Somatic embryogenesis in southern and tropical pine species: Loblolly pine (*Pinus taeda*), Longleaf pine (*P. palustris*) and Oocarpa pine (*P. oocarpa*)

By

Alejandra Monserrath Lara-Chavez

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In Forestry

Ulrika Egertsdotter

Barry Flinn

Thomas Fox

Scott Merkle

Jose Alexander Elvir

August 11th 2010

Danville, VA

Keywords: mineral analysis, basal medium, initiation media, extrusion, maturation, germination, gene expression

Somatic embryogenesis in southern and tropical pine species: Loblolly pine (*Pinus taeda*), Longleaf pine (*P. palustris*) and Oocarpa pine (*P. oocarpa*)

Alejandra M. Lara-Chavez

ABSTRACT

The focus of the current project was to establish an improved and reliable protocol for somatic embryogenesis in 1) *Pinus taeda* and *Pinus palustris*; pine species of high value for commercial applications and germplasm conservation supported through breeding programs at The Virginia Department of Forestry (Chapter III); and 2) *Pinus oocarpa;* an economically important pine species in the southern half of Mexico and Central America (Chapter IV). In addition, 3) the study of the gene expression analysis of developmental stages of both somatic and zygotic embryos of *P. taeda* was compared to assess developmental fidelity at the molecular level (Chapter V). By testing four basal media combined with different plant growth regulator combinations, we have established stable embryogenic cultures from high value families of *P. taeda* and *P. palustris* using the tissue culture medium 1218 (Pullman et al 2005) in combination with an auxin:citokinin ratio at 10:5 (molar). However, optimization of the protocols for the maturation and further conversion of somatic embryos to seedlings requires further work. For *P. oocarpa*, we hypothesized that somatic embryo induction may be possible by mimicking natural seed-embryo developmental conditions, and a new tissue culture medium, based on the mineral content of the seed nutritive tissue (megagametophyte), was formulated. The novel culture medium (PO) was tested in combination with different plant growth regulator concentrations for the initiation of somatic embryogenesis from fresh collections of *P. oocarpa* immature zygotic embryos. Additionally, the established embryogenic cultures were able to mature and germinate, to our knowledge resulting in the first report of the production of *P. oocarpa* plantlets through somatic embryogenesis. PO medium also has the potential to be used successfully for other tropical pine species which today suffer from suboptimal somatic embryogenesis protocols. The fundamental study of molecular regulation of embryo development showed that under the current maturation conditions, *P. taeda* somatic embryos were temporally similar in gene expression to zygotic embryos of the

same species. However, potentially important differences were found and results could potentially explain the low germination success during somatic embryogenesis. More research is still needed to further explore the natural environment of developing seed embryos to improve the somatic embryogenesis protocols and to enable full integration of this clonal propagation method into the breeding programs for pines.

Dedication

I dedicate this dissertation to my grandparents

Angel Manuel Chávez (100 years-old) and Angélica Trinidad Matute (1916-1986)

My parents

Rigoberto Lara and Elsa Cristina Chávez

And my friend always, my brother

Angel R. Lara

Acknowledgements

Table of Contents

List of Abbreviations

½	Half strength
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ADM	desiccation inside six-well plates
aRNA	amplified RNA
BA	6-Benzylaminopurine
CLV	CLAVATA
dw	dry weight
EC	early cotyledonary
ESM	embryogenic suspensor mass
g	gram
HD-Zip I	Homeodomain–leucine zipper class I
IVT	*in vitro* transcription
Kin	kinetin
LEA	late embryogenesis abundant
LC	late cotyledonary
mg/L	milligram per liter
MG	megagametophyte
MS	Murashige and Skoog medium
NAA	naphthalene acetic acid
NPN	non-protein nitrogen
PCIB	2-(p-chlorophenoxy)-2-methylpropionic acid
PE	pro-embryo
PGR	plant growth regulators
PO	*Pinus oocarpa* medium
RG	round/globular
RPN1	26S proteasome subunit S2
SAM	shoot apical meristem
SE	standard error

SD	standard deviation
v/v	volume/volume
WS	whole seed without seed coat
w/v	weight/volume
AL, TX, 1218, DS	culture media
AL, TX, 1218, plant growth regulator-free	plant growth regulators corresponding to previous media (italics)

List of Figures

xv

List of Tables

1 Justification

1.1.   Introduction and motivation for propagation of conifers

Conifers are an ancient group of gymnosperm woody plants, which include two classes: *Gynkgoatae* and *Pinatae*. The latter class encompasses the *Pinaceae* family that due to the broad distribution, number of species and biomass accumulation includes the most economically important conifer species in the northern hemisphere (Fernando *et al.* 2005). The *Pinaceae* family contains a total of eleven genera, with *Pinus* the largest and most economically important. Representatives of the *Pinus* genus can be found from Alaska to Nicaragua, from Scandinavia to North Africa, and from Siberia to Sumatra (Farjon *et al.* 1993). *Pinus* provides some of the most valuable species for timber and paper production; in addition it is used to protect watersheds, thus providing habitats for wildlife and for the construction of shelterbelts. Almost all of the species are evergreens, having needle- to scale-like leaves (Alden 1997).

The United States (US) produces the largest volume of forest products worldwide (FAO 2003), and forest land in the US occupies approximately 33 % (Coleman 2002). The softwoods in the US represent 57 % of the volume of growing stock, and this volume increased from 432 to 492 billion cubic feet between 1953 and 2002 (Coleman 2002). By 2000, the two predominant species of growing stock were Douglas fir [*Pseudotsuga menziessi* (Mir.) Franco] and loblolly pine (*Pinus taeda* L) (FAO 2005). Additionally, other pines, including Longleaf pine (*P. palustris* Mill*)* represent an ecological base of the forest ecosystem and the diverse collection of species that are being threatened by deforestation. A similar situation is found for Oocarpa pine (*P. oocarpa* Schiede). Despite its economic importance in tropical countries in Central and South America, little attention has been paid to germplasm development and conservation. In the current project, we study somatic embryogenesis of three pine species: *P. taeda*, *P. palustris* and *P. oocarpa*. More detail and information about these three species will be found in Chapters III and IV, respectively.

1.2.   The need for the clonal propagation of conifers

Clonal propagation of forest tree species is an alternative for a low-cost and large-scale production of genetically improved germplasm from the breeding programs. Currently, the most used clonal propagation technique is by rooted cuttings. This method has been effective

for hardwood species as compared to pine species; however a common problem is that the rooting ability of the explants decrease with the age of the mother plant (Park 2002). The identification of the superior clones is thus often not completed before the mother plants have become too old to be propagated efficiently by rooted cuttings. In forest tree breeding programs, clonal propagation methods are required to fully capture the genetic gains. Clonal propagation allows for production of a consistent and uniform plant crop from desirable, elite clones. In the future, clonal propagation techniques are needed for propagation of genetically-improved trees carrying introduced desirable genes. For the forest industry, there are four desirable traits in the trees that could be obtained through the introduction of foreign genes: modified lignin content (Merkle and Dean 2000), herbicide and insect tolerance, flowering control and disease tolerance (Sedjo 2004; Trontin *et al.* 2007). Thus, in order to accomplish these goals in the tree breeding programs, alternative approaches for mass clonal propagation are needed. *In vitro* tissue culture techniques offer a suitable tool with the potential for development of economically feasible protocols for the production of large numbers of identical and genetically improved plantlets. In tree species, clonal propagation methods through *in vitro* tissue culture techniques, such as shoot culture, organogenesis and somatic embryogenesis, have been established for many species.

1.3   *In vitro* tissue culture techniques in conifers

In conifer species, two *in vitro* tissue culture techniques (organogenesis and somatic embryogenesis) have shown potential for large scale germplasm multiplication. Micropropagation by either technique offers the rapid and short-time production of reforestation stock, supporting the production of woody biomass and the maintenance of elite and exotic germplasm (Giri *et al.* 2004). The success of these techniques depends on the genetic background of the species, the type and age of the explants, and the nutritional and environmental conditions (Giri *et al.* 2004). Somatic embryogenesis offers the following benefits over organogenesis methods: 1) potential for scale-up through multiplication in bioreactors; 2) somatic embryogenesis tissue can be cryopreseved and stored at a very low temperature, thus maintaining the juvenility of the tissue; and 3) somatic embryogenesis tissue also provides a suitable target tissue for gene transfer (Merkle and Dean 2000). Gene transfer to somatic embryogenesis tissue is facilitated by: 1) the presence of a large amount of competent cells in the culture for transformation (actively dividing cells); 2) the fact that the somatic

2

embryo is usually derived from a single cell thus chimeras are rarely observed; and 3) recovery of transgenic plants from somatic embryos is considerably short (Trontin *et al.* 2007).

1.4   Somatic embryogenesis in tree breeding programs

According to Park (2002) somatic embryogenesis can be implemented in tree breeding programs via the following processes: 1) parents are selected from breeding populations; 2) controlled crosses are made between parents; 3) a small quantity of high quality seeds from these crosses are used for the initiation of somatic embryogenesis; 4) initiated lines are proliferated and cryopreserved; 5) somatic seedlings are tested in the field in replicated clonal field trials; 6) the best performing clones are selected; and 6) their corresponding embryogenic tissue are thawed from cryopreservation (Step 4) and multiplied; somatic seedlings from the best performing clones are used as planting stock for deployment in clonal plantations.

Somatic embryogenesis culture protocols have been generated for most commercially important softwood and hardwood species (Park 2002). However, some of the bottleneck factors hampering the large scale production of plants by somatic embryogenesis in commercial species (especially pine) are: 1) low initiation rates; 2) low culture survival; 3) low maturation, germination and conversion rates and; 4) slow initial growth of somatic seedlings (Pullman and Johnson 2002).

Numerous efforts and research studies have been conducted in order to improve each of the above steps for somatic embryogenesis in pine species. Major emphasis has been placed on the improvement of somatic embryogenesis initiation rates especially in *P. taeda* (Pullman and Johnson 2002; Pullman *et al.* 2005a; Pullman *et al.* 2006). Additionally, these research studies showed that early stage, immature zygotic embryos perform better as initial explants compared to more mature zygotic embryos, and vegetative tissues (Stasolla *et al.* 2003). However, immature zygotic embryos have a developmental window of competence for somatic embryogenesis induction of one to two weeks, thereby decreasing the availability of initial material and the timeframe for successful somatic embryogenesis initiation. Few attempts have been made to use mature zygotic embryos for the initiation of somatic embryogenesis. With seeds of *Pinus strobus,* a maximum initiation rate of 2.7 % was obtained, but without the conversion of embryos to plantlets (Finer *et al.* 1989). Mature zygotic embryos from *P. taeda,* resulted in a high frequency (17 %) of formation of embryogenic tissue (Tang *et al.* 2001) but

attempts to reproduce this procedure in other pine species have not yet succeeded. Recently, the use of a cold treatment on mature tissues (shoot apical meristems) of *Pinus patula* (Malabadi and Van Staden 2005b), *P. kesiya* (Malabadi *et al.* 2005) and *P. roxburghii* (Malabadi and Nataraja 2007b) stimulated the initiation of somatic embryo cultures. These results indicated that successful somatic embryogenesis induction from mature zygotic embryos and mature tissues are difficult, but possible, providing an opportunity to alleviate the disadvantages of using immature zygotic embryos as explants.

Then at the maturation stage improvements of percentage rate have been done with the use of ABA and osmotic agents that mimic the natural conditions of the zygotic embryos. Additionally, the identification and use of molecular markers could help to differentiate the quality of somatic embryos throughout the maturation process by identifying those that closely resemble their zygotic counterparts. These results could help guide the development of potential modification of the culture medium towards a more efficient protocol suitable for the large scale production of seedlings.

1.5. Hypothesis, aims and objectives

1.5.1 Initiation of somatic embryogenesis in *Pinus taeda* and *Pinus palustris* with different basal media and plant growth regulators

Hypothesis: The initiation of somatic embryogenesis in these two species can be enhanced by combining both currently used culture media and plant growth regulator concentrations, with the use of immature zygotic embryos.

Aim: Test different basal culture media and plant growth regulators for the initiation of somatic embryogenesis of *P. taeda* and *P. palustris*.

Objectives:

a. Test four basal media for the initiation of somatic embryogenesis from families of interest of *P. taeda* and *P. palustris* through the use of immature zygotic embryos.

b. Test different plant growth regulators and concentrations for the initiation of somatic embryogenesis from families of interest of *P. taeda* and *P. palustris*.

c. Test different immature zygotic embryo collections for the initiation of somatic embryogenesis from families of interest of *P. taeda* and *P. palustris*.

d. Develop a maturation and germination protocol for the conversion of *P. palustris* and *P. taeda* somatic embryos to maturation phase.

e. Test of ABA, maltose and Phytagel for the maturation and subsequent germination of the *P. taeda* cell line J8189.

1.5.2 Initiation of somatic embryogenesis from immature zygotic embryos of *Pinus oocarpa*

Hypothesis: The development of a culture medium, through a determination of the mineral content in *P. oocarpa* seeds will enhance the development of *P. oocarpa* somatic embryos, compared to somatic embryo development using standard basal media.

Aim: Development of a complete protocol for the *in vitro* propagation of *P. oocarpa* through somatic embryogenesis.

Objectives:

a. Analyze the mineral content of mature seed of *P. oocarpa* for the determination of the major and minor element content.

b. Develop an initiation medium which fulfills the requirement of the embryos based on the analysis of the mineral content for *P. oocarpa*.

c. Develop a protocol for the initiation of *P. oocarpa* somatic embryogenesis through the use of immature zygotic embryos.

d. Develop a maturation and germination media for the conversion of *P. oocarpa* somatic embryos to somatic seedlings.

1.5.3 Analysis of gene expression patterns during somatic and zygotic embryo of *Pinus taeda*

Hypothesis: The expression of genes related to embryo development and maturation is similar in somatic and zygotic embryos in *P. taeda*, and gene expression analyses can be used to assess somatic embryo developmental fidelity.

Aim: Describe the expression of genes of interest in four stages of maturing somatic and zygotic embryos of *P. taeda*.

Objectives:

a. Study the expression of six genes of interest at four stages of maturing somatic embryos of *P. taeda* using real time PCR.

b. Study the expression of six genes of interest in four similar stages of maturing zygotic embryos of *P. taeda* using real time PCR.

c. Comparison of the expression of genes of interest between somatic and zygotic embryos in each of the stages under study.

2   Literature Review

2.1   Zygotic embryogenesis in higher plants

In higher plants, seed plants are comprised of angiosperms and gymnosperms. The floral anatomy of angiosperms and gymnosperms is different and is associated with different processes during fertilization, and the formation of embryos and seeds. The seeds of angiosperms are formed inside the fruit. In contrast, the seeds of gymnosperms are usually formed inside cones, but are not enclosed in a fruit. A recent study on gene expression patterns during embryogenesis in gymnosperms and angiosperms revealed large similarities, as well as differences in expression of genes related to the specificity of the mechanisms of embryogenesis for each taxon (Cairney *et al.* 2006). In both taxa, zygotic embryogenesis begins with the formation of the zygote that passes through a sequence of characteristic stages specific to each taxa, ending with the formation of a mature embryo (von Arnold *et al.* 2002). Therefore, "embryo" is defined as the earliest multicellular stage of an organism followed by the development of the specific structures or organs distinguishing each species (Gray 2005). In general, the process of embryo formation is divided into three phases: morphogenesis, maturation and desiccation (West and Harada 1993). Before describing the somatic embryogenesis process, the basic principles of zygotic embryo development will be outlined for angiosperms and gymnosperms.

2.1.1   Angiosperm zygotic embryo development

During fertilization, the angiosperm megagametophyte containing two synergids and three antipodals, ie two polar nuclei and the egg, it is fertilized by two sperm (Meinke 1991). One fuses with two polar nuclei to produce the triploid endosperm, and the other fuses with the egg to produce the zygote composed of the zygotic embryo and the embryo suspensor (Meinke 1991). This process is known as double fertilization because both sperms fuse with the megagametophyte during embryogenesis. Here we use *Arabidopsis* as a model to explain embryogenesis in angiosperms (Dodeman *et al.* 1997) (Fig. 2-1). After the zygote has formed (Fig. 2-1A), it divides into two asymmetrical cells, the apical cell and the basal cell [the 1-cell stage (Fig. 2-1B)]. The apical cell forms the embryo proper and the basal cell forms the suspensor. The embryo divides to produce a radially symmetrical ball of two groups of four-cells (Fig. 2-1C) and then eight cells above the suspensor which is called the octant stage (Fig.

2-1D). At the octant stage the cells of the embryo proper divides into two tiers, in which the upper most layer gives rise to the shoot apical meristem and most of the cotyledon primordial while the lower layer will develop into the hypocotyl, embryogenic root and the upper cells of the root apical meristem. Meanwhile, the suspensor divides longitudinally. Then at the globular stage (Fig. 2-1E), transverse divisions produce a single outer layer of cells termed the protoderm and will give rise to the epidermis of the plant. Additionally, the hypophysis, defined as the uppermost cell of the suspensor adjacent to the pro-embryo, becomes part of the embryo and form the quiescent centre and central root cap. At the late globular stage, the ground meristem and procambium initiate and specific pattern formation takes place (Fig. 2-1E). Then the heart stage is characterized by the multiplication of cells in the upper half of the embryo which will give rise to cotyledon primordia; this is the first appearance of bilateral symmetry (Fig. 2-1F). Additionally, at this stage the three fundamental tissues of the seedlings, the epidermis, ground tissue and vascular tissue have been established, and the suspensor senesces and is no longer functional. During the torpedo stage, embryos further develop the root and shoot apices and growth of the cotyledons (Fig. 2-1G). Then the cotyledons bend forming the known "walking-stick" or "bent cotyledon" stage (Fig. 2-1H). Thus after the embryo pass throughout these pro-embryo stages, pattern formation and organ expansion and maturation is followed by a quiescent state prior to seed dispersal (Dodeman *et al.* 1997) (Fig. 2-1).

In angiosperms, the endosperm (triploid issue) provides the nutrients to the embryo for its development. Additionally, the suspensor appears to be related to the nutritional demands of the specific species. Depending on the species, the suspensor has different sizes and shapes but later on, the suspensor always degenerates as the embryo matures (Yeung and Meinke 1993).

2.1.2 Gymnosperm zygotic embryo development

Pine species are mostly monoecious, having the male cones (pollen) located in the mid-crown less vigorous branches, and the female (ovule) cones located in the upper, more vigorous branches in the same tree, and wind pollinated (anemophily) (Fernando *et al.* 2005). The cone scales each carry two ovules which usually develop into seeds on their ventral surfaces. Hence these scales are also referred to as ovuliferous scales or seed scales (Fernando *et al.* 2005).
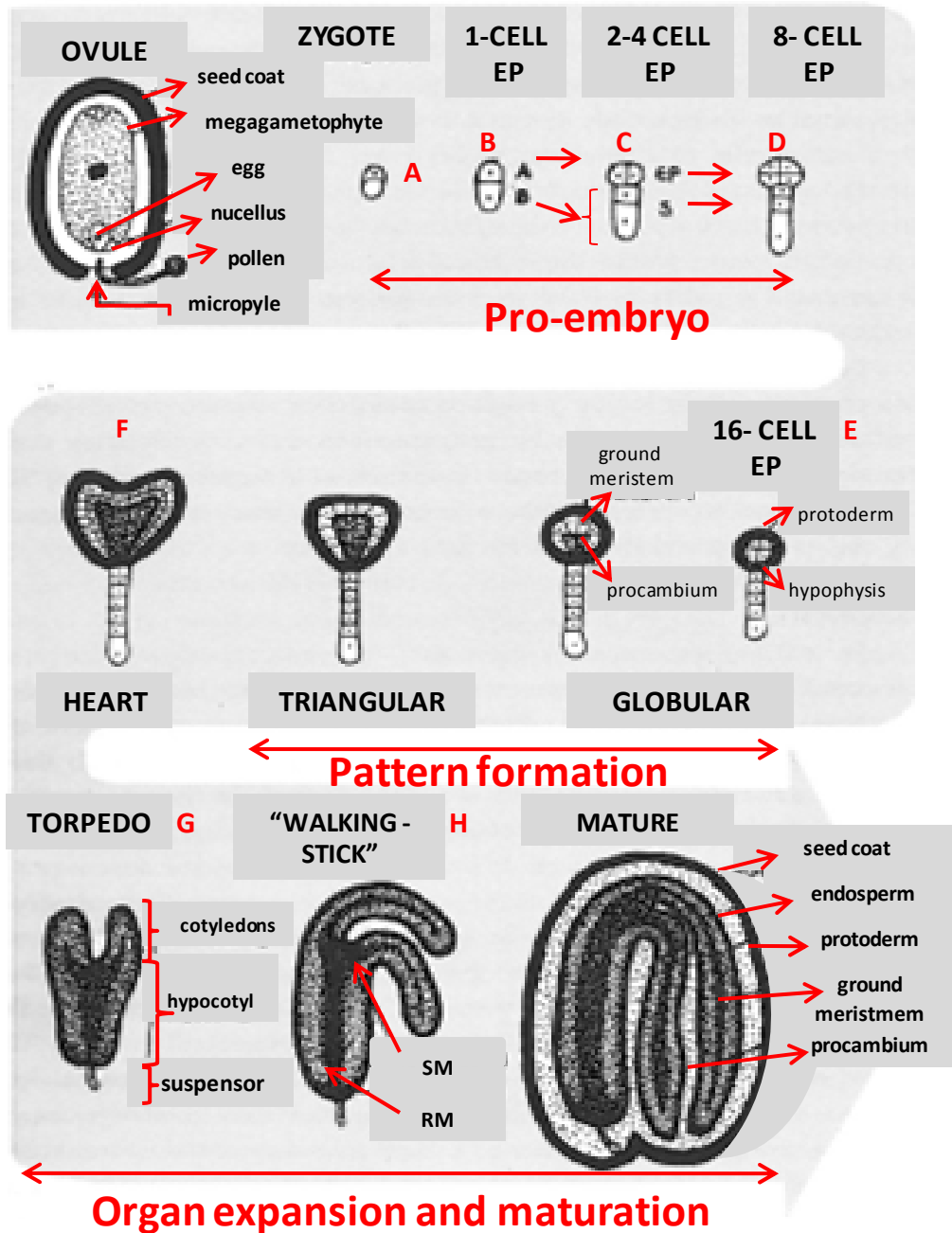
Figure 2-1. Schematic overview of angiosperm embryo development as exemplified by *Arabidopsis thaliana* (see text for details). Abbreviations: A= apical; B= basal cell; EP= embryo proper; and S= suspensor; SM= shoot meristem; RM= root meristem. Modified from Egertsdotter (1996) with permission.

Briefly pollination and fertilization occurs as follow (Pallardy 2008a): pollination take place when a sugary exudation "pollination drop" is deposited in the lower end of the ovule (micropyle). Then the pollen is incorporated into the fluid and sucked into the micropyle until it makes contact with the nucellus tissue. The pollen germinates forming pollen tubes that grow toward the nucellus; each pollen tube elongates, and its generative cell divides to form a stalk cell and the body cell. Then, the body cell divides to form two sperm, where the larger one fuses with the egg nucleus within an archegonia to complete fertilization; meanwhile, the smaller one (sperm) will degenerate.

In gymnosperms, embryos arise from a single fertilization event within the ovule, creating a diploid embryo which develops within a haploid female gametophyte, or megagametophyte. The megagametophyte provides continuity and directness of food supply needed for embryo development (Durzan and Chalupa 1968). Additionally, as postulated previously for angiosperm embryogenesis, the suspensor plays a role in providing the nutrients and growth regulators that modulate embryonic development.

Gymnosperm embryo formation has been divided into three phases: 1) pro-embryogeny; 2) early embryogeny; and 3) late embryogeny (Filonova *et al.* 2000; Raghavan and Sharma 1995). In conifers, the pro-embryogeny phase is characterized by a period of free nuclear division (Filonova *et al.* 2000; Raghavan and Sharma 1995). Pro-embryogeny includes the stages prior to suspensor elongation. It begins after the fertilized egg nucleus divides into two, then four, free nuclei contained within a dense neocytoplasm (dense region of cytoplasm), arranged in the base of the archegonium sac (Fig. 2-2A). Additionally, after division into two tiers, the primary upper tier and the primary embryonal tier are formed, each with four nuclei (Fig. 2-2B). Then, internal division gives rise to two tiers of four cells each: the lower tiers constitute the embryogenial tier, followed by the suspensor tier and the uppermost tier (Fig. 2-2C). The latter tier (uppermost) is often dysfunctional and degenerates. The suspensor tier undergoes abortive meristematic activity to form the primary suspensor but usually is dysfunctional; meanwhile the four upper cells of the embryonal tier elongate to form a functional suspensor tier and the lower four form the embryonal mass (Fig. 2-2D).

10
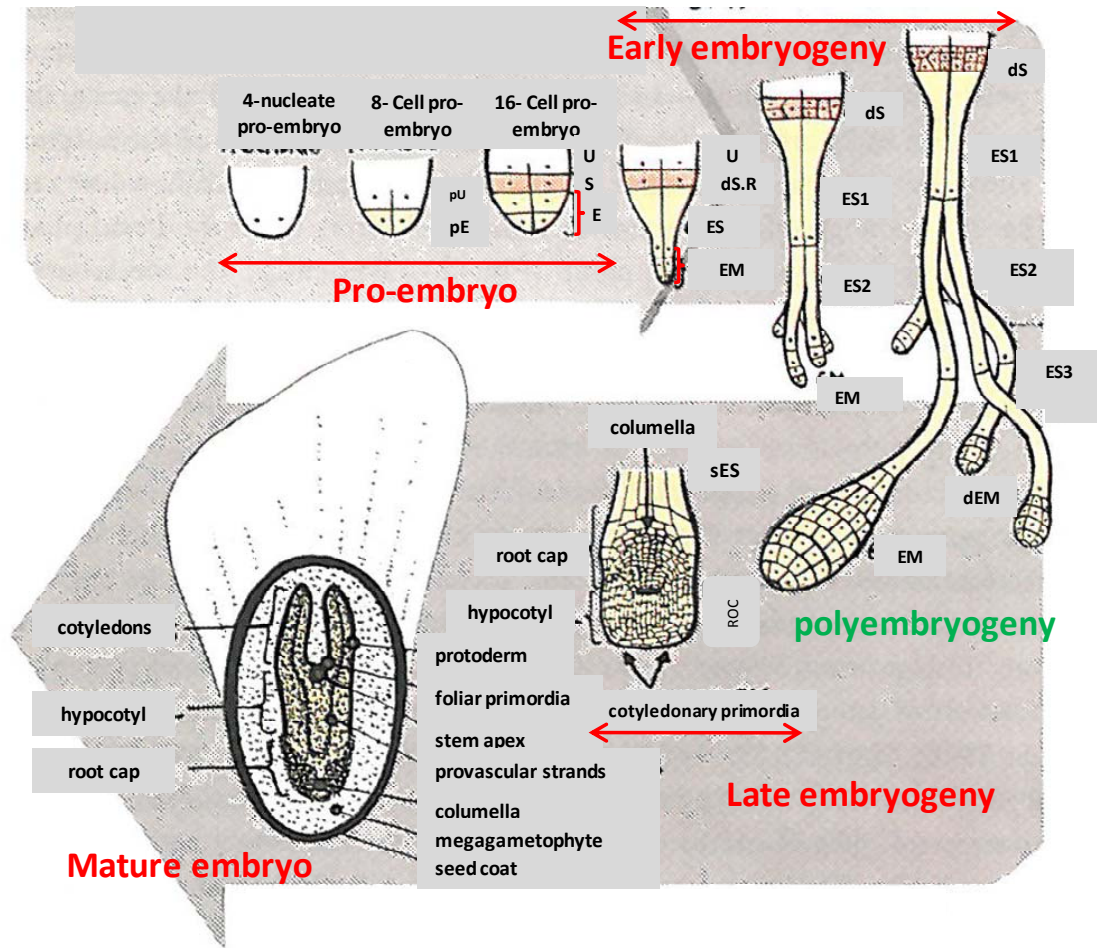
Figure 2-2. Schematic overview of gymnosperm embryo development. Abbreviations: pU= primary upper tier; pE= primary embryonal tier; E= embryogenial tier; U= upper tier; S= suspensor tier; EM= embryonal mass; dS o R= dysfunctional suspensor tier, also known as "Rosette tier"; Es= embryonal suspensor tier; dEM= degenerative embryo mass; sEs= secondary embryonal suspensor cell. Modified from Egertsdotter (1996) with permission.

Early embryogeny includes the stages beginning with the elongation of the suspensor but before the development of the root meristem. The suspensor is formed by elongating cells (Es1 and Es2) from the embryonal functional suspensor cells (Fig. 2-2E), which elongates while carrying the embryonic cell at its tip (Es3) (Fig. 2-2F). In gymnosperms, polyembryony is a common condition found in almost all of the genera, and could be explained by: 1) the fertilization of more than one egg and the development of multiple embryos (simple polyembryony), or 2) the development of multiple embryos from a single zygote (cleavage polyembryony) (Filonova *et al.* 2000; Raghavan and Sharma 1995). In both cases, only the most vigorous embryo will develop to maturity while the other will degenerate.

11

Finally, late embryogeny includes stages with the development of the root and the shoot meristems, and further enlargement of the mature embryo (Filonova *et al.* 2000) (Fig. 2-2G). The enlargement of the embryo gradually fills the corrosion cavity inside the megagametophyte. After the period of cell division and histodifferentiation, the embryo enters the maturation stage where it expands, accumulates storage products and prepares for a desiccation period before germination (Filonova *et al.* 2000).

Based on the previous discussions, the major differences in zygotic embryogenesis between the taxa are:

1. The double fertilization event in angiosperms which give rise to a diploid zygote and triploid endosperm. In contrast, in gymnosperms there is a single fertilization which gives rise to a diploid embryo and a haploid megagametophyte, with smaller male gamete degeneration.

2. In angiosperms, the endosperm (nutritional tissue) forms after fertilization has taken place. Meanwhile in gymnosperms, the megagametophyte (nutritional tissue) originates from the megaspore and develops prior to fertilization.

3. In angiosperms, embryo development is through globular, heart, torpedo and cotyledonary stages for dicots; globular, coleoptiles and juvenile embryo stages for monocots. In gymnosperms, the stages are classified as pro-embryogeny, early and late embryogeny. In both angiosperms and gymnosperms, zygotic embryo formation is followed by a period of maturation.

4. A common feature in gymnosperms is the free nuclei stage which has not been observed in angiosperms.

5. In angiosperms, a single embryo is formed enclosed in the endosperm. In gymnosperms, specifically in *Pinaceae,* the phenomenon of polyembryony is observed in which several embryos can form within a single gametophyte through either simple or cleavage polyembryony.

2.2   Non-zygotic embryo development in higher plants

The production of embryos from a non-zygotic source was first observed in carrot (Stewart *et al.* 1958)*,* where structures described as "embroids" formed when pieces of tissue were cultured

in culture medium. Since then, carrot has been used as model species for the study of somatic embryogenesis, due to its capacity for embryo formation from almost any of its tissues. However, embryogenesis has been observed and induced in different species of both angiosperm and gymnosperm.

The classification of non-zygotic embryogenesis can be done based on the tissue from where it originated (Fig. 2-3); somatic embryogenesis is originated from somatic cells of the plant, apomictic embryogenesis comes from unfertilized cells of the embryo sac, and gametic embryogenesis from megaspore or microspore cells. However, apomictic embryogenesis is sometimes considered somatic embryogenesis because it is initiated from somatic cells (Feher 2006). Due to the purpose of this research, somatic embryogenesis will be defined as the formation of embryos under *in vitro* cultures using somatic cells (Stasolla and Yeung 2003; von Arnold *et al.* 2002) and the literature review will focus on somatic embryogenesis in gymnosperms, more specifically in pine species.
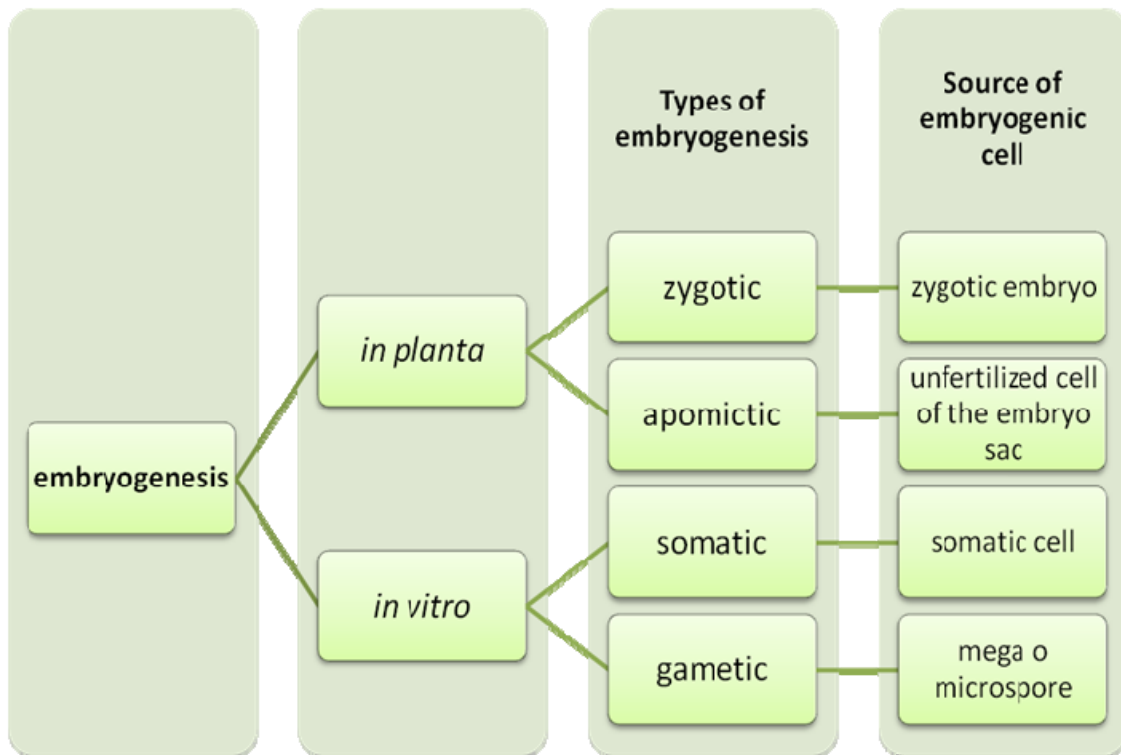
Figure 2-3. Types, sources and multiplication type of embryogenesis in higher plants. Adapted from Feher (2006) and Gray (2005).

2.2.1 Somatic embryogenesis in gymnosperms

By providing the appropriate nutritional and environmental conditions, somatic embryos can develop into complete plantlets by forming shoots and roots (Ikeda and Kamada 2006). Somatic embryogenesis has been recorded for different species across many genera and from a variety of plant tissues. Somatic embryogenesis as a research tool facilitates the understanding of the physiological, biochemical and morphological pathways active during embryogenesis (Zimmerman 1993).

Several reviews (Jimenez *et al.* 2005; Stasolla and Yeung 2003; von Arnold *et al.* 2002) have detailed the advances in somatic embryogenesis for different conifer species. The initiation and further development of somatic embryos depend on the species, genotype response, explants, culture media, plant growth regulator types and concentrations, and environmental conditions. It is thus possible that the developmental limitations of somatic embryos are due to imperfections in the known techniques and/or deficiencies in the composition of the culture media (Pullman *et al.* 2003b). Even though, somatic embryogenesis has been induced in different conifers (Fowke *et al.* 1995), somatic embryogenesis in pine has been more difficult to obtain (Chalupa 1985), thereby representing a bottleneck both for the production of seedlings and for the study of pine embryo development pathways.

The first reports of somatic embryogenesis in gymnosperms were in *Picea abies* (Chalupa 1985; Hakman *et al.* 1985) and *Larix decidua* (Nagmani and Bonga 1985). Currently, there is a large list of protocols for somatic embryogenesis in a variety of gymnosperms including several pine species (Table 2-1). For pine species, the majority of the protocols on somatic embryogenesis have been published in the last ten years and covers approximately 24 species representing approximately 20% of pine species. Perhaps, this list would increase as more improvement and progress in the initiation of pine cultures comes.

Table 2-1. Examples of somatic embryogenesis in different gymnosperm species with emphasis in *Pinus species.*

Specie	Explants	Final stage reached	Author
Pseudotsuga menziesii	immature and mature	plantlets	(Durzan and Gupta 1987)
Abies alba	mature zygotic embryos	plantlets	(Hristoforoglu *et al.* 1995)
A .lasiocarpa	zygotic embryos	plantlets	(Kvaalen *et al.* 2005)
A. nordmanniana	megagametophyte*	matured somatic embryos	(Norgaard and Krogstrup 1991)
Larix decidua and *L. leptolepsis*	immature zygotic embryos	matured somatic embryos	(von Aderkas *et al.* 1990)
L. occidentalis	immature zygotic embryos	plantlets	(Thompson and Aderkas 1992)
Picea glauca	immature zygotic embryos	plantlets	(Lu and Thorpe 1987)
P. glauca/engelmanii hybrid	zygotic embryos	plantlets	(Webb *et al.* 1989)
P. mariana and *P. rubens*	mature zygotic embryos	plantlets	(Iraqi and Tremblay 2001)
Zamia fischeri	megagametophyte*	mature somatic embryos	(Chavez *et al.* 1992)
Z. furfuracea	megagametophyte and zygotic embryo	mature somatic embryos	(Chavez *et al.* 1992)
Z. pumila	megagametophyte and zygotic embryo	plantlets	(Chavez *et al.* 1992)
Pinus armandii	megagametophyte*	plantlets	(Hosoi 2001)
P. banksiana	megagametophyte*	mature somatic embryos	(Von Aderkas *et al.* 2005)
P. bungeana	megagametophyte*	mature somatic embryos	(Zhang 2007)
P. brutia	megagametophyte*	embryo germination	(Yildirim 2006)
P. caribaea	megagametophyte*	plantlets	(Laine 1990)
P. densiflora	megagametophyte*	plantlets	(Maruyama *et al.* 2005a)
P. elliottii	megagametophyte*	plantlets	(Newton *et al.* 2005)
P. gerardiana	mature zygotic embryos	plantlets	(Malabadi and Nataraja 2007c)
P. kesiya	megagametophyte*	plantlets	(Choudhury 2008)
P. koraiensis	mature zygotic embryos	somatic embryo induction	(Bozhkov 1997)
P. nigra	megagametophyte*	plantlets	(Salajova 2005)
P. palustris	megagametophyte*	mature zygotic embryos	(Nagmani 1993)
P. patula	megagametophyte*	plantlets	(Jones and van Staden 2001)
P. pinaster	megagametophyte*	plantlets	(Lelu-Walter *et al.* 1999)
P. pinea	megagametophyte*	plantlets	(Carneros *et al.* 2009)
P. radiata	megagametophyte*	plantlets	(Klimawzseka *et al* 2007)
P. roxburghii	megagametophyte*	mature zygotic embryos	(Arya *et al.* 2000)
P. sylvestris	megagametophyte*	plantlets	(Aronen *et al.* 2009)
P. taeda	megagametophyte*	plantlets	(Pullman *et al.* 2006)
P. thunbergii	megagametophyte*	plantlets	(Maruyama *et al.* 2005b)
P. uncinata	megagametophyte*	somatic embryo induction	(Vejsadova *et al.* 2008)
P. wallichiana	megagametophyte*	plantlets	(Malabadi and Natajara 2007b)

* Megagametophyte containing immature zygotic embryos

Somatic embryogenesis procedures in pine can be divided into six phases (Gupta and Grob 1995): 1) culture initiation; 2) multiplication; 3) cryopreservation; 4) embryo maturation; 5) embryo desiccation; and 6) *in vitro* germination and early growth (Fig. 2-4). The success of pine somatic embryogenesis is affected by culture environment, explant type, and plant growth regulators, which must be optimized at each step in order to maximize embryo yield and development (Stasolla *et al.* 2002a).

During culture initiation, different factors are required for the successful initiation of embryogenic cultures. These include selection of suitable explant, genotype, environmental (light, temperature and humidity) and nutritional factors (Deb and Tandon 2004; Merkle *et al.* 1995). More specifically, the generation of embryogenic cells is limited to the competent cells in explants that have the potential to turn on those genes involved in the processes (von Arnold *et al.* 2002). Once somatic embryogenesis initiation is obtained through the addition of cytokinins and/or auxins, and embryogenic cultures captured, the maintenance and multiplication of the embryogenic masses is made by decreasing the concentration of these two plant growth regulators (Häggman *et al.* 2006) or by maintaining the concentrations used for initiation. To induce maturation of the proliferating somatic embryos in the embryogenic masses, it is necessary to remove the maintenance plant growth regulators (Häggman *et al.* 2006). Morphologically, the embryo masses passes through three characteristic stages (I, II, III) while differentiating into a somatic embryo, and then into a mature embryo (Filonova *et al.* 2000). The initiation of somatic embryo formation is marked with the asymmetric cell divisions within the embryogenic tissue resulting in two cells: one is small and densely cytoplasmic which give rise to the embryo proper, while the other one is composed of larger vacuolated cells which will form the suspensor (Stasolla *et al.* 2002a). Once proliferating somatic embryos form a large globular head and elongated filamentous suspensor, they are ready to be transferred to maturation medium.

Cryopreservation represents one of the advantages of somatic embryogenesis methods for both research purposes and practical applications. Cryopreservation relies on the ability of the embryogenic tissue to be preserved at low temperatures (-140 to -196 ºC) for a period of time, after which the tissue is thawed to resume embryo development (Klimaszewska *et al.* 2007). This maintains the juvenility of the tissue while somatic seedlings are being tested in the field.

SOMATIC EMBRYOGENESIS IN PINE

Superior Tree

Cones: collection and sterilization

megagametophyte with immature zygotic embryo

1. Initiation of Embryogenic Suspensor Mass (ESM)

Low initiation rates

3. Cryopreservation

2. Multiplication of ESM

Low culture survival

Slow initial growth of somatic seedlings

6. Germination

Low germination and conversion rates

5. Desiccation

4. Maturation

Low maturation rates

Figure 2-4. Schematic procedure, stages (blue fonts) and bottlenecks (red fonts) of somatic embryogenesis in pine species (figures not at scale).

Protocols for the cryopreservation of pine species have been reported for *Pinus monticola* and *P. radiata* and applied to other pine species such as *P. strobus* and *P. pinaster* (Klimaszewska *et al.* 2007).

In somatic embryogenesis, maturation is defined as the initiation of the development of somatic embryos to morphological and physiological maturity prior to desiccation and subsequent germination (Stasolla and Yeung 2003). During maturation, different osmotic agents provide a medium with a high osmotic potential (von Arnold *et al.* 2002) to mimic the conditions during seed development. This allows the somatic embryos to accumulate storage substances (including carbohydrates, protein and lipids) that then exhibit similarities or differences in comparison to those of zygotic embryos (Merkle *et al.* 1995). Osmotic potential causes a loss in the turgor pressure of the cell, which is followed by an increase of the *de novo* synthesis of

ABA that induces the expression of specific genes related to ABA such as those for storage protein (Wilen *et al.* 1990).

Observations in zygotic embryogenesis showed that ABA is necessary for maturation of zygotic embryos. Thus, the addition of ABA during somatic embryo maturation mimics the ABA that the megagametophyte supplies to zygotic embryos (Stasolla *et al.* 2002a). The physiological responses during somatic embryo maturation differ among species. In white spruce (*Picea glauca*), the increase of ABA occurs at the same time as an increase in storage protein deposition (lipids and starch), amino acid accumulation (i.e. glutamine, glutamic acid and arginine), ascorbic acid, polyamines and a reduction of precious germination (Stasolla *et al.* 2002a). Thus, the addition or lack of osmotic agents and ABA, as well as their concentrations is likely to affect the expression of genes and the synthesis of molecules involved in the maturation process, and consequently the quality of the embryo (Stasolla *et al.* 2003). Morphologically, the cotyledonary stage is observed as the formation of a ring of cotyledons emerging from the peripheral zone of the shoot apex. A fully developed embryo will have both shoot and root apical meristems with the ability to develop into a complete plantlet.

In some conifer species protocols such as *Pinus thunbergii* (Wilen *et al.* 1990), *P. strobus* (Klimaszewska and Smith 1997) and *P. kesiya* (Choudhury *et al.* 2008), germination has been achieved by placing the embryos directly on a germination medium. In others, a prior desiccation treatment before placement on the germination medium is required, as in *P. glauca/engelmanii* (Roberts *et al.* 1990) and *A. lasiocarpa* (Kvaalen *et al.* 2005). Desiccation allows for a switch in gene expression patterns and reduces endogenous ABA content, changes the sensitivity to ABA or both. Finally, germination and conversion occurs with the development of the root and shoot (Merkle *et al.* 1995). Good growth of the root system is however needed for the success of conversion under *ex vitro* conditions. Germination is usually carried out on a medium that is plant growth regulator-free, supplemented with activated charcoal, and low in sugar and mineral concentrations, thereby allowing somatic embryos to utilize the storage compounds (Häggman *et al.* 2006). Recently, one report showed that germination in *P. sylvestris* increased as a result of inoculation with ectomycorrhizal fungi (Niemi and Häggman 2002). However, interactions between tree species and genotypes with the ectomycorrhizae are highly specific, therefore several interactions between fungus strains and genotypes has to be tested before used in the commercial scale (Häggman *et al.* 2006) .

All of these early developmental stages are critical for optimal development of the future plants *ex vitro* (conversion) since culture initiation can be successful, conversion of somatic embryos to plants could fail (Nehra *et al.* 2005). In pine species, conversion to plants has been achieved for most of the pine species listed in Table 2-1. However, more resources have gone into protocol improvements for the economically important pine species: *P. taeda*, *P. radiata P. banksiana. P. pinaster* and *P. strobus* (Häggman *et al.* 2006; Klimaszewska *et al.* 2007).

For the purpose of this project, we will focus on and discuss the literature associated with the initial explant, culture media and plant growth regulators for each of the developmental stages of somatic embryos in pine.

2.2.1.1 Explants for somatic embryogenesis initiation: mature tissue, mature and immature zygotic embryos

While the initiation of direct somatic embryos in conifers has been more successful in the genus *Picea* (Afele *et al.* 1992) than in *Pinus*, pine somatic embryogenesis has been achieved from different explants such as: 1) isolated mature zygotic embryos of *P. taeda* (Gupta and Durzan 1986; Tang *et al.* 2001) and *P. wallichiana* (Malabadi and Nataraja 2007a), 2) protoplasts from embryogenic cell suspensor derived from immature zygotic embryos of *P. caribaea* (Laine and David 1990) and mature zygotic embryos of *P. taeda* (Gupta and Durzan 1987b); 3) vegetative shoot apices of *P. patula* (Malabadi and Van Staden 2005b), 4) immature zygotic embryos enclosed in the megagametophyte in *P. strobus* (Finer *et al.* 1989), *P. taeda* (Pullman *et al.* 2003b), and *P. bungeana* (Zhang *et al.* 2007) (Table 2.2 for more citation), and 5) isolated immature zygotic embryos of *P. taeda* (Gupta and Durzan 1986)and *P. lambertiana* (Becwar *et al.* 1991) (Table. 2-2). The best results have been obtained with immature zygotic embryos collected during a developmental window of one to two weeks, during which the embryos are highly competent for somatic embryogenesis. The developmental stages of the responsive zygotic embryos corresponds to the stage when the dominant immature zygotic embryos is at pre-cotyledonary stage (Pullman and Johnson 2002). It has been speculated that somatic embryogenesis occurs as a continuation of the polyembryogenic process common in pine species, implicating that the embryogenic tissue originates from more than one genotype (subordinate zygotic embryos) (Becwar *et al.* 1991).

Thus, the development of protocols for somatic embryogenesis using mature zygotic embryos or tissues of mature trees as initial explants would eliminate the practical problems associated with a narrow window in time for explant collection. The use of cold pre-treatment of mature tree tissues of *P. patula* (Malabadi and Van Staden 2005b), *P. kesiya* (Malabadi *et al.* 2005), *P. roxburghii* (Malabadi and Nataraja 2007b) and *P. wallichiana* (Malabadi and Nataraja 2007d) and has recently been shown to stimulate the initiation of somatic embryogenesis. When using mature tissue for somatic embryogenesis initiation, the cells have to undergo a dedifferentiation to become competent for embryogenesis. This can be achieved only if the external and cellular cultural conditions are optimal and allow the expression of the embryogenic programme, allowing differentiation and development of embryos (Feher 2006).

2.2.1.2 Media composition

2.2.1.2.1 Initiation medium

Somatic embryos have to grow and develop *in vitro* supported by the nutrients provided from the culture medium, in contrast to the nutrients provided to the developing zygotic seed embryo by the megagametophyte (Nehra *et al.* 2005; Pullman and Buchanan 2003). Thus, it is believed to be essential to develop tissue culture media and conditions that mimic the natural nutritional environment inside the megagametophyte.

In pine species the most used tissue culture medium is DCR (Gupta and Durzan 1985) (Table 2-2) which is a modification of the basal MS medium (Murashige and Skoog 1962). The DCR medium reduces the concentration of KNO_3, NH_4NO_3 and $CaCl_2 \cdot 2H_2O$ from 18.79, 2.99 and 20.61 to 3.36, 0.58 and 5.00 mM, respectively; and adds $Ca(NO_3)_2 \cdot 4H_2O$ at 2.35 mM (Appendix 2.1). Another common medium used in pine is MSG (Becwar *et al.* 1990) which is a modification of DCR medium (Appendix 2.1), and also decreases KNO_3 from 18.79 to 0.99 mM, lacks NH_4NO_3 but adds KCl at 10.00 mM.

Full-strength LP medium (von Arnold and Eriksson 1981) is rarely used in conifer species. In *P. taeda,* it did not boost the initiation of cultures (Li *et al.* 1998a); rather half-strength has been more effective for the initiation of somatic cultures in *Pinus caribaea* (Laine and David, 1990), as well as in *Picea abies* (Hakman and von Arnold 1988) and *P. sitchensis* (von Arnold and Woodward 1988).

The mineral nutrient composition of developing seeds of *P. taeda* led to development of a novel medium, the P6 medium, for the cell culture of gymnosperm species (Teasdale *et al.* 1986). This medium contained a high concentration of micronutrients (zinc, manganese, iron, boron and iodine) and magnesium; and low concentrations of micronutrients (cobalt, copper and molybdenum) and calcium. Modifications of this culture medium (medium 240) have also been used for the initiation and maturation of embryogenic cultures in *P. taeda* (Pullman and Johnson 2002) and maturation of somatic embryos of *P. patula* (Jones and van Staden 2001).

One of the key compounds in the tissue culture medium is nitrogen. Nitrogen can be added to the culture medium through an organic or inorganic form. However, generally, nitrogen is added to the medium in the inorganic forms nitrate (NO_3) or/and ammonium (NH_4), and because plants prefer nitrate, the amount of this compound is usually greater than that of ammonium. The importance of the amounts and ratios of these two compounds for conifer morphogenesis has been reported for somatic embryogenesis (Tremblay and Tremblay 1991b) and organogenesis (Flinn *et al.* 1986). The DCR medium has approximately 30 % of the total inorganic nitrogen of MS and Litvay media (60.0 mM). On the other hand, MSG medium (Becwar *et al.* 1990) does not have any source of NH_4 and the levels of NO_3 are lower compared with those of MS or DCR medium (Appendix 2.1); but it has been successful for the initiation of somatic culture of *P. gerardiana* (Malabadi and Nataraja 2007c), *P. palustris* (Nagmani *et al.* 1993), *P. patula* (Jones and van Staden 2001) and *P. wallichiana* (Malabadi and Nataraja 2007c). For the establishment of somatic embryogenesis lines of *P. abies,* the optimal ammonium/nitrate molar ratio was about 0.2 with the addition of 3.0 mM L-glutamine, additionally, inorganic forms of nitrogen (asparagine or glutamine) were necessary for cell line establishment and prolonged maintenance (Bozhkov *et al.* 1993). For *P. mariana* and *P. rubens*, an ammonium nitrate concentration of 3.4–10 mM and 3.4–15 mM were optimal for embryo development, respectively (Tremblay and Tremblay 1991b).

Organic forms of nitrogen used in tissue culture medium are amino acids such as proline, aspargine, glycine or glutamine and casein hydrolysate. Amino acids are the basic structures of the proteins. Organic nitrogen is already reduced, so it may be taken up more readily than inorganic nitrogen (Kvaalen *et al.* 2005). The most common form of organic nitrogen used is L-glutamine, which was the only source of nitrogen used in the culture medium for somatic embryogenesis of *Abies lasiocarpa* (Kvaalen *et al.* 2005) and *P. abies* (Verhagen and Wann

1989). Additionally, L-glutamine in combination with casein hydrolysate and other inorganic forms of nitrogen, has generally been used in the different phases of conifer somatic embryogenesis, such as for *P. strobus,* an increased L-glutamine concentration in the maturation medium to 7.3 g/L was beneficial (Garin *et al.* 2000). Similar results were found in the maturation of somatic embryos of *P. patula* (Malabadi and Van Staden 2005a).

Increases in the percentage of somatic embryo initiation in *P. taeda* has been accomplished through adjustments of the concentrations of ABA and silver nitrate (Pullman *et al.* 2003b), brassinolide (Pullman *et al.* 2003a), paclobutrazol (Pullman *et al.* 2005a), organic acids, Vitamins B12 and E (Pullman *et al.* 2006). Additionally, putrescine is added for the initiation of somatic embryo cultures in *P. gerardiana* (Malabadi and Nataraja 2007c), 24-epibrassinolide in *P. wallichiana* (Malabadi and Nataraja 2007a); and triacontanol in *P. roxburghii* (Malabadi and Nataraja 2007b).

After embryos have been initiated, multiplication is usually carried out on the same basal medium on which the cultures were initiated; and the concentration of plant growth regulators usually are kept the same [*Pinus brutia;* (Yildirim *et al.* 2006) and *P. patula;* (Jones and van Staden 2001)]; or decreased [*P. densiflora;* (Taniguchi 2001), *P. thunbergii;* (Maruyama *et al.* 2005b), *P. kesiya* (Choudhury *et al.* 2008) *P. patula* (Malabadi and Van Staden 2005b)] (Table 2-2).

Table 2-2. Explant, culture media and plant growth regulators used in different protocols for initiation and multiplication of somatic embryos in pine species.

Pine Specie	Explant[1]	Media[2]	2,4-D (µM)	BA (µM)	NAA (µM)	Others (µM)[3]	Media	2,4-D (µM)	BA (µM)	NAA (µM)	Others (µM)	Author
		Initiation					Multiplication					
P. armandii	MG	MS	1.0	3.0			MS	1.0	3.0			(Maruyama *et al.* 2007)
P. bungeana	MG	DCR	43.0	17.8			DCR	1.5		1.0		(Zhang *et al.* 2007)
P. brutia	MG	DCR	13.6	2.2			DCR	13.6	2.2			(Yildirim *et al.* 2006)
P. densiflora	MG	EM	10.0	5.0			EM	3.0	1.0			(Maruyama *et al.* 2005a)
P. elliottii	MG	LP	36.0	17.8			LP	4.5	2.2			(Newton *et al.* 2005)
P. gerardiana	MZE	½ MSG	9.0			25.0 Put	½ MSG	2.0			5.7 Put	(Malabadi and Nataraja 2007c)
P. kesiya	MG	DCR	20.0	12.0	12.0		DCR	2.0	0.6	1.2		(Choudhury *et al.* 2008)
P. koraiensis	MZE	LM and DCR	10.0	5.0								(Bozhkov *et al.* 1997)
P. nigra	MG	DCR	9.0	2.2			DCR	9.0	2.2			(Salajova and Salaj 2005)
P. palustris	MG,	MSG	9.0 14.0, 22.5	2.2,0.5 11.0								(Nagmani *et al.* 1993)
P. patula	MG	MSG	9.0	4.5			MSG	9.0	4.5			(Jones and van Staden 2001)
P. patula	SA	DCR	23.0	9.0	27.0		DCR	2.26	2.0	2.68		(Malabadi and Van Staden 2005b)
P. pinaster	MG	LM	9.0	4.4			LM	2.2	2.3			(Lelu-Walter *et al.* 2006)
P. pinea	MG	LM	9.0	4.5			LM	9.0	4.5			(Carneros *et al.* 2009)
P. radiata	MG	EDM6	4.5	2.6			BLG	4.5	2.6			(Walter *et al.* 2005)
P. roxburghii	MG	DCR	10.0	5.0			DCR	5.0				(Arya *et al.* 2000)
P. roxburghii	SNM	DCR	22.6		26.9	5.0 TRIA	DCR	2.3		2.7	2.0 TRIA	(Malabadi and Nataraja 2007b)
P. roxburghii	MG	DCR	50.0	20.0	20.0							(Mathur *et al.* 2000)
P. strobus	MG	LM	2.2	2.2								(Von Aderkas *et al.* 2005)
P. sylvestris	MG	DCR	13.6	2.2			DCR	9.1	2.2			(Aronen *et al.* 2009)
P. taeda	MG	½ P6		2.0	10.7	2.0 Kin	½ P6		2.0	10.74	2.0 Kin	(Pullman *et al.* 2003b)
P. taeda	IZE	LP	45.0	17.8		25.0 IBA and 18.0 Kin	LP	7.6	3.5		5.0 IBA and 3.7 Kin	(Tang *et al.* 2001)
P. thunbergii	MG	½ EM	10.0	5.0			½ EM	3.0	1.0			(Maruyama *et al.* 2005b)
P. uncinata	MZE, MG	DCR		3.5	10.0				0.5	2.3		(Vejsadová *et al.* 2008)
P. wallichiana	MZE	MSG	9.0			2.0 Bras	MSG	2.0			0.5 Bras	(Malabadi and Nataraja 2007a)

[1]MG= megagametophytes; MZE= isolated mature zygotic embryos; IZE= isolated immature zygotic embryos; SNM= secondary needle of mature tree; SA= shoot apices

[2]Some of these basal media have been modified, for more details refer to the citations. DCR= Douglas cotyledonary revised (Gupta and Durzan 1985), LP= (von Arnold and Eriksson 1981); LM= Litvay medium (Litvay *et al.* 1981); MS= (Murashige and Skoog 1962); MSG= MS modified (Becwar *et al.* 1990); EM= (Maruyama *et al.* 2001); EDM6= (Smith 1996); P6= (Teasdale *et al.* 1986)

[3]Put= putrescine; TRIA= triacontanol,; Kin= kinetin; Bras= 2,4-epibrassinolide, and NR= not reported

2.2.1.2.2 Maturation medium components

Maturation is initiated in the somatic embryos on multiplication medium following a pre-maturation treatment, consisting of plant growth regulator-free culture medium to stop the multiplication of somatic embryos (Kvaalen *et al.* 2005) and to switch gene expression patterns to initiate the accumulation of storage proteins (Pinto *et al.* 2002). The major differences in the medium supporting maturation in comparison to the medium supporting multiplication are the lack of auxins and/or cytokinins, addition of ABA and supplement of higher concentration of carbohydrates and/or osmotic compounds. Maturation of somatic embryos is commonly accomplished on solid media (Stasolla and Yeung 2003). With the goal to develop an improved maturation medium, the metal content of *P. taeda* megagametophytes and zygotic embryos, and somatic embryos was analyzed (Pullman *et al*. 2003c). Then, a series of experiments were run in order to find a medium that would produce somatic embryos with similar concentrations of the elements found in zygotic embryos (Pullman *et al.* 2003b). Different types and concentrations of carbohydrate sources have been tested as a source of carbon or osmotic agent (Salaj *et al.* 2004). An osmotic gradient inside the seed allows water movement towards the embryo (Bewley and Black 1994). Different types and concentrations of carbohydrates have proven optimal for different species (Tremblay and Tremblay 1991a). In fir hybrids, 3 % (w/v) of maltose has been shown to promote development of mature embryos (Salaj *et al.* 2004). Up to 6 % (w/v) sucrose was optimal for embryo development in *Picea mariana*, *P. rubens* and *P. glauca* (Tremblay and Tremblay 1991a). Sucrose is hydrolyzed into glucose and fructose, however addition of these two compounds did not result in the same stimulatory effect for somatic embryo formation in *Picea* species as from addition of sucrose (Iraqi and Tremblay 2001). In *Abies alba*, a combination of lactose and sucrose produced the highest percentages of maturation (Schuller *et al.* 2000). The complete or partial substitution of sucrose with alcohol sugars such as mannitol and sorbitol enhanced the percentage of maturation in *P. strobus* (Garin *et al.* 2000). In *P. taeda* (Pullman *et al.* 2003b), the use of 2 % (w/v) maltose gave better embryo yield and quality; an increase of the mannitol concentration to 5 % (w/v) was used for *P. armandii* (Maruyama *et al.* 2007), *P. roxburghii* (Malabadi and Nataraja 2007d) and *P. thunbergii* (Maruyama *et al.* 2005b); 6 % (w/v) for *P. kesiya* (Choudhury *et al.* 2008), *P. densiflora* (Maruyama *et al.* 2005a) and *P. patula* (Jones and van Staden 2001); and up to 9 % (w/v) in *P. nigra* (Salajova and Salaj 2005) (Table 2.3). In contrast, sucrose was used as the

24

only carbohydrate source at concentrations of 2 % (w/v) in *P. serotina* (Mathur *et al.* 2000); 3 % (w/v) in *P. brutia* (Yildirim *et al.* 2006); and 6 % (w/v) in *P. strobus* (Klimaszewska *et al.* 2001) and *P. sylvestris* (Aronen *et al.* 2009) (Table 2.3). Sucrose or maltose are low molecular weight compounds able to penetrate the plasma membrane, allowing for an increase of the water content in the tissue instead of reducing it (Stasolla and Yeung 2003).

Polyethylene glycol (PEG) is a high molecular weight, non-penetrating osmotic agent, that can be added to the medium to create an osmoticum and that exerts water stress similar to natural drying (Attree and Fowke 1993). PEG has been used for somatic embryo maturation in several conifers such as *P. glauca* (Attree *et al.* 1991)*, Abies alba X A. numidica* (Salaj *et al.* 2004), and *P. taeda* (Li *et al.* 1998b). In *P. abies* somatic embryos that had matured on a medium containing 7.5 % (w/v) PEG 4000 and 16 µM ABA resembled the zygotic embryos with low moisture content, quiescence level and ability to survive desiccation to 30 % moisture content (Misra *et al.* 1993). However, it has been shown that PEG can adversely affect the morphology and anatomy of the somatic embryos of *P. abies* (Bozhkov and von Arnold 1998). The effect of PEG in maturation could be due to the enhanced responsiveness of the tissue to growth regulators such as ABA in the initial phases of development (Stasolla *et al.* 2003).

For pine species, experiments with different molecular weight PEGs have shown positive results with 3.75 % (w/v) PEG 4000 for *P. brutia* (Yildirim *et al.* 2006), 7.5 % (w/v) PEG 6000 for *P. taeda* (Tang and Newton 2005b) and *P. elliottii* (Newton *et al.* 2005); also 10 % (w/v) PEG 6000 for *P. thunbergii* (Maruyama *et al.* 2005b), and 10 % (w/v)PEG 8000 for *P. patula* (Table 2-3).

The use of 7.5 % (w/v) PEG 4000 combined with 16 µM ABA increased the levels of storage protein three-fold in somatic embryos of *Picea. glauca* compared with embryos matured on the same ABA concentration without PEG, and the protein profile and protein bodies became similar to mature zygotic embryos (Misra *et al.* 1993). Similarly, the concentration of carbohydrates has been shown to alter the accumulation of storage protein. In *Pinus strobus*, somatic embryos that had matured on 6 % (w/v) sucrose accumulated more storage protein than embryos that matured on 3% sucrose (Klimaszewska *et al.* 2004)

The addition of PEG to the maturation medium induced two major changes in gene expression in immature embryos of *P. glauca*; the first change was observed after transfer of the somatic

embryos to ABA containing medium, and the second when the embryos were fully mature (Stasolla *et al.* 2003). The PEG-sensitive genes included several auxin- and ABA-responsive genes, suggesting that PEG may affect the initial phases of development possibly by altering the responsiveness of the tissue to growth regulators (Stasolla *et al.* 2003).

Another compound that affects water content in the tissue culture medium is the gelling agent, which is a high molecular weight polysaccharide. An increase of the gellan gum concentration from 0.6 % to 1.0 % (w/v) increased the maturation rate of somatic embryos of *Pinus strobus* (Garin *et al.* 2000) and reduced the water potential allowing for higher germination frequency in the same pine species (*P. strobus*) (Klimaszewska *et al.* 2000). Also, the type of gelling agent has been shown to influence the yield of matured embryos per fresh weight, as in *Picea mariana* and *P. rubens* the number of somatic embryos was significantly higher on medium solidified with gelrite than with Difco Bacto-agar (Tremblay and Tremblay 1991b).

For pine species, the use of Phytagel, gelrite and agar are the most common used for the maturation of somatic embryos (Table 2-3).

2.2.1.2.3 Germination and conversion media components

Germination is defined as the extrusion of both shoot and root. However, subsequent growth of the root system after germination is necessary for successful conversion to *ex vitro* conditions. In different pine species protocols, germination has been achieved by placing the embryos directly on a medium, or by a prior desiccation treatment before placement (Roberts *et al.* 1990).

Desiccation treatments can be divided into: 1) partial drying, which improves the frequency of embryos matured on low osmotic conditions, and 2) full drying, which stimulates the post-embryogenic growth of embryos matured under high osmotic conditions (Stasolla *et al.* 2002b).

Partial drying treatment corresponds to a gradual and limited loss of moisture content in the embryos as described for *P. glauca/engelmannii* and (Roberts *et al.* 1990). It is usually carried out by using well-plates where half of the wells are partially filled with water and the remaining wells are used for embryo placement, after which the plates are sealed and placed in darkness. The time frame needed for the loss of water depends on the species. For *P. glauca* an optimal conversion was obtained after 16 days (Roberts *et al.* 1990) and for *P. sitchensis* after 35 days

(Roberts *et al.* 1991). Full drying treatment is usually carried out by placing embryos in plates with saturated salt solutions, which causes a more dramatic loss of water content compared with partial drying (Stasolla and Yeung 2003). Full drying treatment in somatic embryos of *Larix x leptoeuropaea* was carried out by placing individual somatic embryos on nylon filters, then nylons were transferred into three empty wells of a 6-well plate; the three remaining wells were filled to one-fourth with a supersaturated solution of Mg $(NO_3)_2 \cdot 6H_2O$ (6.7 g/ ml distilled water); then plates were sealed and placed in darkness at 4 °C for one week (Dronne *et al.* 1997). High osmotic conditions during maturation likely allows an increase of storage protein content during the maturation phase thus allowing the somatic embryos to survive full drying conditions (Attree and Fowke 1993).

Table 2-3. Culture medium, ABA, gelling agent and carbohydrates used for the maturation of somatic embryos of pine species.

Species	Medium[1]	ABA (µM)	PEG (%)	Gelling agent (%)[3]	CHOS (%)[4]	Author
P. armandii	MS	100	10.0(6)[2]		5Mal	(Maruyama *et al.* 2007)
P. brutia	DCR	80	3.7(4)	1.0 Gel	3Suc	(Yildirim *et al.* 2006)
P. densiflora	EM	100	7.5 (4)	0.5 Gel	6 Mal	(Maruyama *et al.* 2005a)
P. elliottii	LP	15	7.5 (6)	0.6 Agar	1.5 Suc	(Newton *et al.* 2005)
P. gerardiana	½MSG	60		0.8 Phy	6 Mal	(Malabadi and Nataraja 2007c)
P. kesiya	DCR	35		0.5 Phy	6 Mal	(Choudhury *et al.* 2008)
P. nigra	DCR	94.6		0.4 Gel	9 Mal	(Salajova and Salaj 2005)
P. patula	240	40	7.5 or 10 (8)	3.0 Gel	6Mal	(Jones and van Staden 2001)
P. patula	DCR	80		0.9 Phy	6 Mal	(Malabadi and Van Staden 2005b)
P. pinaster	LM	120		1.0 Phy	7 Mal	(Lelu-Walter *et al.* 2006)
P. radiata	BLG	57		0.3 Phy	2 Mal	(Walter *et al.* 2005)
P. roxburghii	DCR	37.84		0.7 Phy	5Mal	(Malabadi and Nataraja 2007d)
P. strobus	LM	120		1.0 Phy	6 Suc	(Klimaszewska *et al.* 2001)
P. sylvestris	DCR	80-90	7.0 (4)	0.8 Phy	6 Suc	(Aronen *et al.* 2009)
P. taeda	½ P6	20	12.0 (8)	0.3 Gel	6 M	(Pullman *et al.* 2003c)
P. taeda	LP	150	7.5 (6)	0.6 Agar	1.5 Suc	(Tang *et al.* 2001)
P. thunbergii	EM	100	10 (6)			(Maruyama *et al.* 2005b)
P. wallichiana	½ MSG	60			6 Mal	(Malabadi and Nataraja 2007a)

[1] Some of these basal media have been modified, for more details refer to the citations. DCR= Douglas cotyledonary revised (Gupta and Durzan 1985), EM= (Maruyama *et al.* 2001); MSG= MS modified (Becwar *et al.* 1990); P6= (Teasdale *et al.* 1986); LP= (von Arnold and Eriksson 1981); LM= Litvay medium (Litvay *et al.* 1981); BLG= (Walter *et al.* 2005).

[2] number in parenthesis stands for molecular weight: 4= 4000, 6= 6000 and 8=8000

[3] Gel= Gelrite and Phy= Phytagel

[4] CHOS= carbohydrates: Suc= sucrose and Mal= maltose

Both types of desiccation treatments induces a switch in gene expression patterns and reduce endogenous ABA content and changes the sensitivity to ABA (Stasolla *et al.* 2002b). In general, mature embryos of conifers need a desiccation period to reach "physiological" maturity in order to be able to grow into viable plantlets (Stasolla and Yeung 2003). Success of the germination process also determines the efficiency of the somatic embryo to develop well-formed shoot and root apical meristems (Yeung *et al.* 1998).

The germination medium consists of a plant growth regulator-free medium with a low salt concentration and the addition of activated charcoal to absorb any growth regulators carried over from maturation. The addition of 2 % (w/v) sucrose has been shown to be essential for the germination of *P. glauca/engelmannii* (Roberts *et al.* 1993). For *Pinus strobus, P. pinaster* and *P. taeda,* the culture of the embryos for seven to ten days in darkness before transferring them to light ensured the elongation of hypocotyls and reduced the anthocyanin synthesis (Klimaszewska *et al.* 2007). Improvements were also observed when the embryos were placed horizontally on the medium and the plates tilted vertically, allowing the roots to develop on the surface of the medium (Klimaszewska *et al.* 2007).

In the past 10 years, the number of protocols for initiation of pine species has increased; however, few of them reported on the survival rate of somatic seedlings transferred to *ex vitro* conditions. In *P. armandii,* 51 % germination and 40 % conversion was reported (Maruyama *et al.* 2007) and 60 and 51 % respectively for *P. thunbergii* (Maruyama *et al.* 2005b). For economically important species, more efforts have been directed towards improving the protocols and the success rates in the somatic embryogenesis processes have for some species been improved significantly. For example, in cell line PM5 of *P. pinaster,* 96 % germination, 89 % conversion and 70 % plant survival was obtained (Klimaszewska *et al.* 2007).

2.2.2 Plant growth regulators during somatic embryogenesis

Hormones are defined as chemical messengers that at low concentration control physiological responses in plants. Analogs to the hormones have been chemically synthesized and shown to have the same or stronger effects (Jimenez 2005). These synthetic compounds are defined as plant growth regulators, which are added exogenously to *in vitro* cultures to modify plant growth (Jimenez 2005). However, the study of plant growth regulator functions can be complex, because of growth regulator interactions, and because of interactions with the

28

endogenous concentrations of the corresponding naturally-occurring hormone in the plant, which changes with season, explants, developmental stages, species and within genotypes (Jimenez 2005).

2.2.2.1 Initiation of somatic embryogenesis

Combinations of auxins and cytokinins are commonly used for induction of somatic embryogenesis (Jimenez *et al.* 2005). Both hormone classes have been shown to participate in cell cycle regulation and cell division (Gaj 2004). There are few cases where the presence of only an auxin (2,4-D at 9.0 µM) plus putrescine was used for somatic embryogenesis induction, such is *P. gerardiana* (Malabadi and Nataraja 2007c) or only a cytokinin was used, such in *A. lasiocarpa* (Kvaalen *et al.* 2005) or *A. nordmanniana* (Norgaard and Krogstrup 1991)

The auxins promote elongation growth by increasing cell wall extensibility (Rayle and Cleland 1992). The primary auxin in plants is indole-3-acetic acid (IAA), however other compounds with auxin activity has been observed, such as indole-3-butyric acid (IBA) (Kende and Zeevaart 1997). The corresponding auxin plant growth regulators most commonly used for somatic embryogenesis in pine species are 2,4-D and 1-naphthaleneacetic acid (NAA) in combination with the cytokinins, benzylaminopurine (BA) or kinetin (Kin) (Table 2.2).

Cytokinins are important for cell division, morphogenesis of shoots and roots, chloroplast maturation, cell enlargement, and senescence delay of detached leaves (Taiz and Zeiger 2006). Cytokinins stimulate protein synthesis and participate in cell cycle control and are most abundant in young, rapidly dividing cells of the shoot and root apical meristems (Taiz and Zeiger 2006). Synthetic versions of cytokinins are commercially available and the most commonly used for somatic embryogenesis is BA. The cytokinin hormones zeatin and 2-isopentenyladenine (2-iP) are currently expensive, thus decreasing their use in commercial labs (Gaba 2005). The effect of cytokinins is most noticeable in tissue cultures where they are used, often together with auxins, to stimulate cell division and control morphogenesis such as embryogenesis (von Arnold *et al.* 2002)

However, in the end, the optimal amounts and kinds of plant growth regulators for initiation are determined by the explants, its developmental stage, genotype and species (Gaj 2004; von Arnold *et al.* 2002).

2.2.2.2 Maturation of somatic embryogenesis

2.2.2.2.1 Role of ABA

ABA plays a major role in water stress, bud dormancy and seed development. Endogenous ABA increases rapidly in water-stressed plants, inducing export of potassium ions and loss of guard cell turgor, hence allowing stomata closure (Taiz and Zeiger 2006). In seed development, ABA prevents precocious germination (Kermode 1995), promotes the accumulation of reserve substances (Cailloux *et al* 1996), regulates the synthesis and deposition of storage and late embryogenesis proteins (Dodeman *et al.* 1997) (Roberts *et al.* 1990a) and promotes embryo maturation (García-Martín *et al.* 2005). Seed dormancy is also controlled by the ratio of ABA to GA. In bud dormancy, increased ABA content is believed to protect the meristem from low temperatures by temporarily inhibiting growth (Taiz and Zeiger 2006).

The effect of ABA on somatic embryo maturation is genotype dependent (Jalonen and von Arnold 1991). For some spruce species (*P. glauca* and *P. mariana*), a concentration of 12 μM ABA is enough to promote embryo development (Attree *et al.* 1991). However, for *Pinus elliottii* and *Picea rubens*, higher concentrations of up to 30 and 40 μM ABA, respectively, are required for normal development (Harry and Thorpe 1991; Liao and Amerson 1995). On the other hand, in *Abies nordmanniana*, ABA alone only yielded a low percentage of maturation (Find *et al.* 2002), but in combination with PCIB (an auxin antagonist), it improved the development of mature embryos; similar to results obtained in *Brassica juncea* (Pradeep *et al.* 2006).

In pine species, relatively high concentrations of ABA have been used to obtain successful maturation of somatic embryos (Table 2-3), ranging from 15 μM in *P. elliottii* (Newton *et al.* 2005), 30 μM in *P. serotina* (Marthur *et al.* 2000) to 100 μM for *P. thunbergii* (Maruyama *et al.* 2005b) and *P. densiflora* (Maruyama *et al.* 2005a); and 120 μM for *P. pinaster* ((Lelu-Walter *et al.* 2006)

ABA induces the accumulation of a variety of reserve substances including amino acids, such as glutamic acid, glutamine, and arginine. These amino acids also play a role in the synthesis of polyamines, such as spermidine (Stasolla and Yeung 2003). Recently, it has been shown that extending the time on maturation media to four months increases the quantitative and qualitative traits of mature embryos of *P. taeda* (Vales *et al.* 2006).

During seed development, the most dramatic morphological and biochemical events occur during maturation (Thomas 1993). ABA has been shown to play a regulatory role in maturation and germination through modulation of gene expression (Leung and Giraudat 1998). Furthermore, over 150 genes from a range of species have been found to be ABA-inducible (Giraudat *et al.* 1994). During the maturation of cotton zygotic embryos, ABA concentration peaks in abundance during late embryogenesis and decreases to low levels during desiccation (Galau *et al.* 1986). It is proposed that this increase of ABA is related with the need of storage product deposition and the inhibition of precocious germination. ABA is supplied to the zygotic embryo through the megagametophyte; the lack of this tissue during *in vitro* conditions has made the supply of ABA essential for the normal maturation of somatic embryos (Stasolla *et al.* 2002b). After transfer of the embryos to ABA-containing maturation medium, morphological changes are observed after weeks, but physiological changes can be observed after hours, such as increased protein, lipid, starch and polyamine content (Stasolla *et al.* 2002b).

Another compound that affects the maturation process is ethylene. Ethylene is a gaseous natural hormone that regulates fruit ripening and processes associated with leaf and flower senescence, leaf and fruit abscission, and reduced stem elongation. The major cause of the overall growth inhibition is cessation or retardation of the mitotic process in the meristems of the root, shoot, and axillary buds (Burg 1973). In addition, ethylene is produced following stresses by chemicals, temperatures, water logging, drought, radiation, insect damage, disease or mechanical wounding (Yu and Yang 1980). Ethylene biosynthesis is also triggered by other compounds and hormones such as auxins (Pallardy 2008b). Currently, there are no synthetic analogs of ethylene, however, some chemicals have been synthesized which are capable of releasing ethylene, such as ethephon (2-chlorethanephosphonic acid) (Gaba 2005). Ethylene is produced by plant tissue cultures growing *in vitro* and may accumulate in large quantities in culture vessels. The accumulation of ethylene influences growth and development, either as a promoter or as a inhibitor, depending on the species used (Biddington 1992). It was suggested that ethylene may be involved in the induction of embryogenic tissue and in the early stages of the embryo maturation of *Picea abies* (Biddington 1992; Kvaalen 1994). In *P. mariana* and *P. glauca*, the embryo production capacity appeared inversely related to the ethylene production rate during maturation (El Meskaoui and Tremblay 2001). The accumulation of ethylene results in the formation of large intracellular air spaces that separates the meristematic cells within the

31

shoot poles. This has a negative effect on the formation of the shoot meristem and decreases germination and conversion rates (Stasolla and Yeung 2003). This problem has been overcome by the use of ethylene inhibitors (Kong and Yeung 1994).

2.4 Markers for somatic embryogenesis

Somatic embryos can be used as a model for studying factors that affect zygotic embryo development. A large number of embryos at defined stages of embryo development can be obtained during any time of the year, in contrast to zygotic embryos which are available under a narrow developmental window. The development of both somatic and zygotic embryos have been well characterized on the morphological level, however it is poorly understood on the molecular level (Stasolla *et al.* 2002b).

It is hypothesized that the more closely the somatic embryos resemble their zygotic embryo counterparts in storage protein content (Klimaszewska *et al.* 2004), mineral content (Pullman *et al.* 2003c) and polyamine levels (Minocha *et al.* 1999), the better their quality and the higher the chances that they will survive and develop into a full plant. Additionally, it has been demonstrated that somatic embryos are indeed similar to zygotic embryos and undergo almost all of the stages, except that they do not become dormant (Suprasanna and Bapat 2005).

The induction process is the major difference between zygotic and somatic embryogenesis where zygotic embryogenesis develops from the fusion between gametes, while somatic embryogenesis is induced through components in the tissue culture medium (von Arnold *et al.* 2002). Thus, in somatic embryogenesis the embryogenic pattern is already determinate in embryogenic cells, while in zygotic embryogenesis is a highly regulated process with the formation of the megagametophyte (endosperm) (Yeung 1995). Then from the globular stage onward some differences may still exist between the two systems, but the developmental strategies should be the very similar (Yeung 1995). For example, it is documented that somatic embryos under optimal conditions have the ability to synthesize the same storage products as the counterpart zygotic embryos such, as in *P. glauca/engelmannii* (Flinn *et al.* 1993), *P. abies* (Hakman *et al.* 1990) and *P. caribaea* (Neutelings *et al.* 1998). Also, both somatic and zygotic embryos have the capability to form cotyledons, although in somatic embryos the number and form could vary depending on the culture medium. Additionally, "normal" somatic embryos have the ability to germinate and become complete plants, with the formation of both shoot

(SAM) and root (RAM) apical meristems. In *P. glauca,* somatic embryos follow the same morphological pattern for the formation of these two (SAM and RAM) as their zygotic counterparts (Yeung *et al.* 1998). "Normal" embryo production has improved with the use of compounds such as ABA and osmoticum during maturation (Jimenez 2005), and/or desiccation treatments (Roberts *et al.* 1990b) which inhibited precocious germination. At the morphological level, a staging system was developed for zygotic and somatic embryos of *P. taeda* and showed that all the stages observed in the zygotic system had counterparts in the somatic system (Pullman and Webb 1994).

During the different stages of somatic embryogenesis, progress through embryo development involves the temporal expression of different sets of genes that allows the normal formation of the embryo (Tang and Newton 2005a). The genes involved in somatic embryogenesis can be classified into three categories: 1) genes involved in cell division; 2) genes involved in organ formation; and 3) genes specific to the process of somatic embryogenesis (Stasolla and Yeung 2003). Gene expression in angiosperms during zygotic embryo development, maturation and germination has been classified into five distinct groups: 1) genes constitutively expressed at all stages and having functions required during normal plant growth; 2) embryo-specific genes whose expression is restricted to the embryo proper, ceasing prior to or at germination. 3) genes highly expressed during early embryogenesis until the cotyledonary stage; 4) genes expressed during the expansion of cotyledons, maturation and accumulation of proteins; and 5) genes expressed abundantly in later stages of embryogenesis until seed maturation, and mainly activated by ABA (Goldberg *et al.* 1994).

Marker genes that are expressed in a pattern that can be related to a specific developmental stage of the embryo are a useful tool for monitoring embryo development, from the acquisition of embryogenic competence through to maturation (Feher *et al.* 2003). These markers should be universal, sensitive, detectable in small amounts of tissue, and reveal specific cellular processes at each of the developmental stages or transitions (Feher *et al.* 2003).

Several markers have been used to distinguish between embryogenic and non-embryogenic cultures, such as SERK, LEC1, FUS3 and ABI3 (Yang and Zhang 2010). Additionally, extracellular protein markers have been described as an aid to determine the embryogenic potential of plant cells, including arabinogalactan (Egertsdotter and von Arnold 1995), non-

specific lipid transfer proteins (LTPs) (Sterk *et al.* 1991), and germin/germin-like proteins (GLPs) (Mathieu *et al.* 2006). Once the embryo has developed, markers such as storage protein genes can monitor the level of maturation achieved (Sterk and de Vries 1993). These markers are a helpful tool for defining embryogenic potential and identify differences among stages in embryo development, and has the potential to be used for the improvement of tissue culture protocols (Merkle *et al.* 1995).

In this study, we will be using four stages during the developmental and maturation of somatic and zygotic embryos to assess the relative expression of the following genes: 1) Legumin-like and vicilin-like (storage proteins); 2) Late embryogenesis abundant (LEA); 3) Clavata-like (meristem development), 4) Homeodomain-leucine zipper I (HD-Zip I); and 5) 26S proteasome regulatory subunit S2 (RPN1). All six genes have been studied in gymnosperm species and/or *Arabidopsis* using different approaches, and shown expression patterns related to embryogenesis. A brief description of each of them will be explained below.

Additionally, the examination of gene expression often results in quantification of the abundance of transcripts. Real-time PCR will be used to examine the relative expression of these genes during both somatic and zygotic embryo development and compared with previously published results. This technique allows for the relative quantification of the real-time expression of genes of interest during embryo development and maturation compared to the expression of genes against housekeeping or endogenous genes (Farrel 2005). Additionally, absolute quantification could be assessed by determining the input copy number, through relating the PCR signal to a standard curve (Livak and Schmittgen 2001).

Four developmental stages of both zygotic and somatic embryos will be chosen following these characteristics: 1) pro-embryos (zygotic) and embryogenic suspensor masses (somatic), 2) round and globular embryos, 3) early and 4) late cotyledonary embryos. In somatic embryos of *P. glauca* these stages have been characterized as follows: 1) immature embryo consisting of an embryonic region with densely cytoplasmic cells and a translucent suspensor which is long and highly vacuolated; 2) globular embryo after 2 weeks on ABA differentiated by prominent and opaque embryos having smooth and glossy surface adjacent to a suspensor which is cream to pale yellow in color; 3) early cotyledonary embryo after 3-4 weeks on ABA were defined when the primordial cotyledons was below the circumference of a prominent central meristem, and it

was cream to pale yellow; and 4) late cotyledonary embryo after 5-6 weeks on ABA with cotyledons extending beyond the central meristem (Dong *et al.* 1997).

2.4.1   Storage Proteins (Legumin-like and Vicilin-like)

Storage compounds such as lipids, carbohydrates and proteins accumulate in the endosperm, the aleurone layer or the embryo depending on the species during embryo maturation (Gatehouse and Shirsat, 1993). Of the proteins, the globulins are the most widely distributed group of storage proteins present in both dicots and monocots and likely to be a prerequisite for achieving vigorous germination. Based on their sedimentation coefficients, they can be divided into two groups: the 11S Legumin-type globulins and the 7S Vicilin-type globulins, representing the first and second subgroups respectively (Shewry *et al.* 1995). In gymnosperm, three major types of embryo storage protein have been identified: 11S Legumin-like in *P. glauca/engelmannii* (Flinn *et al.* 1991) and *P. menziesii* (Leal and Misra 1993b) ; 7S Vicilin in *P. pinaster* (Allona *et al.* 1994), *P. glauca/engelmannii* (Flinn *et al.* 1991) and *P. glauca* (Newton *et al.* 1992); and 2S albumin-like in *P. pinaster* (Allona *et al.* 1994) and *P. menziesii* (Chatthai and Misra 1998). The 11S Legumins are the major storage proteins not only in most legumes, but also in brassicas, composits, cucurbits, oats and rice (Shewry *et al.* 1995).

Profiles of seed storage proteins in gymnosperm have shown similarities to the angiosperm 11S Legumin and 7S Vicilin (Gifford 1988; Leal and Misra 1993b) specifically in their solubility, size and bonding patterns (Newton *et al.* 1992). Additionally, it has been shown that their transcript levels in developing *P. glauca/engelmannii* somatic embryos are similar to those found in zygotic embryos (Flinn *et al.* 1993).

The highest rate of storage accumulation coincides with a high ABA content, thus it is assumed that ABA might have a role in regulating reserve deposition in developing seeds (Yang and Zhang 2010). For example, in *P. glauca/engelmannii*, a concentration for 40 µM ABA was required to promote the synthesis of storage protein similar to levels found in their counterparts zygotic (Flinn *et al.* 1991). Additionally to ABA, osmotic agents play a role in improving the normal accumulation of storage protein in somatic embryos, such as in somatic embryos of *P. glauca* where the protein pattern synthesized was similar to their counterpart zygotic embryos, when somatic embryos were cultured on ABA and at a high osmotic concentration (Misra *et al.* 1993). Thus, the role of ABA and the osmoticum is to prevent precocious germination,

allowing the embryo to fully mature and consequently improving the accumulation of storage protein (Merkle *et al.* 1995).

A high-quality somatic embryo should accumulate storage proteins similarly to zygotic embryos to ensure successful germination, thus the presence of storage proteins are excellent markers to gauge the quality and fidelity of somatic embryos (Merkle *et al.* 1995).

Expression patterns of seed protein genes are highly regulated spatially and temporally during seed development (Dong and Dunstan 2000). The expression of seed storage proteins is tissue specific since it occurs in embryo and endosperm, but never in mature vegetative tissues (Thomas 1993).

Although storage proteins in somatic and zygotic embryogenesis exhibit differences in the kinetics of synthesis and accumulation, somatic embryos under optimal conditions are able to synthesize the same specific storage proteins as their zygotic counterparts (Dodeman *et al.* 1997). Storage proteins are immediately degraded once the zygotic embryos go into desiccation before germination; and similar degradation is observed when somatic embryos are partially desiccated (Dong and Dunstan 2000).

2.4.2   Late embryogenesis abundant (LEA)

During the late stages of zygotic embryogenesis, embryos undergo a period of desiccation, when they become highly dehydrated. During this period, there are proteins that are highly and abundantly synthesized and capable of surviving periods of desiccation, called Late Embryogenesis Abundant (LEA) proteins, because they more abundant during late embryogenesis than in mid-embryogenesis or early germination (Galau *et al.* 1986). Also they can be induced in vegetative tissues exposed to dehydration, osmotic, low temperature stress or with the application of ABA (Dure III *et al.* 1989; Ingram and Bartels 1996). A major problem under severe dehydration is that the loss of water leads to crystallization of cellular components, which damages cellular structures. It is hypothesized that LEA proteins work by stabilizing the cell membrane through conferring preferential hydration at moderate desiccation rates and replacing the water at extreme desiccation rates (Serrano and Montesinos 2003), as well as by serving as protein protectants (Chugh and Khurana 2002). LEA genes were first reported in cotton, where LEA mRNAs accumulated to high levels during embryo development and late embryogenesis and persisted in dry seeds until germination when they rapidly

degraded (Dure III *et al.* 1981). A similar role is played in different species, such as carrots, barley, rape and *Arabidopsis* (Dure III *et al.* 1989). Furthermore, LEA proteins genes have been suggested to confer stress tolerance to plants. After the introduction of the LEA protein gene HVA7 from barley (*Hordeum vulgare* L.) into rice, the transgenic plants showed tolerance to water deficit and salt stress (Xu *et al.* 1996)

LEA proteins have been grouped into various families on the basis of sequence similarity (Chugh and Khurana 2002). Members of the LEA protein families have been reported in angiosperms and gymnosperms. In angiosperms their presence has been confirmed in both zygotic and somatic embryos of carrot (Dodeman *et al.* 1998), as well as for gymnosperms in somatic (Stasolla *et al.* 2003) and zygotic embryos (Dong and Dunstan 1999).

Expression of the LEA genes during zygotic embryogenesis has been shown to be regulated by ABA, with ABI3 (*Arabidopsis*) and VP1 (maize) representing the major transcriptional factors responsible for regulating LEA expression (Leung and Giraudat 1998). Additionally, LEA gene expression was induced when embryos were treated with ABA in somatic embryo cultures of *Arabidopsis* (Ikeda *et al.* 2002) and carrot (C-ABI3), (Shiota *et al.* 1998). Based on these results, LEA gene expression can be considered as markers for endogenous ABA accumulation (Ikeda *et al.* 2006).

2.4.3 Meristem Development (Clavata-like)

In angiosperms, the shoot meristem cells are found in three layers: L1, epidermal cell layer; L2, sub-epidermal cell layer; and L3, under-laying cell layer (Fig. 2-5). The shoot apical meristem contains the pool of undifferentiated cells providing the cell lineage which will differentiate and become organs (Lenhard and Laux 1999). A balanced population of shoot apical meristem cells is maintained by an auto-regulatory feedback loop between *WUSCHEL (WUS)* and a receptor-like kinase pathway comprised of two leucine-rich repeat (LRR) receptor kinases encoded by CLAVATA 1 (CLV 1) and CLAVATA 2 (CLV 2) and their putative ligand encoded by CLAVATA 3 (CLV 3) (Schoof *et al.* 2000). An increase in the number of the undifferentiated cells promotes transcription of CLV 3, which binds to CLV1 suppressing the expression of WUS; subsequently the number of undifferentiated cells decreases, decreasing the level of CLV3 and allowing the expression of WUS (Fig. 2-5). This feedback loop is essential for maintaining the proper balance of undifferentiated cells in the SAM. Over

expression of *WUS* leads to uncontrolled proliferation of undifferentiated cells, whereas over expression of *CLV3* leads to a loss of meristem function (Schoof *et al.* 2000).

The formation of the apical meristem during somatic embryogenesis is one of the most important events defining the quality of the embryo and its ability to germinate (Yeung 1995). However, compared to flowering plants, the molecular mechanisms that regulate conifer embryogenesis are yet poorly understood (Tahir and Stasolla 2006), since for conifer species currently there are only two potential genes, *HBKI* (maize *Knotted* 1) isolated from *P. abies* (Sundås-Larsson *et al.* 1998) and *PgAGO* (ARGONAUTE family) isolated from *P. glauca* (Tahir et al. 2006) to be related to shoot apical meristem development. The expression of both genes was high in the apical notch. Even though CLV has a function during somatic embryo formation, it may not be involved in the acquisition of embryogenic competence (Ikeda and Kamada 2006), thus it cannot be used as marker for embryo induction but for later stages of embryo development. The CLV gene in *Arabidopsis* is expressed in the embryogenic apex, from the heart stage onwards (Souter and Lindsey 2000).

In conifer somatic embryogenesis, manipulation of the maturation medium has been shown to improve the percentage of embryos going through successful development. Low germination and conversion rates could be influenced by nutritional conditions or lack of a functional apical meristem (Yeung 1995). In *P. glauca,* somatic embryos followed the same morphological pattern for the formation of SAM as their zygotic counterparts. However, at the end of the maturation period, some of the somatic embryos showed abnormal meristems disrupted by intercellular spaces (Tahir and Stasolla 2006). It has been suggested that the intracellular spaces are formed due to the accumulation of ethylene inside of the vessel culture, and the use of ethylene inhibitors can thus overcome this problem (Kong and Yeung 1994). Prolonged period of subculture could generate a disruption of the shoot apical meristem formation during *in vitro* culture (Yeung *et al.* 1998). The findings on the disruption of SAM during somatic embryogenesis conclude that this structure is not pre-determined and could be altered by suboptimal conditions such as ethylene accumulation and/or prolonged subcultures.
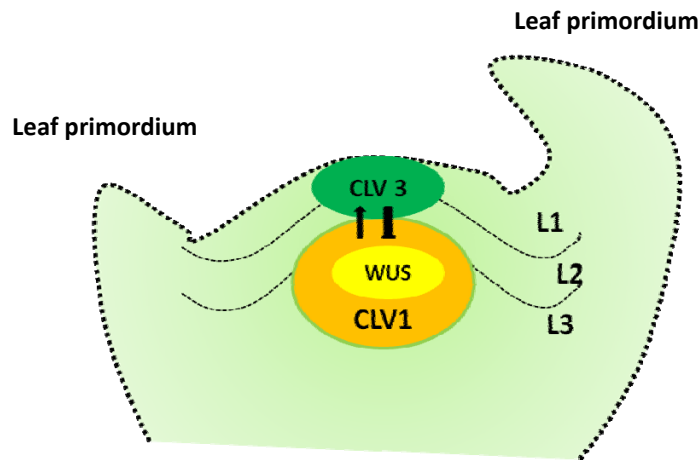
Figure 2-5. Maintenance of shoot apical meristem is controlled by an auto feedback loop between a stimulatory signal produced by cell expression WUSCHEL (arrow line) and inhibitory signal produced by CLAVATA 3 (flat-end line). L1, epidermal cell, L2 sub-epidermal cell and L3, under-laying cells. Adapted from Taiz and Zeiger (2006).

2.4.4 Homeodomain protein: Homeodomain-leucine zipper I (HD-Zip I)

Homeobox genes encode transcriptional factors in plants, which are regulatory proteins involved in the activation of other genes regulating tissue patterning (Chugh and Khurana 2002). Plants, like animals, use homeobox genes as transcription factors to control different developmental processes. The homeobox encode a protein domain, the Homeodomain (HD), composed of a 60-amino acid conserved motif present in transcription factors and folded into a tree-helix structure able to interact specifically with DNA (Ariel *et al.* 2007).

One class of homeodomain proteins unique in plants are the homeodomain-leucine zipper (HD-Zip) group, which differ from the rest because they contain a leucine zipper motif (Zip) adjacent to the homeobox element (Ariel *et al.* 2007) (Fig. 2-6). The Zip motif confers the ability to transcriptionally regulate other genes via a protein-protein interaction. However, the mode of action of HD-Zip is still poorly understood (Elhiti and Stasolla 2009). The homeodomain is responsible for the specific binding to DNA as dimers, whereas the leucine zipper acts as a dimerization motif. The absence of the leucine zipper prevents the binding ability because the relative orientation of the monomers driven by the Zip motif is vital for an efficient recognition of DNA (Ariel *et al.* 2007).

Based on sequence similarity as well as other sequence criteria, the HD-Zip genes of *Arabidopsis* are grouped into four families HD-Zip I to IV (Elhiti and Stasolla 2009). The HD-

Zip I appear to be important regulators of plant development and differentiation in response to environmental factors (Deng *et al.* 2006) and currently there are seventeen members (Ariel *et al.* 2007).

Additionally, of the four HD-Zip classes, class I has been implicated in ABA responses in *Arabidopsis* (Henriksson *et al.* 2005), *Gossypium hirsutum* (Ni *et al.* 2008) and *Picea glauca* (Tahir *et al.* 2008). In *Arabidopsis*, the genes ATHB6, ATHB7 and ATHB12 regulate gene expression in response to ABA, and to environmental conditions known to increase endogenous levels of this plant growth regulator, such as water deficiency (Henriksson *et al.* 2005).

In *P. glauca* a Homeodomain-leucine zipper class I (PgHZ1) was characterized and isolated and found to be related to embryo production *in vitro* (Tahir *et al.* 2008). Phylogenetic analyses with *Arabidopsis* HD-Zip members showed that PgHZ1 belongs to the same monophyletic group of ATHB3, 13, 20, and 23 with which it shares a respective amino acid similarities of 74, 71, 68, and 61 %, respectively (Tahir *et al.* 2008). In *Arabidopsis*, ATHB20 was expressed in 5-days old and 12-days old seedling, roots of 12-days old seedlings, leaves, stem, flowers and siliques, ATHB3 and -23 were expressed in all the previous tissues, except leaves and flowers, respectively; and the ATHB13 was detected in seedlings, leaves, and flowers, but not in roots, stems, or siliques. All of the HD-Zip members in *Arabidopsis* were shown to be responsive to ABA (Henriksson *et al.* 2005).

Expression studies during somatic embryo development showed that the transcript levels of PgHZ1 increased during the late phases of proliferation and remained high during the subsequent embryo growth when the somatic embryos were transferred to ABA. The transcript levels did not increase in a non-embryogenic line, thus suggesting PgHZ1 as a potential molecular marker for embryogenesis (Tahir *et al.* 2008). Furthermore, transformation studies on *Arabidopsis* reveal that PgHZ1 may play a regulatory role related to ABA signaling, as its over-expression enhances the responsiveness of the tissue to ABA, showing plants with a delay in flowering time, smaller in size, reduced apical dominance, lower number of seeds per silique, and embryos with higher accumulation of storage protein compared to those of the wild type (Tahir *et al.* 2008).

Figure 2-6. Schematic representation of the HD-Zip class I. Adapted from Ariel (2007).

2.4.5   26S Proteasome subunit S2 (RPN1)

The 26S proteasome (P) is an ATP-dependent proteolytic system that degrades ubiquitin (Ub) conjugates (Smalle and Vierstra 2004) (Fig. 2-7). The ubiquitin/proteasome system (UPS) controls the balance between degradation and synthesis of proteins regulating the concentration of proteins inside the cell (Dreher and Callis 2007). During ubiquitination, proteins are targeted for degradation by tagging with polymers of Ub (Fig. 2-7A). This process is achieved though three enzymes: 1) E1, Ub-activating enzymes, which modify Ub so that it is in a reactive state; 2) E2, Ub-conjugating enzymes which catalyze the attachment of Ub to the substrate protein; and 3) E3 Ub-ligases, which function in concert with E2 enzymes, in recognizing the substrate protein (Ciechanover *et al.* 1998). Once the proteins are tagged by conjugation with Ub, they are recognized and shuttled to the proteasome for degradation (Dreher and Callis 2007). It is believed that the main role of the Ub is to decrease the rate of dissociation between proteasome and the substrate proteins, allowing the substrate protein to interact with a proteasome for a longer period of time, increasing the probability that the proteasome will degrade it (Ciechanover *et al.* 1998).

The 26S proteasome can be further divided into two particles: the 20S core protease (CP) and the 19S regulatory particle (RP) (Fig. 2-7B). The RP is assumed to trigger the identification of appropriate substrates for breakdown, to remove the attached ubiquitin moieties, to open the alpha-subunit gate, and to direct the entry of unfolded proteins into the core protease cavity for degradation (Ciechanover *et al.* 1998). Peptide bonds are cleaved every 8-9 amino acids and further hydrolyzed to free amino acids (Dreher and Callis 2007). The core protease is cylindrical in shape and formed by the assembly of four stacked heptameric rings of related α and β subunit (Groll et al., 1997) (Fig. 2-7B).

41

Seventeen subunits of the RP have been identified, which form two sub particles: the lid and the base. The base contains three non-ATPase subunits (RPN1, RPN2, and RPN10) and six ATPase subunits (RPT1 to RPT6). The lid binds to the base and contains the other nine subunits (RPN3, RPN5 to RPN9, and RPN11 to RPN13) (Brukhin *et al.* 2005). Mutations affecting specific factors have been shown to block processes as diverse as embryogenesis, hormonal responses, entrainment of circadian rhythms, floral homeosis, photo-morphogenesis trichome differentiation, senescence and pathogen defense (Vierstra 2003). The UPS is essential for the removal of non-functional proteins before they become proteotoxic, accumulate, aggregate and induce cell death (Kurepa and Smalle 2008) and it may be required for the selective destruction of proteins associated with the previous differentiated cell state (Feher *et al.* 2003)

The RPN2 has been shown to play a role in root development (Ueda *et al.* 2004), RPN10 in ABA signaling (Smalle *et al.* 2003) and RPN12 in cytokinins (Smalle *et al.* 2002). The RPN8 play a role in specifying leaf adaxial identity (Huang et al 2006). In embryo development, RPN1 (Brukhin *et al.*, 2005) and RPN5 (Book *et al.* 2009) have been shown to be essential for embryogenesis in *Arabidopsis*, where inactivation of RPN1a arrests embryogenesis at the globular stage (Brukhin *et al.*, 2005). However, a recent study found that mutants lacking RPN1a were viable and had increased cell sizes, thus proposing that RPN1a does not have a specific function in embryo development (Wang *et al.* 2009).
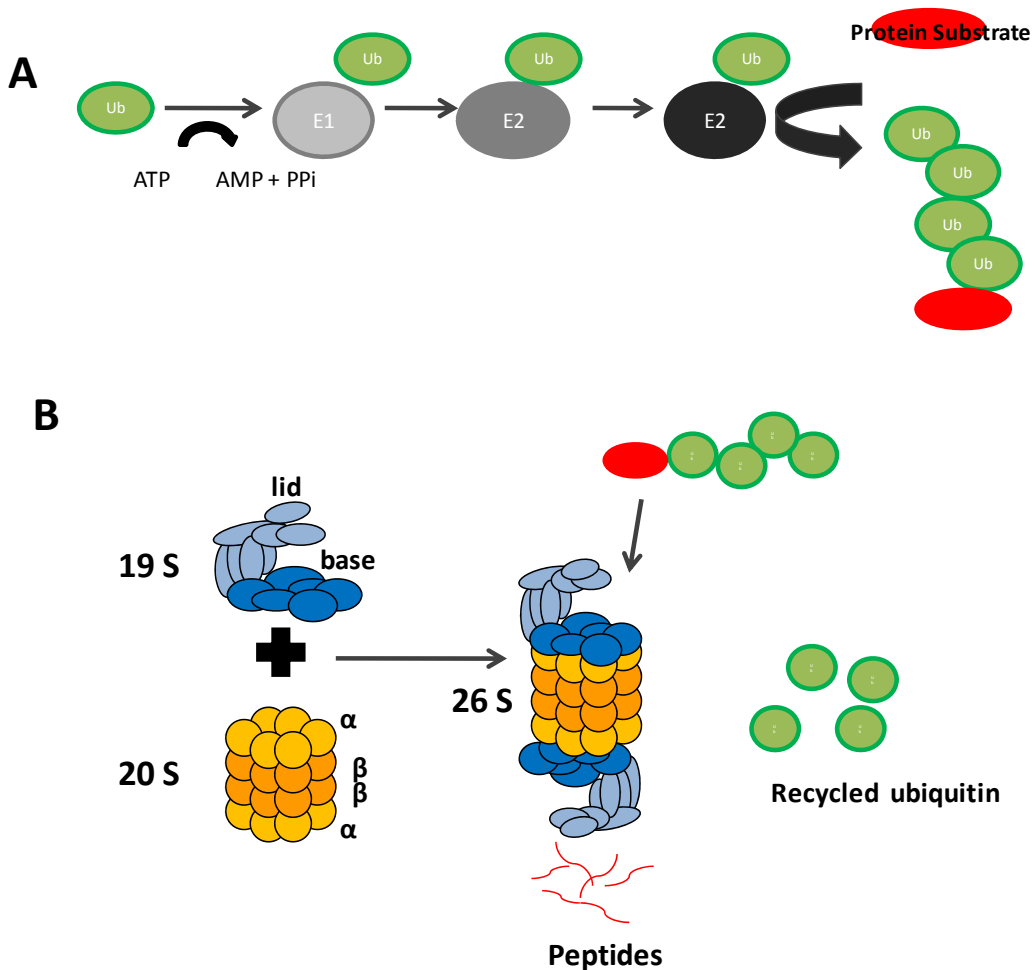
Figure 2-7. Schematic representation of the ubiquitin-proteasome pathway. A) the ubiquitin processes and B) the protein degradation through 26S proteasome, formed by the regulatory particle (19S) and the core protease (20S). Adapted from Ciechanover *et al.* (1998).

2.5   Summary

A large amount of research has been conducted to optimize culture conditions to produce somatic embryos of different coniferous species. However, for some pine species the percentages of initiation, maturation and production of somatic seedlings are still low, thereby reducing the application of this technique for commercial propagation. Production of larger quantities of better quality embryos could be possible by mimicking the natural conditions through the culture medium. Thus, the study of the mineral content in seeds will reveal some of the elements supplied by the megagametophyte. The elemental composition of the megagametophyte will guide us to develop a novel culture medium, supplemented with plant

growth regulators and other compounds reviewed in this chapter to find a protocol for the somatic embryogenesis in *P. oocarpa*. Additionally, existing protocols for somatic embryogenesis in conifers will be tested for the efficiency to initiate somatic embryogenesis in families of interest of *P. taeda* and *P. palustris*.

The quality of *P. taeda* mature somatic embryos will be studied at the gene expression level to compare the transcript levels during somatic embryo development and maturation to transcript levels at the corresponding stages of zygotic embryo development. For this study six genes were chosen which have previously been shown to be related to embryogenesis. The genes related to Legumin-like and Vicilin-like storage proteins, have previously been used as marker genes for embryo maturation. The transcripts have been shown to accumulate during zygotic embryo maturation, as well as somatic embryo maturation under optimal conditions. Expression of another molecular marker is for a Late embryogenesis abundant (LEA) gene, associated with ABA accumulation, and which peaks at the end of the maturation period, to protect the embryo from desiccation damage. The quality of the shoot apical meristem will be studied by tracking the levels of the Clavata-like gene expression. The levels of PgHZ1 transcripts will also be studied. This transcription factor is present in embryogenic tissue and related to ABA sensitivity. And lastly, RPN1 gene expression, which has been associated with embryo development where it plays a role in the degradation of proteins. This information collected would elucidate the fidelity of the somatic embryos matured on the current protocol and guide us in future medium composition improvements.

3   Initiation of somatic embryogenesis in *Pinus taeda* and *Pinus palustris* with different basal media and plant growth regulators

ABSTRACT

Initiation of somatic embryogenesis from immature zygotic embryos was studied from four families of *Pinus taeda* and five families of *Pinus palustris*. Four basal media 1) AL (Kvaalen *et al.* 2005); 2) Dale Smith (DS) (Smith 1996); 3) 1218 (Pullman *et al.* 2005a); and 4) TX (CellFor Inc.) (Attree 2003) were tested, supplemented with different plant growth regulator combinations. For both pine species, the best results were obtained with basal medium 1218 supplemented with auxins and cytokinin at a ratio of 10:5 (molar). The initiation was repeated in the following year (2007) with immature zygotic embryos of *P. palustris* from three families different from those tested the previous year*,* and with the best basal medium from 2006 combined with three concentrations of 2,4-D and BA. The results from the latter experiment showed that the optimal plant growth regulator concentrations for initiation were genotype-dependent. Additionally, the optimal culture medium composition for maturation and germination of somatic embryos of *P. taeda* line J8189 (CellFor Inc.) was tested with different concentrations and combinations of 1) ABA (0, 40, 80 and 120 µM) and maltose [3 and 6 % (w/v)]; and 2) Phytagel [(0.3, 0.6, 0.9 and 1.2 (w/v)]. The number of somatic embryos was statistically different among the ABA concentrations (p=0.003), with the highest number of mature embryos obtained using 40 µM ABA, with more vigorous embryos observed at 6 % (w/v) maltose. In order to increase the number of mature embryos obtained per gram fresh weight of proliferating culture, the best maturation treatment from above was tested in combination with four Phytagel concentrations. The number of mature embryos was significantly increased with the use of 0.6 % (w/v) Phytagel (p >0.0001). Germination of mature embryos was improved by using a desiccation treatment of 10 min. The same ABA, maltose and Phytagel concentrations were tested on the cell lines of *P. taeda* and *P. palustris* established in the above experiments (2006 and 2007). Embryo head formation and some cotyledon initiation were observed, but maturation did not progress further.

Key word: somatic embryogenesis, extrusion, culture medium, 2,4-D, ABA, maltose, Phytagel, desiccation.

3.1   Introduction

The USA is the world's largest consumer and producer of forest products, accounting for about 15 % of world commercialization in forest products (FAO 2003). Currently, the southeastern region of the USA produces approximately one-half of the total world production of products derived forest plantations (Reviewed by Allen *et al.* 2005). Pine plantations such in *P. palustris* are established from seed collected from wild trees, other such as *P. taeda* from open- or control-pollinated parents in seed orchards (Nehra *et al.* 2005) and from rooted cuttings (Park 2002). Seeds from natural tree stands have significant genetic variation, thus the resulting trees differ in performance in the field. Clonal propagation methods are thus required to fully capture the benefits from breeding programs. For some hardwood species, the most used clonal propagation technique is through rooted cuttings; however, this method has been less effective for pine species because the rooting ability of the cuttings decreases with the mother plant age (Park 2002). In the last twenty years, efforts have been made to apply somatic embryogenesis as a clonal propagation method for conifer plant propagation (Klimaszewska *et al.* 2007). There are, however, several bottlenecks that hamper large scale production. In pine species in particular, plant propagation by somatic embryogenesis is limited by low somatic embryogenesis initiation rates, low levels of culture survival, low maturation to germination and conversion rates, and slow initial growth of somatic seedlings (Pullman and Johnson 2002).

Somatic embryogenesis protocols have been published for different pine species, and the optimization of culture media, methodologies, and field studies to determine fidelity of the trees relative to seedlings has been further extended for economically important pine species, such as *P. taeda*, *P. radiata* and *P. banksiana* (Häggman *et al.* 2006). Even though the use of somatic embryogenesis for plant production of high value varieties is at an early stage, it has already been successfully used for the production of millions of somatic seedlings of *P. taeda* by CellFor and planted in the USA (Klimaszewska *et al.* 2007).

Currently, somatic embryogenesis is the only clonal propagation technique with the possibility of capturing the benefits from genetic gain (volume and quality improvement) in conifer species on a large scale. Specifically, somatic embryogenesis propagation from controlled crosses of elite individuals of known genetic value increased the genetic gain in *P. taeda* (Sutton 2002). Somatic embryogenesis also has the advantage of long-term storage under liquid

nitrogen of the clonal material, allowing the clones to be tested in the field and subsequently selected clones can be mass produced from the stored material. Proliferating somatic embryo cultures are also the most suitable tissue for transformation. Despite these advantages, conifers, especially pine species, are still considered recalcitrant to the somatic embryogenesis technique, due to low initiation, maturation and conversion rates and the extensive manual labor associated with the suboptimal culture performance. Thus, different research groups are dedicated to the in depth study of the somatic embryogenesis in commercial species such as *P. taeda* (Li *et al.* 1998a; Pullman and Buchanan 2003; Pullman *et al.* 2006; Pullman and Skryabina 2007). However less has been done for species such as *P. palustris* (Merkle *et al.* 2005; Nagmani *et al.* 1993).

Of the North American pine species, *P. taeda* L. (Loblolly pine) is the most planted species in the southern region of the USA (84 %) and it is one of the most important sources of fiber and timber in both North and South America (McKeand *et al.* 2003). *P. taeda* is found throughout southeastern New Jersey to central Florida and west into Tennessee, Kentucky, Texas, and Oklahoma (Schultz 1997). Additionally, it has been introduced in different countries, including China and Brazil, which currently have the most extensive plantation programs outside of the USA (Schultz 1997). Due to its economic importance, *P. taeda* has been one of the major tree species targeted by breeding programs and biotechnology applications (Fenning and Gershenzon 2002). In 2002, a survey conducted in companies and state forestry agencies belonging to one or more of the South's cooperative tree improvement programs; the Cooperative Forest Genetics Research Program at the University of Florida, the Western Gulf Forest Tree Improvement Program at the Texas Forest Service, and the North Carolina State University–Industry Cooperative Tree Improvement Program, showed that 1,137 million *P. taeda* and 32 million *P. palustris* seedlings are planted annually, and that 61 and 87 % respectively, are designated for marketing (McKeand *et al.* 2003).

P. palustris Mill (Longleaf pine) was once the predominant pine species in the Southern and Coastal plains of the USA. However, over the last two hundred years, it has been drastically reduced (Outcalt and Sheffield 1996) due to intensive exploitation, poor silviculture practices (fire suppression), replacement by other species (*P. elliottii* and *P. taeda*), conversion of land use for other systems (agriculture) and lack of development of the regeneration procedures (Mitchell *et al.* 2006; Schmidtling and Hipkins 1998). The natural range of *P. palustris* extends

from the southeastern tip of Virginia to southern Florida, and westward to eastern Texas (Schmidtling and Hipkins 1998). Interestingly, the *P. palustris* ecosystem is one of the most species-rich plant communities (Outcalt and Sheffield 1996), just behind the Ecuadorian Rainforest system (Mitchell *et al.* 2006). This has caused increasing interest in conservation and restoration of the species due to its historical, commercial and ecological prominence in public and private lands (Merkle *et al.* 2005; Schmidtling and Hipkins 1998). Additionally, *P. palustris* saw timber and poles have been characterized as high-quality products and there is an increasing interest in a better understanding of sustainable timber management (Mitchell *et al.* 2006). Currently, Florida has the largest amount of *P. palustris* forest of all the states, and public agencies own the highest percentage of acreage (Outcalt and Sheffield 1996). However, individual farmers and other private landowners (individual farmers and corporations) own 51 % of the total *P. palustris* acreages; followed by public agencies (33 %) and by forest industries (16 %) (Outcalt and Sheffield 1996).

The Virginia Department of Forestry has an interest in developing a system for somatic embryogenesis propagation of valuable families of *P. taeda* and *P. palustris*. The somatic embryogenesis process is genotype dependent and it is important to test different basal initiation media and other factors to develop an efficient protocol that works across different genotypes (Park 2002). Additionally, the developmental stage of the zygotic embryo used as the explant for somatic embryogenesis initiation is critical, especially in pine species where immature zygotic embryos are required (Park 2002).

The objectives of this project were to: 1) test the effect of four basal media combined with four combinations of plant growth regulator concentrations on the initiation of somatic embryogenesis in *P. taeda* and *P. palustris*, 2) test different immature zygotic embryo collections for the initiation of somatic embryogenesis in *P. taeda* and *P. palustris*, 3) test three concentrations of 2,4-D (5, 10 and 15 µM) combined with three concentration of BA (3, 6 and 9 µM) in a second initiation of somatic embryogenesis in *P. palustris* using immature zygotic embryos, and 4) test the effects of ABA, maltose and Phytagel on the maturation and subsequent germination of somatic embryos in *P. taeda* cell line J8189.

3.2   Materials and methods

3.2.1   Plant material

3.2.1.1   Initiation of somatic embryogenesis of *P. taeda* and *P. palustris* in 2006

Immature *P. taeda* cones were collected from Reynolds Homestead (Critz, VA) (http://www.reynoldshomestead.vt.edu/), and *P. palustris* cones were provided by the Virginia Department of Forestry (http://www.dof.virginia.gov/index.shtml). Immature cones were collected weekly from June 21st to August 1st 2006 (Table 3-1). Cones from families 20-692, 20-615, 20-1036 and 20-639 were collected for *P. taeda*, and cones from families 20-820, 20-803, 20-822, 20-805 and 20-825 were collected for *P. palustris*.

3.2.1.2 Initiation of somatic embryogenesis of *P. palustris* in 2007

A second round of initiations was started during 2007 using three additional *P. palustris* families: 20-807, 20-839 and 20-840. Immature cones were collected weekly from June 29th to August 1st for families 20-839 and 20-840. Cones of family 20-807 were collected once on June 29th (Table 3-1).

3.2.2   Cone disinfection

The cones were gently washed with soap and water. Under aseptic laminar hood conditions, the cones were disinfected for 1-2 min in 70 % (v/v) ethanol, then for 5 min in 2.5 % (v/v) commercial sodium hypochlorite containing 5 drops of Tween-20 per 100 ml of solution, followed by three rinses in sterile deionized water. The seeds were extracted, dissected, and each intact megagametophyte was placed onto initiation media. For both species, the megagametophyte was removed from the seed, and one explant per Petri dish (60 X 15 mm) was placed on the different treatments (3.2.3), and cultured in darkness at 24 ºC. Data was collected 8-9 weeks following initiation and the response of the explants was classified in two categories: 1) Embryogenic cultures, and 2) Non-embryogenic cultures (including callus formation, non-responsive explants, and browning tissues).

Table 3-1. Collection dates for each of the families used in the initiation experiments with *P. taeda* and *P. palustris* in 2006. * indicates dates of collection

Species	Collection dates 2006						
Families	**6/21**	**6/27**	**7/10**	**7/16**	**7/24**	**7/31**	**8/1**
Pinus taeda							
20-692	-	-	*	*	*	-	-
20-615	-	-	*	*	*	-	-
20-1036	-	-	*	*	*	-	-
20-639	-	-	*	*	*	*	*
Pinus palustris							
20-820	*	-	*	*	*	-	*
20-803	*	-	*	-	*	-	-
20-822	-	-	*	-	*	-	-
20-805	-		*	*	*	-	-
20-825	-	*	*	*	*	-	-
	Collection dates 2007						
	6/29	**7/5**	**7/10**	**7/18**	**8/1**		
20-807	*	-	-	-	-		
20-839	*	*	*	*	*		
20-840	*	*	*	*	*		

3.2.3 Initiation of somatic embryogenesis

3.2.3.1 Initiation of somatic embryogenesis of *P. taeda* and *P. palustris* in 2006

Both species were evaluated using four basal media: 1) AL (Kvaalen *et al.* 2005); 2) Dale Smith (DS) (Smith 1996); 3) 1218 (Pullman *et al.* 2005a); and 4) TX (CellFor Inc.) (Attree 2003) (Table 3-2). Each basal medium was tested with four plant growth regulator combinations, belonging to three out of four of the basal media just cited (DS medium is plant growth regulator-free medium): 1) without hormones *(PGR-free)*; 2) AL medium-5.00 µM BA (*AL*); 3) TX medium-4.40 µM BA and 9.79 µM 2,4-D (*TX*); and 4) 1218 medium-2.80 µM BA, 10.74 µM NAA and 2.83 µM Kin (*1218*) (Table 3-3). In addition, the basal media 1218 and DS were also tested with 2.20 µM of BA and 27.14 µM of 2,4-D (*LJ*). There were a total of 18 treatments.

Table 3-2. Basal media for the initiation of somatic embryogenesis of *P. taeda* and *P. palustris* in 2006.

Compound	Concentration (mg/L)			
	AL (Kvaalen *et al.* 2005)	**TX** (Attree 2003)	**1218** (Pullman *et al.* 2005a)	**DS** (Smith 1996)
KNO_3	-	950.00	909.90	715.50
$Ca(NO_3)_2 \cdot 4 H_2O$	-	-	236.20	-
NH_4NO_3	-	-	200.00	-
$NH_4H_2PO_4$	-	-	-	112.50
KH_2PO_4	-	170.00	136.10	-
$K_2HPO_4 \cdot 3H_2O$	570.60	-	-	-
$CaCl_2 \cdot 2H_2O$	-	211.00	-	12.50
$Mg(NO_3)_2 \cdot 6H_2O$	-	-	256.50	-
$MgCl_2 \cdot 6H_2O$	-	-	101.70	-
$MgSO_4 \cdot 7H_2O$	394.20	925.00	246.50	200.00
$CaSO_4 \cdot 2H_2O$	37.80	-	-	-
H_3PO_4	172.00 ml	-	-	-
$NaNO_3$	-	-	-	155.00
$FeSO_4 \cdot 7H_2O$	8.34	27.80	13.90	15.00
$C_{10}H_{14}N_2Na_2O_8$ $2H_2O$	-	37.20	18.65	20.00
KI	0.08	4.15	4.15	0.50
H_3BO_3	2.48	31.00	15.50	4.00
$ZnSO_4 \cdot 7H_2O$	5.76	43.00	14.67	12.50
$MnSO_4 \cdot H_2O$	18.60	21.0	10.50	1.80
$Na_2MoO_4 \cdot 2H_2O$	1.18	1.50	0.13	0.10
$CuSO_4 \cdot 5H_2O$	3.74	0.50	0.17	1.20
$CoCl_2 \cdot 6H_2O$	0.012	0.13	0.12	0.10
$NiCl_2 \cdot 6H_2O$	1.18	-	-	-
$AgNO_3$	-	-	3.39	-
Thiamine HCl (B1)	1.00	0.10	1.00	2.50
Nicotinic Acid	0.50	0.50	0.50	2.50
Pyridoxine HCl (B6)	0.50	0.10	0.50	0.25
Glycine	2.00	-	2.00	-
Myo-Inositol	1,000.00	100.00	20,000.00	500.00
Maltose		-	15,000.00	-
Sucrose	10,000.00	10,000.00	-	10,000.00
Casamino Acids		-	500.00	-
L-glutamine	2,000.00	-	450.00	-
Magnesium citrate	305.00	-	-	-
MES		-	250.00	-
Activated charcoal		-	-	2,000.00
Phytagel	3,000.00	3,000.00	3,000.00	3,000.00

Table 3-3. Plant growth regulators used in the culture medium for the initiation of somatic embryogenesis of *P. taeda* and *P. palustris* in 2006.

Plant growth regulators	Concentration (µM)				
	AL	*DS*	*TX*	*1218*	*LJ*
Auxins					
2,4-D	-	-	9.79	-	27.14
NAA	-	-	-	10.17	-
Cytokinins					
BA	5.00	-	4.40	2.80	2.20
Kin	-	-	-	2.83	-

3.2.3.2 Initiation of somatic embryogenesis of *P. palustris* in 2007

Based on the results from the previous experiment (3.2.3.1), the basal medium 1218 was used for the initiation of somatic embryogenesis from *P. palustris*. The medium was combined with three concentrations of 2,4-D (5, 10 and 15 µM), each combined with three concentrations of BA (3, 6 and 9 µM). A total of nine treatments were tested.

3.2.4 Double staining of tissues

Embryogenic tissue was identified using both morphological characteristics (presence of a white mucilaginous callus) and histological methods (Gupta and Holmstrom 2005). Briefly, for histochemistry the tissue was placed on a glass slide and first stained with 2 % (w/v) acetocarmine. The tissue was divided into smaller pieces, and the slide was heated over a low flame for a few seconds. The tissue was then washed twice with deionized water. The second stain, 0.5 % (w/v) Evan's blue, was added and after 30 seconds was washed twice with water. The tissue was then mounted into glycerol to prevent drying. Observations were made under a zoom stereomicroscope (Olympus America Inc.)

3.2.5 Maturation experiments

3.2.5.1 Maturation of somatic embryos of *P. taeda* and *P. palustris*

The established cell lines of both *P. taeda* (20-692) and *P. palustris* (20-803, 20-839 and 20-840) were tested for their ability to mature using three concentrations of ABA (40, 80 and 120 µM), combined with different compounds: 1) PEG 8000 concentrations of 0, 6 and 12 % (w/v); 2) Phytagel concentrations of 0.3, 0.6, 0.9 and 1.2 % (w/v); and 3) 2,4–D concentrations of 0.1, 1.0 and 10.0 µM). Cultures were incubated for one week on a pre-maturation medium

consisting of the multiplication medium 1218 without plant growth regulators before placement on the different maturation treatments. The maturation medium was the basal medium 927 (Pullman *et al.* 2003c) without PEG 8000 and containing 0.3 % (w/v) Phytagel instead of Gelrite.

3.2.5.2 Maturation experiments with *P. taeda* cell line J8189

P. taeda cell line J8189 was kindly provided by CellFor, Inc. Tissue was multiplied on 1218 medium (Pullman *et al.* 2005a) until enough tissue was available for the maturation experiments. For maturation, three pieces of proliferating culture, each of 400-500 mg, were placed on each 9 cm Petri plate, with three replicate plates per treatment. Cultures were incubated for one week on pre-maturation medium, which consisted of the multiplication medium 1218 without plant growth regulators. Maturation medium was the same as above (3.2.5.1). Two experiments were conducted:

3.2.5.2.1 <u>**Maturation experiment 1:**</u> Maturation medium was tested with two concentrations of maltose, 3 % and 6 % (w/v), each combined with 0, 40, 80 and 120 µM ABA, for a total of 8 treatments.

3.2.5.2.2 <u>**Maturation experiment 2:**</u> The best treatment from the experiments described in 3.2.5.2.1 [40 µM ABA plus 6 % (w/v) maltose] was tested in combination with four Phytagel concentrations 0.3 (control), 0.6, 0.9 and 1.2 % (w/v).

For both experiments, the cultures were transferred to fresh medium after four weeks, and the number of well developed somatic embryos was recorded at week 8 to 10. Embryos were then tested for germination.

3.2.6 Germination experiments

3.2.6.1 Germination experiments with *P. taeda* cell line J8189

Germination was counted as the elongation of both shoot and root. The germination medium consisted of ½ MS medium (M519 Phytotechnology Lab) plus 0.25 mg/L copper sulfate, 100.0 mg/L myo-inositol, 0.3 % (w/v) Phytagel, 0.25 % (w/v) activated charcoal and 2 % (w/v) maltose. Plates were incubated for 7 days in the dark, and then placed under cool white fluorescent light at an intensity of 75.71 µmol m^{-2} s^{-1}, at 25 ºC and a photoperiod of 16/8-h (day/night). Plantlets with epicotyls and roots were transferred to a mixture of sterile

53

vermiculite:perlite (3:1) in GA-7 vessels (Magenta Corporation, Chicago, IL) moistened with sterile liquid germination medium and kept under the same above environmental conditions prior to their transfer to *ex vitro* conditions.

3.2.6.1.1 <u>**Germination experiment 1:**</u> The mature somatic embryos from the Maturation 1 (3.2.5.2.1) experiment were divided and one half placed directly onto germination medium (control) and the second half were placed into a Petri dish and left uncovered under the laminar flood hood for 1 hour (1h desiccation). Embryos were then placed onto germination medium. After 12 weeks, embryos were scored for the percentage of embryos that germinated.

3.2.6.1.2 <u>**Germination experiment 2:**</u> Germination of mature somatic embryos from Maturation 2 (3.2.5.2.2) was carried out on germination medium containing 3 % (w/v) sucrose, after a flow hood desiccation pretreatment of 10 min. After 6-7 weeks, embryos were scored for the percentage of embryos that germinated.

3.2.7 Data collection and statistical analysis

For the analysis of embryogenic cultures, four cultures media were tested with four plant growth regulators combinations. Additionally, two of the basal media (1218 and DS) were tested with one additional combination of plant growth regulators; thus, a total of 16 treatments were evaluated. Percentage of embryogenic cultures was calculated by dividing the number of observation out of the total number of explants used per each family or treatments.

The effect of ABA and maltose was studied using two-way ANOVA and the effect of Phytagel was studied by one-way ANOVA analysis using the statistical program JMP 8.0 (SAS Inc., Cary, NC, USA). Significant differences between means were determined by the Tukey HSD at a significance level of $P<0.05$. Mean values are shown with standard errors (SE).

3.3 Results and discussion

3.3.1 Initiation of somatic embryogenesis

3.3.1.1 Initiation of somatic embryogenesis of *P. taeda* and *P. palustris* in 2006

In *P. taeda*, the two highest extrusion percentages were obtained for families 20-692 and 20-615, with 9.3 and 8.1 %, respectively. For the other two families, the percentage of extrusion was l.0 % or less (Table 3-4). On average, 5 explants out of 100 responded positively; the rest formed either a callus, showed no response, or turned brown. For each of the families, more than 500 megagametophytes containing immature zygotic embryos were dissected; however, almost 95 % of them did not respond with extrusion. Here we observed that the response was genotype dependent, in which extrusion was close to 10 % for some families, while other families showed little response, obtaining low percentages of less than 1 %.

In *P. palustris*, results similar to those in *P. taeda* were observed, in which the extrusion percentage varied among families. The highest percentage was in family 20-822 (10.7 %), followed by family 20-805 with 8.3 %. The lowest percentages were observed in families 20-825, 20-803 and 20-820 with 6.5, 3.6 and 2.3 %, respectively (Table 3-4). In this species, the extrusion average was 6.3 %. Additionally, an average of 552 explants per family was dissected but only 6.3 % of them responded positively.

Here we will discuss the comparison of extrusion of somatic embryogenesis across four commonly used basal media. In both species, the highest percentage of extrusion was obtained using 1218 medium, combined with either *1218* or *TX* plant growth regulator concentrations. The percentages of extrusion in these two treatments were approximately 17 % and 27 % for *P. taeda* and *P. palustris*, respectively (Table 3-5). Both plant growth regulator combinations have an auxin to cytokinin ratio of 10:5 (molar) (Table 3-3). These results agree with previous results, in which the same ratio of auxin to cytokinin has been shown to be more successful for the extrusion of embryogenic tissue in *P. caribaea* (Laine and David 1990), *P. koraiensis* (Bozhkov *et al.* 1997), *P. densiflora* and *P. thunbergii* (Taniguchi 2001). However, the auxin sources were different: 2,4-D for *TX* and NAA for *1218*. On the other hand, the cytokinin source was BA for *TX*, and BA and Kin for *1218* (Table 3-3).

Low percentage of extrusion (5 %) was reported for *P. taeda* (Becwar *et al.* 1990). Improvement and testing of different basal media, combinations of plant growth regulators and other amenities lead to an increase to 17 % extrusion rate when using zygotic embryos enclosed in megagametophytes as explants on BM medium with 13.5 µM 2,4-D and 2.20 µM BA, followed by 4 % multiplication after 12 week from the onset of initiation (Li *et al.* 1998a). An extrusion rate of on average 18 % across 32 open-families of *P. taeda* was obtained on ½ P6 modified medium supplemented with 2.80 µM BA, 2.83 µM Kin plus 10.74 µM NAA (Pullman *et al.* 2003d). In this study, we found similar extrusion percentages as previously reported using the same plant growth regulator combinations and concentrations, 15.5 % across four families of *P. taeda* and a higher percentage (26.5 %) across five families of *P. palustris* (Table 3-5). Through medium and culture procedure optimization, the percentage of initiation in *P. taeda* has now been reported to be 38.5 % across 12 open-pollinated families, and 26.3% across control-pollinated families with the addition of 250 mg/L MES, 0.5 mg/L folic acid, and 0.05 mg/L biotin to modified ½ P6 salts (Pullman *et al.* 2005b).

Extrusion using DS medium was better achieved with *P. taeda* explants than with *P. palustris*. In general, the *P. palustris* response was low (less than 2.5 %). Nevertheless by combining this medium with the highest concentration of plant growth regulators (*LJ*), the percentage of extrusion was 10 % in *P. taeda*.

For *P. palustris* the combinations of 2,4-D and BA at 1) 9.0 and 4.5 µM, 2) 13.6 and 2.2 µM and 22.5 and 11.0 µM, respectively; and 3) 45 µM 2,4-D were effective for extrusion; with an overall extrusion of 3.5 % combining the results of all the treatments (Nagmani *et al.* 1993). To our knowledge this is the third attempt for the initiation and establishment of somatic embryogenic cultures in *P. palustris* in the available literature.

Auxins and cytokinins participate in cell cycle regulation and cell division (Gaj 2004). Both plant growth regulators have been reported necessary for initiation of somatic embryogenesis in different pine species (Jimenez 2005). However, the use of other plant growth regulators in the initiation culture medium such as ethylene, ABA and TDZ alone and in combination with other plant growth regulators has improved the rates of extrusion in conifer species (Stasolla and Yeung 2003).

Table 3-4. Total number and percentage of embryogenic and non-embryogenic explants per species and family in 2006.

Species Families	Embryogenic explants		Non-embryogenic explants	Total explants
	Number	%	Number	
Pinus taeda				
20-692	54	9.3	526	579
20-615	48	8.1	548	595
20-1063	5	1.0	512	517
20-639	2	0.4	545	545
Average per family	**27**	**4.7**	**532**	**559**
Pinus palustris				
20-820	11	2.3	462	473
20-803	21	3.6	567	588
20-822	59	10.7	493	552
20-805	52	8.3	574	626
20-825	34	6.5	492	521
Average per family	**35**	**6.3**	**517**	**552**

Table 3-5. Total number and percentage of embryogenic (EC) and non-embryogenic (NEC) explants per species, culture medium and plant growth regulator combination in 2006.

Medium	plant growth regulator	*Pinus taeda*			*Pinus palustris*		
		EC			EC		
		Number (%)	NEC	Total	Number (%)	NEC	Total
DS	PGR-free	5 (5.2)	91	96	2 (1.7)	116	118
	AL	7 (7.1)	91	98	1 (0.7)	134	135
	1218	3 (2.9)	102	105	1 (0.8)	126	127
	TX	5 (5.0)	95	100	3 (2.3)	127	130
	LJ	5 (10.0)	45	50	0 (0.0)	102	102
1218	PGR-free	8 (6.5)	115	123	8 (6.6)	114	122
	AL	7 (6.0)	110	117	21 (17.5)	99	120
	1218	17 (15.5)	93	110	35 (26.5)	97	132
	TX	22 (18.6)	96	118	36 (27.7)	94	130
	LJ	6 (9.4)	58	64	12 (18.8)	52	64
AL	PGR-free	3 (2.9)	102	105	5 (3.9)	124	129
	AL	0 (0.0)	102	102	2 (1.6)	120	122
	1218	6 (6.4)	88	94	13 (10.7)	109	122
	TX	11 (10.9)	90	101	17 (11.6)	129	146
TX	PGR-free	1 (0.9)	110	111	1 (0.8)	131	132
	AL	0 (0.0)	58	58	3 (2.3)	130	133
	1218	1 (1.2)	80	81	9 (7.6)	109	118
	TX	2 (1.9)	101	103	8 (5.6)	134	142

For somatic embryogenesis initiation in pine species the combination of both 2,4-D and BA are the most frequently used for initiation, such as in *P. bungeana* (Zhang *et al.* 2007), *P. caribaea* (Laine and David 1990), *P. densiflora* (Taniguchi 2001), *P. koraiensis* (Bozhkov *et al.* 1997), and *P. thunbergii* (Maruyama *et al.* 2005b). Concentrations vary depending of the species, thus concentrations as low as 5 µM 2,4-D have been applied for *P. roxburghii* (Arya *et al.* 2000) and up to 43 µM in *P. bungeana* (Zhang *et al.* 2007). Similarly for BA, where the low concentration of 2.2 µM BA is used for *P. brutia* (Yildirim *et al.* 2006), *P. nigra* (Salajova and Salaj 2005) and *P. sylvestris* (Lelu-Walter *et al.* 2008) and up to 18 µM BA for *P. bungeana* (Zhang *et al.* 2007). Initiation of pine embryogenic cultures using cytokinin alone has been reported for *P. elliottii* (Newton *et al.* 2005). In *Abies* species, BA alone is sufficient for the initiation and multiplication of embryogenic tissue in *A. lasiocarpa* (Kvaalen *et al.* 2005) and *A. nordmanniana* (Norgaard and Krogstrup 1991).

Although AL medium supplemented with 5.00 µM BA and TX medium supplemented with 4.40 µM BA and 9.79 µM 2,4-D improved the initiation of somatic embryogenesis in *Abies lasiocarpa* (Kvaalen *et al.* 2005) and *P. menziesii* (Attree 2003), respectively, they did not have the same effect in this study with either *P. taeda* or *P. palustris* (Table 3-5). The AL medium was developed based on a mineral analysis of seed tissues of *A. lasiocarpa*. The lack of response from *P. taeda* and *P. palustris* may be due natural differences in the seed mineral composition of the two genera, such that this medium lacks the essential nutritional compounds needed to trigger the initiation of embryogenic cultures in the *Pinus* species under study. The TX medium showed results similar to the AL medium. The percentages of responses were low for *P. taeda* on this basal medium; however in combination with a 10 µM: 5 µM auxin to cytokinin ratio, it improved initiation in *P. palustris* (Table 3-5). The lack of plant growth regulators with either of these basal media drastically reduced the efficacy of somatic embryo initiation. Also, it has been proposed that the key point in the use of plant growth regulators is the balance between auxin to cytokinin rather than the absolute concentration for the initiation (Li *et al.* 1998a).

By comparing two combinations of plant growth regulators, low (13.5 µM 2,4-D and 2.2 µM BA) (Becwar *et al.* 1996) and high concentrations (50 µM 2,4-D, 20 µM BA and 20 µM Kin) (Gupta and Pullman 1991), it was found that the lower combination statistically increased the extrusion frequency and multiplication in *P. taeda* (Li *et al.* 1998a).

It is believed that the presence of some reduced nitrogen in the culture medium is necessary for the somatic embryogenesis process. This may be supplied in the form of nitrate and/or ammonium. Of the four basal media tested in this study, the 1218 medium has the highest concentration of inorganic nitrogen, followed by TX and DS media (Table 3-1). Additionally, the 1218 medium is rich in calcium, compared with the remaining three media, but TX has the highest concentration of boron and manganese (Table 3-2), possibly compensating in part for the low concentration of calcium, which plays a role in the maintenance of cell integrity (Teasdale *et al.* 1986).

Most of the inorganic nitrogen supplied in the culture medium is converted by plant tissues into amino acids, which are then assimilated into proteins (Pallardy 2008b) . However, the use of ammonium at 30 or 50 mM or for longer periods was toxic for the growth of suspension cultures of *P. menziesii* (Lee and Kirby 1986).

Enhanced embryogenesis and/or improved embryo growth is observed when media have been supplemented with amino acids, in addition to the former compounds, nitrate and ammonium. For the establishment of somatic embryogenesis lines of *P. abies,* the optimal ammonium to nitrate molar ratio was about 0.2 with the addition of 3.0 mM L-glutamine (Bozhkov *et al.* 1993). Somatic embryogenesis also can be obtained on media with amino acids as the only nitrogen source (Garin *et al.* 2000). For *Abies lasiocarpa*, the use of L-glutamine (2 g/L) as the only nitrogen source was sufficient for the initiation of embryogenesis and this is energetically less costly to assimilate than nitrate or ammonium (Kvaalen *et al.* 2005). Similarly, somatic embryos from mature zygotic embryos of *P. abies* were obtained on a modified ½ MS medium without NH_4NO_3 but supplemented with 5 mM L-glutamine, 4.5 µM BA and 10.7 µM NAA or 10.0 µM 2,4-D (Verhagen and Wann 1989). One of the disadvantages of the use of L-glutamine in tissue culture media is that it is heat unstable thus, it cannot be autoclaved and instead must be filter-sterilized, and it degrades rapidly once incorporated in the medium (Barrett *et al.* 1997).

Amino acids are the basic components of proteins. Organic nitrogen is already reduced, and can be taken up more readily than inorganic nitrogen (Kvaalen *et al.* 2005). The most common form of organic nitrogen used is L-glutamine; L-glutamine in combination with casein hydrolysate and inorganic forms of nitrogen, has generally been used in both induction and

maturation of conifer somatic embryogenesis (Garin *et al.* 2000). Conversely, the addition of an inorganic source as the only source nitrogen in the medium in *Picea glauca*, decreased the fresh weight of the cultures compared with the weight of cultures under both sources of nitrogen (Barrett *et al.* 1997).

For the initiation of somatic embryogenesis in pine species, different media have been used in the past, such as DCR (Gupta and Durzan 1985), P6 (Teasdale *et al.* 1986), and LP (von Arnold and Eriksson 1981) and their modifications (Appendix 2.1). However, few reports have compared the initiation of somatic embryogenesis across different media (Li *et al.* 1998a). A comparison between the different media BM (Gupta and Pullman 1991) and DCR (Becwar *et al.* 1996) for the initiation of somatic embryos in *P. taeda* showed that the former exhibited less browning frequency and higher extrusion and multiplication rates. Additionally the LP medium was not effective in the initiations (Li *et al.* 1998a). The main differences between the basal media are: 1) higher concentration of nitrogen in BM medium compared with DCR, even though the ratio $NO_3:NH_4$ was very similar, 2.6 for DCR and 2.7 for BM; 2) a lower concentration of iron and 3) higher concentrations of potassium and boron sources (Appendix 2.1).

Improvements in the extrusion percentages (particularly in *P. taeda)* have been made with the addition of brassinolide (Pullman *et al.* 2003a), ABA and silver nitrate (Pullman *et al.* 2003d), GA_3 (Pullman *et al.* 2005a), biotin and folic acid (Pullman *et al.* 2005b), and, organic acid and vitamins (Pullman *et al.* 2006). Additionally, a recent detailed study of the pH and osmotic profile of megagametophytes during zygotic embryo development suggests that modifying these components during embryo development *in vitro* would mimic natural conditions, and would hence improve embryo yield and quality (Pullman and Johnson 2009a; Pullman and Johnson 2009b).

Immature explants are needed for the initiation of somatic embryogenesis in pine species and the source of this tissue occurs during a temporal window of approximately two weeks. For both pine species studied herein, immature cones were collected from the end of June to the end of July. While embryo stages were not significantly characterized from the excised megagametophytes used as explants, our best guess, based on later *P. taeda* collections (Chapter V) and the literature, suggests that embryos were within the pre-cleavage to early

cotyledonary stages from July 10th to July 24th, which was the time frame that showed the highest percentage of extrusion in both species (Fig. 3-1). The highest positive response (% extrusion) in almost all the families of *P. taeda* in this study were from the cones collected on July 16th (Fig. 3-1A), except for family 20-615, in which the highest percentage of extrusion was from megagametophytes collected on July 24th. Similar results were found with *P. palustris* (Fig. 3-1B). In this study, late cotyledonary stage embryos were not tested in any of the species. However, for two of the families, one of *P. taeda* and one of *P. palustris*, cones collected after July 24th (Table 3-1), showed no extrusion, which lead us to believe that the embryos were already post-cotyledonary and too old for optimal responsiveness.

For both species, cones were collected weekly and stored at 4 ºC until each of them was dissected to remove the megagametophyte. After 8-10 weeks in culture, extrusion was observed. A portion of the tissue was double stained, which indicated well-developed embryonal heads and elongated suspensors, confirming the embryogenic state. Tissue was transferred to the same medium used for initiation until the stable cell lines were established. Figure 3-2 shows photos of what we observed across species and families, using as a model cell line 20-692 of *P. taeda,* showing the megagametophyte (Fig. 3-2A), extrusion (Fig. 3-2B) and double stained tissue showing well developed embryo heads and elongated suspensors (Fig. 3-2C).
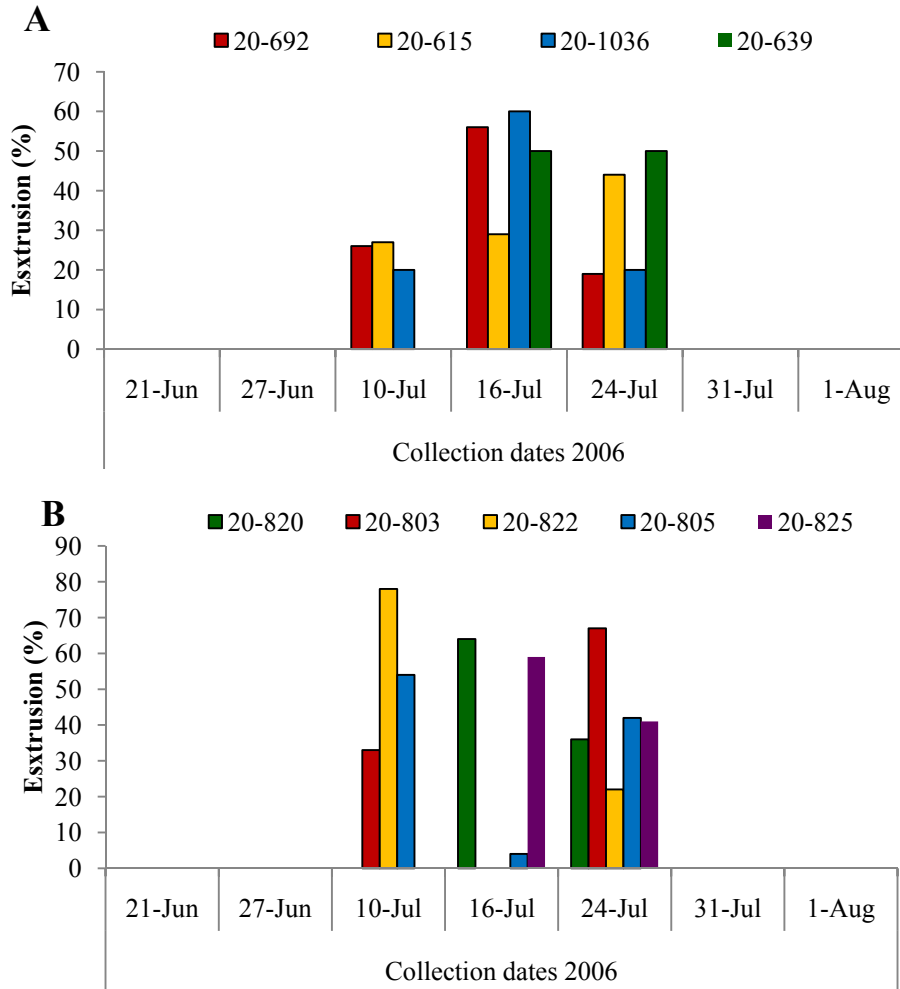
Figure 3-1. Temporal distribution of observed extrusion from total number of megagametophytes for each collection date (day/month) in 2006 and families of: A) *P. taeda* and B) *P. palustris.*
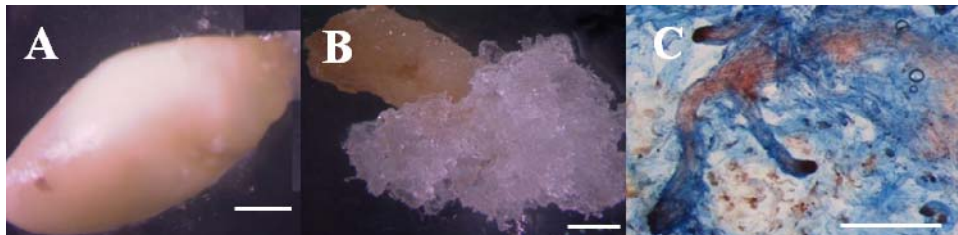
Figure 3-2. Explants, extrusion and multiplication in the somatic embryogenesis of *P. taeda* cell line 20-692 initiated in 2006: A) megagametophyte (bar = 1 mm), B) extrusion of embryogenic tissue (bar = 1mm) and C) double stained tissue (bar = 0.1cm).

However, even though extrusion was observed in all of the families, stable cell line establishment was achieved only in one family of each species: 20-692 (*P. taeda*) and 20-803 (*P. palustris*). Eventually, these two cell lines were maintained on 1218 medium, containing 1218 plant growth regulators, after which these tissues were tested for their ability to mature (3.3.2).

3.3.1.2 Initiation of somatic embryogenesis of *P. palustris* in 2007

The basal medium 1218 supported the best response in the previous experiment (3.3.1.1), and was thus used in a second round of initiation experiments using the immature zygotic embryos of three additional families of *P. palustris* (20-807, 20-839, 20-840). The medium was tested with three concentrations of 2,4-D: 5, 10 and 15 µM, each combined with three concentrations of BA: 3, 6 and 9 µM.

The total numbers of explants evaluated were 101, 275 and 233 for families 20-807, 20-839 and 20-840, respectively (Table 3-6). For this round of initiation experiments, the percentage of extrusion was more than 15 % (Table 3-6), with the highest being 34 % in family 20-807.

Family 20-807 exhibited 34 % extrusion from immature embryos, all of which (100 %) were collected on June 29th (Fig. 3-3). In Family 20-840, the highest percentages of extrusions were observed in the last two collections (18-July and 1-August). Extrusion in family 20-839 was observed in explants from the last four collections (5-July to 1-August). The extrusion percentage slightly increased, peaking on July 18th then decreasing (Fig 3-3).

Table 3-6. Total number and percentage of embryogenic and non-embryogenic explants per families of *P. palustris* in 2007.

Families	Embryogenic explants		Non-embryogenic explants	Total explants
	Number	%	Number	
20-807	34	34	67	101
20-839	41	15	234	275
20-840	52	22	181	233
Average per family	**42**	**24**	**161**	**203**

The genotype influence on the initiation rate was apparent, and embryogenic potential of genotypes or cell lines has been described previously to be influenced by parental crosses (Park *et al.* 2006). A diallel mating using six parent trees of *P. taeda* to look at the parent effect during the initiation of somatic embryos showed that initiation percentage may be increased by choosing a favorable female parent for cross-pollination based on the relative responsiveness of that parent as an open pollinated tree (MacKay *et al.* 2006). In this study, the maternal effects accounted for much of the variation (17.2 %) at the level of extrusion; this could be linked to the use of megagametophyte containing the immature embryo as explants, thus the presence of the megagametophyte (maternal haploid tissue) may enlarge the influence of the mother tree into culture by providing nutritional component and plant growth regulators that improve the extrusion rates (MacKay *et al.* 2006). Similar results were found in *P. sylvestris* where somatic embryogenesis initiation rates were higher using tree A as the female parent crossed with male parents trees B (25 %) and C (20 %); conversely, somatic embryogenesis initiation rates decreased when tree A was used as the male parent and crossed with female parent tree C (5 %), and in selfing (3 %) (Lelu-Walter *et al.* 2008).

Our observation suggested that in 2007, the embryos were within the pre-cleavage to early cotyledonary stages from July 18th to August 1st, as this time frame exhibited the highest percentage of extrusion for two out of the three families. This time frame was a week behind that of 2006. Preliminary climatological data of the National Weather Service Forecast Office showed that for the region of Charlottesville VA, the month of June and July 2006 had an average temperature of 22.2 and 25.3 ºC, respectively; while in June and July 2007 were 21.8 and 23 ºC, respectively (The National Weather Service 2010). Thus these months were colder in 2007, which may have affected embryo development. Curiously, a previous initiation in *P. palustris* showed that megagametophytes containing immature zygotic embryos collected from Gulfport, Mississippi on 14-July 1992 were the most responsive explants for the initiation of embryogenesis (Nagmani *et al.* 1993) compared with the collection of 27-July 1992. In *P banksiana*, the collection of cones for two continuous years showed that due to the cold weather in 2004, zygotic embryo developmental stages were delayed compared with those stages in 2003 (Park *et al.* 2006).

Future somatic embryo initiations in *P. palustris* may be achieved using cones from the same locations as in 2006 and 2007; it is safe to say that cones could be collected from the beginning

of and throughout July; additionally, collections would need to be extended during the month of August to have a better evaluation of explants from these latter dates. However, in addition to looking at collection dates, characterization of embryo developmental stages per collection would be a better indicator to target high initiation rates. We believe that the success of somatic embryogenesis of these two species using cones from the listed locations would be more successful using explants at early stages (pre-cleavage to early cotyledonary) of development before a dominant embryo is defined (Park 2002). However, this needs to be evaluated further before drawing any definitive conclusions.

Out of the various growth regulator treatments, the highest percentage of embryogenic cultures varied within genotype (Fig. 3-4) where: 1) cell line 20-807 showed a higher percentage of extrusion on 15 µM 2,4-D with 3 µM BA, followed by 9 µM 2,4-D with either 5 or 10 µM 2,4-D. 2) Cell line 20-839 had the highest percentage on 15 µM 2,4-D plus 6 µM BA concentration, followed by a reduction of both plant growth regulators at levels of 10 µM 2,4-D plus 3 µM BA. 3) Cell line 20-840 showed the highest percentage on 5 µM 2,4-D combined with either 3 or 6 µM BA, followed by the same 2,4-D concentration combined with the highest concentration of BA used, which was 9 µM BA. 4) Combining the results from all families, the two best treatments were 5 µM 2,4-D and 9 µM BA (lower auxin: higher cytokinin) and 15 µM 2,4-D and 6 µM BA (higher auxin: lower cytokinin).

As previously discussed, genotype has a strong influence in the initiation response. The differences in initiation observed in this study could be due to the different compounds that the megagametophyte could provide to the immature zygotic embryo thus may enhance the initiations rates (Jimenez 2005; MacKay *et al.* 2006). Thus, in the end, the optimal amounts and kinds of plant growth regulators for initiation are determined by the explants (Gaj 2004; von Arnold *et al.* 2002). However, to lower the cost of the procedure and for commercial purpose, it is essential to have a standard combination of plant growth regulators to lower the cost of production, thus based on the above results we suggested that the initiation of somatic embryogenesis in *P. palustris* should be induced with: 1) low concentrations of 2,4-D (5 µM) combined with higher concentration of BA (9 µM) or 2) high concentration of 2,4-D (15 µM) and low concentration of BA (3 or 6 µM).
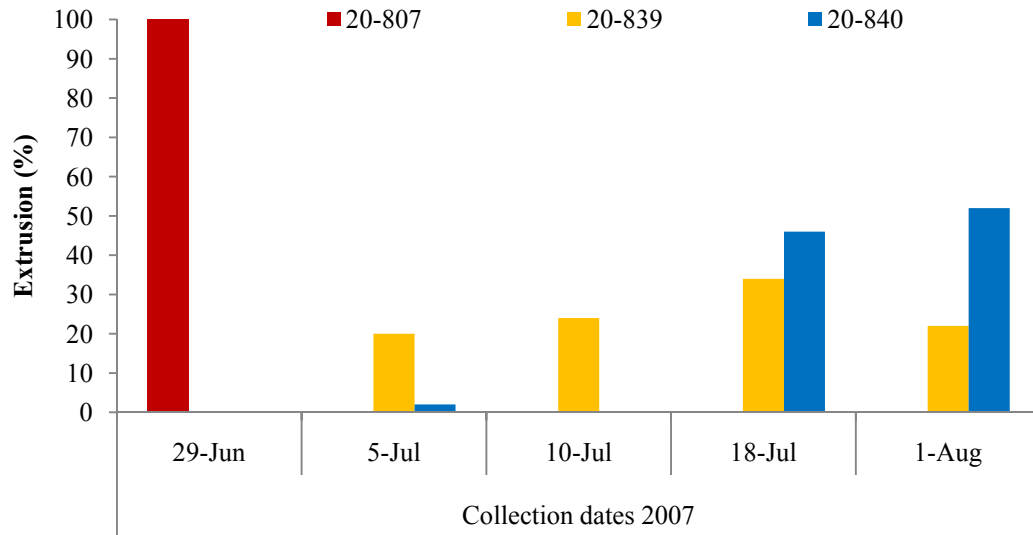
Figure 3-3. Temporal distribution of observed extrusions from total number of megagametophytes for each collection date (day/month) and families of *P. palustris* in 2007.
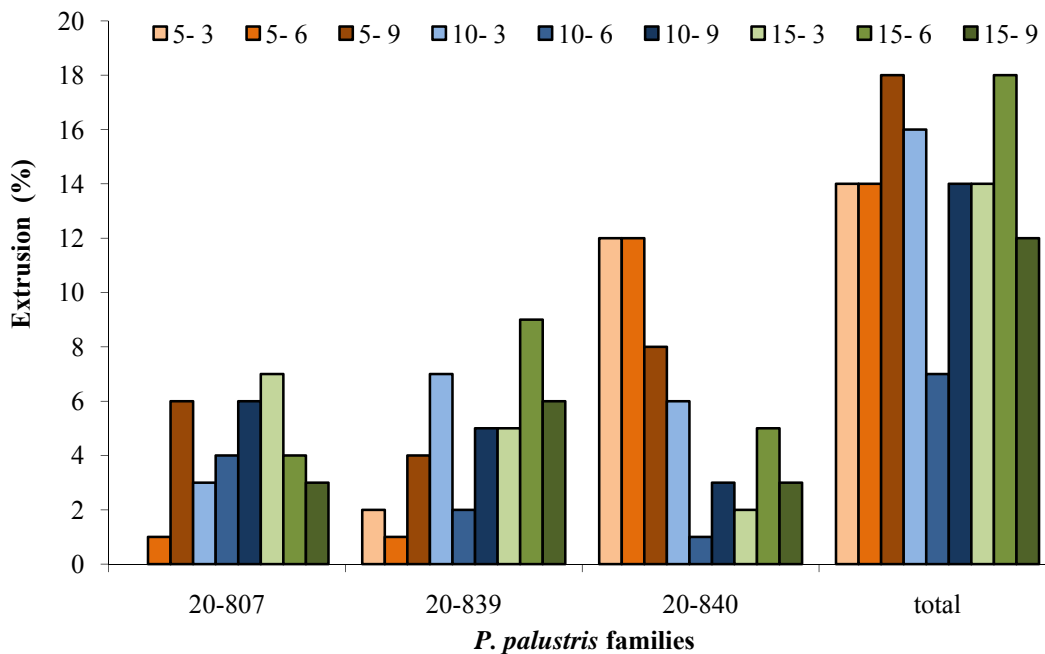
Figure 3-4. Percentage of extrusion per plant growth regulator combination (2,4-D and BA), concentration and family of *P. palustris*. Three 2,4-D concentrations (5, 10 and 15 µM), each combined with three concentrations of BA (3, 6 and 9 µM) were tested.

3.3.2 Maturation experiments

3.3.2.1 Maturation of somatic embryos of *P. taeda* and *P. palustris* from 2006/2007

The embryogenic suspensor mass tissue of all the established cell lines: *P. taeda* (20-692) and *P. palustris* (20-803, 20-839 and 20-840) from the above experiments (3.3.1) was tested for their ability to develop mature somatic embryos. In our attempts to obtain mature embryos, tests of ABA concentrations combined with different PEG, maltose, Phytagel and 2,4-D levels were studied. However, in all of the lines, complete maturation was never reached and no mature embryos were germinated. Cell line 20-840 was the only line that showed maturation of one or two embryos on 80 µM ABA plus 6 % (w/v) maltose after 10 weeks on maturation (Fig. 3-5), but these failed to germinate. Due to these poor results, no conclusion can be drawn for maturation efficiency for these lines and conditions tested (3.2.5.1). Thus, maturation experiments were initiated when the cell lines were established, but up to the writing of this manuscript, the development of mature embryos has not been successful. Future studies on the DNA methylation and polyamine levels in these embryogenic cell lines could help identify possible causes of the failed response during maturation.

In *P. pinaster* the ability of the embryogenic suspensor masses to proliferate and convert into mature embryos was lost after a period of time on the multiplication medium. This period of time can be as short as weeks or as long as months after initiation (Breton *et al.* 2006). In *P. pinaster* cell line PN519, the decrease of maturation performance occurred after 35 weeks maintenance after thawing, and this event was irreversible and definitive (Breton *et al.* 2006). The loss of development potential has previously been associated with changes in the morphology of embryo cultures, the culture being characterized by having smaller suspensor regions (Breton *et al.* 2005). Furthermore, improper manipulation, such as long subcultures time, of the tissue-culture environment can result in the increased formation of free radicals, damaging the DNA and thus causing cellular dysfunction (Benson 2000). It is unknown why the cultures in our experiments exhibited a developmental block.

Two recent studies have shown that genomic DNA global methylation and polyamine levels could be related to the embryogenic potential of cell lines in *P. pinaster* (Klimaszewska *et al.* 2009) and *P. nigra* (Noceda *et al.* 2009), due to their role in cell growth and division.

In *P. pinaster* the study was conducted by looking at three samples of two cell lines (Klimaszewska *et al.* 2009): 1) young (three months on multiplication), 2) old (18 months on multiplication) and 3) proliferation of secondary embryogenic tissue from matured embryos; and it was concluded that: 1) the levels of polyamines were genotype dependent within the two cell lines in study. While in cell line MM25 there was no statistical difference among samples (young, old and secondary proliferation), in cell line NM18, the aged sample showed higher levels of spermidine and spermine. 2) Also, that the global methylation was similar in all the tested samples in both cell lines under study. However, the aged cell line was subject to different concentrations of a hypomethylating drug (5-azaC), which altered the type of methylation in the target sequences depending of the drug concentration and exposure time; and the consequences of these alterations on the successive tissue proliferation remain under study.

On the other hand, in *P. nigra* more positive relationships were found (Noceda *et al.* 2009): 1) there was an inverse relationship between levels of free polyamines (putrescine, spermidine and spermine) and the embryogenic potential. Thus, the most embryogenic potential cell line showed the lowest polyamine levels. 2) The quantification of global genomic DNA methylation showed an inverse relationship between DNA methylation levels and the embryogenic potentials. Thus lower methylation levels were found in the most embryogenic potential cell lines, perhaps due to the fact that DNA methylation is involved in gene silencing.
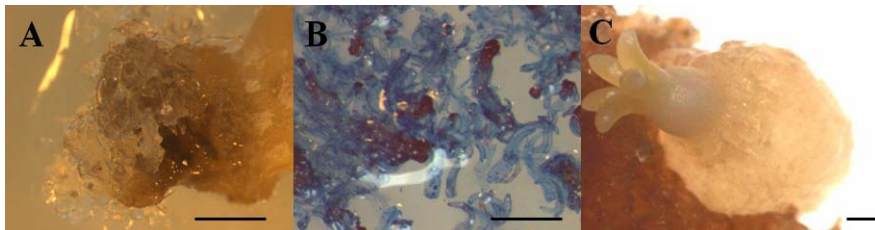
Figure 3-5. Initiation and maturation of cell lines 20-840 of *P. palustris* initiated in 2007: A) extrusion, B) double staining tissue, and C) mature embryo on 80 µM ABA plus 6 % (w/v) maltose (bar = 1mm).

3.3.2.2 Maturation *P. taeda* cell line J8189:

One cell line, kindly provided by CellFor Inc., was used for the experiments to optimize the maturation protocol. This cell line proved to have the ability to mature on almost all treatments, even after a long period of time on multiplication medium which could indicate that genetic background could play a role for the ability of mature.

3.3.2.2.1 Maturation Experiment 1: Two concentrations of maltose [3 and 6 % (w/v)] were tested in combination with four concentrations of ABA (0, 40, 80 and 120 µM). The number of mature embryos obtained did not differ between the two concentrations of maltose tested (p=0.625). However, there was a statistical difference in mature embryo number due to ABA concentration (p=0.003) (Table 3-7). The visual browning of the cultures decreased with an increase of ABA concentration with both levels of maltose. The lack of ABA resulted in abnormal embryos (Fig. 3-6A). The highest number of well-developed mature embryos (Fig. 3-6B-C) was acquired at 40 and 80 µM ABA (Fig. 3-7A). Higher ABA concentrations resulted in fewer mature somatic embryos compared with 40 µM; moreover, embryos showed a poly-embryonic, flat embryo morphology (Fig. 3- 6D) at the higher concentrations.

The use of carbohydrates during maturation have been shown to play a role both as carbon sources and as osmotic agents (Stasolla and Yeung 2003). Different types and concentrations of carbohydrate sources have been tested as a source of carbon or osmoticum (Salaj *et al.* 2004). The optimal types and concentrations vary, according to the species (Tremblay and Tremblay 1991a). In previous reports, maltose has been effective in promoting the maturation of different conifers such as *Abies nordmanniana* (Norgaard 1997), hybrid firs (Salaj *et al.* 2004), and *P. taeda* (Vales *et al.* 2006).

In fir hybrids, 3 % (w/v) of carbohydrates has been shown to promote development of mature embryos (Salaj *et al.* 2004). An increase of up to 6 % (w/v) sucrose was optimal for embryo development in *P. mariana*, *P. rubens* and *P. glauca* (Tremblay and Tremblay 1991a). Even though sucrose was hydrolyzed into glucose and fructose, the addition of these two compounds by themselves did not stimulate the effects of sucrose in the somatic embryo formation in *Picea* species (Iraqi and Tremblay 2001). In *Abies alba*, a combination of lactose and sucrose produced the highest percentages of maturation (Schuller *et al.* 2000). However, for *A.*

nordmanniana the combination of both sucrose and maltose did not improve the maturation, thus a concentration of 3 % (w/v) maltose proved to be better (Norgaard 1997).

In addition, the complete or partial substitution of sucrose with alcohol sugars such as mannitol and sorbitol enhanced the percentage of maturation in *P. strobus;* however, the concentrations were genotype dependent (Garin *et al.* 2000). Similar observations were noted in *P. nigra,* where for cell line E15, well-formed cotyledonary somatic embryos were obtained on either 6 or 9 % (w/v). In contrast, for cell line E16 the addition of 9 % (w/v) maltose yielded few embryos which failed to germinate (Salajova *et al.* 1999).

In *Pinus taeda* (Pullman *et al.* 2003b), the use of 2 % (w/v) maltose gave better embryo yield and quality when combined with 13 % (w/v) polyethylene glycol (PEG) 8000. Similarly, a combination of 4 % (w/v) maltose and 6 % (w/v) PEG yielded an average of around 100 cotyledonary embryos per gram embryogenic tissue of *P. taeda* (Li *et al.* 1998b). The same maltose concentration of 6 % (w/v) combined with 30 µM ABA was the best for somatic embryo maturation in *P. elliotti* (Liao and Amerson 1995).

In pine species either maltose or sucrose are routinely used, and concentrations differ for both, with sucrose beneficial at 3 % (w/v) in *P. brutia* (Yildirim *et al.* 2006) and 6 % (w/v) best for *P. densiflora* (Taniguchi 2001). The optimal concentration of maltose ranged from 5 % (w/v) in *P. roxburghii* (Malabadi and Nataraja 2007d) to 9 % (w/v) in *P. nigra* (Salajova and Salaj 2005).

Table 3-7. Effect of maltose, ABA and their interaction on the number of mature somatic embryos of *P. taeda* cell line J8189 through two-way ANOVA. * represents significance difference at $P < 0.05$

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Maltose	1	1	5.04	0.25	0.625
ABA	3	3	438.46	7.19	0.003*
Maltose*ABA	3	3	33.13	0.54	0.660
Model	7	476.63	68.09	3.35	0.021*
Error	16	325.33	20.33	-	-
C. Total	23	801.96	-	-	-
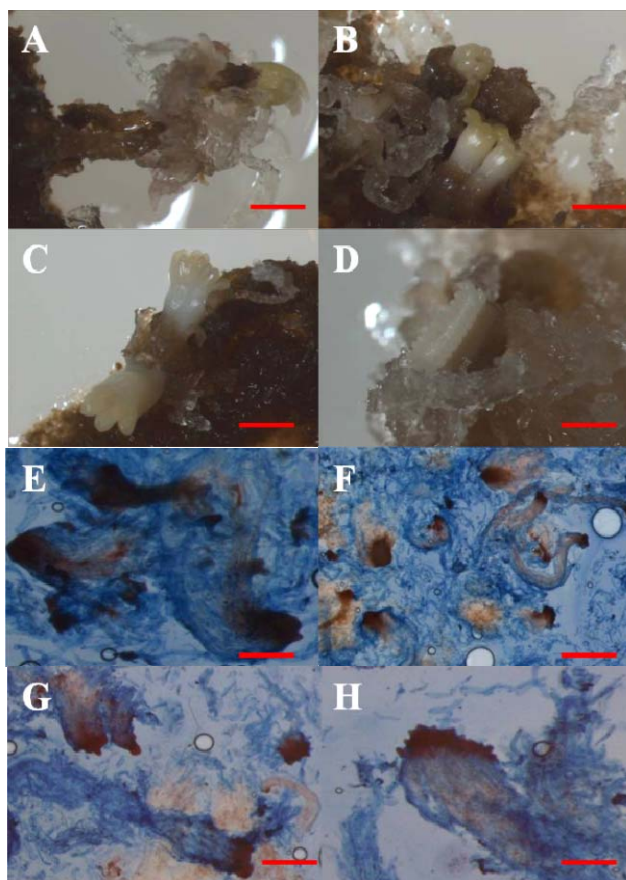
Figure 3-6. The formation of mature embryos on different ABA concentrations after ten weeks on maturation medium. A) 0, B) 40, C) 80 and D) 120 µM (A, B, C, D bar= 1mm) Double staining of tissue from the different ABA concentrations E) 0; F) 40; G) 80 and H) 120 µM (E, F, G, H bar= 0.1mm).

3.3.2.2.2   Maturation Experiment 2: In combination with the best ABA concentrations from the previous experiment (3.3.2.2.1) (40 µM), plus the addition of 6 % (w/v) maltose, three concentrations of Phytagel were tested (0.3, 0.6, 0.9 and 1.2 %). Results showed that an increase in Phytagel to 0.6 % (w/v) tripled the number of mature somatic embryos (23 ± 3) compared with the control ($p < 0.0001$) (Fig. 3-7B). Similar results were found in our lab during the maturation of *Abies koreana* somatic embryos, where Phytagel levels higher [0.9 % (w/v)] than the standard [0.3 % (w/v)] increased the number of cotyledonary embryos (Flinn, unpublished data). The gelling agent is believed to be an inert constituent of the culture medium; however, studies have shown that the agent itself causes variations in growth responses on otherwise identical nutrient media (Beruto *et al.* 1999). Two types of gelling

71

agents (gellan gum and agar) are commonly used in conifer somatic embryogenesis (Klimaszewska *et al.* 2000). In pine species, Phytagel concentrations from 0.5 % (w/v) in *P. kesiya* (Malabadi *et al.* 2004) to 0.9 % (w/v) in *P. patula* (Malabadi and Nataraja 2007d) have yielded positive results.

During maturation, the use of ABA in the medium plus a low osmotic potential are essential to produce "normal" somatic embryos capable of germination; thus the most routinely used osmotic agents for the maturation of conifers are carbohydrates and PEG (Stasolla *et al.* 2002b).

However, in *P. strobus* it was observed that the use of 1 % (w/v) Phytagel (without any other osmotic agent) plus a high concentration of ABA (120 µM) yielded 138 mature embryos per gram of fresh embryogenic tissue without changing the solute concentration (Klimaszewska and Smith 1997). Additionally, the maturation of somatic embryos of *P. strobus* under different gelling agents showed that 1) gel strength increased with gelling agent concentration; 2) that culture media with similar gel strengths but using different gelling agent brands, yielded different numbers of mature somatic embryos. For example, Difco agar produced more embryos than MBI agar; and 3) maturation on 0.8 and 1 % (w/v) Phytagel showed mature cotyledonary embryos with higher germination rates and more normal phenotypes than somatic embryos matured with 0.4 and 0.6 % (w/v) (Klimaszewska *et al.* 2000). This result were also confirmed by Garin *et al.* (2000), where maturation of *P. strobus*, using gellan gum at 1 % (w/v) yielded higher numbers of mature embryos per fresh weight than at 0.6 % (w/v).

In the maturation of *P. taeda*, the use of 1 % (w/v) Phytagel resulted in a high level of explant browning and the lowest extrusion frequency, while increasing the Phytagel up to 4 % (w/v) induced some dry embryogenic extrusions. Thus, a concentration of 2 % (w/v) Phytagel was regarded as the best level for initiation of embryogenic cultures (Li *et al.* 1998a). Our results indicated that Phytagel concentrations lower than 1 % (w/v) was better, yielding higher numbers of normal mature embryos than higher concentration, such as 1.2 % (w/v).
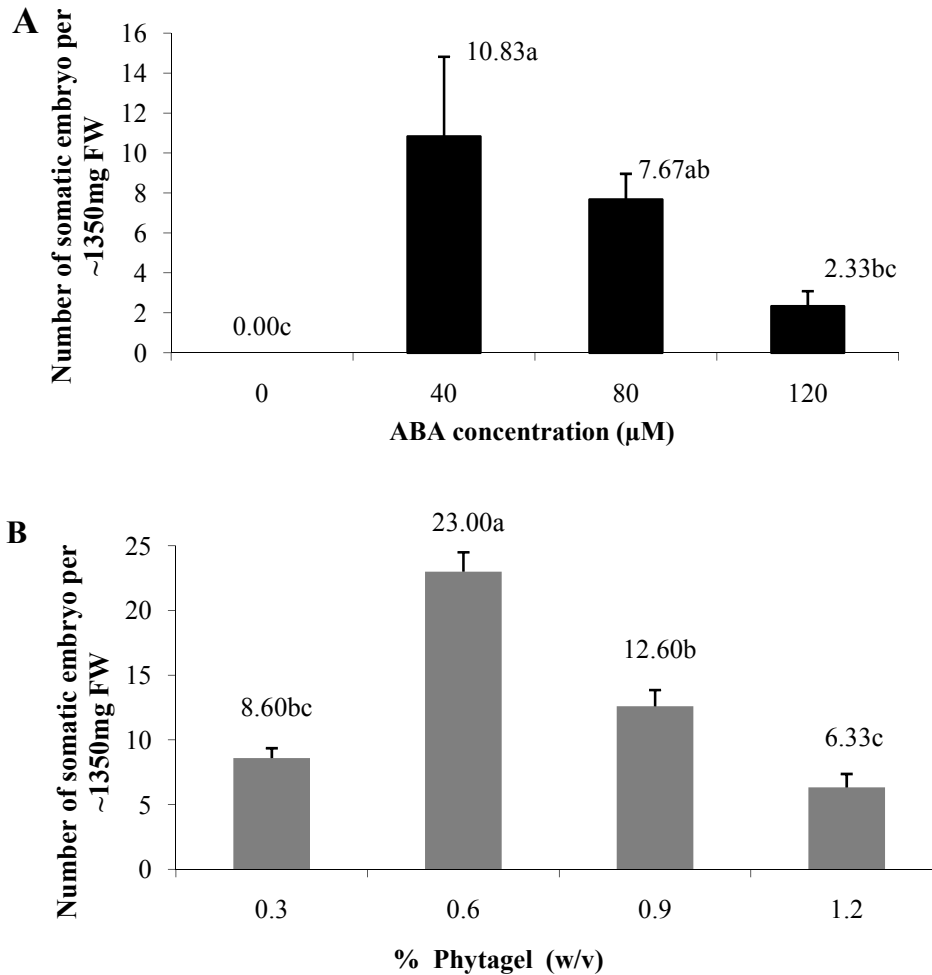
Figure 3-7. Mean number of mature somatic embryos of *Pinus taeda* line J8189 per 1350 mg FW of embryogenic tissue on A) different ABA concentrations and B) different Phytagel concentrations. Each value represents mean + SE from three replicates for each treatment. Analysis through one-way ANOVA. Treatments with the same lower case letter are not significantly different according to Tukey HSD at a significance level of $P<0.05$.

3.3.3 Germination experiments

3.3.3.1 Germination of *P. taeda* cell line J8189

3.3.3.1.1 <u>Germination Experiment 1:</u> Embryos from Maturation Experiment 1 (3.3.2.2.1) were tested for their ability to germinate. We tested one period of desiccation drying time, 1 h under the laminar hood and no desiccation (control). Embryos were germinated in 2 % (w/v) maltose for the first six weeks, and embryos showed only shoot development, but no root elongation. Thereafter, embryos were transferred to the same germination medium but

with 3 % (w/v) sucrose instead of maltose. After six weeks (week 12 total), embryos had developed roots, and germination was recorded for each of the treatments (Table 3-8). No statistical differences were found, but the highest percentage of germination was from embryos matured on 3 % (w/v) maltose plus 80 µM ABA and without desiccation treatment, followed by embryos matured on 6 % (w/v) maltose and 40 µM ABA, but with the desiccation treatment (Table 3-8).

During maturation of cell line J8189, early cotyledonary (Fig. 3-8A) through late cotyledonary (Fig. 3-8B-C) stages were observed. Germination was observed with the elongation of root and hypocotyls (Fig. 3-8D), after which the germinants were transferred to GA-7 vessels containing autoclaved vermiculite:perlite (3:1) moistened with liquid medium, and incubated for four weeks before transfer to *ex vitro* conditions (Fig. 3-8E).

Table 3-8. The percentage of mature somatic embryos that germinated after different maturation treatments. Maturation was twelve weeks of culture and different maltose and ABA combinations were tested.

Maturation treatments		Germination		Germination	
Maltose (%)	ABA (µM)	treatments	Embryos	#	(%)
3	40	Control	22	1	5
		1h desiccation	16	1	6
	80	Control	12	3	25
		1h desiccation	12	0	0
	120	Control	9	0	0
		1h desiccation	7	0	0
6	40	Control	27	1	4
		1h desiccation	14	2	14
	80	Control	16	1	6
		1h desiccation	14	1	7
	120	Control	9	0	0
		1h desiccation	7	0	0
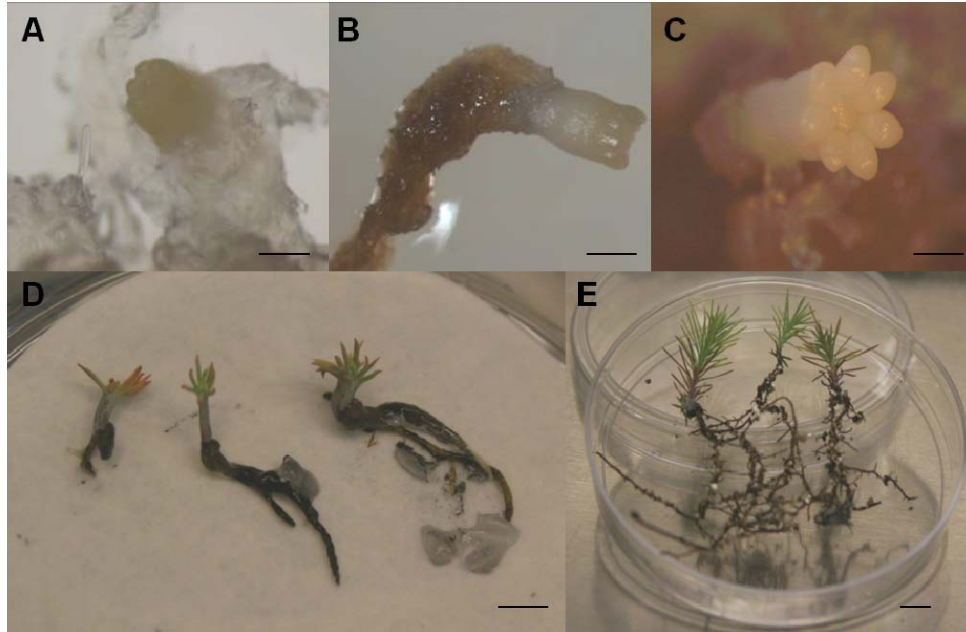
Figure 3-8. Maturation and germination of *P. taeda* cell line J8189. Different somatic embryo stages observed during maturation: A) observation of cotyledons formation after five weeks, B) mature embryo after seven weeks and, C) fully mature after 10 weeks. Subsequent germination of *Pinus taeda* embryos after: D) 12 weeks before transfer to vermiculite:perlite (3:1) and E) 16 weeks before transfer to *ex vitro* conditions. (A, B, C bar= 1mm and D, E bar= 1cm).

3.3.3.1.2 <u>**Germination experiment 2:**</u> Germination of mature somatic embryos from Maturation 2 (3.3.2.2.2) was carried out on germination medium containing 3 % (w/v) sucrose, after a flow hood desiccation pretreatment of 10 min. After 6-7 weeks, embryos showed a germination level of 46 %. Germinated embryos were transferred to GA-7 vessels boxes containing autoclaved vermiculite:perlite (3:1) moistened with liquid medium, and cultured for two weeks before transfer to *ex vitro* conditions.

Desiccation treatments of somatic embryos have been shown to be essential for successful germination in conifer species (Merkle *et al.* 1995). By maturing somatic embryos on a medium with high concentration of the gelling agent for a longer period, this intermediate step is avoided resulting in a significant saving of cost and labor (Klimaszewska *et al.* 2000).

Germination of somatic embryos of pine species has been achieved either by transferring them directly to a germination medium or by a desiccation treatment to mimic the natural conditions of the seeds (Roberts *et al.* 1990b). However, in general, mature embryos of conifers need a desiccation period to reach physiological maturity and be capable to grow into viable plantlets

(Stasolla and Yeung 2003). Additionally, for some species such as *P. strobus, P. pinaster* and *P. taeda,* the culture of the embryos for seven to ten days in darkness before transferring them to light ensures the elongation of hypocotyls and reduces anthocyanin synthesis (Klimaszewska *et al.* 2007).

Based on our results, we found that mature embryos of *P. taeda* cell line J8189 also need a period of desiccation in order to increase the efficiency of transfer to *ex vitro* conditions. Here we found that desiccation under a laminar hood for a period of 10 min improved the germination of somatic embryos; however, more procedures would need to be tested to have a synchronized germination such as the partial drying at high relative humidity used for *P. glauca /engelmanii* (Roberts *et al.* 1990b) and *A. lasiocarpa* (Kvaalen *et al.* 2005).

It is necessary that the maturation process is complete for the somatic embryo to develop a functional shoot apical meristem (Yeung *et al.* 1998) and to utilize the accumulated storage proteins to switch to an autotrophic system (Merkle *et al.* 1995). Germination of fully mature embryos is usually carried out on a medium without plant growth regulators but supplied with carbohydrates such as in: *P. strobus* supplied with sucrose at 2 % (w/v) (Klimaszewska and Smith 1997), *P. densiflora* plus sucrose at 3 % (w/v) (Maruyama *et al.* 2005a) and *P. nigra* plus maltose at 2 % (w/v). Still, there are some protocols that lack any source of carbohydrates such as in *P. thunbergii* (Maruyama *et al.* 2005b), *P. kesiya* (Malabadi *et al.* 2005) and *P. roxburghii* (Malabadi and Nataraja 2007b).

Here we found that a higher concentration of sucrose [3 % (w/v)] improved germination (both shoot and root elongation) of the somatic embryos. We also observed an improvement when the embryos were placed horizontally on the medium and then plates were tilted vertically to allow the roots to develop on the surface of the medium (Klimaszewska *et al.* 2007).

During germination of somatic embryos of *P. taeda*, red wavelengths provided by LEDs increased the percentage of germination and conversion to 64 and 50 %, respectively (Merkle *et al.* 2005). In *P. abies,* the effect of light quality was studied on the growth of embryogenic tissue and it was found that to be strongly genotype dependent, because tissue proliferation was strongly inhibited by blue and red light in genotype 164-4 and 86-52, while genotype 186-3 was insensitive to light quality and grew fast in all light conditions tested (Latkowska *et al.* 2000)

Then acclimatization of somatic seedlings is successful after they have acquired an autotrophic growth, which can be identified by the formation of the epicotyls (Roberts *et al.* 1993). In the past 10 years, the number of protocols for initiation of pine species has increased; however, few of them reported the transfer of somatic seedling to *ex vitro* conditions and the survival rate. In *P. armandii* 51 % of germination and 40 % conversion was reported (Hosoi and Ishii 2001). Similar percentage were obtained in *P. thunbergii,* with 60 % germination on ½ EM medium without plant growth regulators and 2 % w/v activated charcoal, and 51 % conversion (Maruyama *et al.* 2005b). In economically important species for which protocols have been improved in the past years, these percentage are pretty high, for example in *P. pinaster* cell line PM5, 96, 89 and 70 % of germination, conversion to plants, and survival was obtained (Klimaszewska *et al.* 2007).

3.4 Summary

Initiation of somatic embryogenesis in both *P. taeda* and *P. palustris* during 2006 was successful on all 16 treatments and for all of the families. The highest percentage of initiation for both pine species was on medium 1218, combined with both auxin and cytokinin at a ratio of 10:5 molar (*1218* or *TX* plant growth regulators concentrations). The 1218 medium supported better growth and facilitated the establishment of stable cultures of families 20-692 (*P. taeda*) and 20-803 (*P. palustris*). Additionally, the initiation frequency depended on the collection date in both species (*P. taeda* and *P. palustris*). The second somatic embryogenesis initiation frequency series of three additional families of *P. palustris* also depended on the embryo developmental stage and the concentrations of 2,4-D and BA. Stable proliferating cultures were established from families 20-839 and 20-840. The cultures of all cell lines established in both years (2006 and 2007), were tested for maturation. Results showed that the embryos did not pass the pre-cleavage or early cotyledonary stages; hence the development of complete mature late cotyledonary embryos was arrested. Additional tissue culture, biochemical and/or molecular studies will be needed to determine the causes of this developmental arrest.

On the other hand, for the maturation of the well established model cell line J8189 somatic embryos, the best ABA concentration was 40 µM. Maturation was increased three-fold by raising the Phytagel concentration from 0.3 % to 0.6 % (w/v). The highest percentages of

germination were from embryos matured on 80 µM ABA plus 3 % (w/v) maltose, without desiccation pretreatment, followed by embryos on 40 µM ABA plus 6 % (w/v) maltose with a 1h desiccation pretreatment. The percentage of germination was increased by reducing the desiccation time to 10 min.

3.5 Acknowledgements

4   Initiation of somatic embryogenesis from immature zygotic embryos of *Pinus oocarpa*

ABSTRACT

The focus of the current project was to study somatic embryogenesis in the tropical pine species *P. oocarpa*, using immature zygotic embryos as explants. We hypothesize that somatic embryo induction may be possible via mimicking natural seed-embryo developmental conditions through the development of a tissue culture medium formulation based on the mineral content of the seed nutritive tissue (megagametophyte). A novel culture medium (PO) was tested in combination with different plant growth regulator concentrations and compared with standard media 1218 (Pullman *et al.* 2005a) and 1250 (Pullman *et al.* 2006) both for *P. taeda* for the initiation of somatic embryogenesis from immature zygotic embryos. Extrusion rate was related to embryo explant stage and basal medium composition, with 11 % and 7 % extrusion rates for mother tree genotypes 3 and 5, respectively. Multiplication of the extruded tissue was improved by lowering the concentrations of plant growth regulators (2.5 µM 2,4-D, 1.0 µM BA, 1.0 µM Kin and 2.5 µM ABA). Established embryogenic cultures matured on two concentrations of ABA (40 µM and 80 µM). Germination was preceded by partial desiccation for a period of two to three weeks before transferring the embryos to germination medium. Germination was observed after seven days under low light, and apical primordia slowly expanded after transfer to *ex vitro* conditions. To our knowledge, this is the first report on the production of somatic seedlings in *P. oocarpa* through the use of immature zygotic embryos. Additionally, the initiation of somatic embryogenic cultures was tested with the use of shoot apical meristems used as explants which generated a white, mucilaginous tissue resembling immature somatic embryo tissue; however proliferating embryogenic cultures could not be captured.

Key words: Mineral analysis, medium development, initiation, somatic seedling, *Pinus oocarpa*, somatic embryogenesis

4.1 Introduction

Pinus oocarpa var. oocarpa Schiede (Pinophyta, Pinales, Pinaceae subgenero *Pinus*) is the most common pine species in the southern half of Mexico and in Central America (Dvorak *et al.* 2009). It is found from southeastern Sonora, Mexico, to northern Nicaragua (Dvorak *et al.* 2000). In Honduras, this species constitutes approximately two-thirds of the pine forest and represents an important timber export commodity (Dvorak *et al.* 2000). Its wood is used for plywood, construction lumber, packing boxes, soft drink boxes, broomstick handles, popsicle sticks, railroad ties and posts, fuel wood, kindling and resin products (Zamora 1981). It is primarily found in shallow, sandy, clay soils of moderate acidity (pH 4.0-6.5) and badly drained and non-fertile soil (Dvorak *et al.* 2000). It has an average genome size of 21.74 pg/C (Hall *et al.* 2000), larger than that of *Pinus taeda* (21.27 pg/C), but smaller than that of *P. radiata* (22.43 pg/C) (Wakamiya *et al.* 1993).

Reviews by Dvorak (2000) and Greaves (1982) show the potential of this species for commercial plantations through provenance studies in South America, Africa and New Zealand. Although the potential of this species for commercial plantations and carbon sequestration (Alberto and Elvir 2008) is known, along with its ability to hybridize with other pine species (Greaves 1982), little attention has been paid to development of tissue culture protocols for the micropropagation of this species (Schwarz *et al.* 1991).

P. oocarpa should not be considered as a cold-tolerant pine species; indeed, its best development is found in eastern Guatemala, Honduras and northern Nicaragua (Dvorak *et al.* 2000). Some of the qualities of *P. oocarpa* are its excellent wood quality; it hybridizes with a number of species (increasing the number of hybrids); it is well adapted to nursery conditions; it is tolerant to moderate drought conditions and fire; and it is resistant to *Fusarium subglutinans* (Dvorak *et al.* 2000) and *F. circinatum* (Dvorak *et al.* 2009). The latter has received much attention as even though this fungus does not threaten natural stands of pines in the Mesoamerica (southern part of Mexico, Guatemala, El Salvador and western part of Honduras and Nicaragua), it is a serious problem in pine nurseries and plantations, especially for *P. radiata* and *P. patula* (Dvorak *et al.* 2009). Additionally, there is evidence that *P. oocarpa* can grow in soil with no organic material and with a pH near 5.8, which has been documented in Siguatepeque, Honduras (Dvorak *et al.* 2000).

More than 90 % of Honduran wood consumption comes from pine, with pine lands found almost all over Honduras, and *P. oocarpa* the dominant species in the highest part, and *P. caribaea* on the coasts (AFE-COHDEFOR 2000b). Additionally, *P. oocarpa* represents an important timber export commodity (Dvorak *et al.* 2000). In Honduras, this species has been subjected to high exploitation and high-grading for the use of timber, fuel wood, and for new agricultural sites and cattle ranches (Sunderlin and Rodríguez 1996),

In Mexico, this species is the most important pine species for resin production (Sáenz-Romero *et al.* 2006). However, despite the ecological and economic importance of this species in Mexico, natural populations are rapidly vanishing due to uncontrolled forest fires, illegal harvesting, and deforestation for the use of lands for agriculture, fruit production and pasture (Sáenz-Romero *et al.* 2003). Similarly, in Honduras, approximately 37 % of the total forest land is used for agriculture including shrimp culture, livestock and coffee production (AFE-COHDEFOR 2000a). Proportionally out of the total pine forests, this species represents approximately 50 % percent in Guatemala, 90 % in Nicaragua and 60 % in El Salvador (Greaves 1982). Even with extensive exploitation, the conservation status of the species is not considered to be vulnerable (with the exception of the southern part of Nicaragua) because of its extensive range of distribution (Dvorak *et al.* 2000). Perhaps because *P. oocarpa* in natural habitats responds to fire conditions by producing numerous sprouts and adapting to a very wide range of conditions, it is not yet seen as a vulnerable species.

In the tropical and subtropical areas of Brazil, Colombia and Africa, there are commercial plantations of *P. oocarpa* (Dvorak *et al.* 2000; Greaves 1982; Wright *et al.* 1993). Some studies suggest that superior clones from rooted cuttings of this species have shown high wood quality, more so than other *Pinus* species (Iwakiri 2002; Viveros-Viveros *et al.* 2005) and hybrids developed with *P. oocarpa* could improve wood quality and the capacity for vegetative propagation (Dvorak *et al.* 2000).

However, given the importance of this species in its natural habitats and potential use in commercial plantation as stated above, no or few resources are dedicated to its research thus more research is needed and justified (Schwarz *et al.* 1991).

Concerning the *in vitro* micropropagation in *P. oocarpa,* plantlet regeneration has been reported via adventitious buds induced on explants obtained from 7- and 10-day old seedling

tissues using a modified MS medium plus 0-25 nM NAA and 25 and 50 µM BA (Franco and Schwarz 1985), and from the formation and culture of callus using protoplasts from 11-day old seedlings using 5 µM NAA and 10 µM zeatin (Laine *et al.* 1988). Nevertheless, to our knowledge, no studies have been conducted on the initiation of cultures of somatic embryos. Clonal propagation of forest species is a prerequisite for low-cost and large-scale production of genetically improved germplasm. However, the price and efficiency of the production process for a specific species depends on the availability of protocols for efficient clonal propagation. Clonal propagation methods are also required to capture the genetic gains obtained through traditional breeding or the introduction of desirable genes via genetic modification into commercial varieties of both crop and tree species. The most viable clonal propagation method currently is through rooting of cuttings (Park 2002). However, micropropagation offers rapid and short-time production of reforestation stock, the production of woody biomass and the maintenance of elite and exotic germplasm (Giri *et al.* 2004).

Somatic embryogenesis has been studied for different economically important species, with the purpose of improving protocols for mass propagation of plants and targeting the transfer of genes of interest (Reviewed in Chapter II). The goal for this study was to develop a protocol for somatic embryogenesis in *P. oocarpa*. To build up to this goal the objectives were to: 1) analyze the mineral content of mature seeds of *P. oocarpa* for the determination of the major and minor element contents; 2) develop an initiation medium based on the analysis of the mineral content for *P. oocarpa*; 3) initiate somatic embryogenesis from immature zygotic embryos and shoot apical meristems; and 4) develop maturation and germination media for the conversion of *P. oocarpa* somatic embryos to plants.

4.2 Materials and Methods

4.2.1 Analyses of the mineral content in megagametophytes and whole seeds of *P. oocarpa*

Mature *P. oocarpa* seeds stored at 4 ºC, collected in 1994 and with an original germination rate of 91 %, were provided by the "National School of Forest Sciences" (ESNACIFOR), Honduras. The seeds from Las Botijas, Francisco Morazán, Honduras were dissected; the seed coats were removed, and either whole seeds or megagametophytes were collected. One gram of fresh weight (approximately 80-90 seeds) of each tissue type was collected. Three replicates per analysis were conducted. The tissue was prepared following the "Dry ash procedure for foliar analysis of total cations and phosphorous" procedures from the Forestry Nutrition Laboratory at Virginia Tech (http://www.forestry.vt.edu/ResearchFacilities.html): one gram of fresh tissue was dried at 65 ºC for at least 24 hours and was then ground. Samples corresponding to 0.500 g +/- 0.0002 g of dry weight were used for the analysis. Each sample was ashed using the muffle furnace, by gradual temperature increase. The samples were kept at 100 ºC for approximately one hour, then at 300 ºC for an hour and 500 ºC for at least 12 hours, until the samples were ashed. Then, 10.0 ml 6N HCl were added to each sample, which was left to sit for at least 6 hours. Finally, 40.0 ml of dH_2O were added to the sample, which was shaken thoroughly and finally filtered using Whatman filter paper #2.

Samples were analyzed through the Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) at the Soil Testing Laboratory at Virginia Tech (http://www.soiltest.vt.edu). The following elements were determined: silver (Ag), aluminum (Al), boron (B), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel, (Ni), phosphorus (P), sulfur (S) and zinc (Zn).

4.2.2 Analysis of the total nitrogen, non-protein nitrogen and nitrate content in whole seeds and megagametophytes of *P. oocarpa* and *P. taeda*

Mature *P. taeda* seeds were provided by the Virginia Department of Forestry. Two samples (one of 3 g and the other 5 g) of each megagametophyte (MG) and whole seeds without seed coats (WS) of both *P. oocarpa* and *P. taeda* were used for the analysis of the total nitrogen,

non-protein nitrogen and nitrate. The analyses were conducted at the AandL Eastern Agriculture Laboratories, Inc., in Richmond, Virginia (http://al-labs-eastern.com).

In order to develop a tissue culture medium for somatic embryogenesis based on an analysis of the mineral content for *P. oocarpa*, the results from the elemental analyses of *P. oocarpa* seeds were compared to previously published data on mineral contents in *P. taeda* (Pullman and Buchanan 2003). The concentration of elements in the new formulation medium "PO medium" (Table 4-2) were adjusted to reflect the differences in *P. oocarpa* and *P. taeda* elemental content, with reference to a standard *P. taeda* culture medium (1218) (Pullman *et al.* 2005a).

4.2.3 Initiation of somatic embryogenesis from immature zygotic embryos of *P. oocarpa*

4.2.3.1 Plant material

Initiation of somatic embryogenesis from *P. oocarpa* was attempted using plant material from Mexico (University of Guadalajara), Honduras [National School of Forest Science (ESNACIFOR)], and Hawaii (Na'Aina Kai Botanical Garden and Sculpture Park, Kauai). Cones from Mexico were collected in Zapopan, Jalisco, Mexico from June to September 2007. Cones were green upon arrival in our laboratory (Fig. 4-1A), and contained embryos at the pre- and late cotyledonary stages (Fig. 4-3E and F). Cones from Honduras were collected in January 2008 from Villa San Antonio, Comayagua, Honduras. Cones were brown upon arrival in our laboratory (Fig. 4-1B), and contained embryos at the late cotyledonary stage (Fig. 4-3D). Cones from Hawaii were collected from the Na'Aina Kai Botanical Garden and Sculpture Park, in Kauai (http://www.naainakai.org) in June of 2008 and 2009 from two mother trees: 3 and 5. Cones were green in color upon arrival. For the 2008 Hawaii collection, the cones contained embryos in pre- or late cotyledonary stages. Similarly, cones collected in 2009 contained zygotic embryos at the pre-cleavage stage, embryos at the cleavage stage and late cotyledonary (Fig. 4-3). Observations were made under a zoom stereomicroscope (Olympus America Inc.). For all plant materials, the length, width and number of seeds per cone were recorded and averages were calculated (Fig. 4-1B-C). Also, the number of filled (Fig. 4-1D-E) and unfilled seeds was recorded (Fig 4-1F).

84
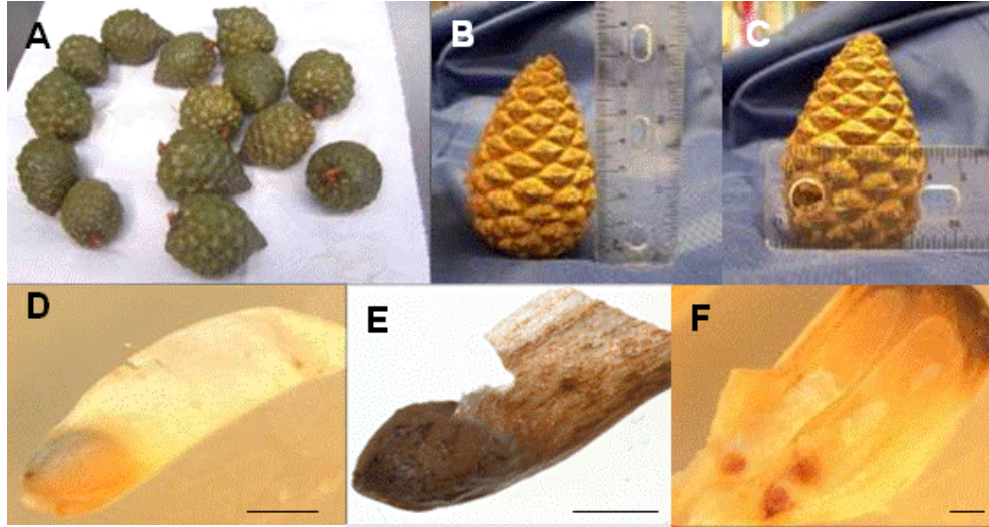
Figure 4-1. Cones of *P. oocarpa* collected for seed extraction and initiation of somatic embryogenesis from zygotic seeds: A) Green cones from Mexico containing immature embryo stages. Brown cones from Hawaii containing mature embryo stages showing measurement of length (B) and width (C), D) yellow seed with immature zygotic embryo (bar = 7 mm); E) brown seed with mature zygotic embryo and F) unfilled seeds (bar = 5 mm).

4.2.3.2 Cone disinfection

Seeds were extracted from cones containing embryos at the late cotyledonary stages and placed into a Petri plate at 4 °C until disinfection. Seeds were put inside a tea ball, rinsed with tap water for 3 minutes, soaked in 70 % (v/v) ethanol for two minutes (min), and rinsed once with sterile deionized water. Seeds were disinfected by soaking for 15 min under stirring in a 5 % (v/v) commercial bleach (sodium hypochlorite) solution supplemented with a drop of Tween-20, then rinsed four times for two minutes with sterile deionized water. The seed coat was removed, and the megagametophyte was dissected out. The zygotic embryo was carefully partially removed, allowing the embryo to remain connected to the tissue. Embryos were placed with the megagametophyte facing down on the respective media.

Cones containing immature zygotic embryos (pre-cleavage and pre-cotyledonary stages) were gently washed with soap and water and disinfected for 1-2 min in 70 % (v/v) ethanol followed by 5 min in 2.5 % (v/v) commercial bleach (sodium hypochlorite) solution supplemented with 5 drops of Tween-20 per 100 ml of solution, followed by three rinses in sterile deionized water. The seeds were extracted, and intact megagametophytes were placed onto initiation media.

4.2.3.3 Culture media

For the first three initiations, two basal media were tested—1218 (without brassinosteroids and activated charcoal) and PO—and 7 explants were placed per Petri dish. In 2007, each of the two basal media was tested with the plant growth regulator concentrations of the 1218 medium (2.83 µM BA, 2.80 µM Kin and 10.74 µM NAA) (Pullman *et al.* 2005a) and TW (17.80 µM BA, 18.60 µM Kin and 45.24 µM NAA) (Tang *et al.* 2001). For 2008, only 1218 plant growth regulators were studied. In 2009, PO and 1250 (Pullman *et al.* 2006) were tested with one explant per Petri dish. For this year, each of the media was tested with 0.5, 1 and 2X of the plant growth regulator concentrations (1X= 5 µM 2,4-D, 2 µM BA, 2 µM Kin and 5 µM ABA), for a total of six treatments. Salt concentrations, other compounds and plant growth regulators of each of the media in study per year are detailed in Table 4-1.

4.2.3.4 Induction of somatic embryogenesis

Extrusions appeared after four to six weeks in culture. For the last induction in 2009 using megagametophyte containing immature zygotic embryos, extrusions were also observed and recorded every two weeks after the first data collection during the subsequent 8 weeks. Captured cultures were transferred to the same medium composition on which they were initiated (4.2.4 and Table 4.1 for more details)

4.2.3.5 Double staining of tissues

Embryogenic tissue was identified by both morphological characteristics as translucent to white mucilaginous white tissue and histological methods (Gupta and Holmstrom 2005). Briefly, the tissue was placed on a glass slide and first stained with 2 % (w/v) acetocarmine until the tissue was completely submerged. The tissue was divided, and the slide was heated over a low flame for a few seconds. The tissue was then washed twice with deionized water. The second stain, 0.5 % (w/v) Evan's blue, was added and after 30 seconds was washed twice with water. The tissue was then mounted into glycerol to prevent drying. Observations were made under a zoom stereomicroscope (Olympus America Inc.).

Table 4-1. Concentrations of salts, other components and plant growth regulators used for the initiation of somatic embryogenesis in *P. oocarpa.*

	Concentration in basal media (mg/L)		
Components	**PO**[a,b,c]	**1218**[a,b] (Pullman *et al.* 2005a)	**1250**[c] (Pullman *et al.* 2006)
Salts			
NH_4NO_3	200.00	200.00	603.80
$Ca(NO_3)_2 \cdot 4\,H_2O$	637.00	236.00	236.00
$CaCl_2.2H_2O$	73.51	-	-
$FeSO_4 \cdot 7H_2O$	-	13.90	6.95
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	-	18.66	9.33
$C_{10}H_{12}N_2NaFeO_8$	91.73	-	-
$AgNO_3$	3.39	3.39	-
$AlCl_3$	58.90	-	-
Others			
Biotin	0.05[b]	-	0.05
Folic acid	0.50[b]	-	0.50
Myo-inositol	20,000.00	20,000.00	1000.00
Maltose	15,000.00	15,000.00	-
Sucrose	-	-	30,000.00

	Concentration (µM)				
Plant Growth				**1250**[c]	
Regulators	**TW**[b]	**1218**[a,b]	**0.5X**	**1X**	**2X**
NAA	45.24	10.74	-	-	-
2,4-D	-	-	2.5	5.0	10.0
BA	17.80	2.83	1.0	2.0	4.0
Kin	18.60	2.80	1.0	2.0	4.0
ABA	-	-	2.5	5.0	10.0

[a] tested in 2007
[b] tested in 2008
[c] tested in 2009

4.2.4 Multiplication of somatic embryogenesis

Using the inductions from year 2009, multiplication was tested. After three months from the onset of induction, two cell lines originated from explants from Hawaii, [mother tree 5, cone 1 and seed 12 (T5C1S12) and mother tree 5, cone 2 and seed 1 (T5C2S01) initiated on 1250-1X and 1250-2X respectively], were placed on six treatments to test the multiplication rate. Two basal media, 1250 and PO were combined with three concentrations of different plant growth regulators (Table 4-2). A similar amount of tissue (average 0.065 g) was placed per plate per treatment and was recorded as the initial weight. Three subcultures were performed with ten

day intervals (Subcultures 1, 2 and 3) and the tissue weight was recorded before each subculture. The final weight was taken after 40 days on multiplication medium. To calculate the growth rate, the fresh weight of embryogenic suspensor masses (ESM) was determined at the beginning of each subculture by subtracting the weight of a fresh medium-containing dish from the weight of the same fresh medium-containing dish, but including the embryogenic tissue. The same procedure was used to calculate the rate growth in *P. pinea* (Carneros *et al.* 2009). The relative growth increase rate was defined as the fresh weight at the end of that period minus the initial weight, divided by the initial weight. Three replicates per treatment were set up. Small Petri dishes (60 X 15 mm) and 10 ml of medium per plate were used.

4.2.5 Maturation and germination of somatic embryos

4.2.5.1 Experiment 1: Maturation of two cell lines T5C1S12 and T5C2S01

After three months from induction, two embryogenic cell lines, T5C1S12 multiplied on 1250-1X and T5C2S01 on 1250-2X, were tested for their ability to mature. Cultures were placed on a pre-maturation medium for five days and were then transferred to two treatments for maturation. The basal maturation medium was 927 (Pullman *et al.* 2003c) plus 6 % (w/v) maltose, 12 % (w/v) PEG 8000, 0.6 % (w/v) Phytagel combined with two concentrations of ABA, 40 and 80 µM. Approximately 300 mg of tissue was placed on a filter paper #4 (Whatman) and into a 60X15 mm Petri dish. Each Petri dish was considered as a replicate, and three replicates per treatment were arranged. The number of mature embryos was recorded after 10 weeks on culture, and a subculture was made once in week 5. Cultures were kept in the dark. For germination, embryos were pre-desiccated for ten minutes inside the laminar hood airflow and then transferred directly to the germination medium.

4.2.5.2 Experiment 2: Maturation of somatic embryogenic tissue of cell lines T5C1S12 and T5C2S01 under different multiplication treatments

The tissue from the multiplication treatment (4.2.4) was placed on the best treatment from the previous maturation experiment (4.2.5.1). The procedure and media preparation were the same as above.

4.2.5.3 Experiment 3: Germination of cell line T5C1S12

Mature embryos of cell line T5C1S12 from experiment PEG 0 % (w/v) plus 40 µM ABA were used to test different germination procedures:

1. No desiccation, embryos transferred directly to the germination medium.

2. No desiccation, embryos transferred directly to the germination medium but placed on a Whatman filter paper # 4 disc.

3. Five minutes desiccation then transferred to the germination medium.

4. Ten minutes desiccation then transferred to the germination medium.

The desiccation treatments were performed by placing the embryos on an empty Petri dish and leaving it open in the hood for a specified period of time (5 or 10 min). Then, filter paper holding the embryos was placed on the germination medium. The germination medium consisted of ½ MS medium (M519 Phytotechnology Lab), with 3 % (w/v) sucrose and 0.25 % (w/v) activated charcoal. There were five embryos per plate and three plates per treatment. The experiment was repeated twice. Germination was considered to be the elongation of both the root and shoot. Data were recorded 30 and 45 days later. Seedlings were transferred to GA-7 vessels containing autoclaved peatmoss:vermiculite (3:1) moistened with liquid germination medium, and incubated for 11 days under cool white fluorescent light at an intensity of 75.71 µmol m^{-2} s^{-1}, at 25 ºC and a photoperiod of 16/8-h (day/night) before being moved to *ex vitro* conditions.

4.2.5.4 Experiment 4: Germination of matured somatic embryos of cell lines T5C1S12 and T5C2S01 previously multiplied under different multiplication treatments

Mature embryos from experiment 4.2.5.2 were used to test different desiccation treatments before transferring embryos to germination. Briefly, six embryos were placed into 3 wells of 6-well plates, where the other three wells were filled with 3 ml of sterile water (ADM) (Roberts *et al.* 1990b). The plates were sealed and stored in the dark for one, two, three and four weeks, after which embryos were transferred to germination medium which was the same as in 4.2.5.3. Observations were recorded every week for each of the treatments and germination was considered the elongation of both the root and shoot.

4.2.6 Attempts to initiate somatic embryogenic cultures from shoot apical meristems of *P. oocarpa*

4.2.6.1 Plant material

Apical shoots harvested from five different *P. oocarpa* trees were used for the initiation. Disinfection of shoots was according to Method 2 "Mercuric Chloride" (Egertsdotter 2009, personal communication), where needles were removed from the shoots and the length of the shoot cut back to 1 cm including the top. The trimmed shoot sections were rinsed under tap water for several minutes. Shoot sections were soaked in 4 % (v/v) hypochlorite for 5 min, followed by soaking in 0.2 % (w/v) mercuric chloride for 5 min, then rinsed in water 3 times for 5 min each, by replacing the liquids with a pipette. The trimmed shoots were then transferred to a plastic sterile Petri plate. All sterilization procedures were done with sterile glassware, water and tools in a laminar flow hood. After the scales had been peeled away, the remaining bud was dipped in 70 % (v/v) ethanol for 20 seconds before dissected on a sterile filter paper. The scales of the buds were peeled off one by one by forceps and the shoot stalk trimmed back to 5 mm total length. Buds were sliced using a scalpel with blade #10. The slicing was transverse when the shoot apical meristem was the target. Explants were placed in 1218 medium with no plant growth regulator for 4 days at 4 ºC, then transferred to basal medium PO plus 0.2 g/L PVP, 1.0 g/L L-glutamine, 1.0 g/L casein hydrolysate, 1.0 g/L myo-inositol, 15.0 g/L maltose, 20 µM 2,4-D, 23 µM NAA, 9 µM BA (Malabadi and Van Staden 2005b) and 3 % (w/v) Phytagel. Plates were placed under darkness and observations were recorded.

4.2.7 Data collection and analysis

For the mineral and nitrogen analysis, a Means and Coefficient of Variance (CV) analysis was performed. Because initiation of the culture was obtained only with the 2009 collection, a statistical analysis was performed using Poisson log linear models at a significance level of $P<0.05$ to test the respective effect of the mother tree, the stage of zygotic embryo development, the media composition and plant growth regulators on extrusion and establishment of somatic embryogenesis. For the multiplication treatment, relative growth data of embryogenic suspensor masses were transformed using the normal logarithm before Analysis of Variance. The statistical program JMP 8.0 (SAS Inc., Cary, NC, USA) was used.

Significant differences between means were determined by the Tukey HSD at a significance level of P<0.05. Mean values are shown with standard errors (SE).

4.3   Results and discussion

4.3.1   Analysis of the mineral content in megagametophytes and whole seeds of *P. oocarpa*

The megagametophyte provides the nutrients for the development and growth of the zygotic embryo. Thus, it is important to study the mineral content in the megagametophyte to provide a target for the development of a medium that supplies those nutrients. It is believed that the more closely the nutritional and environmental conditions resemble the megagametophyte, the more likely it is to develop a truly high-quality somatic embryo (Pullman and Buchanan 2003). For a discussion of these results, we compared the mineral content of megagametophytes and whole seeds without seed coats (WS) of *P. oocarpa* (Table 4-2) with megagametophytes and embryos of other coniferous species (Table 4-3). The unit for comparison was the µg/g of dry weight (dw).

Table 4-2. Concentration of minerals (µg/g dry weight ± SD) in seeds without seed coats (WS) and megagametophyte (MG) of *P. oocarpa*. Each value represents a mean ± SD of three determinations.

Mineral	WS	MG
Ag	<0.003	<0.003
Al	48.4 ± 24.3	59.2 ± 23.7
B	30.8 ± 9.4	30.5 ± 3.4
Ca	787.3 ± 90.4	809.2 ± 94.0
Co	<0.005	<0.005
Cu	13.1 ± 2.1	12.9 ± 2.1
Fe	176.3 ± 17.9	184.3 ± 23.3
K	11031.6 ± 503.7	11765.7 ± 1281.6
Mg	6267.4 ± 202.7	6587.6 ± 680.4
Mn	248.9 ± 18.6	316.4 ± 55.4
Mo	<0.013	<0.013
Na	523.7 ± 81.1	613.7 ± 28.7
Ni	2.0 ± 1.5	3.2± 0.6
P	15159.8 ± 476.1	15911.2 ± 1625.2
S	99.0 ± 14.1	123.7 ± 8.8
Zn	195.7 ± 7.7	207.3 ± 24.2

Table 4-3. Comparison of mineral contents (µg/g dry weight) of seeds without seed coats (WS), megagametophyte (MG) and zygotic embryos of *P. oocarpa* and other coniferous species. NS= not studied.

Species	*P. oocarpa*		*P. taeda* (Pullman and Buchanan 2003)		Pinus (West and Lott 1993)		*Pseudotsuga menziesii* (Litvay *et al.* 1981)		*Picea glauca* (Reid *et al.* 1999)		*Abies lasiocarpa* (Kvaalen *et al.* 2005)	
Replicates	3		5 sites*		11 species**		1		4		1	
Mineral	WS	MG	Embryo	MG	Embryo	MG	Embryo	MG	Embryo	MG	Embryo	MG
Ag	<0.003	<0.003	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Al	48	59	NS	NS	NS	NS	NS	NS	NS	NS	67	242
B	30	30	6	22	NS	NS	50	<50	NS	NS	4	17
Ca	787	809	192	254	140	274	250	150	94	NS	108	349
Co	<0.005	<0.005	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cu	13	12	26	20	NS	NS	<50	<50	NS	NS	13	39
Fe	176	184	243	72	129	73	100	75	242	144	75	78
K	11031	11765	12312	9248	10004	10342	4000	10000	7738	8236	7843	7473
Mg	6267	6587	7672	5118	4585	5294	2400	2800	3708	4910	2897	2890
Mn	284	316	83	221	56	228	100	250	NS	NS	126	246
Mo	<0.013	<0.013	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Na	523	613	6	3	NS	NS	100	400	NS	NS	NS	NS
Ni	2	3	2	2	NS	NS	NS	NS	NS	NS	13	13
P	15159	15911	16500	12327	11607	11927	5700	2400	10697	11630	12489	9001
S	99	123	2531	5450	2533	5507	NS	NS	NS	NS	1647	2301
Zn	195	207	132	139	98	147	<50	200	149	195	50	53

* (1) BC-1 (S4PT6) Boise Cascade 1995, Lake Charles, Louisiana; (2) UC5-1036, Union Camp 1995, Bellville, GA; (3) UC10-1003, Union Camp 1995, Rincon, GA; (4) UC10-14, Union Camp 1995, Rincon, GA; and 5) 7-56, Westvaco 1995, Summerville, SC.

**P. banksiana, P. contorta, P. coulteri, P. koraiensis, P. mugo, P. nigra, P. ponderosa, P. resinosa, P. sabiniana, P. strobe, P. sylvestris*

The concentration of all of the minerals analyzed in this study was higher in the megagametophyte than in the whole seed of *P. oocarpa*, with the exception of copper, which was lower, as well as boron and phosphorus, which showed similar concentrations in both tissues (Table 4-2). The four most abundant minerals in both whole seed and megagametophyte were phosphorus, followed by potassium, magnesium and calcium. These results are in agreement with results from previous studies in other *Pinus* species (West and Lott 1993) and *P. taeda* (Pullman and Buchanan 2003), where the same elements were the three showing the highest concentrations in the megagametophyte (Table 4-3). However, the concentrations of these three minerals were slightly higher in *P. oocarpa* than in the averages of the eleven species (West and Lott 1993) and *P. taeda* (Pullman and Buchanan 2003).

Analyses of megagametophytes for other coniferous species, such as *Abies lasiocarpa* (Kvaalen *et al.* 2005) and *Picea glauca* (Reid *et al.* 1999) showed that three elements—phosphorus, potassium and magnesium—from highest to lowest, respectively, were also the most abundant. In contrast, in *Pseudotsuga menziessi,* the most abundant element was potassium, followed by magnesium and phosphorus (Litvay *et al.* 1981). Since these species belong to different genera, the requirements of each element could differ; as well as it could differ among species and environmental conditions such as soil and fertilization during growth.

Of the microelements in *P. oocarpa,* sodium was the most abundant, followed by manganese, iron and zinc. The sodium concentration was 3.9 and 400 µg/g dw in *P. taeda* and *P. menziesii*, respectively, (Litvay *et al.* 1981; Pullman and Buchanan 2003); in *P. oocarpa,* this concentration was 153 times higher than the concentration in *P. taeda*. In C4 and CAM plants sodium has been shown to be essential element for their photosynthesis pathway (Pilon-Smits *et al.* 2009). Although the manganese average concentration in *Pinus* was 228.8 µg/g dw, concentrations ranged from 65.3 µg/g dw in *P. coulteri* to slightly more than 600 µg/g dw in *P. resinosa* (West and Lott 1993). *P. oocarpa*'s magnesium concentration (Table 4-2) was within the range of the *Pinus* species, close to the concentration of *P. strobus* (310.1 µg/g dw) (West and Lott 1993). In *P. taeda*, *Pseudotsuga menziessi* and *Abies lasiocarpa*, the range of magnesium was 221.0 to 246 µg/g dw (Kvaalen *et al.* 2005; Litvay *et al.* 1981; Pullman and Buchanan 2003).

The concentration of iron ranged between 72 and 78 µg/g in *P. taeda* (Pullman and Buchanan 2003), *Pseudotsuga menziessi* (Litvay *et al.* 1981) and *Abies lasiocarpa* (Kvaalen *et al.* 2005); 144 µg/g dw in *Picea glauca* (Reid *et al.* 1999) and 184.4 µg/g dw *P. oocarpa* (Table 4-3). The concentration of zinc was slightly higher in *P. oocarpa* than in the concentrations of the other coniferous species, which were lower than the concentrations in *P. banksiana* (231.3 µg/g dw) and *P. contorta* (215.5 µg/g dw) (West and Lott 1993). In both, the whole seed and megagametophyte amounts of silver, cobalt and molybdenum were not detected by the ICP (Table 4-3). Data for these minerals have not been collected for coniferous species, such as *Pinus* (Pullman and Buchanan 2003; West and Lott 1993), *Pseudotsuga menziessi* (Litvay *et al.* 1981), *Picea glauca* (Reid *et al.* 1999) and *Abies lasiocarpa* (Kvaalen *et al.* 2005).

In *P. oocarpa,* the concentration of sulfur was 44 times lower than the concentration of the average of the *Pinus* species (including *P. taeda*) (5478.5 µg/g dw). The aluminum (Al) concentration in the megagametophyte of the *Pinus* species has not been reported; though, it was present in *P. oocarpa* at high concentrations, but lower than the concentration of *A. lasiocarpa* (242 µg/g dw). These could be due to differences of general requirements and growing conditions, such as soil mineral composition. The bioavailability of aluminum is highest in acidic soils and enhances herbivore defense, prevent iron toxicity and may promote phosphorus uptake (Pilon-Smits *et al.* 2009). The copper concentration was 1.5 times higher in *P. taeda* than in *P. oocarpa* (Table 4-3).

The previous results in the mineral analysis of the megagametophyte of *P. oocarpa* showed that calcium, iron, zinc and sodium were 3.2, 2.6, 1.5 and 157.0 times respectively more abundant in *P. oocarpa* than in the megagametophyte of *P. taeda*. Conversely, the concentration of copper was 1.5 times higher, while sulfur was 44 times higher in the megagametophyte of *P. taeda* than in the megagametophyte of *P. oocarpa*.

The results found in this analysis of minerals of the megagametophyte of *P. oocarpa* were used to modify the 1218 basal medium in order to adjust for these requirements in an attempt to improve the percentage of somatic embryogenesis initiation, using both mature and immature zygotic embryos.

4.3.2 Analysis of the total nitrogen, non-protein nitrogen and nitrate content in whole seeds and megagametophytes of *P. oocarpa* and *P. taeda*

In this study, the total nitrogen, non-protein nitrogen (NPN) and nitrate were evaluated in the megagametophyte and whole seeds (without seed coat) for two *Pinus* species: *P. oocarpa* and *P. taeda*. The average of the total nitrogen was higher in the megagametophyte than the average in the whole seeds (Table 4-4).

Table 4-4. Comparison (µg/g dry weight ± SD) of total nitrogen (N), non-protein nitrogen (NPN) and nitrate in the megagametophyte (MG) and seeds without seed coats (WS) in *Pinus oocarpa* and *P. taeda.*

Mineral	Total N	NPN	Nitrate
P. oocarpa			
WS	78000 ± 1838.48	270 ± 113.14	16 ± 2.83
MG	87500 ± 0.00	185 ± 21.21	<10
P. taeda			
WS	86550 ± 120.00	235 ± 49.50	<10
MG	90550 ± 3747.67	170 ± 14.14	<10

Note: Each value represents a mean ± SD of two determinations. Two separate analyses, 3 g and 5 g of pool samples of each MG and WS of each species.

Of the total nitrogen, non-protein nitrogen represented 0.002 % and 0.003 % for megagametophyte and whole seed, respectively. In addition, nitrate was detected only in the whole seed of *P. oocarpa*. As the nitrogen content in both species was similar, the $NO_3:NH_4$ ratio was not altered in the PO medium.

Generally, nitrogen is added to the medium in the inorganic forms nitrate (NO_3) or/and ammonium (NH_4), and because plants prefer nitrate, the amount of this compound is usually greater than that of ammonium. In addition, organic nitrogen is taken up by plants in the form of amino acids. Studies have shown that the pine megagametophyte contains the highest percentage of storage proteins in the seed (Gifford 1988), and is rich in arginine, which will be released during germination and the early stages of seedling growth (King and Gifford 1997). The importance of ammonium and nitrate and their ratios for conifer *in vitro* morphogenesis has been reported for somatic embryogenesis (Tremblay and Tremblay 1991b) and organogenesis (Flinn *et al.* 1986).

4.3.3 A novel tissue culture medium for somatic embryogenesis based on analysis of the mineral content for *P. oocarpa*

Modifications for the development of the initiation medium for *P. oocarpa* were based on a comparison of the concentrations of each mineral element in the megagametophyte of *P. taeda* (Pullman and Buchanan 2003) and the megagametophyte of *P. oocarpa*. The basal medium modified was 1218 (Pullman *et al.* 2005a), which itself is a modification of the LP medium (von Arnold and Eriksson 1981) and has been used for somatic embryogenesis initiation in *P. taeda*. We have also used this modified medium (1218) for the initiation of somatic embryogenesis cultures in families of *P. taeda* and *P. palustris* (Chapter III). We hypothesized that the composition of mineral elements in proportions similar to those found in the megagametophyte of *P. oocarpa* can provide an optimum tissue culture medium for the initiation and/or multiplication of embryogenic cultures of this species.

Taking into account all of the above results, changes in the new medium were as follows: 1) calcium was increased by increasing the concentration of $Ca(NO_3)_2 \cdot 4H_2O$ and adding an additional source: $CaCl_2 \cdot 2H_2O$. 2) To increase the concentration of iron, EDTA iron (III) sodium salt was used instead, which also increased the sodium concentration. High concentrations of iron (97.65 mg/L) were supplied by using two sources: $FeSO_4 \cdot 7H_2O$ and EDTA disodium for the maturation of *Pinus taeda* cultures (Pullman *et al.* 2003c). 3) $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ were eliminated in order to decrease the concentration of sulfur. Zinc was supplied with $Zn(NO_3)_2 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$, copper with $CuSO_4 \cdot 5H_2O$, and the last two compounds supplied sulfur. Magnesium and manganese were added with the use of $Mg_3(C_6H_5O_7)_2 \cdot 9H_2O$ and $MnCl_2 \cdot 4H_2O$, respectively. 4) Aluminum is an element found to be toxic for plant development. The presence of aluminum concentration in the megagametophyte of *P. oocarpa* could be due to the growth conditions of the mature seeds. Even though the concentration of this mineral in *A. lasiocarpa* (Kvaalen *et al.* 2005) was higher than in the concentration in *P. oocarpa,* no aluminum source was added to that medium. It is unusual to find an Al source in the tissue culture medium for almost any plant. For orchids, $AlCl_3 \cdot 6H_2O$ was added in low levels of 0.0561 mg/L (Lindemann *et al.* 1970). For *P. oocarpa*, aluminum was supplied with $AlCl_3 \cdot 6H_2O$, adding 58.90 mg/L. 5). In addition, nitrate concentrations increased, so in order to keep the same $NO_3 \colon NH_4$ ratio, $NH_4H_2PO_4$ was added to raise the concentration of ammonium.

Changes in the chemical composition of the element of interest (calcium, iron, zinc, sulfur, copper and aluminum) led to changes in the concentration of other ions. The new medium PO was tested for the initiation of both mature and immature zygotic embryos.

The success of plant tissue culture protocols depends on a balance of environmental (light, temperature and relative humidity) and nutritional conditions (Niedz and Evens 2007). Nutritional requirements will be supplied by a tissue culture medium and will contribute to defining the response of the explants used. The majority of the culture media have been developed empirically by varying (either by decreasing or increasing) the concentration of the mineral composition of the basal medium (Staikidou *et al.* 2006), such as MS (tobacco) (Murashige and Skoog 1962), Gamborg's B5 (soybean) (Gamborg *et al.* 1968), Nitsch's (anther culture) (Nitsch and Nitsch 1969) and the SH (callus culture of monocots and dicots) (Schenk and Hildebrandt 1972) medium, among others.

Recently, analyses of elemental mineral compositions of different plant tissues, such as the leaves of *Passiflora edulis* (Monteiro *et al.* 2000), peach almond hybrids (Terrer and Tomas 2001) and *Ceratonia siliqua* (Gonçalves *et al.* 2005b); the bulbs of *Galanthus* spp (Staikidou *et al.* 2006); the seeds of *Pinus* spp. (Litvay *et al.* 1981; Pullman and Buchanan 2003; West and Lott 1993), *Picea glauca* (Reid *et al.* 1999), *Corylus avellana* L. (Nas and Read 2004), and *Abies lasiocarpa* (Kvaalen *et al.* 2005) have led to modification of the mineral composition of current basal media in order to supply the mineral at more and less of that rates found in the corresponding tissue under study (Staikidou *et al.* 2006).

Additionally, by supplying each of the minerals in concentrations similar to those found in plant tissue analyses, the *in vitro* responses of *Passiflora edulis* (Monteiro *et al.* 2000), *Corylus avellana* L. (Nas and Read 2004) and *Galanthus* spp. (Staikidou *et al.* 2006) have improved. The elemental composition of a medium should be developed, taking into account four different considerations (Spaargaren 1996): 1) the elemental composition of the plant tissue should be included in the medium composition; 2) the explants should be able to absorb and metabolize the molecular form of the elements; 3) the lesser quantity of chemicals should be used to supply the element in order to facilitate lab preparation; and 4) low-cost chemicals (without hampering the quality) should be used in order to make the experiment commercially feasible.

4.3.4 Initiation of somatic embryogenic cultures from immature zygotic embryos of *P. oocarpa*

4.3.4.1 Plant material

The average number of seeds per cone was similar in the collections from Honduras and Hawaii, more or less 20 seeds per cone (Table 4-5). However, the number of unfilled or empty seeds was higher in the collection from Honduras. Cones from Mexico had a low total number of seeds, with an average of 4.2 seeds per cone. This agreed with previous studies, where seed production or seeds per cone depended on the year, mother tree and location, among other factors (Wolffsohn 1984). In the natural stands of *P. oocarpa,* the percentage of filled seeds dispersed is 12-15 %, and the remaining percentage is a mix of empty and aborted seeds, as well as fragments (Wolffsohn 1984). Taking into account all of the collections in this study, the number of seeds per cone ranged from 0 to 63 seeds per cone. Studies in Honduras and Guatemala have found roughly 15 to 35 filled seeds per cone (Wolffsohn 1984), with a potential of *P. oocarpa* being approximately 140 seeds per cone (reviewed by Dvorak *et al.* 2000). The average lengths were 6.0, 8.4 and 7.0 cm for cones from Mexico, Honduras and Hawaii, respectively. However, the average cone width was 4.0 cm for Honduras and Mexico and 3.0 cm for the Hawaii collection.

Table 4-5. Number of cones, average cone length and width, total and average seed per cone in the different collection of *P. oocarpa* for the initiation of somatic embryogenesis.

Location year	Number of cones	Cone average (cm)		Number of total seeds (empty or unfilled seeds)	Average seeds per cones
		Length	Width		
Mexico 2007	24	6.0	4.0	101 (not recorded)	4.2
Honduras 2008	26	8.4	4.1	561 (183)	21.5
Hawaii					
2008	18	6.9	3.2	401 (50)	22.3
2009	30	6.6	3.1	565 (91)	18.8

4.3.4.2   Induction of somatic embryogenesis

The first attempt at the initiation of somatic embryogenesis in *P. oocarpa* was with the use of megagametophyte-enclosed immature zygotic embryos from Mexico. The cones were collected from June to September 2007; one month later cones were dissected and plated for initiation in both media 1218 and PO, each combined with 2.83 µM BA, 2.80 µM Kin and 10.74 µM NAA. Perhaps due to the prolonged storage after collection and the low temperature of cone storage, the explants failed to respond, and in less than one month, the embryos turned brown. A second attempt was performed with the use of a fresh collection of mature zygotic embryos of cones from Honduras. A total of 234 were used: 39 late cotyledonary embryos attached to the megagametophyte were placed in five treatments: the control, 1218, PO with and without aluminum (PO-Al), each combined with two plant growth regulators concentrations, 1218 (2.83 µM BA, 2.80 µM Kin and 10.74 µM NAA) (Pullman *et al.* 2005a) and TW (17.80 µM BA, 18.60 µM Kin and 45.24 µM NAA) (Tang *et al.* 2001). The zygotic embryos responded to all of the treatments, forming white mucilaginous tissue from the cotyledons. The highest percentage of white mucilaginous tissue was found in 1218 (38 %) and PO plus 1218 plant growth regulators (21 %) and less than 10 % on PO-Al (data not shown). However, explants on the later treatment (PO-Al) exhibited more browning in both the medium and around the explants. Browning due to phenolic compounds has been an issue in the micropropagation of woody plants including pine tissue (Zimmerman 1993).

Even though some of the embryos had white mucilaginous tissue formation around them, this white tissue failed to become embryogenic. Cultures were transferred every three to four weeks into fresh medium so as to pursue embryogenic tissue, but the white tissue became a hard white callus. In conifers, several factors affect the frequency of induction of somatic embryogenesis, including the genotype, the developmental stage of the initial zygotic embryo explant, and the culture medium (Tautorus *et al.* 1991). Additionally, for most pine species, initiation of somatic embryogenesis cultures is restricted to immature embryos limited to a developmental window of 1-2 weeks during which the embryos are highly competent for somatic embryogenesis. Combinations of auxins and cytokinins are most commonly used for the initiation of somatic embryogenesis (Jimenez 2005), which could be due to the fact that these plant growth regulators participate in cell cycle regulation and cell division. Moreover, the optimal amounts

and types of plant growth regulators for initiation are determined by the explants used (Gaj 2004; von Arnold *et al.* 2002).

Based on previous findings, a source of immature tissue was needed to achieve somatic embryogenesis initiation for *P. oocarpa*. A third initiation was set up using immature zygotic embryos of cones from Hawaii. Due to a small amount of explants, we decided to test the same PO and 1218 basal media plus 1218 plant growth regulators (2.83 µM BA, 2.80 µM Kin and 10.74 µM NAA). The extrusion of somatic embryogenesis was achieved by using immature zygotic embryos at the pre-cotyledonary stage. The percentages of extrusion were 13 and 10 % for mother trees 3 and 5, respectively (Fig. 4-2) and the response was higher in the PO medium than in 1218 for both trees (data not shown). However, embryogenic tissue was not captured in either of the trees or the culture media.
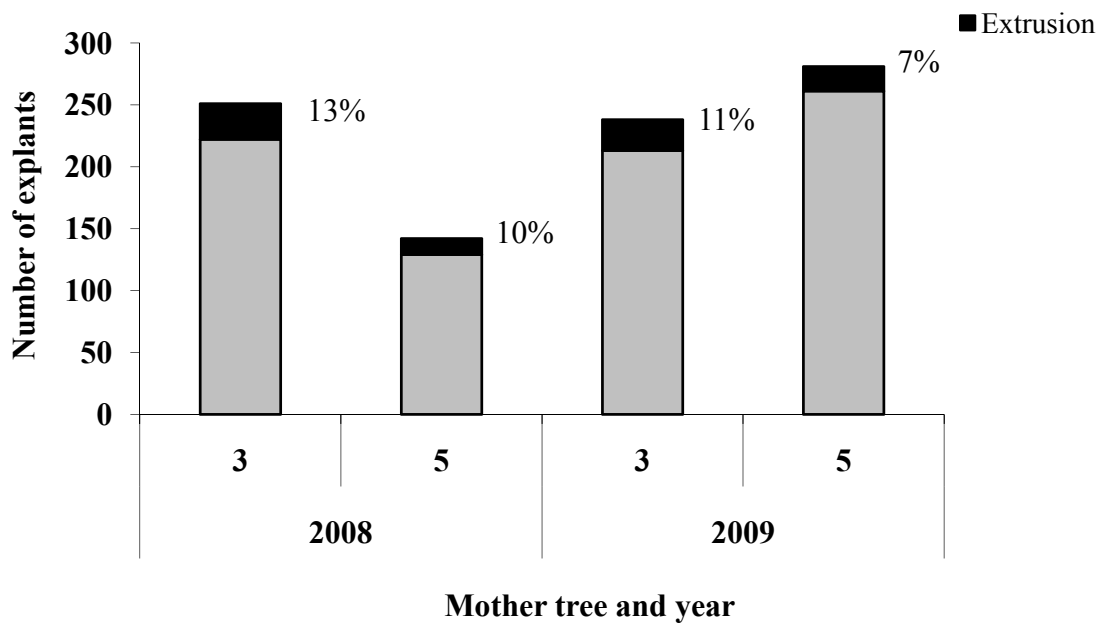
Figure 4-2. Number of explants on initiation and number and percentage of extrusion per mother tree and year in the initiation of somatic embryogenesis in *P. oocarpa.*

In 2009, cones from the same mother trees in Hawaii were harvested for a fourth *P. oocarpa* initiation. Two basal media were tested: PO and 1250 (Pullman *et al.* 2006) (Table 4-1). Each of the media was tested with 0.5, 1 and 2X of the plant growth regulators concentrations (1X = 5 µM 2,4-D, 2 µM BA, 2 µM Kin and 5 µM ABA), with a total of six treatments (Table 4-1). The total amount of seeds per stage of two mother trees 3 and 5 were split into the six treatments. Seeds from each cone were classified into three stages: small white seeds (Fig. 4-3A), light-yellow seeds (Fig. 4-3B), and brown seeds (Fig. 4-3C). From these seeds, embryo stages were identified as: the embryo stage pre-cleavage (found mainly in the small white seeds) (ES1) (Fig. 4-3D); the cleavage stage (ES2) (found in the light-yellow seeds) (Fig. 4-3E); and the late embryo stage (LE) (found in the brown seeds) (Fig. 4-3F).

After six weeks the number and percentages of extrusions were calculated per tree, cone, embryo stage and treatment. Extrusions were recorded every two weeks after the first data collection and for a period of eight weeks, for a total of four collection dates (Fig. 4-4). The larger part of the extrusion was observed in the first data collection date (8-12-2009), followed by a decline in the next three observation data dates (Fig. 4-4). However, the number of observations in the last three observation dates summed half of the total observations in each of the mother trees. After October 7th, no further new extrusions were observed from explants, but browning started to appear from those explants that did not respond. Initiation of somatic embryogenesis usually occurs after 6-10 weeks, however this is not synchronized and can be observed between 2 and 16 weeks (Klimaszewska *et al.* 2007).
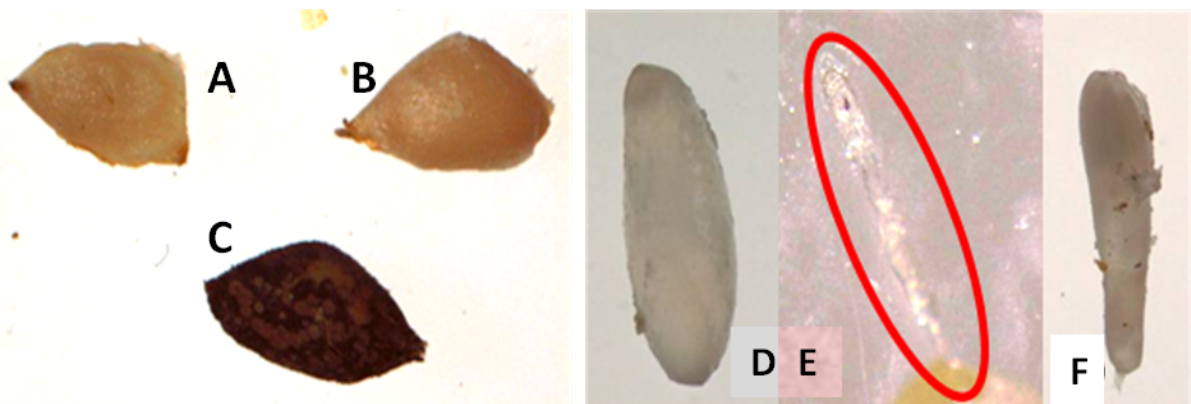
Figure 4-3. Explants used for the initiation of somatic embryogenesis of *P. oocarpa*. Three seed stages: A) white small seeds, B) light-yellow seeds and C) brown seeds. Embryo stages: D) ES1, megagametophyte containing immature zygotic embryo (found mainly in white small seeds), E) ES2, early stage (found in light-yellow seeds) and F) LE, late embryo stage (found in brown seeds).
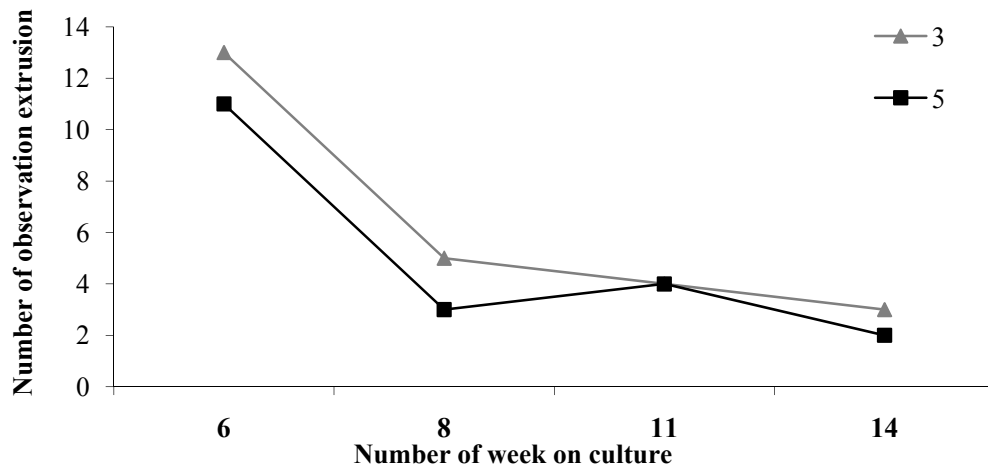
Figure 4-4. Number of extrusions per number of weeks on culture of two mother trees (3 and 5) in the initiation experiments for somatic embryogenesis of *P. oocarpa.*

The number of mother trees tested in this study was relatively low. However, differences in the extrusion frequency of somatic embryogenesis among various mother trees have been observed in *P. pinea* (Carneros *et al.* 2009), *P. banksiana*, *P. strobus*, *P. pinaster* and *P. sylvestris* (Park *et al.* 2006). Moreover, the percentage of extrusion was lower in 2009, compared with 2008 (Fig. 4-3). Thus, the identification and manipulation of factors that mark the differences in the percentages of extrusions and that capture the period between both years 2008 and 2009 could improve the success in the establishment of more lines.

A statistical analysis indicated that for the 2009 trials, the embryo stage and the basal medium had a significant effect on the initiation of somatic embryogenesis (Table 4-6). For both mother trees, the LE stage did not initiate embryogenic cultures, but instead a white callus or germination of the initial zygotic embryo failing to follow the pathway toward somatic embryogenesis as previously observed in *P. pinaster* (Lelu-Walter *et al.* 1999). In mother tree 3, megagametophytes enclosing embryos at either stages ES1 or ES2, the percentage of extrusion was similar; however, in mother tree 5, the highest percentage was using megagametophyte enclosing ES2 (Fig. 4-5A). This finding revealed that mother trees have a significant interaction for the potential to initiate somatic embryogenesis (Table 4-6). In *P. taeda* it was found that the maternal effects account for much of the variation (17.2 %) at the level of extrusion (MacKay *et al.* 2006). For *P. oocarpa,* somatic embryogenesis initiation was obtained with the use of pre-cleavage and cleavage stages, similar to stages reported in other

103

pine species where embryos at the cotyledonary stage were less successful in the achievement of somatic embryogenesis. However, for *P. oocarpa,* more studies are needed in order to describe the effect of developmental stage of the initial explants for the initiation of somatic embryogenesis.

Statistically a difference was observed between the two basal media, but not among plant growth regulator concentrations (Table 4-6). The percentages of extrusions were 11 and 7 % for mother trees 3 and 5, respectively. Additionally, in both years, mother tree 3 had a higher extrusion percentage, compared with mother tree 5 (Fig. 4-5A). The highest percentage of extrusion was obtained in the medium 1250 plus the 2X plant growth regulator concentration, followed by 0.5X and 1X (Fig. 4-5B). The 2X plant growth regulator concentration has 10.0 μM of both 2,4-D and ABA, plus 1 μM of both BA and Kin, close to the well-known standard concentration for the initiation of pine embryogenic cultures of 10 μM auxin and 5 μM cytokinin (Klimaszewska and Cyr 2002). Explants in the basal medium PO responded better in combination with lower plant growth regulator concentrations in the study (0.5X concentrations), followed by 2X and 1X (Fig. 4-5B). The 0.5X concentration has 2.5 μM 2,4-D plus 1.0 μM of both BA and Kin and 2.5 μM ABA, similar to low plant growth regulator concentrations, which has been shown to have a better response for the initiation of *P. strobus* and *P. monticola* somatic embryogenesis (Klimaszewska and Cyr 2002).

Table 4-6. Effect of mother tree, embryo stage, basal medium, plant growth regulator concentration and order 2- interactions on the extrusion of somatic embryogenesis in *P. oocarpa.* Extrusion was adjusted to a Poisson log-linear model. * represents significant difference at P<0.05

Source of variation	DF	χ^2	Prob>χ^2
Mains Effects:			
Mother tree	1	0.1042687	0.7468
Embryo stage	2	39.179071	<0.0001*
Basal medium	1	11.611572	0.0007*
Plant growth regulator concentration	2	4.2407293	0.1200
Interactions:			
Mother tree-Basal medium	1	0.6793737	0.4098
Embryo stage-Basal medium	2	11.149534	0.0038*
Mother tree-Embryo stage	2	6.7917162	0.0335*

Combining the extrusion of mother trees, culture media and zygotic embryo development stages in 2009, only 10 % of the cultures extruded obtainable tissue, which represents 45 extrusions out of 474 total explants on initiation. Extrusion percentage varies among species; it has been as low as 3.9 % for *Pinus banksiana* and as high as 76.2 % for *P. pinaster* (Park *et al.* 2006). Subsequently, the establishment of ten cell lines (2 %) was recorded. The only two factors that showed statistical influence during establishment were the embryos stage and the basal medium (Table 4-7). Similar percentage of established lines were observed on *P. taeda* with 2.8 % (Li *et al.* 1998a), 2.4 % in *P. roxburghii* (Arya *et al.* 2000), 1.1 % in *P. densiflora* (Maruyama et al. 2005a) and 2.3 % in *P. thunbergii* (Maruyama et al. 2005b). All of these results showed that in *Pinus* species low levels of establishment are obtained, thus more studies toward its improvements need to be done.

Table 4-7. Effect of mother tree, embryo stage, basal medium, plant growth regulator concentration on the establishment of somatic embryogenesis in *P. oocarpa*. Establishment was adjusted to a Poisson log-linear model. * represents significant difference at P<0.05

Source of variation	DF	χ^2	Prob>χ^2
Mother tree	1	1.6335296	0.2012
Embryo stage	2	10.399904	0.0055*
Basal medium	1	6.3998424	0.0114*
Plant growth regulator concentration	2	0.5036698	0.7774
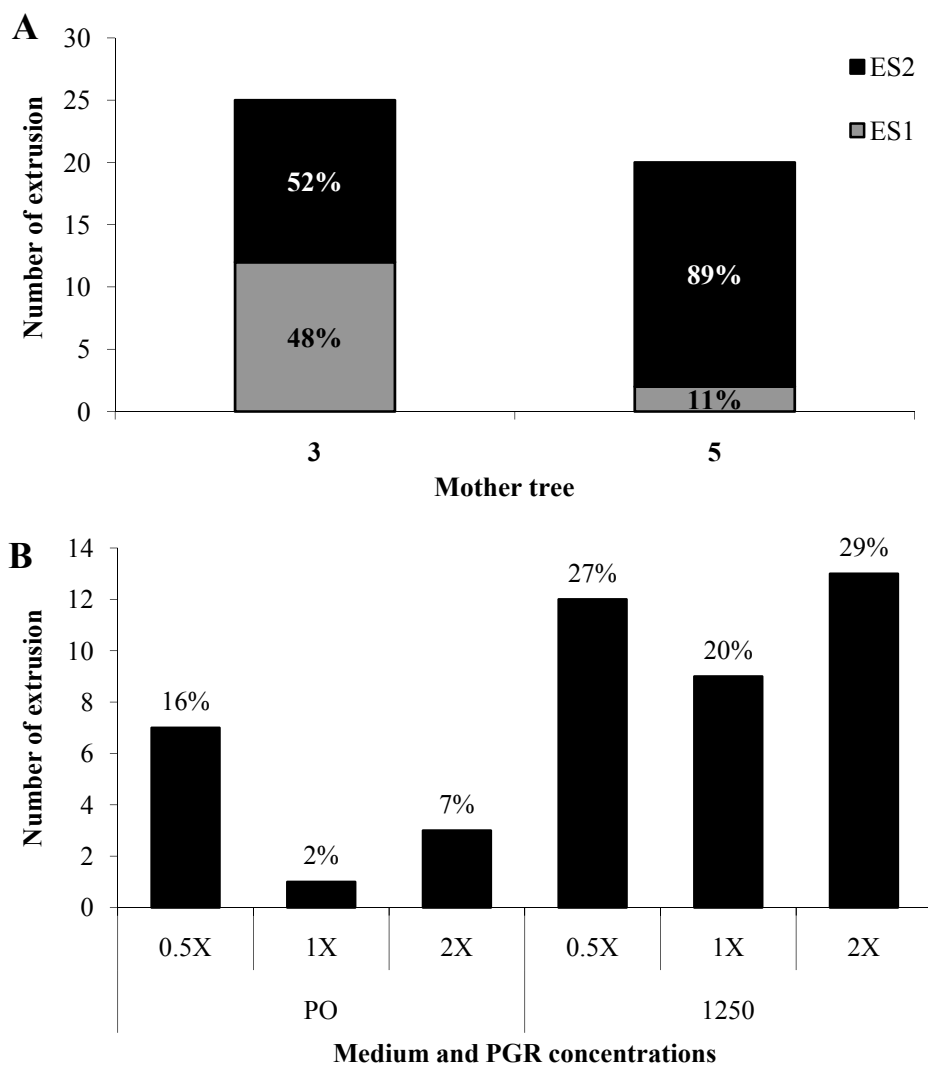
Figure 4-5. Number and percentage of extrusions in the initiation of somatic embryogenesis in *P. oocarpa:* A) by embryo stage per mother tree with ES1= pre-cleavage stage and ES2=cleavage stage. B) By treatment (basal media and plant growth regulators concentration). 1X= 5 µM 2,4-D, 2 µM BA, 2 µM Kin and 5 µM ABA.

In all of the initiations for 2008 and 2009, extrusion of embryogenic tissue was observed six weeks after initiation (Fig. 4-6A). Proliferated cells and somatic embryos appeared in the extruded tissue (Fig. 4-6B). Once the lines were established and embryogenic suspensor masses (ESM) were formed (Fig. 4-6C), a portion of this tissue (ESM) was used to confirm the embryogenic state by using the double staining procedure (Fig. 4-6D-E). In some cell lines, large embryonic heads composed of dense cytoplasmic cells were observed, while other heads were smaller and with long vacuolated suspensors. For each of the mother trees, faster-growing lines were easily identified.

106
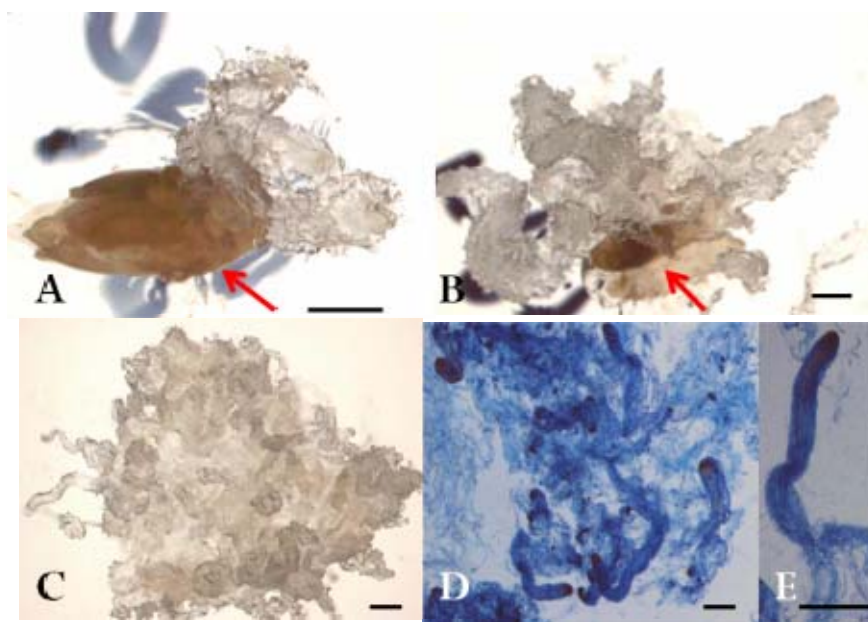
Figure 4-6. Initiation of somatic embryo growth in *P. oocarpa*. Somatic embryo tissue: A) 6 weeks and B) 8 weeks on initiation medium (arrow indicates MG). C) Established embryogenic culture (ESM), and double staining of ESM showing, D) mass of embryos and E) single embryo (bar=1 mm).

Studies have reported that some pine species are nutrient-dependent for the initiation of somatic embryogenesis (Carneros *et al.* 2009). We believe that *P. oocarpa* is also nutrient-dependent for the success of somatic embryogenesis establishment. Although extrusion was obtained in the three basal media studied, the highest percentage was obtained in the 1250 medium, which differs from the PO medium by having a higher concentration of inorganic nitrogen, NH_4/NO_3, a lower calcium concentration and a lower iron source concentration (Table 4-1). These media composition differences could also suggest a better, but non-significant response of explants in PO medium, compared with 1218 in 2008.

Additionally, silver nitrate was added to the PO medium. Silver nitrate, an ethylene inhibitor, increased the initiation in *P. taeda* when added to the medium in the range of 20 µM to 30 µM, especially when combined with ABA (Pullman *et al.* 2003d). In this study, PO and 1218 media were supplemented with 20 µM silver nitrate, but not in the medium 1250 (Table 4-1). The role of ethylene in the somatic embryogenesis of pine has yielded different results, depending on the species. Some results have suggested that ethylene may be involved in the induction of embryogenic tissue and in the early stages of the embryo maturation of *Picea abies* (Kvaalen 1994). On the other hand, in *P. mariana* and *P. glauca*, embryo production capacity may be

inversely related to the ethylene production rate during maturation (El Meskaoui *et al.* 2000; El Meskaoui and Tremblay 2001). The accumulation of ethylene results in the formation of large intracellular air spaces, which separate the meristematic cells within the shoot poles of developing embryos (Stasolla and Yeung 2003).

Additionally, by using the immature zygotic embryos of *P. taeda*, both extrusions and initiations increased with the addition of MES to the medium (medium 1218), and increased even higher with the addition of both MES and the vitamins biotin and folic acid (medium 1250) (Pullman *et al.* 2006). In our study, by using both of the above media, extrusion was achieved, but only on 1250 medium were somatic embryogenic cultures established.

4.3.5 Multiplication of somatic embryogenic masses

In order to find out the best multiplication medium, the two fastest-growing cell lines from the same mother tree but different cones (T5C1S12 and T5C2S01) were tested on six treatments. These treatments were the same treatments used for initiation. In both cell lines, the significant effects of the treatments ($p<0.005$), the subcultures ($p<0.005$) and their interactions ($p<0.005$) for relative weight increase were found (Fig.4-7). For cell line T5C1S12, statistical differences started to be observed in the second subculture between the two basal media. In the third subculture, treatment 1250-0.5X significantly increased the growth by 30-fold, but in the last subculture (40 days on multiplication medium), the basal medium 1250 with either of the plant growth regulator concentrations obtained almost the same relative growth increase (Fig. 4-7A). However, the highest relative growth rate was obtained on 1250-0.5X by approximately 35-fold. Cell line T5C2S01 growth was also better on 1250-0.5X medium. The second subculture's statistical differences were observed, and more than a 10-fold growth was obtained; this increase was close to 60-fold after 40 days on multiplication medium (Fig. 4-7B). The second best treatment was 1250-1X, in which the relative growth increase was close to 45-fold. No statistical differences were observed among the rest of the treatments; indeed, PO-2X showed better growth than 1250-2X, in which the growth rate was similar to the rest of the treatments on PO medium (05 and 1X).
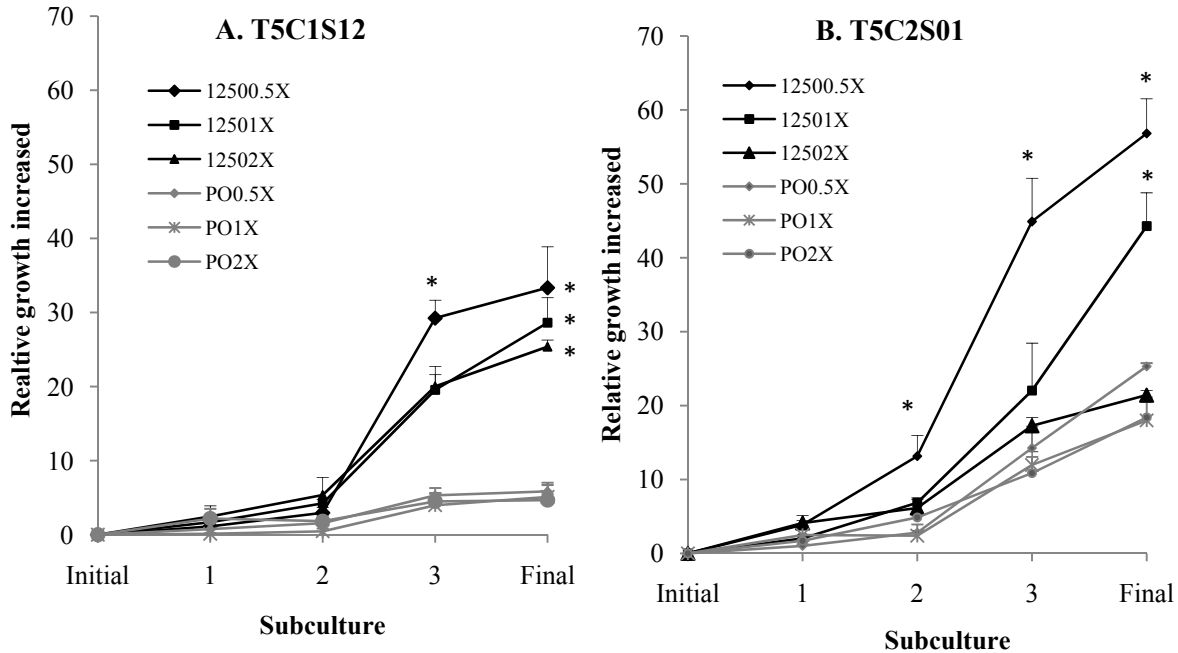
Figure 4-7. Relative growth increase (%) during the multiplication of two *P. oocarpa* cell lines A)T5C1S12 and B)T5C2S01 on two basal medium (PO and 1250) plus three plant growth regulator concentrations (0.5X, 1X and 2X). 1X= 5 μM 2,4-D, 2 μM BA, 2 μM Kin and 5 μM ABA. Each value represents a mean + SE of three replicates. * means significance difference at $P<0.05$

Differences were also observed between the two cell lines, the relative growth rate of cell line T5C2S01 increased almost twice as much as cell line T5C1S12 when both were placed on the same treatments. Regarding the PO medium, all of the plant growth regulators had almost the same increase within the cell lines. Again, cell line T5C2S01 obtained twice the growth compared to T5C1S12 on the same treatments.

Due to tissue availability, only two cell lines were tested for the multiplication experiments. The results showed that, independent of the medium in which they were initiated, the decrease of the plant growth regulator concentration was favorable for the increased growth of the tissue. Additionally, the increase of the tissue was different between cell lines. Even when both were initiated from the same mother tree and cone, the ability to grow was different.

By visually evaluating the plates where the cultures were kept, differences were observed in growth. It was obvious and easy to distinguish those cultures of both cell lines on 1250 medium, as they had increased the amount of tissue compared with lines on PO (Fig. 4-8A and C). Also noticeable was that even though PO increased the tissue at a lower rate, the cultures still grew and no necrosis was observed. Also, in all of the cultures, a continuous formation of

embryogenic structures with a smooth and spiky morphology was observed. After the completion of the experiment (40 days), a representative embryogenic suspensor mass sample from each of the treatments for each cell line was double stained to observe the embryo morphology. It was observed that in cell line T5C1S12, cultures were composed of embryos with nice cytoplasmic heads stained in red and with defined vacuolated suspensors stained in blue (Fig. 4-8B). Similar morphology was observed in the tissue of cell line T5C2S01 on PO medium but not on 1250 medium (Fig. 4-8D). The later tissue was formed by large embryo heads with many, albeit not well-defined associated vacuolated suspensor cells.

The multiplication process in somatic embryogenesis is also known as secondary, recurrent or repetitive embryogenesis. This process is usually carried out using the plant growth regulators 2,4-D, BA, and occasionally ABA (Klimaszewska *et al.* 2007). During multiplication the concentrations of these plant growth regulators are reduced or are kept the same as the concentrations used for initiation (Häggman *et al.* 2006). Additionally, multiplication of somatic embryogenesis cultures on a plant growth regulator-free medium has been possible (autoembryony), in which it is believed to help maintain early embryogenic competence (Breton *et al.* 2005). Thus, deciding which concentration to use will be determined by the species. Conversely, ABA is usually added at a high concentration for the development of somatic embryos, but a low concentration during multiplication could promote early embryo development and synchronization (Breton *et al.* 2005).

Different techniques have been shown to improve the multiplication rates in pine species, such as the use of the dispersal of tissue instead of clumps on a semisolid medium (Carneros *et al.*, 2009). However, the use of liquid cultures is usually preferred for large-scale propagation. In both semisolid and liquid, cultures are maintained in the dark (Stasolla *et al.* 2002b). In some species, multiplication can occur indefinitely, as well as the ability to mature. However, in pine species, the loss of embryogenic potential has been observed during prolonged multiplication, which could be associated with somaclonal variation (von Arnold *et al.* 2002). Subcultures are usually transferred every 10–14 days on solid medium, or less (7 days) in liquid medium, to avoid browning. To avoid adverse effects from prolonged culture, established cell lines can be cryopreserved, and only thawed when needed for propagation or experiments.

Additionally, manipulation of the multiplication medium, such as the addition of pre-conditioned medium, which has been demonstrated to be composed of proteins such as arabinogalactans, lipo-chito-oligosacharides and chitinase-like proteins (Egertsdotter and von Arnold 1995; reviewed by Stasolla and Yeung 2003), could improve the organization of embryo heads, consequently shortening the period required for embryo development.
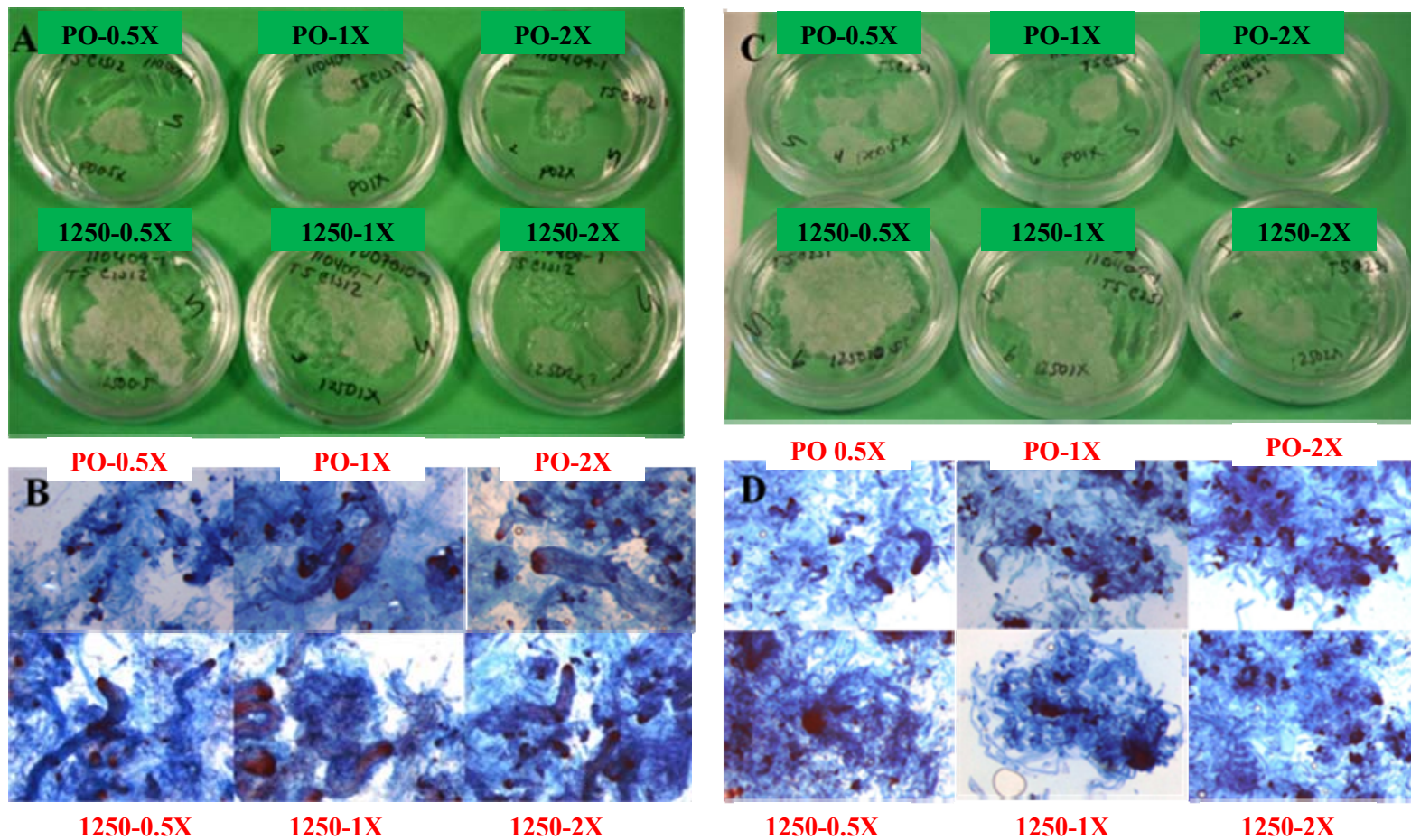
Figure 4-8. Multiplication and double staining of *P. oocarpa* somatic embryogenic tissues. Basal medium: PO and 1250, plus three plant growth regulator concentrations (0.5X, 1X and 2X were tested). A-B) Cell line T5C1S12, and C-D) cell line T5C2S01. 1X= 5 μM 2,4-D, 2 μM BA, 2 μM Kin and 5 μM ABA.

4.3.6 Maturation and germination of somatic embryos

4.3.6.1 Experiment 1: Maturation of two cell lines T5C1S12 and T5C2S01

Alongside the multiplication experiment, the same cell lines (T5C1S12 multiplied on 1250-1X and T5C2S01 on 1250-2X) were tested for maturation using the basal maturation medium 927 (Pullman *et al.* 2003c) plus 6 % (w/v) maltose, 12 % (w/v) PEG 8000, 0.6 % (w/v) Phytagel combined with two concentrations of ABA, 40 and 80 µM. After two weeks on maturation, embryos with small yellow heads were observed (Fig. 4-9A), which enlarged and developed into complete mature embryos after ten weeks. Embryos went through round-globular, early and late cotyledonary stages (Fig. 4-9C-D).

However, relatively few numbers of embryos were obtained from the two cell lines in the study. In cell line T5C1S12, the total number of mature embryos was the same in the two concentrations of ABA, (Table 4-8). On the other hand, in cell line T5C2S01, mature embryos were obtained only under 40 μM ABA with an average number of 10 mature embryos per 300 mg of fresh weight (FW) (Table 4-8). These results agreed with previous results, in which the response of the tissue to the ABA concentration was genotype dependent. Mature embryos obtained from this experiment were desiccated for 10 min inside the laminar hood before being transferred to the germination medium.

Unfortunately, all of them failed to germinate. The main reason was attributed to physical damage made while picking up the embryos after they were partially dry and transferring them to the medium. Shoot elongation was observed after fifteen days, but no root elongation. However, although the shoots elongated and were green in color, vitrification was observed, which ultimately led to the browning and death of the tissue.

Table 4-8. Average number of mature somatic embryos of two cell lines of *P. oocarpa* after 10 weeks on basal maturation medium 927 (Pullman *et al.* 2003c) plus two concentrations of ABA.

		Mature embryos	
Cell line	ABA (µM)	Total Number	Mean ± SE
T5C1S12	40	4	1.3 ± 1.3
	80	4	1.3 ± 0.6
T5C2S01	40	31	10.3 ± 0.6
	80	0	0.0 ± 0.0

300 mg of fresh weight of embryogenic tissue per plate and three replicates per treatment
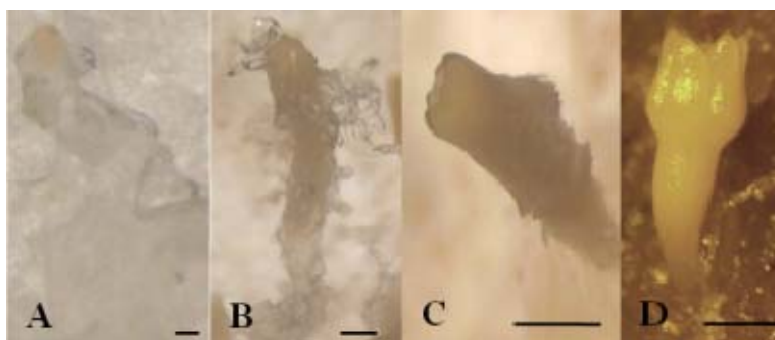
Figure 4-9. Maturation of somatic embryos of *P. oocarpa*: A) yellow head formation after two weeks on maturation, B) embryos at round stage, C) early cotyledonary, and D) mature embryos after ten weeks on maturation (bar = 1mm).

4.3.6.2 Experiment 2: Maturation of somatic embryogenic tissue of cell lines T5C1S12 and T5C2S01 under different multiplication treatments

Based on the results from the previous experiment (Table 4-8), tissue from the different multiplication treatments (4.3.5) were placed on the best ABA concentration (40 µM) for the advanced development of embryos. Results showed that there was an interaction between the cell line and multiplication treatment for the maturation of embryos. While in cell line T5C1S12 mature embryos were obtained from tissue multiplied on 1250 basal medium with either of the plant growth regulator concentrations, cell line T5C2S01 maturation was obtained only on tissue coming from multiplication basal medium PO (Fig. 4-10). The highest number of embryos was 21/mg FW and 3.1/ mg FW for the former and latter cell lines, respectively.

As shown previously, the morphology of embryos of cell line T5C1S12 was similar across treatments (Fig. 4-8A), but in cell line T5C2S01, embryos on PO showed better-looking embryos than on 1250 basal medium. Additionally, the growth of this line (T5C2S01) was higher on 1250 medium and twice that of the other cell line; it was composed of a large amount of vacuolated cells and no well-defined embryos, which failed to mature. Thus, the selection with higher probabilities to mature will be more effective by looking at the micromorphology level (i.e. double stained), compared to that of the macromorphology level (visual observation) (Breton *et al.* 2005).

114
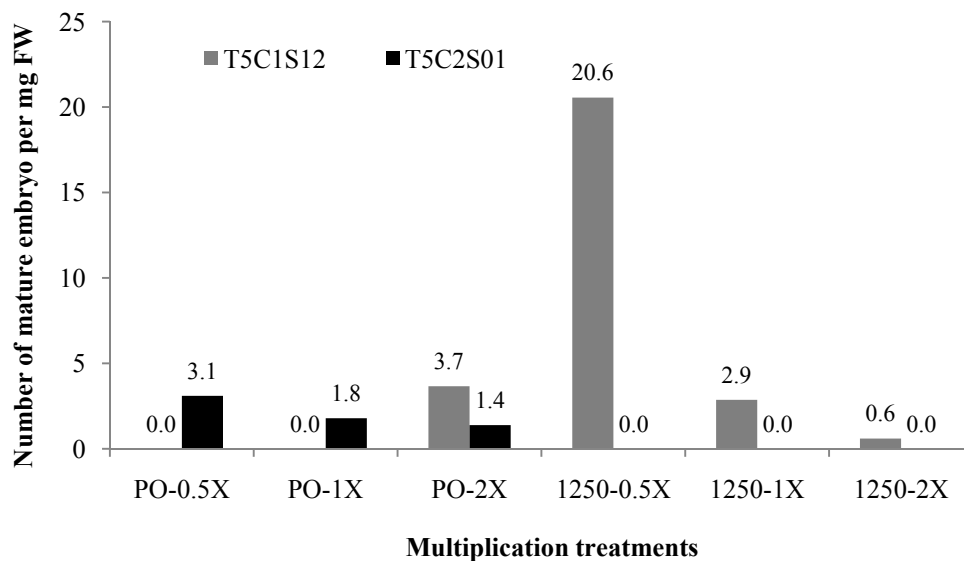
Figure 4-10. Number of mature embryos per mg of fresh weight (FW) in two cell lines of *P. oocarpa* coming from different multiplication treatments and matured in basal medium 927 (Pullman *et al.* 2003c).

Multiplication of cell lines of *P. oocarpa* could be improved by: 1) modifying the PO basal medium, mainly changing those major and minor minerals that differ with *P. taeda's* (4.3.1-4.3.3) and 2) initiating the culture on 1250 and then proliferating on PO; in order to obtain better embryo morphology for maturation. PO medium along with ABA and PEG, were also tested for the maturation of these two cell lines, but the tissue failed to mature, and multiplication was observed.

The maturation of embryos of cell line T5C1S12 was achieved but with the observation of tissue browning, while cell line T5C2S01 on PO medium did not show any browning (Fig. 4-11). Perhaps this is linked to the high amount of vacuolated cells in tissue. Embryos were transferred to germination following desiccation treatments (4.2.5.4).

In this study, maturation was tested with only two cell lines; the objective was to demonstrate that embryo development could be obtained for the two faster-growing cell lines of *P. oocarpa*. As the results showed, maturation was possible in both cell lines but we believe that more research is needed to test more compounds, such as those previously tested including gelling agents and their concentrations, carbon sources and their concentrations, as well anti-auxin (Carneros *et al.* 2009), among others.

In pine species for instance *P. nigra* (Salajova and Salaj 2005), *P. taeda* (Tang and Newton 2005b), *P. brutia* (Yildirim *et al.* 2006), *P. pinaster* (Lelu-Walter *et al.* 2006), *P. armandii* (Maruyama *et al.* 2007), *P. wallichiana* (Malabadi and Nataraja 2007a) and *P. sylvestris* (Aronen *et al.* 2009) maturation has been routinely achieved by the addition of ABA and an increase of the osmolarity to achieve normal embryo development (formation of cotyledons and suppression of precocious germination). ABA has been demonstrated to be essential in the further development of the embryos; however, the concentration required is both species and genotype dependent. To make this hormone more available to the tissue, a reduction of auxin and cytokinin levels is achieved by culturing embryogenic suspensor masses for seven days on a basal medium PGR-free prior to ABA treatment. ABA induces the accumulation of a variety of reserve substances including amino acids, such as glutamic acid, L-glutamine, and arginine, which also play a role in the synthesis of polyamines, such as spermidine (Stasolla and Yeung 2003); they also play a role in the accumulation of the storage proteins and the late embryogenic abundant protein.

The effect of ABA is genotype dependent (Jalonen and von Arnold 1991). For some spruce species (*Picea glauca* and *P. mariana*), a concentration of 12 µM ABA is enough to promote embryo development (Attree *et al.* 1991). However, for species such as slash pine (*Pinus elliottii*) and red spruce (*Picea rubens*), high concentrations of up to 30 and 40 µM ABA, respectively, are necessary in order to allow normal development (Harry and Thorpe 1991; Liao and Amerson 1995). On the other hand, in *Abies nordmanniana*, ABA alone yielded a low percentage of maturation (Find *et al.* 2002), but in combination with PCIB (an auxin antagonist), it improved the development of mature embryos. Similar results were obtained in *Brassica juncea* (Pradeep et al. 2006). In pine species, high concentrations have been used from 30 µM in *P. kesiya* (Choudhury *et al.* 2008) up to 120 µM for *P. pinaster* (Lelu-Walter *et al.* 1999).

Concentrations (ranging from 12 µM to 161 µM) have yielded maturation in conifers species (Stasolla and Yeung 2003). Here we studied only two concentrations (40 and 80 µM), selected from previous work in *P. taeda* (Chapter III), which yielded better embryo morphology and germination. Our results showed that the optimal concentration was genotype dependent.

Additionally, maturation has been routinely achieved by combining both PEG (non-permeating osmotic agent) and maltose (permeating osmotic agent) in several conifer genera, including pine species (Stasolla and Yeung 2003). Thus, we also tested three concentrations of PEG [0, 6 and 12 % (w/v)], combined with three concentrations of ABA (40, 80 and 120 µM) using the same two cell lines (T5C1S12 and T5C2S01). For this experiment cell lines (T5C1S12 was multiplied on 1250-1X and T5C2S01 on 1250-2X) were kept in the same medium as initiated and maturation was tested after six month on multiplication.

Results showed that maturation was obtained in cell line T5C1S12 on 0 % (w/v) PEG with either 40 or 120 µM ABA. Conversely, in cell line T5C2S01, maturation was observed in cultures on 12 % (w/v) PEG plus 40 µM ABA. Because the highest amount of mature embryos was obtained in cell line T5C1S12 on 0 % (w/v) PEG with 40 µM ABA, these embryos were tested for germination under different procedures (4.3.6.4). When embryos are matured with the use of PEG, they require a pre-treatment before germination, consisting of either a cold treatment and/or partial drying (Klimaszewska *et al.* 2007).

Additionally, a gelling agent had been added to reduce water availability, but the type and concentration of the agent was shown to affect the embryo quality and quantity (Stasolla *et al.* 2002b). In the beginning, the success of pine maturation was achieved by using the existing protocol for spruce and other conifers. Recently, however, work in *Pinus*, specifically *P. taeda*, showed that during maturation, an increase of $FeSO_4.7H_2O$ and Na_2EDTA to 41.7 and 55.95 mg/L, respectively, resulted in a statistically significant increase in cotyledonary embryo yields (Pullman *et al.* 2003c).
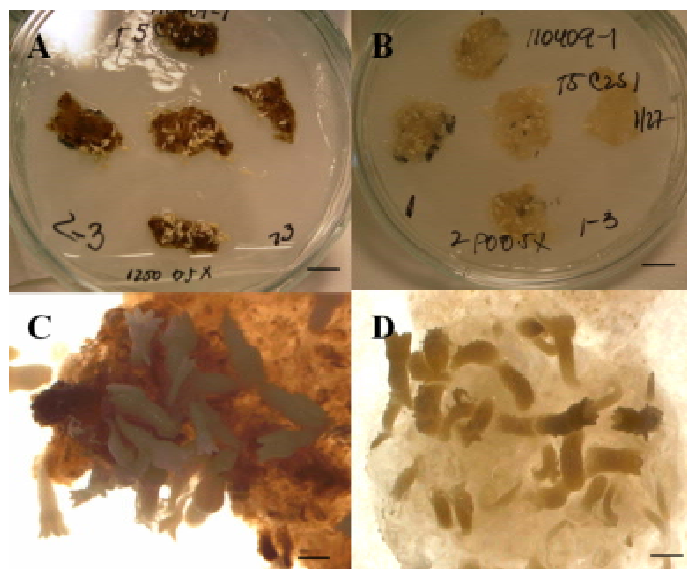
Figure 4-11. Maturation of *P. oocarpa* on basal medium 927 (Pullman et al. 2003c): A and C) cell line T5C1S12 multiplied on 1250-0.5X; B and D) cell line T5C2S01 multiplied on PO-0.5X. (A-B bar= 1cm and C-D bar= 1mm).

4.3.6.3 Experiment 3: Germination of cell line T5C1S12

For this experiment, we tested the ability of embryos of cell line T5C1S12 matured without PEG plus 40 µM ABA to germinate either by transferring them directly to a germination medium or with a desiccation treatment before germination (Table 4-9). After 30 days, the highest amount of germination was obtained when embryos were placed on the medium, but supported by the filter paper (Table 4-9), instead of putting them directly onto the medium (Fig. 4-12). In both of the previous treatments, embryos were able to produce better shoot elongation, compared with the two desiccation treatments; however vitrification plus no root elongation was observed when embryos were placed directly on the medium. On the other hand, by using either five or ten minutes of desiccation, the amount of germination was similar (Table 4-8), showing a longer root, but less shoot elongation (Fig. 4-12). After 45 days, the number of somatic seedlings with one root longer than 1 cm was recorded. Here, we found that embryos with 5 min desiccation treatment reached the highest number of somatic seedlings with roots longer than 1 cm. Although the somatic seedlings across treatments were similar in length, the total number in treatment 1, 2 and 4 was similar, approximately four (Table 4-9).

Even without desiccation treatment, the elongation of shoots was observed; the conversion to somatic seedlings was arrested at all or in a high percentage. Usually the tissue starts to turn

118

brown, which eventually leads to death. Also, the growth was slower, producing somatic seedlings with poor quality, making them less likely to survive *ex vitro* conditions. Before transferring the somatic seedlings to *ex vitro* conditions, they were placed in GA-7 vessels containing autoclaved peatmoss:vermiculite (3:1) for 11 days, to enhance the apical development and growth. After 66 days of germination, the percentage of germination was 0, 5, 10 and 2 for treatment, 1 to 4, respectively (Table 4-9). In all treatments, except for the 5 min desiccation treatment, the number of germinated embryos decreased with the time. Additionally, the average lengths of both root and shoot slightly increased compared with their counterpart at 45 days.

From this experiment, embryos after 15 days in the light did not show any root formation (Fig. 4-13A), but after 21 days both root and shoot growth was noticeable (Fig. 4-13B). Somatic seedlings grew for the next 24 days (Fig. 4-13C-D) on germination medium and then were transferred for 11 days to GA-7 vessels for growth of apical primordia and needles (Fig. 4-13E-F) before being moved to *ex vitro* conditions (Fig. 4-13G).

Table 4-9. Number and percentage of germination of mature embryos of cell line T5C1S12 of *Pinus oocarpa* after: 30, 45 and 66 days germination treatment

Treat[1]	Num. of embryos[2]	After 30 days		After 45 days			After 66 days		
		Germination [3]	Total embryos germinated (%)[4]	Num. seedlings (%) [4,5]	Length (cm) root	shoot	Num. seedlings (%)[4,5]	Length (cm) root	shoot
1.	30	0.0±0.0c	0 (0)	3 (10)	2.2±0.7	1.2±0.1	0 (0)	-	-
2.	30	2.7±0.2a	16 (53)	5 (16)	2.1±0.4	1.2±0.1	5 (16)	2.2±0.5	1.3±0.1
3.	30	1.3±0.4b	8 (27)	11(36)	1.7±0.2	1.1±0.1	10 (30)	1.9±0.3	1.3±0.1
4.	30	1.2±0.3b	7 (23)	4 (13)	1.6±0.6	1.1±0.2	2 (6)	2.0±1.1	1.6±0.6

[1] Treatments:
1. No desiccation, directly onto the medium
2. No desiccation, placed on filter paper and then onto the medium
3. Five minutes desiccation
4. Ten minutes desiccation

[2] Five embryos per replicate, three replicate per treatment. Total number 30 embryos

[3] Each number is the mean ± SE of three replicates, repeated twice. Letter with different letters represent statistical difference Tukey HSD $p<0.05$

[4] Percentage calculated based on total number germinated out of embryos on germination treatment

[5] Somatic seedlings with root longer than 1cm
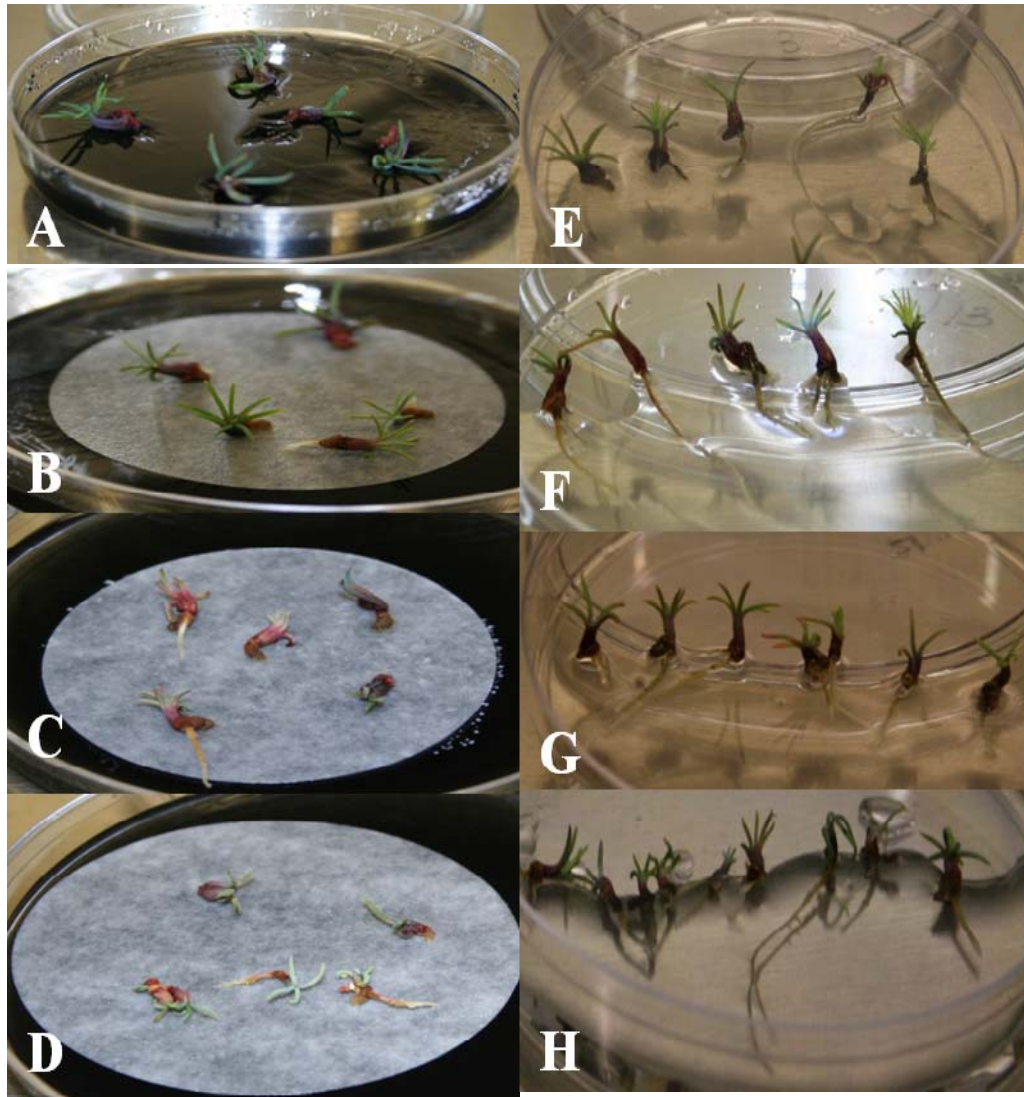
Figure 4-12. Germination of *P. oocarpa* mature embryos on different germination treatments after: A-D 30 days and E-H) 45 days. A and E) Germination without desiccation treatment; B and F) germination without desiccation treatment but embryos held on filter paper; C and G) germination with 5 min desiccation treatment; and D and H) germination with 10 min desiccation treatment.
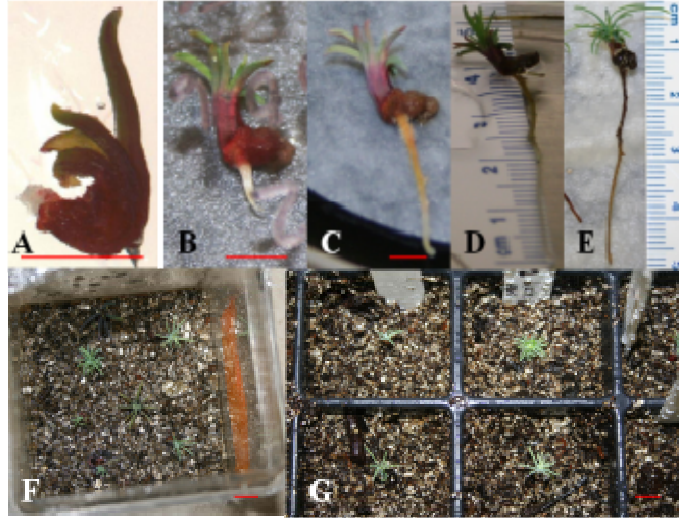
Figure 4-13. Germination of somatic embryos of *P. oocarpa* using desiccation treatments under the hood after: A) 15 days, B) 21 days C) 28 days, D) 45 days and E) 66 days. F) Somatic seedlings in GA-7 vessels before transfer to G) *ex vitro* conditions (bar = 0.5 cm).

4.3.6.4 Experiment 4: Germination of matured somatic embryos of cell lines T5C1S12 and T5C2S01 previously multiplied under different multiplication treatments

Embryos of the two cell lines were multiplied in different treatments (4.3.5) and matured with PEG and 40 µM ABA (4.3.6.2). These embryos were tested for germination using desiccation treatments (Table 4-10). Desiccation treatments consisted of placing the embryos in 3 wells of a six-well plate, with the remaining wells filled with 3 ml of sterile water (ADM). Plates were kept in the dark for one, two, three and four weeks, after which the embryos were transferred to germination medium. These plates were cultured in the dark one more week and then placed under light with a photoperiod of 16h/8h (day/night).

In the cell lines studied, two or three weeks on ADM improved the germination, and root extrusion was observed after seven days following embryo transfer to light (Table 4-10). When embryos were desiccated for only one week, it took 21 days to germinate (Table 4-10). A four week desiccation period was too long, completely inhibiting any subsequent growth.

Table 4-10. Percentage of germination per treatment of mature embryos of *P. oocarpa* after 1-4 weeks on desiccation treatments and after 7-14 and 21 days under light (see text for more details)

Cell line	Multiplication treatment	Weeks on Desiccation	Germination (%) after days under light[2]		
			7	14	21
T5C1S12	1250-0.5X	1	0	0	20±0.06
		2	100±0.0	Contamination	
		3	100±0.0	100±0.0	100±0.0
		4	0	0	0
T5C2S01	PO-2X	1	0	60±0.2	60±0.2
		2	100±0.0	Contamination	
		3	86±0.2	86±0.2	86±0.2
		4	0	0	0
T5C2S01	PO-0.5X	1[1]	-	-	-
		2	90±0.1	Contamination	
		3	100±0.0	100±0.0	100±0.0
		4	0	0	0
T5C2S01	PO-1X	1[1]	-	-	-
		2	100±0.0	Contamination	
		3	53±0.06	75±0.06	75±0.06
		4	0	0	0

[1] not tested

[2] five embryos per well and three wells per cell line and treatments. After desiccation embryos were placed on germination medium and plates were cultured in the dark for seven days then transferred to light. Percentage is based on five embryos per plate and three plates per treatment.

ADM treatment shortened the period of germination compared with the desiccation treatments in Experiment 3 (4.3.6.3) performed under the laminar hood. After 7 days, root development was observed (Fig. 4-14B) and elongation of both root and shoot was observed after 14 days (Fig. 4-14C) and synchronized root and shoot elongation was observed (Fig. 4-14D). Somatic seedlings were moved to GA-7 vessels before transfer to *ex vitro* conditions.
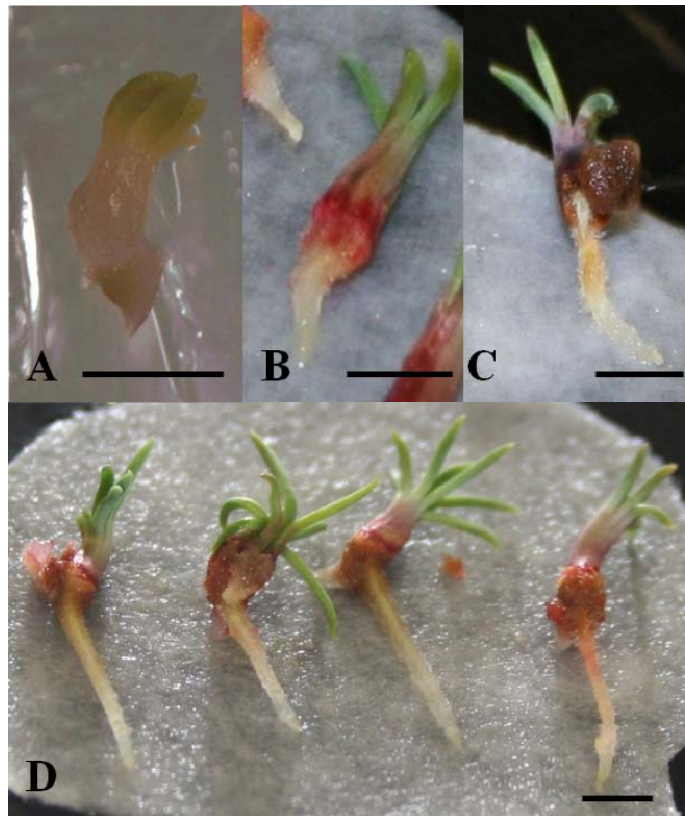
Figure 4-14. Germination of somatic embryos of *P. oocarpa* using desiccation treatment ADM: A) seven hour, B) 7 days C) 14 days and D) 28 days (bar = 0.5 cm).

Germination of somatic embryos of pine species has been achieved either by transferring them directly to a germination medium or by a desiccation treatment to mimic the natural conditions of the seeds (Roberts *et al.* 1990b). However, in general, mature embryos of conifers need a desiccation period to reach "physiological" maturity, and they must be able to grow into viable plantlets (Stasolla and Yeung 2003). Additionally, for some species such as *P. strobus, P. pinaster* and *P. taeda,* the culture of the embryos for seven to ten days in darkness before transferring them to light ensures the elongation of hypocotyls and reduces anthocyanin synthesis (Klimaszewska *et al.* 2007), which is visible once the embryos are transferred to light. In our experiments, we found that mature embryos of *P. oocarpa* also need a period of desiccation in order to increase their efficiency to grow in *ex vitro* conditions. Perhaps due to the small size of the somatic embryos, the optimal time of desiccation was two to three weeks. Without desiccation, abnormal germination was observed, followed by vitrification and death. Similar results were observed in spruce were synchronized elongation of the hypocotyls and radicle were observed when embryos were desiccated using the ADM treatments for three

124

weeks (Roberts *et al.* 1990b). Success of the maturation is also counted for the efficiency of the somatic embryo to develop a well formed shoot apical meristem (Yeung *et al.* 1998) and the accumulation of storage protein during maturation to broken and assimilated while embryos switch to an autotrophic system (Merkle *et al.* 1995).

Additionally, germination of fully mature embryos is usually carried out on a medium without plant growth regulators (Klimaszewska *et al.* 2007), however the supply of sucrose at a 2 % (w/v) level has been shown to be essential for the germination of *P. glauca/engelmanii* (Roberts *et al.* 1993). Improvement also is observed when embryos are placed horizontally on the medium and the plates tilted vertically allowing the roots to develop on the surface of the medium and without medium penetration (Klimaszewska *et al.* 2007).

The acclimatization of somatic seedlings has been more successful after they acquired autotrophic growth, which can be identified by the formation of the epicotyls (Roberts *et al.* 1993). In the past 10 years, the number of protocols for initiation of pine species has increased; however, few of them reported the transfer of somatic seedlings to *ex vitro* conditions and the survival rate. In *P. armandii,* 51 % of germination and 40 % conversion was reported (Hosoi and Ishii 2001). Similar responses were obtained in *P. thunbergii,* with 60 % germination on ½ EM medium without plant growth regulators and 2 % (w/v) activated charcoal, and 51 % conversion (Maruyama *et al.* 2005b). In economically important species where protocols have been improved in the past years, these percentage are pretty high, for example in *P. pinaster,* cell line PM5 exhibited 96, 89 and 70 % germination, conversion to plants, and survival, respectively (Klimaszewska *et al.* 2007).

Protocol for the somatic embryogenesis of *P. caribaea,* another tropical pine was achieved by the use of immature zygotic embryos. The highest percentage of initiation (5 %) was obtained by using megagametophytes containing immature zygotic embryos on LP medium plus 10 µM 2,4-D and 5 µM BA. Maturation was achieved by increasing the concentration of ABA to 15 µM ABA and subsequently germination was obtained on plant growth regulator-free medium (Laine and David 1990). However, improvement to this protocol has not been reported.

The identification of proteins has been done in whole seeds (without seed coat) of *P. oocarpa* and showed that 72 % of the total protein was insoluble, with the remainder being soluble (Gifford 1988). The insoluble proteins under reducing conditions migrated in two mass ranges

of 31-35 kDa and 21.5-22.5 kDa, while the soluble proteins had a molecular mass of 27-29.5 kDa (Gifford 1988). These proteins could be used in the future for a comparison of protein accumulation in somatic embryos with zygotic embryos, which could provide us with more information about the quality of the somatic embryos under maturation treatments, tested in the study, and guide us in the improvement of protocols for maturation.

4.3.7 Attempts to initiated somatic embryogenic cultures from shoot apical meristems of *P. oocarpa*

Sections of shoot apical meristems of *P. oocarpa* (Fig. 4-15A) were tested for their ability to initiate embryogenic tissue. After 15 days on the initiation medium (Fig. 4-15B), callus formation was observed around the explants. Afterwards, sample tissue was double stained to assess for embryogenesis (Fig. 4-15C). A high contamination rate was also observed, although mercuric chloride was used for disinfection. Recently, the use of cold treatments on mature tissue of *Pinus patula* (Malabadi and Van Staden 2005b) and *P. kesiya* (Malabadi *et al.* 2005) explants has been shown to stimulate the initiation of somatic embryo cultures. A similar procedure was applied for *P. oocarpa,* but beyond the formation of white callus from the center or peripheral part of the explants, embryogenesis was not obtained, in contrast to the above results. White callus was transferred to a fresh medium biweekly, but after six weeks browning was observed, and the callus became harder and yellowish.

In pine, few successes have been reported in the initiation of somatic embryogenic cultures from mature zygotic embryos or mature tissue as explants. High percentages (up to 16.9 %) of white translucent, glossy mucilaginous embryogenic tissue were initiated from mature *P. taeda* seeds on LOB callus induction medium, plus 45.24 µM 2,4-D, 17.8 µM BA and 18.6 µM Kin with a pH of 5.8 (Tang *et al.* 2001). Additionally, initiation has been reported using shoot apices of *P. patula* on DCR medium plus 23 µM 2,4-D, 27 µM NAA and 9 µM BA (Jones and van Staden 2001) and secondary needles of *P. roxburghii* on DCR medium plus 22.6 µM 2,4-D 26.9 µM NAA and 5.0 µM triacontanol (Malabadi and Nataraja 2007d). Subsequently, this embryogenic tissue produced somatic seedlings.
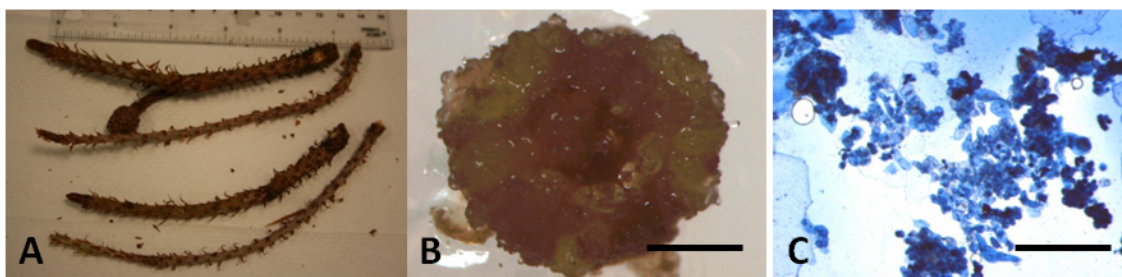
Figure 4-15. Attempts to initiate somatic embryogenesis of *P. oocarpa* using shoot apical meristems: A) shoot apices of mature *P. oocarpa*; B) cold-pretreated shoot apical section after 15 days on the initiation medium (bar= 0.3 mm) and C) double staining to assess for the presence of embryogenic tissue from callus (bar= 0.1 mm).

These results indicate that successful somatic embryogenesis induction from mature zygotic embryos and tissue is difficult, but possible, providing an opportunity to alleviate the disadvantages of using immature zygotic embryos as explants. However, replication of these above results using mature tissue has been not published; thus, pine species are still considered as recalcitrant in terms of somatic embryogenesis initiation through mature tissue, which is the major obstacle in clonal propagation (Bonga *et al.* 2010).

The initiation of white translucent tissue from mature zygotic embryos was tested using different techniques (data not shown): infiltration, the use of conditioned medium, different pH levels for the culture medium, and pre-treatments with ABA to decrease or reduce the germination and increase the percentage of white tissue. All of the treatments yielded the same result; the formation of non-embryogenic callus.

4.4   Summary

To our knowledge, this is the first report of the initiation of somatic embryogenesis in *P. oocarpa* using immature zygotic embryos. This was accomplished by studying the mineral composition of the megagametophyte of mature seeds which was used to modify a currently-used culture medium, combined with plant growth regulators. Additionally, it was observed that similar to other pine species, for somatic embryogenesis induction in *P. oocarpa,* a more immature source of zygotic embryos resulted in a higher embryogenic response. Maturation was then accomplished with the use of ABA, and normal germination was promoted by a partial desiccation of the embryos (Fig. 4-16). However, more culture initiation needs to be tested, and the number of explants per embryogenic developmental stage has to be increased in order to augment the percentage of successful inductions. Additionally, our study was limited

by the number of mother trees; thus, a more extensive study is needed to test additional mother trees and to look at the maternal effect on initiation and maturation. The number of mature somatic embryos obtained is still low, compared with the number reported in other pine species. Therefore, tests of other agents such as Phytagel, maltose and anti-auxins may improve the results. Somatic embryogenesis has been studied for different economically important species, with the purpose of increasing multiplication rates and targeting the transfer of genes of interest. We believe that this technology could be used, in combination with traditional breeding of *P. oocarpa,* to improve the qualities of the actual hybrid clones, especially for those countries in which a high percentage of the population depends on its products. Furthermore, the introduction of this technology will help increase the economies of these countries through the development of a better quality product.

Figure 4-16. Schematic representation of stages of somatic embryogenesis in *P. oocarpa* (Figures not a scale).

4.5   Acknowledgments

5   Analysis of gene expression patterns during embryo development in somatic and zygotic embryos of *Pinus taeda*

ABSTRACT

The relative expression of developmentally regulated genes was analyzed during somatic and zygotic embryo development and maturation in *P. taeda*. Four developmental stages of embryos were studied: zygotic pro-embryos and somatic embryogenic suspensor masses; the following stages of both zygotic and somatic stages: round/globular, early cotyledonary and late cotyledonary were chosen. Differential gene expression between developmental stages and between somatic and zygotic embryos of similar stages was detected during the early stages of embryo development through to maturation. The relative transcript levels of six genes of interest were tested: Legumin-like and Vicilin-like, LEA, Clavata-like, RPN1 and HD-Zip I. Relative transcript abundance for ABA-inducible genes (Legumin-like/Vicilin-like storage proteins and LEA) accumulated gradually through somatic embryo maturation, in contrast to zygotic embryos, where the accumulation was more dramatic at the late cotyledonary stage. Transcripts for Clavata-like (involved in shoot meristem regulation) and RPN1 (26S proteasome activity) were higher during the round/globular stage in both somatic and zygotic embryos, indicating that distinct changes in gene expression occur during the transition from pro-embryo to later stage embryos. The relative expression of HD-Zip I (Homeodomain leucine zipper I) differed between somatic and zygotic embryo systems, and was attributed to compositional differences between the embryogenic suspensor masses of somatic embryos and zygotic pro-embryos. These results are in agreement with the results of previous studies.

Key word: storage protein, LEA, Clavata-like, RPN1, HD-Zip I, relative transcript abundance, somatic embryo, zygotic embryo

5.1   Introduction

In higher plants, the process of zygotic embryo formation is divided into three phases: morphogenesis, maturation and desiccation (West and Harada 1993). These stages of zygotic embryogenesis correspond to the following events during embryo development: 1) definition of the shoot-root plant body pattern, 2) formation of storage organs (such as cotyledons), and 3) conversion to dormancy until conditions are favorable for germination (Goldberg *et al.* 1994).

Somatic embryogenesis is described as the process in which cells within the plant are induced to form somatic embryos through the manipulation of culture medium components and plant growth regulators (von Arnold *et al.* 2002). Somatic embryos pass through similar stages of development as they are exposed to culture conditions that attempt to mimic the conditions of their zygotic embryo counterparts (Ikeda and Kamada 2006). Thus, somatic embryogenesis has been used as a model system to study the physiological, biochemical and morphological pathways during zygotic embryogenesis (Zimmerman 1993), and to reveal novel pathways and gene interactions during embryo development (Quiroz-Figueroa *et al.* 2006; Stasolla *et al.* 2002a). An advantage to using somatic embryogenesis as a model system is that large amounts of somatic embryos at specific developmental stages can be produced and collected all year around and the growth conditions can be manipulated *in vitro*. In contrast, zygotic embryos at different stages have to be individually dissected from seeds, a process which is time consuming and temporally limited, as cones are available only once per year for temperate pine species.

Furthermore, somatic embryogenesis is currently the only available clonal propagation technique suitable for the large-scale production of conifer seedlings that has the potential to become a low-cost method (Stasolla and Yeung 2003). Somatic embryogenesis has been recorded for different species across many genera and from a variety of plant tissues. The first report on somatic embryogenesis was from carrot (*Daucus carota*) (Stewart *et al.* 1958). Since then, carrot in particular has been used as a model for the study of somatic embryogenesis in angiosperms, revealing a better understanding of embryo development. In conifers, the first report on somatic embryogenesis was with *P. abies* (Chalupa 1985; Hakman *et al.* 1985), and since then, protocols for the propagation of other conifers through the use of somatic embryogenesis have been developed (Stasolla *et al.* 2002a). The first protocol for somatic

embryogenesis in *P. taeda* was reported more than 20 years ago (Gupta and Durzan 1987a), and several improvements have been made to increase the number of "normal" plants produced, with major emphasis placed on the improvement of initiation rates especially in *P. taeda* (Pullman and Johnson 2002a; Pullman *et al*. 2003a*;* Pullman *et al.* 2005; Pullman *et al.* 2006)*.* However, many of the current protocols for somatic embryogenesis report low numbers of mature embryos per gram of fresh weight, and in some cases, somatic embryos do not fully mature, resulting in slow germination and initial growth (Pullman *et al.* 2003b).

A better understanding of the underlying physiological differences between zygotic and somatic embryo development through the analysis of gene expression patterns has the potential to be used to improve *in vitro* protocols (Bonga *et al.* 2010). The developmental pathways of somatic embryos have been described on the cellular level for *Picea abies* (Filonova *et al* 2000). However, the lack of sequence information for conifer species and the absence of an efficient mutagenesis system hamper the development of embryo-specific mutants for studies of gene function.

Differences between somatic and zygotic embryos can be related to their nutritional status. Zygotic embryos develop inside the megagametophyte, which is rich in nutrients and storage reserves. On the other hand, somatic embryos mature within an open environment, and their nutrition has to come from the artificial culture medium.

Currently, the most efficient explant for the initiation of somatic embryos in pine is the immature zygotic embryos associated with the megagametophyte, at the stage where subordinated embryos at the cleavage stage are present. When these explants are placed on culture medium, the subordinate embryos extrude from the micropylar end of the ovule (Cairney *et al.* 2006). It is possible to distinguish embryogenic tissue from non-embryogenic tissue through visual observations, or through the use of histochemical stains. The expression patterns of SERK, LEC1, FUS3 and ABI3 are differentially expressed between embryogenic and non-embryogenic cultures (Yang and Zhang 2010). Following the induction and multiplication of embryogenic tissue, maturation of somatic embryos can be monitored by other markers such as storage protein gene expression and storage protein accumulation (Sterk and de Vries 1992). Furthermore, the presence of LEA proteins has been found to be associated

with the accumulation of ABA during maturation, as well as associated with embryo protection during desiccation (Stasolla *et al.* 2002b).

Gene expression analyses can be a useful tool for monitoring embryo development from the acquisition of embryogenic competence through to maturation (Feher *et al.* 2003). Ideal "marker" genes should be universal, sensitive, detectable in small amounts of tissue, and reveal specific processes characteristic of each of the developmental stages or transitions (Feher *et al.* 2003). Out of the plant growth regulators, ABA has been shown to regulate several essential processes during embryo maturation, and over 150 genes from a range of species are found to be ABA-inducible (Giraudat *et al.* 1994). ABA is supplied to the zygotic embryo through the megagametophyte, and the lack of this tissue during *in vitro* developmental conditions has made the application of ABA essential for the normal maturation of somatic embryos, also in pine species.

In somatic embryos of *P. glauca,* development and maturation stages have been characterized as follows: 1) immature embryo consisting of an embryonic region with densely cytoplasmic cells and a translucent suspensor which is long and highly vacuolated; 2) globular embryo differentiated by prominent and opaque embryos having smooth and glossy surface adjacent to a suspensor which is cream to pale yellow in color; 3) early cotyledonary embryo where the primordial cotyledons are below the circumference of a prominent central meristem, and it was cream to pale yellow; and 4) late cotyledonary embryo with cotyledons extending beyond the central meristem (Dong *et al.* 1997). We used this classification to collect the stages in both somatic and zygotic embryos.

The aim of this study was to assess the relative expression of genes related to embryo development and maturation in four developmental stages of somatic and zygotic embryos, respectively. This would serve to demonstrate the fidelity of the somatic embryos and could aid in identifying changes that could be made to culture medium and conditions to enhance the quality of the somatic embryos. The genes targeted for analysis encode: 1) Legumin-like and Vicilin-like (storage proteins), 2) Late embryogenesis abundant or LEA protein (late embryo development and desiccation tolerance), 3) Clavata-like protein (meristem development), 4) Homeodomain-leucine zipper I (HD-Zip I) protein transcription factor and 5) 26S proteasome regulatory subunit S2 (RPN1) (ubiquitin-mediated proteolysis). For gene expression analysis,

133

we used the real-time PCR technique, which has become a routine technique allowing for gene expression analysis in plants under different experimental conditions, in different tissues within the plant, and/or in different developmental stages of the plant.

5.2 Materials and methods

5.2.1 Plant Material

5.2.1.1 Collection of zygotic embryo samples

Eight cones of Loblolly pine *(P. taeda)* were collected weekly from the Reynolds Homestead (Critz, VA) (http://www.reynoldshomestead.vt.edu/), starting on June 10th 2009 to September 15th 2009. Cones were opened and seeds were collected for isolation of zygotic embryos. Average number of seeds per cone, cone length and width was noted for each collection. The megagametophyte was opened and embryo or mass of embryos were observed under a stereoscope to determine the developmental stage of the embryos. Zygotic embryos were classified in four stages: 1) pro-embryo (PE), round/globular (RG), early cotyledonary (EC) and late cotyledonary (LC) (Fig. 5-1A-D). Collected samples were frozen in liquid nitrogen and kept at -80 ºC until RNA was extracted.

5.2.1.2 Collection of somatic embryo samples

P. taeda line 637.1 (courtesy of Dr. Scott Merkle) was used to produce different developmental stages of somatic embryos. Classification of somatic embryo stages was based on morphological similarities with their counterpart zygotic embryos (5.2.1.1): 1) embryogenic suspensor mass (ESM), 2) RG, 3) EC, and 4) LC (Fig. 5-2A-D). ESM was collected from cultures on maintenance medium. Meanwhile, for the collection of maturation stages, ESM cultures were placed on pre-maturation medium (same multiplication medium but plant growth regulator-free) for one week. Then, cultures were re-suspended with plant growth regulator-free maintaining medium at 20 % (w/v) and 2 ml were spread per plate, and the remaining liquid medium removed from each plate. Maturation medium consisted of 927 basal medium (Pullman *et al.* 2003c) (no PEG 8000 and 0.3 % (w/v) Phytagel instead of Gelrite), 6 % (w/v) maltose and 40 μM ABA. Collected samples were frozen in liquid nitrogen and kept at -80 ºC until RNA was extracted.

All the observations for the collection of the tissue in both somatic and zygotic embryogenesis were made under a zoom stereomicroscope (Olympus America Inc.)
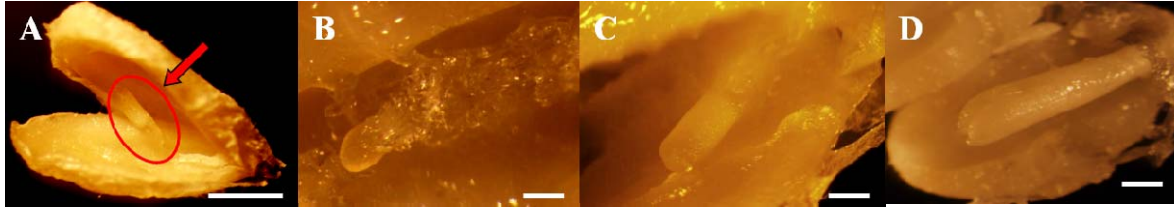
Figure 5-1. Developmental stages of mature zygotic embryos in *P. taeda:* A) megagametophyte (MG) containing the embryo (bar = 1 mm); B) round/globular (RG) stage; C) early cotyledonary (EC) stage, and D) late cotyledonary (LC) stage. The arrow points to the embryo. (B, C, D bar = 0.1 mm).
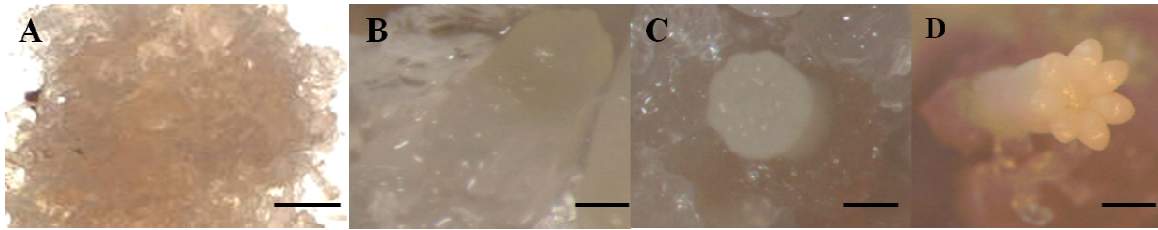
Figure 5-2. Developmental stages of mature somatic embryos in *P. taeda:* A) Embryogenic suspensor mass (ESM) (bar = 1 mm); B) round/globular (RG) stage; C) early cotyledonary (EC) stage and D) late cotyledonary (LC) stage. (B, C, D bar = 0.1 mm).

5.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from zygotic and somatic embryo samples using the Concert ™ Plant RNA Reagent (Invitrogen, Carlsbad, CA). To eliminate any residual genomic DNA present in the samples, RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's protocol. The quantity of isolated RNA both before and after DNAase treatment was measured using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA was quantified and the integrity was verified by agarose gel electrophoresis. Thereafter, for zygotic embryo samples, total RNA from each sample underwent one round of amplification using the MessageAmp II aRNA kit (# AM1751, Ambion, Austin, TX), according to the manufacturer's protocol, using an *in vitro* transcription time of 14h. Subsequently, RNA of each sample was used for cDNA synthesis using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules) for somatic embryo samples, and SuperScript III, First-Strand Synthesis System for RT-PCR (Invitrogen) for zygotic embryo samples, with the use of random hexamers. Subsequently, cDNA was checked via PCR using Tubulin primers (Table 5-1). The PCR conditions were: 95 ºC for 4 min (1X); 95 ºC for 30 sec, 55 ºC for 45 sec, 72 ºC for 45 sec (34X); and 72 ºC for 5 min (1X).

5.2.2 Relative Real-time PCR

The relative transcript abundance of the six genes of interest (Table 5-1) was analyzed by real time PCR. The reactions were carried out using a Bio-Rad iQ5 Multicolor Real Time Detection system under the same conditions used by Ratnaparkhe *et al.*(2009). Briefly, reactions were carried out in a 20 µl volume, containing 10 µl iQ SYBR green Supermix (Bio-Rad), 1 µl (100 nM) forward and 1 µl (100 nM) reverse primer, 2 µl cDNA template and 6 µl nuclease-free water. Tubulin and *Pinus taeda* Ubiquitin conjugating enzyme 1 [Pta-UBC1- similar to *P. abies* UBC1 (Palovaara and Hakman 2008)] genes (Table 5-1) were used as endogenous controls for normalization. The transcript levels were normalized using 1) Pta-UBC1 for the gene expression within each embryo developmental series and 2) Tubulin, for the gene expression comparison between individual somatic and zygotic embryo stages. Each sample was subjected to three technical replicates and the relative transcript level expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Mean values are shown with standard errors (SE).

Table 5-1. Description of genes and primer sets used for real time PCR.

Gene	Accession number	Description	Primer pair (forward/reverse, 5'→3')	Product size (bp)
Endogenous controls				
Tubulin	Spruce X57980.1 (GENBANK)	Essential for the structure and kinetics of the cytoskeleton	ACAGCTGATCTCGGGCAAAGAAGA/ TGCAGTTATCGGCTAGCTTCCTCA	119
PtaUBC1	Spruce AY639585 (GENBANK)	Ubiquitin-conjugating enzyme 1	GAGCAGTGGAGTCCTGCCT/ AGCTCTTGCTGTCTCCTCATAACGA	147
Genes in study				
Legumin-like	Pine TC58155 (TIGR GI)	Storage protein	CAGCGCTTCTGTTGCTCTAC/ CTCCGACATGCTGATGATCT	133
Vicilin-like	Pine TC57029 (TIGR GI)	Storage protein	AGCTTACGTGCACCAGAATG/ AGACTGGTAATGCGAAGGCT	129
LEA	Pine TC57074 (TIGR GI)	Late Embryogenesis Abundant	AGATCCTGGGTCAGTGAAGG/ GTAAGGCCAATCTCCCATGT	138
Clavata-like	Spruce DQ530597 (GENBANK)	Meristem development	TCTTCGTGGATGCTGACTTC/ TCCGTCGCTCACAATAACAT	96
Pg HD-Zip I	Spruce DQ201170 (GENBANK)	Homeodomain-leucine zipper-like	GCCATGATCGAACGTATGAG/ TCATGAGAAGCTGCAAGTCC	109
RPN1	Pine TC57100 (TIGR GI)	Similar to 26S proteasome regulatory subunit S2	CAATGCTGGATTTGGTCAAG/ TCCCAAAGGAGAATCAAACC	144

5.3   Results and discussion

5.3.2   Zygotic embryo samples

Cones were collected at random locations on the tree every week during the summer of 2009 (Fig. 5-3A). Megagametophytes were excised, and zygotic embryos were dissected at different stages (Fig. 5-1A-D). The length and width of each cone was recorded every week (Fig. 5-3B-C, respectively) as well as the number of seeds per cone (Fig. 5-3D), and then averages were calculated. In the first two collections, small, white translucent seeds were observed without any possible distinction between the megagametophyte and the embryo (Fig. 5-3E). White and light yellow seeds were observed in the fourth and fifth collections, respectively (Fig. 5-3F). Yellow seed coats were observed in the seventh collection (Fig. 5-3G), and light brown seeds at the ninth collection and onwards (Fig. 5-3H). Megagametophytes were distinguished from the seeds by the fourth collection, and were white and translucent with a yellow knob (Fig. 5-3I), while white megagametophytes were observed starting in the fifth (Fig. 5-3J), light yellow in the seventh (Fig. 5-3K) and yellow in the ninth through to the last collection (Fig. 5- 3L).
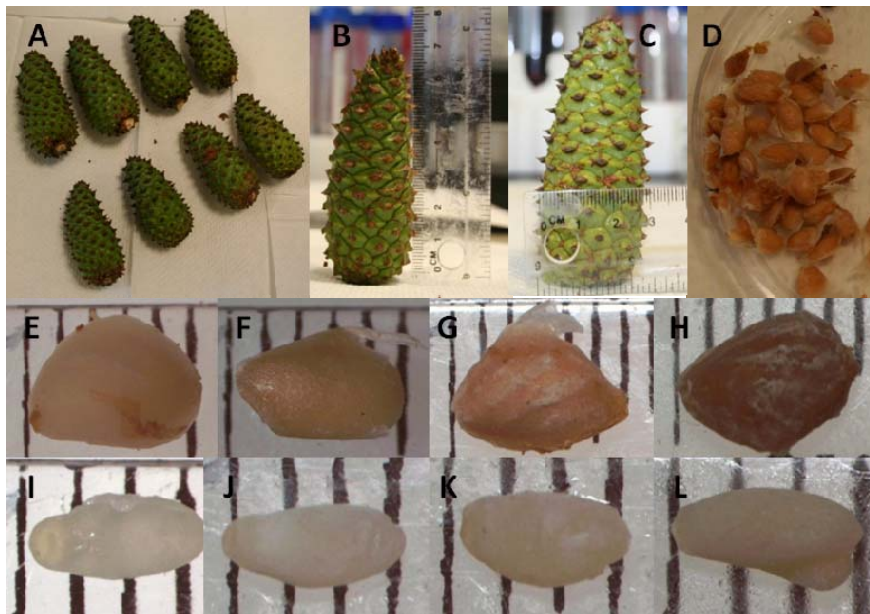
Figure 5-3. Collection of cones and extraction of megagametophytes from the seeds of *P. taeda* during 2009: A) eight cones were collected each week; B) length measurements and C) width measurements were made. D) Seeds collected from cones. Seed development during: E) first, F) fifth, G) seventh and H) ninth collection. Megagametophytes: I) translucent white, J) white, K) light yellow, and L) yellow.

From June 24th to July 8th (third to fifth collection) megagametophytes, presumably containing very immature embryos, were observed, without any distinguishable embryos. Observations were made with the use of a zoom stereomicroscope. Beginning with cones collected on July 14th, embryos were dissected and identified as pro-embryos (PE) and were also collected during the following week (July 28th). Round-globular, early cotyledonary and late cotyledonary stages were observed and collected from August 5th, August 12th and August 25th, respectively. Tissue was extracted from the cone samples until the last collection, where the majority of the embryos were at the late cotyledonary stage.

The average of both length and width of the cones increased during cone development (Fig. 5-4A). On the other hand, the average number of seeds per cone decreased after the first collection (Fig. 5-4B) with two low points; one during the collection of June 24th, and the second a month later. Visual assessment methods showed unfilled seeds after the seventh collection, when the pro-embryo stage was observed, and this number increased until the last collection, when embryos were at the late cotyledonary stage (Fig. 5-4B). This is in agreement with previous studies, which showed that *P. taeda* embryo death counts occurred from fertilization onwards, with a linear relationship to time (Williams 2008). The rate of abortion has been attributed to either self-pollination or climate fluctuations which also affect the ovule at different maturations stages of the embryo (Lelu-Walter *et al.* 1999).

5.3.3   Somatic embryo samples

The embryogenic line 637.1 was used for the collection of developmental stages and further gene expression analysis. This line was shown to produce fully mature embryos capable of germination. Four different stages resembling their zygotic counterparts were selected for this study (Fig. 5-2A-D). Briefly, the stages are characterized as follows: 1) Embryogenic suspensor masses, similar to zygotic pro-embryos, which were collected from callus on a maintenance medium and consisting of small cytoplasmic cells and long vacuolated suspensor-like cells; 2) Round/globular stages, which were collected after cultures were transferred to maturation medium, and where the heads of the embryos were opaque and round-shaped; 3) Early cotyledonary embryos which were characterized by the observation of primordial cotyledons below the circumference of a prominent central meristem; and 4) Late cotyledonary embryos were fully mature in appearance with well-formed, elongated cotyledons, but were not dry.
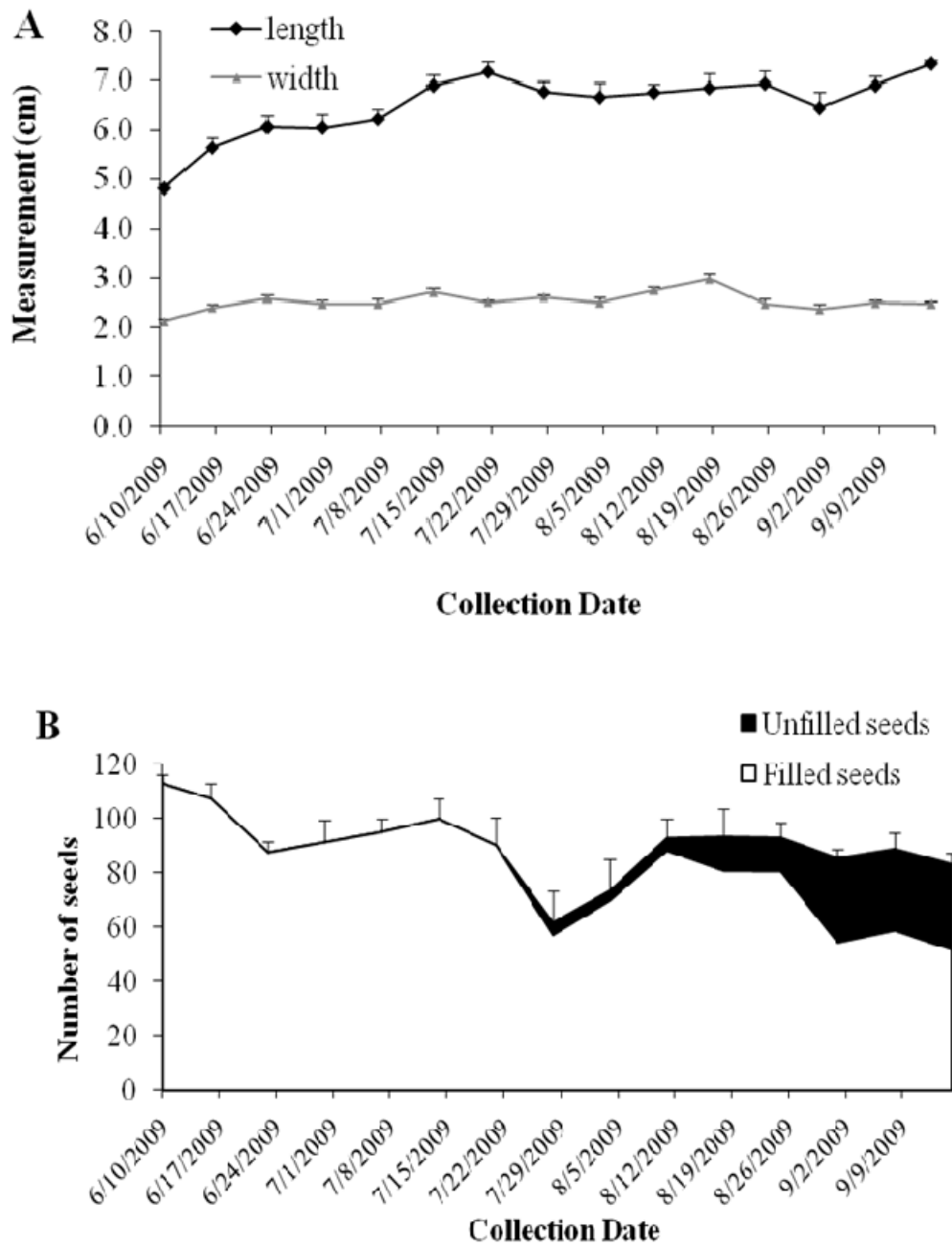
Figure 5-4. *P. taeda* cone collection, Summer 2009: A) Average length and width per cone; B) average number of seeds per cone at different collection dates. Each number represents mean ± SE of 8-10 cones per collection.

5.3.4 RNA amplification from zygotic samples

Due to the small amount of tissue collected from the early stages of zygotic embryogenesis, the amount of RNA was too limiting to proceed through the entire range of experiments. Thus, the samples were subjected to *in vitro* amplification using the T7 RNA Polymerase amplification method (van Gelder *et al.* 1990), which has been shown to amplify starting RNA by up to 200-fold (Spiess *et al.* 2003). After the first amplification (1aRNA), the increase of the RNA concentration was 48-, 17-, 37- and 21-fold for pro-embryo, round/globular, early cotyledonary and late cotyledonary stages, respectively (Table 5-2). Additionally, samples showed a 260/280 ratio close to 2.0. The maximum *in vitro* transcription (IVT) incubation time of 14h was used. This technique has been used for the amplification of RNA from roots for the study of genes regulated by ammonium in *P. pinaster* (Canales *et al.* 2010), for the characterization of xylem of *P. pinaster* (Paiva *et al.* 2008), as well as for the amplification of secondary xylem tissue from *P. taeda* (Wadenback *et al.* 2005). It offers a tool for the amplification of RNA when only small amounts of sample is available, such as samples from the early stages of embryogenesis (Thomas 1993).

Table 5-2. Concentrations of RNA in the samples after the primary *in vitro* transcription amplification (aRNA). Final volume for all the samples was 200 µl. PE= pro-embryo, RG= Round/globular, EC= Early cotyledonary and LC= Late cotyledonary.

Samples	Starting amount for amplification (ng)	Concentration ng/µl	260/280	Total aRNA (ng)	Fold
HeLa (control)[1]	1000	883.52	2.23	176704	176.7
PE	100	24.05	1.51	4810	48.1
RG	784	67.25	2.08	13450	17.2
EC	700	128.73	2.08	25746	36.8
LC	500	52.89	2.20	10578	21.1

[1]According to the kit's manual "the positive control should produce ≥50 µg of aRNA.

5.3.5 Relative gene expression analysis

Real-time PCR was used to investigate the relative transcript abundance levels of six genes of interest for morphologically-similar somatic and zygotic embryo developmental stages of *P. taeda* (Table 5-1). Two endogenous controls were tested for the normalization of the data: *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) and Tubulin. Endogenous controls are usually housekeeping genes involved in basic cellular processes (Jain 2009), and it is assumed that their expression is unaffected by experimental conditions. The most appropriate internal control would be one that has the least variation in its expression under various experimental conditions and in different tissue types (Gonçalves *et al.* 2005a). However, numerous studies have shown that the expression of even the housekeeping genes can vary under given experimental conditions (Gonçalves *et al.* 2005a; Jain 2009). Of the two endogenous controls tested in this study, Pta-UBC1 was more stable across samples within a developmental series compared to Tubulin. The Ct values of Pta-UBC1 were around 12 to 14 across zygotic samples; conversely Tubulin Ct values were 16 to 20 for same samples. During somatic embryogenesis, Pta-UBC1 Ct values were 19 to 21 and for Tubulin 20 to 22. Similar results were obtained for the study of the expression of the WOX2 gene during somatic embryogenesis in *Picea abies,* where of the four endogenous controls tested, the *P. abies* Ubiquitin-conjugating 1 enzyme was the most stable gene across samples (Palovaara and Hakman 2008). Thus, the analysis of the results in the present study was based on the normalization using Ubiquitin-conjugating 1 enzyme as the endogenous control gene.

The induction process is the major difference between zygotic and somatic embryogenesis, where zygotic embryogenesis develops from the fusion between gametes, while somatic embryogenesis is induced through components in the tissue culture medium (von Arnold *et al.* 2002). Hence, the pro-embryo and embryogenic masses were not chosen for the relative comparison baseline. Conversely, from the globular stage onward some differences may still exist between the two systems, but the developmental strategies should be very similar (Yeung 1995). For example, it is documented that somatic embryos under the optimal condition have the ability to synthesize the same storage product as their counterparts, the zygotic embryos, such as in *P. glauca/engelmannii* (Flinn *et al.* 1993), *P. caribaea* (Neutelings *et al.* 1998) and *Picea abies* (Hakman *et al.* 1990). Additionally, "normal" somatic embryos have the ability to germinate and become complete plants, with the formation of both shoot (SAM) and root

(RAM) apical meristems (Yeung *et al.* 1998). At the morphological level, both somatic and zygotic embryos have the capability to form cotyledons (Pullman and Webb 1994).

Due to the similarities described above between the late cotyledonary stage somatic embryos with the late cotyledonary zygotic embryos, the transcript levels were compared relative to this stage for both the somatic and zygotic samples.

5.3.5.1 Expression of genes for storage proteins (Legumin-like and Vicilin-like)

Storage proteins accumulate in the embryos during maturation to provide carbon and nitrogen reserves during germination, until the germinant switches to autotrophic growth. The relative transcript quantification describes the change in expression of the gene of interest relative to a reference gene, this expression could be referring to the amount and time of appearance of the functional gene product (Livak and Schmittgen 2001).

Expression patterns of seed protein genes are highly regulated spatially and temporally during seed development (Dong and Dunstan 2000), because storage protein genes are expressed abundantly during the cell expansion phase which occurs at mid through late embryo development (Yeung 1995). These genes are expressed in a tissue-specific manner, occurring in embryo and endosperm, but never in mature vegetative tissues (Thomas 1993).

The transcript levels for two storage proteins, Legumin-like and Vicilin-like, were studied. The results showed that during both somatic and zygotic embryo development, Legumin-like followed the same pattern of expression, where the relative transcript levels increased during the round-globular stage, decreased somewhat in the early cotyledonary stage and significantly increased at the late cotyledonary stage of development with maximal levels at this stage (Fig. 5-5).

A similar expression pattern was observed for Vicilin-like in zygotic embryo developmental stages; although Vicilin-like transcripts showed a somewhat higher increase of the relative transcript levels in the early cotyledonary stage (Fig. 5-6). In both cases, somatic and zygotic, the maximum relative expression of both storage proteins was in the late cotyledonary stage. Similar results were found in *Picea glauca* where the storage protein Vicilin-like was detected early in somatic embryos with seven days on maturation medium and accumulated gradually during embryo maturation; and that during the late stage, this protein (Vicilin-like) was the

most dominant protein (Lippert *et al.* 2005). Thus, these results showed that the amount of a particular storage product, as well as the timing of its accumulation can differ between somatic and zygotic embryos (Yeung 1995).

Quantification and identification of storage protein has been done in different conifer species in both zygotic and somatic embryos. In *Pseudotsuga menziesii,* a Legumin-like storage protein (pseudotsugin) was observed in mature zygotic embryos (Leal and Misra 1993b). Later, four full-length cDNA clones (PM2S1, PM2S2, PM2S3 and PM2S4) encoding isoforms of 2S seed storage proteins were present in *P. menziesii* somatic embryos [matured on 40 μM ABA, 19 % (w/v) PEG and 6 % (w/v) sucrose] at the same developmental stages as those in the zygotic embryos (Chatthai and Misra 1998).

In somatic embryos of *P. glauca,* the protein pattern was similar to their counterpart zygotic embryos, when somatic embryos were cultured on ABA and high osmoticum (Misra *et al.* 1993). In contrast, in *P. pinaster* the presence of PEG resulted in strong inhibition of further embryo development and affected the accumulation of storage proteins (Tereso *et al.* 2007).

The globulins are the most widely distributed group of storage proteins present in both dicots and monocots, and likely to be a prerequisite for achieving vigorous germination. Based on their sedimentation coefficients, they can be divided into two groups: the 11S Legumin-type globulins and the 7S Vicilin-type globulins, representing the first and second subgroups respectively (Shewry *et al.* 1995).

In gymnosperms, three major types of embryo storage proteins have been identified: 11S Legumin-like in *Pseudotsuga menziesii* (Leal and Misra 1993b), 7S vicilin-like in *P. glauca* (Newton *et al.* 1992), and 2S albumin-like in *P. pinaster* (Allona *et al.* 1994).

The 11S Legumins are the major storage proteins not only in most legumes, but also in brassicas, composits, cucurbits, oats and rice (Shewry *et al.* 1995). In *Arabidopsis*, 2S and 12S proteins are the major seed storage proteins (Pang *et al.* 1988).
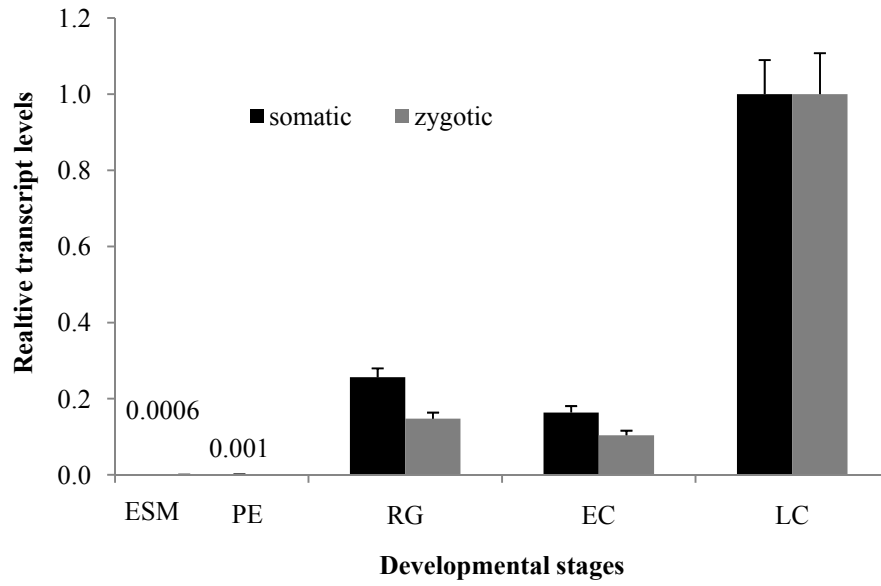
Figure 5-5. Legumin-like transcript levels at four developmental stages of somatic and zygotic embryos using *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.
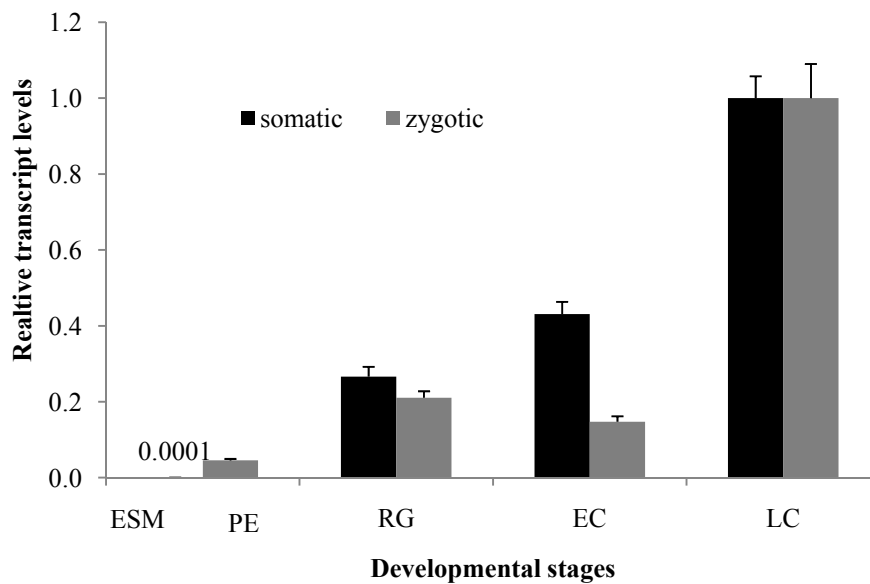
Figure 5-6. Vicilin-like transcript levels at four developmental stages of somatic and zygotic embryos using *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.

146

Profiles of seed storage proteins in conifers have shown similarities to the angiosperm 11S Legumin and 7S Vicilin (Gifford 1988; Leal and Misra 1993b) specifically in their solubility, size and bonding patterns (Newton *et al.* 1992). Additionally, it has been shown that their transcript levels in developing conifer somatic embryos are similar to those found in zygotic embryos, and major accumulation occurred when cotyledons developed in both somatic and zygotic embryos (Flinn *et al.* 1993). In *P. glauca,* storage proteins accumulated gradually and constantly during somatic embryo maturation, while during zygotic embryo maturation the accumulation was more temporally distinct. These differences may be due to the continuous exposure of ABA through the culture medium during somatic embryo maturation (Flinn *et al.* 1991).

In *P. strobus*, somatic embryos accumulated storage proteins in a similar manner to zygotic embryos, showing the highest accumulation at late cotyledonary stage, and that the most abundant were the 11S Legumin and the 7S Vicilin (Klimaszewska et al. 2004). In *P. abies*, storage protein accumulation in the megagametophyte occurred a few weeks earlier than in the zygotic embryo and during organ differentiation. Somatic embryos displayed similar patterns of storage protein accumulation (Hakman 1993). An explanation for the appearance of storage proteins in the megagametophyte before the zygotic embryos could be due to the fact that in conifers, the embryo is fed mainly by the megagametophyte (Minocha *et al.* 1999).

In mature seed of *P. taeda*, the megagametophyte stores 1.9 mg out of the 2.1 mg of total protein, while the embryo contains the remaining 0.2 mg protein (Stone and Gifford 1997). Additionally the major storage proteins in mature zygotic embryos are a 47-kDa globulin and 37.5 and 22.5 kDa glutein-like protein (Groome *et al.* 1991). During somatic embryogenesis, an increase in the above proteins occurred throughout development (Brownfield *et al.* 2007). Globulin production gradually accumulated during embryo development, reaching its maximum at the end of maturation after 10-12 weeks on maturation medium, while glutein-like protein reached its maximum at early stage of maturation after 4-6 weeks on maturation medium (Brownfield *et al.* 2007). By comparing desiccated somatic mature embryos with mature zygotic embryos, it was also observed that somatic embryos produced more protein overall, especially globulin, than zygotic embryos on a dry weight basis (Brownfield *et al.* 2007).

In *Arabidopsis*, during the maturation phase, a rapid increase in seed dry weight was observed with storage proteins accounting for approximately 40 % of dry matter at the end of this stage (Baud *et al.* 2002). However, in some plants, the accumulation of storage proteins is not observed during somatic embryo development, and this feature has been used to distinguish between somatic and zygotic embryos (Stasolla and Yeung 2003). Thus, a high-quality somatic embryo includes the accumulation of storage proteins analogous to those of zygotic embryos. The accumulation of these proteins is essential for normal zygotic embryo development, thus it represent a excellent marker to check the quality and fidelity of somatic embryos (Merkle *et al.* 1995). Differences in storage protein patterns between somatic and zygotic embryos have been detected in *Brassica* where the zygotic embryos contain the 12S storage protein at the cotyledonary stage while in somatic embryos this protein is detected at the globular and heart stages and at a lower level (Crouch 1982). These results indicate that although they may exhibit differences in their synthesis kinetics and accumulation rates, somatic embryos are able to synthesize the specific storage proteins of their zygotic counterparts (Dodeman *et al.* 1997).

ABA has been suggested to play a role in regulating reserve deposition in developing seeds based on the observation in many species that the highest rate of reserve accumulation coincides with a high ABA content (Yang and Zhang 2010). In *P. glauca,* a positive relationship was found between storage accumulation and ABA concentration, where low concentration (10 µM) embryos germinated precociously, while at the higher concentration (40 µM), normal embryo formation was observed containing significant amounts of storage protein (Roberts *et al.* 1990a). Somatic embryos develop in the absence of the megagametophyte tissue. Instead the culture medium must provide the essential elements and regulators to ensure the development of vigorous somatic embryos. In *P. glauca,* a concentration of 40 µM ABA was required to promote storage protein synthesis to similar levels found in their zygotic counterparts (Flinn *et al.* 1991). In addition to ABA, osmotic agents play a role in improving the accumulation of storage proteins in somatic embryos. For example, when somatic embryos of *P. glauca* were cultured on ABA at a high osmotic concentration, the protein patterns were similar to their zygotic embryos counterparts (Misra *et al.* 1993). Thus, ABA and osmoticum aid in the prevention of precocious germination, and also allow the embryo to fully mature by improving the accumulation of storage proteins (Merkle *et al.* 1995). In *P. strobus,* somatic embryos matured on 6 % (w/v) sucrose accumulated more storage protein than those matured

on half of the former concentration. However, a reduction of protein content was found after an extended period of maturation of 12 weeks instead of 9 weeks (Klimaszewska *et al.* 2004).

These observations of somatic embryos show that under optimal culture conditions, storage proteins are localized in the same organs, and exhibit the same molecular weight and biochemical characteristics as those in their zygotic embryo counterparts (Merkle *et al.* 1995).

In conifers, the most dramatic changes in protein accumulation during zygotic embryo development occur between the stage of mid-embryogenesis and the beginning of late embryogenesis, which coincides with an increase in fresh and dry weight (Dong and Dunstan 2000). Protein transcripts are immediately degraded once the zygotic embryos go into desiccation prior to germination; a similar degradation is observed when somatic embryos are partially desiccated (Dong and Dunstan 2000). Moreover, accumulation levels in somatic embryos are strongly influenced by the concentration of ABA, as well as osmotic agents in the medium, which mimic the nutritional and environmental conditions of the megagametophyte.

Here, we found that in *P. taeda,* the relative transcript levels of these two storage proteins (Legumin-like and Vicilin-like) are present in both zygotic and somatic embryo developmental stages, and genes for both followed a similar expression pattern. Thus, the actual maturation conditions used for somatic embryo development were sufficient to stimulate their expression.

5.3.5.2   Late embryogenesis abundant (LEA)

LEA genes, ''late embryogenesis abundant'' proteins, are more abundant during late embryogenesis than during mid-embryogenesis and increases during the desiccation state, in preparation for dry seed dispersal (Galau *et al.* 1986). Studies have found that these genes are expressed at the late cotyledonary stage during zygotic embryogenesis, and they are considered as a marker gene for the quality of the mature somatic embryo before undergoing germination (Zimmerman 1993). LEA protein levels gradually fall several hours after embryos begin to imbibe water (Ingram and Bartels 1996).

During this study, LEA transcript levels were present in the ESM and gradually increased during somatic embryo maturation (Fig. 5-7). In contrast, the relative transcript levels in zygotic embryos appear at the round/globular stage, the levels remained the same during early cotyledonary stage, and then rapidly increased at late cotyledonary stage (Fig. 5-7). However,

while the temporal patterns of transcript accumulation were somewhat different, in both cases the maximal levels of expression were observed in late cotyledonary embryos.

LEA proteins were first described from embryos of mature *Gossypium hirsutum* (cotton) (Dure III *et al.* 1981). Although these genes were first identified from developing seeds, many LEA-like genes are induced only by ABA or environmental stresses (Galau *et al.* 1986). Due to the nature of this work, we will focus only on those LEA genes related to embryogenesis and/or ABA-induction.

During embryogenesis, the accumulation of storage proteins is followed by the accumulation of LEA proteins (Dodeman *et al.* 1997). In carrot, *in situ* localization of the LEA EMB-1 revealed that its expression pattern in somatic embryos was analogous to its expression pattern in zygotic embryos. mRNA begins to accumulate uniformly in both somatic and zygotic globular embryos, which is significantly earlier in development than the time of maximum production of LEA proteins during the late cotyledonary stage (Wurtele *et al.* 1993). However, EMB-1 accumulates to higher levels in zygotic embryos than in somatic embryos, perhaps due to an external signal from either the maternal tissue of the seed or the endosperm, which could be responsible for the enhanced expression levels (Wurtele *et al.* 1993).
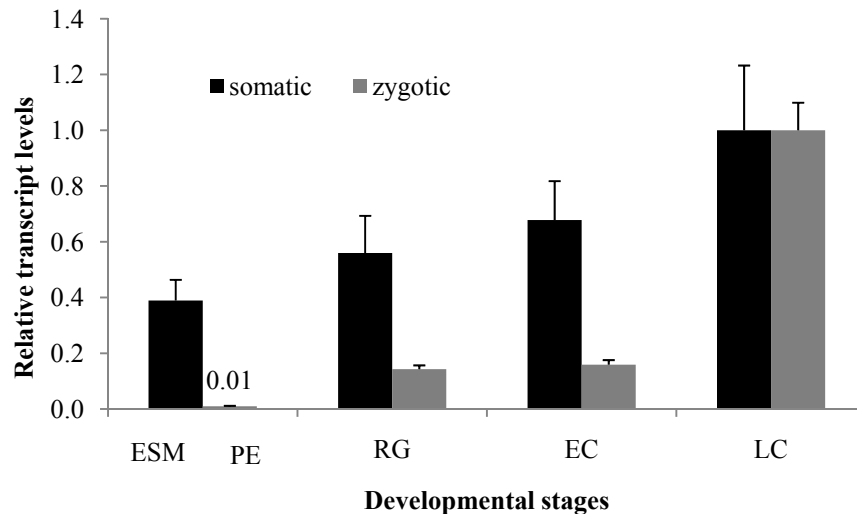
Figure 5-7. LEA transcript levels at four developmental stages of somatic and zygotic embryos using *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.

These EMB-1 LEAs accumulated only in embryos, starting at the early stages of embryo development, remaining at low levels through the globular stages and reaching maximum accumulation as the embryo matured at the torpedo stage (Wurtele *et al.* 1993). Similarly, it was found that LEA B19 mRNA in barley was expressed only in zygotic and somatic embryos, and young seedlings but not in root or leaves, and was induced by ABA and mannitol (Espelund *et al.* 1992). Furthermore, the *Arabidopsis* LEA *AtECP31 (*similar to carrot ECP31) mRNA was expressed only in mature seed, but not in immature siliques, thus suggesting its involvement in maturation and desiccation tolerance of the seed (Yang *et al.* 1996).

Recently, the isolation and characterization of QrEm (a LEA gene in *Quercus robus*) indicated that expression of this gene was first occurring during early cotyledonary stage of zygotic embryos, with gradual accumulation throughout mid-maturation, and a subsequent decline towards the end of seed development (Šunderlíková *et al.* 2009). In somatic embryos, a strong promotion of QrEM transcript accumulation was observed after induction of maturation on 6 % sorbitol (w/v) (no ABA) for 5 weeks, then increased after partial desiccation (somatic embryos at 25 % lost of moisture) and declined when embryos lost more than 30 % of their moisture (Šunderlíková *et al.* 2009).

LEA genes were induced in excised young embryos of cotton cultured on ABA (Galau *et al.* 1986). Additionally, LEA gene expression has been found to be induced in cotton somatic embryos when they are treated with ABA (Ikeda and Kamada 2006). In *P. glauca*, a LEA-like cDNA (PgEMB21) was cloned from somatic embryos and transcript levels accumulated after the somatic embryos were transferred to ABA (Dong and Dunstan 1996). Similar results were found with the clones PgEMB8 and PGEMB7, which were abundant after 14 days following transfer of somatic embryos to maturation. However, transcript levels were not detected in zygotic embryos at any developmental stages, perhaps due to undetectably low transcript levels or somatic embryo gene expression induced by the *in vitro* culture conditions (Dong and Dunstan 1999). In *P. taeda*, the significance of LEA genes during embryo/seed desiccation was illustrated by the identification of a transcript from root tissue under water stress strongly affected by drought (Lorenz *et al.* 2005). The transcript was highly related to the genes EMB32, EMB28 and EMB11 first identified in developing embryos of *P. glauca* (Lorenz *et al.* 2005).

In somatic embryos of *P. glauca*, the transcript levels of many LEA genes increased in the presence of PEG during the late stages of embryo maturation compared with embryos matured without PEG. This may enhance the desiccation tolerance of the embryos, thus improving postembryonic growth (Stasolla *et al.* 2003). Northern analysis of *P. glauca* RNA from developing zygotic embryos using a heterologous probe p8B6 (a desiccation protein from radish), showed that accumulation begun just prior to late cotyledonary phase, reached maximal level in the fully mature embryo and was stable in dry seeds (Leal and Misra 1993a).

Previous reports have identified two peaks of ABA during the embryo development of *P. taeda* (Vales *et al.* 2006). However, depending on the mother tree, the two peaks could be observed during late development zygotic embryos, or one during mid-development, and a second during late development of zygotic embryos of *P. taeda* (Kapik *et al.* 1995). Thus, the ABA peaks in these mother trees did not necessarily occur at the same stage of embryo development. In this present study, we did not monitor the endogenous ABA-levels throughout the collection of different stages, thus this could be targeted in future studies and related to the relative expression of the genes under study.

In this study, the continual exposure of somatic embryos to ABA during maturation most likely accounts for the earlier and more gradual accumulation of LEA transcripts in the somatic embryos compared with the zygotic embryos observed in this study (Fig. 5-7), similar to what was observed for the storage protein genes (Fig. 5-5 and 5-6).

5.3.5.3 Homeodomain protein: Homeodomain-leucine zipper I (HD-Zip I)

Homeobox genes encode transcriptional factors in plants, which are regulatory proteins involved in the activation of other genes controlling tissue patterning (Chugh and Khurana 2002). The homeodomain-leucine zipper I protein (HD-Zip I) has been implicated in ABA responses in plants and stress responses (water and light stress) (Elhiti and Stasolla 2009). In this study, the expression of HD-Zip I in both somatic and zygotic embryo development decreased in the late cotyledonary stage from peak levels in the round/globular stage (somatic embryos) or in pro-embryos (zygotic embryos) (Fig. 5-8). This pattern partially agreed with the relative expression observed in *P. glauca* somatic embryos, where PgHZ1 increased after seven days on maintenance medium, but remained high afterwards during the maturation stage (Tahir *et al.* 2008). Unfortunately, the expression of PgHZ1 during zygotic embryogenesis was not

addressed in that study. In zygotic embryos, the current study observed the highest relative expression of HD-Zip I was in pro-embryos and remained high during the early stages of maturation, finally decreasing at the late cotyledonary stage. In contrast, the lowest level of expression in somatic embryos was in the somatic embryogenic suspensor mass stage, peaking in the round/globular stage and then declining. Hence, there was a higher and more prolonged expression relative to the late cotyledonary stage in the zygotic embryos than there was in the somatic embryos. A phenotypic analysis of plants with higher levels of HD-Zip I gene expression showed that elevated levels could be related to cotyledon formation (Hanson *et al.* 2001) and that the expression was responsive to ABA and water deficit stress (Henriksson *et al.* 2005).

In cotton, the accumulation of GhHB1 mRNAs was significantly increased in roots and seedlings after ABA treatment (Ni *et al.* 2008). Another tomato HD-Zip I gene, VaHox1, is expressed in the phloem during secondary growth and may participate in the regulation of processes specific to secondary phases of phloem development (Tornero *et al.* 1996). In resurrection plants (*Craterostigma plantagineum*) the expression of an HD-Zip I protein (CpHB7- similar to *Arabidopsis* transcription factor ATHB6) was characterized and ectopic expression experiments showed that transgenic plants displayed reduced sensitivity towards ABA during seed germination and stomatal closure (Deng *et al.* 2006). This work, and the work described earlier regarding ABA responses suggests a role for HD-Zip 1 in embryo-associated ABA responses.

The mode of action of HD-Zips is poorly understood, however, these genes appear to be important regulators of plant development and differentiation in response to environmental factors (Deng *et al.* 2006). The described HD-Zip I in *P. glauca* (PgHZI), exhibits similarities with ATHB3 and ATHB23 in a 74 and 61 % of the sequence information, respectively, and higher expression levels of these two genes in *Arabidopsis* yielded plants exhibiting pointed-cotyledon phenotypes, suggesting at least one role in embryo morphogenesis (Tahir *et al.* 2008).
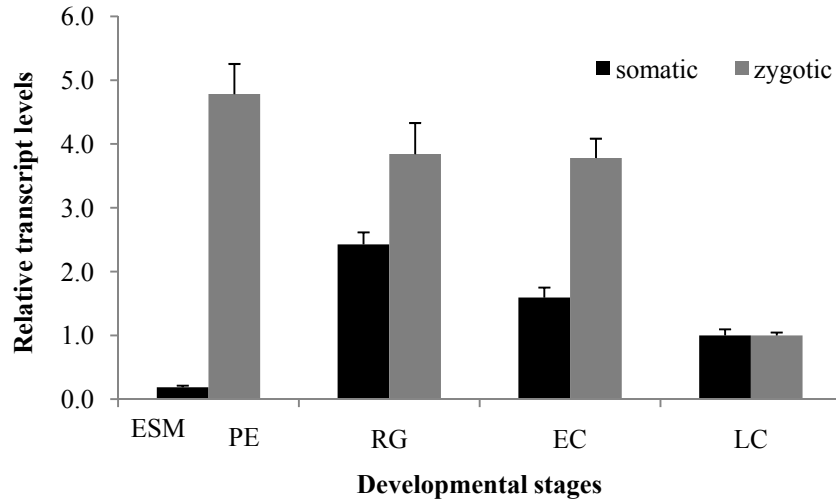
Figure 5-8. HD-Zip I transcript levels at four developmental stages of somatic and zygotic embryos using *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.

Here we found that HD-Zip I was expressed during pine embryogenesis, perhaps increasing the sensitivity to ABA and promoting embryogenesis *in vitro* as previously suggested (Tahir *et al.* 2008). It is interesting that HD-Zip I transcripts increased in somatic embryos upon transfer of ESM tissues to ABA-containing maturation medium. In contrast, during zygotic embryogenesis, the gene was highly expressed at the early pro-embryo stages, and decreased at the end of embryo development. It is possible that the very early expression in pro-embryos may have some relationship to endogenous ABA levels in the seed, but this requires further analysis.

5.3.5.4   26S Proteasome subunit S2 (RPN1)

The 26S proteasome is an ATP-dependent proteolytic system that degrades ubiquitin (Ub) conjugates (Smalle and Vierstra 2004). Inactivation of RPN1 arrests embryogenesis at the globular stage in *Arabidopsis*, suggesting that it is a key gene involved in controlling embryo development and maturation. In this study, both the zygotic and somatic embryo developmental stages exhibited their highest relative expression at the round/globular stage. However, in the somatic embryo system it maintained a high level until it decreased at the late cotyledonary stage (Fig. 5-9). In zygotic embryos, its expression decreased at the early cotyledonary stage and then slightly increased at the late cotyledonary stage.
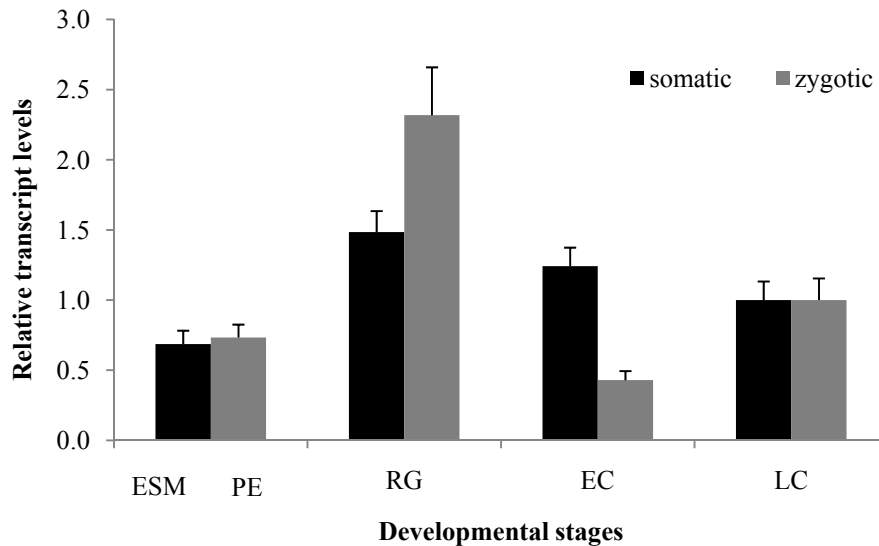
Figure 5-9. 26S Proteasome subunit S2 (RPN1) transcript levels at four developmental stages of somatic and zygotic embryos using *P. taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.

The expression of plant 26S subunit genes is regulated by levels of hormones, which vary during plant development (Santner and Estelle 2010); thus, different embryo developmental stages (including somatic embryos) could require different proteasome activity levels (Stasolla *et al.* 2004). Mutations affecting another proteasome subunit, RPN10, confer hypersensitivity to ABA by selectively stabilizing the ABA-INSENSITIVE5 (ABI5) protein and preventing either their recognition or degradation by the proteasome (Smalle *et al.* 2003) .

Proteasome subunits RPN1 and RPN5 have been shown to be essential for embryogenesis in *Arabidopsis* (Book *et al.* 2009; Brukhin *et al.* 2005). In *Arabidopsis*, two genes encode the RPN1 subunit, RPN1a and RPN1b, both of which are strongly expressed from the pro-embryo to the late globular stage, and then decrease from the heart through the walking stick stages (Brukhin *et al.* 2005). In addition to embryo development, expression of the two RPN1 genes in different tissues of *Arabidopsis* showed that RPN1a was highly expressed in callus, cell suspension, seeds and shoot apices, while RPN1b was expressed in the same tissues with the exception of seeds (Kurepa and Smalle 2008). Disruption of RPN1a causes embryo lethality, characterized by premature division of the hypophyseal cell, abnormalities in the suspensor region, and disturbances in protoderm and procambium cell division. Therefore, RPN1a plays a

role in the control of cell cycle progression and differentiation during embryogenesis (Brukhin *et al.* 2005). In contrast, RPN1b disruption did not show embryo lethally, indicating that even though both genes express functionally equivalent proteins, they do not act redundantly during embryogenesis (Brukhin *et al.* 2005).

Similar to RPN1, RPN5 is encoded by two genes, RPN5a and RPN5b, which play a role during embryogenesis (Book *et al.* 2009). Single *rpn5a* mutants showed abnormal embryogenesis, partially de-etiolated development in the dark, a severely dwarfed phenotype when grown in the light, and infertility (Book *et al.* 2009). Additionally, most of the rpn5a-2, rpn5a-3, and rpn5a-4 embryos, after the late heart-shape stage, exhibited fewer hypocotyls cells and limited cotyledon development. However, in contrast to RPN1, the *rpn5a* phenotypes could be rescued by ectopic expression of either RPN5a or RPN5b, demonstrating that the two isoforms can act redundantly (Book *et al.* 2009).

In both cases, RPN1 and -5 have been related to auxin signaling, because mutant phenotypes resembled known auxin mutants (such as *bdl*, *mp*, *hbt* and *rpn1a*), all of which showed defects in the development of the hypophyseal cell (Brukhin *et al.* 2005). Furthermore, an analysis of gene expression in RPN5a mutants indicated drastically altered expression patterns for PIN4, QC25, and SCR, genes which are also related to auxin (Book *et al.* 2009). However, it is well known that 26S proteasome function is involved in many other cellular processes, including cell cycle progression, stress responses and differentiation which may also be the cause of defects during embryogenesis (Smalle and Vierstra 2004).

In some cases, the inhibition of 26S proteasome activity, such as via RPN10 (Smalle *et al.* 2003) and RPN5 (Book *et al.* 2009), has been shown to increase the level of ubiquitinylated proteins presumably awaiting breakdown (Santner and Estelle 2010).

Our results showed that the expression of the *P. taeda* RPN1 gene plays a role during the round-globular stage of both somatic and zygotic embryos, but its relative expression decreases at the end of the maturation stage, with the transition from round/globular stage to cotyledonary stage. It is possible that the round/globular stage is where the major morphological and physiological changes start, and thus the degradation of proteasome-targeted proteins is increased, with RPN1 participating in the target specificity of them and overall proteasome regulation at this crucial stage (Brukhin *et al.* 2005).

5.3.5.5 Meristem Development (Clavata-like)

The Clavata (CLV) gene is known to regulate the activity of the shoot apical meristem (SAM), which is formed and develops during embryogenesis, and which is required to produce new cells and tissues during post-germination growth (Tahir and Stasolla 2006). The relative expression of Clavata-like in both somatic and zygotic embryos was shown to be high during the round/globular stage in this present study (Fig. 5-10). Furthermore, we found that in zygotic embryos, the level of gene expression relative to the late cotyledonary stage was significantly greater in the round/globular and early cotyledonary stages, while expression in zygotic pro-embryos was low, comparable to the level in late cotyledonary embryos (Fig. 5-10). These results contrasted with the somatic embryos where minimal Clavata-like gene expression was observed in the embryogenic suspensor masses tissue, but transcript levels increased somewhat and remained relatively constant from round/globular through to late cotyledonary stages. Hence, the major increase in Clavata-like transcripts in the mid-developmental stages observed for zygotic embryos was not observed with somatic embryos.
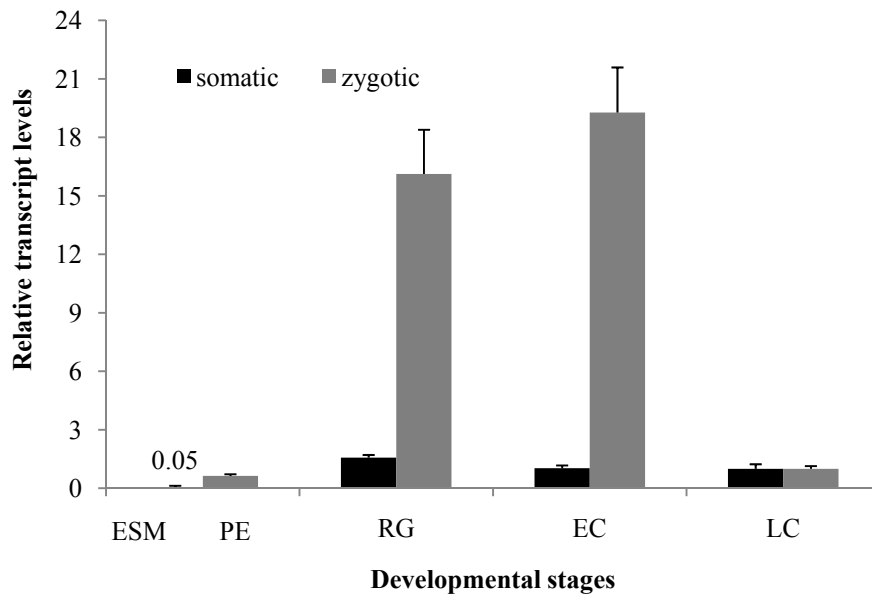
Figure 5-10. Clavata-like transcript levels at four developmental stages of somatic and zygotic embryos using *Pinus taeda* Ubiquitin-conjugating enzyme 1 (Pta-UBC1) as endogenous control. ESM= embryogenic suspensor masses, PE= pro-embryo, RG= round/globular, EC= early cotyledonary and LC= late cotyledonary. Values were normalized to the value of LC set at 1. Each value represents a mean ± SE of three technical replicates.

An anatomical description of SAM formation in *Picea glauca* using zygotic embryos was recorded (Yeung *et al.* 1998) and the formation of the SAM in somatic embryos of *P. glauca* followed the same pattern as their counterpart zygotic embryos (Tahir and Stasolla 2006). The same morphology observations were also found for the formation of the shoot apical meristem during both zygotic and somatic embryogenesis of *P. pinaster* (Tereso *et al.* 2007).

Although the shoot apical meristem initial is present in somatic embryos, it is less defined and is typically smaller than those identified in zygotic embryos (Yeung *et al.* 1998). This has been attributed to the disruption of the somatic embryo SAM by the presence of intercellular air spaces, which suggested that the manipulation of culture conditions are needed to promote normal SAM development (Stasolla and Yeung 2003). Improvement has been achieved through the use of ethylene inhibitors such as silver nitrate ($AgNO_3$), cobalt chloride ($CoCl_2$) and aminoethoxyvinylglycine (AVG), which prevent the accumulation of ethylene in the culture vessels, which is the causative agent of the enlarged intercellular spaces (Kong and Yeung 1994). In addition, in *P. glauca,* modification of the glutathione redox state improves the apical meristem structure of mature embryos. The addition of 0.1 mM reduced glutathione (GSH) to somatic embryo cultures during maturation, and after seven days the subsequent transfer transfer to 0.1 mM oxidized glutathione (GSSG)-containing medium, increased the number of embryos with more than four cotyledons and a higher ability for conversion of embryogenic tissue (Belmonte *et al.* 2005). Further evidence for the impact of medium composition on meristem development and organization was indicated when expression profiles of a *P. abies* gene homologous to the *Arabidopsis* CLV 1 differed between embryos matured on medium with PEG, compared with the non-PEG control medium control (Stasolla *et al.* 2003). These results suggested that addenda to somatic embryo maturation media have the potential to impact on CLV gene expression and resultant meristem development and fidelity.

Development of the SAM in conifer embryos is an early event; however, the molecular mechanism that regulates the SAM *in planta* is unknown (Tahir and Stasolla 2006). Through the use of *Arabidopsis* mutants, our understanding of the formation and maintenance of the SAM has improved (Bowman and Eshed 2000). In *Arabidopsis*, WUSCHEL is the first SAM molecular marker expressed at early stages, and is localized in the four inner apical cell of the 16-cell embryos (Bowman and Eshed 2000). Then, the formation of the stem cells are demarked by the expression of SHOOT MERISTEMLESS and the CLAVATA which occur at

the globular and heart-stages, respectively (Tahir and Stasolla 2006). Since CLAVATA is initiated later, it might be involved in regulating the size of the central zone (Bowman and Eshed 2000); moreover, the *clavata* mutants accumulate excess cells in the central zone (Clark 2001). In conifers, the major genes described above involved in the regulation of the SAM through CLAVATA/WUSCHEL signaling are present in the spruce EST data (Palovaara and Hakman 2008) as well as in the EST collections of *P. taeda* (Cairney *et al.* 2006). They most likely also play a key role in *P. taeda* somatic embryo morphological development. An additional gene WOX (WUS-related homeobox), a transcription factor related to meristem regulation, was identified in *P. abies* somatic embryos and exhibited highest expression in samples after six days on maturation medium, then declined once embryos formed cotyledons. Furthermore, its expression was not detected in non-embryogenic cells (Palovaara and Hakman 2008). While WOX was not assessed in this current study, this gene and the others described above represent additional markers that could be used with CLAVATA to assess the developmental fidelity and meristem quality/organization during *P. taeda* somatic embryo development.

A poor development of the SAM during maturation would lead to a decrease in germination and conversion frequency due to the inability to sustain new growth (Kong and Yeung 1994). Furthermore, disruption of the SAM would prevent the development of fully mature embryos with normal cotyledon formation, and may result in the production of fused or collar-like cotyledons that are often seen in aberrant embryos (Liu *et al.* 1993). As the SAM in somatic embryos arises through the tissue culture process, it is possible that this structure can be disrupted if the culture conditions are not the favorable (Tahir and Stasolla 2006).

Here, we studied one of the genes related to SAM regulation and our results showed that Clavata was expressed during both somatic and zygotic embryo development, thus playing a role during embryogenesis. However, differences were observed in the expression patterns between systems, where more constant relative expression was observed during somatic embryo development stages. In contrast, zygotic embryos exhibited a peak level of expression during the round/globular and early cotyledonary stages. The significance of these differences between zygotic and somatic embryos is unknown, as our somatic embryos exhibited SAM domes and cotyledonary development. However, we did not assess SAM structure microscopically, nor did we assess subsequent germination and growth of the embryos. It is

possible that the differences in relative expression levels observed during somatic embryo development might be reflected in poorer germination. This remains to be determined. It could suggested that an improvement of our current maturation medium would need to be done to obtain a more similar pattern expression in both systems as previously suggested (Stasolla and Yeung 2003).

5.3.6   Comparison between developmental stages of somatic and zygotic embryos

The previously described work assessed the relative transcript level changes within somatic or zygotic embryo systems during development, relative to the level in late cotyledonary embryos. However, these did not provide an assessment of the relative transcript differences between the somatic and zygotic embryos themselves at different stages of development. In order to do this, transcript levels were compared at each developmental stage between zygotic and somatic embryo stages, using levels of the housekeeping gene Tubulin for normalization. Tubulin was chosen for normalization as it exhibited less variation between the somatic and zygotic embryo stages than Pta-UBC1.

While comparing the relative expression levels of the genes in this study between the pro-embryo and the embryogenic suspensor mass, it was noticed that with all of the genes, with the exception of Legumin-like and RPN1, the zygotic pro-embryo had a relatively higher expression levels compared to embryogenic suspensor mass (Fig. 5-11). The expression of Legumin-like and RPN1 in embryogenic suspensor mass was 1.3 and 2.2 higher than in pro-embryo, respectively (Fig. 5-11). Additionally, it was noticed that in both systems, these early stage pro-embryos and embryogenic suspensor masses already expressed many of the genes that would continue throughout embryo development.

For the remaining three developmental stages (round/globular, early cotyledonary and late cotyledonary), the somatic embryo stages showed higher relative expression levels than the zygotic embryo stages, except for the LEA gene (Fig. 5-12 to 5-14).

The expression levels of Vicilin-like, Clavata-like, HD-Zip I, Legumin-like and RPN1 were approximately 5.7, 2.0, 1.4, 30.0 and 12.0 times higher in somatic round/globular stage than in the same zygotic stage (Figure 5-12).
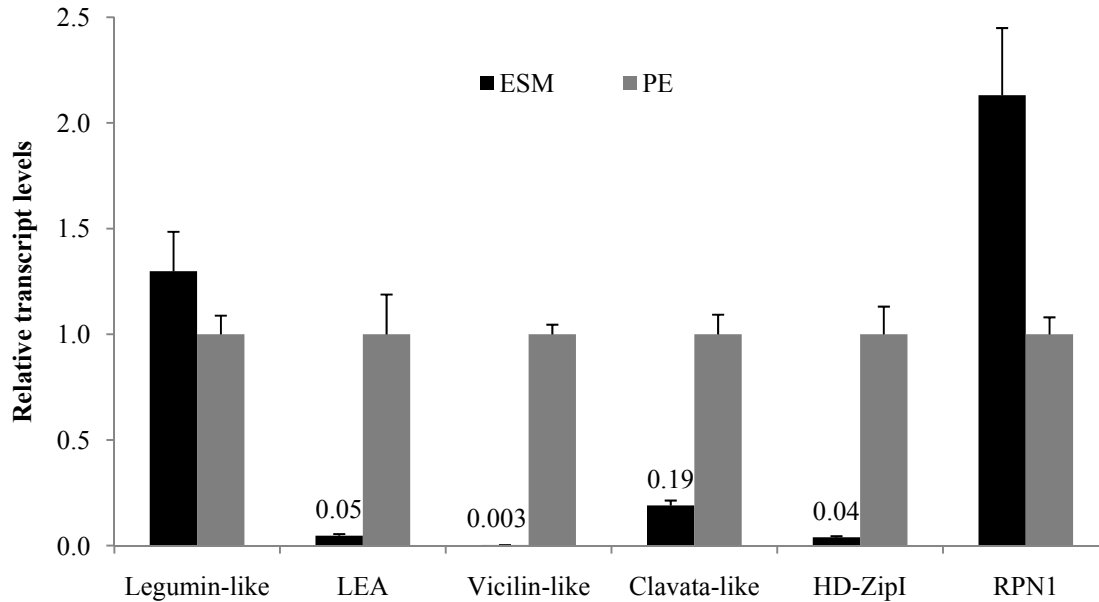
Figure 5-11. Comparison of relative transcript levels between somatic embryogenic suspensor masses (ESM) and zygotic pro-embryos (PE) using Tubulin as endogenous control. Values were normalized to the value of PE set at 1. Each value represents a mean ± SE of three technical replicates.

During the early cotyledonary stage, the levels of Vicilin-like, Clavata-like, HD-Zip I, Legumin-like and RPN1 were approximately 30.0, 2.0, 9.0, 49.0 and 90.0 times higher respectively, in the somatic embryos compared with the zygotic embryos (Fig. 5-13). This trend continued through to the late cotyledonary stage, where the relative transcript levels for Vicilin-like, Clavata-like, HD-Zip I, Legumin-like and RPN1 were 38.0, 55.0, 40.0, 100.0 and 45 times respectively, greater in the somatic embryos (Fig. 5-14).

Thus, for almost all of the genes in the study, the fold-differences in gene expression between somatic and zygotic embryos increased during maturation. For example, for Legumin-like the somatic embryo round/globular transcript level was 30-fold higher compared to the zygotic embryo round/globular, and this fold difference increased to 50- and 100-fold at the early cotyledonary and late cotyledonary stages, respectively. These relatively higher gene expression levels in the somatic embryo stages compared to their counterpart zygotic stages meant that the transcripts could be detected more readily in the somatic embryos compared to the zygotic.

For RNA extraction, the total tissue of the embryogenic suspensor mass was used which characterized by the continued multiplication of cell masses composed of aggregates of small,

rapidly dividing cells and vacuolated non-dividing cells (Palovaara and Hakman 2008). These small, immature embryos highly resemble the early stages of zygotic embryos, designated in this study as pro-embryo (PE). For RNA extraction, the total tissue of the embryogenic suspensor mass was used, in contrast intact pro-embryos were collected with the aid of a dissection microscope. Hence, actual transcript levels in the embryogenic cells of the embryogenic suspensor masses were diluted by the surrounding accessory cells.

For somatic embryos, it seems that the expression of storage protein genes is similar temporally to their zygotic counterparts (Fig. 5-5 and 5-6). Zygotic embryos develop surrounded by the maternal megagametophyte tissue which could be providing the signals for the synthesis of storage protein (Brownfield *et al.* 2007). In contrast, for somatic embryos the stimulus for the synthesis of storage proteins has to be provided by the culture medium (Stasolla *et al.* 2002b); thus, if low gene expression and gene product levels are observed, it is probably due to inappropriate conditions that are required for their synthesis (Flinn *et al.* 1991).

During maturation from the round/globular stage onwards, differences in both somatic and zygotic embryos could be observed, but the basic developmental strategies are likely similar (Yeung 1995). The differences observed in the relative gene expression of LEA in this study could be related to embryogenic environment, such as the constant supply of ABA or osmotic agents during maturation.

While this current study used a small number of genes to assess developmental gene expression fidelity, a larger gene expression analysis study comparing both somatic and zygotic embryos of *P. taeda* using differential display and DNA arrays found: 1) genes that were expressed during the late stage of zygotic embryos but not in the same stage of somatic embryos; 2) genes expressed in early to middle stages of zygotic embryos that were not expressed until the late stages of somatic embryos; and 3) genes expressed in somatic embryos but not expressed in zygotic embryos (Cairney *et al.* 1999).
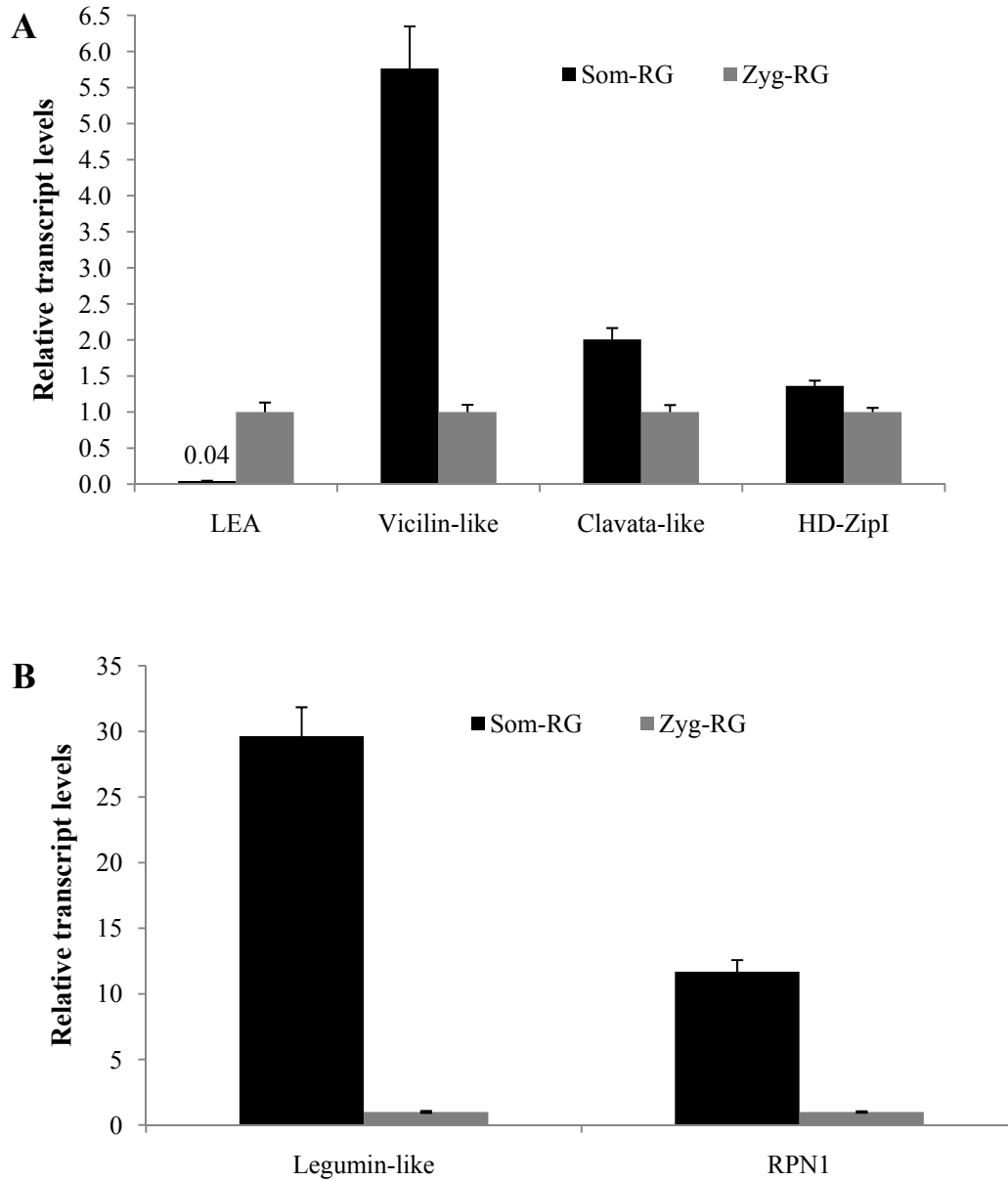
Figure 5-12. Comparison of relative transcript levels between somatic round/globular (Som-RG) and zygotic round/globular (Zyg-RG) stages using Tubulin as endogenous control for A) LEA, Vicilin-like, Clavata-like, HD-Zip I; and B) Legumin-like and RPN1. Values were normalized to the value of Zyg-RG set at 1. Each value represents a mean ± SE of three technical replicates.
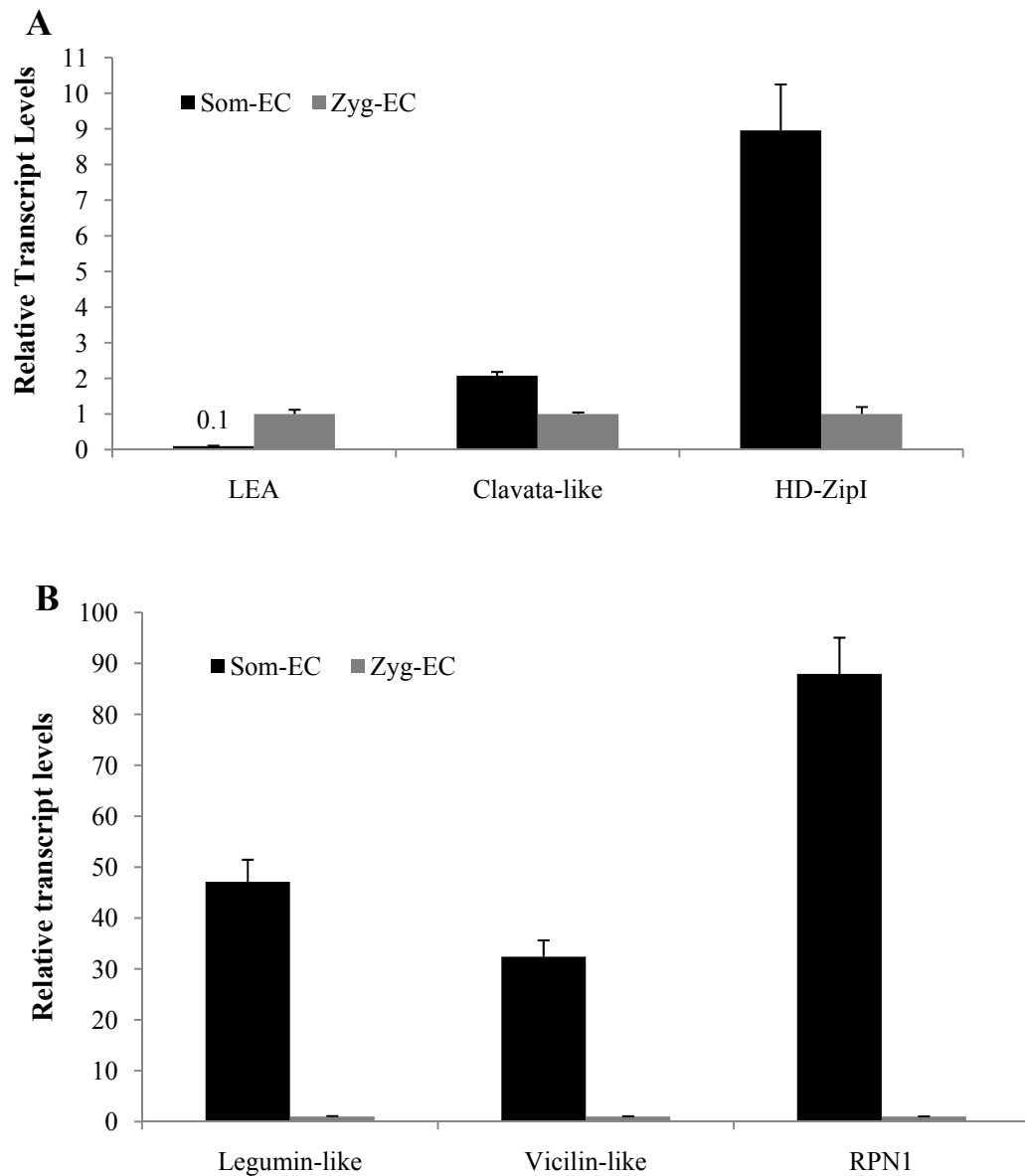
Figure 5-13. Comparison of relative transcript levels between somatic early cotyledonary (Som-EC) and zygotic early cotyledonary (Zyg-EC) embryos using Tubulin as endogenous control for A) LEA, Clavata-like and HD-Zip I; and B) Legumin-like, Vicilin-like and RPN1. Values were normalized to the value of Zyg-EC set at 1. Each value represents a mean ± SE of three technical replicates.

164
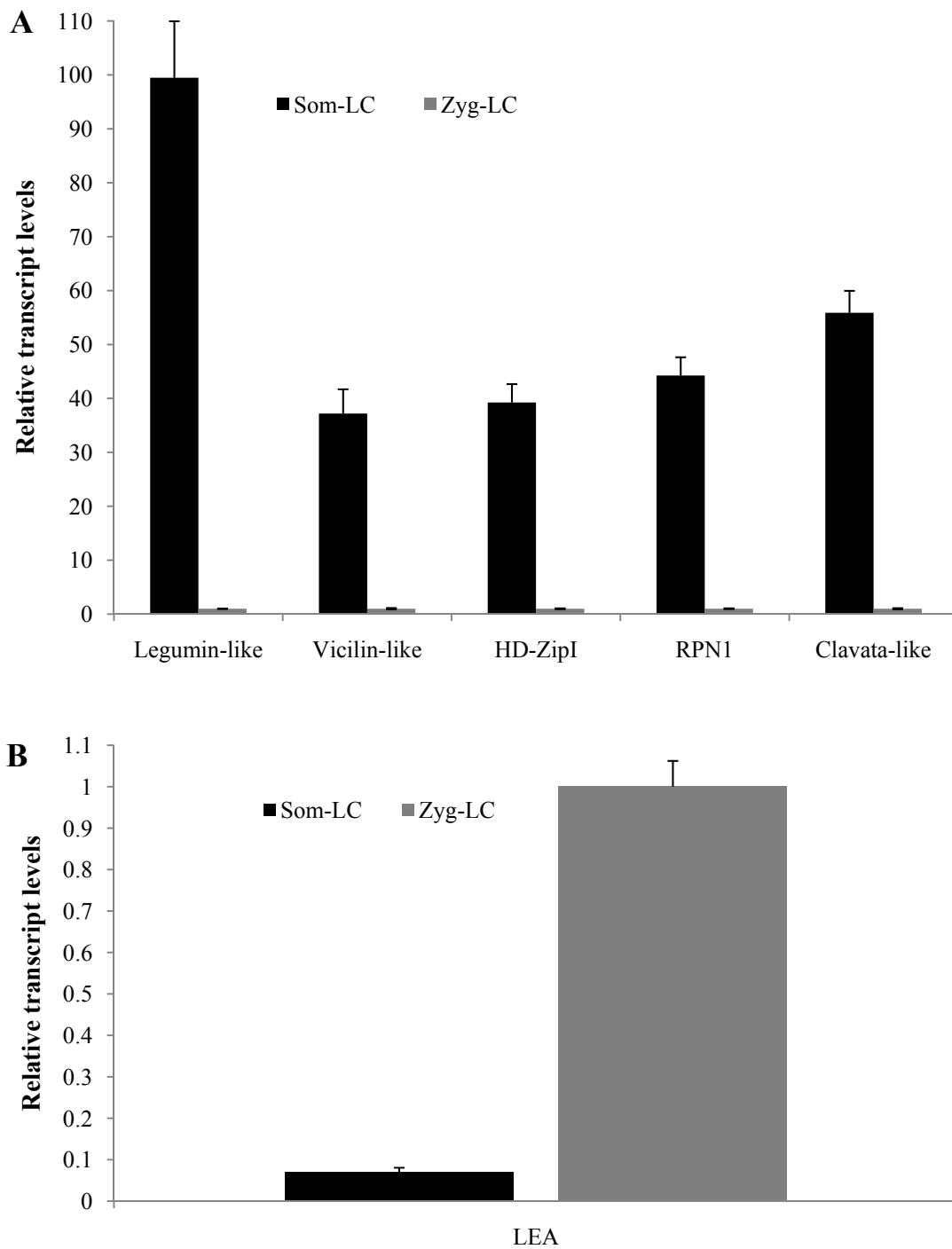
Figure 5-14. Comparison of relative transcript levels between somatic late cotyledonary (Som-LC) and zygotic late cotyledonary (Zyg-LC) in A) Legumin-like, Vicilin-like, HD-Zip I, RPN1 and Clavata-like; and B) LEA. Values were normalized to the value of Zyg-LC set at 1. Each value represents a mean ± SE of three technical replicates.

5.4   Summary

The results from this study are in agreement with results obtained using other method such as microscopy for SAM development, biochemical quantifications and characterizations of storage proteins, mutant analysis (RPN1) and relative expression (HD-Zip I). Namely, that somatic embryos exhibited developmental gene expression profiles generally similar to their zygotic counterparts, although some differences were noted and that our use of real time PCR was reliable.

1) The SAM under *in vitro* conditions could be interrupted by culture conditions such as ethylene formation (Yeung 1995) and hence the differences of the relative transcribed levels between somatic and zygotic embryo maturation observed in this study could be due to current suboptimal culture conditions for its expression and improvements need to be made. 2) The relative transcript levels of storage protein followed the same expression pattern in somatic and zygotic embryo maturation. Additionally, higher transcript levels were observed at early stages in somatic embryos compared with zygotic embryos. If this is translated to gene product accumulation, somatic embryos could accumulate more storage protein because of the lack of the megagametophyte (Brownfield *et al.* 2007). 3) The highest relative transcripts level of the RPN1 was observed at the globular/round stage of somatic and zygotic embryo; the same stage where *Arabidopsis* mutants were arrested during embryogenesis (Brukhin *et al.* 2005). 4) The transcript levels of HD-Zip I during somatic embryo development showed the same pattern as during somatic embryo maturation of *P. glauca* (Tahir et al. 2008). However, comparison with zygotic stages could not be made due to the lack of this information in *P. glauca*.

Additionally, in this study the somatic embryos of *P. taeda* displayed similar gene expression patterns as zygotic embryos for several embryo developmental marker genes (Fig. 5-15). The expression of storage protein genes for Vicilin-like and Legumin-like, and the desiccation-associated, ABA-responsive LEA gene, showed distinct accumulation patterns during embryo development in both somatic and zygotic embryos. In zygotic embryos there was a steep increase in transcript levels between early and late cotyledonary stages in contrast to somatic embryos where these transcripts showed more gradual increases during embryo maturation. Peak levels of expression were still reached in the late cotyledonary stage. These prolonged expression patterns were attributed in part to the continuous supply of ABA in the medium

during the maturation of somatic embryos. Similarly, in zygotic embryos, there is believed to be a steep ABA-peak in the late stages of maturation, which would have coincided with the peak accumulation of the above gene transcripts. In summary, for the above genes known to be induced by ABA, it was observed that the relative transcript levels gradually increased during *in vitro* conditions; meanwhile for *in planta* conditions, the levels were low until the late cotyledonary stage, when the maximum levels of ABA were anticipated to occur (Fig. 5-15).

For the gene encoding the 26S proteasome subunit (RPN1) and the gene encoding Clavata-like, the maximum relative transcript levels were observed at the round/globular stage in both the somatic and zygotic embryos and then decreased at the end of the maturation stage (Fig. 5-15). In contrast to these results, the relative expression pattern of the homeodomain leucine zipper class I (HD-Zip I) gene significantly differed between zygotic pro-embryo and somatic embryogenic suspensor mass samples. In somatic embryos, the expression was higher during the round/globular stage, while in zygotic embryos maximal expression was during the pro-embryo stage. This expression pattern difference may reflect the meristematic composition of the cells comprising the two developmental stages. The zygotic embryo pro-embryo stage contains defined embryonal and suspensor cells. In contrast, the somatic embryo embryogenic suspensor mass tissue contains a large number of aggregates of small, rapidly dividing cells and vacuolated non-dividing cells, which may serve to dilute the HD-Zip 1 transcript levels of the actual somatic pro-embryos.

Our study used a very small set of molecular markers for genes associated with key aspects of the embryo developmental process. However, the development of additional markers that could differentiate the quality of embryos from different cell lines and under different degrees of embryo maturation would facilitate the screening or identification of those somatic embryos that more closely resemble their zygotic counterparts. Theoretically, this could have an increased potential for conversion into plantlets and subsequent survival. Our results suggest that under the current maturation conditions, somatic embryos are temporally similar to the zygotic embryos. However, as we observed some key developmental expression differences between somatic and zygotic embryos for the Clavata-like gene, improvements may need to be made in our culture system towards enhancing the expression of genes related to SAM development. Additionally, the gene expression results could potentially explain the low

germination success during somatic embryogenesis. Furthermore, they point to areas were modifications could be made to *in vitro* protocols using these gene expression markers.

5.5 Acknowledgment
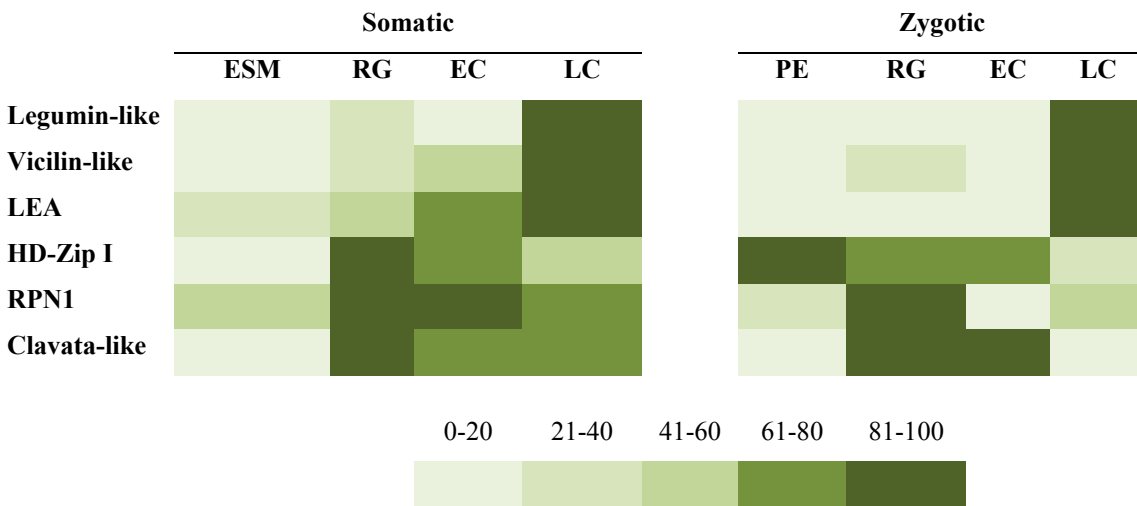
Figure 5-15. Summary of relative expression of different genes during embryo development and maturation in somatic and zygotic embryos. Heat map in green shade coding gives relative values of expression levels (darker color represents higher expression).

6   Appendix

Appendix 2-1. Culture basal media used for initiation of somatic embryogenesis in pines and other conifer species.

Components	MS (Murashige and Skoog 1962)		DCR (Gupta and Durzan 1985),		LP (von Arnold and Eriksson 1981)		MSG (Becwar et al. 1990)	
	mg/L	mM	mg/L	mM	mg/L	mM	mg/L	mM
KNO_3	1900.00	18.79	340.00	3.36	1900.00	18.79	100.00	0.99
$Ca(NO_3)_2 \cdot 4 H_2O$			556.00	2.35		0.00		
$CaCl_2 \cdot 2H_2O$	440.00	2.99	85.00	0.58	1760.00	11.97	440.00	2.99
NH_4NO_3	1650.00	20.61	400.00	5.00	1200.00	14.99	0.00	0.00
KH_2PO_4	170.00	1.25	170.00	1.25	340.00	2.50	170.00	1.25
$Mg(NO_3)_2 \cdot 6H_2O$								
$MgCl_2 \cdot 6H_2O$								
$MgSO_4 \cdot 7H_2O$	370.00	1.50	370.00	1.50	370.00	1.50	370.00	1.50
KCl							745.00	9.99
$FeSO_4 \cdot 7H_2O$	27.80	0.10	27.80	0.10	13.90	0.05	27.80	0.10
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	37.30	0.10	37.30	0.10	18.70	0.05	37.30	0.10
$ZnNa_2$ EDTA					4.05	0.00		
KI	0.83	0.01	0.83	0.01	0.75	0.00	0.83	0.01
H_3BO_3	6.20	0.10	6.20	0.10	63.00	1.02	6.20	0.10
$ZnSO_4 \cdot 7H_2O$	8.60	0.03	8.60	0.03			8.60	0.03
$MnSO4 \cdot H2O$	22.30	0.13	22.30	0.13	2.23	0.01	16.90	0.10
$CuSO_4 \cdot 5H_2O$	0.03	0.00	0.03	0.00	0.00	0.00	0.03	0.00
$CoCl_2 \cdot 6H_2O$	0.03	0.00	0.03	0.00	0.00	0.00	0.03	0.00
$NiCl_2$			0.03	0.00				
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.00	0.25	0.00	0.03	0.00	0.25	0.00
$AgNO_3$								

Appendix 2-1. Continued

Components	1218 (Pullman *et al.* 2005a)			1/2P6 (Teasdale *et al.* 1986)			Litvay (Litvay *et al.* 1981)			BM (Gupta and Pullman 1991)		
	mg/L	mM		mg/L	mM		mg/L	mM		mg/L	mM	
KNO_3	909.90	9.00		909.90	9.00		1900.00	18.79		909.90		9.00
$Ca(NO_3)_2 \cdot 4H_2O$	236.20	1.00		236.20	1.00					236.20		1.00
$CaCl_2 \cdot 2H_2O$							22.00	0.15				
NH_4NO_3	200.00	2.50		603.80	7.54		1650.00	20.61		603.80		7.54
KH_2PO_4	136.10	1.00		136.10	1.00		340.00	2.50		136.10		1.00
$Mg(NO_3)_2 \cdot 6H_2O$	256.50	1.00		256.50	1.00					256.50		1.00
$MgCl_2 \cdot 6H_2O$	101.70	0.50								50.00		0.25
$MgSO_4 \cdot 7H_2O$	246.50	1.00		246.50	1.00		1850.00	7.51		246.50		1.00
KCl												
$FeSO_4 \cdot 7H_2O$	13.90	0.05		6.95	0.02		27.80	0.10		6.95		0.02
$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	18.65	0.05		9.33	0.03		37.30	0.10		9.33		0.03
$ZnNa_2$ EDTA												
KI	4.15	0.03		4.15	0.03		4.15	0.03		4.15		0.03
H_3BO_3	15.50	0.25		15.50	0.25		31.00	0.50		15.50		0.25
$ZnSO_4 \cdot 7H_2O$	14.67	0.05		14.40	0.05		43.00	0.15		14.40		0.05
$MnSO4 \cdot H2O$	10.50	0.06		10.50	0.06		21.00	0.12		10.50		0.06
$CuSO_4 \cdot 5H_2O$	0.17	0.00		0.13	0.00		0.50	0.00		0.13		0.00
$CoCl_2 \cdot 6H_2O$	0.12	0.00		0.13	0.00		0.13	0.00		0.13		0.00
$NiCl_2$												
$Na_2MoO_4 \cdot 2H_2O$	0.13	0.00		0.13	0.00		1.25	0.01		0.13		0.00
$AgNO_3$	3.39	0.02										

7   Literature Cited

AFE-COHDEFOR. (2000a) El sector forestal en Honduras http://www.cohdefor.hn/sector_forestal/. Date accessed 10-10-07.

AFE-COHDEFOR. (2000b) Principales ecosistemas asociados con Honduras http://www.cohdefor.hn/documentos/principales_ecosistemas.shtml. Date accessed 10-10-07.

Afele JC, Senaratna T, McKersie BD, Saxena PK. (1992) Somatic embryogenesis and plant regeneration from zygotic embryo culture in blue spruce (*Picea pungens* Engelman.). Plant Cell Reports 11(5):299-303.

Alberto DM, Elvir JA. (2008) Acumulación y fijación de carbono en biomasa aérea de *Pinus oocarpa* en bosques naturales en Honduras. Investigación Agraria: Sistemas y Recursos Forestales 17(1):67-78.

Alden HA. (1997) Softwoods of North America. In: United States Department of Agriculture, Forest Service.

Allen H, Fox T, Campbell R. (2005) What is ahead for intensive pine plantation silviculture in the south? Southern Journal of Applied Forestry 29(2):62-69.

Allona I, Collada C, Casado R, Aragoncillo C. (1994) 2S arginine-rich proteins from *Pinus pinaster* seeds. Tree Physiology 14(2):211.

Ariel F, Manavella P, Dezar C, Chan R. (2007) The true story of the HD-Zip family. Trends in Plant Science 12(9):419-426.

Aronen T, Pehkonen T, Ryynänen L. (2009) Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. Scandinavian Journal of Forest Research 24(5):372-383.

Arya S, Kalia R, Arya I. (2000) Induction of somatic embryogenesis in *Pinus roxburghii* Sarg. Plant Cell Reports 19(8):775-780.

Attree S, Moore D, Sawhney V, Fowke L. (1991) Enhanced maturation and desiccation tolerance of white spruce [*Picea glauca* (Moench) Voss] somatic embryos: Effects of a non-plasmolysing water stress and abscisic acid. Annals of Botany 68(6):519-525.

Attree S, Fowke L. (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell, Tissue and Organ Culture 35(1):1-35.

Attree S. (2003) Increasing levels of growth regulator and/or water stress during embryo development. Canada. USA patent 6,627,441 B1. Issued September 30 2003

Barrett JD, Park YS, Bonga JM. (1997) The effectiveness of various nitrogen sources in white spruce [*Picea glauca* (Moench) Voss] somatic embryogenesis. Plant Cell Reports 16(6):411-415.

Baud S, Boutin J, Miquel M, Lepiniec L, Rochat C. (2002) An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiology and Biochemistry 40(2):151-160.

Becwar MR, Nagmani R, Wann SR. (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Canadian Journal of Forest Research 20(6):810-817.

Becwar MR, Blush TD, Brown DW, Chesick EE. (1991) Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. Plant Cell, Tissue and Organ Culture 26(1):37-44.

Becwar MR, Chesick EE, Handley III L, Rutter M. (1996) Method for regeneration of coniferous plants by somatic embryogenesis. USA patent No 5413930. Issued October 14 1997.

Belmonte MF, Donald G, Reid DM, Yeung EC, Stasolla C. (2005) Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). Journal of Experimental Botany 56 (419):2355-2364.

Benson E. (2000) Special symposium: *In vitro* plant recalcitrance: An introduction. In Vitro Cellular and Developmental Biology - Plant 36(3):141-148.

Beruto M, Beruto D, Debergh P. (1999) Influence of agar on in vitro cultures: I. Physicochemical properties of agar and agar gelled media. In Vitro Cellular and Developmental Biology - Plant 35(1):86-93.

Bewley J, Black M. (1994) Seeds: physiology of development and germination. Second ed. Springer. New York, USA.445p.

Biddington NL. (1992) The influence of ethylene in plant tissue culture. Plant Growth Regulation 11(2):173-187.

Bonga J, Klimaszewska K, von Aderkas P. (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell, Tissue and Organ Culture 100(3):241-254.

Book A, Smalle J, Lee K, Yang P, Walker J, Casper S, Holmes J, Russo L, Buzzinotti Z, Jenik P. (2009) The RPN5 subunit of the 26S proteasome is essential for gametogenesis, sporophyte development, and complex assembly in *Arabidopsis*. The Plant Cell 21(2):460-478.

Bowman JL, Eshed Y. (2000) Formation and maintenance of the shoot apical meristem. Trends in Plant Science 5(3):110-115.

Bozhkov P, Mikhlina S, Shiryaeva G, Lebedenko L. (1993) Influence of nitrogen balance of culture medium on Norway spruce [*Picea abies* (L.) Karst] somatic polyembryogenesis: high frequency establishment of embryonal-suspensor mass lines from mature zygotic embryos. Journal of Plant Physiology 142(6):735-741.

Bozhkov P, Ahn I, Park Y. (1997) Two alternative pathways of somatic embryo origin from polyembryonic mature stored seeds of *Pinus koraiensis* Sieb et Zucc. Canadian Journal of Botany 75(3):509-512.

Bozhkov P, von Arnold S. (1998) Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiologia Plantarum 104 (2):211-224.

Breton D, Harvengt L, Trontin J, Bouvet A, Favre J. (2005) High subculture frequency, maltose-based and hormone-free medium sustained early development of somatic embryos in maritime pine. In Vitro Cellular and Developmental Biology-Plant 41 (4):494-504.

Breton D, Harvengt L, Trontin J, Bouvet A, Favre J. (2006) Long-term subculture randomly affects morphology and subsequent maturation of early somatic embryos in maritime pine. Plant Cell, Tissue and Organ Culture 87(1):95-108.

Brownfield D, Todd C, Stone S, Deyholos M, Gifford D. (2007) Patterns of storage protein and triacylglycerol accumulation during loblolly pine somatic embryo maturation. Plant Cell, Tissue and Organ Culture 88(2):217-223.

Brukhin V, Gheyselinck J, Gagliardini V, Genschik P, Grossniklaus U. (2005) The RPN1 subunit of the 26S proteasome in *Arabidopsis* is essential for embryogenesis. Plant Cell 17(10):2723-2737.

Burg S. (1973) Ethylene in plant growth. Proceeding of the National Academy Sciences of the United State of America 70(2):591-597.

Cairney J, Xu N, Pullman GS, Ciavatta VT, Johns B. (1999) Natural and somatic embryo development in loblolly pine. Applied Biochemistry and Biotechnology 77(1):5-17.

Cairney J, Zheng L, Cowels A, Hsiao J, Zismann V, Liu J, Ouyang S, Thibaud-Nissen F, Hamilton J, Childs K, Pullman GS, Zhang Y, Oh T, Buell CR. (2006) Expressed sequence tags from loblolly pine embryos reveal similarities with angiosperm embryogenesis. Plant Molecular Biology 62(4-5):485-501.

Canales J, Flores-Monterrosso A, Rueda-López M, Avila C, Cánovas F. (2010) Identification of genes regulated by ammonium availability in the roots of maritime pine trees. Amino Acids- Online.11 p.

Carneros E, Celestino C, Klimaszewska K, Park YS, Toribio M, Bonga JM. (2009) Plant regeneration in Stone pine (*Pinus pinea* L.) by somatic embryogenesis. Plant Cell, Tissue and Organ Culture 98(2):165-178.

Chalupa V. (1985) Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Communicationes Instituti Forestalis Cechosloveniae 14:57-63.

Chatthai M, Misra S. (1998) Sequence and expression of embryogenesis-specific cDNAs encoding 2S seed storage proteins in *Pseudotsuga menziesii* [Mirb.] Franco. Planta 206(1):138-145.

Chavez VM, Litz RE, Norstog K. (1992) Somatic embryogenesis and organogenesis in *Zamia fischeri*, *Z. furfuracea* and *Z. pumila*. Plant Cell, Tissue and Organ Culture 30(2):99-105.

Choudhury H, Kumaria S, Tandon P. (2008) Induction and maturation of somatic embryos from intact megagametophyte explants in Khasi pine (*Pinus kesiya* Royle ex. Gord.). Current Science 95(10):1433-1438.

Chugh A, Khurana P. (2002) Gene expression during somatic embryogenesis-recent advances. Current Science 83(6):715-730.

Ciechanover A, Everett R, Orr A, Preston C, Arnold I, Pfeiffer K, Neupert W, Stuart R, Schägger H, Wouters F. (1998) The ubiquitin–proteasome pathway: on protein death and cell life The EMBO Journal 17:7151-7160.

Clark S. (2001) Cell signalling at the shoot meristem. Nature Reviews Molecular Cell Biology 2(4):276-284.

Coleman MA. (2002) An economic overview of the United State solid woods Industry: USDA Foreign Agricultural Service, Forest and Fishery Products Division.

Crouch M. (1982) Non-zygotic embryos of *Brassica napus* L. contain embryo-specific storage proteins. Planta 156(6):520-524.

Deb CR, Tandon P. (2004) Factors influencing initiation of embryogenic cultures in *Pinus kesiya* Royle ex Gord. Indian Journal of Biotechnology 3(4):589-593.

Deng X, Phillips J, Bräutigam A, Engström P, Johannesson H, Ouwerkerk P, Ruberti I, Salinas J, Vera P, Iannacone R. (2006) A homeodomain leucine zipper gene from Craterostigma plantagineum regulates abscisic acid responsive gene expression and physiological responses. Plant Molecular Biology 61(3):469-489.

Dodeman VL, Ducreux G, Kreis M. (1997) Zygotic embryogenesis versus somatic embryogenesis. Journal of Experimental Botany 48(8):1493-1509.

Dodeman VL, Le Guilloux M, Ducreux G, de Vienne D. (1998) Somatic and zygotic embryos of *Daucus carota* L. display different protein patterns until conversion to plants. Plant Cell Physiology 39(10):1104-1110.

Dong JZ, Dunstan DI. (1996) Expression of abundant mRNAs during somatic embryogenesis of white spruce [*Picea glauca* (Moench) Voss]. Planta 199(3):459-466.

Dong JZ, Perras MR, Abrams SR, Dunstan DI. (1997) Gene expression patterns, and uptake and fate of fed ABA in white spruce somatic embryo tissues. Journal of Experimental Botany 48(2):277.

Dong JZ, Dunstan DI. (1999) Cloning and characterization of six embryogenesis-associated cDNAs from somatic embryos of *Picea glauca* and their comparative expression during zygotic embryogenesis. Plant Molecular Biology 39(4):859-864.

Dong JZ, Dunstan DI. (2000) Molecular biology of somatic embryogenesis in conifers. In: Molecular Biology of Woody Plants. Jain SM and Minocha SC, eds.1:51–87.

Dreher K, Callis J. (2007) Ubiquitin, Hormones and Biotic Stress in Plants. Annals of Botany 99:787–822.

Dronne S, Label P, Lelu M-A. (1997) Desiccation decreases abscisic acid content in hybrid larch (*Larix X leptoeuropaea*) somatic embryos. Physiologia Plantarum 99(3):433-438.

Dure III L, Greenway S, Galau G. (1981) Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. Biochemistry 20(14):4162-4168.

Dure III L, Crouch M, Harada J, Ho T, Mundy J, Quatrano R, Thomas T, Sung Z. (1989) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Molecular Biology 12(5):475-486.

Durzan DJ, Chalupa V. (1968) Free sugars, amino acids, and soluble proteins in the embryo and female gametophyte of jack pine as related to climate at the seed source. Canadian Journal of Botany 46(4):417-428.

Durzan DJ, Gupta PK. (1987) Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. Plant Science (Limerick) 52(3):229-235.

Dvorak WS, Gutierrez EA, Osorio LF, Hodge GR, Brawner JT. (2000) *Pinus oocarpa.* In WS Dvorak, GR Hodge, JL Romero, WC Woodbridge, eds. Conservation and testing of tropical and subtropical forest tree species by the CAMCORE Cooperative", College of Natural Resources, NCSU, Raleigh, NC, USA. p 128-147.

Dvorak WS, Potter K, Hipkins V, Hodge G. (2009) Genetic diversity and gene exchange in Pinus oocarpa, a Mesoamerican pine with resistance to the pitch canker fungus (*Fusarium circinatum*). International Journal of Plant Science 170(5):609-626.

Egertsdotter U, von Arnold S. (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). Physiologia Plantarum 93(2):334-345.

Egertsdotter U. (1996) Regulation of somatic embryo development in Norway spruce (*Picea abies*). In: Forest Genetics Uppsala. Swedish University of Agricultural Science. 60.

El Meskaoui A, Desjardins Y, Tremblay FM. (2000) Kinetics of ethylene biosynthesis and its effects during maturation of white spruce somatic embryos. Physiologia Plantarum 109: 333-342.

El Meskaoui A, Tremblay FM. (2001) Involvement of ethylene in the maturation of black spruce embryogenic cell lines with different maturation capacities. Journal of Experimental Botany 52(357):761-769.

Elhiti M, Stasolla C. (2009) Structure and function of homodomain-leucine zipper (HD-Zip) proteins. Plant Signaling and Behavior 4(2):86-89.

Espelund M, Sæbøe-Larssen S, Hughes D, Galau G, Larsen F, Jakobsen K. (1992) Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. The Plant Journal 2(2):241-252.

FAO. (2003) Forest and the forestry sector. In: http://www.fao.org/forestry/site/23747/en/usa/ Date accessed 10-10-2007.

FAO. (2005) Growing stock statistic USA http://www.fao.org/forestry/site/32183/en/usa Date accessed 07-20-10.

Farjon A, Page CN, Schellevis N. (1993) A preliminary world list of threatened conifer taxa. Biodiversity and Conservation 2(3):304-326.

Feher A, Pasternak T, Dudits D. (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell, Tissue and Organ Culture 74(3):201-228.

Feher A. (2006) Why Somatic Plant Cells Start to form Embryos? In: Somatic Embryogenesis. Mujib A and Samaj J, eds. Springer. 2:85-101.

Fenning T, Gershenzon J. (2002) Where will the wood come from? Plantation forests and the role of biotechnology. Trends in Biotechnology 20(7):291-296.

Fernando D, D. , Lazzaro M, D. , Owens J, N. . (2005) Growth and development of conifer pollen tubes. Sexual Plant Reproduction 18(4):149-162.

Filonova LH, Bozhkov PV, von Arnold S. (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. Journal of Experimental Botany 51(343):249-264.

Find J, Grace L, Krogstrup P. (2002) Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmanns fir (*Abies nordmanniana*). Physiologia Plantarum 116(2):231-237.

Finer JJ, Kriebel HB, Becwar MR. (1989) Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Reports 8(4):203-206.

Flinn BS, Webb DT, Georgis W. (1986) In vitro control of caulogenesis by growth regulators and media components in embryonic explants of eastern white pine (*Pinus strobus*). Canadian Journal of Botany 64(9):1948-1956.

Flinn BS, Roberts DR, Taylor I. (1991) Evaluation of somatic embryos of interior spruce. Characterization and developmental regulation of storage proteins. Physiologia Plantarum 82(4):624-632.

Flinn BS, Roberts DR, Newton CH, Cyr DR, Webster FB, Taylor IEP. (1993) Storage protein gene expression in zygotic and somatic embryos of interior spruce. Physiologia Plantarum 89(4):719-730.

Fowke LC, Atree SM, Binarova P, Galway ME, Wang H. (1995) Conifer somatic embryogenesis for studies of plant cell biology In Vitro Cellular and Developmental Biology 31(1):1–7.

Franco EO, Schwarz OJ. (1985) Micropropagation of two tropical conifers: *Pinus oocarpa* Schiede and *Cupressus lusitanica* Miller. Plenum Press 195-213.

Gaba V. (2005) Plant growth regulators in plant tissue culture and development. In: Plant Development and Biotechnology. Trigiano R and Gray D, eds. Boca Raton: CRC Press. 87-100

Gaj MD. (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulators 43(1):27-47.

Galau G, Hughes D, Dure L. (1986) Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. Plant Molecular Biology 7(3):155-170.

Gamborg OL, Miller RA, Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50(1):151-158.

García-Martín G, Manzanera JA, González-Benito ME. (2005) Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. Plant Cell, Tissue and Organ Culture 80(2):171-177.

Garin E, Bernier-Cardou M, Isabel N, Klimaszewska K, Plourde A. (2000) Effect of sugars, amino acids, and culture technique on maturation of somatic embryos of *Pinus strobus* on medium with two gellan gum concentrations. Plant Cell, Tissue and Organ Culture 62(1):27-37.

Gifford DJ. (1988) An electrophoretics analysis of the seed proteins from *Pinus monticola* and eight other species of pine. Canadian Journal of Botany 66(9):1808-1812.

Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J, Morris P, Bouvier-Durand M, Vartanian N. (1994) Current advances in abscisic acid action and signalling. Plant Molecular Biology 26(5):1557-1577.

Giri CC, Shyamkumar B, Anjaneyulu C. (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees-Structure and Function 18(2):115-135.

Goldberg RB, De Paiva G, Yadegari R. (1994) Plant embryogenesis: zygote to seed. Sciences 266(5185):605-615.

Gonçalves S, Cairney J, Maroco J, Oliveira M, Miguel C. (2005a) Evaluation of control transcripts in real-time RT-PCR expression analysis during maritime pine embryogenesis. Planta 222(3):556-563.

Gonçalves S, Correia PJ, Martins-Loução MA, Romano A. (2005b) A new medium formulation for in vitro rooting of carob tree based on leaf macronutrients concentrations. Biologia Plantarum 49(2):277-280.

Gray DJ. (2005) Propagation from non-meristematic tissues - Non-zygotic embryogenesis. In: Plant development and Biotechnology. Trigiano R, Gray D, eds. Boca Raton: CRC Press. 187-200.

Greaves A. (1982) *Pinus oocarpa*. Forestry Abstracts 43(9):503-532.

Groome MC, Axler SR, Gifford DJ. (1991) Hydrolysis of lipid and protein reserves in loblolly pine seeds in relation to protein electrophoretic patterns following imbibition. Physiologia Plantarum 83(1):99-106.

Gupta PK, Durzan DJ. (1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Reports 4(4):177-179.

Gupta PK, Durzan DJ. (1986) Somatic polyembryogenesis from callus of mature sugar pine embryos. Nature Biotechnology 4(7):643-645.

Gupta PK, Durzan DJ. (1987a) Biotechnology of somatic polyembryogenesis and plantlet regeneration in Loblolly pine. Nature Biotechnology 5(2):147-151.

Gupta PK, Durzan DJ. (1987b) Somatic embryos from protoplasts of Loblolly pine proembryonal cells. Nature Biotechnology 5(7):710-712.

Gupta PK, Pullman G. (1991) Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. USA patent 5036007. Issued August 17 1993.

Gupta PK, Grob JA. (1995) Somatic embryogenesis in conifers. In: Somatic Embryogenesis in Woody Plants. Jain SM, Gupta PK, Newton RJ, eds. Klumer Academic Publishers. 81-98.

Gupta PK, Holmstrom D. (2005) Double staining technology for distinguishing embryogenic Cultures. In: Protocol for Somatic Embryogenesis in Woody Plants. Jain SM and Gupta PK, eds. Springer. 77:573-575.

Häggman H, Vuosku J, Sarjala T, Jokela A, Niemi K. (2006) Somatic embryogenesis of pine species: from functional genomics to plantation forestry. In: Somatic Embryogenesis. Mujib A and Samaj J, eds. Springer. 2:119-140.

Hakman I, Fowke LC, von Arnold S, Eriksson T. (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway Spruce). Plant Science 38(1):53-59.

Hakman I, von Arnold S. (1988) Somatic embryogenesis and plant regeneration from suspension cultures of *Picea glauca* (white spruce). Physiologia Plantarum 72(3):579-587.

Hakman I, Stabel P, Engström P, Eriksson T. (1990) Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). Physiologia Plantarum 80(3):441-445.

Hakman I. (1993) Embryology in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. Physiologia Plantarum 87(2):148-159.

Hall SE, Dvorak WS, Johnston JS, Price HJ, Williams CG. (2000) Flow cytometric analysis of DNA content for tropical and temperate new world pines. Annals of Botany 86(6):1081-1086.

Hanson J, Johannesson H, Engström P. (2001) Sugar-dependent alterations in cotyledon and leaf development in transgenic plants expressing the HDZhdip gene ATHB13. Plant Molecular Biology 45(3):247-262.

Harry IS, Thorpe TA. (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of Red spruce. Botanical Gazette 152(4):446-452.

Henriksson E, Olsson A, Johannesson H, Johansson H, Hanson J, Engstrom P, Soderman E. (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. Plant Physiology 139(1):509-518.

Hosoi Y, Ishii K. (2001) Somatic embryogenesis and plantlet regeneration in *Pinus armandii* var. amamiana. Progress in Biotechnology 18:313-318.

Hristoforoglu K, Schmidt J, Bolhar-Nordenkampf H. (1995) Development and germination of *Abies alba* somatic embryos. Plant Cell, Tissue and Organ Culture 40(3):277-284.

Ikeda M, Satoh S, Kamada H. (2002) Establishment of a reproducible tissue culture system for the induction of *Arabidopsis* somatic embryos. Journal of Experimental Botany 53(374):1575-1580.

Ikeda M, Kamada H. (2006) Comparison of molecular mechanisms of somatic and zygotic embryogenesis. In: Somatic Embryogenesis. Mujib A and Samaj J, eds. Springer. 2:51-68.

Ikeda M, Umehara M, Kamada H. (2006) Embryogenesis-related genes; Its expression and roles during somatic and zygotic embryogenesis in carrot and *Arabidopsis*. Plant Biotechnology 23(2):153-161.

Ingram J, Bartels D. (1996) The molecular basis of dehydration tolerance in plants. Annual Review of Plant Biology 47(1):377-403.

Iraqi D, Tremblay FM. (2001) The role of sucrose during maturation of black spruce (*Picea mariana*) and white spruce (*Picea glauca*) somatic embryos. Physiologia Plantarum 111(3):381-388.

Iwakiri S. (2002) Produção de compensados de *Pinus taeda* L. E *Pinus oocarpa* Schiede com diferentes formulações de adesivo uréia formaldeído: Scielo Brasil. Revista Árvore, Viçosa 8(2):92-97.

Jain M. (2009) Genome-wide identification of novel internal control genes for normalization of gene expression during various stages of development in rice. Plant Science 176 (5):702-706.

Jalonen P, von Arnold S. (1991) Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. Plant Cell Reports 10(8):384-387.

Jimenez V. (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regulators 47(2):91-110.

Jimenez VM, Guevara E, Herrera J, Bangerth F. (2005) Evolution of endogenous hormone concentration in embryogenic cultures of carrot during early expression of somatic embryogenesis. Plant Cell Reports 23(8):567-572.

Jones N, van Staden J. (2001) Improved somatic embryo production from embryogenic tissue of *Pinus patula*. In Vitro Cellular and Developmental Biology - Plant 37(5):543-549.

Kapik R, Dinus R, Dean J. (1995) Abscisic acid and zygotic embryogenesis in *Pinus taeda*. Tree Physiology 15(7-8):485.

Kende H, Zeevaart J. (1997) The Five" Classical" Plant Hormones. Plant Cell 9(7):1197-1210.

King JE, Gifford DJ. (1997) Amino acid utilization in seeds of Loblolly pine during germination and early seedling growth (I. Arginine and Arginase Activity): Plant Physiology 113:1125-1135.

Klimaszewska K, Smith DR. (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiologia Plantarum 100 (4):949-957.

Klimaszewska K, Bernier-Cardou M, Cyr D, Sutton B. (2000) Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cellular and Developmental Biology - Plant 36(4):279-286.

Klimaszewska K, Park Y-S, Overton C, Maceacheron I, Bonga J. (2001) Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cellular and Developmental Biology - Plant 37(3):392-399.

Klimaszewska K, Cyr DR. (2002) Conifer somatic embryogenesis: I. Development. Dendrobiology 48:31-39.

Klimaszewska K, Morency F, Jones-Overton C, Cooke J. (2004) Accumulation pattern and identification of seed storage proteins in zygotic embryos of *Pinus strobus* and in somatic embryos from different maturation treatments. Physiologia Plantarum 121(4):682-690.

Klimaszewska K, Trontin J, Becwar M, Devillard C, Park Y, Lelu-Walter M. (2007) Recent progress on somatic embryogenesis of four *Pinus* spp. Tree and Forestry Science and Biotechnology 1:11-25.

Klimaszewska K, Noceda C, Pelletier G, Label P, Rodriguez R, Lelu-Walter M. (2009) Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). In Vitro Cellular and Developmental Biology - Plant 45(1):20-33.

Kong L, Yeung EC. (1994) Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. Plant Science 104(1):71-80.

Kurepa J, Smalle J. (2008) Structure, function and regulation of plant proteasomes. Biochimie 90(2):324-335.

Kvaalen H. (1994) Ethylene synthesis and growth in embryogenic tissue of Norway spruce: Effects of oxygen, 1-aminocyclopropane-1-carboxylic acid, benzyladenine and 2,4-dichlorophenoxyacetic acid. Physiologia Plantarum 92(1):109-117.

Kvaalen H, Dæhlen OG, Rognstad AT, Grønstad B, Egertsdotter U. (2005) Somatic embryogenesis for plant production of *Abies lasiocarpa*. Canadian Journal of Forest Research 35(5):1053-1060.

Laine E, David H, David A. (1988) Callus formation from cotyledon protoplasts of *Pinus oocarpa* and *Pinus patula*. Physiologia Plantarum 72(2):374-378.

Laine E, David A. (1990) Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea*. Plant Science 69:215-224.

Latkowska M, Kvaalen H, Appelgren M. (2000) Genotype dependent blue and red light inhibition of the proliferation of the embryogenic tissue of Norway spruce. In Vitro Cellular and Developmental Biology - Plant 36(1):57-60.

Leal I, Misra S. (1993a) Developmental gene expression in conifer embryogenesis and germination. III. Analysis of crystalloid protein mRNAs and desiccation protein mRNAs in the developing embryo and megagametophyte of white spruce (*Picea glauca* (Moench) Voss). Plant Science 88(1):25-37.

Leal I, Misra S. (1993b) Molecular cloning and characterization of a legumin-like storage protein cDNA of Douglas fir seeds. Plant Molecular Biology 21(4):709-715.

Lee MS, Kirby EG. (1986) Growth parameters of cell suspension cultures of *Pseudotsuga menziesii* and effects of nitrogen sources on growth. New Zealand Journal of Forestry Science 16(3):369-376.

Lelu-Walter MA, Bastien C, Drugeault A, Gouez ML, Klimaszewska K. (1999) Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. Physiologia Plantarum 105(4):719-728.

Lelu-Walter MA, Bernier-Cardou M, Klimaszewska K. (2006) Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Reports 25(8):767-776.

Lelu-Walter MA, Bernier-Cardou M, Klimaszewska K. (2008) Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell, Tissue and Organ Culture 92(1):31-45.

Lenhard M, Laux T. (1999) Shoot meristem formation and maintenance. Current Opinion in Plant Biology 2(1):44-50.

Leung J, Giraudat J. (1998) Abscisic acid signal transduction. Annual Review of Plant Physiology and Plant Molecular Biology 49(1):199-222.

Li X, Huang F, Gbur Jr E. (1998a) Effect of basal medium, growth regulators and Phytagel concentration on initiation of embryogenic cultures from immature zygotic embryos of loblolly pine (*Pinus taeda* L.). Plant Cell Reports 17(4):298-301.

Li X, Huang F, Murphy J, Gbur E. (1998b) Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine (*Pinus taeda* L.). In Vitro Cellular and Developmental Biology-Plant 34(1):22-26.

Liao YK, Amerson HV. (1995) Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis II. Maturation of somatic embryos and plant regeneration. New Forests 10(2):165-182.

Lindemann EGP, Guwckel JE, Davidson OW. (1970) Meristem Culture of Cattleya. American Orchid Society Bulletin 39:1002-1004.

Lippert D, Zhuang J, Ralph S, Ellis DE, Gilbert M, Olafson R, Ritland K, Ellis B, Douglas CJ, Bohlmann J. (2005) Proteome analysis of early somatic embryogenesis in *Picea glauca*. Proteomics 5(2):461-473.

Litvay JD, Johnson MA, Verma D, Einspahr D, Weyrauch K. (1981) Conifer suspension culture medium development using analytical data from developing seeds. The Institute of Paper Chemistry . Techinical Paper Series 115:1-7.

Liu C, Xu Z, Chua N. (1993) Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. The Plant Cell 5(6):621-630.

Livak K, Schmittgen T. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods 25(4):402-408.

Lorenz W, Sun F, Liang C, Kolychev D, Wang H, Zhao X, Cordonnier-Pratt M, Pratt L, Dean J. (2005) Water stress-responsive genes in loblolly pine (*Pinus taeda*) roots identified by analyses of expressed sequence tag libraries. Tree Physiology 26(1):1-16.

Lu CYI, Thorpe TA. (1987) Somatic embryogenesis and plantlet regeneration in cultured immature embryos of *Picea glauca*. Journal of Plant Physiology 128(3):297-302.

MacKay J, Becwar M, Park Y, Corderro J, Pullman G. (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genetics and Genomes 2(1):1-9.

Malabadi RB, Choudhury H, Tandon P. (2004) Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. Scientia Horticulturae 102(4):449-459.

Malabadi RB, Gangadhar SM, Nataraja K. (2005) Plant regeneration via somatic embryogenesis in *Pinus kesiya* (Rolye ex. Gord.) influenced by triacontanol. Acta Physiologiae Plantarum 27(4):531-537.

Malabadi RB, Van Staden J. (2005a) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. In Vitro Cellular and Developmental Biology - Plant 41(2):181-186.

Malabadi RB, Van Staden J. (2005b) Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. Tree Physiology 25(1):11-16.

Malabadi RB, Nataraja K. (2007a) 24-epiBrassinolide Induces Somatic Embryogenesis in *Pinus wallichiana* A. B. Jacks. Journal of Plant Sciences 2(2):171-178.

Malabadi RB, Nataraja K. (2007b) Plant regeneration via somatic embryogenesis using secondary needles of mature trees of *Pinus roxburghii* sarg. International Journal of Botany 3:40-47.

Malabadi RB, Nataraja K. (2007c) Putrescine influences somatic embryogenesis and plant regeneration in *Pinus gerardiana* Wall. American Journal of Plant Physiology 2(2):107-114.

Malabadi RB, Nataraja K. (2007d) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* AB Jacks. Journal of Plant Sciences 2(1):45-53.

Maruyama E, Tanaka T, Hosoi Y, Ishii K, Morohoshi N. (2001) Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese cedar (*Cryptomeria japonica* D. Don). Plant Biotechnology -Tokyo 17(4):281-296.

Maruyama E, Hosoi Y, Ishii K. (2005a) Propagation of Japanese red pine (*Pinus densiflora* Zieb. et Zucc.) via somatic embryogenesis. Propagation of Ornamental Plants 5(4):199-204.

Maruyama E, Hosoi Y, Ishii K. (2005b) Somatic embryo production and plant regeneration of Japanese black pine (*Pinus thunbergii*). Journal of Forest Research 10(5):403-407.

Maruyama E, Hosoi Y, Ishii K. (2007) Somatic embryogenesis and plant regeneration in yakutanegoyou, *Pinus armandii* Franch. var. amamiana (Koidz.) Hatusima, an endemic and endangered species in Japan. In Vitro Cellular and Developmental Biology - Plant 43(1):28-34.

Mathieu M, Lelu-Walter MA, Blervacq AS, David H, Hawkins S, Neutelings G. (2006) Germin-like genes are expressed during somatic embryogenesis and early development of conifers. Plant Molecular Biology 61(4-5):615-627.

Mathur G, von Arnold S, Nadgauda R. (2000) Studies on somatic embryogenesis from immature zygotic embryos of chir pine (*Pinus roxburghii* Sarg.). Current Science 79:999-1004.

McKeand S, Mullin T, Byram T, White T. (2003) Deployment of genetically improved loblolly and slash pines in the south. Journal of Forestry 101(3):32-37.

Meinke DW. (1991) Embryonic mutants of *Arabidopsis thaliana*. Developmental Genetics 12(6):382-392.

Merkle SA, Parrott WA, Flinn BS. (1995) Morphogenic aspects of somatic embryogenesis. In: In vitro embryogenesis in plants. Dordrecht: Kluwer Academic Publishers. 155-203.

Merkle SA, Dean JFD. (2000) Forest tree biotechnology. Current Opinion Biotechnology 11(3):298-302.

Merkle SA, Montello P, Xia X, Upchurch B, Smith D. (2005) Light quality treatments enhance somatic seedling production in three southern pine species. Tree Physiology 26(2):187-194.

Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC. (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiologia Plantarum 105(1):155-164.

Misra S, Attree S, Leal I, Fowke L. (1993) Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Annals of Botany 71(1):11-22.

Mitchell R, Hiers J, O'Brien J, Jack S, Engstrom R. (2006) Silviculture that sustains: the nexus between silviculture, frequent prescribed fire, and conservation of biodiversity in longleaf pine forests of the southeastern United States. Canadian Journal of Forest Research 36 (11):2724-2736.

Monteiro A, Higashi E, Gonçalves A, Rodriguez A. (2000) A novel approach for the definition of the inorganic medium components for micropropagation of yellow passionfruit (*Passiflora edulis* sims. F. Flavicarpa Deg.). In Vitro Cellular and Developmental Biology - Plant 36(6):527-531.

Murashige T, Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15(3):473-497.

Nagmani R, Bonga JM. (1985) Embryogenesis in subcultured callus of *Larix decidua*. Canadian Journal of Forest Research 15:1088-1091.

Nagmani R, Diner A, Sharma G. (1993) Somatic embryogenesis in longleaf pine (*Pinus palustris*). Canadian Journal of Forest Research 23(5):873-876.

Nas MN, Read PE. (2004) A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. Scientia Horticulturae 101(1/2):189-200.

Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang S, Wilde HD, Kodrzycki RJ, Zhang C, Gause KC. (2005) Invited Review: forest biotechnology: innovative methods, emerging opportunities. In Vitro Cellular and Developmental Biology-Plant 41(6):701-717.

Neutelings G, Domon JM, Membre N, Bernier F, Meyer Y, David A, David H. (1998) Characterization of a germin-like protein gene expressed in somatic and zygotic embryos of pine (Pinus caribaea Morelet). Plant Molecular Biology 38(6):1179-1190.

Newton CH, Flinn BS, Sutton BCS. (1992) Vicilin-like seed storage proteins in the gymnosperm interior spruce (*Picea glauca/engelmanii*). Plant Molecular Biology 20(2):315-322.

Newton R, Tang W, Jain S. (2005) Slash Pine (*Pinus elliottii* Engelm.). In: Protocol for Somatic Embryogenesis in Woody Plants. Jain SM and Gupta PK, eds. Springer. 77:1-10.

Ni Y, Wang X, Li D, Wu Y, Xu W, Li X. (2008) Novel cotton homeobox gene and its expression profiling in root development and in response to stresses and phytohormones. Acta Biochimica et Biophysica Sinica 40(1):78-84.

Niedz R, Evens T. (2007) Regulating plant tissue growth by mineral nutrition. In Vitro Cellular and Developmental Biology - Plant 43(4):370-381.

Niemi K, Häggman H. (2002) Pisolithus tinctorius promotes germination and forms mycorrhizal structures in Scots pine somatic embryos in vitro. Mycorrhiza 12(5):263-267.

Nitsch JP, Nitsch C. (1969) Haploid Plants from Pollen Grains. Science. 163 (3862):85.

Noceda C, Salaj T, Perez M, Viejo M, Canial MJ, Salaj J, Rodriguez R. (2009) DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. Trees: Structure and Function 23(6):1285-1293.

Norgaard JV, Krogstrup P. (1991) Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. Plant Cell Reports 9(9):509-513.

Norgaard JV. (1997) Somatic embryo maturation and plant regeneration in *Abies nordmanniana* Lk. Plant Science 124(2):211-221.

Outcalt K, Sheffield R. (1996) The longleaf pine forest: trends and current conditions. Resources Bulletin. United State Department of Agriculture, Forest Service, Southern Research Station. North Carolina.28 p.

Paiva JAP, Garcés M, Alves A, Garnier-Géré P, Rodrigues JC, Lalanne C, Porcon S, Le Provost G, da Silva Perez D, Brach J. (2008) Molecular and phenotypic profiling from the base to the crown in maritime pine wood-forming tissue. New Phytologist 178(2):283-301.

Pallardy S. (2008a) Reproductive growth. In: Physiology of Woody Plants. Third Edition. San Diego: Academic Press. 87-106.

Pallardy S. (2008b) Plant hormones and other endogenous growth regulators. In: Physiology of Woody Plants. Third Edition. San Diego: Academic Press. 454.

Palovaara J, Hakman I. (2008) Conifer WOX-related homeodomain transcription factors, developmental consideration and expression dynamic of WOX2 during *Picea abies* somatic embryogenesis. Plant Molecular Biology 66(5):533-549.

Pang P, Pruitt R, Meyerowitz E. (1988) Molecular cloning, genomic organization, expression and evolution of 12S seed storage protein genes of *Arabidopsis thaliana*. Plant Molecular Biology 11(6):805-820.

Park YS. (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Annals of Forest Sciences 59:651-656.

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM. (2006) Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell, Tissue and Organ Culture 86(1):87-101.

Pilon-Smits E, Quinn C, Tapken W, Malagoli M, Schiavon M. (2009) Physiological functions of beneficial elements. Current Opinion in Plant Biology. 12 (3):267-274.

Pinto G, Santos C, Neves L, Araújo C. (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Reports 21(3):208-213.

Pradeep KA, Parinita A, Jan BMC, Chun-ming L, Bhojwani SS. (2006) PCIB an antiauxin enhances microspore embryogenesis in microspore culture of *Brassica juncea*. Plant Cell, Tissue and Organ Culture 86(2):201-210.

Pullman G, Webb D. (1994) An embryo staging system for comparison of zygotic and somatic embryo development. In Proceedings of the TAPPI R and D Division Biological Sciences Symposium, October 3-6, Minneapolis, MN. Technical Association of the Pulp and Paper Industry Press, Atlanta, GA, pp 31-34.

Pullman GS, Johnson S. (2002) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation rates. Proceedings of the Wood 59:663-668.

Pullman GS, Zhang Y, Phan BH. (2003a) Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Reports 22(2):96-104.

Pullman GS, Johnson S, Peter G, Cairney J, Xu N. (2003b) Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. Plant Cell Reports 21(8):747-758.

Pullman GS, Montello P, Cairney J, Xu N, Feng X. (2003c) Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: maturation improvements by metal analyses of zygotic and somatic embryos. Plant Science 164(6):955-969.

Pullman GS, Buchanan M. (2003) Loblolly pine (*Pinus taeda* L.): stage-specific elemental analyses of zygotic embryo and female gametophyte tissue. Plant Science 164(6):943-954.

Pullman GS, Namjoshi K, Zhang Y. (2003d) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation with abscisic acid and silver nitrate. Plant Cell Reports 22(2):85-95.

Pullman GS, Mein J, Johnson S, Zhang Y. (2005a) Gibberellin inhibitors improve embryogenic tissue initiation in conifers. Plant Cell Reports 23(9):596-605.

Pullman GS, Johnson S, Tassel SV, Zhang Y. (2005b) Somatic embryogenesis in loblolly pine (*Pinus taeda*) and Douglas fir (*Pseudotsuga menziesii*): improving culture initiation and growth with MES pH buffer, biotin, and folic acid. Plant Cell, Tissue and Organ Culture 80(1):91-103.

Pullman GS, Chopra R, Chase K-M. (2006) Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: Improvements in embryogenic tissue initiation by supplementation of medium with organic acids, Vitamins B12 and E. Plant Science 170(3):648-658.

Pullman GS, Skryabina A. (2007) Liquid medium and liquid overlays improve embryogenic tissue initiation in conifers. Plant Cell Reports 26(7):873-887.

Pullman GS, Johnson S. (2009a) Loblolly pine (*Pinus taeda*) female gametophyte and embryo pH changes during seed development. Tree Physiology 29(6):829-836.

Pullman GS, Johnson S. (2009b) Osmotic measurements in whole megagametophytes and embryos of loblolly pine (*Pinus taeda*) during seed development. Tree Physiology 29(6):819-827.

Quiroz-Figueroa F, R., Rafael R-H, Rosa MG-A, Víctor ML-V. (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell, Tissue and Organ Culture 86(3):285-301.

Raghavan V, Sharma KK. (1995) Zygotic embryogenesis in gymnosperms and angiosperms. In: In: In Vitro Embryogenesis in Plants. Current Plant Science and Biotechnology in Agriculture. Thorpe TA, ed. Kluwer Academic Publisher. 20:73–115.

Ratnaparkhe S, Egertsdotter E, Flinn B. (2009) Identification and characterization of a matrix metalloproteinase (Pta1-MMP) expressed during Loblolly pine (*Pinus taeda*) seed development, germination completion, and early seedling establishment. Planta 230(2):339-354.

Rayle D, Cleland R. (1992) The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiology 99(4):1271-1274.

Reid DA, Lott JNA, Attree SM, Fowke LC. (1999) Mineral nutrition in white spruce (*Picea glauca* [Moench] Voss) seeds and somatic embryos. I. phosphorus, phytic acid, potassium, magnesium, calcium, iron and zinc. Plant Science 141:11-18.

Roberts DR, Flinn BS, Webb DT, Webster FB, Sutton BCS. (1990a) Abscisic acid and indole-3-butyric acid regulation of maturation and accumulation of storage proteins in somatic embryos of interior spruce. Physiologia Plantarum 78(3):355-360.

Roberts DR, Sutton BCS, Flinn BS. (1990b) Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. Canadian Journal of Botany 68(5):1086-1090.

Roberts DR, Lazaroff WR, Webster FB. (1991) Interaction between maturation and high relative humidity treatments and their effects on germination of Sitka spruce somatic embryos. Journal of Plant Physiology 138:1-6.

Roberts DR, Webster FB, Flinn BS, Lazaroff WR, Cyr DR. (1993) Somatic embryogenesis of spruce. In Synseeds: Applications of Synthetic Seeds to Crop Improvements. Redenbaugh K, eds. CRC Press, Boca Raton. 427-450.

Sáenz-Romero C, Snively AE, Lindig-Cisneros R. (2003) Conservation and Restoration of Pine Forest Genetic Resources in Mexico. Silvae Genetica 52(5-6):233-236.

Sáenz-Romero C, Guzmán-Reyna RR, Rehfeldt GE. (2006) Altitudinal genetic variation among Pinus oocarpa populations in Michoacán, Mexico: Implications for seed zoning, conservation, tree breeding and global warming. Forest Ecology and Management 229(1-3):340-350.

Salaj T, Matusova R, Salaj J. (2004) The effect of carbohydrates and polyethylene glycol on somatic embryo maturation in hybrid fir *Abies alba× Abies numidica*. Acta Biologica Cracoviensia Series Botanica 46:159-167.

Salajova T, Salaj J, Kormutak A. (1999) Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. Plant Science 145(1):33-40.

Salajova T, Salaj J. (2005) Somatic embryogenesis in *Pinus nigra*: embryogenic tissue initiation, maturation and regeneration ability of established cell lines. Biologia Plantarum 49(3):333-339.

Santner A, Estelle M. (2010) The ubiquitin-proteasome system regulates plant hormone signaling. The Plant Journal 61(6):1029-1040.

Schenk RU, Hildebrandt AC. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany 50:199-204.

Schmidtling R, Hipkins V. (1998) Genetic diversity in longleaf pine (*Pinus palustris*): influence of historical and prehistorical events. Canadian Journal of Forest Research 28(8):1135-1145.

Schoof H, Lenhard M, Haecker A, Mayer K, Jürgens G, Laux T. (2000) The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100(6):635-644.

Schuller A, Kirchner-Neß R, Reuther G. (2000) Interaction of plant growth regulators and organic C and N components in the formation and maturation of *Abies alba* somatic embryos. Plant Cell, Tissue Organ and Culture 60(1):23-31.

Schultz R. (1997) Loblolly pine: the ecology and culture of loblolly pine (*Pinus taeda* L.). Agriculture Handbook 713, Forest Service, USDA, Washington, DC.

Schwarz O, Beaty R, Franco E. (1991) Egg-cone pine (*Pinus oocarpa* Schiede). Biotechnology in agriculture and forestry 16:305-316.

Sedjo RA. (2004) Biotechnology and the Global Wood Supply. The Bioengineered Forest: Challenges for Science and Society.

Serrano R, Montesinos C. (2003) Molecular bases of desiccation tolerance in plant cells and potential applications in food dehydration. Food Science and Technology International 9(3):157-161.

National Weather Service Climate. (2010) Preliminary Monthly Climate Data http://www.weather.gov/climate/index.php?wfo=lwx (date accessed 7/05/2010).

Shewry P, Napier J, Tatham A. (1995) Seed storage proteins: structures and biosynthesis. The Plant Cell Online 7(7):945-956.

Shiota H, Satoh R, Watabe K, Harada H, Kamada H. (1998) C-ABI3, the carrot homologue of the *Arabidopsis* ABI3, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. Plant Cell Physiology 39(11):1184-1193.

Smalle J, Kurepa J, Yang P, Babiychuk E, Kushnir S, Durski A, Vierstra R. (2002) Cytokinin growth responses in *Arabidopsis* involve the 26S proteasome subunit RPN12. The Plant Cell 14(1):17-32.

Smalle J, Kurepa J, Yang P, Emborg T, Babiychuk E, Kushnir S, Vierstra R. (2003) The pleiotropic role of the 26S proteasome subunit RPN10 in *Arabidopsis* growth and development supports a substrate-specific function in abscisic acid signaling. The Plant Cell 15(4):965-980.

Smalle J, Vierstra RD. (2004) The ubiquitin 26S proteasome proteolytic pathway. Annual Review of Plant Biology 55:555-590

Smith D. (1996) Growth medium: USA patent No 5565355. Issued October 15 1996.

Souter M, Lindsey K. (2000) Polarity and signalling in plant embryogenesis. Journal of Experimental Botany 51(347):971.

Spaargaren DH. (1996) The design of culture media based on the elemental composition of biological material. Journal of Biotechnology 45(2):97-102.

Spiess A-N, Mueller N, Ivell R. (2003) Amplified RNA degradation in T7-amplification methods results in biased microarray hybridizations. BMC Genomics 4(1):44.

Staikidou I, Selby C, Hanks GR. (2006) Development of a medium for in vitro culture of Galanthus species based on the mineral composition of bulbs. Journal of Horticultural Science and Biotechnology 81(3):537-545.

Stasolla C, Kong L, Yeung EC, Thorpe TA. (2002a) Maturation of somatic embryos in conifers: Morphogenesis, physiology, biochemistry, and molecular biology. In Vitro Cellular and Developmental Biology-Plant 38(2):93-105.

Stasolla C, Kong L, Yeung E, Thorpe T. (2002b) Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. In Vitro Cellular and Developmental Biology-Plant 38(2):93-105.

Stasolla C, Zyl Lv, Egertsdotter U, Craig D, Liu WB, Sederoff RR. (2003) The effects of polyethylene glycol on gene expression of developing white spruce somatic embryos. Plant Physiology 131(1):49-60.

Stasolla C, Yeung EC. (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell, Tissue and Organ Culture 74(1):15-35.

Stasolla C, Bozhkov PV, Chu TM, Van Zyl L, Egertsdotter U, Suarez MF, Craig D, Wolfinger RD, Von Arnold S, Sederoff RR. (2004) Variation in transcript abundance during somatic embryogenesis in gymnosperms. Tree Physiology 24(10):1073-1085.

Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC. (1991) Cell-Specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3(9):907-921.

Sterk P, de Vries S. (1992) Molecular markers for plant embryos. In: Synseeds: Applications of Synthetic seeds to Crop Improvement. Redenbaugh K, eds. CRC Press, Boca Raton, Florida. 115-132.

Stewart F, Mapes M, Hears K. (1958) Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. American Journal of Botany 45:709-713.

Stone SL, Gifford DJ. (1997) Structural and Biochemical Changes in Loblolly Pine (*Pinus taeda* L.) Seeds During Germination and Early-Seedling Growth. I. Storage Protein Reserves. International Journal of Plant Sciences 158(6):727-737.

Sundås-Larsson A, Svenson M, Liao H, Engström P. (1998) A homeobox gene with potential developmental control function in the meristem of the conifer *Picea abies*.

Proceedings of the National Academy of Sciences of the United State of America 95(25):15118.

Šunderlíková V, Salaj J, Matušíková I, Wilhelm E. (2009) Isolation and characterization of an embryo-specific Em-like gene of pedunculate oak (*Quercus robur* L.) and its temporal and spatial expression patterns during somatic and zygotic embryo development. Trees - Structure and Function 23(1):135-144.

Sunderlin WD, Rodríguez JA. (1996) Cattle, broadleaf forests and the agricultural modernization law of Honduras. CIFOR. Occasional Paper No 7 E. 33p.

Suprasanna P, Bapat V. (2005) Differential gene expression during somatic embryogenesis. Somatic Embryogenesis 305-320.

Sutton B. (2002) Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Annals of Forest Sciences 59:657-661.

Tahir M, Law DA, Stasolla C. (2006) Molecular characterization of PgAGO, a novel conifer gene of the ARGONAUTE family expressed in apical cells and required for somatic embryo development in spruce. Tree Physiology 26(10):1257.

Tahir M, Stasolla C. (2006) Shoot apical development during in vitro embryogenesis. Botany 84(11):1650-1659.

Tahir M, Belmonte MF, Elhiti M, Flood H, Stasolla C. (2008) Identification and characterization of PgHZ1, a novel homeodomain leucine-zipper gene isolated from white spruce (*Picea glauca*) tissue. Plant Physiology and Biochemistry 46(12):1031-1039.

Taiz L, Zeiger E. (2006) Plant Physiology. 4th ed.

Tang W, Guo Z, Ouyang F. (2001) Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. In Vitro Cellular and Developmental Biology-Plant 37(5):558-563.

Tang W, Newton R. (2005a) Genome-Wide expression analysis of genes involved in somatic embryogenesis. In: Somatic Embryogenesis. Mujib A and Samaj J, eds. Springer. 2:69-83.

Tang W, Newton R. (2005b) Loblolly Pine (*Pinus taeda*). In: Protocol for somatic embryogenesis in woody plants. Mohan JS and Gupta PK, eds. 95-106.

Taniguchi T. (2001) Plant regeneration from somatic embryos in *Pinus thunbergii* (Japanese black pine) and *Pinus densiflora* (Japanese red pine). Progress in Biotechnology 18:319-324.

Tautorus T, Fowke L, Dunstan D. (1991) Somatic embryogenesis in conifers. Canadian Journal of Botany 69(9):1873-1899.

Teasdale RD, Dawson PA, Woolhouse HW. (1986) Mineral nutrient requirements of a loblolly pine (*Pinus taeda*) cell suspension culture. Evaluation of a medium formulated from seed composition data. Plant Physiology 82(4):942-945.

Tereso S, Zoglauer K, Milhinhos A, Miguel C, Oliveira M. (2007) Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study. Tree Physiology 27(5):661-669.

Terrer JC, Tomas DF. (2001) Determination of macronutrients to be included in in vitro culture media according to leaf concentrations. The Journal of Horticultural Science and Biotechnology 76:484-488.

Thomas T. (1993) Gene expression during plant embryogenesis and germination: an overview. The Plant Cell 5(10):1401-1410.

Thompson R, Aderkas P. (1992) Somatic embryogenesis and plant regeneration from immature embryos of western larch. Plant Cell Reports 11(8):379-385.

Tremblay L, Tremblay FM. (1991a) Carbohydrate requirements for the development of black spruce (*Picea mariana* (Mill.) B.S.P.) and red spruce (*P. rubens* Sarg.) somatic embryos. Plant Cell, Tissue and Organ Culture 27(1):95-103.

Tremblay L, Tremblay FM. (1991b) Effects of gelling agents, ammonium nitrate, and light on the development of *Picea mariana* (Mill) B.S.P. (black spruce) and *Picea rubens* Sarg. (red spruce) somatic embryos. Plant Science 77(2):233-242.

Trontin J, Walter C, Klimaszewska K, Park Y, Lelu-Walter M. (2007) Recent Progress in Genetic Transformation of Four Pinus spp. Transgenic Plant Journal 1:314-329.

Ueda M, Matsui K, Ishiguro S, Sano R, Wada T, Paponov I, Palme K, Okada K. (2004) The HALTED ROOT gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of *Arabidopsis* meristems. Development 131(9):2101.

Vales T, Feng X, Ge L, Xu N, Cairney J, Pullman GS, Peter GF. (2006) Improved somatic embryo maturation in loblolly pine by monitoring ABA-responsive gene expression. Cell Biology and Morphogenesis 26(2)133-143.

van Gelder R, von Zastrow M, Yool A, Dement W, Barchas J, Eberwine J. (1990) Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proceedings of the National Academy of Sciences of the United State of America 87:1663-1667.

Vejsadová H, Vla, íová H, Havel L. (2008) Prereservation of a rare bog pine genotypes using micropropagation techniques. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 56(4):197-206.

Verhagen SA, Wann SR. (1989) Norway spruce somatic embryogenesis: high-frequency initiation from light-cultured mature embryos. Plant Cell, Tissue and Organ Culture 16(2):103-111.

Vierstra R. (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends in Plant Science 8(3):135-142.

Viveros-Viveros H, Sáenz-Romero C, Guzmán-Reyna RR. (2005) Genetic control of *Pinus oocarpa* seedlings growth traits in the nursery. Revista Fitotecnia Mexicana 28(4):333-338.

von Aderkas P, Klimaszewska K, Bonga JM. (1990) Diploid and haploid embryogenesis in *Larixleptolepis, L. decidua*, and their reciprocal hybrids. Canadian Journal of Forest Research 20(1):9-14.

von Aderkas P, Coulter A, White L, Wagner R, Robb J, Rise M, Temmel N, MacEacheron I, Park Y, Bonga J. (2005) Somatic embryogenesis via nodules in *Pinus strobus* L. and *Pinus banksiana* Lamb.–dead ends and new beginnings. Propagation of Ornamental Plants 5:3-15.

von Arnold S, Eriksson T. (1981) In vitro studies of adventitious shoot formation in *Pinus contorta*. Canadian Journal of Botany 59(5):870-874.

von Arnold S, Woodward S. (1988) Organogenesis and embryogenesis in mature zygotic embryos of *Picea sitchensis*. Tree Physiology 4(3):291.

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L. (2002) Developmental pathways of somatic embryogenesis. Plant Cell, Tissue and Organ Culture 69(3):233-249.

Wadenback J, Clapham D, Craig D, Sederoff R, Peter G, von Arnold S, Egertsdotter U. (2005) Comparison of standard exponential and linear techniques to amplify small cDNA samples for microarrays. BMC Genomics 6(1):61.

Wakamiya I, Newton RJ, Johnston JS, Price HJ. (1993) Genome size and environmental factors in the genus *Pinus*. American Journal of Botany 80(11):1235-1241.

Walter C, Find J, Grace L. (2005) Somatic embryogenesis and genetic transformation in *Pinus radiata.* In: Protocol for Somatic Embryogenesis in Woody Plants. Jain SM and Gupta PK, eds. Springer. 77:11-24.

Wang S, Kurepa J, Smalle J. (2009) The *Arabidopsis* 26S proteasome subunit RPN1a is required for optimal plant growth and stress responses. Plant and Cell Physiology 50(9):1721-1725.

Webb DT, F. Webster, B. S. Flinn, D. R. Roberts, Ellis DD. (1989) Factors influencing the induction of embryogenic and caulogenic callus from embryos of *Picea glauca* and *P. engelmanii*. Canadian Journal of Forest Research 19(10):1303-1308.

West MAL, Harada JJ. (1993) Embryogenesis in higher plants: an overview. American Society of Plant Biology 5(10)1361-1369.

West MM, Lott JNA. (1993) Studies of mature seeds of eleven Pinus species differing in seed weight. I: Element concentrations in embryos and female gametophyte. Canadian Journal of Botany 71(4):570-576.

Wilen R, Mandel R, Pharis R, Holbrook L, Moloney M. (1990) Effects of abscisic acid and high osmoticum on storage protein gene expression in microspore embryos of *Brassica napus*. Plant Physiology 94(3):875.

Williams C. (2008) Selfed embryo death in *Pinus taeda*: a phenotypic profile. New Phytologist 178(1):210-222.

Wolffsohn A. (1984) Estudios silviculturales de *Pinus oocarpa* Schiede en la Republica de Honduras. In: Publicacion miscelanea (Honduras): ESNACIFOR. 55p.

Wright JA, Ladrach WE, No. (1993) Ten-year evaluation of a field demonstration in Colombia of pine, cypress and eucalyptus transmission poles and fence posts treated with CCA. 5p.

Wurtele E, Wang H, Durgerian S, Nikolau B, Ulrich T. (1993) Characterization of a gene that is expressed early in somatic embryogenesis of *Daucus carota*. Plant Physiology 102(1):303-312.

Xu D, Duan X, Wang B, Hong B, Ho T, Wu R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiology 110 (1):249-257.

Yang H, Saitou T, Komeda Y, Harada H, Kamada H. (1996) Late embryogenesis abundant protein in *Arabidopsis thaliana* homologous to carrot ECP31. Physiologia Plantarum 98(3):661-666.

Yang X, Zhang X. (2010) Regulation of somatic embryogenesis in higher plants. Critical Reviews in Plant Sciences 29(1):36-57.

Yeung E, Meinke DW. (1993) Embryogenesis in angiosperms: development of the suspensor. Plant Cell 5(10):1371-1381.

Yeung E. (1995) Structural and developmental patterns in somatic embryogenesis. In: In vitro embryogenesis in plants. Thorpe TA, ed. Springer. 205-248.

Yeung E, Stasolla C, Kong L. (1998) Apical meristem formation during zygotic embryo development of white spruce. Canadian Journal of Botany 76(5):751-761.

Yildirim T, Kaya Z, I ik K. (2006) Induction of embryogenic tissue and maturation of somatic embryos in *Pinus brutia* TEN. Plant Cell, Tissue and Organ Culture 87(1):67-76.

Yu Y, Yang S. (1980) Biosynthesis of wound ethylene. Plant Physiology 66(2):281-285.

Zamora S. (1981) Algunos aspectos sobre *Pinus oocarpa* en el estado de chiapas. Ciencia Forestal 6(32):3-5.

Zhang C-X, Li Q, Kong L. (2007) Induction, development and maturation of somatic embryos in Bunge's pine (*Pinus bungeana* Zucc. ex Endl.). Plant Cell, Tissue and Organ Culture 91(3):273-280.

Zimmerman JL. (1993) Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5(10):1411-1423.