are generally located in the lower branches of the tree. Whilst the ovulate cones develop on the ends of long shoot buds located in the higher branches of the tree (Weier *et al.* 1982).

The following descriptions of *Pinus radiata* embryology are based on observations and research conducted by Lill (1976), Lill and Sweet (1977) and Chandler and

Young (1995). In southern Australia the staminate cones are initiated in late autumn to winter with pollen shed from August to October. The development of the female cone takes longer. From early summer to autumn several cycles of cone primordia development occur. Over winter the cone bud primordia differentiate and result in the development of two ovules each enclosing a single megasporangium on the upper surface of the ovuliferous scales of the cone. By the end of winter the cones are receptive, appearing red in colour and 10-20mm in length (Chandler and Young 1995). From late winter to early spring pollination occurs with the transfer of pollen by air currents to the female cone. From here the pollen moves into the cone to the micropyle of the ovules (Lill and Sweet 1977). The receptive time for the female cone lasts for about two weeks or even less if rain occurs. After which time the scales close leaving no gaps and the cone enlarges. Within the female cone the pollen starts forming a pollen tube in order to reach the ovules. The changes initiated by pollination occur within the female cone over a period of one year. The ovules enlarge and the megagametophyte undergoes meiosis to form the female gametophyte/megaspore. The megaspore enlarges and undergoes differentiation to form 1-4 archegonia, each with an egg cell (Chandler and Young 1995). By the next spring to early summer the pollen tubes containing the pollen microspore have recommenced development and reach the receptive female egg cells. Fertilization takes place to produce the diploid zygotic embryo (refer to Figure 1.2 for schematic interpretation of *Pinus* embryology).

After fertilization the development of the zygotic embryo takes about five months, during which time it undergoes two types of embryo development. Initially, simple polyembryony, whereby fertilization of more than one ovule within the seed occurs. In simple polyembryony each proembryo is the result of fertilization from separate pollen grains therefore each proembryo is genetically different. This stage is then followed by cleavage polyembryony where multiple embryos form from the one fertilized egg (Gupta 1988; Chandler *et al.* 1989; Tautorus *et al.* 1991). In this case the individual proembryo divides into four genetically identical embryos one of which becomes dominant (refer to Figure 1.3). The other embryos cease to develop and are usually absorbed by the gametophytic tissue (Filonova *et al.* 2000). Cleavage polyembryony is seen as an effective way of eliminating unfit embryos (Tautorus *et al.* 1991). Becwar *et al.* (1991) described the complexity of the embryology within

the *Pinus* species. If an ovule contained three fertilized egg cells and the resulting embryos undergo cleavage polyembryony then the ovule would contain twelve developing embryos with three different genotypes. One dominant embryo outgrows the rest (Becwar *et al.* 1991).

The natural development of the *Pinus* seed consists of six stages according to Smith (1994). The first stage involves the embryo consisting of one to three cells attached to the archegonium. These are positioned within the corrosion cavity of the seed. Secondly, the embryos multiply and develop each having less than 64 cells (zygotic polyembryogenesis). The third stage sees the growth of the embryo away from the archegonium and towards the end of the corrosion cavity. The axis of the embryo elongates and assumes a cylindrical shape with suspensor cells attached at one end. This stage denotes the "bullet stage" of development. Stage 4 sees the development of cotyledonary tissue at the shoot apex and further development at the root end. In stage 5 further development and maturation of the embryo occurs with the formation and greening of the cotyledons, development of the epicotyl and hypocotyl. This stage ends with the emergence of the root (radicle), hence the germination stage. Finally stage 6 sees the establishment of the germinated embryo as a plant capable of growing in the soil (Smith 1994). Pullman and Webb (1994) also described the natural developmental stages of the seed for *Pinus taeda* and *Picea abies*. The descriptions for each seed stage are slightly more detailed than Smith's (1994) with nine stages described instead of six. Dogra (1978) had another in depth analysis of conifer embryogeny in an earlier publication.

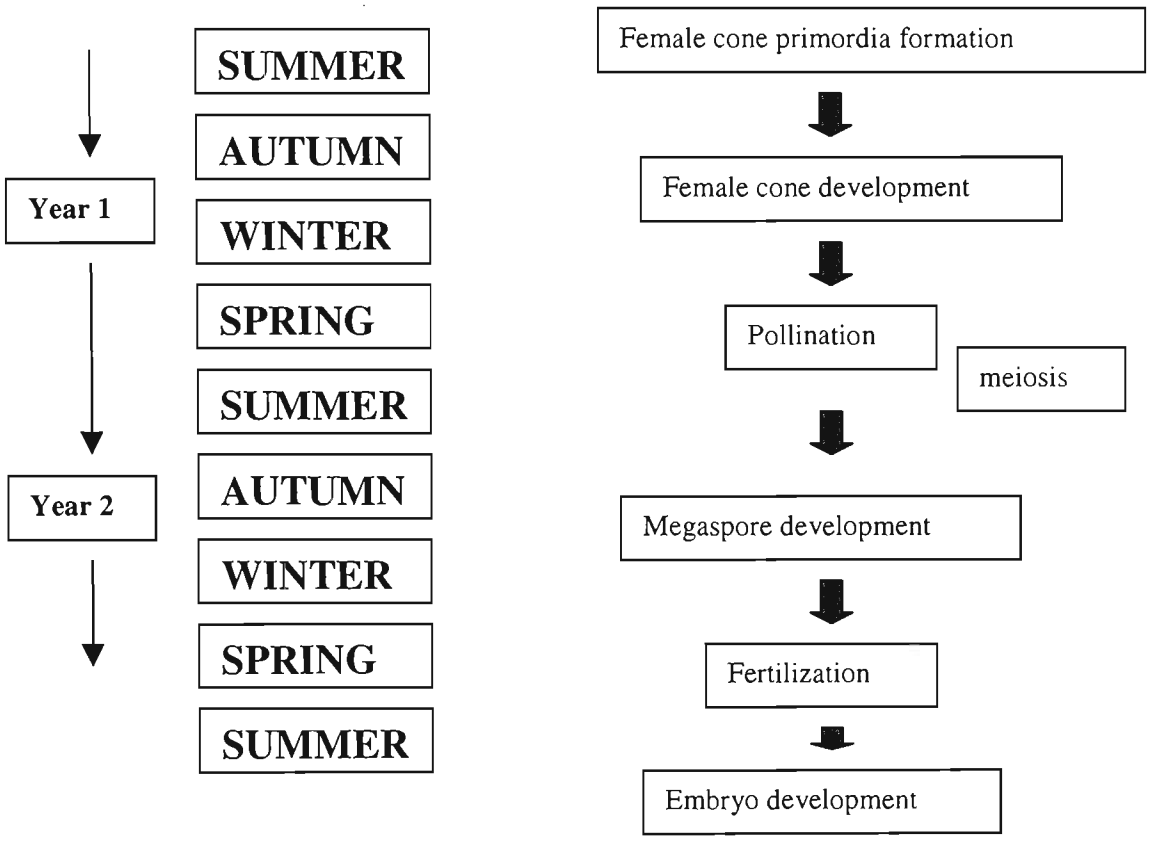


Figure 1.2 Schematic representation of the embryology of *Pinus radiata* in southern Australia

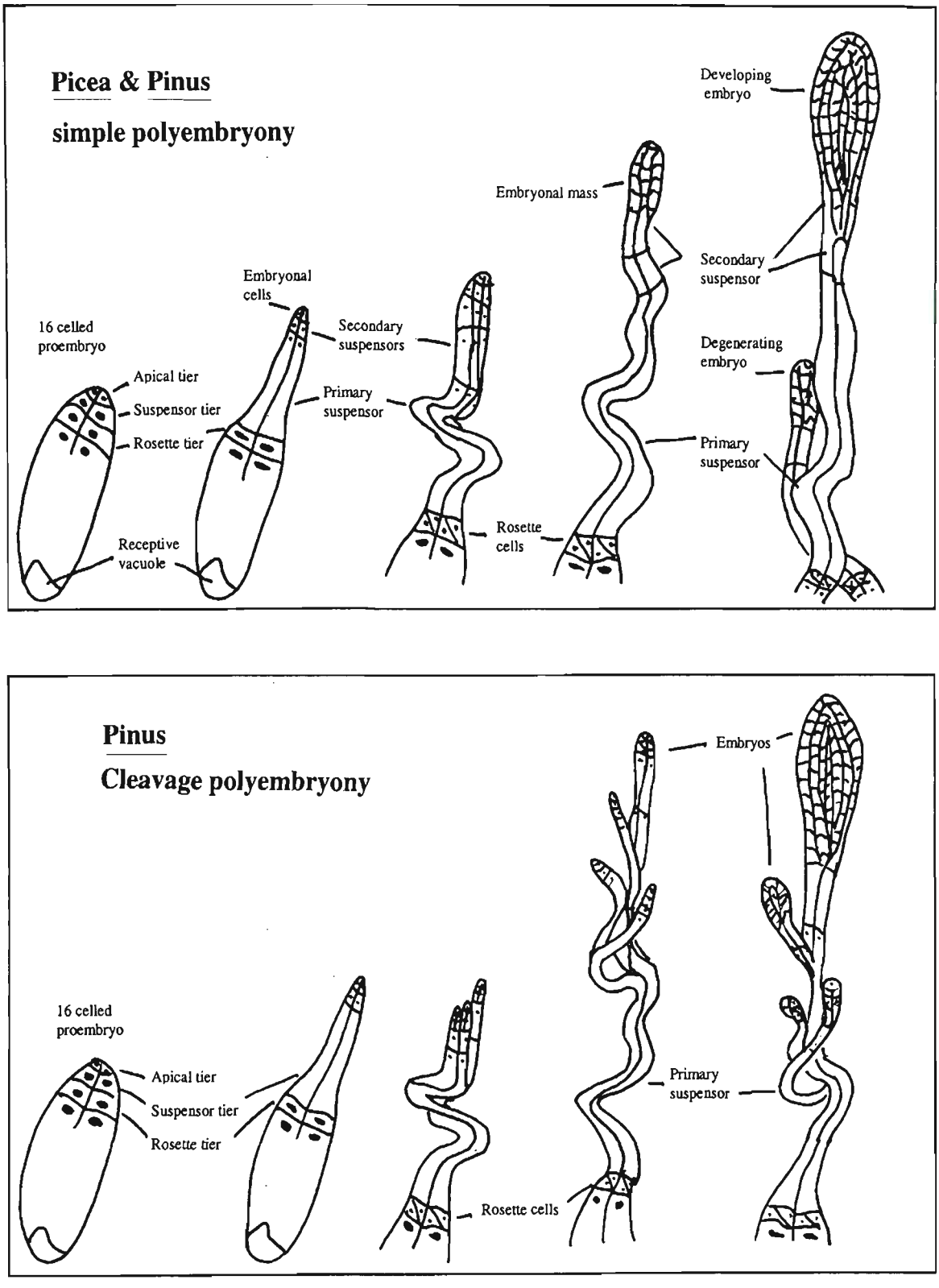


Figure 1.3 Simple polyembryony and cleavage polyembryony of *Pinus* species

(With permission from Owens and Blake 1985)

1.6 Somatic embryogenesis in *Pinus radiata*

The majority of work on somatic embryogenesis in conifers has been achieved with *Picea* species and *Larix* species. Experimental work conducted on *Pinus* species has also been widespread however, there is limited published research on somatic embryogenesis in *Pinus radiata*. Table 1.1 provides a summary of somatic embryogenesis in *Pinus*.

Table 1.1 Summary of somatic embryogenesis in *Pinus* species

Species	Explant used for induction	Reference
Pinus caribaea Morelet. – Caribbean pine	IZE	Lainé and David 1990; Lainé *et al.* 1992
P. elliottii Engelm. – Slash pine	IZE	Jain *et al.* 1989; Liao and Amerson 1995
P. nigra Arn. – European black pine	IZE	Salajova and Salaj 1992; Salajova *et al.* 1995; Salajova *et al.* 1999
P. radiata D. Don. – Monterey pine, Radiata pine	IZE	Chandler *et al.* 1989; Chandler & Young 1990; Smith *et al.* 1994; Smith 1994; Aitken-Christie 1995; Chandler and Young 1995; Maddocks *et al.* 1995
P. strobus L. – Eastern white pine	IZE MZE	Finer *et al.* 1989; Klimaszewska and Smith 1997; Garin *et al.* 1998.
P. taeda L. – Loblolly pine	IZE	Becwar and Pullman 1995; Gupta and Durzan 1987; Becwar *et al.* 1990; 1991; Handley *et al.* 1994; Pullman *et al.* 1995; Li and Huang 1996; Li and Huang 1997; Li *et al.* 1998
P. serotina	IZE	Becwar *et al.* 1988
P. patula – Mexican weeping pine	IZE	Jones *et al.* 1993; Jones and van Staden 1995
P. pinaster – Maritime pine	IZE	Bercetche & Pâques 1995; Lelu *et al.* 1999; Ramarosandratana *et al.* 2001
P. *sylvestris* L. – Scots pine	IZE	Hohtola 1995; Keinonen-Mettälä *et al.* 1996; Häggman *et al.* 1999; Lelu *et al.* 1999;
P. koraiensis – Korean pine	MZE	Bozhov *et al.* 1997
P. palustris – Long leaf pine	IZE	Nagmani *et al.* 1993
P. massoniana - Masson pine	MZE	Huang *et al.* 1995

IZE = immature zygotic embryo, MZE = mature zygotic embryo.

New Zealand forestry companies have long adopted tissue culture approaches using the somatic embryogenic method, where their usefulness in clonal propagation programs has been recognized. Forest Research in New Zealand was the first group to regenerate *Pinus radiata* by somatic embryogenesis (Chandler and Young 1995). Research began in 1983-84 with the first plants being produced in 1987. Research papers from 1994 show trial plantations in the nursery, greenhouse and field have yielded over 4000 somatic seedlings (Smith *et al.* 1994). With improvements in techniques the numbers of somatic seedlings have increased. A client of Metagenetics (a company in New Zealand) planted about 100,000 *Pinus radiata* plants that originated from somatic embryos in 1999-2000. These plants were established as cuttings taken from somatic seedlings used as stool plants (Ropati (2001) pers. comm.). Detailed protocols of the regeneration procedures of somatic embryogenesis in *Pinus radiata* were not published until recently due to industry support necessitating confidentiality. Patents have now been awarded to the methods and media used (Smith 1997).

1.6.1 Integration of somatic embryogenesis into *Pinus* breeding programs

The integration of somatic embryogenesis into breeding programs for *Pinus radiata* was intended to be through the direct seedling or synthetic seed delivery system, where mature somatic embryos, encapsulated in a hydrated, oxygenated gel, could be directly sown in the field (Gupta *et al.* 1993; Horgan 1993). Due to the limitations of the finite life of embryogenic cell lines, the generation of competent cotyledonary stage somatic embryos and the unreliability of producing large numbers of somatic embryos from individual genotypes, this approach may not be viable (Smith 1997; Ropati (2001) pers. comm.). Instead, somatic embryogenesis can be used to initially produce large numbers of plants which then can be used to establish stool beds from which cuttings can be taken for nursery plantings (Smith 1997; Menzies and Aimers-Halliday 1997). This 'hybrid' delivery system is currently being trialed in New Zealand (Aimers-Halliday *et al.* 1997; Menzies and Aimers-Halliday 1997; Ropati (2001) pers. comm.). Cost of production is also seen as a limiting factor with the use of direct sowing of somatic seeds.

Clonal forestry will only be effective if techniques are cost effective, work for most genotypes and give large numbers of uniform plants per genotype without the

problems of aging (Aimers-Halliday *et al.* 1997). There is also the issue of somaclonal variation in the regeneration from callus or cell-suspensions possibly leading to genetic variations in the regenerated plants (Larkin and Scowcroft 1981). The variations may appear as genotypic or phenotypic changes, and often have several advantages or disadvantages to an *in vitro* plant regeneration system. Somaclonal variation may lead to characteristics that can be enriched during *in vitro* culture. These include resistance to disease, herbicides and tolerance to environmental stresses. However, a disadvantage could occur in operations that require clonal uniformity such as in the forestry industry. In this case, tissue culture employed for rapid propagation of elite genotypes, has no desire for aberrant genotypes or phenotypes. Data suggests that embryos recovered via somatic embryogenesis do not undergo any pronounced genetic changes (Jain *et al.* 1989; Attree and Fowke 1993). This may be due to the formation of organized structures, such as somatic embryos which minimizes the changes of somaclonal variation (Isabel *et al.* 1996). Mo *et al.* (1989) found that Norway spruce somatic embryos did not undergo any gross changes in DNA content, as too Eastman *et al.* (1991) with Interior spruce. However, Isabel *et al.* (1996) regenerated a small percentage of white spruce emblings showing *variegata* phenotypes. These arose form embryogenic tissue that had been held in subculture for up to 20 months. This length of time in subculture has been recognized as increasing the risks of somaclonal variation (Isabel *et al.* 1996).

For *in vitro* propagation systems to be adapted into breeding programs they must be more cost competitive than the conventional methods. As yet there is no evidence that somatic embryogenesis is more cost effective than the other *in vitro* propagation technique of micropropagation. Both are labour intensive and require a few manual transfer steps. Cuttings and seedlings are still seen as less labour intensive and cheaper in plant establishment costs. Table 1.2 shows a comparison of cloning methods for *Pinus radiata*. Information for the Table is based on New Zealand plantings, as no comparable information is available from Australian plantings (Smith 1997).

Table 1.2 Comparison of cloning methods for *Pinus radiata* (Smith 1997)

	Seedlings	Stem Cuttings	Fascicles	Micro-propagation	Embryo-genesis
Genotypes within families for which the technique will work	95%+	95%+	95%+	60-90%+	2-30%
Production cost per thousand ($NZ)	$100[a]	$150-200[b]	$200-300[c]	$550-650[c]	$600-1000[c]

[a]The base cost for open pollinated seedlings. Inclusion of seed costs for best control pollinated seedlings increases cost of production to $400-450 per thousand.
[b]From commercial operations
[c]Best estimates

As mentioned previously somatic seedlings are being utilized in a 'hybrid' delivery system with plants established as stool beds and cuttings taken from them. If this system is more widely adopted, plant establishment costs will be reduced, making cuttings from somatic stool plants comparable with cuttings taken from control pollinated seedlings (Ropati (2001) pers. comm.). In the future the use of hybrid systems are likely to be used for delivering transgenic plants regenerated through somatic embryogenesis (Menzies and Aimers-Halliday 1997).

1.7 The role of somatic embryogenesis of *Pinus radiata* in Australian forestry

Studies in Australia into somatic embryogenesis of *Pinus radiata* have been limited. Research was conducted at Florigene (previously Calgene Pacific) in Victoria in the late 1980s to study somatic embryogenesis in *Pinus radiata* (Chandler *et al.* 1989). Explants suitable for embryogenic tissue initiation were investigated as well as embryo development and germination. Results showed limited success with infrequent and occasional abnormal maturation of somatic embryos (Chandler *et al.* 1989). Since then no other groups in Australia have tackled the optimization of somatic embryogenesis in *Pinus radiata.*

The lack of interest in advancing the development in new technologies for *Pinus radiata* propagation in Australia may be due to several reasons. The major factors

could be lack of dependency on the forestry resource, the cost of the implementation of the technology and the unreliability of somatic embryogenesis across the genotypes. In 1997-98 the forestry industry contributed about 2% to Australia's GDP (McLennan 2000). This figure makes this sector one of the lowest contributors to the GDP for that time period. Even though there is a contribution by the forestry industry to Australia's economic well being, the economy is by no means dependent upon it. This lack of dependency should be seen as an incentive to increase and adopt new technologies in order to develop a competitive advantage within the Asia-Pacific region. Such is the example in New Zealand, where the forestry sector is an indispensable part of the economy. Besides being self sufficient the balance of the wood harvested is exported to countries such as Japan and Australia. Exports contribute significantly to the New Zealand economy with $NZ2.5 billion of forest products exported in the year to June 1999 (Lewis and Ferguson 1993; MAF NZ 2001).

As Aimers-Halliday *et al.* (1997) mentioned, for clonal forestry to work the techniques have to be cost effective, work for most genotypes and give large numbers of uniform plants per genotype without the problems of aging. The cost of establishment of somatic embryogenesis into the breeding program in a commercial environment may be another reason why this technique has not been applied. Due to the level of manual handling and equipment necessary for the development of somatic plants initial costs are quite high and are less comparable than other vegetative techniques such as cuttings (Smith 1997). The cost of the establishment of a laboratory for the use of *in vitro* techniques such as somatic embryogenesis and micropropagation would be considered to be uneconomical for most Australian forestry organizations. As a result industry would probably find it cheaper and more practical to obtain the technology and information from other organizations. An example of this is the push of several New Zealand organizations, such as Forest Research and Carter Holt Harvey Forests Ltd (CHH) into the Australian forestry market (Aitken-Christie 2000 pers. comm; FRI Annual report 2000). Rather than "reinvent the wheel" these organizations hope to take advantage of the future considerations of the application of *in vitro* techniques into plantation establishments as well as planting clonal trials with Australian partners (Aitken-Christie (2000) pers. comm; Barometer 2000).

Genotype variations and the ability to produce large numbers of somatic embryos from individual cell lines play a role in the establishment of somatic seedlings in the field. Currently somatic embryogenesis does not work for 100% of the genotypes therefore the selection of superior genotypes may be limited. Horgan (1993) noted that a low success rate could erode the genetic gains. Frequencies of less than 5% to around 30% of genotypes have been found for embryogenic tissue initiation in *Pinus radiata* (Menzies and Aimers-Halliday 1997; Smith 1997). Though, through further efficacy improvements the technology should work with a larger number of desired genotypes (Menzies and Aimers-Halliday 1997).

1.8 Summation

Plantation forestry plays a large role in the Australian environment and economy. By utilizing timber from plantations native forests can be protected from ecological and environmental damage. Pressure from conservationists and the public have prompted the government to initiate programs to build up the numbers of plantations in order to meet the increased demands for timber and timber products such as paper. The introduced species, *Pinus radiata*, is the preferred softwood timber used in plantations. Extensive breeding has been conducted over the decades in Australia and New Zealand to produce trees with desired characteristics and timber of high quality. Vegetative propagation techniques have been developed such as cuttings to aid the establishment of *Pinus radiata* plantations. *In vitro* propagation methods have also been developed to further aid breeding programs and circumvent some of the problems of long rotations and early maturation of plants. Somatic embryogenesis, an *in vitro* technique, has the potential to revolutionize breeding in all conifers, not just *Pinus radiata*, by producing unlimited numbers of somatic embryos and ultimately plants from cell lines and genotypes. Therefore plants from genotypes with superior qualities can be rapidly multiplied and selected for placement into the field. Embryogenic tissue provides a suitable target for genetic manipulation to introduce useful genes into trees as well as cryopreservation, which enables tissue to be maintained for the duration of field trials in a juvenile state. Therefore somatic embryogenesis would make an attractive addition to breeding programs. However, some of its potential has yet to be realized for *Pinus radiata*. High production costs,

the unreliability of the technique working across all genotypes and the potential for somaclonal variation require more research before it replaces cuttings as the preferred method. In Australia somatic embryogenesis has not been adopted in the forestry sector, with the aforementioned reasons probably being the explanation. In New Zealand several forestry organizations have developed somatic embryogenesis in *Pinus radiata* and are researching means to overcome some of the problems in order to commercialize the technique. To reduce costs an integration of somatic embryogenesis and the cuttings method have been established to produce cuttings from somatic stool plants. This hybrid system will probably be the preferred use for somatic plants due to the direct sowing of somatic embryos being quite a distance in the future. To overcome the unreliability of the technique further research needs to be conducted. Also, in order to develop a 'home-grown' basis for this technology, experimentation with the plant material within Australian conditions is necessary.

CHAPTER 2

The initiation of *Pinus radiata* embryogenic tissue

2.1 Introduction

The initiation of the embryogenic tissue within the somatic embryogenesis pathway is a vital step. The initiation process refers to the formation of extruded embryogenic tissue (Becwar *et al.* 1988). The extruded embryogenic tissue is similar in all conifer species being white to translucent and mucilaginous in appearance (Chandler *et al.* 1989; Jalonen and von Arnold 1991; Fowke *et al.* 1995). Microscopic examination shows that the embryogenic cultures consist of a mixture of early stage somatic embryos containing an embryonal mass (globular clumps of densely cytoplasmic cells) with attached suspensor-like cells (Attree and Fowke 1993; Fowke *et al.* 1995; Misra 1994). This tissue development was initially referred to as 'embryogenic callus' but this term is no longer used as there is in fact an organized arrangement of the cells (Attree and Fowke 1993). Other terms have been used to describe the embryogenic tissue, including stage 1 somatic embryos (von Arnold and Hakman 1988), embryonal suspensor masses (ESM) (Gupta *et al.* 1991) and embryonal masses (Misra 1994).

Efficiency rates

The success of the initiation of embryogenic tissue in *Pinus* species has been quite low compared to other conifer species such as *Picea* (Becwar *et al.* 1988; Tautorus *et al.* 1991; Fowke *et al.* 1995). Initiation frequencies have been relatively high in *Picea* species, up to and greater than 75%, and consistently low in *Pinus* species, generally below 15% (Becwar *et al.* 1988; Chandler *et al.* 1989; Fowke *et al.* 1995; Li *et al.* 1998). Combined with other difficulties such as low efficiency of maturation and plantlet establishment, *Pinus* species, including *Pinus radiata,* have been regarded as recalcitrant to developing somatic embryogenesis as a viable technique (Tautorus *et al.* 1991; Li *et al.* 1998). Therefore the main challenge is to overcome the low frequency of embryogenic tissue initiation and to deal with or devise methods to narrow these variations (Handley *et al.* 1994; Misra 1994).

The differences observed in response to embryogenic tissue initiation are most likely due to several factors, acting either independently or in combination. These factors include: explant selection, the developmental stage of the zygotic embryo or time of collection, genotypic differences between and among clones and the components of the initiation medium (Harry and Thorpe 1991; Huang *et al.* 1995; Li *et al.* 1998). All these factors have been reported to affect the initiation of embryogenic tissue in conifer species, in particular *Pinus* species.

Explant selection

Explant selection has been shown to be critical to the successful initiation of embryogenic tissue. The establishment of the immature intact embryo-suspensor complex or the fertilized ovules in the female gametophyte, as the explant for embryogenic tissue initiation was first undertaken in radiata pine (Smith *et al.* 1985). Since then many other plant structures have been experimented with to ascertain their embryogenic potential, most with limited success. These include the isolated zygotic embryo (Hakman *et al.* 1985; Chandler *et al.* 1989; Lelu *et al.* 1999), mature zygotic embryos (Jalonen and von Arnold 1991; Park *et al.* 1998; Garin *et al.* 1998), seedlings (Mo and von Arnold 1991; Ruaud *et al.* 1992) and the female gametophyte (Chandler and Young 1995). However, the immature megagametophyte-embryo complex is still the most common explant used. It has been suggested that the relative success achieved using this explant is due to the fact that as the gametophyte is attached to the embryo there is a reduction in excision stress. The gametophyte provides the embryo with some of its natural nutrients and phytohormones, thus making culture easier (Tautorus *et al.* 1991; Bonga and von Aderkas 1992). It has also been found to be less awkward and time consuming to culture the whole megagametophyte rather than dissect the embryo.

Time of explant selection

The importance of the time of explant collection suggests that there is a developmental period during which zygotic embryos are most responsive to forming embryogenic tissue (Becwar *et al.* 1988). This has been observed in most *Picea* and *Pinus* species that have shown somatic embryogenesis (Chandler *et al.* 1989; Jones 1990; Tautorus *et al.* 1991; Fowke *et al.* 1995; Pullman and Webb 1994). Smith *et al.* (1994) observed that the *in vitro* capture of embryogenic tissue from the immature zygotic embryo of *Pinus radiata* occurred during a period termed a 'window of

competence' occurring in early summer. After this time the embryo matured and initiation frequencies decreased. This study postulated that if tissue was collected at the correct time, up to 100% of whole megagametophyte explants could give rise to embryogenic tissue, though the best demonstrated result was 32.9%. Studies with *Picea glauca* show that often one collection out of several from different times proves to be better for embryogenic tissue initiation. This suggests that the timing of tissue collection influences initiation success (Hakman and Fowke 1987; Lu and Thorpe 1987). It also indicates that the tissue from the same plant cultured at various stages of development can often differ in their initiation response (Tautorus *et al.* 1991). These results indicate that in order to obtain the best initiation frequencies for embryogenic tissue, the right explant at the right developmental stage must be chosen.

As stated previously, the preferred explant for *Pinus* species is the immature zygotic embryo-gametophyte complex. The stage of development classified for this explant has been termed pre-cotyledonary, whereby cones containing the immature explant are collected 4-8 weeks post-fertilization (Chandler *et al.* 1989). At this time the natural zygotic embryos become multicellular and more responsive to initiation medium (Jones 1990). In *Picea* species the post-cotyledonary embryos are classified as the optimal developmental stage for embryogenic tissue initiation (Fowke *et al.* 1995).

Genotypic influence

It has also been found that genotype selection is also very important for successful embryo initiation in *Pinus* species (Huang *et al.* 1995). This observation is in agreement with that of Jain *et al.* (1989) for *Pinus elliotti* and Becwar *et al.* (1990) for *Pinus taeda*. Genotypic differences between and within families have shown wide variations in embryogenic capacity, with some genotypes exhibiting quite low initiation frequencies (Li *et al.* 1998). Cheliak and Klimaszewska (1991) found significant differences among open-pollinated families of *Picea mariana* (black spruce) where up to 85% of the families successfully initiated embryogenic tissue but the within family frequency varied from 0-18%. Handley *et al.* (1994) observed similar variations in *Pinus taeda*. It has been suggested that using parental genotypes with a known and consistently high embryogenic initiation frequency in breeding programs and modifying media to increase initiation, will reduce these variations and lead to a consistently higher success rate (Handley *et al.* 1994).

Effects of the source of explant material have also been noted (Hakman and von Arnold 1985; Tremblay 1990). Differences were observed in the somatic embryogenesis of *Picea glauca* (white spruce) from several seed locations (Tremblay 1990). Whilst Hakman and von Arnold (1985) also noted that the ability to produce embryogenic tissue varied among plant material collected from different localities. The effect of different growing locations is due to genetic differences between families, observed through variations in physical characteristics such as seed shape and embryo development. Go *et al.* (1993) reported that embryos of *Pinus caribaea* from two fast growing locations showed earlier development of shoot development and more shoots per surviving embryos compared with embryos from two slow growing locations.

Differences in embryogenic initiation ability may be caused by more than genetic characteristics, if seed families are present in more than one area. Climatic conditions may influence the development of the plant material by either increasing or decreasing the rate of maturation of the zygotic embryo, therefore providing variation (Dogra 1978; Keinonen-Mettälä *et al.* 1996). It has been noted that the optimal time for embryogenic tissue initiation ('window of competence') may be extended with the use of plant tissue from the same families grown in two different growing regions (Pascoe *et al.* 1998).

Media selection

Modifications to the embryogenic tissue initiation medium also impacts on initiation frequencies. Many basal media have been utilized to aid the initiation process. Some researchers have used the standard tissue culture salts of Murashige and Skoog (MS) (Murashige and Skoog 1962) as well as modifications of this medium such as MSG (Becwar *et al.* 1990) in which ammonium nitrate (NH_4NO_3) is replaced with glutamine and the potassium nitrate (KNO_3) level is reduced. Other researchers have developed media specifically suited to conifer embryogenic tissue initiation. These include Douglas Fir medium (DCR) (Gupta and Durzan 1985), Hakman medium (LP) (Hakman and von Arnold 1985), P21 (Chandler (1995) pers. comm.) and the New Zealand Forest Research Institute's (now known as Forest Research (FR)) media for a variety of conifer species including *Pinus radiata* and *Pinus taeda* (Smith 1994). Modifications to medium components such as sucrose concentration, replacement of sucrose with other carbohydrate sources, organic nitrogen levels, gelling agents and

their concentrations and mineral elements such as potassium and activated charcoal have all been researched to some degree to determine their influence on embryogenic tissue initiation (Tautorus *et al.* 1991; Attree and Fowke 1993; Misra 1994; Smith 1994; Pullman *et al.* 1995; Li *et al.* 1998).

The presence or absence of plant growth regulators such as auxins and cytokinins are important in the initiation process. Plant growth regulators may occasionally enhance the growth of tissue by stimulating cell division, however, in some instances there is minimal dependence upon plant growth regulators. Embryogenic tissue initiation can occur in the absence of plant growth regulators, especially at the right developmental stage for the zygotic embryo (Chandler *et al.* 1989; Smith 1994). A low concentration of plant growth regulators such as 2,4- dichlorophenoxy acetic acid (2,4-D) and N^6-Benzyladenine (BA) has generally prompted a better initiation response in species such as in *Pinus taeda* (Li *et al.* 1998).

2.1.1 Objectives

The work in this chapter is aimed at:

- examining the embryogenic tissue initiation frequencies by comparing different basal media.

- observing the influence of basal media modifications such as the inclusion of plant growth regulators, activated charcoal, and seed extracts.

- understanding the initiation process.

  In doing so, the developmental stages of the zygotic embryo would be recorded at each collection time and initiation frequency variations monitored between genotypically different families.

- observing the differences in location of source material (New Norfolk and Tasmania) on the initiation frequencies during the 1997-98 initiation period.

2.2 Methods

2.2.1 Plant material

Maryvale material

Immature green pinecones were collected from open-pollinated stock in a breeding arboretum at Maryvale (Victoria, Australia). (Refer to Figure 2.1 for locations of the Albury, Maryvale and New Norfolk). Collections were conducted over a three year period, in December 1995 to February 1996; December 1996 to February 1997 and December 1997 to February 1998 (refer to Table 2.1 for collection dates and Table 2.2 for clones used). The clones selected had a diverse genetic background (Table 2.3). Four green cones were delivered for each collection date (refer to Figure 2.2).

Albury and New Norfolk material

Immature green pinecones were collected from control-pollinated stock in nurseries at New Norfolk (Tasmania, Australia) and Albury (New South Wales, Australia) (refer to Table 2.4 for clones). Two to three green cones were collected for each genotype at each collection date. Collections were conducted fortnightly from December 1997 to February 1998 (refer to Table 2.5).

Upon arrival the immature green cones were washed in 70% ethanol and air-dried in a laminar flow cabinet, then placed into paper bags and put into cold storage until dissection. Green cones were stored for a maximum of 2 weeks.

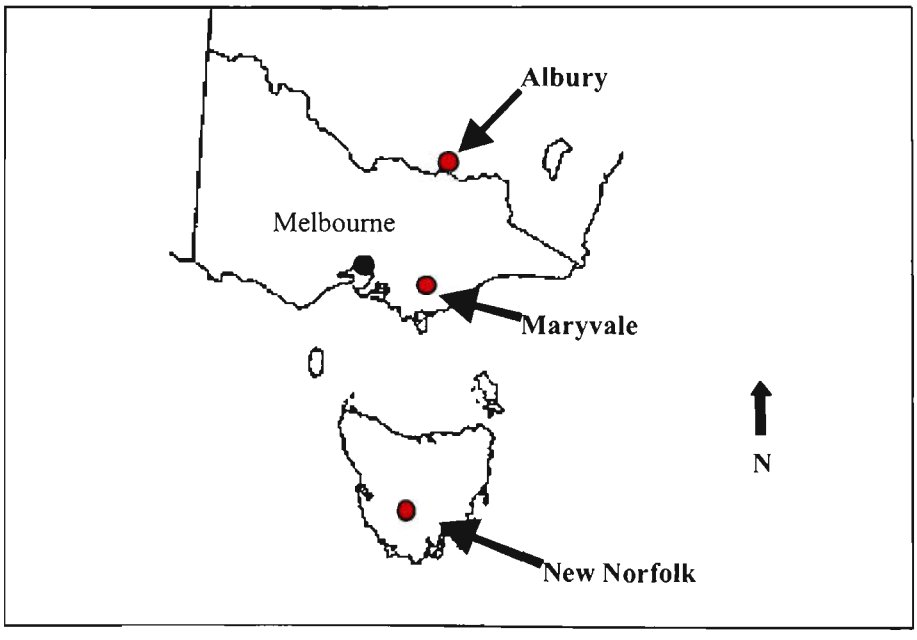


Figure 2.1 Location of source material for embryogenic tissue initiation experiments

Figure 2.2 Green pinecones used for embryogenic tissue initiation Experiments (bar = 1cm)

Table 2.1 Immature green cone collection dates from Maryvale over a 3-year period

Year 1 **1995-96**	**Year 2** **1996-97**	**Year 3** **1997-98**
6/12/95	16/12/96	15/12/97
8/1/96	6/1/97	22/12/97
24/1/96	19/1/97	5/1/98
5/2/96	3/2/97	12/1/98
19/2/96	-	20/1/98
-	-	27/1/98

Table 2.2 Maryvale clones used for embryogenic tissue initiation experiments over the 3 years

Year 1 1995-96	Year 2 1996-97	Year 3 1997-98
-	36013	-
-	36014	36014
-	36055	36055
-	36057	36057
38001	-	-
38002	38002	38002
38010	-	-
38011	38011	-
38012	38012	38012
38031	38031	38031
-	-	35114

Table 2.3 Background pedigree information on the Maryvale clones selected for embryogenic tissue initiation experiments

1st generation selections	
36013	There is no information on the parents of these trees. They are elite trees chosen from a routine plantation established with open pollinated seed from unknown females.
36014	
36055	
36057	
Early 2nd generation selections	
38001	Half-sib. 80085 x wind
38002	Half-sib. 80091 x wind
38010	Full-sib. 80055 x 80121
38011	Full-sib. 30007 x 80055
38012	Full-sib. 30020 x 80055
Advanced 2nd generation selection	
38031	Half-sib. 35078 x wind
35114	Information not given

Table 2.4 Albury and New Norfolk Clones used for embryogenic tissue initiation experiments

Albury clones	New Norfolk clones
33 x 31	12349x51255
	Clone 3
9 x 31	38048x51255
	Clone 4
11 x 70*	50048x31087
	Clone 1
11 x 35*	50048x70052

- Clone 11 x 70 was mistakenly replaced with Clone 11 x 35 for Collections 5/1/98 18/1/98 and 3/2/98. There is no New Norfolk clone to make any comparisons with.

Table 2.5 Collection dates and clones from Albury and New Norfolk

Collection date	Albury clones	Collection date	New Norfolk clones
19/12/97	11 x 70	24/12/97	Clone 1
	33 x 31		Clone 3
	9 x 31		Clone 4
5/1/98	-	8/1/98	Clone 1
	33 x 31		Clone 3
	9 x 31		Clone 4
	11 x 35		-
18/1/98	-	24/1/98	Clone 1
	33 x 31		Clone 3
	9 x 31		Clone 4
	11 x 35		-
3/2/98	-	6/2/98	Clone 1
	33 x 31		Clone 3
	9 x 31		Clone 4
	11 x 35		-

2.2.2 Media

The media used varied over the three-year period. Initiations in year one (1995-96) were based on the identification of an appropriate basal medium (refer to Tables 2.6 and 2.7). Media used included DCR (Gupta and Durzan 1985), WPM (Lloyd and McCown 1981), LP (Hakman and von Arnold 1985), and P21 (Chandler (1995) pers. comm.). An additive of 4.4g/l glutamine (Sigma) was also included in each basal medium, therefore two treatments of each basal medium was utilized. The media

were solidified with 4g/l Gelrite (Sigma). The pH of media was adjusted to 5.8 before autoclaving at 121°C for a time period dependent on the medium volume.

Initiations in year two (1996-97) investigated additives included in the media to optimise and improve initiation rates (refer to Tables 2.6 and 2.8). These additives included presence or absence of 0.25% (w/v) activated charcoal, 3% and 6% (w/v) seed extract (extracted from mature *Pinus radiata* seeds according to a method by Egertsdotter and von Arnold 1995), and presence or absence of a combination of the plant growth regulators 2,4-D (Sigma) and N⁶-Benzyladenine (BA) (Sigma). The hormone stock solutions were filter sterilized and added to the medium after autoclaving.

Initiations for the material from Maryvale in Year three (1997-98) utilized the media of the previous two years (DCR hormone-free with activated charcoal) (refer to Tables 2.6 and 2.9). Other media treatments included DCR with and without hormones, DCR with 6% (w/v) seed extract (with and without hormones) and Forest Research's standard embryogenic tissue capture medium (Smith 1994) (known in this study as NZ).

Initiations for the material from Albury and New Norfolk in 1997-98 utilized only three media. These included the DCR hormone-free medium, DCR hormone-free medium with activated charcoal and the NZ standard embryogenic tissue capture medium (refer to Table 2.9 for details).

Table 2.6 Media used over the 3 years for initiation experiments from Maryvale (a complete list of ingredients for the basal media is listed in Appendix I)

1995-96	1996-97	1997-98	Basal medium	Additives	Code
✓			DCR	Glutamine	DCR+
✓			DCR		DCR-
✓			LP	Glutamine	LP+
✓			LP		LP-
✓			P21	Glutamine	P21+
✓			P21		P21-
✓			WPM	Glutamine	WPM+
✓			WPM		WPM-
	✓	✓	DCR	Hormone-free	DCR-
	✓	✓	DCR	Activated charcoal	AC
	✓		DCR	3% seed extract	SEED3
	✓	✓	DCR	6% seed extract	SEED6
	✓		DCR	2,4-D & BA	2.0/1.0
	✓	✓	DCR	2,4-D & BA	2.2/1.1
		✓	DCR	6% seed extract & 2,4-D & BA	SE6:2.2/1.1
		✓	FR		NZ

Table 2.7 Year one (1995-96) initiation media for Maryvale experiments

	LP+/-	DCR+/-	WPM+/-	P21+/-
Macronutrients	LP major	DCR major	WPM major	P21 major
Micronutrients	LP minor	DCR minor	WPM minor	P21 minor
Vitamins	LP vits	DCR vits	WPM vits	0
Iron stock	LP iron stock	DCR iron stock	WPM iron stock	P21 iron stock
Sucrose (mg/l)	34 200	30 000	30 000	30 000
Myo inositol (mg/l)	1000	200	100	100
Glycine	0.2	2	0	2
Amino acids	LP acids	0	0	0
Glutamine (mg/l)(+)	4400	4400	4400	4400
(-)	0	0	0	0
Activated Charcoal (mg/l)	2500	2500	2500	2500
Gelrite (mg/l)	4000	4000	4000	4000

Table 2.8 Year two (1996-97) initiation media for Maryvale experiments

	DCR-	AC	SEED3	SEED6	2.2/1.1	2.0/1.0
Macronutrients	DCR major ions for all media					
Micronutrients	DCR minor ions for all media					
Vitamins	DCR vitamins for all media					
Iron stock	DCR iron stock for all media					
Sucrose (mg/l)	30 000	30 000	30 000	30 000	30 000	30 000
Glycine (mg/l)	2	2	2	2	2	2
Myoinositol (mg/l)	200	200	200	200	200	200
2,4-D (mg/l)	0	0	0	0	2.2	2.0
BA (mg/l)	0	0	0	0	1.1	1.0
Activated Charcoal (mg/l)	0	2500	0	0	0	0
Seed Extract (%)(w/v)	0	0	3	6	0	0
Gelrite (mg/l)	4000	4000	4000	4000	4000	4000

Table 2.9 Year three (1997-98) initiation media for Maryvale and Albury/New Norfolk experiments

	DCR-	2.2/1.1	SEED6	SE6: 2.2/1.1	AC	NZ
Macronutrients	DCR	DCR	DCR	DCR	DCR	NZ
Micronutrients	DCR	DCR	DCR	DCR	DCR	NZ
Vitamins	DCR	DCR	DCR	DCR	DCR	NZ
Iron stock	DCR	DCR	DCR	DCR	DCR	NZ
Myoinositol (mg/l)	200	200	200	200	200	500
Sucrose (mg/l)	30 000	30 000	30 000	30 000	30 000	10 000
Glycine (mg/l)	2	2	2	2	2	0
2,4-D (mg/l)	0	2.2	0	2.2	0	0
BA (mg/l)	0	1.1	0	1.1	0	0
Activated Charcoal (mg/l)	0	0	0	0	2500	2000
Seed Extract (%) (w/v)	0	0	6	6	0	0
Gelrite (mg/l)	4000	4000	4000	4000	4000	0
Difco Bacto agar (mg/l)	0	0	0	0	0	8000

2.2.3 Initiation of embryonal masses

Seeds were removed from the scales of the cones from the base towards the cone apex (refer to Figure 2.3). The seeds were surface sterilized in 1g/l Mercuric chloride ($HgCl_2$) (0.1% SDS (Sodium dodecyl sulfate)) for 20 minutes then washed 4 times with sterile distilled water. The seed coat and integuments were removed from the megagametophyte under a binocular microscope prior to culture. For each genotype and collection date, the developmental stage of the zygotic embryo was evaluated. Five to ten seeds, chosen at random, were dissected and examined under a light microscope or stereo microscope, depending on the age of the immature zygotic embryo(s).

The whole megagametophyte containing the immature zygotic embryo served as the explant. The number of explants per petri dish varied depending on the availability of the seeds from each genotype. The megagametophyte complex was placed horizontally on the culture medium. Each plate was sealed with Parafilm®. The dishes were cultured in darkness at a constant temperature of 24°C +/- 1°C.

Figure 2.3 Dissection of pinecone showing seeds located under each scale (bar = 1cm)

2.2.4 Maintenance of embryonal masses

Embryogenic tissue exuded from the micropylar end of the megagametophyte onto the medium over the next twelve weeks.

To promote proliferation, once the exuded embryogenic tissue was about 8-10mm in diameter it was removed from the original explant on the initiation media and placed onto a medium of hormone-free DCR (same medium as for initiation but with 0.6% gelrite, refer to Table 2.7). In year 3 most exuded embryogenic tissue was placed onto the NZ standard embryogenesis medium (embryogenic tissue maintenance medium) (Smith 1994). Embryogenic tissue was subcultured at least monthly and sooner if proliferation occurred rapidly, and incubated under the same conditions as for initiation.

To aid in the selection of cell lines, a screening method developed by Maddocks *et al.* (1995) at Carter Holt Harvey in Rotorua, New Zealand was used. This screening method employed a staining procedure developed by Gupta and Durzan (1987) and a grading system with four types of embryo initials. Grades 1 and 2 embryogenic cell lines possessed embryo initials with some degree of organization and maturity (Figure 2.4) whilst Grades 3 and 4 embryogenic cell lines possessed little to no organization of embryo initials (Figure 2.5) (Maddocks *et al.* 1995). It was found that Grade 1 and 2 embryogenic cell lines generally produced mature high quality embryos whilst Grade 3 and 4 cell lines had little to no embryo-forming ability. The staining system was employed because the acetocarmine or aceto-orcein stains enabled a reliable and quick observation between embryogenic tissue and non-embryogenic tissue. This was due to the complete staining of the inner components of the embryonic region (Bellarosa *et al.* 1992).

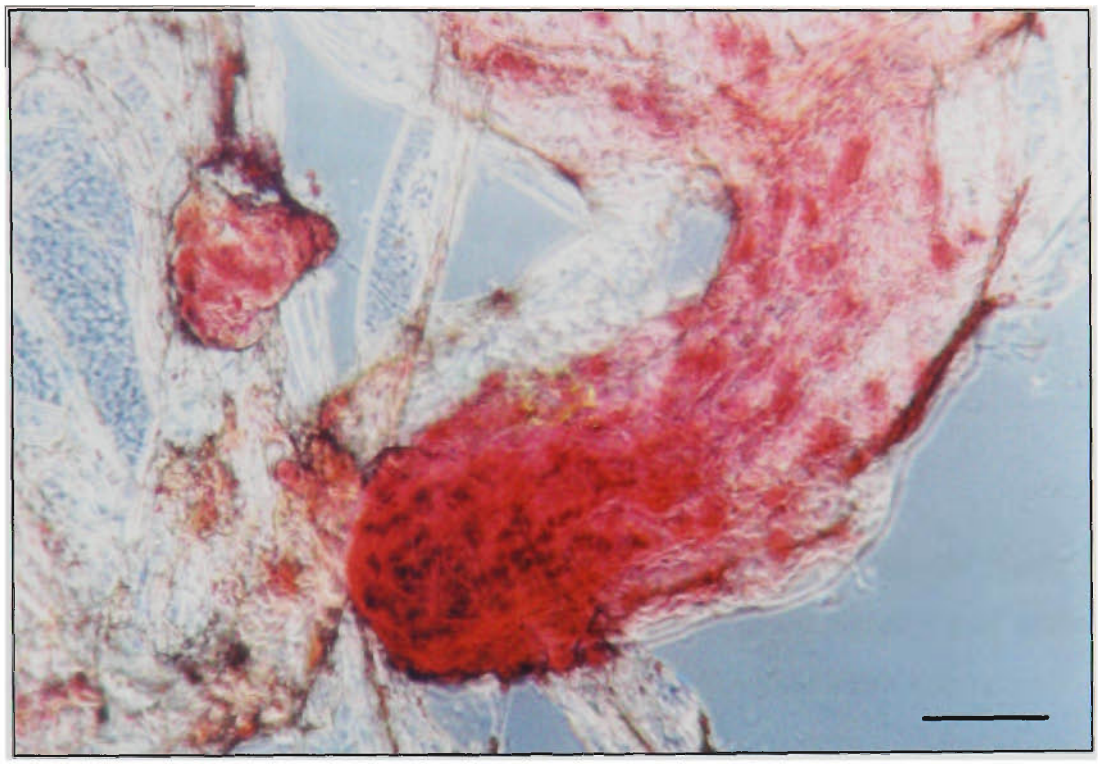


Figure 2.4 Histochemically stained embryogenic cells depicting Grade 1 and 2 cell lines (bar = 0.1mm)

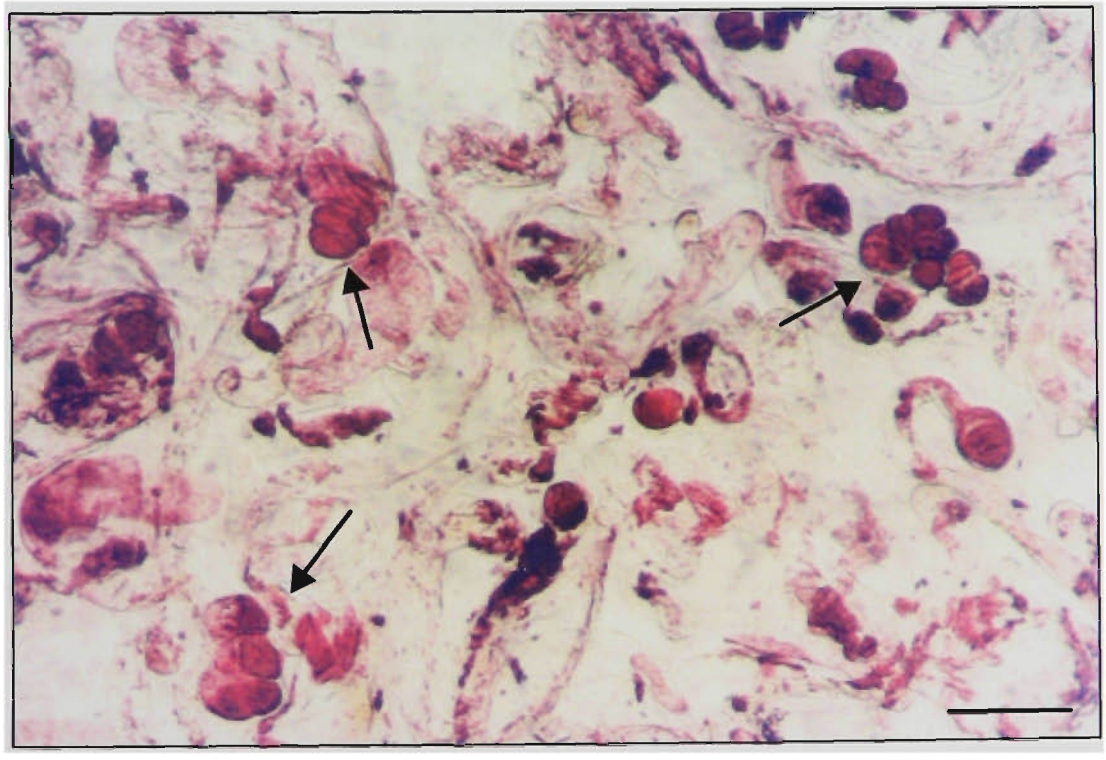


Figure 2.5 Histochemically stained embryogenic cells, depicting Grade 3 and 4 cell lines. Arrows show small clusters of embryogenic cells (bar =0.1mm)

2.2.5 Histochemical staining

The presence of embryonal masses was determined by staining with 2%(w/v) acetocarmine (Gupta and Durzan 1987) or with aceto-orcein (2.2% aceto-orcein in glacial acetic acid) (Lainé and David 1990). Aceto-orcein neither stains the cytoplasm, nucleoli nor overstains the chromosomes (Lelu *et al.* 1990). All tissue preparations were stained for two minutes, rinsed with water and gently squashed with a cover slip. The preparations were examined under a light microscope and photographed when appropriate.

2.2.6 Data Collection

Cultures were monitored on a monthly basis for three months for the numbers of extrusion of the embryogenic tissue. Final estimation of the frequency of formation of embryogenic tissue was made after three months on initiation medium.

2.2.7 Data analysis

The frequency of extrusion provided a measure of the number of explants that possessed the potential for the initiation of embryogenic tissue. The data obtained from each year was analyzed separately due to the differences in structure between each experiment. The initiation data was (binomial distribution) analyzed using a Generalized Linear Model (GLM) with Genstat Release 4.2, Fifth edition (Lawes Agricultural Trust, Rothamsted Experimental Station 2000) and a logit link function. Figures do not possess error bars due to the method of statistical analysis. Statistical significance of data is measured at $p < 0.05$ unless otherwise stated.

2.3 RESULTS

2.3.1 Zygotic embryo development

Development of Maryvale zygotic embryos

Zygotic embryo development varied over the three-year period. Throughout the time length of the cone collections the normal developmental process of the seed occurred. In 1995-96 the stage at which polyembryogenesis occurred was around late December to early January (as shown in Table 2.10). For the following two years of cone collections this stage occurred around mid-December to mid-January and mid-December through to early January, respectively. Variations in zygotic embryo development between the three periods of collections were probably due to clonal differences and climatic conditions, which are known to influence the process of fertilization and maturation of the zygotic embryo (Dogra 1978; Keinonen-Mettälä 1996). Most of the clones throughout the three-year period showed early globular stage development in the December collections (refer to Figure 2.6), but variations in development occurred between the clones in the January collections. This can be observed in the example of the early January collections seen in Table 2.11. By the end of the collection period, early February, the zygotic embryos had possessed cotyledonary initials prior to germination. This stage being classified as cotyledonary or stage 4 (Smith 1994; Chandler and Young 1995) (refer to Figure 2.7).

Table 2.10 Zygotic embryo development at the time of collection

Year	Collection date	Embryo stage[a]	Length of dominant embryo (mm)	Length of megagametophyte (mm)
1995-96	6/12/95	Early globular (1)	No dominant embryo	5.0-5.5
	8/1/96	Early globular-globular (1-2)	<1.0	4.9-6.7
	24/1/96	Early cotyledonary – cotyledonary (3-4)	1.0-3.0	5.7-6.7
	5/2/96	Cotyledonary (4)	3.0-6.0	5.6-6.8
	19/2/96	Cotyledonary (4)	5.0-6.7	5.7-7.0
1996-97	16/12/96	Early globular (1)	No dominant embryo	4.0-6.1
	6/1/97	Early globular-globular (1-2)	<1.0	4.4-6.9
	19/1/97	Globular-early cotyledonary (2-3)	1.0-3.0	4.4-6.5
	3/2/97	Cotyledonary (4)	2.0-6.0	4.8-6.6
1997-98	15/12/97	Early globular (1)	No dominant embryo	4.2-5.6
	22/12/97	Early globular (1)	No dominant embryo	4.0-5.7
	5/1/98	Globular (2)	< 1.0	4.7-6.2
	12/1/98	Globular-early cotyledonary (2-3)	1.0-3.0	4.5-5.5
	20/1/98	Early cotyledonary (3)	2.0-5.0	4.3-5.7
	27/1/98	Early cotyledonary-cotyledonary (3-4)	2.0-5.0	4.5-5.9

a: classification of stages of zygotic embryo development from Smith (1994) and Chandler and Young (1995).

Table 2.11 Clonal differences in zygotic embryo development in immature cones collected in early January

Year	Clone	Stage	Year	Clone	Stage	Year	Clone	Stage
95-96	38031	1-2	**96-97**	38031	2	**97-98**	38031	2
	38012	2-3		38012	2		38012	2
	38010	2		36013	1-2		35114	2
	38002	2-3		38002	1-2		38002	2
	38001	1-2		36014	2		36014	2
	38011	2-3		38011	1-2			
				36055	2		36055	2
				36057	1-2		36057	2

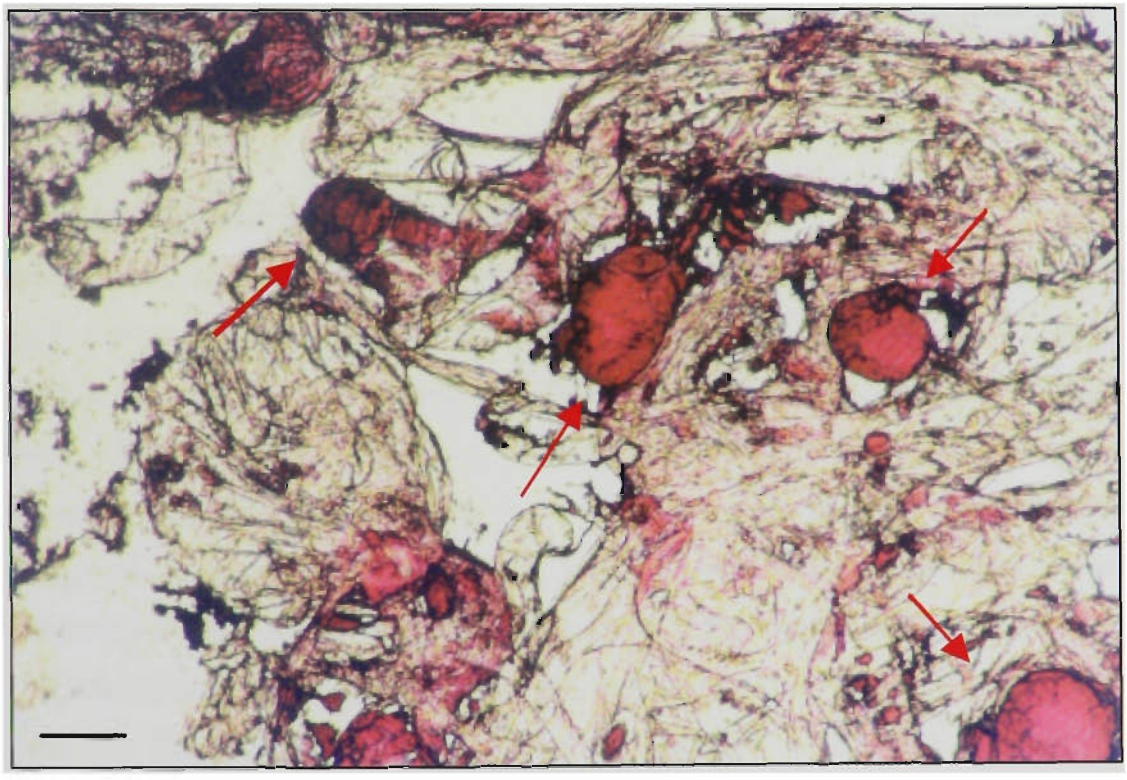


Figure 2.6 Early globular stage of zygotic embryo development from 15th December 1997 collection. Arrows show the multiple embryos due to cleavage polyembryogenesis (bar = 0.1mm)

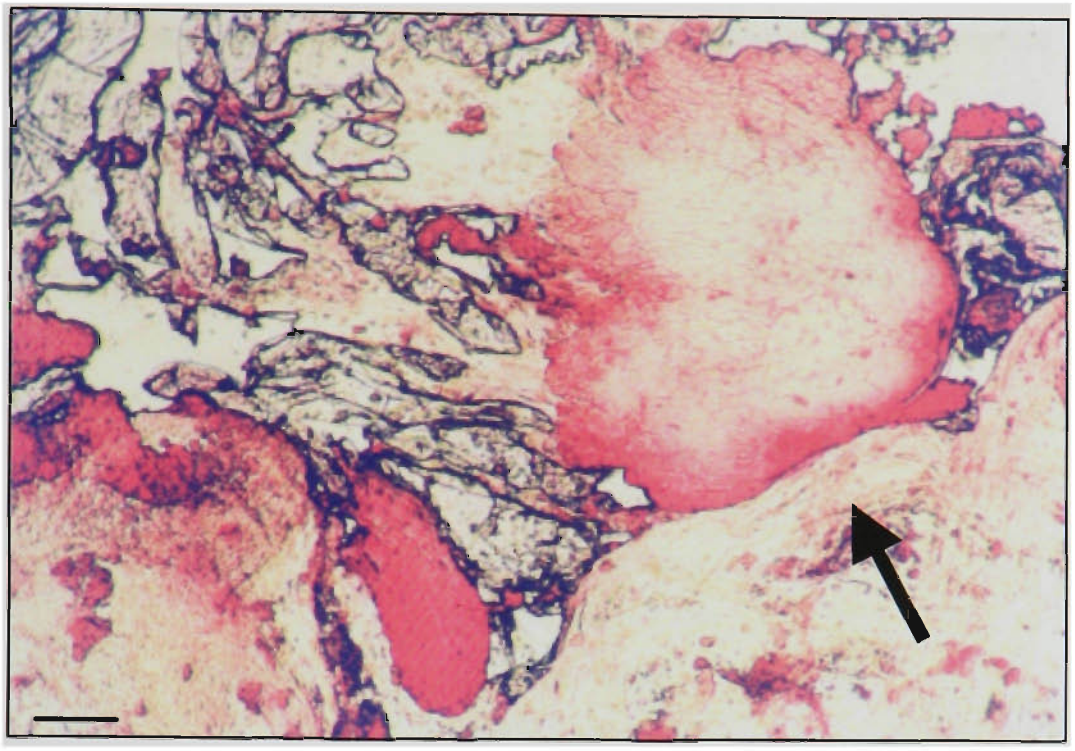


Figure 2.7 Pre-cotyledonary stage of zygotic embryo development from 5th January 1998 collection. Arrow shows the dominant embryo present (bar = 0.1mm)

Development of Albury and New Norfolk zygotic embryos

Overall the zygotic embryo development varied quite dramatically between the New Norfolk cones and the Albury cones, as can be observed in Table 2.12. The first collection in late December from the New Norfolk cones showed no dominant embryo within the megagametophyte, however, on staining the cells showed evidence of cleavage polyembryogenesis with 3 – 4 embryo initials present. From the same time period of cone collection, the Albury clones showed globular to early cotyledonary embryo development. Observations of the zygotic embryo development over the collection period showed the natural maturation of the embryos from both regions. As Table 2.12 shows, throughout the duration of the collections the New Norfolk embryos varied from early globular to globular stage embryos in December through to precotyledonary and cotyledonary stage by early February. For the same period the Albury embryos were more advanced with embryos maturing to the cotyledonary stage by mid January.

Table 2.12 Zygotic embryo development from Albury and New Norfolk immature cones

Region	Collection date	Embryo stage[a]	Length of dominant embryo (mm)
New Norfolk	24/12/97	early globular – globular (1-2)	No dominant embryo
	8/1/98	globular (2)	<1.0
	24/1/98	early cotyledonary (3)	1.0 - 3.0
	6/2/98	early cotyledonary – cotyledonary(3-4)	3.0
Albury	19/12/97	globular – early cotyledonary (2-3)	2.0 – 3.0
	5/1/98	early cotyledonary (3)	3.0
	18/1/98	cotyledonary (4)	5.0
	3/2/98	cotyledonary (4)	5.0

a: classification of stages of zygotic embryo development from Smith (1994) and Chandler and Young, (1995).

2.3.2 The effect of collection date on initiation frequencies

Maryvale initiation experiments

Embryogenic tissue was successfully initiated from explants over the three-year period. Tissue extrusions from the micropylar end of the megagametophyte started to appear as early as two to three weeks after culture (refer to Figure 2.8). Tissue appeared translucent to white and mucilaginous and was determined to be embryogenic when stained with aceto-orcein. Embryogenic tissue contained embryonic structures that varied in the level of organization. Some embryogenic tissue possessed clusters of small cells whilst others contained definite embryo heads with suspensor cells. The embryogenic cells stained deep red with the acetocarmine or aceto-orcein (refer to Figures 2.4 and 2.5). Not all of the early tissue extrusions proliferated over the following few months. The proliferation of conifer embryogenic tissue can be arrested due to poor seed quality, early browning of the megagametophyte, inappropriate nutrient levels for sustained proliferation or some other unidentified factor (Lelu *et al.* 1999).

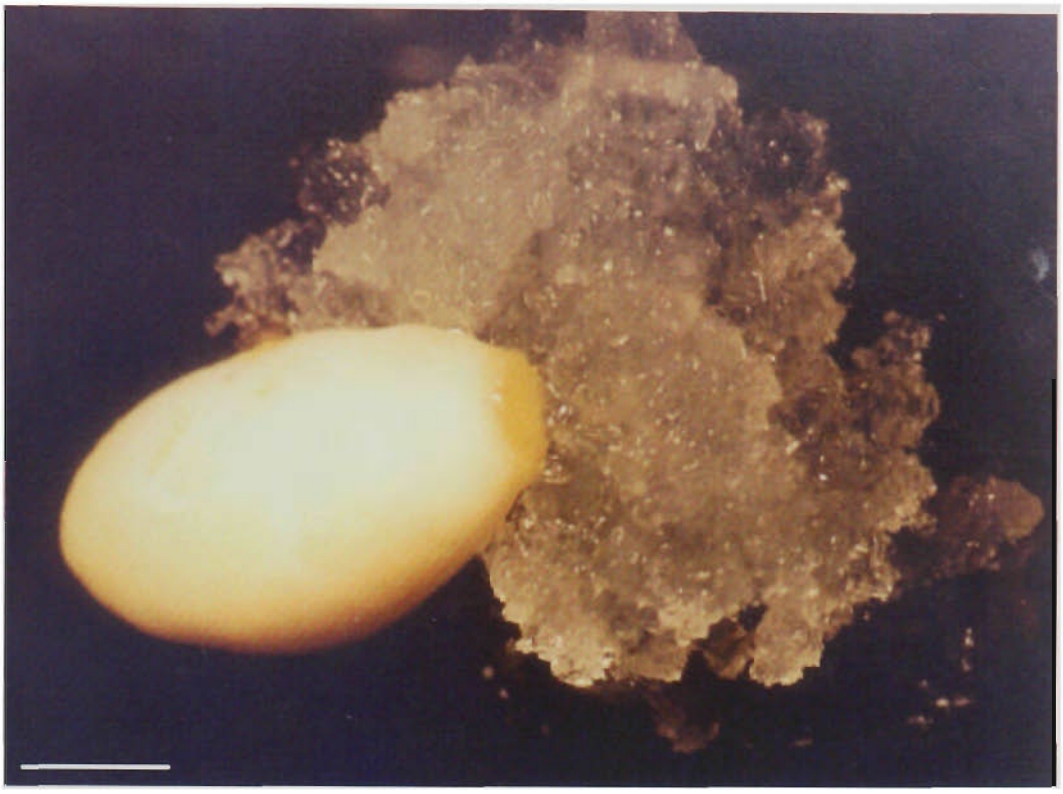


Figure 2.8 Embryogenic tissue extrusion from the micropylar end of the megagametophyte (bar = 1cm)

Overall the average initiation frequency (at three months) of embryogenic tissue initiated across the families for 1995-96 was 7.13%, in 1996-97 it was 4.33% and in 1997-98 it was 14.03%. Table 2.13 shows the average initiation frequencies for each cone collection over three years. The period of time when embryogenic tissue initiation was at its peak did not vary greatly over the three-year period. In 1995-96, the peak period or 'window of competence' was around late December to early January, as can be seen by the average frequency of initiated embryogenic tissue, 21.79% for the 8th January collection. In 1996-97, the same peak period was observed this being late December to early January, with an average frequency of 8.16%. The peak period was slightly different in the 1997-98 collections with a lengthening of the competent phase. Excellent levels of embryogenic tissue were initiated in early to mid December (average of 21.66% from the 15th December collection) right through to early January (average of 11.72% from the 5th January collection). However, the peak of induction appeared around late December with an average of 36.19% initiated from the 22nd December collection (refer to Table 2.13).

Table 2.13 Average initiation frequencies (three-month analysis) of embryogenic tissue from 1995-96, 1996-97 and 1997-98

	1995-96[a]			1996-97[b]			1997-98[c]	
Date	**% initiation**	% germination	**Date**	**% initiation**	% germination	**Date**	**% initiation**	% germination
6th Dec	3.48	0	16th Dec	4.36	0	15th Dec	21.66	0.9
8th Jan	21.79	14.5	6th Jan	8.16	2.2	22nd Dec	36.19	6.9
24th Jan	7.57	37.0	19th Jan	0.68	1.3	5th Jan	11.72	7.8
5th Feb	1.92	64.4	3rd Feb	4.85	47.8	12th Jan	5.88	27.9
19th Feb	0.44	62.1				20th Jan	7.66	28.9
						27th Jan	6.49	52.0
Average total	**7.13**			**4.33**			**14.03**	

a,b,c: p-value <0.001 (p-values for a, b, c indicate some significance between the collection dates, however due to incomplete pairwise comparison, accurate assessments cannot be determined. Only observational differences can be made.)

The differences in initiation rates over the duration of the cone collections (December to February) appeared to follow the developmental stages of the zygotic embryo. That is, the normal maturation of the embryo. As can be observed in Tables 2.11 and 2.13 the average frequencies from each collection over the three years corresponded with the stages of embryo development. For each year the 'window of competence' occurred when the zygotic embryo was in the early globular to globular stage or stages 1 to 2, normally when the embryo was going through the cleavage polyembryogenesis process. These stages of development occurred in late December to early January for 1995-96 and 1996-97 and mid December to early January during 1997-98.

The maturation of the zygotic embryo can also be observed with the germination rates presented in Table 2.13. An increase in the germination rate usually coincided with a decrease in the initiation rate of embryogenic tissue. This can be observed throughout the three years of cone collections (refer to Table 2.13). For example, during 1997-98 the average initiation rate during the 15th December collection was 21.66%, whilst the corresponding germination rate was 0.9% when the embryo was at its most immature

(stage 1). The contrasting example can then be observed when the embryo was most mature (stage 4) with an initiation rate of 6.49% and germination at 52.0%. Figure 2.9 shows the results of a germination experiment with the whole gametophyte and the isolated embryo from the 1996-97 collection on hormone-free medium. The zygotic embryos were too immature to germinate in the 16th December collection. However, in the 3rd February collection the zygotic embryo had reached the cotyledonary stage and tended to germinate.

Figure 2.9 Germination potential from whole megagametophytes and isolated zygotic embryos from the 16th December collection and the 3rd February collection (1996-97) (bars = 3 cm)

Variations in the overall average initiation frequencies can be observed between the years. Initiation frequencies are known to fluctuate over the years due to various reasons including genotype differences and weather conditions during the fertilization and seed maturation periods (Dogra 1978; Keinonen-Mettälä *et al.* 1996; Lelu *et al.* 1999). In 1995-96 the overall initiation rate of 7.13% was probably lower due to the overlooked late December cone collection (refer to Table 2.13). This collection was

amongst the peak period of embryogenic tissue initiation therefore, was likely to have produced a high average initiation frequency. The overall initiation frequency during 1996-97 was low (4.33%) due to the extreme weather conditions experienced during the seed maturation stages. Hot weather was experienced during January and February of 1996, usually around the periods of harvesting the cones and transfer to the laboratory. For example, the temperature on the 19th January 1997 collection date was 35.8°C, whilst during the days of transfer the temperatures reached 39.8°C and 38.5°C, respectively. This can be compared with weather data from the previous year, when on the 24th January collection, the temperatures were substantially cooler with 27.3°C, 20.3°C and 25.5°C for the days of collection and transfer (Bureau of Meteorology 1998). Keinonen-Mettälä *et al.* (1996) found that the temperature during the summer, when the seed developed, affected initiation rates. Whilst Jones and van Staden (1995) observed that moisture stress might affect the seed development and indirectly influence the embryogenic capability of the explants. This was also observed in this study.

Albury and New Norfolk initiation experiments

Embryogenic tissue was successfully initiated in explants from Albury and New Norfolk. Overall frequencies of initiation of embryogenic tissue for Albury and New Norfolk were 5.6% and 17.97%, respectively. Table 2.14 shows the initiation frequencies for each sampling period and the overall averages. Differences in the initiation frequencies were observed between the regions. The period of time when embryogenic tissue initiation was at its peak for the New Norfolk region was from late December to early January (26.4%, 34.8%). However, the peak period for Albury may have been prior to the first collection made in December.

Table 2.14 Overall initiation frequencies of embryogenic tissue from Albury and New Norfolk (%)

	Late Dec.	Early Jan	Mid Jan.	Early Feb.	Overall average
New Norfolk[a]	26.4	34.8	0.85	0.29	17.97
Stage of zygotic embryo[c]	1-2	2	3	3-4	
% germination	0.4	5.1	60	64	
Albury[b]	13.3	7.3	0.8	0	5.6
Stage of zygotic embryo[c]	2-3	3	4	4	
% germination	54	55	57	95	

a:p-value <0.001; **b**:p-value <0.001 (p-values for a and b indicate some significance between the collection dates, however due to incomplete pairwise comparison, accurate assessments cannot be determined. Only observational differences can be made.)

c: classification of stages of zygotic embryo development from Smith (1994)

The differences in the initiation frequencies between the two regions also appear to be due to the developmental stage of the zygotic embryo. The influence of the stage of development of the embryo can be observed in the embryogenic tissue initiation frequencies and the level of germination of the zygotic embryos observed over the time period of cone collections from the two regions (refer to Table 2.12).

As noted previously, the peak period of embryogenic tissue initiation in the New Norfolk region was late December to early January. The collections made during this period also coincided with the optimal developmental stage, early globular. This indicated that the developmental stage of the zygote was at its optimal state for good embryogenic tissue initiation. Unfortunately the peak initiation period for the Albury region was not captured in this study, but by assessing the stage of development of the zygotic embryo and the initiation rates the approximate time frame could be determined. Based on the results, the optimal time to capture the peak initiation in the Albury region should be late November to mid December. As in the Maryvale initiations, the level of germination corresponded with the maturation of the zygotic embryo. The results in Table 2.14 show that as the embryo's capacity to produce embryogenic tissue decreased its capacity to germinate tended to increase.

2.3.3 The effect of media on embryogenic tissue initiation

The media effect on Maryvale explants

Embryogenic tissue was successfully initiated on all media regardless of additional media additives used throughout the three years. As Table 2.15 shows, the overall average frequencies of embryogenic tissue initiation varied with the different media. In 1995-96, the effects of basal media, in combination with glutamine, on the initiation rates were analyzed. Average initiation frequencies varied amongst the different treatments, with DCR+ showing the highest frequency of 9.75%. Statistical analysis showed that a significance difference was observed between the LP- medium compared with the rest of the media tested (*P* value <0.001). The remaining media showed no significance between initiation frequencies (Table 2.15). There was also no significant effect of glutamine on initiation frequencies (chi pr. 0.080). As Figure 2.10 shows, media with or without glutamine produced high initiation frequencies during the peak period around early January. As Table 2.16 shows, media during this initiation period showed significant differences when compared with the same types of media used for later initiation periods (24th January and 5th February collections). Examples of the media used on 8th January collection, shown in Table 2.16, are statistically significant from the other collection dates of 24th January and 5th February (shown in the right columns). For example; DCR- with a frequency of 21.2% was significantly different from P21+ (24th January) with a frequency of 9.2%, as the t-probability value is less than 0.05.

Table 2.15 Overall average effect of media on initiation frequencies*

95-96	**DCR+**	**DCR-**	**LP+**	**LP-**	**P21+**	**P21-**	**WPM+**	**WPM-**
%	9.75^{a}	6.97^{a}	6.63^{a}	3.74^{b}	7.34^{a}	7.36^{a}	7.84^{a}	7.75^{a}
96-97	**2.0/1.0**	**2.2/1.1**	**AC**	**DCR-**	**SEED3**	**SEED6**		
%	4.10^{a}	5.21^{ab}	8.25^{b}	2.26^{a}	2.91^{a}	3.53^{a}		
97-98	**AC**	**DCR-**	**2.2/1.1**	**NZ**	**SEED6**	**SE6: 2.2/1.1**		
%	14.58^{ab}	12.83^{ab}	14.30^{ab}	17.90^{b}	11.94^{ab}	17.44^{b}		

*Numbers with the same letter are not significant within each row for each year.

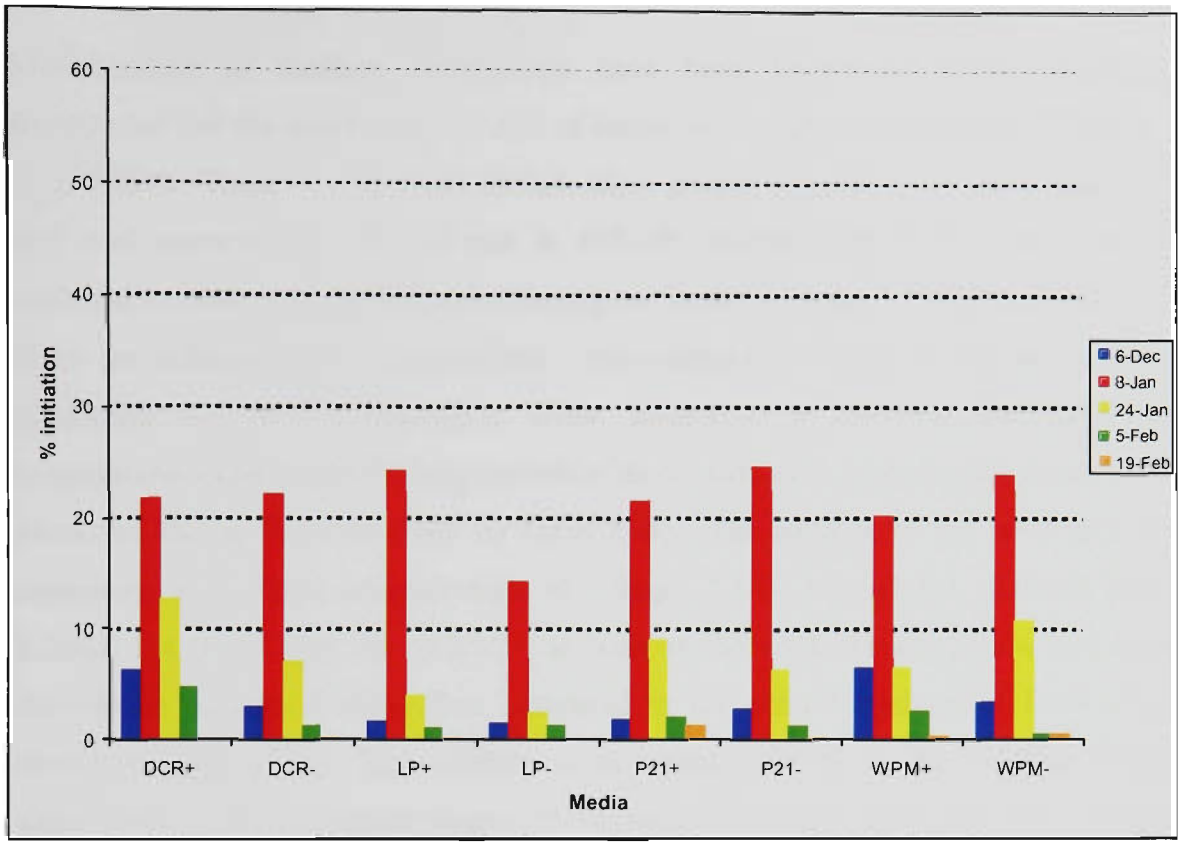


Figure 2.10 Effect of media on average initiation frequencies at each cone collection during 1995-96

Table 2.16 Statistical significance of t-probabilities of pairwise differences between a selection of the treatments of the 8th January collection 1995/96 with other collections

Treatment	%		Treatment	%	t-prob. value
DCR-	21.2	Significantly	P21+ (24th Jan.)	9.2	0.005
LP+	23.2	different	P21- (24th Jan.)	7.2	0.010
P21+	20.4	to	DCR+(24th Jan.)	12.1	0.005
P21-	23.5		DCR+(24th Jan.)	12.1	0.004
WPM-	23.2		LP+(24th Jan.)	3.8	0.000
DCR-	21.2		DCR+(5TH Feb.)	4.1	0.000

For further embryogenic tissue initiation experiments, DCR- was selected based on its good performance during the peak period and its consistent performance over the other collection dates.

Modifications to medium components have been known to affect initiation frequencies and the quality and the rate of initiation of embryogenic tissue (Tautorus *et al.* 1991). These modifications include plant growth regulators, activated charcoal and seed extracts. In 1996-97 and in 1997-98 modifications to the media were analyzed to observe the effects on embryogenic tissue initiation. Tables 2.8 and 2.9 show the different media compositions. Environmental conditions were not highly favourable for good embryogenic tissue initiations in 1996-97, due to high temperatures experienced for long periods of days. However, embryogenic tissue was generated during this time (refer to Table 2.15). The addition of the plant growth regulators, at different concentrations of 2.0mg/l 2,4-D, 1.0mg/l BA (2.0/1.0) and 2.2mg/l 2,4-D, 1.1mg/l BA (2.2/1.1), as well as the seed extracts at 3% and 6% showed no significant differences compared to the control medium of DCR- (as shown in Table 2.15). The addition of activated charcoal to the medium (AC) significantly enhanced embryogenic tissue initiation when compared with DCR- (8.25% compared to 2.26%). It was also significantly different when compared to the hormone medium, 2.0/1.0, and the seed extract media of Seed3 and Seed6. However, no significance was noted between the AC medium and the hormone medium of 2.2/1.1 (refer to Table 2.15). These differences were probably due to the ability of activated charcoal to absorb most impurities within the medium that might otherwise interfere with the initiation process (Gupta *et al.* 1993).

The effect of the media on the average initiation frequencies at each collection during the 1996-97 experiments showed little variation. As Figure 2.11 shows, a treatment of activated charcoal (AC) at the 6th January collection showed an average frequency of 18.1%. However, statistical analysis of t-probabilities of pairwise differences showed no significance between this media and the collection date that it was used on, compared with the other media and collection dates. This may have been due to the unfavourable initiation conditions experienced during this time.

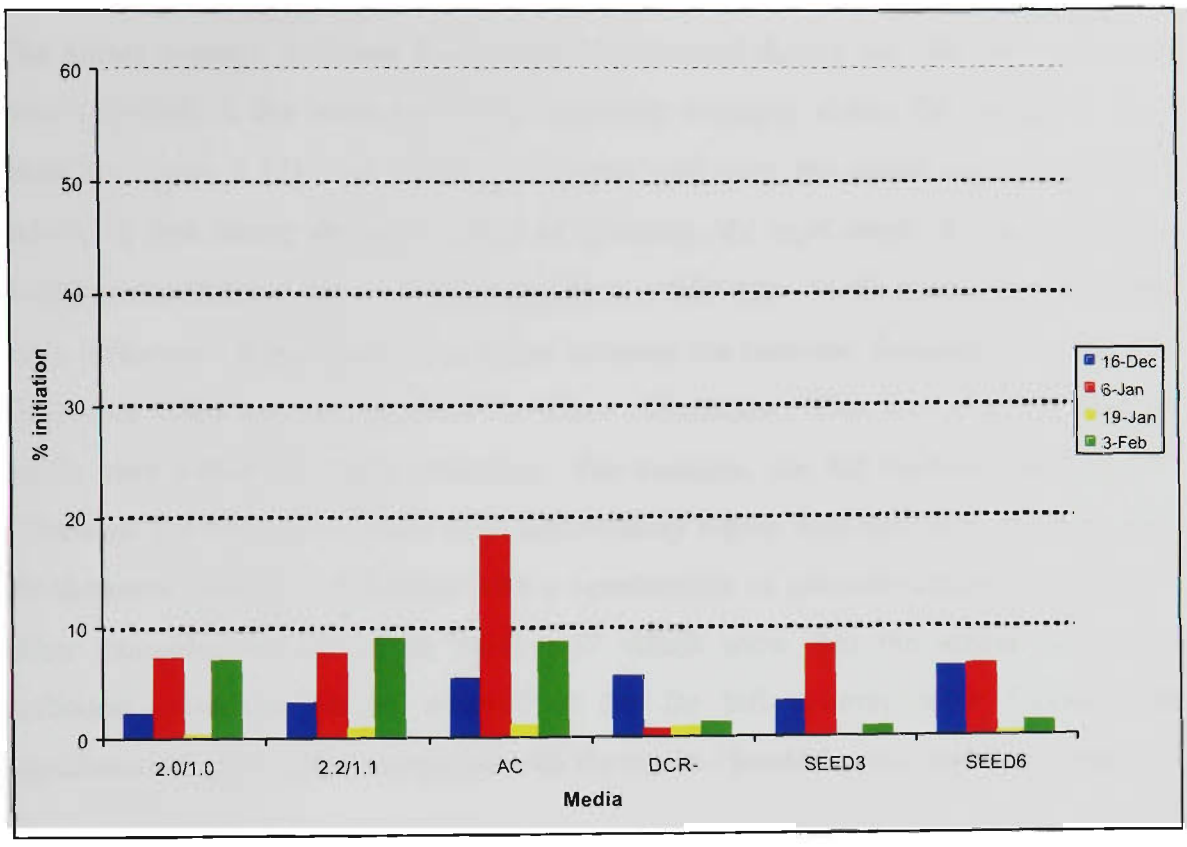


Figure 2.11 Effect of media on average initiation frequencies at each cone collection during 1996-97

Further modifications were made to the media for the 1997-98 embryogenic tissue initiation experiments (Table 2.9). The control medium, DCR-, and the activated charcoal medium, AC, were included, as well as several combinations of hormones and seed extract. The higher concentration of 2,4-D and BA (2.2/1.1) was included as well as a combination with the seed extract concentration of 6% (SE6:1.1/2.2). The standard 6% seed extract medium, Seed6, was also included (Table 2.9). Another medium utilized in the experiments was the New Zealand standard embryogenic tissue capture medium (NZ). Scientists at Forest Research in New Zealand developed this medium for somatic embryogenesis in *Pinus radiata* (Smith 1994). It was included to make comparisons of embryogenic tissue initiation with the other media. As Table 2.15 shows, all media successfully initiated embryogenic tissue. The lowest average frequency observed was 11.94% on 6% seed extract medium, whilst the highest frequency observed was on the NZ medium (17.9%), followed by SE6:2.2/1.1 (17.44%). These two media (NZ and SE6:2.2/1.1) showed no statistical differences between each other, though they were marginally significant from the other media used. No statistical significance was observed between the other media used for this year of initiations (Table 2.15).

The higher average initiation frequencies experienced during the 1997-98 collections were reflected in the break-up of the treatment averages across the collection dates (refer to Figure 2.12). All media performed well over the entire collection period, indicating that during the peak period of initiation, the right stage of zygotic embryo development dictated the level of frequencies, whilst later on the media may have had more influence. Significance was found between the initiation frequencies of the 22nd December collection and most of the other collections. This was also the case for media used within the same collection. For example, the NZ medium used on 22nd December (39.48%) was found to be significantly higher than the DCR- medium used for the same collection (27.63%) with a t-probability of pairwise difference of 0.016. Other examples are shown in Table 2.17 which show that the media used for a particular collection period, situated in the far left column, were found to be significantly higher when compared with the media (located in the middle column).

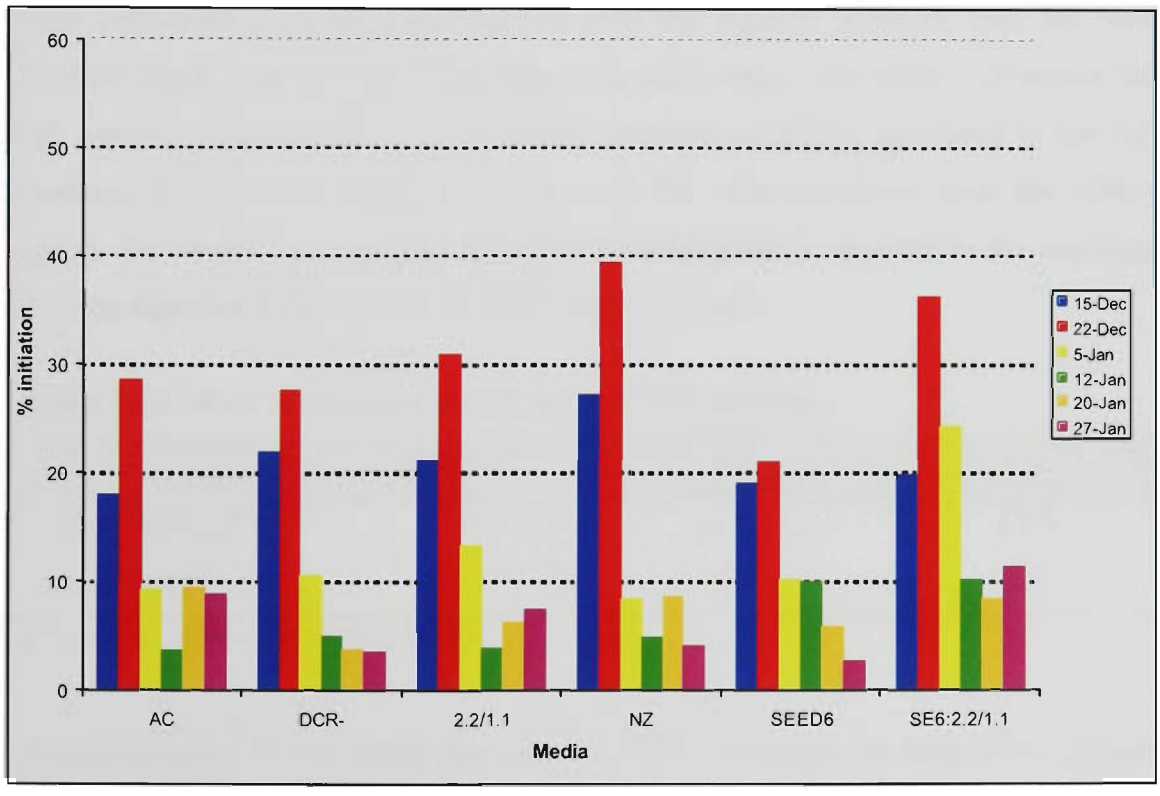


Figure 2.12 Effect of media on average initiation frequencies at each cone collection during 1997-98

Table 2.17 Examples of statistical significance between different media treatments from the 1997-98 collections

Treatment	Date	%		Treatment	Date	%	t-prob. value
NZ	22nd Dec	39.48	Significantly	DCR-	15th Dec	22.07	0.001
NZ	22nd Dec	39.48	different	SEED6	15th Dec	19.0	0.000
AC	22nd Dec	28.52	to	AC	15th Dec	18.11	0.002
SE6:2.2/1.1	22nd Dec	36.20		SEED6	22nd Dec	21.10	0.001
SE6:2.2/1.1	22nd Dec	36.20		2.2/1.1	20th Jan	6.41	0.000
NZ	22nd Dec	39.48		AC	20th Jan	9.50	0.000

The media effect on Albury and New Norfolk explants

The initiation of embryogenic tissue varied with the media used over the course of the cone collections. The initiation response from the explants collected from the New Norfolk region was significant between each media type. As Table 2.18 shows the NZ medium produced the greatest overall response of 22.8% compared to the AC medium, at 17.7% and DCR- medium, at 12.7%. For the clones from the Albury region the media type had less influence on the initiation frequencies for explants, with no significant differences found between treatments.

Table 2.18 Effect of media on overall initiation frequencies

	DCR-	AC	NZ
New Norfolk[a]	12.7	17.7	22.8
Albury[b]	5.1	4.6	7.1

a: p-value <0.001; **b**:p-value 0.453

The influence of the collection date and hence the developmental stage of the zygotic embryo and the media used for embryogenic tissue initiation can be observed in Figures 2.13 and 2.14. As previously mentioned the media did not significantly affect the initiation frequency in explants from the Albury region. It can be observed that the combination of collection date 19th December, with the NZ medium produced an initiation frequency of 19.4%. This was a higher response compared to the other media but it was not significantly different (t-probabilities of pairwise differences ranged from 0.366 to 0.991, showing no significant differences between the media

and the collection dates). Media and collection date influences on initiation frequencies from the New Norfolk region showed no significant differences between most of the media used. However, significant differences were observed between the NZ medium (45.2%) and the other media used on the 8th January collection (t-probabilities of pairwise difference of 0.003 for DCR- and 0.001 for AC). Differences can also be observed with this media/collection date combination compared with other combinations of collection dates and media, for example NZ media used for the 24th December collection (27.3%) produced a t-probability of pairwise difference of 0.001. These differences are more likely due to collection date differences and hence zygotic embryo developmental stages, rather than the media used.

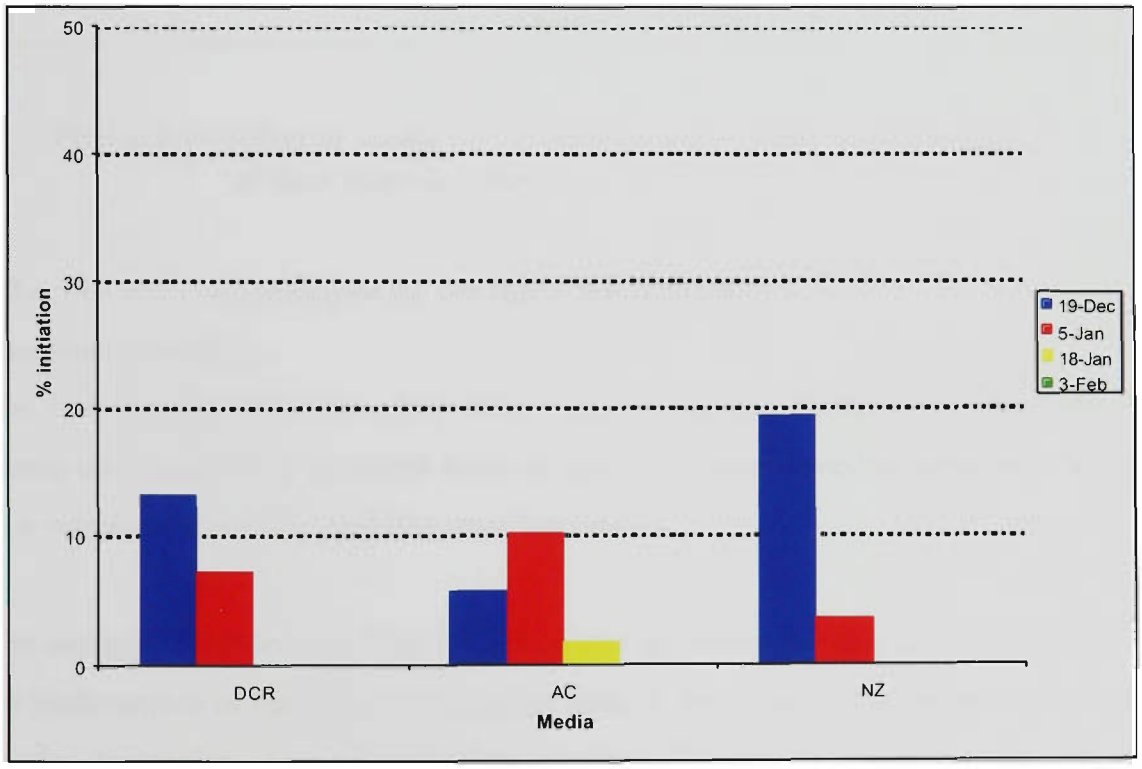


Figure 2.13 Effect of media on the embryogenic initiation frequencies of Albury collections

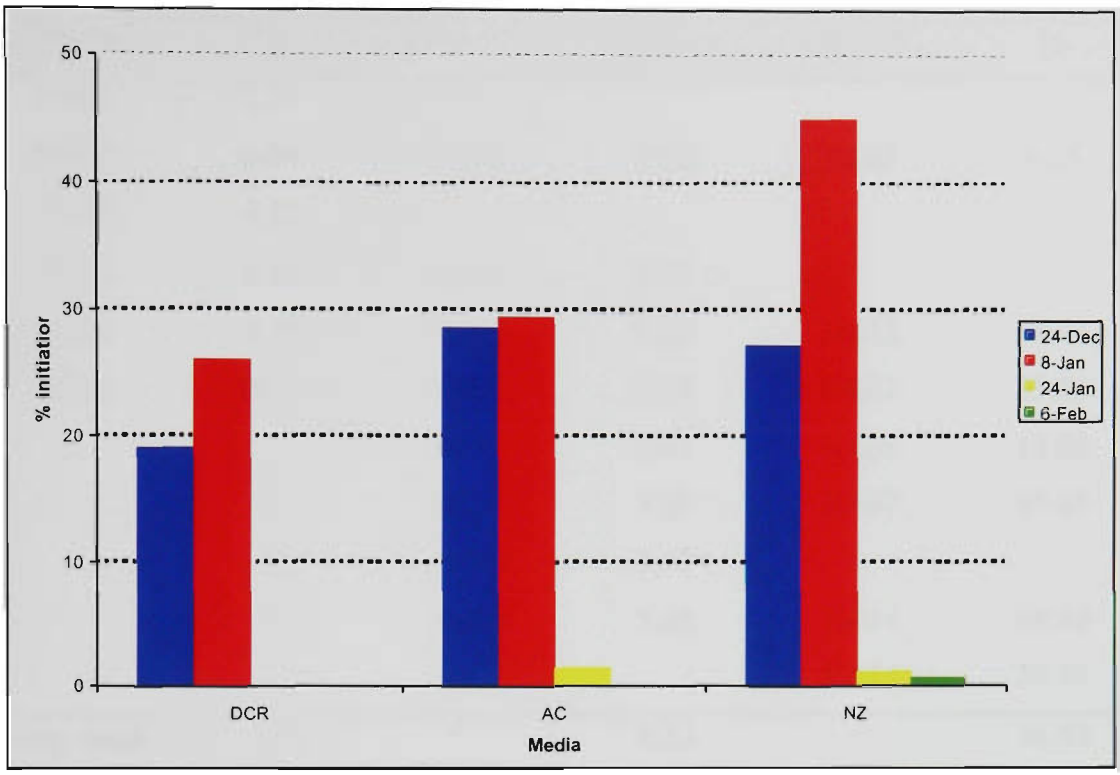


Figure 2.14 Effect of media on the embryogenic initiation frequencies of New Norfolk collections

2.3.4 The effect of genotype on the initiation frequencies

Maryvale genotypes

The clones selected for this study have a diverse genetic background. As Table 2.3 shows the clones were collected from an open pollinated breeding arboretum as well as a variety of controlled and uncontrolled crosses.

The average initiation frequency of each clone is shown in Table 2.19. Variations in the performance of the clones occurred in each of the collection periods, however due to the incomplete t-probabilities of pairwise differences generated, an accurate measure of significance between the clones could not be conducted. Clones that were used in two or three yearly collections also showed variations in initiation frequencies over the three years (refer to Table 2.19). General observations show that in 1995-96, the average initiation rates varied from 1.57% (38001) to 16.71% (38031). In 1996-97 the rates varied from 1.62% (38012) to 9.07% (38031) and in 1997-98 they varied from 9.25% (38002) to 23.26% (38031).

Table 2.19 Overall genotypic variations in the average initiation frequencies

1995-96[a]	%	1996-97[b]	%	1997-98[c]	%
38001	1.57				
38002	6.04	38002	4.50	38002	9.25
38010	4.52				
38011	6.16	38011	2.57		
38012	8.28	38012	1.62	38012	18.03
38031	16.71	38031	9.07	38031	23.26
		36055	5.61	36055	13.66
		36057	7.39	36057	10.85
		36013	1.99		
		36014	3.65	36014	18.63
				35114	10.46
Avg. total	**7.13**		**4.33**		**14.03**

a,b,c: p-value <0.001 (incomplete pairwise differences did not enable an accurate measurement of significance between clones.)

The influence of the collection date can also be taken into account with the initiation performance of the clones. As Figures 2.15, 2.16 and 2.17 show differences were evident amongst the collections. In 1995-96 the peak collection period around 8th January 1996 showed the best performance for initiation rates with the clones (refer to Figure 2.17). During this period the initiation frequencies varied from 2.61% (38001) to 48.63% (38031). Statistical significance was also observed between a variety of clones at each collection date. For example, 38031 at 8th January collection (48.63%) and 38002 at the same collection date (14.7%) showed a t-probability value of 0.000. Whilst the same t-probability value (0.000) was also observed with 38010 at the same collection (13.76%). Other examples are shown in Table 2.20. During the 1995-96 collections, clone 38031 was undoubtedly the best performing clone.

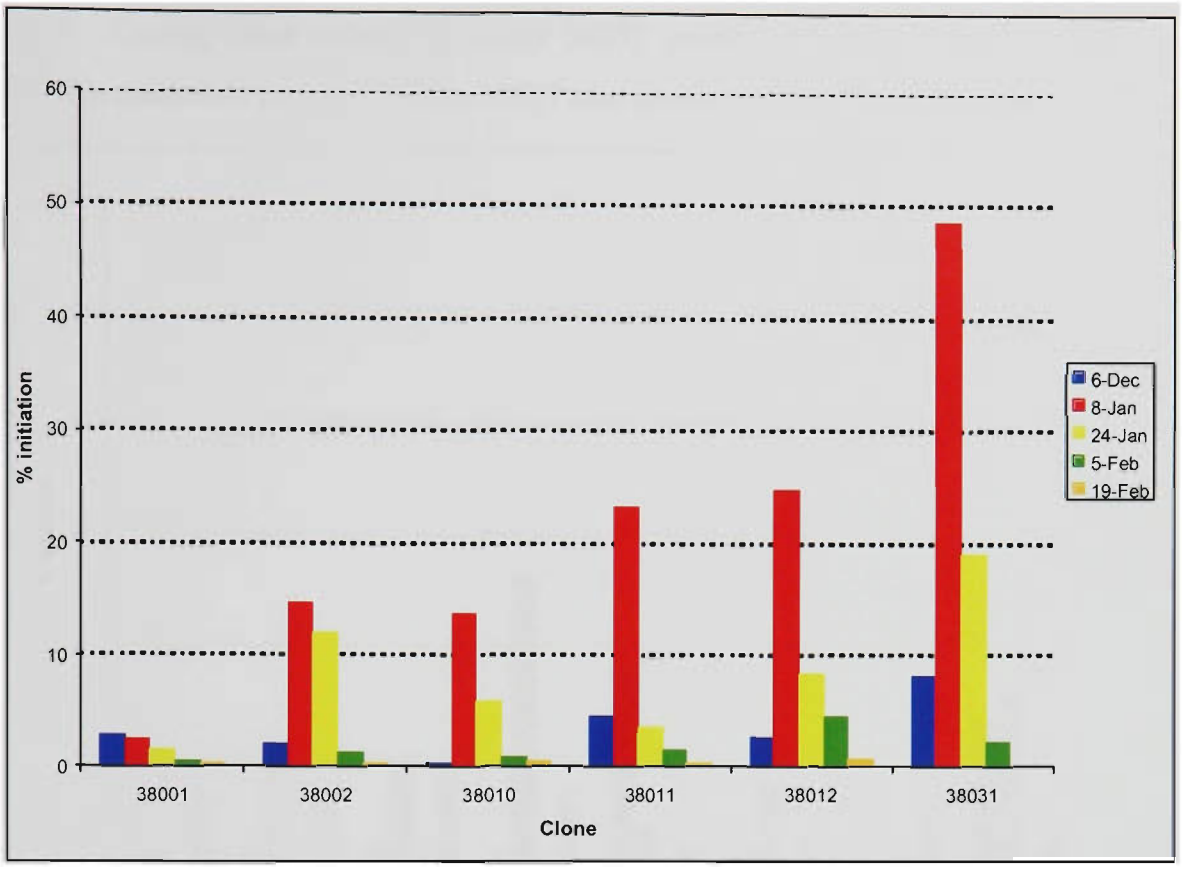


Figure 2.15 Average initiation frequencies of each family at cone collections conducted during 1995-96

Table 2.20 Examples of statistical significance between clones and collection dates during 1995-96

Clone	Date	(%)		Clone	Date	(%)	t-prob. Value
38031	8th Jan	48.63	Significantly	38010	24th Jan	6.1	0.000
38011	8th Jan	23.6	different	38010	8th Jan	13.1	0.010
38010	8th Jan	13.1	to	38012	5th Feb	4.5	0.000
38031	24th Jan	18		38010	24th Jan	6.1	0.002
38012	8th Jan	24.78		38012	5th Feb	4.5	0.000

The unfavourable initiation conditions during 1996-97 influenced the initiation frequencies of the clones at each collection date with lower frequencies compared to 1995-96 (refer to Figure 2.16). The peak initiation period around the 6th January collection showed the influence of the developmental stage of the embryo. During this period 36057 and 38031 performed well with 26.21% and 15.67% initiation frequencies, respectively. Clone 36057 (6th January) showed significant differences between a few clones/collection dates. These included 36055 (6th January) with a

pairwise difference of 0.001and 38031 (16th December) with a pairwise difference of 0.031. During these collections clone 38031 continued to show good consistency over the collection periods. Clone 36057 also produced good initiation rates.

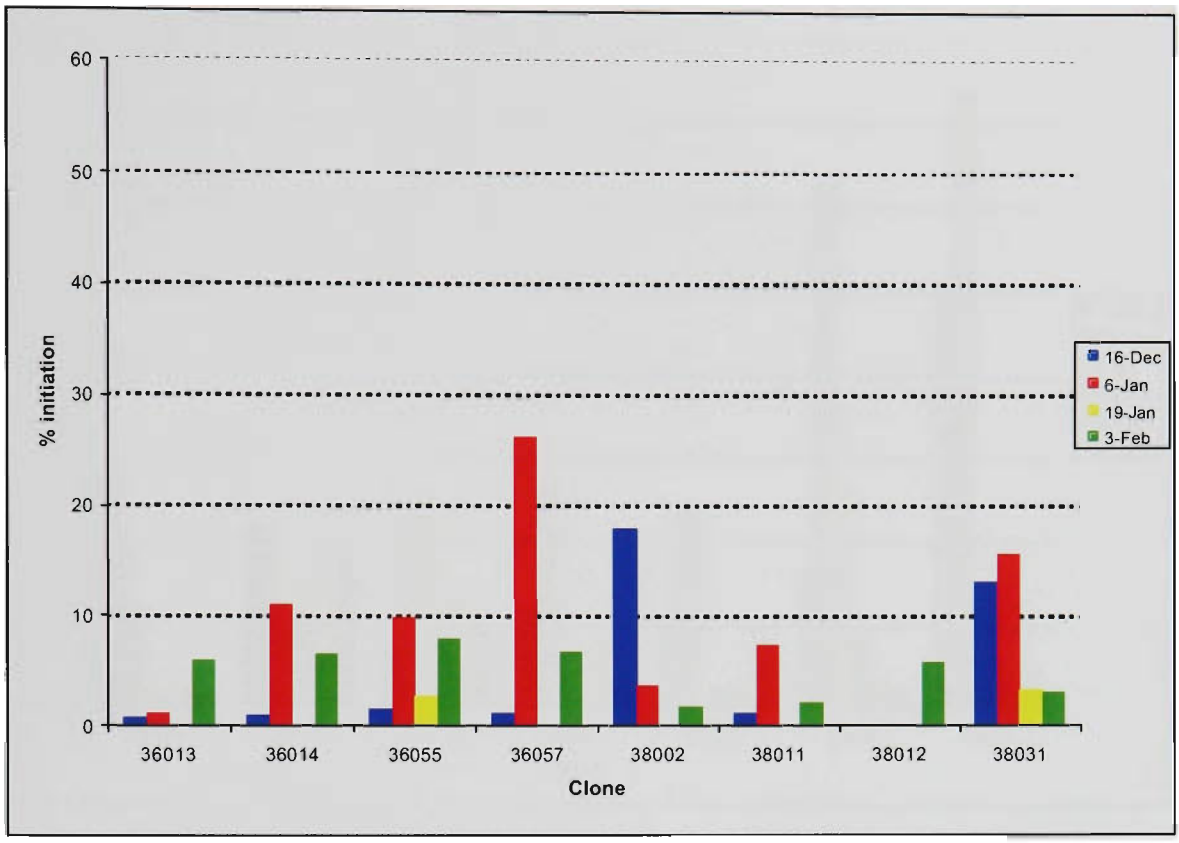


Figure 2.16 Average initiation frequencies of each family at cone collections conducted during 1996-97

During the initiation experiments conducted in 1997-98 initiation frequencies were much higher than for the previous two collection years (as seen in Table 2.19). As can be seen in Figure 2.17, the peak period of mid December to early January clearly showed the highest frequencies of embryogenic tissue initiation. Again this was largely due to the most responsive stage of embryo development being evident during this time (refer to Table 2.11). Clones 38031 and 38012 produced the highest initiation frequencies during the 22nd December collection with 73.87% and 63.86%, respectively. Clone 38031 also produced the highest initiation frequency during the 15th December collection with 49.83% (refer to Figure 2.17). Consistently good initiation frequencies of above 20% were also observed in several clones (Figure 2.17). Statistical significance was found between a large number of initiation frequencies. Several examples are shown in Table 2.21. These examples show that the clones located on the far-left column are significantly different compared to the clones shown in the middle column. For example, clone 38031 from 22nd December collection produced an average initiation frequency of 73.87%. This value was

significantly higher than the frequency produced from clone 38002, 22nd December collection, which was 24.18%.

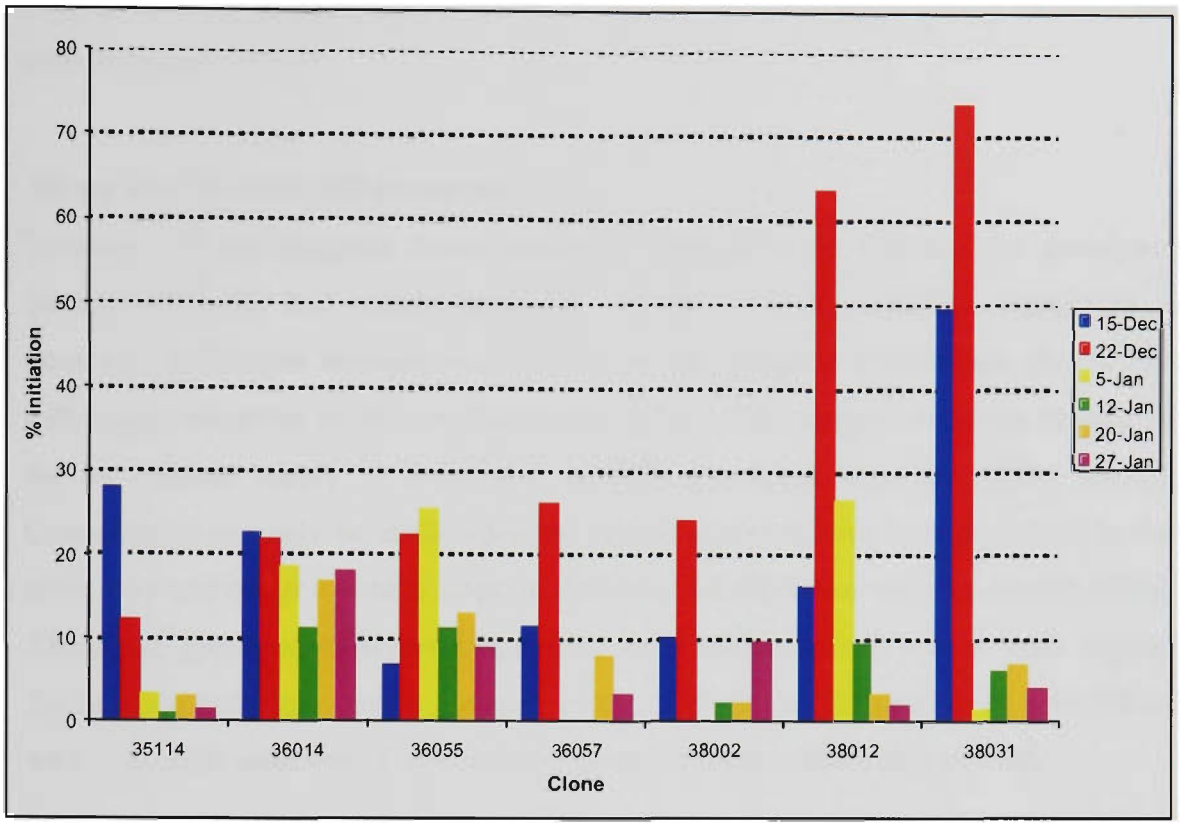


Figure 2.17 Average initiation frequencies of each family at cone collections conducted during 1997-98

Table 2.21 Examples of statistical significance between clones and collection dates during 1997-98

Clone	Date	%		Clone	Date	%	t-prob. Value
38031	22nd Dec	73.87	Significantly	38002	22nd Dec	24.18	0.000
38031	22nd Dec	73.87	different	36014	22nd Dec	21.99	0.000
35114	15th Dec	28.05	to	35114	22nd Dec	12.37	0.000
36055	5th Jan	25.53		36014	12th Jan	11.27	0.001
38012	5th Jan	26.79		36057	20th Jan	8.08	0.003

The results clearly showed how genotypic differences greatly influence the initiation rates of embryogenic tissue. The influence of the stage of embryo development was also seen in the results in Figures 2.15, 2.16 and 2.17. Lower performing clones decreased the overall averages of the initiation frequencies, however, these results varied from year to year (as can be seen in Table 2.19). Clone 38031 produced the

highest initiation rates throughout most of the initiation experiments as well as consistent rates through the collections in the individual years. Other clones such as 38012, 36014 and 36055 also showed consistent initiation rates during 1997-98 (as seen in Figure 2.17).

Albury and New Norfolk genotypes

Variations in embryogenic tissue initiation were observed between the genotypes (clones) from the two regions analyzed. It was difficult to make comparisons of genotypic influences between each region on the initiation frequencies, due to the differences observed in the developmental stages of the zygotic embryos as well as the inconsistent supply of cones and the right genotypes from the Albury region. Comparisons can only be made when the zygotic embryos have been pretreated in the same way and are at a similar stage of development (Hakman and von Arnold 1985). Therefore, genotype initiation frequencies were analyzed only within each region. To be more comprehensive a greater number of clones would need to be sampled as well as a wider selection of clones (superior and average performing clones).

The average initiation frequencies for the clones in each region are shown in Table 2.22. No significant differences were observed among the Albury clones' ability to produce embryogenic tissue, however, clones from New Norfolk showed some significant differences. Clones 1 and 3 were similar in initiation frequencies (23.2 and 21.5% respectively), but both were significantly different from clone 4 (9.4%).

Table 2.22 Overall genotypic variation in the initiation frequencies for each region

New Norfolk clones	Initiation frequencies[a] (%)	Albury clones	Initiation frequencies[b] (%)
3	21.5[b]	33 x 31	3.0[a]
4	9.4[a]	9 x 31	10.9[a]
1	23.2[b]	11 x 70*	7.3[a]
		11 x 35*	0.5[a]
Average total	**17.97**		**5.6**

*Clone 11 x 70 was only supplied for the first collection. All subsequent collections used clone 11 x 35.

a,b: Numbers with same letters in the same column are not significantly different.

The performance of the clones at each collection date can be observed in Figures 2.18 and 2.19. No significant differences could be found between the clones across the collection dates for the Albury region (p =0.753). In the New Norfolk region the clones performed well throughout the duration of the collections (as Table 2.22 shows). Clone 3 showed the highest initiation frequency from the 8th January collection with 46.5% initiation of embryogenic tissue. This frequency was significant compared to most of the other collections and clones including Clone 4, 8th January collection (t-prob. pairwise difference, 0.001) and Clone 1, 24th December collection (t-prob. pairwise difference 0.041) (refer to Figure 2.19).

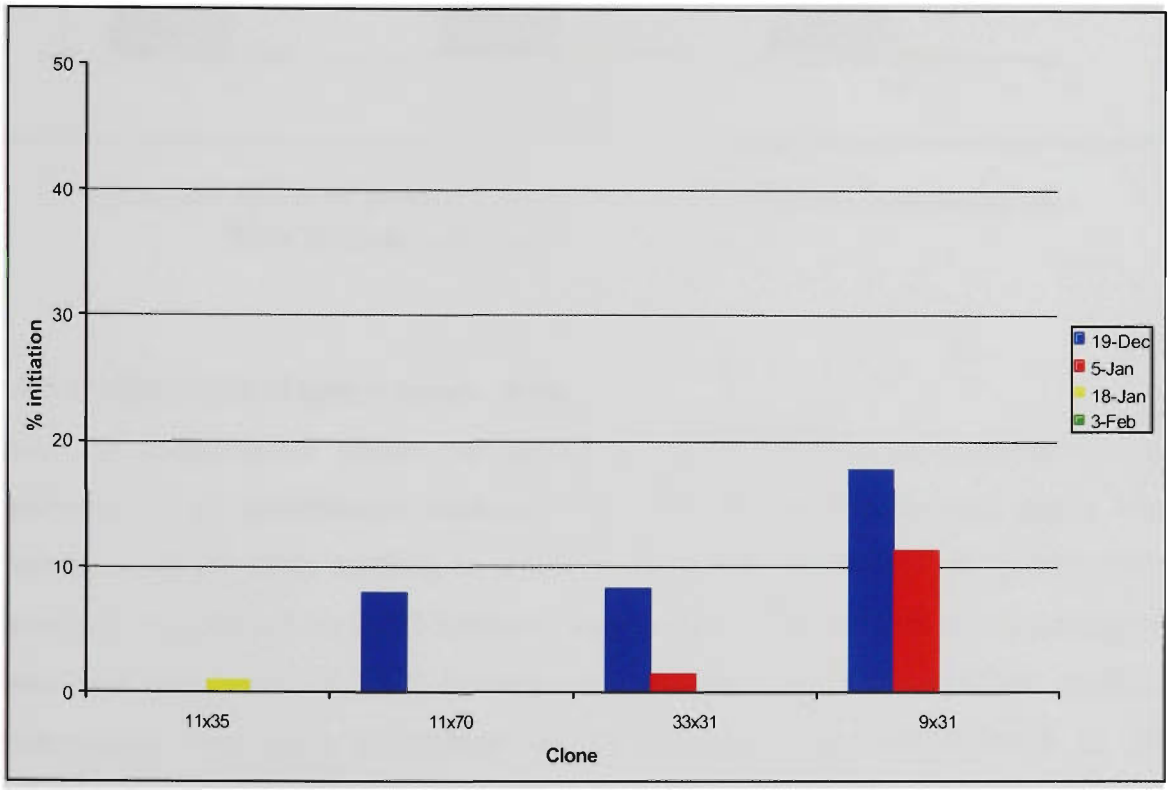


Figure 2.18 Effect of genotype on the embryogenic initiation frequencies of Albury collections

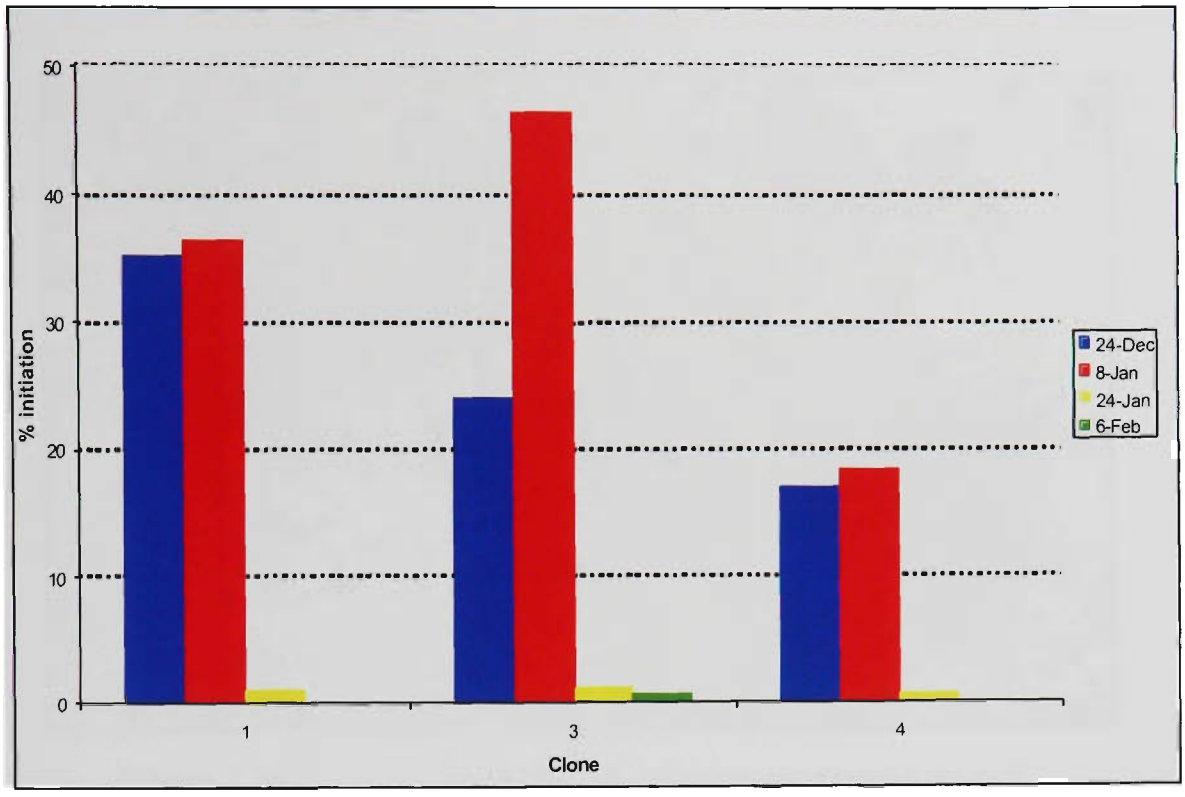


Figure 2.19 Effect of genotype on embryogenic initiation frequencies of New Norfolk collections

2.3.5 Maintenance of embryogenic tissue

Once the embryogenic tissue had grown to about 8-10mm in diameter it was transferred to a maintenance medium. In 1995-96 the embryogenic tissue was maintained on the same medium as it was initiated on. In 1996-97 the embryogenic tissue was maintained on DCR hormone-free medium. In 1997-98 the embryogenic tissue was maintained on DCR hormone-free medium or NZ maintenance medium. Embryogenic cell lines maintained on DCR medium were transferred to NZ maintenance medium after one month, due to the better quality of tissue that was produced when the cell lines were placed on this medium.

Several tissue types were produced. Some tissue, typically cream to yellow in appearance and quite friable, proved to be non-embryogenic when stained with acetocarmine. Cells were small, circular or sausage shaped and only the nucleus stained intensely, rather than the whole cell. The embryogenic tissue could be easily distinguished from the non-embryogenic tissue. It was more translucent and mucilaginous in appearance and when stained with either acetocarmine or aceto-orcein showed small cells that were intensely red, sometimes in small clusters or subtended by long, highly vacuolated cells (refer to Figures 2.4 and 2.20).

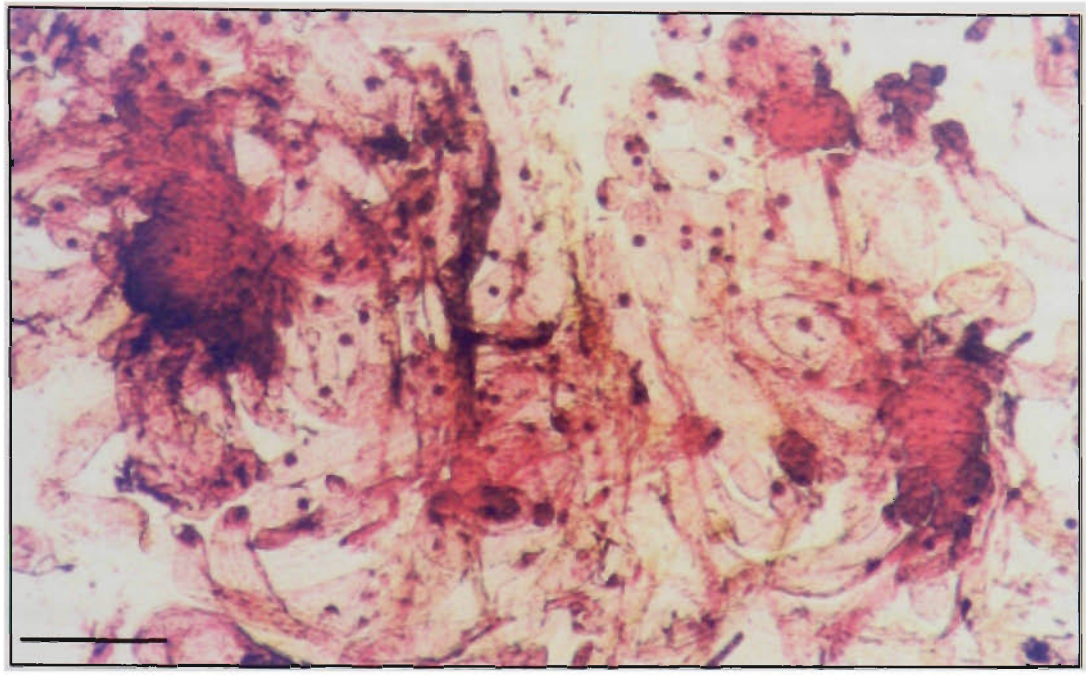


Figure 2.20 Histochemically stained embryogenic tissue from cell line 58 (bar = 0.3mm)

From the 1995-96 Maryvale initiation experiments 309 cell lines were isolated and placed onto maintenance medium. Embryogenic tissue remained in a 'primitive' state and with little further development. The embryogenic tissue was regularly subcultured onto fresh medium and most tissue was maintained for six months. During this time tissue browned and died or failed to proliferate. Variations in the embryogenic cell structures of the isolated cell lines were evident. Embryogenic cell lines were classified by the system developed by Maddocks *et al.* (1995). This staining system is based on the classification of the morphology of the embryogenic structures. Embryogenic cell lines with structures showing embryo initials with some degree of organization, with or without suspensors, were classified as Grade 1 or 2 (refer to Figure 2.4 and 2.20). Whilst cell lines with embryonic structures with no defined organization were classified as Grade 3 or 4 (refer to Figure 2.5). The majority of the embryogenic cell lines were classified as either grade 3 or 4, therefore were thought to have little potential for further maturation. As a result these grades of tissue were discarded. Cell lines classified as grade 1 or 2 were maintained for maturation experiments.

From the 1996-97 Maryvale initiation experiments 231 cell lines were isolated and maintained on DCR hormone-free medium. Embryogenic tissue continued to proliferate on the initiation media and was re-isolated several times. Re-isolation occurred once from 77 initial cell lines, twice from 43 cell lines, three times from 17 cell lines and four times from 5 cell lines. For example, cell line 32, which was from clone 38031, initiated on activated charcoal medium from the 16th December collection, had embryogenic tissue removed four times from the original initiation plate. Cell line 44, which was clone 38002, initiated on 6% seed extract medium from the 16th December collection, had embryogenic tissue removed three times.

Of the 231 cell lines, 61 developed mature somatic embryos that were usually abnormal in shape (thin and long) and either remained white or pale green. Hence, precocious germination occurred. Some of the somatic embryos were isolated and placed through the pre-germination experiments (refer to section 3.3.6). Figure 2.21 shows the maintenance of established embryogenic cell lines. Some of the tissue continued the maturation of the somatic embryos. Embryogenic cell lines were maintained for up to eight months. At that stage a lot of the tissue (223 cell lines) had started to discolour and the tissue had lost its embryogenic capacity, which was determined by staining. However, throughout the time of maintenance, embryogenic cell lines were utilized for maturation experiments.

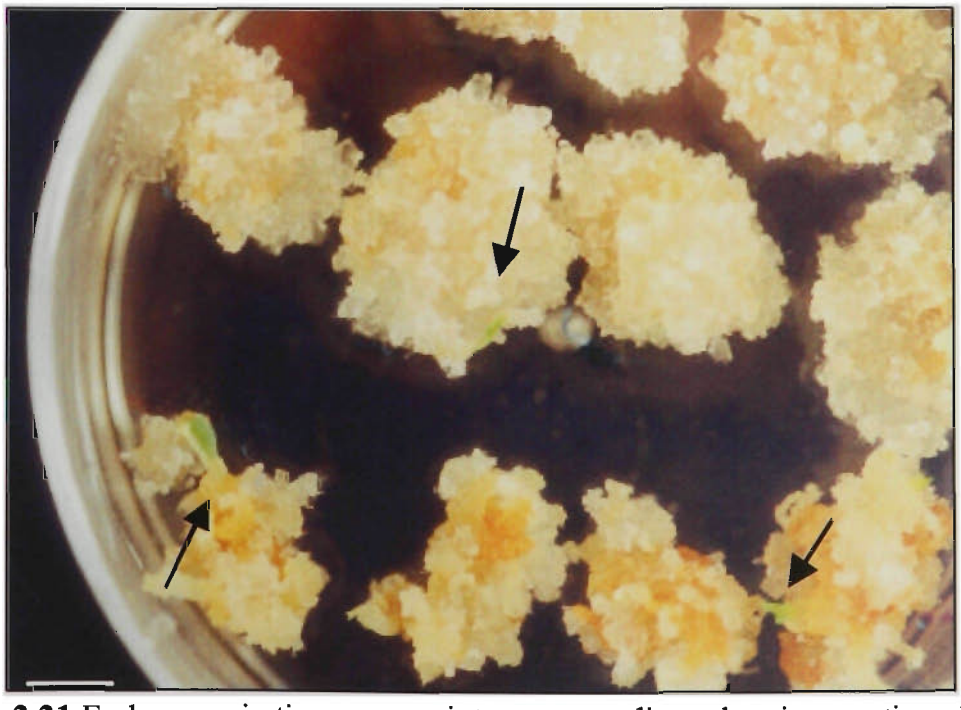


Figure 2.21 Embryogenic tissue on maintenance medium showing continued maturation of somatic embryos (as shown by the arrows) (bar = 0.5cm)

Over several months 192 embryogenic cell lines were isolated from the 1997-98 Maryvale initiation experiments (refer to Appendix 1). The colour of the embryogenic tissue varied from white, crystalline to cream, light orange and quite mucilaginous. When stained with aceto- orcein and classified by the Maddocks *et al.* (1995) method, about 54 cell lines were discarded because of poor to no organization of embryonic structures. Cell lines that were discarded corresponded to tissue that was brown and very mucilaginous in colour, normally with a poor proliferation rate. During this time, some cell lines with a high level of embryonic organization continued to produce early to late stage somatic embryos, some of which were transferred to a pre-germination treatment. All the remaining embryogenic cell lines were utilized for maturation experiments.

Of the 296 extrusions (from 1647 explants) from the New Norfolk initiation experiments and the 38 extrusions (from 675 explants) from the Albury initiation experiments 45 and 17 embryogenic cell lines were established, respectively (refer to Appendix 2). The texture and appearance of the embryogenic tissue was the same as those observed from the Maryvale initiations. Embryogenic cell lines were subcultured, stained, classified and discarded in a similar fashion to the Maryvale cell lines.

2.4 DISCUSSION

Initiation of embryogenic tissue

Embryogenic tissue from *Pinus radiata* has been successfully initiated in the experiments conducted in this study. Over the three-year period, overall average initiation frequencies from the Maryvale region varied from 4.33% to 14.03%. When the explant was at its most responsive, initiation frequencies were consistently over 20% and at one stage as high as 73.87% (in 1997-98 collections) during this study. The initiation experiments conducted from material from New Norfolk and Albury showed an overall average frequency of 17.97% and 5.6% respectively. The embryogenic initiation frequencies obtained for *Pinus radiata* in this study were in agreement with other initiation experiments conducted with *Pinus radiata.* Maddocks *et al.* (1995) obtained overall initiation frequencies of 5.2% and 7.2% in two capture periods. They also found that the initiations varied during the 'window of competence' from 14% to 40%. In studies conducted by Smith (1994) it was surmised that if explants were placed onto the appropriate medium (the New Zealand patented medium) when the explants were at their most responsive, frequencies of up to 100% could be achieved. To date, no results have been published that acknowledge the achievement of these levels. Obtaining 100% embryogenic tissue initiation is a possibility, but this would have to be further investigated. By using various cone collection timings, such as collections conducted during the mornings or evenings and also carrying out collections from different parts of the tree, initiation rates could be further analyzed. The best initiation frequency that was obtained in this study for the same medium and at the most responsive explant stage was 76.7% using clone 38012.

The capacity for *Pinus* species to produce embryogenic tissue has been considered to be quite low, typically less than 3% as Becwar *et al.* (1988) and Fowke *et al.* (1995) suggested. Though Li *et al.* (1998) observed a slightly higher average of 10%. In *Picea* species, average initiation frequencies have been relatively high, from 40% to 75% (Becwar *et al.* 1988; Fowke *et al.* 1995; Misra 1994; Li and Huang 1996; Lelu *et al.* 1999). Embryogenic tissue initiation frequencies have varied amongst *Pinus* species as Table 2.22 shows.

Table 2.23 Initiation frequencies of *Pinus* species

***Pinus* species**	**Initiation frequency**	**Reference**
Pinus caribaea	0.32%	Lainé & David 1990
Pinus koraiensis	5%	Bozhkov *et al.* 1997
Pinus patula	2.6%, 0.99%	Jones *et al.* 1993; Jones & van Staden 1995
Pinus pinaster	6.8%	Lelu *et al.* 1999
Pinus radiata	5.9%, 5.2-7.2%	Smith *et al.* 1994; Maddocks *et al.* 1995
Pinus serotina	4.4%	Becwar *et al.* 1988
Pinus strobus	36%, 3.6%,14%	Finer *et al.* 1989; Klimaszewska & Smith 1997; Garin *et al.* 1998
Pinus sylvestris	0.2%-0.9%, 1.1%, 1.5%	Keinonen-Mettälä *et al.* 1996; Häggman *et al.* 1999; Lelu *et al.* 1999
Pinus taeda	1.3%, 7%, 0.96-1.2%, 9%	Gupta & Durzan 1987; Handley et al. 1994; Li & Huang 1996; Li *et al.* 1998

The origin of somatic embryogenesis from the explant has continued to be of some controversy. Most studies in conifer somatic embryogenesis have suggested that embryogenic tissue resulted from the continuation of the cleavage polyembryogenesis process (Jones *et al.* 1993; Fowke *et al.* 1995; Lelu *et al.* 1999). Gupta and Durzan (1987) suggested this theory for *Pinus taeda*, however, for the same species Becwar *et al.* (1991) observed the development of embryogenic tissue prior to somatic embryo formation. Filonova *et al.* (2000) suggested that the origin of embryogenic tissue might be due to three factors. These include the cleavage polyembryogenesis multiplication of somatic embryos as suggested earlier, the asymmetrical division of a single cell to form somatic embryos and the direct formation of somatic embryos from meristematic cells within suspensor-like cells (Filonova *et al.* 2000). Tautorus *et al.* (1991) also suggested these processes with a more detailed explanation of them. In this study no detailed analyses of the origin of embryogenic tissue was conducted, but through observations of early developmental stage embryos the theory of the continuation of the cleavage polyembryogenesis seems to be the most plausible.

Choice of explant for initiation of embryogenic tissue

The development of the zygotic embryo plays an important part in determining the success of embryogenic tissue initiation (Smith 1994; Chandler and Young 1995; Garin *et al.* 1998). Peak embryogenic tissue initiation performance usually occurs at a stage in the natural development of the embryo. During this stage the embryos are actively multiplying and developing with embryos having from 64 to 100 cells. This growth stage coincides with the process of cleavage polyembryogenesis, as multiple embryos are produced. The dominant embryo may or may not as yet be obvious (Smith 1994; Keinonen-Mettälä 1996). The term used for this stage varies between researchers, with Stage 1 embryos (Smith 1994), and early globular stage (Chandler and Young 1995) two of the terms used. However, they all agree that the peak period for embryogenic tissue initiation coincides with cleavage polyembryogenesis. Chandler and Young (1995) observed that embryos, which were collected at the globular and early cotyledonary stage (stages 2 and 3 from Smith (1994)) also produced embryogenic tissue, with the highest mean number of embryo initials around the early globular to globular stage. The stages discussed are also referred to collectively as precotyledonary stages due to the absence of cotyledonary primordia (Garin *et al.* 1998).

In *Picea* species the post-cotyledonary embryo is considered the optimum explant for high embryogenic potential, whilst in *Pinus* species, the pre-cotyledonary embryo has been the most responsive stage of development (Fowke *et al.* 1995). The presence of this stage limits the period for initiation to a few weeks each year. This observation of stage limitation is in agreement with many researchers of pine somatic embryogenesis (Becwar *et al.* 1991; Klimaszewska and Smith 1997; Garin *et al.* 1998). Jones *et al.* (1995) noted that the number of embryos undergoing cleavage began to decline as the presence of cotyledonary primordia occurred. It was also noted with studies conducted on *Pinus strobus* (Garin *et al.* 1998). The initiation frequencies have also been noted to decrease as storage proteins accumulate in the embryo (Roberts *et al.* 1989; Tautorus *et al.* 1991). Mature conifer seeds accumulate storage protein reserves to aid in germination and seedling growth, a normal feature of the maturing embryo (Tautorus *et al.* 1991; Misra 1994).

Several studies on *Pinus* somatic embryogenesis have determined the time of fertilization to aid in the selection of the correct stage of zygotic embryo development.

However, this period is more difficult to establish than determining the stage of the zygotic embryo. This is due to the variations in development that can arise among different clones and during different weather conditions around the time of fertilization and seed maturation (Dogra 1978; Keinonen-Mettälä *et al.* 1996; Lelu *et al.* 1999). Most researchers place a time frame of a certain amount of weeks after fertilization of the most responsive stage of embryo development and hence peak embryogenic tissue initiation. For establishment of pine embryogenic cultures, Becwar *et al.* (1991) suggested 3-5 weeks, for *Pinus radiata* it is 4-8 weeks after fertilization (Chandler *et al.* 1989), for *Pinus patula* it is 3-5 weeks after fertilization (Jones *et al.* 1993) and for *Pinus sylvestris,* 2 weeks after fertilization (Lelu *et al.* 1999). Observations from this study found that the time scale for optimal *Pinus radiata* embryogenic tissue establishment varied from 3-5 weeks over the three years of collections. These results were based on observations of zygotic embryo development (refer to Table 2.10).

The 'window of competence' for *Pinus radiata* was found to vary between the regions where cones were collected for this study. The peak time usually occurs over two weeks around early January (in the southeastern region of Australia). However, this was only observed in the New Norfolk. The zygotic embryos from Albury were found to mature a few weeks earlier. The differences in the zygotic embryo development between the regions may be quite beneficial. By using the same clone grown in different locations, the peak period may be extended to a greater length of time. Immature cones could be collected from the Albury region when the zygotic embryos are within the competent period, then collected from the New Norfolk region (or any other region where the same clones are grown) when the zygotic embryos are at the right stage of development. This could possibly extend the peak period by two to three weeks (therefore about five weeks in total). The zygotic embryos from Maryvale followed a similar time frame as the New Norfolk explants even though different clones were analyzed.

Variations of growing location for source material

The regions where cone collections were conducted experienced different weather conditions. Climatic conditions have been known to influence the process of fertilization and the maturation of the zygotic embryo (Dogra 1978; Keinonen-Mettälä 1996). This influence can be clearly seen in the differences observed in the

developmental stages of the zygotic embryos from each of the regions from which material was sourced. The zygotic embryos from New Norfolk were from a region that had higher rainfall and lower temperatures compared to the climatic conditions in the Albury region. Favourable climatic conditions for zygotic embryo development within the Albury region were found to have occurred earlier than for the New Norfolk region, as shown by the maturation of the zygotic embryo. Keinonen-Mettälä *et al.* (1996) also found that *Pinus sylvestris* seed development corresponded not only to the time of the year, but was also influenced by the temperature during summer, when development occurred. This may be due to water availability requirements for the physiological and biochemical processes that occur during fertilization and seed development. Jones and van Staden (1995) provided circumstantial evidence of the effect of environmental factors such as rainfall and temperature on induction frequencies, especially with reduced initiations during the summer period. As stressful conditions, such as high temperatures, may influence the initiation rates of embryogenic tissue, collections of pinecones during the cooler hours of the day (i.e., early morning or late evening) would be more feasible.

Choice of medium for initiation of embryogenic tissue

Culture medium can also significantly affect the success of embryogenic tissue initiation. This is largely due to different cell lines and clones requiring different nutritional conditions (Tautorus *et al.* 1991; Häggman *et al.* 1999). Many basic and modified versions of initiation media have been used in an attempt to achieve high initiation frequencies. Some media have been more successful than other types and the many trials of different media have enabled researchers to develop new media compositions. One of the most common media used for embryogenic tissue initiation is DCR basal medium (Gupta and Durzan 1985). This medium, as well as modified versions, has been utilized with many *Pinus* species including *Pinus sylvestris, Pinus patula, Pinus taeda* and *Pinus strobus* (Jones *et al.* 1993; Keinonen-Mettälä *et al.* 1996; Li *et al.* 1998; Häggman *et al.* 1999). The other basal media used in the 1995-96 collections have all been used at some time to analyze the efficiency of somatic embryogenesis in pines. Li *et al.* (1998) found that LP basal medium was not effective in the initiation of embryogenic tissue in *Pinus taeda.* Modified LP and modified half strength LP were used more frequently than the original LP medium composition (Li *et al.* 1998). P21 basal medium is a modification of the P6 medium developed by Teasdale *et al.* (1986) for cell suspension experiments with *Pinus taeda*

and also tested with *Pinus radiata* cell suspension cultures. No comparisons have been made with this medium and other media until this study. Overall average initiation frequencies of embryogenic tissue using P21 were comparable with the other media used in this study (Table 2.15).

It is common to include growth regulators in the initiation medium to aid somatic embryogenesis. All *Picea* species require growth regulators such as 2,4-D and BA for the induction of somatic embryogenesis, as do some *Pinus* species (Fowke *et al.* 1995; Misra 1994). Li *et al.* (1997) found that high concentrations of plant growth regulators were not essential for initiation, though it was considered that the combination of plant growth regulators was more important than the actual concentrations. Smith (1994) found that the unique salt formula in the NZ medium allowed for the capture of embryogenic tissue without the need for plant growth regulators. Though the presence of auxins and cytokinins may on occasions enhance the tissue development, if the explant was captured at the appropriate stage of development they were not essential. This is also in agreement with Chandler and Young (1990) in the case of *Pinus radiata* and Attree *et al.* (1994) with Pond pine. During this study embryogenic tissue was initiated successfully on media without hormones however, during the 1997-98 collection, media with growth regulators (DCR-2.2/1.1 and SE6-2.2/1.1) showed consistent initiation frequencies when compared with the other media types (Table 2.15 and Figure 2.12).

Charcoal in the medium adsorbs growth regulators as well as any impurities that might otherwise affect the initiation process (Gupta *et al.* 1993). Chandler and Young (1995) found that the addition of activated charcoal to the medium provided a two to three-fold increase in responding explants. Media with activated charcoal used during the 1996-97 collections (AC medium) and the 1997-98 collections (AC and NZ media) (Table 2.15) showed comparable results with the other media types.

Mature *Pinus radiata* seed extract was used as an additive in the last two years of media experiments (refer to Tables 2.8 and 2.9). Conifer seeds have been analyzed in the past to ascertain the levels of nutrients within the seed. Teasdale *et al.* (1986) used some of this analysis to develop new media, such as P6 medium for cell suspensions of *Pinus taeda*. The inclusion of the seed extract supplemented any deficiencies in the original basal medium. The combinations of the seed extract and

plant growth regulators in the media experiments in 1997-98 were to enhance the initiation frequencies. Judging by the results in Table 2.15 and Figure 2.12, this may have been the case. The nutrient medium for embryogenic tissue initiation has frequently included nitrogen additives such as glutamine (Hakman *et al.* 1985; Durzan and Gupta 1987; Simola and Santanen 1990; Jones and van Staden 1995). This has been observed to stimulate callus growth especially in the presence of low levels of inorganic nitrogen (Simola and Santanen 1990). This was observed to a slight degree in the 1995-96 initiation experiments.

Jain *et al.* (1989) suggested that the culture medium affected the behaviour of the genotypes for somatic embryogenesis. They postulated that individual genotypes might exert a genetic control on somatic embryogenesis by one or more genes, which were only expressed under certain conditions. However, the genes under consideration have not been identified (Jain *et al.* 1989). The study discussed here showed that all the nutrient media tested were suitable for the initiation of embryogenic tissue. Though due to the differences between the media, as well as other factors such as developmental stage and genotype, initiation frequencies varied.

Genotypic influence on embryogenic tissue initiation frequencies

It is well known that the genotype of the explant has an influence on the embryogenic frequency (Huang *et al.* 1995; Park *et al.* 1998). Proper genotype selection is seen as a critical factor in achieving successful embryogenic tissue initiation in *Pinus* species (Jain *et al.* 1989; Becwar *et al.* 1990; Huang *et al.* 1995). Lainé and David (1990) noted that even small genetic differences between half-sib material were enough to produce large changes in embryogenic potential, which were also observed in this study. The response of different genotypes to various culture media may account for the lack of repeatability of certain experiments, as well as the differences observed in the initiation rates over the three years of initiation experiments. Handley *et al.* (1994) noted that the major limitation with Loblolly pine (*Pinus taeda*) was the widely variable initiation frequencies observed from different genetic families.

Due to the differences in the genetics and ability of genotypes to produce embryogenic tissue, it is expected that some will contribute more whilst others less to the overall yield of embryogenic tissue. Ekberg *et al.* (1993) suggested that embryogenesis *in vitro* can be significantly improved by adapting the method used to

each genotype. However, to overcome the relatively low initiation frequencies observed amongst quite a few *Pinus* species and the significant effect of the genotypes of explants it might be possible to use explants from controlled crossings between competent genotypes. Developing more specific media for the important seed families might achieve this improvement.

Maintenance of embryogenic tissue

During maintenance of embryogenic tissue, differences were observed in the somatic embryo morphology between the embryogenic cell lines. Some cell lines contained well-formed somatic embryos, similar to zygotic embryos, with polar or radial symmetry and with or without suspensor cells, but a few cell lines contained cell aggregates arranged loosely or compact, mostly without organization. The unorganized cell lines were considered low in their potential maturation performance whilst the organized cell lines were considered to have a medium to high maturation potential (Maddocks *et al.* 1995). Classification systems such as the one described by Maddocks *et al.* (1995) have been useful in rating embryogenic cell lines and their maturation potential. Jalonen and von Arnold (1991) used a similar system to classify the somatic embryo morphology in *Picea abies.* Embryogenic tissue with polar embryos (A1 type) was considered highly likely to mature and germinate. Polar somatic embryos with radial symmetry (A2 type) were likely to mature but sometimes germinated precociously, whilst tissue with less developed embryos and cell aggregates (type B) were highly likely not to mature properly (Jalonen and von Arnold 1991). The classification of somatic embryos before the maturation stage enables low quality cell lines to be discarded, therefore saving the time and effort required to maintain them.

The embryogenic cell lines isolated from the initiation media showed varying levels of embryogenic capability initially, but over time the embryogenic potential declined. A decrease such as this has also been reported in both *Picea* and *Pinus* species with rates of decline varying between species (Garin *et al.* 1998). The physiological factors causing the decline are not fully understood. The low quality of the embryogenic tissue, such as grades 3 and 4 or type B classifications, also corresponded with the colour of the tissue (Jalonen and von Arnold 1991; Maddocks *et al.* 1995). Embryogenic tissue that turned yellow or brown corresponded with the low quality classifications and usually meant a loss of embryogenic capability. This situation was

also found in *Pinus taeda* with colour being an important factor to distinguish embryogenic tissue (Li and Huang 1996). Differences in tissue types that were extruded from the zygotic embryo have been noted in many cases (Jain *et al.* 1989; Li *et al.* 1998). Fortunately the majority of the proliferating tissue was embryogenic and could be easily distinguished from non-embryogenic tissue through staining methods (Gupta and Durzan 1987; Lainé and David 1990).

The further development of somatic embryos has been observed within tissue growing on hormone-free media, such as the DCR maintenance medium. Precocious germination of the somatic embryos occurred quite frequently. This has been observed in a few cases of conifer embryogenic tissue maintenance (Lainé and David 1990). Lelu *et al.* (1999) recorded this occurrence in *Pinus sylvestris* embryogenic tissue with the observation of abnormal somatic embryo development as a result of the spontaneous maturation These somatic embryos were referred to as shooty embryos due to their abnormal appearance, which was pale green to white in colour, long and thin, and usually with fused cotyledons (Lelu *et al.* 1999). These types of mature somatic embryos, as well as the 'normal' appearing somatic embryos were also observed in this study

Summation

The significant effect of the genetic background of the explants and the initiation medium reveal that it may be possible to improve the embryogenic initiation rates. This may be done by choosing the right stage of development from controlled crosses between competent genotypes and by developing more specific media to aid the differences in nutritional requirements (Häggman *et al.* 1999). Handley *et al.* (1994) also suggested that if the process of somatic embryogenesis is to be used routinely, ways must be found to deal with the variations observed between genotypes, media and localities. Tautorus *et al.* (1991) suggested that medium optimization should be conducted for each species using several cell lines, as different genotypes within a species have different nutritional requirements.

For this study, each of the three main factors, explant maturity, genetic background and the media/component type played a role in the frequency of embryogenic tissue. Of the three, explant maturity had the greatest influence on the initiation rates. Efficiency within a breeding program would be to exploit the immature embryo at its

most favourable stage of development for embryogenic tissue initiation. Indicating that during the peak time period harvesting of immature pinecones would have to be conducted on a daily basis. This would enable a reasonable amount of embryogenic tissue to be developed, irrespective of genotype, as has been shown in this study. Even though further work is needed to fully develop the potential of somatic embryogenesis, when the individual and combined issues are optimized then *Pinus* embryogenic tissue initiations will no longer be thought of as being recalcitrant. Therefore, the technique can be accepted more readily into conifer breeding programs.

CHAPTER 3

Maturation of *Pinus radiata* somatic embryos and conversion to plants

3.1 Introduction

Embryogenic tissue initiation has been successful in a variety of conifer species including *Pinus radiata.* Embryogenic cultures of all conifer species have a similar appearance; white to translucent tissue, mucilaginous in texture and consisting of many small, undeveloped somatic embryos when stained (Jalonen and von Arnold 1991). The level of embryogenesis of cell lines varies quite significantly between genotypes, as discussed in many papers and in chapter 2. Maturation and germination of the somatic embryos in *Pinus* species has been observed to be generally low and unpredictable (Jalonen and von Arnold 1991; Misra 1994; Li and Huang 1997; Lelu *et al.* 1999). This may be due to somatic embryos not attaining the appropriate level of maturity and hence not developing further. It is also reasonable to assume that the regeneration ability varies significantly between different genotypes even under the same culture conditions (Jalonen and von Arnold 1991; Aitken-Christie 1995).

Developmental influence of somatic embryos on maturation

The level of development that the somatic embryos need to reach is critical to the success of germination. Both the embryogenic capability of the individual cell line and the maturation treatments applied, could affect the quality as well as the distribution of the embryos across the different developmental stages (immature, mature cotyledonary, germinating somatic embryos) (Tremblay and Tremblay 1991a; Li and Huang 1997). Pullman and Webb (1994) noted that only early stage embryos with morphological organization continue on to produce cotyledonary embryos, therefore, a stage-related criterion must be met. Embryos should resemble the zygotic embryo prior to germination; therefore, they should be white, opaque, bi-polar and have the characteristic bullet shape. Incomplete or abnormal germination can occur when embryos are too immature (Lelu *et al.* 1994; Smith *et al.* 1994; Smith 1997).

As Klimaszewska and Smith (1997) observed, embryo maturation involves several distinct phases, including the accumulation of storage material, the desiccation of the

embryo and the arrest of metabolic activity. *In planta* this process occurs in an environment that is nutritionally and hormonally complex - the megagametophyte. Therefore, to mimic the normal maturation process *in vitro*, the embryogenic tissue must be treated in a similar way.

The influence of Abscisic acid on maturation

Abscisic acid (ABA) has been recognised as an important hormone, with a role in early to late maturation. In the normal development of the seed ABA accumulates during mid to late stages of development preventing the embryos from germinating precociously (Attree and Fowke 1993; Misra 1994). The racemic form, (±) ABA, has been used successfully in somatic embryo maturation experiments. This form is an equal mixture of natural (+) ABA and the synthetic (-) ABA (Dunstan *et al.* 1991; Find 1997). Exogenous applications have shown that ABA regulates the course of maturation and the accumulation of storage products, such as lipids, storage proteins and triacylglycerols (von Arnold and Hakman 1988; Roberts *et al.* 1990a; Dunstan *et al.* 1991). At higher levels, ABA inhibits precocious germination within conifer somatic embryos as well as preventing cleavage polyembrony and promoting synchronous maturation of the embryos (Attree and Fowke 1993; Misra 1994; Kong and Yeung 1995). Somatic embryos exposed to low levels of ABA, or unable to utilize the available ABA, often mature abnormally, resulting in lower numbers of cotyledonary embryos, abnormal morphology and poor germinability (Kong and Yeung 1995; Gutmann *et al.* 1996). This is due to the decreasing sensitivity to ABA that embryos exhibit during the maturation process (Attree and Fowke 1993). Therefore, higher concentrations are required to prolong the maturation and prevent precocious germination. The age of the embryogenic cell lines undergoing maturation may also impact on ABA requirements needed for maturation. It has been observed that younger cell lines grow more rapidly therefore, require longer durations of ABA, compared to older cell lines that grow more slowly (von Arnold and Hakman 1988; Afele *et al.* 1992)

Influence of osmotica on maturation

Improvement in maturation frequencies and subsequent germination has been attained for somatic embryos with the use of a combination of ABA and an osmoticum (Find 1997). The addition of an osmoticum *in vitro* imposed an osmotic stress on the

somatic embryo via water stress or desiccation (Roberts *et al.* 1990b; Kong and Yeung 1995; Klimaszewska and Smith 1997). A natural drying period occurs in the later stage of seed maturation, as a preparation stage prior to germination. Imposing an osmotic stress in the medium is seen as important for further embryo development (Attree and Fowke 1993; Kong and Yeung 1995). Permeating and a non-permeating osmotica have been studied on many conifer species including *Pinus taeda* (Li and Huang 1997), *Pinus strobus* (Finer *et al.* 1989; Klimaszewska and Smith 1997), *Pinus sylvestris* (Keinonen-Mettälä *et al.* 1996), *Picea abies* (Find 1997), *Picea rubens* (Tremblay and Tremblay 1991b), *Picea glauca* (Attree *et al.* 1995; Kong and Yeung 1995) and hybrid larch (Lelu *et al.* 1994). The differences between the types of osmotica relates to the size of the compound and its ability to enter the plant cell. Permeating osmotica, such as sucrose, mannitol and other simple sugars, impose an osmotic stress on the plant cell by causing plasmolysis, but prolonged exposure often leads to osmotic recovery of the cells due to the entry of the molecules into the cell (Attree *et al.* 1991; Tremblay and Tremblay 1991b). Non- permeating osmotica, such as polyethylene glycol (PEG), stimulates a non-osmotic moisture stress at the cellular level due to the large size of the molecules, preventing passage into the cells. The effects of the non-permeating PEG are similar to water stress in drought conditions (Attree *et al.* 1991; Kong and Yeung 1995).

Another way to stimulate the effect of water stress or desiccation is through the use of high concentrations of gellan gum or Gelrite™ within the maturation medium (Klimaszewska and Smith 1997; Lelu *et al.* 1999). Gellan gum enables water availability to the somatic embryos to be limited from the maturation medium (Klimaszewska and Smith 1997). Klimaszewska and Smith (1997) noted in *Pinus strobus* that media with a high gel rigidity (0.8 to 1.0% gellan gum) supported the growth of high mature somatic embryo yields as well as high germination conversions. Lelu *et al.* (1999) found similar results when using maturation medium with gellan gum (1%) on *Pinus pinaster* and *Pinus sylvestris* somatic embryos. Smith (1994) also used high gellan gum (0.6%) in the first stage of maturation of *Pinus radiata* somatic embryos, then for the second stage a water vapour permeable film was incorporated to further limit the available moisture to the maturing somatic embryos.

The aim of the modifications to the maturation medium through the use of ABA, osmotica or gellan gum levels, is to provide an environment *in vitro* similar to the embryo's natural seed environment. By doing so the natural processes of maturation and desiccation of the embryo can be promoted in order to produce good quality somatic embryos that will undertake the final processes required for germination.

Classification of mature somatic embryos

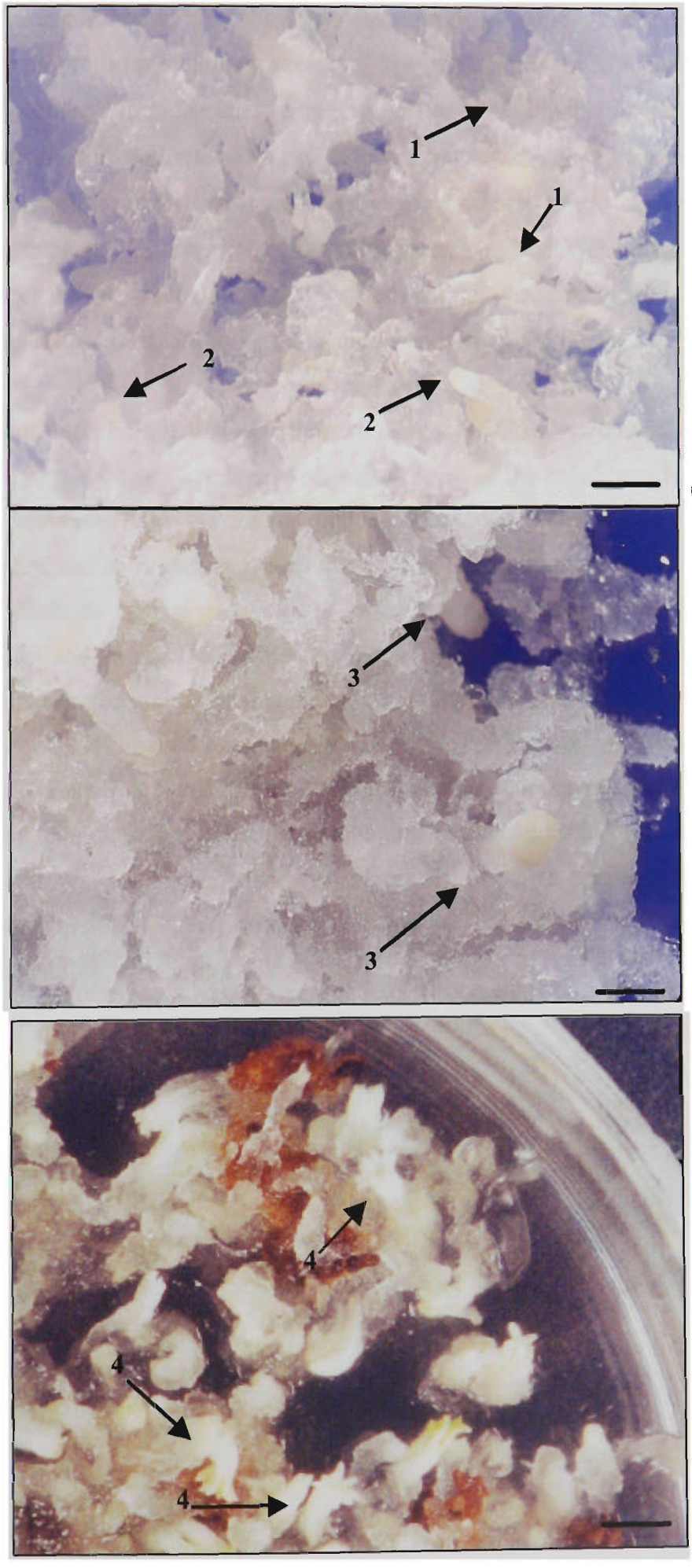
In attempting to compare somatic embryo development with zygotic embryo development there is often inconsistency in the terminology used for the various stages. The term 'proembryo' is sometimes used to describe the early stage somatic embryo however, this term applies more accurately to an early stage within cleavage polyembryony (Tautorus *et al.* 1991). A more useful means to classify the stages of somatic embryo development was developed by von Arnold and Hakman (1988). The embryo stages are classified according to their level of development (Figure 3.1). A stage 1 embryo is a somatic embryo with translucent suspensor, densely appearing embryonal apex and slightly irregular outline. A stage 2 embryo is more prominent, smooth in outline and opaque, cream to pale yellow in appearance and subtended by a suspensor. A stage 3 embryo has small cotyledons clustered around a central meristem, with or without a suspensor. The stage 3 embryo starts to resemble the zygotic embryo which is cream to pale green in color. At stage 4 the embryo has taken on the appearance of an early plantlet with distinct, partly elongated cotyledons clustered around a central meristem, with the hypocotyl slowly elongating. At this stage the plantlet has definite elongating green cotyledons and hypocotyl, with rudimentary radicle development, hence germination is imminent (von Arnold and Hakman 1988; Tautorus *et al.* 1991).

Figure 3.1 Maturation stages of somatic embryos. Arrows refer to stages of maturation development

A) **(1) Stage 1** embryos consist of a very small embryonic region subtended by an elongated suspensor. The embryo is translucent with a slight globular shape.
(2) Stage 2 embryos have thickening of the embryonic region, are slightly opaque and are globular shaped with a suspensor. (bar = 0.5mm)

B) **(3) Stage 3** embryos have an opaque, slightly elongated embryonic region with short cotyledon development. (bar = 1mm)

C) **(4) Stage 4** embryos have well formed cotyledons, slightly elongated hypocotyl with very short or absent suspensors. The bullet shape is dominant.
(bar = 0.7cm)


1
1
2
2
(A)
3
3
(B)
4
4
4
(C)

When the mature somatic embryos reach the appropriate stage of development they are transferred to a germination medium. The medium is usually hormone-free, enabling the embryo to utilise its own biochemical resources to aid germination. Over time the mature embryo germinates and develops roots, and is then transferred to soil and acclimatised in glasshouse conditions. Germination of the mature somatic embryo is indicated by the partly elongated green cotyledons and hypocotyl with rudimentary radicle development, finally resulting in the emergence of the root through the basal cells of the embryo (Tremblay and Tremblay 1991b).

The influence of a high relative humidity treatment (HRH) on germination

It has been suggested that a treatment of partial drying of the embryos at high relative humidity following ABA maturation improves germination. This stage of partial drying or desiccation is seen to reduce the level of endogenous ABA. This has been observed within the normal maturation of an embryo prior to germination (Roberts *et al.* 1990b; Li and Huang 1997). The drying may enhance germination by converting protein synthesis from a developmental to a germinative mode (Attree *et al.* 1995). Roberts *et al.* (1990b) used this technique with *Picea glauca-engelmannii* complex and *Picea sitchensis* embryos. Smith (1994) used water vapour permeable films in a similar way to successfully enhance germination. It is clear that by modifying the somatic embryo development so that it resembles more closely the zygotic development beneficial effects on the maturation and germination of the embryo can be encouraged.

3.1.1 Objectives

The work in this chapter is aimed at:

- optimising the maturation efficiencies of somatic embryogenic tissue of *Pinus radiata* through experimental modifications of medium using osmotica, different ABA concentrations and other components.

- optimising conditions for germination and plant establishment of the *Pinus radiata* somatic emblings.

3.2 Methods

3.2.1 Plant Material

The embryogenic cell lines used for all the maturation experiments were initiated from explants during the initiation experiments of 1995-96, 1996-97 and 1997-98 (as described in chapter 2). The embryogenic cell lines utilized were isolated from explants collected from Maryvale, New Norfolk and Albury. After initiation, embryogenic cell lines were maintained on, either DCR hormone-free medium (Gupta and Durzan 1985) or NZ maintenance medium (SEM) (refer to Appendix 2) (Smith 1994). Plates were incubated in the dark at 21 to 22°C.

3.2.2 Media

The basal medium, DCR (Gupta and Durzan 1985) was used for the maturation experiments unless otherwise stated. The components that were investigated for their effect on the maturation of the somatic embryos are shown in Figure 3.2. Several experiments investigated the effect of the standard basal salts, which have been used for other maturation media. These included MS (Murashige and Skoog 1962), SH (Schenk and Hildebrandt 1972), GD (Gresshoff and Doy 1975), P21 (Chandler (1995) pers. comm), DCR (Gupta and Durzan 1985), NZ-EMM (Smith 1994) and LP (Hakman and von Arnold 1985). Other components investigated for their effect on the maturation of the somatic embryos were ABA (Sigma), polyethylene glycol (PEG), Gelrite (Sigma), glutamine, potassium chloride (KCl) (BDH), sucrose (Sigma) and seed extract (extraction based on methods developed by Egertsdotter and von Arnold 1995). The media was autoclaved at 121°C for 20 minutes. Seed extract and freshly prepared ABA solutions (dissolved by 1N NaOH and pH adjusted to 5.5) were filter sterilized and added to the culture medium after they had cooled to about 50°C.

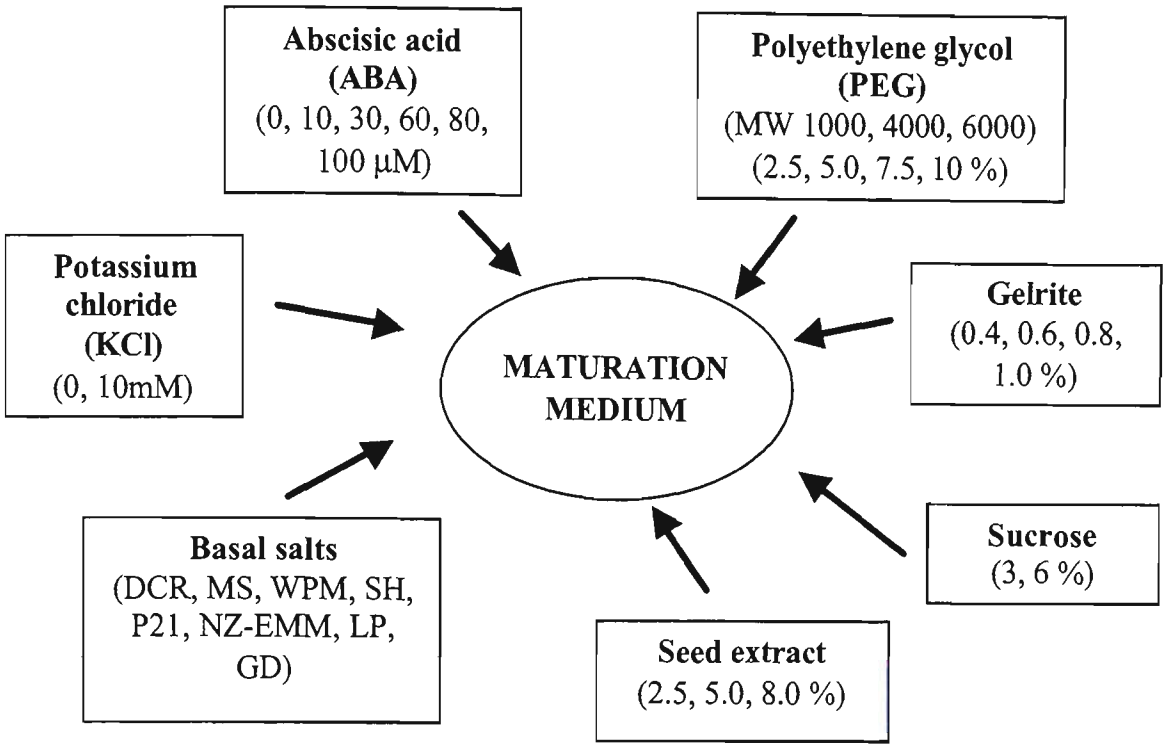


Figure 3.2 Components of the maturation media tested

3.2.3 Experimental set-up

Clumps of developing embryos (1cm maximum) were placed on the media containing the appropriate chemical combinations for each maturation experiment. On most occasions three to four clumps of each cell line were placed onto each of three Petri dishes. Three to four cell lines were used for each maturation experiment. In total, twelve to sixteen clumps of tissue were present on each dish. Dishes were incubated in darkness at a constant temperature of 24°C+/- 1°C.

3.2.4 Classification of mature somatic embryos

Throughout the maturation experiments, the analysis of the maturing somatic embryos was carried out using the system developed by von Arnold and Hakman (1988). Stage 1, 2, 3 and 4 embryos are defined in Figure 3.1.

3.2 5 Further development of mature somatic embryos

Stage 3 and stage 4 somatic embryos from each maturation experiment were transferred to the next phase of the experiment for continued development towards plantlet establishment.

3.2.6 Treatment of mature somatic embryos prior to germination

Mature somatic embryos underwent the High Relative Humidity (HRH) treatment as described by Roberts *et al.* (1990b). The mature embryos were placed into 6 wells of a 12 well Elisa plate (Labserv) and 4 ml of sterile distilled water was placed into the remaining 6 wells (refer to Figure 3.3). The plate was sealed with paraffin and wrapped in aluminium foil. Plates were placed in a 27°C growth cabinet for 7 to 14 days.

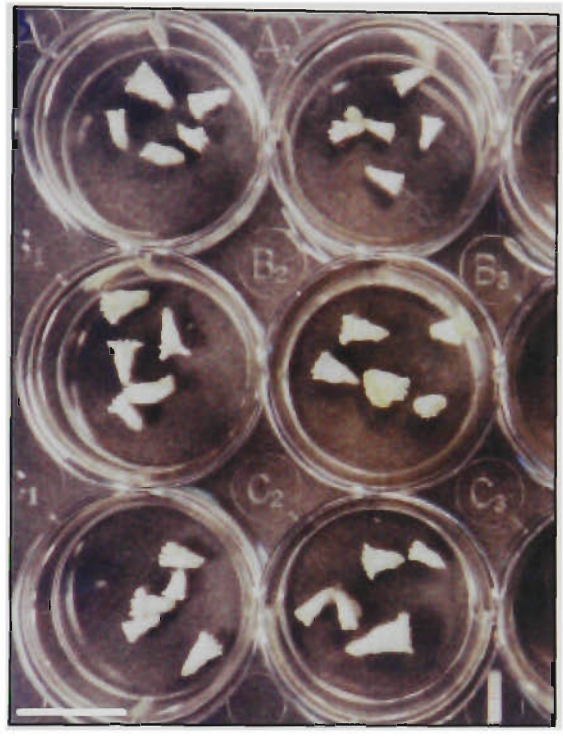


Figure 3.3 Demonstration of HRH treatment with mature somatic embryo (bar = 1cm)

3.2.7 Germination of mature somatic embryos

On completion of the HRH treatment mature somatic embryos were aseptically removed and placed onto germination medium (without hormones). The germination media used were DCR and NZ based media (Smith 1994). Germination of the somatic embryos was conducted under a light regime of 12 hours day light and 12 hours darkness at 22-24°C. Germination of the somatic embryo was based on the emergence of the radicle from the base of the hypocotyl. This was normally preceded by a pink colouring at the base of the hypocotyl. As the root continued to lengthen, somatic emblings were transferred to jars holding 50ml of germination medium for further growth.

3.2.8 Transfer of somatic plantlets to glasshouse conditions

When somatic emblings showed good primary and secondary needle growth, good extension of the root system and appropriate elongation of the stem they were transferred to Growool® and placed into trays on a mist bed in the glasshouse to acclimatise. Prior to planting out the Growool was soaked in plant starter (Yates) with the appropriate nutrients. Plantlets were left on the mistbed for approximately three months. Plantlets that survived and continued to grow were transferred to soil/peat/perlite mix (2:1:1) in 5-inch pots and placed in the glasshouse to continue growth.

3.2.9 Data collection

Cultures were assessed at three weeks and then finally at six to nine weeks. The presence of mature somatic embryos was described using the classification system detailed in section 3.2.4. The total numbers of somatic embryos at the different stages of maturation were counted from each cell line from each plate of each treatment and added together. Several cell lines were used in the maturation experiments to overcome variations in maturation capabilities between cell lines.

3.2.10 Data analysis

The data from each experimental area were analyzed separately due to the differences in treatments and cell lines. The maturation stages for each experiment were analyzed separately from one another. The maturation data was analyzed using a General Analysis of Variance (ANOVA) to obtain an F-probability value in Genstat Release 4.2, Fifth edition (Lawes Agricultural Trust, Rothamsted Experimental Station 2000). Comparisons of least significant differences of means were conducted. Figures show bars with +½ least significant difference (LSD). Statistical significance of data is measured at < 0.05 unless otherwise stated.

3.3 Results

3.3.1 The effect of ABA on the maturation of *Pinus radiata* somatic embryos

Maturation experiments utilizing ABA were conducted to ascertain the concentration at which maturation of the somatic embryos occurred. The different stages of maturation of the somatic embryos and the overall yield of somatic embryos were analyzed to observe the influence of different concentrations of ABA. Figure 3.4 shows the influence of ABA on the maturation capability of somatic embryos at the different stages of maturation.

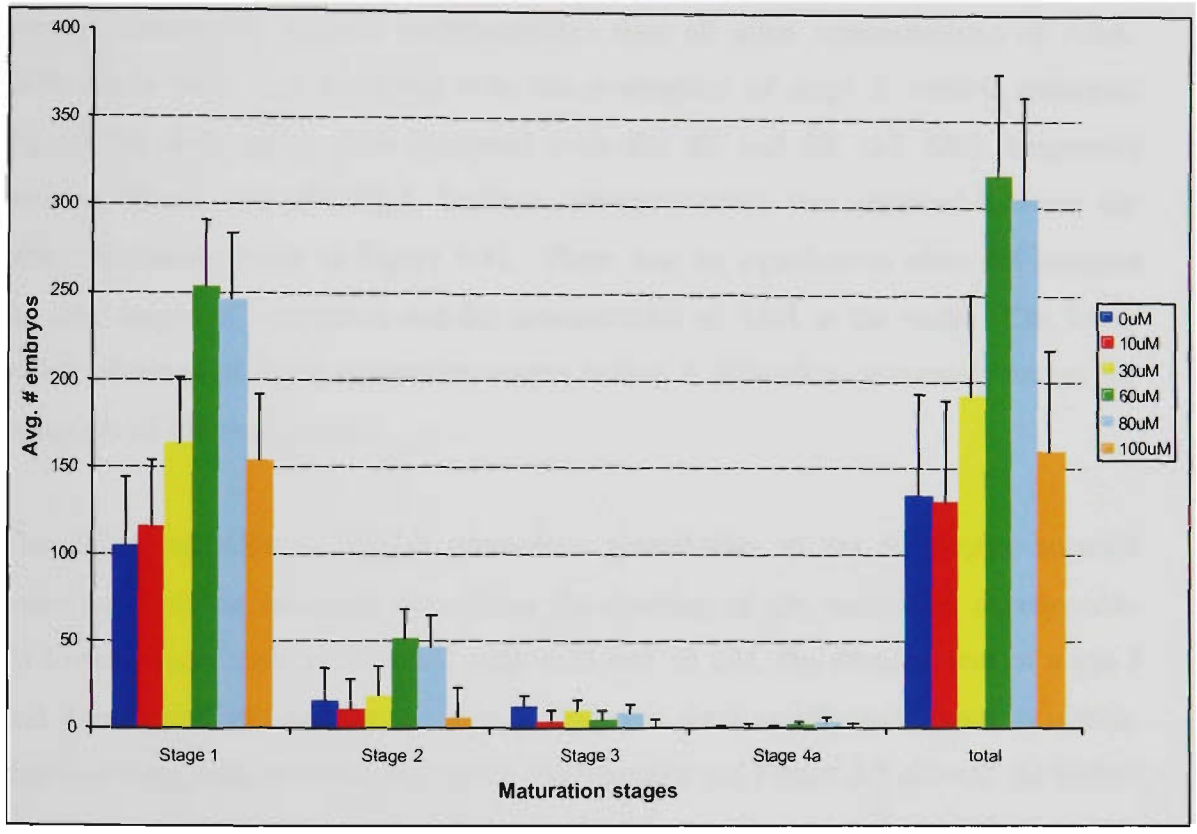


Figure 3.4 Effect of ABA on the maturation capacity of somatic embryos.
(bars are + ½ LSD)

Maturation of the somatic embryos was obtained with the inclusion of ABA in the maturation medium. The majority of somatic embryos obtained over the maturation duration were stage 1 embryos (as classified in Figure 3.1). Maturation to stages 2, 3 and 4 embryos occurred but to a lesser degree, as can be seen in figure 3.4. The embryogenic tissue was clear and crystalline to white in appearance and after about three or four weeks on the maturation media showed some level of embryo

organization. The tissue remained of this same appearance for its entire duration on the maturation media however, the level of embryo maturation changed with time. The amount of embryos at each of these stages of development and the total yield of embryos varied amongst the different maturation treatments applied.

The effect of the ABA concentration on the maturation of the somatic embryos peaked at 60-80 μM, followed by a decrease in yield and stage development at 100 μM. Significance was observed between the different ABA concentrations in the appearance of stage 1 somatic embryos (F probability 0.001). At 60 and 80 μM ABA the number of stage 1 somatic embryos were significantly higher (253 and 246 average number of somatic embryos/plate) than all other concentrations of ABA. Differences were also observed with the production of stage 2 somatic embryos. Significant differences were observed with the 60 and 80 μM ABA treatments between 10 and 100 μM ABA, however, no significance was observed between the other treatments (refer to Figure 3.4). There was no significance observed between the later stages of maturation and the concentration of ABA in the media. The lower yields obtained at these maturation stages makes it difficult to accurately assess the influence of the treatments.

The ability of ABA to inhibit precocious germination in the developing somatic embryos could be observed throughout the duration of the maturation experiments. At lower concentrations of ABA, such as 0 and 10 μM, the development of stage 3 and 4 embryos was usually abnormal. Embryos were usually pale green in colour, thin and long with minimal cotyledon development (as Figure 3.5 shows). At higher concentrations of ABA such as 60 and 80 μM, the mature somatic embryos were white and typically bullet shaped with cotyledon development (as Figure 3.6 shows). Sometimes the somatic embryos that matured at the lower concentrations were similar to the white, bullet shaped mature embryos but were often smaller and thicker in the hypocotyl. The structural variation between mature somatic embryos derived from the different concentrations of ABA shows that the higher quality mature somatic embryos were achieved mostly at higher concentrations of ABA.

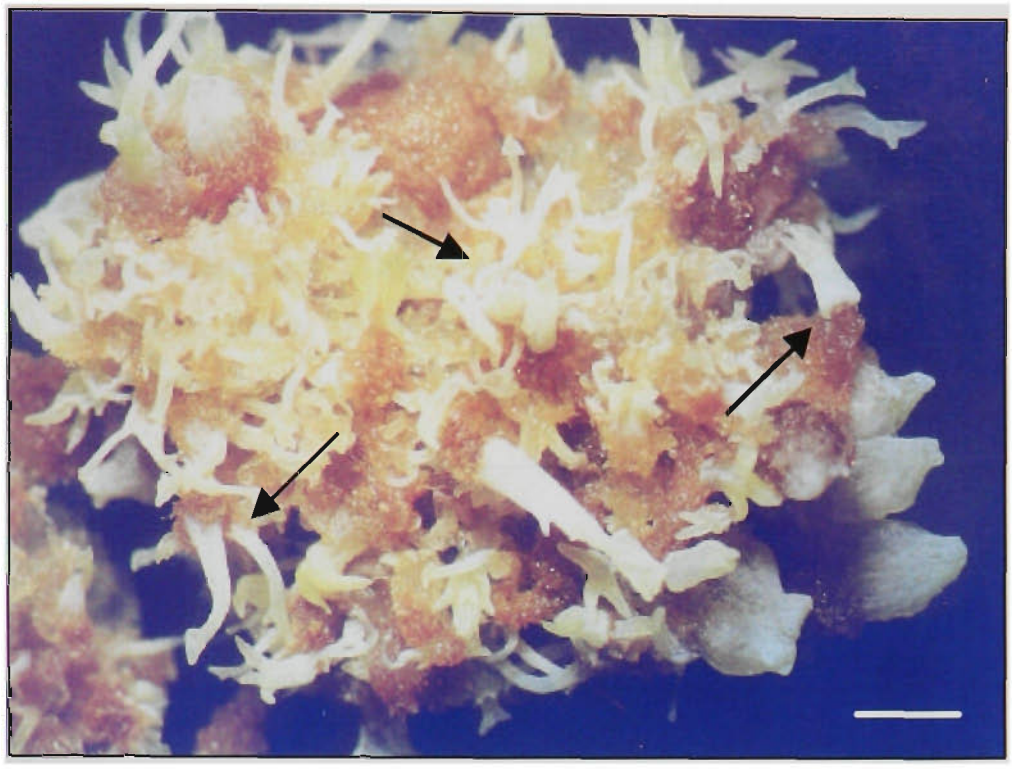


Figure 3.5 Abnormal somatic embryo development. Note the long, thin, pale green embryos (bar = 0.5cm)

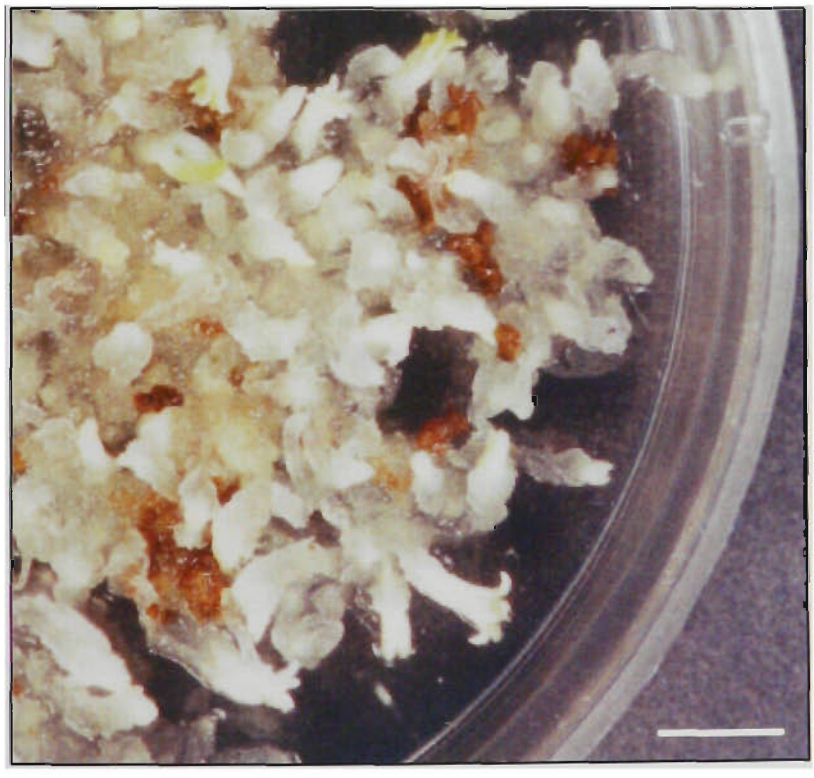


Figure 3.6 Normal somatic embryo development (bar = 0.7 cm)

3.3.2 The effect of osmotica on the maturation of somatic embryos

3.3.2.1 Permeating osmotica – sucrose

Sucrose can be used as an osmoticum within the maturation medium. Unlike PEG, sucrose is classified as a permeating osmoticum that is able to penetrate into the cells. If used for long time periods, the osmotic effect is reversed, enabling the cell to retain water instead of removing it. Different concentrations of sucrose were incorporated into the maturation media to observe the effects on somatic embryo maturation. Figure 3.7 shows the results of this experiment.

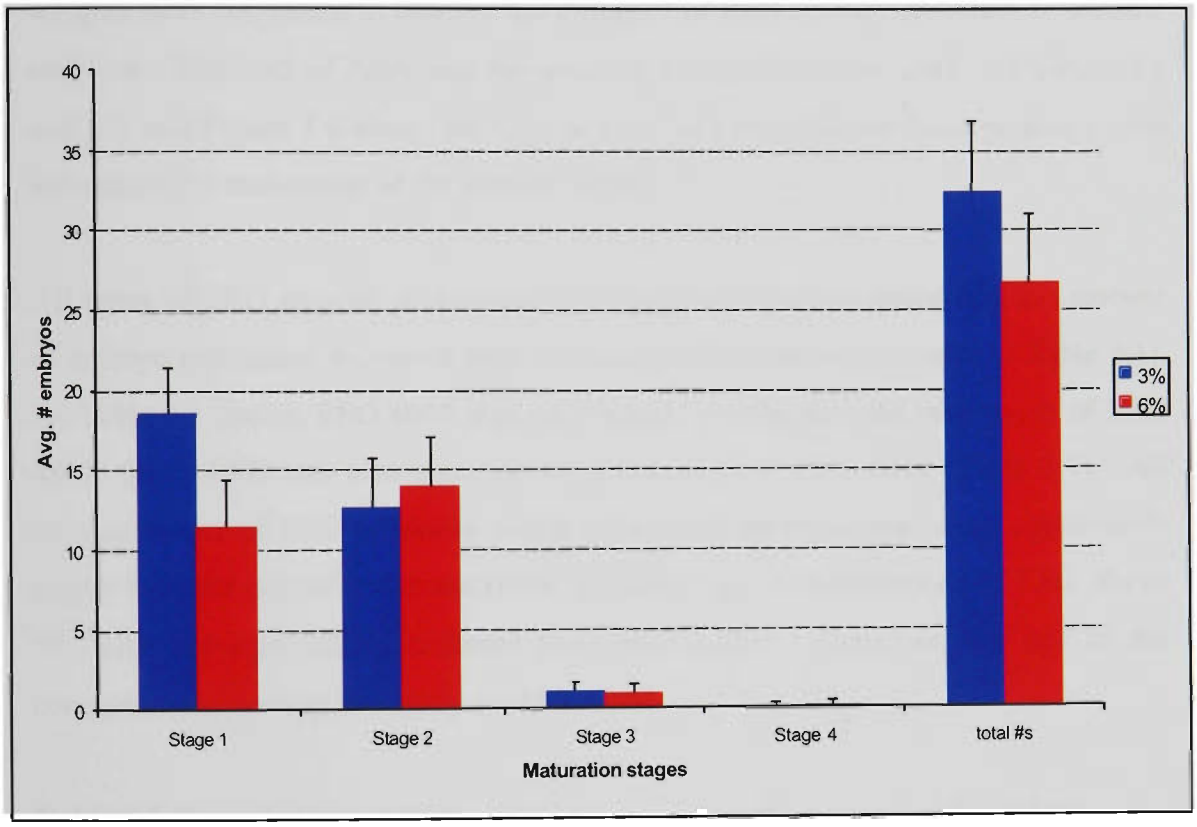


Figure 3.7 Effect of sucrose on the maturation of somatic embryos (bars are + ½ LSD)

The standard concentration at which sucrose is included in maturation media is 3-4%, whilst 6% sucrose may provide a greater osmotic effect and therefore, enhance maturation. As Figure 3.7 shows, yields of somatic embryos were low. Significant differences were observed between the two concentrations of sucrose for stage 1 development (F. prob: stage 1: 0.014). However, no significance was obtained for any of the other maturation stages between the treatments (F. prob: stage 2: 0.691, stage 3: 0.949, stage 4: 0.366). Overall yields of somatic embryos at all stages were quite low. Total average yields of somatic embryos at 3% sucrose were 32.5, whilst at 6% they were 26.7. No significant differences were observed between the overall

yields for each treatment. Sucrose at 6% showed a slightly better yield of stage 2 somatic embryos than 3% sucrose, however, as mentioned earlier, no significance between the treatments was obtained. Based on these results, sucrose in the maturation medium could be used at either 3% or 6%, though further experimentation would be necessary to make more accurate assumptions of performance.

3.3.2.2 Non-permeating osmotica – polyethylene glycol (PEG)

Maturation experiments incorporating PEG at different concentrations and molecular weights were conducted to observe the influence of PEG on the maturation of somatic embryos. The level of ABA was the same for all treatments (60 μM). As Tables 3.1 and 3.2 and Figure 3.8 show, the type of PEG and the different concentrations used influenced the maturation of the somatic embryos

All types of PEG showed an increase in somatic embryo maturation and the amount of embryo maturation increased with increasing molecular weight (refer to Table 3.1). As Table 3.1 shows, PEG 6000 was significantly greater than the other types of PEG used. PEG 4000 was also significantly different from PEG 1000 (Table 3.1). All concentrations of PEG produced yields of somatic embryos that were significantly higher than the control treatment (refer to Table 3.2). Concentrations of PEG above 5% were not significantly different from one another. Therefore, any one of the concentrations used in this study could be used.

Table 3.1 Overall effect of PEG molecular weight on the average total yield of somatic embryos per plate of maturation media

MW	1000	4000	6000
Avg. somatic embryos	58^{a}	102^{b}	173.5^{c}

Numbers with the same letter are not significantly different.

Table 3.2 Overall effect of PEG concentration on the average total yield of somatic embryos per plate of maturation media

% PEG	0	2.5	5.0	7.5	10.0
Avg. somatic embryos	26.7^{a}	90.8^{b}	143.3^{c}	135.8^{c}	159.2^{c}

Numbers with the same letter are not significantly different.

When the concentrations of PEG and the molecular weight are shown in combination, the average total yield of somatic embryos per treatment can be observed. For each molecular weight, the different concentrations produced varying numbers of somatic embryos (refer to Figure 3.8). PEG 1000 used at 5% was significantly different from the control treatment and several other concentrations used (2.5 and 7.5%) (refer to Figure 3.8). With increasing concentrations, PEG 4000 showed increasing yields of somatic embryos. Higher yields may have been achieved if higher concentrations had been investigated. For PEG 6000, the highest yields of somatic embryos were achieved when concentrations of 5%, 7.5% or 10% were used. These yields were significantly different when comparing them with PEG 6000 at 0% and 2.5%, but they were not significantly different to each other (refer to Figure 3.8).

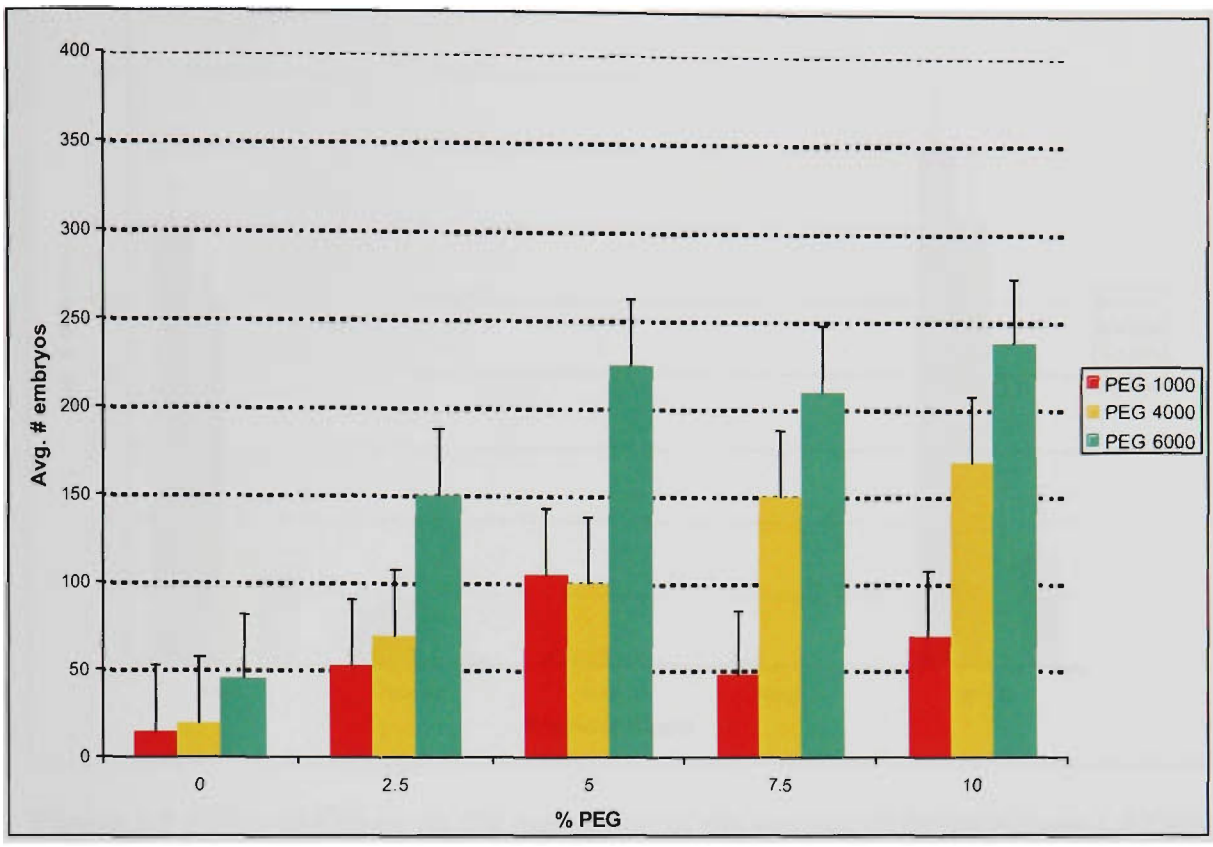


Figure 3.8 Effect of the molecular weight and concentration of PEG on the average total yield of somatic embryos per plate of maturation media (bars are + ½ LSD)

These results indicate that PEG can be incorporated into the maturation medium to achieve somatic embryo maturation. The level of PEG used may vary between PEG 4000 and PEG 6000 as the yields for somatic embryo maturation may increase with higher concentrations. With this series of maturation experiments, PEG at concentrations of at least 5% produced the highest yields of mature somatic embryos.

3.3.3 The effect of Gelrite® on the maturation of somatic embryos

The different concentrations of Gelrite within the maturation media appeared to affect the maturation of the somatic embryos (refer to Figure 3.9). Gelrite used at 0.4% and 0.6% produced the highest yields of somatic embryos at all stages of maturation. Significant differences were evident with the production of stage 1 embryos on media containing 0.4% and 0.6% Gelrite when compared to the higher concentrations of 0.8% and 1.0% (F prob. <0.001). An average yield of stage 1 embryos per plate of media containing 0.4% and 0.6% was 295 and 244 embryos, whilst at 0.8% and 1.0% only 82 and 69 stage 1 embryos were produced.

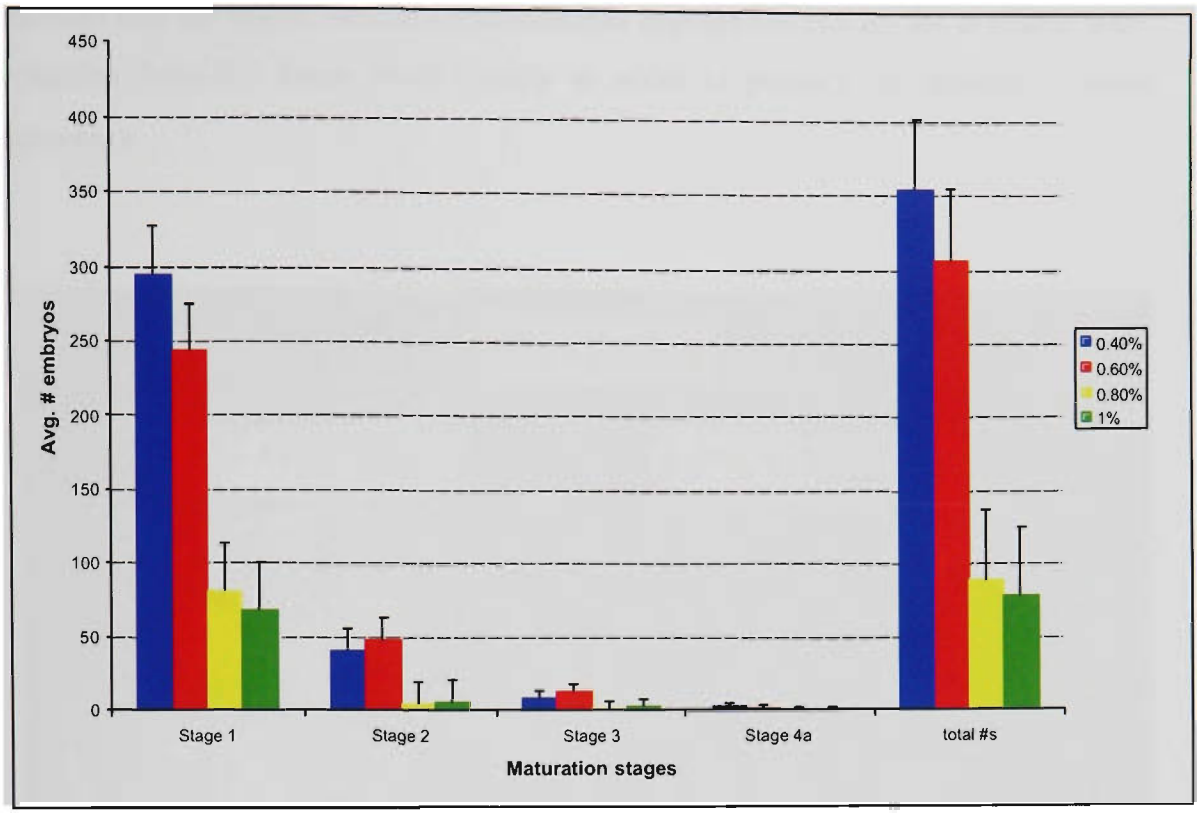


Figure 3.9 Effect of Gelrite on the maturation of the somatic embryos (bars are + ½ LSD)

The maturation of stage 2 embryos also showed significant differences from the lower concentrations of Gelrite, when compared to the higher levels. At 0.4% and 0.6% Gelrite, an average of 41 and 48 stage 2 embryos were produced per plate whilst on 0.8% and 1.0% Gelrite media, an average of 4 and 6 embryos were produced. The maturation of stage 3 somatic embryos showed differences between treatments. The 0.6% Gelrite treatment was marginally different from the higher concentrations of Gelrite (0.8% and 1.0%), but on this occasion 0.4% Gelrite was not significantly different from any other concentration used. Stage 4 somatic embryos showed no appreciable differences between the Gelrite concentrations (as Figure 3.9 shows). The total yield of somatic embryos produced showed significant differences between Gelrite concentrations (F prob. <0.001), however, this was a reflection of the production of stage 1 somatic embryos at 0.4 % and 0.6% Gelrite.

The quality of the embryogenic tissue reflected the differences observed amongst the Gelrite concentrations. Tissue at the lower concentrations, such as 0.4% and 0.6% was translucent and crystalline in appearance whilst at the higher concentrations of 0.8% and 1.0%, the tissue appeared white and desiccated (refer to Figure 3.10). This

indicated that the higher Gelrite concentrations appeared to extract the available water molecules from the tissue more readily in order to produce the desiccated tissue appearance.

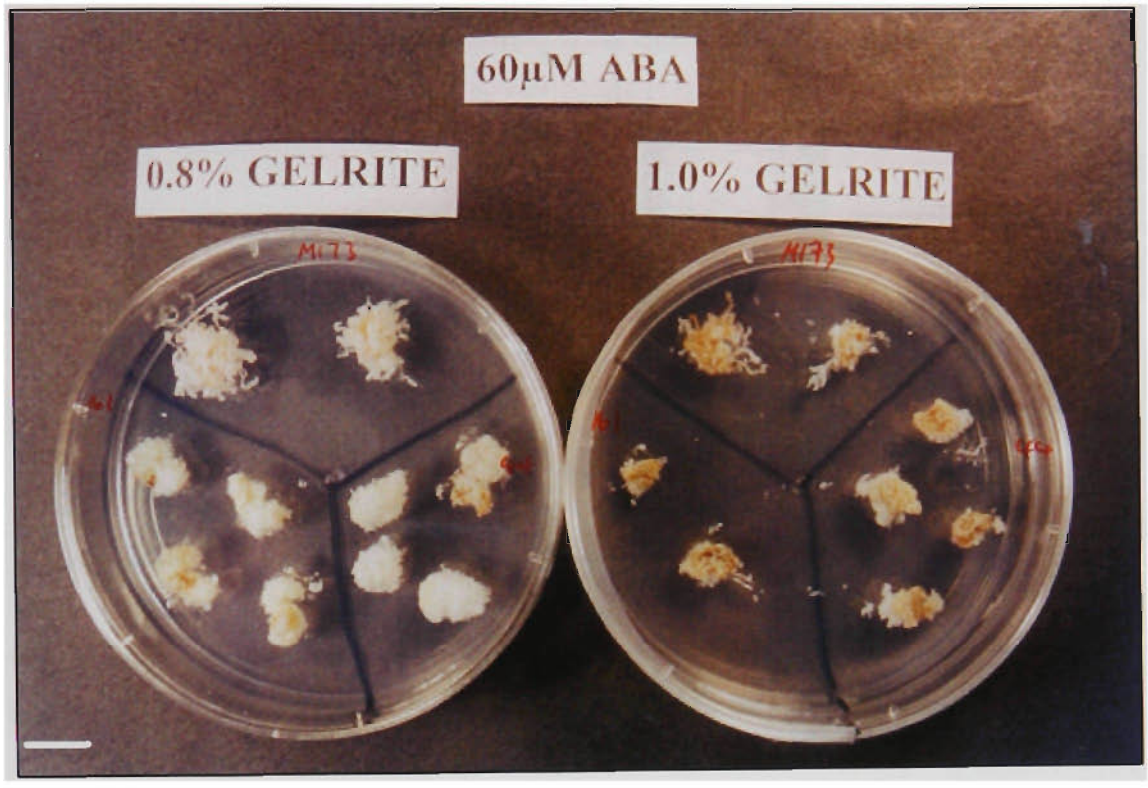


Figure 3.10 Embryogenic tissue quality due to higher Gelrite concentrations (bar = 1cm)

When the different Gelrite concentrations were tested with ABA concentrations, the results obtained supported the results previously detailed in section 3.3.1 and the previous Gelrite experiment. As Figure 3.11 shows, the ABA concentration and the Gelrite concentration affected the maturation of the somatic embryos. In combination the highest yields of mature somatic embryos obtained were when 60 μM and 80 μM ABA was incorporated with 0.4% or 0.6% Gelrite. These yields were significantly different from all other treatments, except 30 μM ABA and 0.4% Gelrite (refer to Figure 3.11). At each of these ABA concentrations over 430 somatic embryos were produced per plate of medium, with 80 μM ABA/0.6% Gelrite producing an average of 575 somatic embryos (as Figure 3.10 shows). A good yield of somatic embryos was also obtained with 30 μM and 0.4% Gelrite (average of 462 somatic embryos per plate).

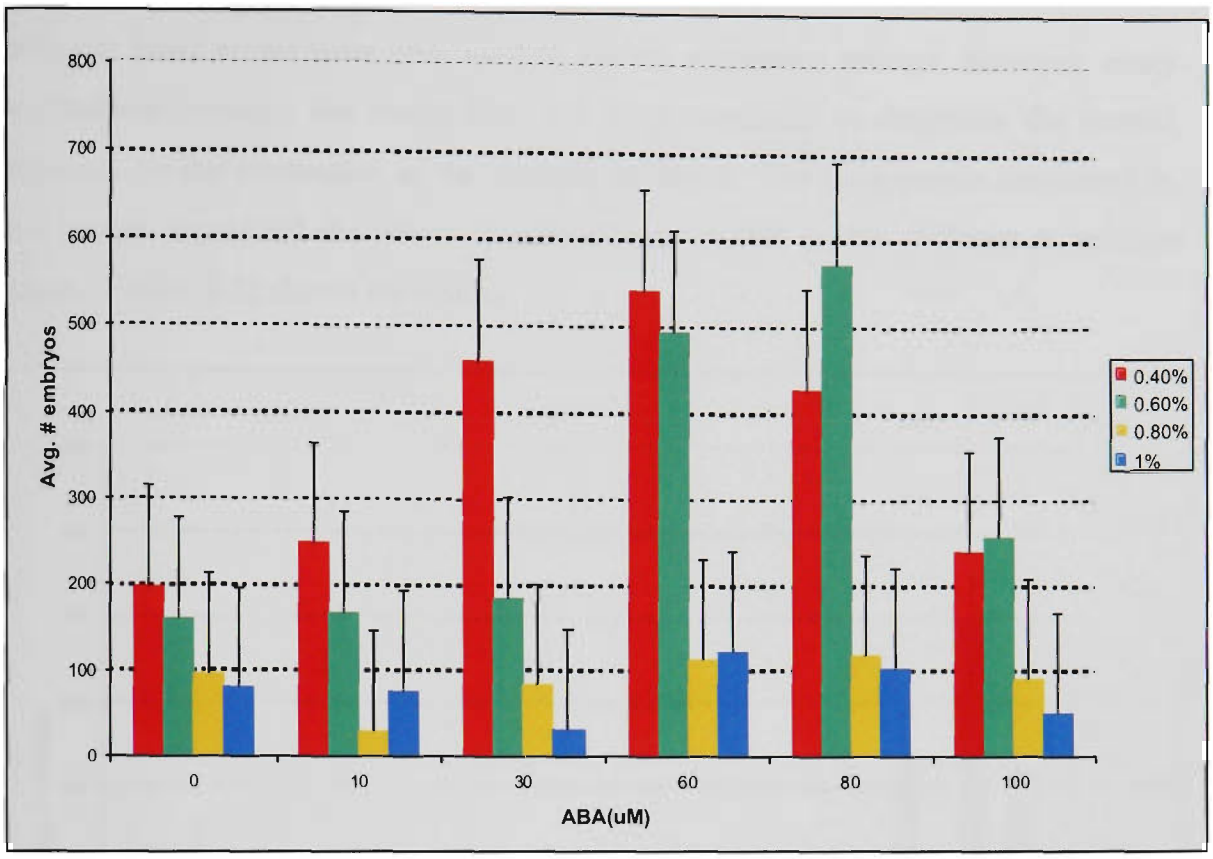


Figure 3.11 Effect of ABA and Gelrite combination on the total number of mature somatic embryos (bars are + ½ LSD)

The results from the experiments investigating the effect of Gelrite on the maturation capabilities of the somatic embryos suggest that concentrations of 0.4% and 0.6% Gelrite are favourable for good yields of mature somatic embryos. When used at the appropriate concentrations of ABA (60 μM and 80 μM) high yields of mature somatic embryos can be produced.

3.3.4 The effect of basal media on the maturation of somatic embryos

3.3.4.1 Broad comparison of basal media

Different basal media have been used to aid the maturation process, however, many comparisons between the media have not been conducted to determine the overall influence on the maturation of the somatic embryos. The experiments conducted in this section examined the effect of several basal media on the different maturation stages. Figure 3.12 shows the results.

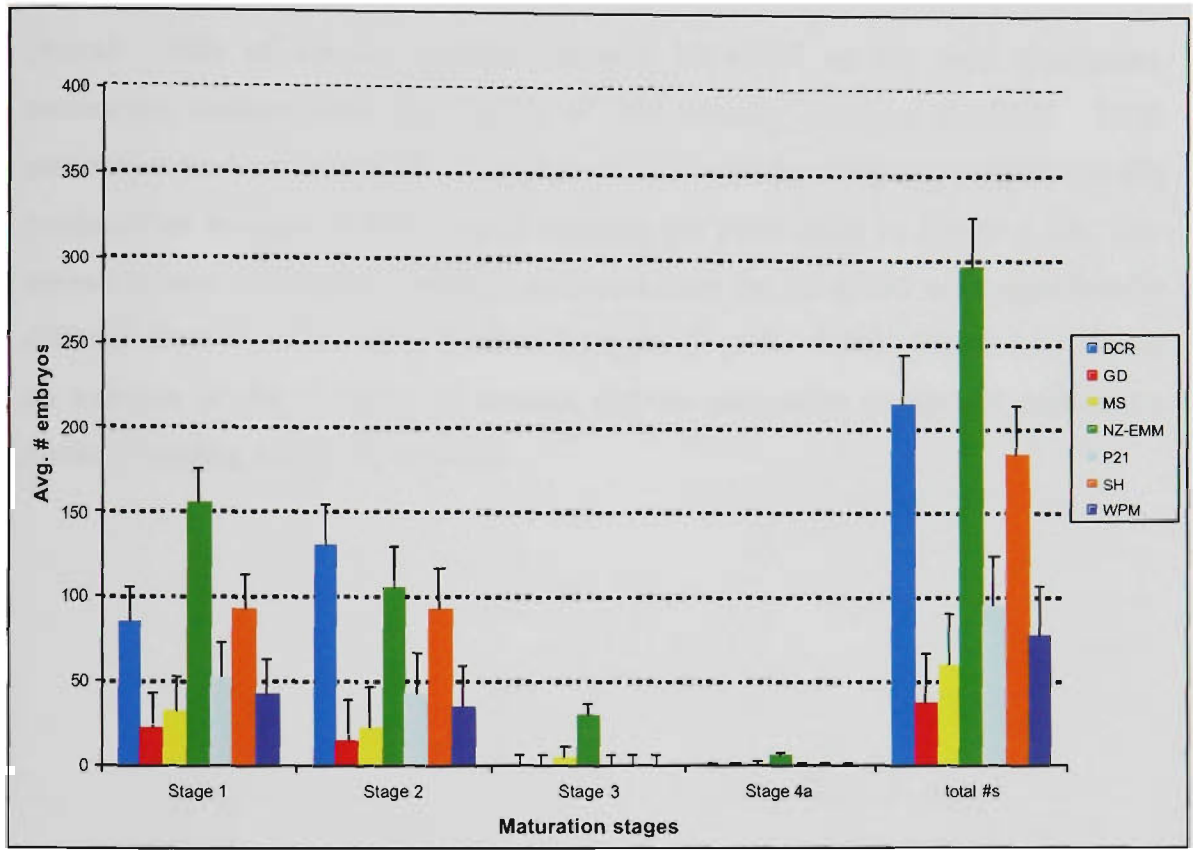


Figure 3.12 Effect of different basal media on the maturation stages of the somatic embryos (bars are + ½ LSD)

The majority of the media treatments produced stage 1 embryo development with NZ, SH and DCR media where the average numbers per plate were 155, 92 and 85, respectively. The average numbers produced from NZ-EMM were significantly different from all other media types used (F. prob. 0.001). Stage 2 embryo development was also produced in low amounts with the DCR and SH based medium and NZ-EMM (130, 92 and 105 average number per plate) showing the highest levels (Figure 3.12). DCR based medium was not significantly different from the numbers produced on NZ-EMM and SH maturation media (F. prob. 0.004) but was significant when compared to the other types of basal media (refer to Figure 3.12). Later stage

somatic embryos, stages 3 and 4 were produced on only two media, NZ-EMM and MS based maturation media. Numbers produced were low with 30 stage 3 embryos and 7 stage 4 embryos produced on NZ-EMM and 5 stage 3 and 1 stage 4 embryo produced on MS based medium. The yields on NZ-EMM at the later stages were significantly different from the MS medium (Stage 3: F prob. 0.011. Stage 4: F prob. 0.013). Other maturation media failed to produce any late stage somatic embryos (refer to Figure 3.12).

Overall yields of somatic embryos showed NZ-EMM as the most productive maturation medium with an average of 296 somatic embryos obtained. DCR maturation medium produced an average of 215 somatic embryos per plate and SH produced an average of 185 somatic embryos per plate (refer to Figure 3.12). The overall numbers of somatic embryos produced from the NZ-EMM were significantly different from all of the other basal media types (F prob. <0.00). Figure 3.13 shows an example of the diversity of somatic embryo maturation on several maturation media, including NZ-EMM and GD.

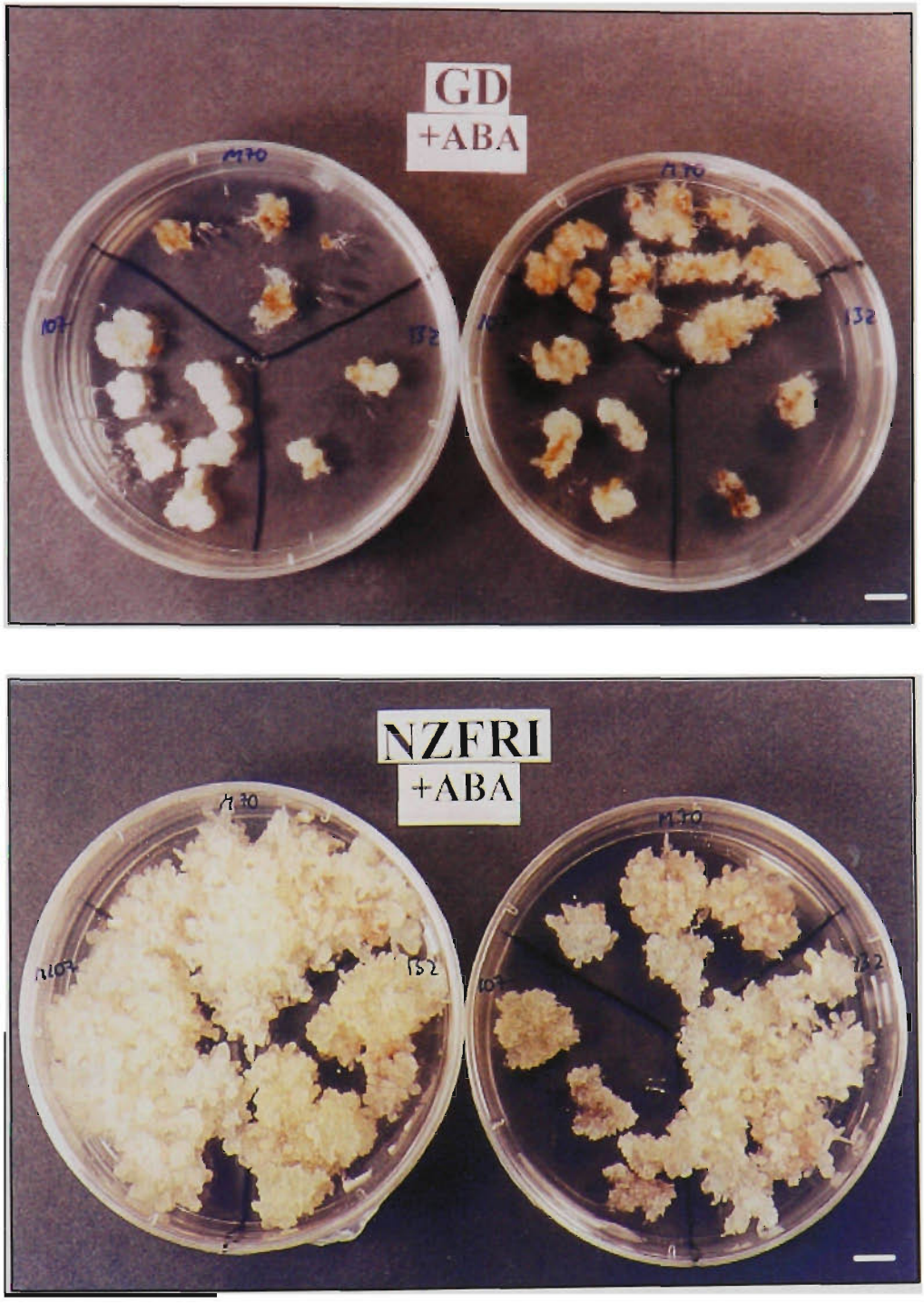


Figure 3.13 Comparison of somatic embryo maturation between several maturation media (NZFRI is NZ-EMM) (bars = 1cm)

3.3.4.2 Comparison between DCR and NZ -EMM

A comparison between the DCR maturation medium and the FR's maturation medium (NZ-EMM) further supported the consistent performance of the NZ-EMM on the maturation of the somatic embryos. Figure 3.14 shows the results of the comparison.

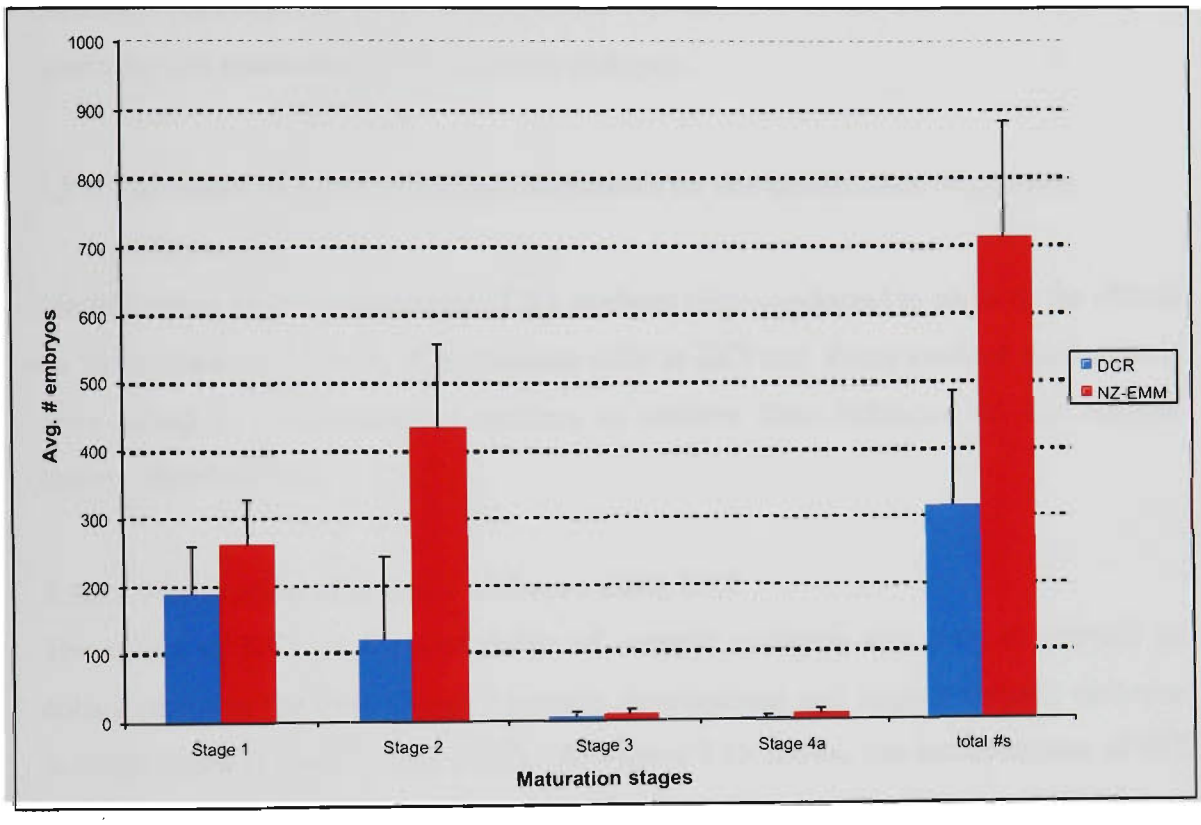


Figure 3.14 Effect of DCR and NZ-EMM on the different stages of maturation of the somatic embryos (bars are + ½ LSD)

The NZ-EMM out-performed the DCR maturation medium in all stages of somatic embryo development. There was no significant difference observed between the treatments of stage 1 somatic embryos, even though the yields appeared quite different. An average of 192 somatic embryos developed on DCR medium and 262 on NZ-EMM. Significant differences were observed between stage 2 somatic embryos obtained on both media. NZ-EMM showed a greater number of somatic embryos than the DCR medium with an average of 434 stage 2 embryos compared to 120 stage 2 embryos (refer to Figure 3.14). Stage 3 and 4 somatic embryos also developed on the maturation media, though average numbers were low on both (4.2 and 9 stage 3 embryos on DCR and NZ-EMM, 0.8 and 9.3 stage 4 embryos on DCR and NZ-EMM). No significant difference between the media was observed for the later maturation stages. Overall yields of somatic embryos favoured the NZ-EMM with

almost double the numbers of somatic embryos developed on this medium compared to the DCR medium (average of 714 to 317 somatic embryos). Again NZ-EMM was appreciably different from DCR medium. Results of observations obtained from the DCR/NZ comparison and the basal media maturation experiments supported the consistent and superior performance of the NZ-EMM over the use of the other basal media for the maturation of the somatic embryos.

3.3.5 The effect of other media modifications on the maturation of somatic embryos

Modifications to the components of the medium were conducted to observe the effects on the maturation process. Components such as KCl and *Pinus radiata* seed extract were added to the maturation medium to observe their influence on the somatic embryo development.

3.3.5.1 Maturation of somatic embryos using KCl

The effect of KCl on the maturation of somatic embryos was seen to provide an enhancement to the initial stage 1 embryo development and stage 2 somatic embryos in *Pinus taeda* (Li and Huang 1997). As Figure 3.15 shows, the incorporation of KCl into the maturation medium at 10mM slightly enhanced the production of stage 1 somatic embryos. Significant differences were observed between the control and the incorporation of 10mM KCl. Stage 2 somatic embryos were reasonably comparable between treatments, with no significance obtained. Later stage embryo maturation did not occur for either treatment. Therefore, it appears that KCl did not enhance the maturation of stage 3 somatic embryos. Overall yields of somatic embryos showed significance between treatments, but that was most likely a reflection of the stage 1 yields. The results showed that KCl may have enhanced the development of early stage somatic embryos but no significance between treatments of later stage maturation was obtained.

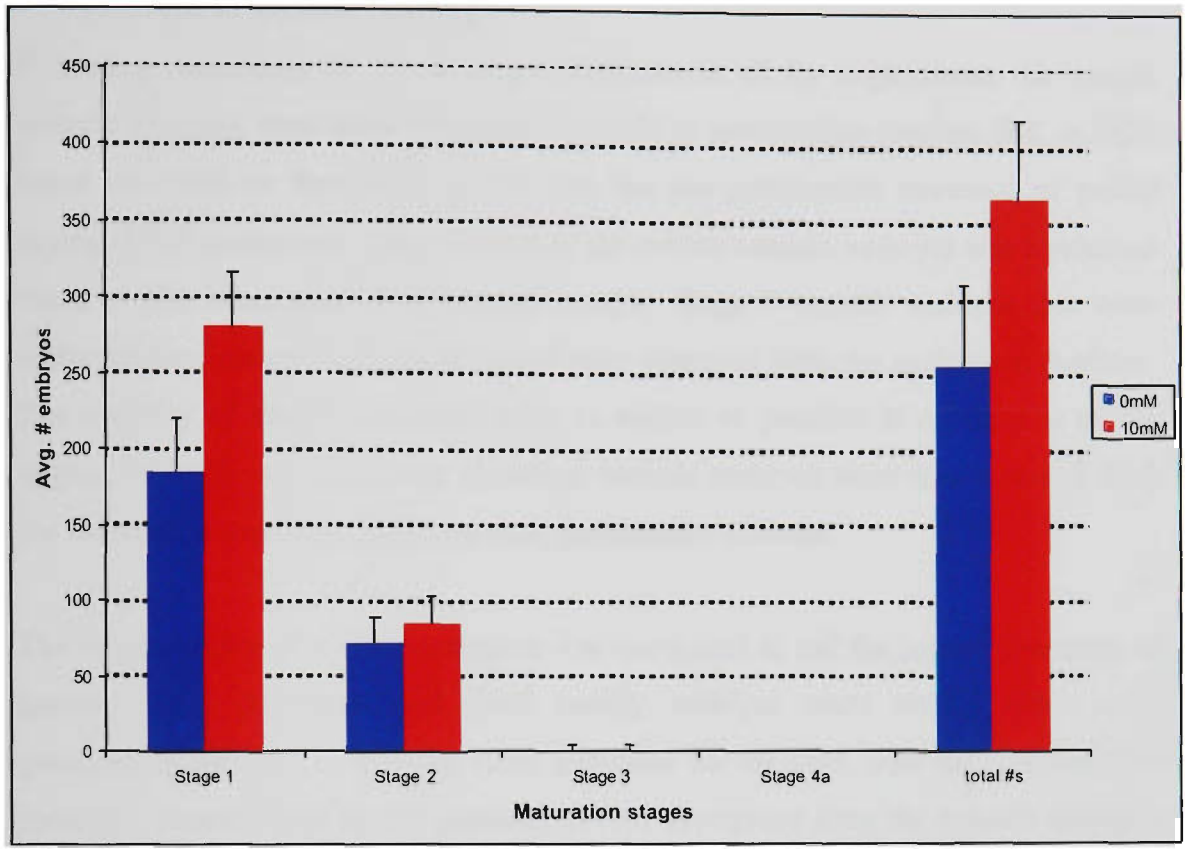


Figure 3.15 Effect of KCl on the maturation stages of somatic embryos (+ 60 µM ABA) (bars are + ½ LSD)

3.3.5.2 Maturation of somatic embryos using seed extract

The mature *Pinus radiata* seed contains compounds that could theoretically enhance the maturation of somatic embryos, including endogenous ABA, storage proteins, arabinogalactins and lipids. The incorporation of different concentrations of seed extract and ABA (at 60 µM) into the maturation medium was conducted to observe the effect on embryo maturation. No significant differences were observed between the treatments using different concentrations of seed extract (no data shown). The results obtained in this study indicated that the incorporation of the seed extract at different concentrations did not enhance the development of mature somatic embryos. Further work would need to be considered to determine the full extent of seed extract usage.

3.3.6 Pre-germination experiments and the conversion of mature somatic embryos to somatic emblings

Following maturation by the different treatments in all the experiments, the mature somatic embryos were transferred either straight to germination medium (NZ or DCR based medium) or they were placed into the pre-germination treatment of partial drying (HRH treatment). The selection of the mature somatic embryos was conducted based on the maturation developmental stage. Stage 3 somatic embryos that were white/creamy, opaque and bullet shaped were removed from the maturation medium. The majority of embryos selected were as similar as possible in appearance to the zygotic embryo. On occasions, abnormal somatic embryos were also removed from the maturation medium and placed onto germination medium.

The incorporation of a HRH treatment was conducted to aid the natural processes of germination. Abnormal and good quality embryos were placed either onto germination medium or into the HRH treatment for 10 days, then onto germination medium. Examination for the presence of root emergence from the somatic embryos was quite low in both treatments, however, germination occurred in somatic embryos that were subjected to the HRH treatment (Table 3.3). Figure 3.16 shows a comparison of mature somatic embryos from both with and without HRH treatments. The successful germinants were 'good' quality somatic embryos, whilst the 'abnormal' somatic embryos failed to germinate. These somatic embryos browned, died or become quite swollen (thickened hypocotyls), but failed to produce any roots. After several weeks the abnormal somatic embryos were subjected to the HRH treatment for 10 days then placed back onto germination medium. Germination occurred with only 1 somatic embryo. Again this germinant was from a good quality somatic embryo. Whilst the abnormal somatic embryos failed to produce any roots, these embryos continued an abnormal development with continued tissue proliferation and irregular cotyledon development. Figure 3.17 shows examples of abnormal development of mature somatic embryos.

Table 3.3 Pre-germination experiment with and without HRH treatment with mature somatic embryos*

	# Embryos		# Germinated		
Treatment	**'Good'[a]**	**'Abnormal'[b]**	**'Good'**	**'Abnormal'**	**total**
With HRH	8	14	5	0	5
Without HRH	1	13	0	0	0

*No statistical analysis conducted.

a: somatic embryos that are white and resemble a torpedo shape.

b: somatic embryos that do not resemble a torpedo shape. May be slightly green or yellow, elongated hypocotyl, thin or small or have abnormal shaped cotyledons.

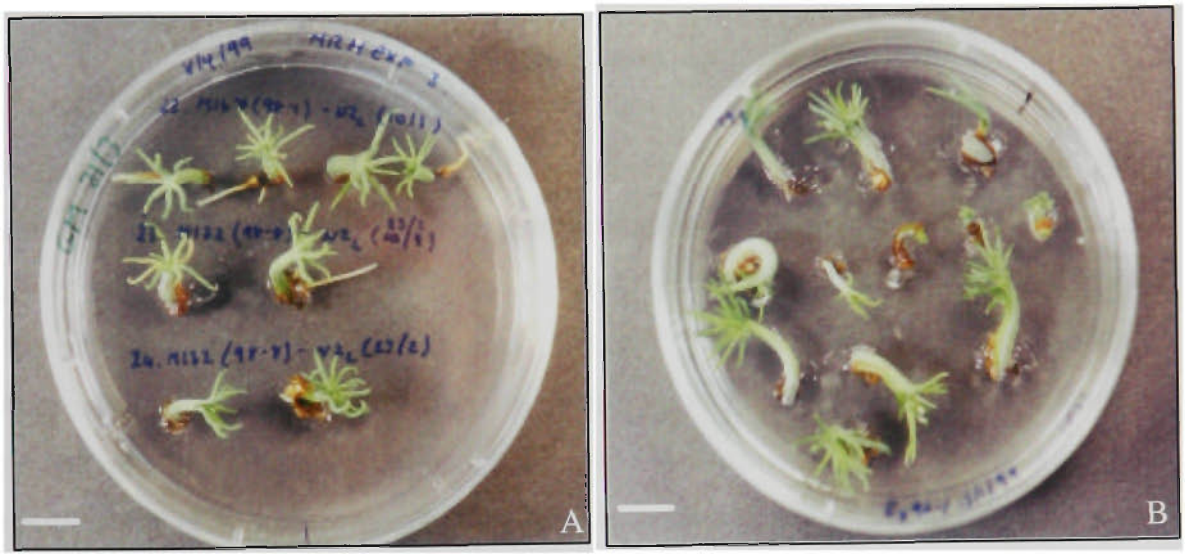


Figure 3.16 Mature somatic embryos subjected to the HRH treatment (A) and the embryos without the HRH treatment (B) (bars = 1cm)

Figure 3.17 Examples of abnormal or failed mature somatic embryos after maturation and pre-germination treatment (bar = 1cm)

Following successful germination of mature somatic embryos after the HRH treatment, all somatic embryos were subjected to this pre-germination step. Table 3.4 shows the selected mature somatic embryos removed from the maturation experiments and their progression through to survival in the glasshouse. Approximately 312 somatic embryos were removed from all of the maturation experiments and from maintenance medium. As mentioned in chapter 2 (2.3.5), the continuation of somatic embryo development occurred while the embryogenic tissue was on hormone-free maintenance medium. As a result some of the somatic embryos were also transferred to the pre-germination stage.

Table 3.4 Status of selected somatic embryos from maturation experiments

Selected	HRH	Germinated	Growool	Soil
312	119	82	78	19
%of HRH treated		68.9	65.5	16
Overall%	38.1	26.3	25	**6.1**

Of the 312 somatic embryos about 119 were subjected to HRH treatment. Of these, germination occurred in 82 somatic embryos (68.9% of the HRH treated embryos). Most embryos produced roots and good shoot development, however, some only produced continued shoot development or root development without further shoots (refer to Figure 3.18 and Figure 3.19).

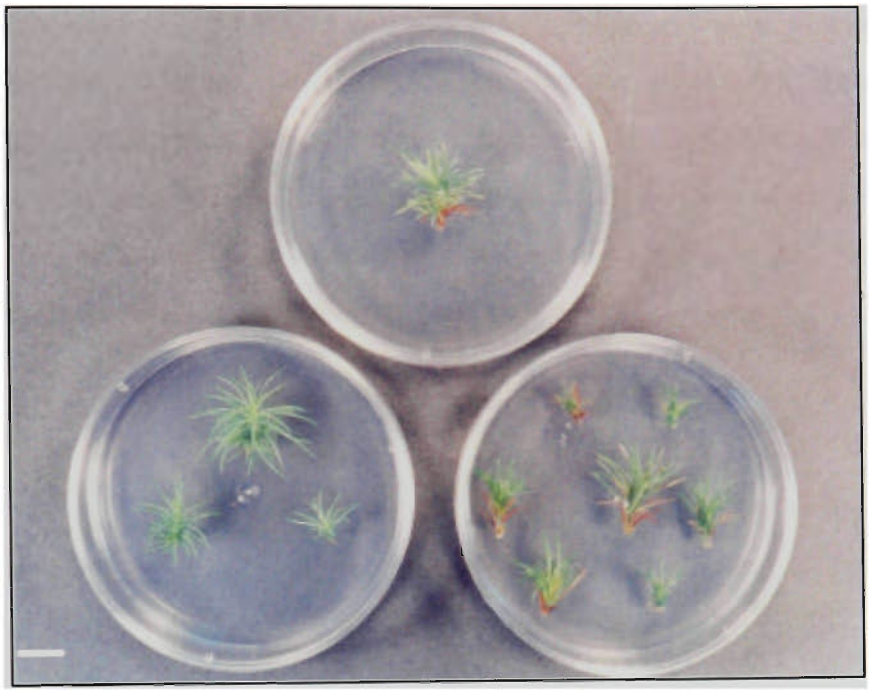


Figure 3.18 Development of 'shooty' somatic embryos without root emergence (bar = 1cm)

Figure 3.19 Somatic emblings with good shoot and root development (bar = 1cm)

Somatic emblings were transferred to jars of germination medium when good secondary shoot growth and root development had occurred. After about two months 78 somatic emblings were transferred to Growool® for acclimation in the glasshouse (refer to Table 3.4). From the 78 somatic emblings only 19 survived acclimation and were transferred to a soil/peat/perlite mixture, still in glasshouse conditions. These somatic plants showed good needle and root development and were approximately 5 to 10 centimetres in height. As Table 3.4 shows the overall conversion of the selected mature somatic embryos removed from the maturation experiments was 6.1%. This result is quite low when compared with other *Pinus* species and *Pinus radiata* studies indicating that further work is needed to optimise and improve the acclimation rate of *Pinus radiata* somatic emblings.

3.4 Discussion

The maturation of the embryogenic tissue into individual somatic embryos was the most difficult and unpredictable stage within the process. As mentioned earlier, the initiation of embryogenic tissue appears to be quite unproblematic when captured at the right time, whilst the maturation and germination of the resultant somatic embryos consistently creates challenging problems. Many components are involved in the maturation of somatic embryos. These different components can influence the level of maturation as well as the overall yield of mature somatic embryos obtained. During the course of the study, a variety of media amendments were assessed. These included ABA, Gelrite, PEG, sucrose, KCl, seed extract and basal media.

The influence of Abscisic acid on maturation

Abscisic acid or ABA is an important component of the maturation media. Its influence on the development of the somatic embryo is vital to its overall progression towards maturity. Zygotic embryos produce ABA during their development. However, excised embryos rapidly lose ABA to their environment and germinate prematurely. To overcome these problems, somatic embryos are placed onto ABA containing maturation medium (Bonga and von Aderkas 1992). The importance of ABA for the maturation of somatic embryos was recognized in earlier studies on angiosperms and has continued to be used for the development of many plant species (Ammirato 1983). Earlier studies on conifer maturation were attempted using little or no plant hormones, however, maturation was infrequent and the quality of emblings were poor (Hakman *et al.* 1985; Lu and Thorpe 1987).

ABA of varying concentrations has been used to promote maturation in conifers. Low levels (<12 μM) were found to be beneficial for some conifers. However, as research progressed higher levels above 40 μM have achieved greater yields of mature somatic embryos (Attree and Fowke 1993). Higher concentrations of ABA have been applied due to the decreasing sensitivity of somatic embryos to ABA. These levels of ABA also decrease the risk of precociously germinating somatic embryos (Attree and Fowke 1993; Misra 1994). During embryo development, ABA levels within the seed

decrease as maturation reaches the final stages. In doing so, the embryo is able to germinate (Attree and Fowke 1993; Misra 1994).

Observations from this study on *Pinus radiata* showed that ABA was most effective at the higher concentrations of 60-80 µM. Klimaszewska and Smith (1997) and Garin *et al.* (1998) used 80 µM for maturation of *Pinus strobus* somatic embryos. They also found that 60 µM ABA also enhanced the development of stage 3 or cotyledonary somatic embryos of *Pinus pinaster* and *Pinus sylvestris* (Lelu *et al.* 1999). Li and Huang (1997) observed ABA levels higher than 20mg/l (75 µM) were required to produce Stage 3 or cotyledonary somatic embryos of *Pinus taeda*. They also found that if development was arrested for whatever reason that ABA at greater than 40 mg/l (150 µM) would overcome this. Earlier studies in maturation with *Pinus radiata* somatic embryos used various concentrations up to 25 mg/l (95 µM) ABA with only small numbers of globular somatic embryos developed on media with less than 10mg/l ABA (38µ M) (Chandler and Young 1995). Studies by Smith (1994) on *Pinus radiata* also used 15mg/l (60 µM) ABA for the maturation of somatic embryos.

Spontaneous somatic embryo development was observed on embryogenic cell lines during maintenance of the tissue prior to maturation treatments (refer to 2.3.5). This was also observed in some cell lines of *Pinus caribaea* that spontaneously produced stage 1 and 2 somatic embryos (Lainé and David 1990), as well as in *Pinus patula* cell lines on ABA free medium. In this case, Jones and van Staden (1995) noticed that the embryogenic tissue rapidly senesced and embryo development rarely exceeded stage 1.

Low ABA concentrations failed to allow the full maturation of the somatic embryos. Structural variations were observed between somatic embryos that were exposed to high and low levels of ABA. Maturation at low levels of ABA (<10 µM) produced abnormally developed somatic embryos usually thin, pale green with elongated or fused cotyledons (refer to Figure 3.6). At higher ABA levels (60+ µM), somatic embryo maturation resembled the bipolar, creamy/white appearance of the zygotic embryo (refer to Figure 3.5). Jones and van Staden (1995) observed similar poor quality somatic embryos of *Pinus patula* matured on media with 5 µM and 19 µM ABA. Lainé and David (1990) noticed better differentiation of *Pinus caribaea*

somatic embryos with 15 μM ABA, but with a mixture in development of normal and abnormal quality embryos. Media with 7.5 μM ABA produced poor quality somatic embryos that failed to survive (Tremblay and Tremblay 1991a). Roberts *et al.* (1990a) observed the development of aberrant elongated green embryos (or 'shooty' embryos) at ABA levels of 1-10 μM. These aberrant embryos were inhibited at higher concentrations of ABA. Above 30 μM ABA well-organized bipolar somatic embryos developed however, from 10-20 μM ABA somatic embryos still germinated precociously (Roberts *et al.* 1990a).

The abnormal development of the mature somatic embryos exposed to low levels of ABA are most likely due to the incomplete biochemical development within the somatic embryo. ABA helps promote storage product accumulation, including storage proteins, lipids and triacylglycerols (Roberts *et al.* 1990a; Dunstan *et al.* 1991). If incomplete maturation occurs, then there is only a partial accumulation of the storage products resulting in poor quality somatic embryo maturation. Observations from maturation studies conducted over the last decade have shown that good quality somatic embryos with complete storage product accumulation are produced with higher concentrations of ABA in the maturation medium. Research from this study into *Pinus radiata* somatic embryo maturation also supports these observations.

The influence of an osmoticum on maturation

In combination with ABA, improvements in maturation frequencies have been attained for somatic embryos with an osmoticum (Find 1997). Attree and Fowke (1993) found that ABA and moisture stress were important for the maturation of many angiosperms, so it would seem logical to apply this theory to conifers. Water stress on the somatic embryos can be imposed by either elevating the osmolarity of the medium (through the use of non-permeating or permeating osmotica) or by limiting the amount of water availability from the medium (Klimaszewka and Smith 1997).

Permeating osmotica include low molecular weight compounds such as sucrose, that are able to penetrate into the cell. As a result, they create an osmotic effect with water withdrawal from the cell. However, if cultured for too long on media with these compounds cell death can eventually result due to the osmotic recovery of the cell (ie.

solutes are able to move back into the cell) (Attree and Fowke 1993; Tremblay and Tremblay 1991b). In this study, sucrose at different concentrations was analysed for the effect on the maturation of the somatic embryos. No significance was found between the overall average yield of somatic embryos at each concentration of sucrose (3% and 6%). Significance was only observed between the stage 1 somatic embryos produced on the 3% sucrose media compared to those matured on 6% sucrose media. On the 3% sucrose media higher average levels of somatic embryos were produced than on the 6% media. Based on these results either one or the other concentration would be suitable for the maturation of somatic embryos.

In almost all cases the use of higher concentrations of sucrose have been found to be more effective at inducing mature somatic embryos. Chandler and Young (1995) found that 6% sucrose was more effective than 3% sucrose on *Pinus radiata* somatic embryo differentiation. Tremblay and Tremblay (1991b) found similar results with *Picea mariana* and *Picea rubens* somatic embryos, as too Lelu *et al.* (1994) for hybrid larch. Incorporation of higher levels of sucrose, above 6%, was observed to rapidly decrease the production of somatic embryos (Tremblay and Tremblay 1991b, Lelu *et al.* 1994; Attree *et al.* 1995). This was probably due to the increased uptake of solutes after prolonged culture (Attree *et al.* 1995). The enhancing effect of higher sucrose concentrations on the development somatic embryos was suggested as being osmotic in nature (Lu and Thorpe 1987).

Other somatic embryo maturation experiments have shown good success with lower levels of sucrose (around 3%). These studies have included *Picea abies* (Filonova *et al.* 2000), *Picea pungens* (Afele *et al.* 1992) and *Pinus strobus* (Garin *et al.* 1998). There is continuing debate over the appropriate concentration of sucrose or other permeating osmotica to use within maturation media. Optimisation of each species is needed to fully obtain the benefits for maturation of somatic embryos.

Non-permeating osmotica have been shown to be more effective than the permeating osmotica. This is due to the larger sizes of the non-permeating osmotica being excluded from entry into the plant cells. As a result, a moisture stress is imposed on the cell (Attree and Fowke 1993). Polyethylene glycol or PEG has been the non-permeating osmotica of choice for many maturation studies (Attree *et al.* 1991; Gupta

et al. 1993; Misra 1994; Kong and Yeung 1995; Find 1997). PEG has been found to aid the promotion of storage products in the maturing somatic embryo as well as aiding desiccation tolerance towards germination (Attree *et al.* 1995; Kong and Yeung 1995; Find 1997). In all cases, PEG has shown to enhance the maturation of somatic embryos.

The effect of the different PEG molecular weights and each component at different concentrations on the maturation of the somatic embryos were analysed in this study. The higher the molecular weight of PEG in the maturation media, the greater the average number of somatic embryos produced. PEG 6000 was found to be significantly higher than the other PEG molecular weights of 1000 and 4000. The concentrations of PEG used showed that the lower levels (0 and 2.5%) produced significantly fewer somatic embryos than the other concentrations of 5%, 7.5% and 10%. These levels were not found to be significant from each other. Overall combinations of the molecular weights and concentrations of PEG showed that PEG 4000 or 6000 would be appropriate at the higher concentrations (10%), though in this study PEG 6000 at 10% appeared to produce the highest yields of mature somatic embryos.

Attree *et al.* (1995) observed good maturation rates of *Picea glauca* somatic embryos with higher molecular weight PEGs at around 7.5%. They found that the somatic embryos matured on PEG 1000 media were small and failed to survive. Whilst somatic embryos matured on PEG 4000 media were of better quality (Attree *et al.* 1995). Klimaszewska and Smith (1997) used PEG 4000 at 10% and PEG 8000 at 5% with the production of relatively normal *Pinus strobus* somatic embryos up to stage 2 development. However, those somatic embryos callused over and eventually arrested development. Li and Huang (1997) noted that PEG used at 5-7.5% showed better maturation of *Pinus taeda* somatic embryos than PEG at 10%, however, they only assessed the maturation capability to stage 1 somatic embryos. In this study, the stage 1 somatic embryo maturation followed the overall average numbers of somatic embryos produced (refer to Figure 3.7).

Besides elevating the osmolarity of the medium, through the use of non-permeating or permeating osmotica, enhancement of maturation of somatic embryos can be achieved

by limiting the amount of water availability from the medium. In this study, Gelrite (or gellan gum) was used in the maturation medium to produce the moisture stress. Gelrite incorporated at 0.4% and 0.6% produced significantly higher average numbers of somatic embryos than the other treatments with 0.8% and 1.0% Gelrite. The quality of the tissue was also reflected with the different concentrations of Gelrite used. Embryogenic tissue became more desiccated with increasing Gelrite concentration. This was due to the higher Gelrite concentrations limiting the water available to the somatic embryos, though based on other studies this has been noted to improve somatic embryo maturation.

Klimaszewska and Smith (1997) found that the higher the gel rigidity, the higher the average numbers of mature *Pinus strobus* somatic embryos produced. Their studies showed higher mean numbers of mature somatic embryos at 1% Gelrite. These figures did not support the results presented in this study. Differences may have been due to the basal media used or even the culture duration for maturation. Ramarosandratana *et al.* (2001) also noted that Gelrite used at 0.9% promoted better development of maritime pine somatic embryos than the other treatment which used 0.45% Gelrite. However, differences between the two media included the carbohydrate source (maltose instead of sucrose) and the inclusion of PEG. Smith (1994) showed that maturation media with either 0.45% Gelrite or 0.6% Gelrite were ideal to stimulate maturation in *Pinus radiata* and other species. Garin *et al.* (1998) found similar results with 0.45% and 0.6% Gelrite in the maturation media for *Pinus strobus* somatic embryos. It can be seen that altering the concentration of Gelrite in the maturation does influence the numbers of mature somatic embryos produced. However, the quality of the embryogenic tissue needs to be considered when decisions on the appropriate Gelrite concentrations are made. Other studies have shown that higher levels of Gelrite are capable of producing high numbers of mature somatic embryos however, in all of the maturation experiments conducted in this study quality of embryogenic tissue and production of somatic embryos was maintained mostly on the mid-range Gelrite concentrations of 0.4-0.6%.

Li and Huang (1997) found that potassium chloride (KCl) may also act as a slight osmoticum within the maturation media. The KCl effect on the maturation of *Pinus taeda* somatic embryos only enhanced the initial response or stage 1 and 2

development. In studies conducted with *Pinus radiata* somatic embryos similar effects were also observed. Significant differences were observed between the development of stage 1 somatic embryos treated with 10mM KCl and the control treatment. However, no differences were observed with the subsequent maturation stages. These results showed that KCl may have enhanced the early stages of maturation, but for further maturation to occur, higher molecular weight osmotica were probably needed. A difference noted between the results from the *Pinus radiata* studies and the results from the *Pinus taeda* studies (Li and Huang 1997) were the yields of stage 1 and 2 somatic embryos developed. The yields obtained by Li and Huang (1997) were very much lower than the yields produced from these studies (79 stage 1 somatic embryos compared to 231 stage 1 somatic embryos). Differences in yields may have been due to embryogenic cell line differences, genotype of cell line or even the culture environment. In this study only one concentration of KCl was trialled, therefore, further experiments would be necessary with different concentrations to ascertain the full effect of KCl on *Pinus radiata* somatic embryo maturation.

The influence of basal media on maturation

The influence of the basal media appears to be more pronounced in the initiation phase of somatic embryogenesis. The extra components within the maturation media such, as ABA, osmotica and gelling agents show a greater effect on the maturation ability of the somatic embryos. The differences between the basal media are within the macronutrient and micronutrient concentrations as well as the strength at which the media is used (eg. half, full, double strength). Klimaszewska and Smith (1997) noticed that basal salt formulations affected the gel strength. They found that large differences in gel hardness were observed when 0.6% Gelrite was used to solidify half-LM as compared with NZ-EMM this was due to the ion-gel interaction within the medium. Liao and Amerson (1995) found that by increasing the mineral nutrients of the media, elements that could have been limiting in the original strength medium may be provided. As well the doubling of the macronutrients encouraged the differentiation of stage 1 somatic embryos to stage 2 and 3 somatic embryos of *Pinus caribaea* (Lainé and David 1990). For the comparisons of basal media conducted for the maturation studies, only full strength concentrations were analyzed.

In the broad comparison of basal media the NZ-EMM), DCR and SH media showed the highest levels of maturation development between the stages. The NZ-EMM also showed significant differences with the overall average yield of somatic embryos compared to other well performing media, DCR and SH. The consistent maturation performance of the NZ-EMM was further shown with a direct comparison with DCR maturation media (used for most of the maturation experiments). NZ-EMM out-performed the DCR maturation media in all stages of somatic embryo development. Large yields of stage 2 somatic embryos were developed (over 400), which were significantly different to the same stage development on DCR media (120). Klimaszewska and Smith (1997) used the NZ-EMM with good maturation efficiencies in *Pinus strobus* somatic embryos. Garin *et al.* (1998) also observed good results of NZ -EMM with *Pinus strobus* somatic embryos. Smith (1994) noted that the NZ-EMM would be suitable for *Pinus radiata, Pinus taeda, Pinus elliottii* and *Pseudotsuga menziesii.*

To aid somatic embryo production researchers based the development of new nutrient media on the mineral analysis of different parts of conifer seeds (Litvay *et al.* 1981; Simola and Santanen 1990). Other analyses of conifer somatic embryos have found that they possess proteinaceous compounds that can influence the morphology of the somatic embryo (Egertsdotter and von Arnold 1995). Egertsdotter and von Arnold (1995) stimulated the somatic embryos of less developed embryogenic tissue to develop further to resemble the morphology of more advanced embryogenic tissue. This response was tested with *Pinus radiata* somatic embryos. The seed extract did not appear to stimulate any effect on the embryogenic cell lines to produce good quality somatic embryos. To exert a greater positive effect on the somatic embryos, extracts from more developed embryogenic cell lines may have been more preferable than seed extract isolated from mature, zygotic seeds.

The influence of pre-germination treatments on germination frequencies

Pre-germination treatments for somatic embryos have been found to be necessary to improve the frequency of soil-established plantlets. Most conifer somatic embryos require partial drying at high relative humidity (RH>95%) for enhancement of germination. Partial drying (HRH treatment) was first reported to affect germination of mature somatic embryos of interior spruce by Roberts *et al.* (1990b) and has been

used to promote germination in *Picea rubens* (Harry and Thorpe 1991), *Picea mariana* (Tremblay and Tremblay 1991a), *Picea glauca* (Kong and Yeung 1992) and *Picea abies* (Bozhkov *et al.* 1992). This treatment has also been used in *Pinus* species including *Pinus patula* (Jones and van Staden 1995), *Pinus taeda* (Li and Huang 1997) and *Pinus nigra* (Salajova *et al.* 1999). During the partial drying treatment, the endogenous levels of ABA decrease dramatically, therefore, on re-hydration the somatic embryos are capable of germination (Find 1997). As Roberts *et al.* (1990b) and Attree *et al.* (1991) mentioned, embryo drying enables the transition from the developmental stage to the germinative mode. In most cases the mature somatic embryos are removed from the media for the HRH treatment, however, the application of a water permeable film over the culture plate has allowed desiccation to occur whilst the mature somatic embryos are still in contact with the media (Smith 1994).

During this study, mature somatic embryos were either placed straight onto germination media without the partial drying treatment or subjected to a 10 day HRH treatment. Somatic embryos on germination media without HRH treatment were very limited in their germination. On most occasions the hypocotyl swelled, distorted and the base usually callused over. The incorporation of the HRH treatment aided the germination process. This was clearly seen with the comparisons made in the small pre-germination experiment in the results (Section 3.3.6., Table 3.3). Overall the frequency of germination from all of the somatic embryos from the maturation experiments subjected to HRH treatment were quite good (68.9%) though this result is slightly lower than observed in some other pre-germination experiments. Harry and Thorpe (1991) observed that the HRH treatment enabled the highest rates of synchronous germination with 83% - 100% germination when compared to other treatments. Find (1997) noted that no somatic embryos of *Picea abies* germinated without the HRH treatment irrespective of the maturation media. It is clear that by exposing the somatic embryos to treatments or environments that can modify their development to resemble more closely the zygotic development then the beneficial effects on the maturation and germination of the embryo can occur.

The quality of mature somatic embryos

The quality of the mature somatic embryos is vital for normal germination to occur. Hakman and von Arnold (1988) recommended that stage 3 somatic embryos were developmentally mature enough to be transferred to the germination phase. This stage of somatic embryo development usually resembled the zygotic embryo development with a bipolar, torpedo shaped appearance. Harry and Thorpe (1991) observed that the selection of vigorous, creamy-white somatic embryos were critical for germination and plantlet establishment. Tremblay and Tremblay (1991b) also noticed the critical selection of appropriate mature embryos of *Picea mariana* and *Picea rubens* for germination success. Lower stage (stage 2) or precociously germinated somatic embryos are unable to germinate properly resulting in abnormally developed emblings with fused or irregular cotyledons, long thin hypocotyls and no roots. This is most likely due to the incomplete accumulation of storage products, proteins and carbohydrates necessary for the conversion to the plantlet stage of development. Findings from this study on *Pinus radiata* somatic embryos observed similar physical variations in the germination phase. Immaturely and precociously developed somatic embryos germinated abnormally or failed to germinate even after HRH treatment. Besides the typical germinated embryos (roots and shoots) there were incompletely germinated embryos with no roots, but good cotyledon development and hypocotyl elongation and 'shooty' embryos with no cotyledon development. These observations were supported by Park *et al.* (1998) with germination of both normal mature somatic embryos and precociously germinating embryos developing in different ways. They noted that 22% germinated normally (roots and shoots), 36% produced germinants without roots *26*% were misshapen and 16% died. Liao and Amerson (1995) also noticed different embryo germination patterns with *Pinus elliottii* somatic embryos. In most cases the incomplete germination of the somatic embryos was from mature somatic embryos that were developmentally immature. Aitken-Christie (1995) recognised that somatic embryo quality was often a constraint with 50 – 60% of mature somatic embryos below standard.

The establishment of somatic emblings to soil is another area where further work is necessary. Lelu *et al.* (1994) noted that plant regeneration from somatic embryos was still a problem with only a few reports indicating the establishment of plants in soil. From our studies the conversion rate to soil was of somatic emblings from overall

mature somatic embryos was 6.1%. From the HRH treated somatic embryos, the conversion rate was 16%. These rates are low when compared to other studies. Khlifi and Tremblay (1995) observed conversion rates varying from 9% to 40% for *Picea mariana* plantlets and Hristoforoglu *et al.* (1995) noted a 70% survival rate of *Abies alba* plantlets to the greenhouse. Good conversion rates for *Pinus radiata* emblings have been achieved by Aitken-Christie (1995) noting that emblings for field trials are well into the thousands.

Summation

A variety of factors have been found to affect the maturation of *Pinus radiata* somatic embryos. In this study, the different treatments to improve maturation affected either the total number of mature somatic embryos produced as well as the distribution of embryos among the different developmental stages. ABA levels were assessed to observe the optimum concentration for good quality mature somatic embryos. Permeating and non-permeating osmotica were analyzed in the presence and absence of ABA to enhance maturation frequencies. Observations of mature somatic embryo development on basal media were also undertaken to investigate the basic medium which would fulfill the maturation requirements and other media additives such as seed extract and potassium chloride were assessed on their maturation capabilities. Overall, somatic embryo maturation occurred to some degree on most maturation media analyzed. The optimal factors included the NZ-EMM, higher ABA levels (60-80 μM) and Gelrite concentrations between 0.4% and 0.6%.

Pre-germination treatments of partial drying enabled the somatic embryos to follow the natural process of development after which germination was easily achieved. The success of the germination was determined by the quality of the somatic embryo produced from the maturation treatments. Good quality mature somatic embryos, resembling white/creamy, torpedo shaped zygotic embryos produced normal germinants with good root and cotyledon development. More often than not, immature stage somatic embryos (stage 2) or precociously germinated embryos either failed to germinate or germinated abnormally with either no cotyledons or no roots. Observations made from this study showed the importance of the quality of the mature somatic embryos for germination success, as well as the practicality of the partial drying treatment for *Pinus radiata* somatic embryos. Further optimisation

work is required for good plantlet conversion frequencies to the soil environment, before commercialisation of the technology of somatic embryogenesis can considered.

CHAPTER 4

General Discussion

4.1 Introduction

Pinus radiata has had a significant impact on the Australian Forestry industry since its introduction for plantation timber in the mid-1800s. The recognition of its importance as a plantation species has lead to the development of extensive breeding programs aimed at gaining greater genetic and economic benefits (Chandler and Young 1995). Traditional breeding methods using open-pollinated and control pollinated seed have been overtaken by vegetative propagation methods (field or *in vitro* based) which substantially reduce the time required to assess the success of the genetic characteristics. Each vegetative propagation method (cuttings, fascicle cuttings and micropropagation) has different advantages and disadvantages, making each attractive to the forestry industry (Menzies and Aimers-Halliday 1997).

One vegetative propagation technique, somatic embryogenesis, has been seen as an attractive option for mass propagation due to its high multiplication rates and rejuvenation potential. Another aspect of somatic embryogenesis is its use in conjunction with genetic engineering (Merkle and Dean 2000). The genetic manipulation of embryogenic tissue offers the opportunity to exploit genes that may influence developmental pathways for productivity gains (Smith *et al.* 1994). Somatic embryogenesis of plants has been used since the late 1950s and improvements in techniques continue to make progress in cereals, angiosperms and conifers (Steward 1958; Tisserat *et al.* 1977; Vasil 1985; Tautorus *et al.* 1991). Since the first researchers reported somatic embryogenesis in conifers in 1985, many coniferous species have successfully produced embryogenic tissue and somatic emblings (Hakman *et al.* 1985; Chalupa 1985; Tautorus *et al.* 1991).

Somatic embryogenesis of *Pinus radiata* has been achieved to a certain degree in New Zealand, but its application commercially has not been fully achieved. Field trials of

somatic emblings have been established with research into their viability and success currently being conducted (Smith *et al.* 1994; Ropati. (2001) pers. comm.). For integration into a breeding program, somatic embryogenic techniques need to be cost effective, work for most genotypes and provide large numbers of uniform plants per genotype without the problems of aging (Aimers-Halliday *et al.* 1997).

The aim of this research was to provide a greater understanding of the factors that influence the initiation of embryogenic tissue within the somatic embryogenesis process, in order to optimise the conditions for initiation. This work was also undertaken to further understand the maturation and germination conditions necessary to optimise the efficiencies of conversion from embryogenic tissue through to established emblings. Another critical objective was to assess the potential of somatic embryogenesis in Australia using different growing locations, genotypes and collection dates.

The explants used in this study were sourced mainly from the Maryvale region in Victoria (all three years). However, in the third year the material was also collected from New Norfolk (Tasmania) and Albury (New South Wales).

4.2 Main Findings

This study describes the optimisation of somatic embryogenesis in *Pinus radiata*. The main findings of this study were:

(a) Initiation of embryogenic tissue of *Pinus radiata* was readily achievable. Many factors influenced the frequencies of embryogenic tissue initiation. These included genetic background, media types, media components and explant maturity (Chapter 2).

(b) The explant maturity provided the greatest influence on embryogenic tissue initiation. The immature, pre-cotyledonary zygotic embryo was the preferred explant. This

developmental stage occurred at different dates from year to year, but seemed to roughly correspond to mid-December through to early January (Chapter 2).

(c) The overall frequency of somatic embryogenesis, irrespective of the varied conditions of initiation, over the three years of explants from the Maryvale region were 7.13% (1995-96), 4.33% (1996-97) and 14.03% (1997-98) (Chapter 2).

(d) Initiation frequencies differed between the regions of New Norfolk and Albury. Again the explant maturity influenced the peak period of embryogenic tissue initiation. The difference of several weeks observed between the two regions would provide a beneficial extension of the explant competency period to obtain a greater amount of embryogenic tissue by using clones grown in different locations (Chapter 2).

(e) Maturation of somatic embryos was achieved after analysing a variety of media components. The factors that provided the greatest influence on maturation quality and yields were the New Zealand maturation medium (NZ-EMM), abscisic acid (ABA) at 60 or 80 μM and Gelrite concentrations at 0.4% or 0.6% (Chapter 3).

(f) Pre-germination treatment of partial drying at high relative humidity (HRH) was necessary to promote germination. The germination rate of HRH treated somatic embryos was 68.9% (Chapter 3).

(g) Quality of the mature somatic embryo was vital for successful germination. Germination of poor quality or immature somatic embryos was usually incomplete or abnormal, whilst germination of good quality somatic embryos was normal and complete (Chapter 3).

(h) Establishment of somatic emblings to glasshouse conditions was low. The overall conversion rate of germination of mature somatic embryos from the maturation

experiments was 6.1%. However, the conversion frequency from the HRH treated somatic embryos was 16% (Chapter 3).

4.3 Somatic embryogenesis in *Pinus radiata*

4.3.1 Initiation of embryogenic tissue

Initiation of embryogenic tissue from *Pinus* species has previously been considered quite low. Compared with *Picea* species difficulties have still remained in the process to combat the low efficiencies of initiation (Li et *al.* 1996). In this study the initiation of embryogenic tissue from *Pinus radiata* was successfully achieved and appeared not to be a limiting factor. Overall averages from initiation experiments conducted from 1995/96 through to 1997/98 varied from 4.33% to 14.03% (Chapter 2). When the explant was at its most responsive, initiation frequencies were consistently over 20% and were observed as high as 73.87%. These initiation frequencies appear to be higher than those obtained from most other *Pinus* species. Early reports showed lower frequencies of 0.32% and 2.6% (Lainé and David 1990; Jones *et al.* 1993). However, as methods become more refined frequencies have varied from 1.1% to higher than 10% (Li *et al.* 1998; Lelu *et al.* 1999).

Explant selection was vital to the initiation success of *Pinus radiata* in this study. The explant to show the highest initiation response was the pre-cotyledonary zygotic embryo. The other stages of zygotic development were analysed for their embryogenic potential, but a response was not achieved. If too immature (globular stage) embryos were used, no embryogenic suspensor masses developed. If too mature (post-cotyledonary) were used, germination of the zygotic embryo tended to occur. These responses have also been observed with other *Pinus* species (Becwar *et al.* 1991; Jones and van Staden 1995; Garin *et al.* 1998). In order to obtain the right stage of embryo development for embryogenic tissue initiation regular or even daily collections of immature cones would have to be conducted throughout the time period of zygotic embryo competency. Other explants, such as cotyledons, hypocotyls and mature tissue were not investigated in this study as good initiation rates were achieved with the immature zygotic embryo. However, it

would be beneficial to study the efficiency of embryogenic tissue initiation on other explants in order to obtain a greater source of explant tissue.

Embryogenic tissue initiation was variable between genotypes with some more highly embryogenic than others (Chapter 2). This response has been found in all cases of somatic embryogenesis with conifers, so its occurrence in this study was consistent. A correlated efficiency of initiation in individual genotypes was evident in one clone (38031) over the three years of initiation experiments (Chapter 2). This observation of lack of consistency over time has not been shown in any other study, as most initiation experiments were carried out over only one or two seasons. Definite genetic barriers exist between genotypes in the initiation of embryogenic tissue, making selection of superior clones difficult for breeding programs. Unfortunately, the highly desired, selected genotypes are not necessarily highly embryogenic genotypes. This is due to the differences in the genetics of the clone and the expectation that some will contribute more embryogenic tissue than others. Though in order to obtain an established culture from most genotypes only a few seeds would probably be needed to produce embryogenic tissue. However, the quality of the embryogenic tissue would have to be assessed.

Observations were made on the locality effects of embryogenic tissue initiation frequencies of *Pinus radiata.* Differences emerged in the initiation frequencies between New Norfolk (Tasmania) and Albury (New South Wales). Maryvale explants were comparable in their initiation frequencies with the New Norfolk explants and the Albury explants (Chapter 2). Differences were observed in the explant maturity with Albury explants maturer than New Norfolk explants collected around the same time. Influences of location have been observed with other species, such as *Pinus sylvestris* (Keinonen-Mettälä *et al.* 1996). These are largely due to the environmental factors on the growth of the zygotic embryo (Jones and van Staden 1995; Keinonen- Mettälä *et al.* 1996). The differences in explant maturity observed in this study could be exploited to provide a longer period of responsive explants for embryogenic tissue initiation.

Initiation of embryogenic tissue varied according to the embryogenic tissue capture medium used. In this study a wide variety of media and components were analysed for an influence on initiation of embryogenic tissue. The basal medium, DCR was found to be the most influential in the earlier experiments, but in the final year of initiation experiments a clear superiority and consistency of embryogenic frequencies was found with the FR's standard embryogenic tissue capture medium (NZ). Another medium to perform consistently well in this study over the initiation period of 1997-98 was the 6% seed extract with 2, 4-D and BA. This media produced similar results to the NZ initiation medium (Chapter 2). For a more comprehensive comparison, these two media need to be further analyzed. The improved effect of this medium may have been due to the seed extract acting in combination with the plant growth regulators. The inclusion of the seed extract may have supplied the zygotic embryo with nutrients that were otherwise missing from the standard medium (Litvay *et al.* 1981; Teasdale *et al.* 1986). Even though it is common to include plant growth regulators in the initiation medium, this study showed that they are not necessary. Comparisons between media with and without plant growth regulators showed similar results (Chapter 2). The initiation frequencies of NZ medium, without hormones and the seed extract medium, with plant growth regulators were significantly different from the other media tested in this study.

It is reasonable to conclude that many factors affected the initiation of embryogenic tissue from *Pinus radiata* in this study. The three main factors - explant development, genetic background and media/component type - each played a role in the frequency of embryogenic tissue development. Of all the variables tested, explant development provided the greatest influence on initiation frequencies. Many factors influence the initiation of embryogenic tissue from this study, areas have been identified that could further aid the optimisation of somatic embryogenesis in *Pinus radiata*.

4.3.2 Maturation and conversion of mature somatic embryos to somatic emblings

In this study maturation of embryogenic tissue was undertaken, with many variables investigated (Chapter 3). These variables included ABA, permeating and non-permeating osmotica, such as sucrose, PEG and Gelrite, basal media and other media additives such

as seed extract and KCl. Some level of maturation was attained in most experiments, however, early stage development was predominantly observed in this study (Chapter 3). Later stage (stages 3 and 4) mature somatic embryos developed throughout the experiments but to a lesser extent and with less consistency. This was the main problem observed in the maturation experiments of this study. Therefore, further work is necessary to provide reliable conversions to good quality somatic embryos.

ABA has been recognized to promote the maturation of somatic embryos and this was also observed in this study. Various concentrations of ABA were screened to observe the appropriate level at which optimal maturation occurred. Higher levels of ABA (60 to 80 μM) were shown to produce good quality somatic embryos (stage 3) that resembled the white, bullet shaped zygotic embryo (Chapter 3). Lower levels of ABA produced abnormal somatic embryos that germinated precociously. These observations were in accordance with many other ABA experiments conducted on conifers (Lainé and David 1990; Tremblay and Tremblay 1991, Jones and van Staden 1995). This study confirmed that, the higher levels of ABA (60 to 80 μM) were the most effective concentrations to promote maturation of *Pinus radiata* somatic embryos.

In this study, observations of maturation frequencies were also undertaken using different types of osmotica. Moisture stress, in the form of permeating and non-permeating osmotica were applied in combination with ABA. Permeating osmotica such as sucrose has been found useful in the maturation of other conifer species (Lu and Thorpe 1987; Tremblay and Tremblay 1991b, Afele *et al.* 1992; Lelu *et al.* 1994; Garin *et al.* 1998; Filonova *et al.* 2000). Higher levels of sucrose have been found to be more influential than lower levels but this did not appear to be the case in this study where 3% and 6% sucrose gave similar results. On the other hand, non-permeating osmotica such as PEG showed a greater influence on the maturation of *Pinus radiata* somatic embryos (Chapter 3). Various concentrations and molecular weights of PEG were analysed in this study with PEG 6000 at 10% appearing to produce the highest yields of mature somatic embryos. Overall concentrations of PEG did not significantly differ in effect at the mid to high ranges of 5%, 7.5% and 10%. These figures have been observed in other

experiments with PEG 4000 or 6000 used at 5-10% producing good quality somatic embryos (Attree *et al.* 1995; Klimaszewska and Smith 1997; Li and Huang 1997).

The availability of water from the medium to the embryogenic tissue was analysed for its effect on maturation frequencies. Altering the Gelrite concentration within the maturation medium can influence the number of somatic embryos produced. In this study, the Gelrite concentration found to produce good yields of somatic embryos as well as good quality tissue, was either 0.4% or 0.6% (Chapter 3). These figures show consistency with other findings of maturation of *Pinus strobus* somatic embryos (Klimaszewska and Smith 1997; Garin *et al.* 1997). Higher levels of Gelrite were seen to desiccate the embryogenic tissue, which then tended to produce no mature somatic embryos (Chapter 3).

Another component within the media that was analysed was the influence of KCl on early stage somatic embryos. In this study, the inclusion of KCl was seen to enhance the early stage maturation of *Pinus radiata* somatic embryos (Chapter 3). No further differences were observed in the later stages of maturation. Li and Huang (1997) also noted similar observations with *Pinus taeda* somatic embryos. The addition of seed extract from *Pinus radiata* seeds was seen to have the opposite effect with no influence whatsoever on maturation frequencies (Chapter 3). Possibly to exert a more positive effect on the somatic embryos, extracts from more developed embryogenic cell lines may have been more beneficial than the extracts isolated from mature, zygotic seeds.

Along with individual components, the basal media was also analysed for its influence on maturation frequencies. During this study DCR, SH and the NZ-EMM showed the highest levels of maturation (Chapter 3). Significant differences were observed with NZ-EMM showing higher average yields of somatic embryos compared to the rest of the basal media. Further consistency in results of maturation frequencies with NZ-EMM were also observed in a direct comparison with DCR media. The quality of the mature somatic embryos was also better on the NZ-EMM than the DCR medium. Klimaszewska

and Smith (1997) used the NZ-EMM with good maturation efficiencies of *Pinus strobus* somatic embryos, as did Garin *et al.* (1998).

The pre-germination treatment of HRH, as developed by Roberts *et al.* (1990b) aided germination of the mature somatic embryos obtained throughout the maturation experiments (Chapter 3). This step was needed to enable the somatic embryo to follow the natural zygotic embryo development process, where the embryo goes through a desiccation step prior to germination. Success with this pre-treatment was quite high compared to the untreated mature somatic embryos that went straight to germination medium. It was also successful in aiding germination with older mature somatic embryos that did not occur without the HRH treatment.

The success of germination was dependent on the quality of the mature somatic embryo. Stage 3 somatic embryos are thought to be developmentally mature enough to be transferred to the germination phase (Hakman and von Arnold 1988). Observations in this study showed that lower stage somatic embryos (stage 2) or precociously germinated somatic embryos, mostly from the maintenance medium or maturation medium with low levels of ABA, were unable to germinate normally (Chapter 3). On these occasions, incomplete germination usually occurred with either a lack of roots or a lack of shoots. Generally, stage 3 somatic embryos, resembling white, bullet shaped zygotic embryos, germinated normally with good root and shoot development (Chapter 3). Similar physical variations have been observed in other studies (Tremblay and Tremblay 1991; Liao and Amerson 1995; Park *et al.* 1998).

Establishment of the somatic emblings to glasshouse conditions was quite low in this study compared to results with other conifers (Hristoforoglu *et al.* 1995; Khlifi and Tremblay 1995). Conversions of 6.1% of surviving emblings to soil from the total number of selected mature somatic embryos were observed in this study compared to survival rates of 9% to 40% for *Picea mariana* emblings and 70% for *Abies alba* emblings (Hristoforoglu *et al.* 1995; Khlifi and Tremblay 1995). Much higher acclimation rates, closer to 100%, would be necessary to provide a target more applicable

for commercialisation. Clearly further work is necessary to obtain more consistent acclimation of *Pinus radiata* somatic emblings.

Overall, somatic embryo maturation occurred to some degree on most maturation media. In this study the different treatments to improve maturation affected the overall yield of somatic embryos as well as the distribution of embryos among the different developmental stages. Of the components tested, specific recommendations for further somatic embryo maturation experiments would be to use NZ-EMM, higher levels of ABA (60-80 µM), Gelrite concentrations at 0.4% to 0.6% and PEG 4000 or 6000 at either 7.5% or 10%. Pre-germination treatments have shown that partial drying by HRH treatment is necessary to promote germination of the mature somatic embryo, therefore, incorporation of this step would be vital.

4.4 Future work

Even though the initiation of *Pinus radiata* embryogenic tissue was easily achieved when the most responsive explant and the right selection of genotype and media were used, a few other factors need to be further investigated to ensure more consistent and reliable initiation. Further work is needed to investigate the application of somatic embryogenesis on different types of explants, including cotyledons and mature needle tissue. Smith (1999) has demonstrated the rejuvenation of *Pinus radiata* with mature tissue through somatic embryogenesis. The use of mature tissue for somatic embryogenic systems would be the ideal source of material to overcome the barriers of rejuvenation. However, information on methods from Smith's experiments is unavailable (Merkle and Dean 2000). The use of other explants has also been demonstrated in other conifers but further optimisation is needed.

Other locations of sources of material should also be further investigated. The differences observed between the plantation growing regions suggested that explant material could be sourced from a variety of areas to extend the competent period of the

zygotic embryo. Therefore, all plantation areas should be surveyed to make available a greater amount of embryogenic tissue over a longer time period.

Genes have been identified relating to lignin production, male cone formation and specific biochemical pathways in conifers. However, the identification of the gene or genes that relate to the differences between genotypes and their responsiveness to somatic embryogenesis would enable the genetic barriers to be diminished. This would encourage the wider application of somatic embryogenesis, as it would be seen as eliminating one of the limitations of incorporation of somatic embryogenesis into a breeding program.

Further factors which might increase the frequency of maturation of *Pinus radiata* somatic embryos also remain to be examined. The use of extracts from highly embryogenic cell lines onto lower developed cell lines would be worth investigating. A clear stimulation of embryogenic potential was observed in the research conducted by Egertsdotter and von Arnold (1995) on *Picea abies*, therefore, a study with *Pinus radiata* embryogenic cell lines might provide beneficial results.

The maturation of the somatic embryos was observed to stall after a period of time on some maturation media in several cases for no apparent reason. Often the somatic embryos would mature to about stage 2 development and not proceed any further. Factors such as transfer to fresh medium and individual somatic embryo culturing failed to push the maturation process along. Li and Huang. (1997) observed a similar problem and partially overcame the arrest with the addition of ABA at concentrations higher than 120 μM. This hormone stress, or any other possible stress, such as the unavailability of water, could be further examined if an arrest in maturation occurred. Hopefully the application of the stress would allow the maturation process to continue.

4.5 Conclusion

Somatic embryogenesis of *Pinus radiata* was achieved throughout the study. This study has highlighted the complexities of obtaining consistent and reliable initiation of

embryogenic tissue and the subsequent conversion into mature somatic embryos and established emblings. The study has aided the optimisation of somatic embryogenesis in *Pinus radiata* by identifying the areas of initiation and maturation that influence their respective frequencies. As well as identifying areas throughout the whole process that need further investigation.

As yet there is no evidence that somatic embryogenesis of *Pinus radiata* is more cost effective than other propagation techniques. Also, to date no commercial production of somatic embryos have been undertaken for direct planting due to several reasons. These include the genotypic variations in the ability to produce embryogenic tissue. This has limited the further selection of superior genotypes and the potential for somatic embryogenesis to produce unlimited numbers of somatic embryos and plants from each cell line has not been realised (Menzies and Aimers-Halliday 1997; Merkle and Dean 2000; Smith (2001) pers. comm.; Sutton (2001) pers. comm.).

Direct sowing of encapsulated somatic embryos has not met the cost effectiveness and ease needed for integration into commercial breeding programs the only avenue for use of this technology on *Pinus radiata* in the forestry industry would be through a hybrid system. A hybrid system that uses two vegetative propagation methods may overcome some of the barriers to commercial use of this technology. If genetic barriers, proper selection of media and components, embryo quality issues and better survival rates of emblings could be further addressed then somatic embryogenesis of *Pinus radiata* would be an attractive addition to forestry breeding programs.

On a broad view, this study has provided several factors that can aid the implementation of somatic embryogenesis in an Australian breeding program. The technique of somatic embryogenesis has been shown to work on Australian breeding clones that possess different standards of embryogenic tissue forming capacity. Even the low embryogenic initiation clones were able to produce a small amount of tissue mainly during the peak initiation time. The study has also shown that material sourced from different regions within Australia is capable of producing embryogenic tissue and mature plants. This

indicates that the technique of somatic embryogenesis is readily applicable to Australian conditions.

The accessibility of different conifer media from commercial suppliers and the formulations within the public domain would enable somatic embryogenesis to be applied to *Pinus radiata* within Australian forestry companies. The New Zealand developed media has shown to be reproducible with Australian breeding clones therefore, there would be no need to repeat these experiments as this study has shown reliable results. Small-scale laboratories at the appropriate forestry sites could be set up to ascertain the suitability of somatic embryogenesis with *Pinus* species. Superior clones from individual companies could be initiated into tissue culture and maintained. This could be achieved either through cryopreservation or subsequent subculture of embryogenic tissue. Though based on cost effectiveness, time and longevity of testing superior clone quality, cryopreservation would probably be more suitable. The cost of laboratory set up, employment and tissue initiation and maintenance would need to be appraised by individual companies for its cost effectiveness. However, based on a two person team and the use of 'low cost' tissue culture equipment that is becoming more available, the initiation of embryogenic tissue, maintenance, maturation and plantlet conversion could be achievable. An investment into this technology over long term would prove very promising.

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Appendix 1: Composition of plant cell culture basal salt mixtures used in the initiation and maturation experiments.

Constituent	Concentration (mg/l)						
	DCR	**LP**	**WPM**	**GD**	**SHR6**	**MS**	**P21**
Ammonium Nitrate	400	1200	400			1650	
Ammonium Phosphate Monobasic					300		230
Ammonium Sulfate				200			
Boric Acid	6.2	0.63	6.2	5	5	6.2	3.72
Calcium Chloride Anhydrous	64.14	180	72.5	1150	151	332.2	
Calcium nitrate	386.31		386				354
Cobalt Chloride.$6H_2O$	0.025	0.002		0.2	0.1	0.025	0.02
Cupric Sulfate.$5H_2O$	0.25	0.025	0.25	0.2	0.2	0.025	1.25
Na_2EDTA	37.3		37.3	40	20	37.26	
Ferric Chloride.$6H_2O$							1.35
FeNaEDTA		19.24			19.24		19.24
Ferrous Sulfate.$7H_2O$	27.8		27.8	30	15	27.8	
Magnesium Sulfate	180.7	370	180.7	250	195.4	180.7	
Manganese Chloride.$4H_2O$	22.3						
Manganese Sulfate.H_2O		1.84	22.3	20	10	16.9	1.69
Molybdic Acid(Sodium salt).$2H_2O$	0.25	0.025	0.25	0.2	0.1	0.25	0.024
Nickel Chloride.$6H_2O$	0.025						0.02
Potassium Chloride				300			
Potassium Iodide	0.83	0.75		1	1	0.83	0.33
Potassium Nitrate	340	1900		1000	2500	1900	128
Potassium Sulfate			990			170	174
Potassium Phosphate Monobasic	170	340	170				
Sodium Chloride							5.8
Sodium Nitrate							
Sodium Phosphate monobasic.$2H_2O$				100			
Sodium Phosphate dibasic				30			
Zinc Sulfate.$7H_2O$	8.6	2.3	8.6	1	1	8.6	5.75
$ZnNa_2EDTA$		4.05					

Appendix 1 cont'

	DCR	LP	WPM	GD	SHR6	MS	P21
Myo-inositol		100			250	100	100
Thiamine HCl	1	5	1	5	5	0.1	
Nicotinic acid	0.5	2	0.5	5	5	0.5	
Pyridoxine HCl	0.5	1	0.5	0.5	0.5	0.5	
Arginine-HCl		0.01			1000		
Glycine		2	2			2	
Glutamine		0.4					
L-alanine		0.05					
L-cysteine.HCl		0.02					
L-phenylalanine		0.01					
L-tyrosine		0.01					
L-leucine		0.01					

Appendix 2: Composition of plant cell culture basal salt mixtures of NZ medium used in the initiation and maturation experiments (Smith 1994).

Constituent	Concentration (mg/l)				
	NZ	SEM	EDM	NZ-EMM	NZ-EMMb
Ammonium Phosphate Monobasic	112	225	225	225	225
Boric Acid	4	8	8	8	8
Calcium Chloride Anhydrous	12.5	25	25	25	25
Cobalt Chloride.$6H_2O$	0.1	0.2	0.2	0.2	0.2
Cupric Sulfate.$5H_2O$	1.2	2.4	2.4	2.4	2.4
Ferrous Sulfate.$7H_2O$	15	30	30	30	30
Magnesium Sulfate	200	400	400	400	400
Manganese Sulfate.H_2O	1.8	3.6	3.6	3.6	3.6
Molybdic Acid(Sodium salt).$2H_2O$	0.1	0.2	0.2	0.2	0.2
Na_2EDTA	20	40	40	40	40
Potassium Iodide	0.5	1	1	1	1
Potassium Nitrate	715	1431	1431	1431	1431
Sodium Nitrate	155	310	310	310	310
Zinc Sulfate.$7H_2O$	12.5	25	25	25	25
Myo-inositol	500	1000	1000	1000	1000
Thiamine HCl	1	2	2	2	2
Nicotinic acid	1	2	2	2	2
Pyridoxine HCl	0.1	0.2	0.2	0.2	0.2
Arginine-HCl		35	175	35	35
Glutamine		110	550	365	365
Asparagine		105	510	105	105
Alanine		2.5	12.5	50	50
Citrulline		4.9	25	98	98
Ornithine		4.7	24	95	95
Lysine		3.4	17	68	68
Proline		2.1	11	44	44

NZ: Standard embryogenic tissue capture medium, SEM:Embryogenic maintenance medium,

EDM: Embryo development medium, NZ-EMM/EMMb: Embryo maturation medium

Appendix 3: Embryogenic cell lines from Maryvale initiation experiments 1997-98.

Cell line	Clone	Date	Medium	Cell line	Clone	Date	Medium
M1	35114	5/1	HM	M51	38012	12/1	SE
M2	35114	5/1	HM	M52	38012	12/1	DR
M3	36014	5/1	DR	M53	38012	12/1	SE+
M4	36014	5/1	SE	M54	38012	12/1	HM
M5	36014	5/1	SE	M55	36014	12/1	SE
M6	36014	5/1	SE	M57	36014	12/1	DR
M7	36014	5/1	SE	M58	36014	12/1	SE+
M8	36014	5/1	SE	M59	36014	12/1	SE+
M9	38031	5/1	AC	M60	36014	12/1	AC
M10	38031	5/1	NZ	M61	36014	12/1	NZ
M11	36013	5/1	SE+	M62	36014	12/1	NZ
M12	36013	5/1	SE+	M63	36055	20/1	HM
M13	36013	5/1	NZ	M64	36055	20/1	HM
M14	36013	5/1	NZ	M65	36055	20/1	SE+
M15	36013	5/1	SE+	M66	36055	20/1	SE+
M16	36013	5/1	SE	M67	36055	20/1	SE+
M17	36055	5/1	HM	M68	36055	20/1	AC
M18	36055	5/1	HM	M69	36055	20/1	AC
M19	36055	5/1	HM	M70	36055	20/1	NZ
M20	36055	5/1	HM	M71	36055	20/1	NZ
M21	36055	5/1	SE	M72	36055	20/1	NZ
M22	36055	5/1	AC	M73	36055	20/1	NZ
M23	36055	5/1	NZ	M74	36055	20/1	NZ
M24	36055	5/1	NZ	M75	36055	20/1	NZ
M25	36055	5/1	DR	M76	36055	20/1	SE
M26	36055	5/1	DR	M77	38031	20/1	SE
M27	36055	5/1	DR	M78	38031	20/1	SE
M28	36055	5/1	DR	M79	38031	20/1	DR
M29	36055	5/1	SE+	M80	38031	20/1	HM
M30	36055	5/1	SE+	M81	38031	20/1	HM
M31	36055	5/1	SE+	M82	38031	20/1	HM
M32	38012	5/1	SE	M83	38002	20/1	HM
M33	38012	5/1	SE+	M84	38031	20/1	AC
M34	38012	5/1	SE+	M85	38031	20/1	NZ
M35	38012	5/1	HM	M86	38031	20/1	NZ
M36	38012	5/1	HM	M87	38031	20/1	NZ
M37	38012	5/1	HM	M88	38031	20/1	NZ
M38	38012	5/1	AC	M89	38031	20/1	NZ
M39	38012	5/1	NZ	M90	38031	20/1	NZ
M40	38012	5/1	NZ	M91	38031	20/1	NZ
M41	38031	12/1	NZ	M92	38031	20/1	NZ
M42	36055	12/1	SE+	M93	38002	20/1	NZ
M43	36055	12/1	SE+	M94	38002	20/1	SE+
M44	36055	12/1	SE	M95	35114	20/1	AC
M45	35114	12/1	AC	M96	35114	20/1	AC
M46	35114	12/1	NZ	M97	35114	20/1	HM
M47	35114	12/1	NZ	M98	35114	20/1	NZ
M48	38002	12/1	AC	M92	38031	20/1	NZ
M49	38002	12/1	SE+	M93	38002	20/1	NZ
M50	38002	12/1	HM	M94	38002	20/1	SE+

Appendix 3 cont'

Cell line	Clone	Date	Media	Cell line	Clone	Date	Media
M95	35114	20/1	AC	M144	35114	12/1	NZ
M96	35114	20/1	AC	M145	36013	22/12	AC
M97	35114	20/1	HM	M146	36014	22/12	AC
M98	35114	20/1	NZ	M147	36014	22/12	SE+
M99	35114	20/1	SE+	M148	36014	22/12	DR
M100	38012	20/1	HM	M149	36055	15/12	DR
M101	38012	20/1	AC	M150	36055	15/12	NZ
M102	38012	20/1	AC	M151	38002	15/12	NZ
M103	38012	20/1	AC	M152	38012	15/12	AC
M104	38012	20/1	AC	M153	36057	15/12	NZ
M105	38012	20/1	AC	M154	36055	20/1	AC
M106	38012	20/1	AC	M155	38031	20/1	AC
M107	36057	20/1	SE	M156	38031	20/1	AC
M108	36057	20/1	AC	M157	38031	20/1	AC
M109	36057	20/1	AC	M158	38031	20/1	DR
M110	36057	20/1	AC	M159	38031	20/1	SE
M111	36057	20/1	NZ	M160	38031	20/1	AC
M112	36057	20/1	NZ	M161	38031	20/1	DR
M113	36057	20/1	NZ	M162	38031	20/1	DR
M114	36057	20/1	HM	M163	38031	20/1	AC
M115	36057	20/1	DR	M164	36014	20/1	DR
M116	36057	20/1	SE+	M165	36057	27/1	NZ
M117	36057	20/1	SE+	M166	38012	27/1	SE+
M118	36014	20/1	NZ	M167	36014	27/1	SE+
M119	36014	20/1	NZ	M168	36014	27/1	HM
M120	36014	20/1	NZ	M169	38031	27/1	NZ
M121	36014	20/1	NZ	M170	38031	27/1	NZ
M122	36014	20/1	NZ	M171	38002	27/1	DR
M123	36014	20/1	NZ	M172	38002	27/1	AC
M124	36014	20/1	NZ	M173	38002	27/1	SE+
M125	36014	20/1	NZ	M174	38002	27/1	SE+
M126	36014	20/1	SE+	M175	38002	27/1	SE+
M127	36014	20/1	SE+	M176	38002	27/1	SE+
M128	36014	20/1	SE+	M177	38002	27/1	SE+
M129	36014	20/1	SE+	M178	36055	27/1	NZ
M130	36014	20/1	SE+	M179	36055	27/1	SE+
M131	36014	20/1	SE+	M180	36055	27/1	SE+
M132	36014	20/1	SE+	M181	36055	27/1	NZ
M133	36014	20/1	SE+	M182	36055	27/1	NZ
M134	36014	20/1	AC	M183	36055	27/1	AC
M135	36014	20/1	AC	M184	38031	12/1	DR
M136	36014	20/1	AC	M185	36055	20/1	AC
M137	36014	20/1	AC	M186	36055	20/1	AC
M138	36014	20/1	AC	M187	36055	20/1	AC
M139	36014	20/1	DR	M188	36014	27/1	HM
M140	36014	20/1	SE	M189	36014	27/1	HM
M141	36014	20/1	SE	M190	36014	27/1	HM
M142	36014	20/1	HM	M191	36055	27/1	SE
M143	36014	20/1	HM	M192	36057	27/1	NZ

AC- Activated Charcoal; DR- DCR medium; HM- Hormone medium; SE- Seed extract medium; SE+ - Seed extract medium with hormones.

Appendix 4: Embryogenic cell lines from Albury and New Norfolk (N.N) initiation experiments 1997-98.

Albury	Clone	Date	Media	N. N.	Clone	Date	Media	N. N.	Clone	Date	Media
A1	11x70	19/12	DR	T1	4	24/12	DR	T26	3	24/12	AC
A2	11x70	19/12	NZ	T2	4	24/12	AC	T27	3	24/12	AC
A3	33x31	19/12	DR	T3	4	8/1	NZ	T28	3	24/12	NZ
A4	33x31	19/12	NZ	T4	4	8/1	NZ	T29	3	24/12	NZ
A5	9x31	19/12	DR	T5	4	8/1	AC	T30	3	24/12	NZ
A6	9x31	19/12	DR	T6	4	8/1	AC	T31	3	24/12	NZ
A7	9x31	19/12	DR	T7	4	8/1	AC	T32	3	8/1	NZ
A8	9x31	19/12	NZ	T8	4	8/1	NZ	T33	3	8/1	NZ
A9	9x31	19/12	AC	T9	1	24/12	DR	T34	3	8/1	DR
A10	9x31	5/1	AC	T10	1	24/12	DR	T35	3	8/1	DR
A11	9x31	5/1	AC	T11	1	24/12	DR	T36	3	8/1	AC
A12	9x31	5/1	AC	T12	1	24/12	DR	T37	3	8/1	CA
A13	9x31	5/1	AC	T13	1	24/12	DR	T38	3	8/1	AC
A14	9x31	5/1	NZ	T14	1	8/1	DR	T39	3	8/1	AC
A15	9x31	5/1	NZ	T15	1	8/1	DR	T40	3	8/1	AC
A16	11x35	18/1	AC	T16	1	8/1	DR	T41	3	8/1	AC
A17	11x35	18/1	NZ	T17	1	8/1	AC	T42	3	8/1	NZ
				T18	1	8/1	AC	T43	3	8/1	NZ
				T19	1	8/1	AC	T44	4	24/1	AC
				T20	1	8/1	NZ	T45	3	6/2	NZ
				T21	3	24/12	DR				
				T22	3	24/12	DR				
				T23	3	24/12	AC				
				T24	3	24/12	AC				
				T25	3	24/12	AC				