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Douglas fir megagametophyte development in situ and in vitro

by

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B.Sc., University of Guelph, 1994

A Dissertation Submitted in Partial Fulfillment of the Requirements for the
Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming to the required standard

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ABSTRACT

Megagametophyte development *in situ* and *in vitro* was investigated in Douglas fir to address the following questions: 1) Do endogenous levels of plant hormones change during megagametophyte development and are they associated with morphological changes? 2) Can megagametophytes be cultured prior to fertilization? 3) Can embryos be rescued from megagametophytes cultured soon after fertilization?

A histochemical study of storage reserve deposition during megagametophyte development was performed with material isolated weekly for 11 weeks. Prior to fertilization, starch was detected in the neck cells of megagametophytes analyzed 9 weeks after pollination (WAP). During embryogenesis, starch was deposited in the central region of megagametophytes. Proteins and lipids were first detected in the prothallial cells in the periphery of megagametophytes isolated 14 and 15 WAP, respectively. With further development, starch was deposited in prothallial cells around the corrosion cavity, while proteins and lipids were spatially localized to prothallial cells in the periphery. In the embryo, starch accumulation was preferentially localized in the root cap and the embryonal suspensor cells at 17 WAP.

A parallel study quantifying the endogenous levels of plant hormones: IAA, IAAsp, Z, ZR, iP, IPA, ABA and ABA-GE, in megagametophytes was performed. Hormones were extracted, purified and fractionated using HPLC. To correct for losses due to procedures, radiolabelled standards were added prior to extraction. The hormones were quantified using an ELISA method. On a dry weight basis, Z levels were highest in megagametophytes at the late central cell stage (8 WAP). During embryogenesis, Z levels peaked during week 13. ZR peaked twice at 13 and 17 WAP. The iP content of megagametophytes increased at 10, 13 and 17 WAP while iPA concentration increased at 13 and 17 WAP. Prior to fertilization, the free IAA was highest in megagametophytes at 9 WAP. During embryogenesis, the major IAA accumulations occurred at 11, 13 and 15 WAP. IAAsp concentrations reached their highest levels at 10, 14 and 18 WAP. ABA content increased at 11, 13 and 17 WAP. In contrast, ABA-GE levels were relatively constant over the 11 weeks analyzed.

Megagametophytes were isolated weekly from 7-10 WAP and cultured on a modified half-strength Litvay's medium supplemented with one of three auxins (NAA, IBA or 2,4-D) and a cytokinin (2 mg/L BAP). Each auxin was tested at three levels: 0.1, 1.0 or 10 mg/L. The objective was to determine whether the megagametophytes would continue to grow in culture. Megagametophytes increased in length after 9 and 18 days of culture. Auxin and cytokinin supplements had a significant effect on growth for material isolated 7 or 10 WAP. However, the viability of the archegonia rapidly declined on all the media tested. The most optimal treatment for each auxin type (BAP in combination with 0.1 mg/L NAA, 1.0 mg/L IBA or 1.0 mg/L 2,4-D) was used to initially culture pollinated megagametophytes in the embryo rescue experiment.. After 21 days, megagametophytes were transferred to media containing ABA concentrations of 0, 5, 20 or 40 µM. A majority of the rescued embryos were developmentally arrested at the globular stage. Only three embryos, containing over 30 cotyledons each, matured on ABA concentrations of 5, 20 or 40 µM.

In conclusion, the prothallial cells of the pre-fertilization megagametophytes could be cultured for long periods and their growth was not dependent on the presence of viable archegonia. The endogenous levels of plant hormones varied with megagametophyte development and were associated with morphological changes. This information has implications for growing megagametophytes for *in vitro* fertilization and embryo rescue experiments. The endogenous levels of plant hormones could be used to design culture media for rescuing embryos resulting from *in vitro* fertilization in Douglas fir.

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Acknowledgements

Completing this study in the Biology Department at the University of Victoria is one of the most fulfilling experiences of my life, and this would have been impossible without the help of the following people. I will forever be grateful to my supervisor, Dr. Patrick von Aderkas, for his guidance, unwavering support and consideration throughout my studies as well as in editing this dissertation. I wish to thank Drs. Louise Page, Barbara Hawkins and Terry Pearson, my committee members, for their constructive suggestions throughout my study and on this dissertation.

I am deeply grateful to Dr. Joe Webber of the BC Ministry of Forests at the Glyn Road Research Station for providing the plant material for this study and Dr. Philippe Label for supplying the conjugated hormones and antibodies and their working dilutions for the hormonal analysis experiment. I wish to thank Drs. Nancy Sherwood and Will Hintz for allowing me to use their laboratory equipment for some of my experiments, Heather Down and Tom Gore for their assistance in the Advanced Imaging Laboratory, Luke Chandler for taking the time out of his studies to help me with the plates in this dissertation and Marlies Rise for her help with the embedding and sectioning of specimens (You are a true angel Mar.!!).

I wish to thank Cathy Leary, who was a work study student in our laboratory, for her help with the megagametophyte culture study. I also wish to thank Brett Poulis, Dan O'neil and Carol for their help with the Beckman HPLC, my lab mate Stephen O'Leary for just being Stephen, and the people in the Graduate Centre for Forest Biology for

providing such a supportive and truly enjoyable working environment within the department. Finally, I wish to thank my friends Brian . Nadja. Erica Fradinger, Marlies Rise, my family members Doug Chiwocha, Cleopatra Makoni and little Timmy for always being there for me. The financial support for this study was provided by the operating grant of the Natural Science and Engineering Research Council of Canada held by Dr. Patrick von Aderkas.

CHAPTER 1

INTRODUCTION

Douglas fir (*Pseudotsuga menziesii*) is a member of the Pinaceae and has a wide distribution in the Pacific Northwest. Its natural range extends from the southern half of British Columbia through the United States into Mexico (Owens, 1973). Because of its economic importance as a lumber species, methods to ensure a constant supply of superior reforestation stock which can be produced in a short period of time are necessary.

The reforestation of many conifer species has encountered several impediments. Conifers have long juvenile periods (10-20 years), before they become reproductive (Pharis and King, 1985). Gibberellic acid (GA) can dramatically shorten the juvenile period such that flowering can be induced in plants 3-12 months old (Pharis and King, 1985). Different classes of GA are required to induce flowering in different conifer families. Polar GAs such as GA_3 are useful in species belonging to the Taxodiaceae and Cupressaceae whereas non-polar GAs such as GA_9 and $GA_{4/7}$ are effective in members of the Pinaceae (Pharis and King, 1985), including Douglas fir (Owens, 1991). This effect of GAs has been widely exploited in commercial forestry where it is used to obtain seed from young trees that have not developed to the reproductive phase.

Another common problem is the periodicity of cone crop production, which in Douglas fir has been found to average five years (Owens, 1973). GA used alone or in conjunction with other treatments that promote flowering can overcome this obstacle in seed orchards. Girdling, which is believed to limit the downward transport of carbohydrates from the crown, enhances flowering in several species (see Owens, 1991 for review). Moisture stress treatment, which is achieved by either drought or root pruning, has been successful in *Pinus*, *Picea*, *Abies*, and *Pseudotsuga* (see Owens, 1991 for review).

An additional problem is that Douglas fir has a reproductive cycle that spans seventeen months, limiting the harvesting of mature seed to once every two years (Owens, 1973). Production of seedlings from other methodologies, such as in vitro culture, may increase the production of selected genotypes in a shorter period, and in turn, speed up the reforestation process for Douglas fir. These novel breeding strategies include micropropagation, somatic embryogenesis and in vitro fertilization (IVF). One form of micropropagation which has been successful in both conifers and angiosperms is organogenesis, in which foliage explants, e.g. individual conifer needles, are initially cultured to stimulate adventitious shoot development. The shoots are then rooted, allowing plants to be produced in vitro (see Bonga and von Aderkas, 1992 for review). Somatic embryogenesis is a form of vegetative propagation in which embryos are produced from cells that are not naturally embryogenic following induction of the primary explants (usually immature zygotic embryos in conifers) with plant hormones (see Bonga and von Aderkas, 1992 for review).

In vitro fertilization requires the introduction of a male gamete to a female gamete and is therefore a form of sexual propagation. The type of IVF in conifers involves direct microgametophyte / megagametophyte interaction. Steps towards in vitro production of embryos from IVF were made recently in Douglas fir (Takaso et al, 1996; Dumont-Béboux and von Aderkas, 1997; Fernando et al, 1997). The first report of successful IVF in any conifer was by Fernando et al. (1998) in Douglas fir. Although this is a major breakthrough in conifer research, the ultimate goal is to rescue the embryos resulting from this technology and grow them into plants. Embryo rescue is a method that is used to raise embryos that would otherwise abort due to post-zygotic selection (Ramming, 1990). In conifers, embryo rescue of zygotic and proembryonic stages has not been accomplished (Sterling, 1949; Gates and Greenwood, 1991). In part, this could be due to the lack of basic physiological information on early embryo development, including levels of plant hormones.

Plant hormones are defined as "a group of naturally occurring organic compounds which influence physiological processes at low concentration" (Davies, 1995). Nine classes of organic substances have been identified that fall under this broad definition. They are auxins, gibberellins, cytokinins, ethylene, abscisic acid, polyamines, jasmonates, salicylic acid and brassinosteroids. For historical reasons, most studies have concentrated on the first five hormones. These have been shown to affect physiological processes that occur during seed development in several species of angiosperms and conifers. Of these five hormones, abscisic acid (ABA) is the most studied. Very few studies have investigated changes in plant hormone levels during seed development in conifers. ABA levels have been quantified during zygotic embryogenesis in loblloly pine (Kapik et al., 1995) and more recently in white spruce (Carrier et al., 1999). Endogenous auxin, gibberellins and ABA levels in developing white spruce seeds were published by Kong et al. (1997).

Embryo rescue of early stage embryos requires the optimization of culture media and culture conditions to promote further growth of the embryos. Prior knowledge of endogenous hormone levels may be invaluable in the design of culture media for use in such studies. Imitation of conditions in the natural seed environment may promote further development of the cultured embryos (Ramming, 1990). Techniques which are currently available for the quantification of plant hormones include high pressure liquid chromatography (HPLC), gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS) and enzyme immunoassays (EIs) (Hock et al., 1992). Because of the small amounts of hormones in plant tissue samples, methods with high sensitivity are required for quantification. The high affinity and specificity of EIs makes them particularly suitable for such work.

Plant hormone studies during seed development in Douglas fir have been restricted to investigating the role played by ABA in the establishment of dormancy in mature seeds (Jarvis et al., 1997; Bianco et al., 1997). Hormone levels during earlier stages have not been studied. This information is essential in light of the recent report by Fernando et al. (1998) of IVF in this species. The reported success of somatic embryogenesis technology in conifers (see Bonga et al., 1995; Becwar and Pullman, 1995; Chandler and Young, 1995 for reviews) could be applied to embryos resulting from IVF studies. The rescued embryo could be multiplied by this method, providing a quick way for propagation.

My research focused on megagametophyte development and was designed to address the following questions:

Question 1. Do plant hormone levels change during megagametophyte development?

Question 2. Are morphological and physiological changes during development independent of one another i.e. are there morphological markers of physiological change?

Question 3. Can megagametophytes be cultured prior to fertilization?

Question 4. Can embryos be rescued from megagametophytes cultured soon after fertilization?

CHAPTER 2

LITERATURE REVIEW

2.1 Conifer seed development

Embryogeny starts with the establishment of the haploid megagametophyte well before fertilization. This is followed by the formation of structures bearing the female gametes (the eggs) within the megagametophyte tissue (Figure 1a). In conifers, fertilization is a single fusion event between a male nucleus and the egg nucleus. The embryo initially develops within the archegonium (Figure 1b) until suspensor elongation pushes it into the megagametophyte (Figure 1c) where it matures surrounded by the haploid prothallial cells (Figure 1d; Singh, 1978).

2.1.1 Embryogeny

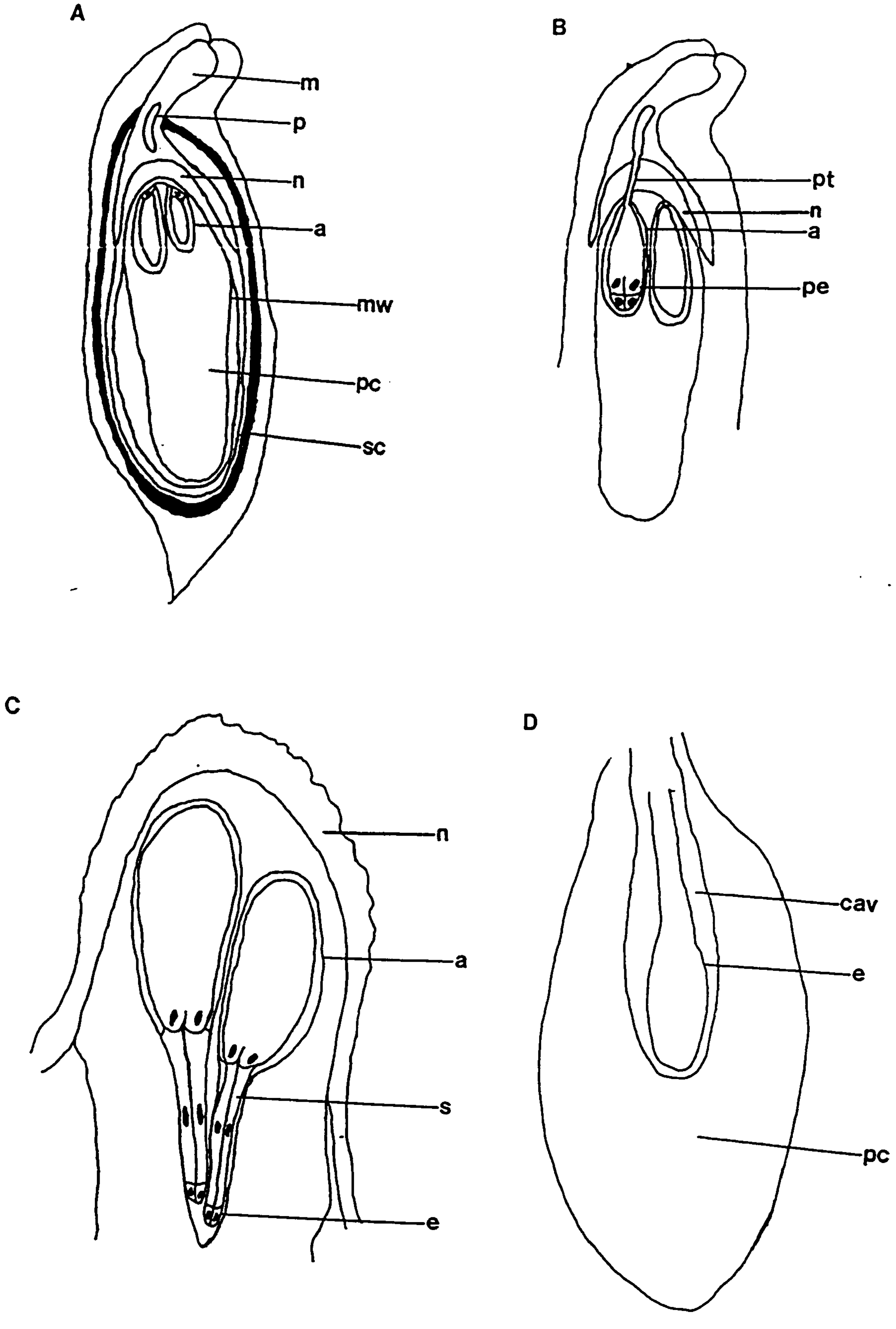
The developmental events leading to egg cell formation in the prefertilization megagametophyte have been extensively studied by light and electron microscopy for various conifers (Allen and Owens, 1972; Owens and Morris, 1990; Runions and Owens, 1999a; see Singh, 1978 for review). Briefly, development starts with differentiation of the megaspore mother cell in each ovule. This cell undergoes meiosis, resulting in four daughter cells, three of which degenerate. The remaining cell, the functional megaspore, undergoes a number of free nuclear divisions, giving rise to a large multinucleate cell. The nuclei are suspended in a thin layer of parietal cytoplasm surrounding a large central vacuole. Cellularization of the ceonocyte follows in a process called alveolation (Singh, 1978). Archegonial initials differentiate at the micropylar end of this structure. These cells enlarge and divide to form a small primary neck cell and a larger central cell. The primary neck cell undergoes a few mitotic divisions to form a short neck. The central cell

Figure 1A Schematic diagram of an ovule prior to fertilization in Douglas fir. The pollen (p) is trapped in the micropyle (m) where it elongates while megagametophyte development progresses. The haploid megagametophyte is surrounded by the megaspore wall (mw) and is made up of prothallial cells (pc) and the archegonia (a) located at the micropylar end. The archegonia contain central cells prior to fertilization and mature egg cells at fertilization. The wall of the ovule, the seed coat (sc) develops from the integument. The nucellus (n) is a diploid tissue through which pollen tubes grow in order to inseminate the eggs at fertilization.

Figure 1B After pollen germination, the pollen tube (pt) grows through the nucellus (n) to penetrate the archegonium, releasing the male gametes at fertilization. The zygote divides to give rise to the proembryo (pe) within the archegonium.

Figure 1C The nucellus begins to degenerate as elongation of the suspensor (s) pushes the embryos (e) out of the archegonium and into the prothallial cells of the megagametophyte before corrosion cavity formation.

Figure 1D Cells in the central region of the megagametophyte degenerate to form the corrosion cavity (cav) as the suspensor pushes the dominant embryo deeper into the megagametophyte tissue. The embryo matures in this cavity, surrounded by the haploid prothallial cells.


A
m
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enlarges and then divides to give rise to the egg cell and a ventral canal cell which are both housed within an archegonium (Allen and Owens, 1972; see Singh, 1978 for review). The prothallial cells surrounding the archegonia differentiate to form the jacket cells. The number of archegonia formed is species dependent within a conifer family, ranging from 1 up to 100 in some species (see Singh, 1978 for review). In Pinaceae for example, at fertilization, each Douglas fir megagametophyte has 4-6 archegonia (Allen and Owens, 1972), *Larix* has 3-4 (Schopf, 1943) whereas interior spruce has 2-3 (Runions and Owens, 1999a). *Podocarpus totara*, which is a member of the Podocarpaceae family, has 4-6 archegonia (Wilson and Owens, 1999) and in Taxaceae, *Taxus brevifolia* has an average of 4 archegonia per ovule (Anderson and Owens, 1999).

The megagametophyte may be involved in more than just egg production. Regulation of pollen behavior has been implied in a series of recent studies. In Douglas fir, the presence of an ovular secretion in the micropyle prior to fertilization has been reported by Takaso et al. (1996) and von Aderkas and Leary (1999). Formation of the ovular secretion was dependent on the developmental stage of the megagametophyte. Takaso et al. (1996) found that the secretion was absent in ovules containing central cell stage megagametophytes, only being produced a week before fertilization when the egg cells were nearly mature. However, von Aderkas and Leary (1999) recently reported the appearance of this secretion for a two week period, starting as early as the central cell stage. In this species, pollination occurs about two months before fertilization (Owens and Morris, 1990), when megagametophyte development is just beginning. The pollen grains remain trapped in the micropyle while megagametophytes progress through the various developmental stages leading to egg cell formation. During this time, the pollen grains shed their exines and slowly elongate over a period of 6 weeks (Owens and Morris, 1991). An hypothesized secretion from the egg and prothallial cells into the micropyle caused dissolution of the pollen grain wall concurrent with pollen tube formation (Takaso and Owens, 1994; 1996; Takaso et al., 1996). Owens and Morris

(1990) reported that the nucellar tip in Douglas fir was actively secretory at the time of pollen tube initiation. Cessation of fluid production just before fertilization (Takaso et al., 1996) was accompanied by the appearance of transparent areas throughout the egg cytoplasm, suggesting that the secretion contained material resulting from cytoplasmic degradation of the egg cells (Takaso and Owens, 1994). Indirect evidence that the secretion originated from the megagametophyte was provided by Takaso et al. (1996) in their experiment with megagametophyte homogenates. Similar ovular secretions have been reported in *Larix* (Barner and Christiansen, 1960; Said et al., 1991; Owens et al. 1994). Although conifers are generally not believed to have any prezygotic selection mechanisms (Owens, 1991), Takaso and Owens, (1994) reported a high percentage of distorted pollen grains in self-pollinated ovules compared to cross-pollinated ovules, suggesting that there may be some form of pollen recognition mechanism prior to pollen tube germination.

Events occurring in the archegonia of several conifers just before as well as soon after fertilization have been described ultrastructurally (Owens and Morris, 1990; 1991; Runions and Owens, 1999a; 1999b; Anderson and Owens, 1999) and by light microscopy (Allen and Owens, 1972, Wilson and Owens, 1999). In particular, cytoplasmic inheritance of chloroplasts and mitochondria in Douglas fir has been studied (Owens and Morris, 1990; 1991). As a mechanism, these authors suggested that transformation of plastids into large inclusions, which begins in the central cell and is complete at the mature egg cell stage, functions to exclude the migration of maternal plastids into the early embryo. In interior spruce, this is followed by further spatial isolation of the modified plastids from the perinuclear zone that surrounds the egg nucleus (Runions and Owens, 1999a). Maternal plastid transformation prior to fertilization has also been reported for *Pinus* and *Larix* (Camefort, 1962; 1967 respectively, cited in Owens and Morris, 1990) as well as *Taxus* (Anderson and Owens, 1999). Thus chloroplasts are exclusively of paternal origin in the above genera. Although aggregation of mitochondria in the perinuclear zone of the egg nucleus prior to fertilization results in a large maternal contribution of this organelle into the embryo, approximately 10% of mitochondria may be of paternal origin in *Pinus* (Wagner et al., 1992) and *Pseudotsuga* (Owens and Morris, 1991). Mixed inheritance of mitochondria was also recently reported in *Taxus brevifolia* by Anderson and Owens (1999). In *Picea*, genetic studies have confirmed that mitochondria are strictly of maternal origin (Sutton et al., 1991; David and Keathley, 1996). This result agrees with what was suggested from an ultrastructural study by Runions and Owens (1999b).

Once formed, the pollen tube must penetrate the nucellus before it can enter the egg. The mechanism by which a pollen tube grows through the nucellus to find the neck of an archegonium has been the subject of much speculation. Runions and Owens (1999a) reported lipid accumulation in the archegonial chamber above each neck which may direct pollen tube growth toward an archegonium, although they did not think that such a signal was strong enough to transverse the nucellus. Indeed, neck cells have characteristics of secretory cells in Douglas fir (Owens and Morris, 1990). However, results from a recent study by Dumont-Béboux et al. (1998) did not support this proposed function of the neck cells. The pollen tube penetrated the egg cell through the side of the archegonium *in vitro* (Dumont-Béboux et al., 1998). Furthermore, Anderson and Owens (1999) reported degeneration of neck cells prior to fertilization in *Taxus brevifolia*. In angiosperms, directional growth of pollen tubes toward the ovary is believed to be a chemotropic response (for review, see Mascarenhas, 1993). Whether a chemical signal does exist in conifers and whether it is produced by the neck or egg cells or both has yet to be determined. The pollen tube tip releases many proteins with hydrolytic properties, which aid pollen tube penetration of the nucellus (Pettitt, 1985; Runions and Owens, 1999a). Shafer and Kriebel (1974) reported that nucellar cells around the growing pollen tube showed an increase in cytoplasmic RNA and suggested that the pollen tubes may produce a hormone-like substance which stimulates RNA and protein synthesis in these cells.

2.1.2 Fertilization and zygotic embryogenesis

The fertilization process in angiosperms is different from that in conifers. In angiosperms, seed development is a result of double fertilization (Taiz and Zeiger, 1991). One male gamete fuses with an egg cell, which is housed within an embryo sac, to form the zygote. The second unites with the diploid central cell of the embryo sac to form a triploid endosperm, which develops into a nutritive tissue. In conifers, only one pollen tube will penetrate each egg cell, and the deposition of a pectinaceous substance at the site of pollen tube entry into the egg may inhibit insemination by other pollen or the release of egg contents (Runions and Owens, 1999b). Ultrastructural studies of fertilization in conifers have demonstrated that although two male gametes are deposited into the egg cell upon pollen tube penetration, only the leading nucleus fuses with the egg nucleus, while the trailing gamete remains in the micropylar end of the egg cell cytoplasm and eventually degenerates (Owens and Morris, 1990; Runions and Owens, 1999b; Wilson and Owens, 1999; see Singh, 1978 for review). Pollen tube penetration is primarily through the neck cell of each archegonium, although Runions and Owens (1999b) reported one instance when a pollen tube grew through the side of an egg cell.

In the Pinaceae, mitotic divisions of the zygote nucleus gives rise to four free nuclei which migrate to the chalazal end of the archegonium and are arranged in a single tier. Divisions by these nuclei quickly give rise to eight nuclei. Cell wall formation results in a two tiered proembryo, in which the upper cells are open to the egg cytoplasm. In Douglas fir, divisions by cells in the lower tier results in a mature proembryo comprised of 12 cells in three tiers; an open tier, a suspensor tier in the middle and a lower embryo tier (Allen and Owens, 1972; Owens and Morris, 1991). In most Pinaceae, the mature proembryo is comprised of 16 cells in 4 tiers (see Singh, 1978 for review).

Elongation of the suspensor cells pushes the early embryo into a corrosion cavity which forms in the megagametophyte tissue soon after fertilization. The corrosion cavity is absent in Douglas fir megagametophytes before suspensor elongation (Owens et al., 1993). Although the mechanism of cavity formation is still unknown, Shafer and Kriebel (1974) described an increase in cytoplasmic RNA accumulation by prothallial cells in the region of the megagametophyte that later formed this fluid filled structure in eastern white pine. This increase was evident in pollinated megagametophytes at or before fertilization, as well as in pollinated unfertilized explants (in which the egg cells were degenerating) collected several days past fertilization period. The conifer embryo develops further and matures inside the corrosion cavity (Singh, 1978).

Detailed descriptions of zygotic embryogenesis in conifers have been published (see Singh, 1978 for review). This process in Douglas fir has been thoroughly described at the light microscope level (Allen, 1946; 1947a; 1947b, Allen and Owens, 1972, Grob et al., 1999) and at the ultrastructural level (Owens et al., 1993). Development can be divided into the proembryo, early, late and mature embryo stages (Allen and Owens, 1972). The term proembryo encompasses the free-nuclear and cellular embryo stages within the archegonia prior to suspensor elongation. This is followed by the early embryo stage, in which embryonal masses are pushed into the corrosion cavity following suspensor elongation (Krasowski and Owens, 1993). Further divisions result in a club shaped embryo, in which polarity is established subsequent to development of the proximal and distal meristems (Krasowski and Owens, 1993). Histodifferentiation occurs during the late embryo stage. This period is characterized by formation of the root and shoot apical meristems, development of the hypocotyl-shoot axis and by the initiation and elongation of cotyledons (Krasowski and Owens, 1993). Further development results in the mature dormant embryo (Allen and Owens, 1972).

2.1.3 Storage reserves during conifer seed development

Prior to fertilization, conifer megagametophytes contain little storage material and are translucent. Accumulation of storage products commences during early embryogenesis (Schopf, 1943). Although the major storage reserves in mature conifer seeds are lipids (Owens et al., 1993; Stone and Gifford, 1999), a majority of studies have concentrated on biochemically characterizing the nature of storage proteins in mature seeds (Lammer and Gifford, 1989; Hakman et al., 1990; Baker et al., 1996; Misra and Green, 1994) and mobilization of these proteins during germination (Gifford et al., 1989; Lammer and Gifford, 1989; Gifford and Tolley, 1989; King and Gifford, 1997). It has been suggested that studies focusing on seed storage proteins are invaluable because they provide insight into DNA regulated events during embryogenesis (Owens, 1995).

Histochemical and ultrastructural studies focusing on the accumulation of storage products in conifer megagametophytes and embryos during embryogenesis have been published for Douglas fir (Owens et al., 1993) and white spruce (Krasowski and Owens, 1993). Starch was the only storage product detected in the corrosion cavity region of megagametophytes at fertilization through proembryo development in Douglas fir (Owens et al., 1993). The archegonia may initially provide nourishment for the developing proembryo and early embryo through the suspensor (Owens and Morris, 1991). Once the early embryo is forced into the prothallial tissue by suspensor elongation, rapid hydrolysis of starch in the corrosion cavity region occurred (Owens et al., 1993). Lipid and protein deposition was first detected in megagametophytes containing early embryos. By the club-shaped embryo stage, protein and lipid content had increased in the combined embryo and megagametophyte. After histodifferentiation, when cotyledons and the root cap had formed and the meristems were established, starch accumulation was primarily in the root cap while lipids and proteins were more uniformly distributed in both the megagametophyte and embryo (Owens et al., 1993).

Similar accumulation patterns were reported for *Picea glauca* (Krazowski and Owens, 1993).

Most molecular and biochemical studies commence after histodifferentiation (Owens et al, 1993) and these studies characterize the nature of storage proteins in conifer seed. Gifford (1988) identified crystalloid proteins in mature seeds of *Pinus monticola* and several other *Pinus* species as the major storage proteins. The wide occurrence of crystalloids in mature conifer seeds has since been demonstrated by similar studies in lodgepole pine (Lammer and Gifford, 1989); Norway spruce (Hakman et al., 1990), interior spruce (Flinn et al., 1991), Douglas fir (Misra and Green, 1990; 1991) and eastern white pine (Baker et al., 1996). A large proportion of these proteins is located in the megagametophyte and the remainder in the embryo.

Misra and Green (1991) showed that crystalloid protein synthesis is developmentally regulated during embryogenesis in Douglas fir, with maximum synthesis occurring during the mid-to-late cotyledonary stages. Similar results were obtained for interior spruce by Flinn et al. (1991). Northern blot analysis with megagametophytes at various stages of development found mRNAs encoding crystalloids were maximal from the club-shaped through to cotyledonary stages and then declined during later maturation in both the embryo and the megagametophyte (Leal and Misra, 1993). Similar results were obtained by Baker et al. (1996) in eastern white pine.

It appears that crystalloid gene expression is both developmentally regulated and tissue specific. In Douglas fir, mRNAs encoding crystalloids were not detected in mature dry seed or in leaves and roots of 2 week old seedlings by DNA-RNA hybridization (Leal and Misra, 1993). Baker et al. (1996) also found differential gene expression within the ovules; mRNAs encoding crystalloids were not detected by either DNA-RNA hybridization, tissue printing or RNA filter hybridization in sporophytic tissue (i.e. inner and outer integuments as well as the nucellus).

The evolutionary relationship between crystalloid proteins of gymnosperms and the 11s globulins of angiosperms has been investigated. White spruce crystalloids were immunologically related to those of several conifers in Pinaceae and to 11s globulins of several angiosperms (Misra and Green, 1994). Baker et al. (1996) also found mRNAs in eastern white pine ovules that had significant sequence homology to the mRNAs of 11s globulins of several angiosperms. There was no immunological relationship between 11s globulins of legumes (legumins) with crystalloid proteins of white spruce (Misra and Green, 1994) or *Pinus* (Gifford, 1988). However, a comparison of legumin precursor cDNA with Douglas fir crystalloid cDNA showed 29-38.5% identity, suggesting that these storage protein genes may have a common ancestry but have diverged during the course of evolution (Leal and Misra, 1993).

In addition to storage proteins, deposition of sugars and lipids also occur during conifer seed development. Gates and Greenwood (1991) reported that increases in hexose sugar, soluble protein and lipid concentration paralleled the increase in seed dry weight from the club-shaped embryo to the early cotyledonary stages during embryogenesis of *Pinus resinosa.* An ultrastructural and histochemical study by Krasowski and Owens (1993) yielded similar results for protein and lipid accumulation during seed development in white spruce. However, a more recent study in white spruce utilizing gas-chromatography detected lipids prior to fertilization, when megagametophytes were still aqueous (Carrier et al., 1999). Stone and Gifford (1999) recently reported that lipids comprised 59% of the total storage reserves in mature seeds of loblolly pine.

2.2 *In vitro* megagametophyte culture studies

The conifer megagametophyte is comprised of a variety of cells, namely the prothallial, jacket, neck and either central cells prior to fertilization or egg cells at fertilization. Few studies have been published that were carried out to establish culture conditions for IVF of megagametophytes isolated prior to fertilization. Douglas fir megagametophytes cultured prior to fertilization on media supplemented with auxin and cytokinin grew to lengths not normally achieved *in situ* by 9 days in culture (von Aderkas et al, 1997). Ma et al. (1998) reported multiplication of prothallial, neck and jacket cells in cultured Douglas fir megagametophytes isolated at fertilization. However, egg cell viability could only be maintained for 3-4 days in the same species (Fernando et al., 1997). Fernando et al. (1997) and von Aderkas et al. (1997) also found that growth of prothallial cells continued even after the egg cells had degenerated.

Gates and Greenwood (1991) reported that fertilized megagametophytes of *Pinus resinosa* cultured on media supplemented with sucrose concentrations of up to 21% resulted in megagametophytes that resembled material *in situ*. However, they found that development of embryos within the megagametophytes was arrested on these same media.

Until recently, the primary focus of *in vitro* culture of megagametophytes in conifers has been the induction of haploid cultures for embryogenesis. This is historical, partly because of the successful creation of haploid based breeding programs for angiosperms. Haploid plants have been produced from both anthers and unfertilized ovules in angiosperm tree species, (see Chalupa, 1995; Gosal et al., 1995 for reviews). In gymnosperms, the first and only tissue to produce haploid cultures was megagametophytes (Bonga et al., 1994; see Bonga and von Aderkas, 1992 for review). Although seeds of selected genotypes are currently produced in seed orchards from tree improvement programs, the production of breeding lines that are homozygous for desirable traits by inbreeding is restricted by the long juvenile periods and the long reproductive cycles in conifers. In vitro production of haploid plants would therefore be a particularly valuable system in conifers (Bonga and Fowler, 1970; Simola and Honkanen, 1983; Pattanavibool et al., 1995). It has been suggested that the haploid plants could be diploidized artificially by colchicine treatment, allowing the production of homozygous breeding lines (Bonga and Fowler, 1970). A flow cytometric and chromosome study by

Pattanavibool et al. (1995) reported diploidization after 2-9 years in culture of haploid lines of *L. decidua* megagametophytes. These authors emphasized the benefits of regenerating plants from these diploidized cultures as this would eliminate the need to artificially double chromosome number as originally suggested by Bonga and Fowler (1970).

Haploid callus tissue proliferation has been induced in a number of conifer species from explants of megagametophytes grown on media supplemented with exogenous plant hormones. Major factors that had an impact on successful induction of embryogenic cultures from such explants were genotype and collection date within a species. Bonga and Fowler (1970) obtained haploid callus tissue from megagametophyte explants of *Pinus resinosa* isolated between fertilization and embryo elongation only. In *L. decidua*, embryogenic callus was produced with megagametophytes isolated a few weeks after fertilization (von Aderkas et al., 1987).

In *Picea abies*, culture of megagametophytes from immature seed resulted in callus tissue with a poor capacity for organogenesis (Simola and Honkanen, 1983). In a later study using the same species, Hakman et al. (1985) were unable to induce haploid callus formation from megagametophyte explants. Further studies in *P. abies* revealed the importance of hormone levels and nitrogen source on haploid embryoid regeneration from megagametophyte callus although the embryos they produced failed to mature *in vitro* (Simola and Santanen, 1990). Successful production of haploid embryoids which were able to develop into embryos was first achieved in *Larix decidua* (Nagmani and Bonga, 1985). Work in other species of *Larix* also produced haploid plantlets but these only exhibited limited growth after potting (von Aderkas et al., 1990). Further studies in L. *decidua* reported the first successful production of plants from a haploid megagametophyte-derived culture line that later survived greenhouse conditions (von Aderkas and Bonga, 1993).

2.3 Embryo rescue

In plants, post-zygotic selection can cause poor embryo development (Ramming, 1990). This is evident in some interspecific crosses in which the hybrids fail due to embryo abortion. Post-zygotic selection has been overcome in fruit breeding programs by the use of embryo culture (see Ramming, 1990 for review). Seeds fail to develop due to cessation of embryo development soon after fertilization in seedless varieties of grapes (Emershad and Ramming, 1984). In early ripening *Prunus* (Ramming, 1985) spontaneous abortion results in the production of seedless fruit. Breeding plants from such genotypes have been produced by culturing embryos prior to abortion. Because of the small size of the embryos, whole ovules were initially cultured. This allowed further development of the embryos within the ovule which were then subcultured and grown into plants (Ramming, 1990). Depending on the genotype, multiple embryos developed in some cultured ovules as a result of somatic embryogenesis by the zygotic embryos (Ramming, 1990). Such work has been successful in early ripening *Prunus* (Ramming, 1985) and seedless varieties of *Vitis* (for review, see Ramming, 1990).

A few recent reports on work done in maize have opened up the possibility of using this technology to rescue embryos resulting from *in vitro* fertilization (IVF) experiments. The first report of IVF in maize was by Kranz et al. (1991a, b). Since then, embryo regeneration into plants has been achieved by *in vitro* culture of embryo sacs soon after fertilization in this angiosperm (Campernot et al., 1992; Möl et al., 1993; 1995; Matthys-Rochon et al., 1998). This latest study by Matthys-Rochon et al. (1998) validated the significance of the types and the levels of hormones used, the genotype, as well as the carbon source on successful plant regeneration.

In conifers, seedlings were produced by culturing mature embryos resulting from interspecific crosses between the blister rust susceptible *Pinus lambertiana* with pollen from the blister rust resistant *P. armandi* or *P. koraiensis* (Stone and Duffield, 1950). Healthy seedlings were also reported from cultured embryos of *P. lambertiana* (Sacher, 1956; Haddock, 1954). In larch, Sterling (1949) attempted embryo rescue by isolating early embryos from megagametophytes and only obtained limited growth after two months of culture. He also observed cleavage embryogenesis by a few of the cultured larch embryos, which does not occur *in situ*. This result led Sterling (1949) to suggest that removal of the megagametophyte may have released the embryos from developmental constraints that embryos *in situ* would naturally encounter during development. Based on work in *Pinus resinosa*, osmotic potential in the corrosion cavity of the megagametophyte does not appear to play a critical regulatory role during embryo development. Gates and Greenwood, (1991) reported that only slight changes occurred in supernatants obtained from megagametophyte containing embryos at the zygote up to cotyledonary stages. Although embryo rescue of early stages has not been achieved in conifers, tremendous progress has been made in developing somatic embryogenesis systems in a number of economically important species. Immature zygotic embryos are commonly used as the primary explants for conifers making these studies a form of embryo rescue.

2.3.1 Somatic embryogenesis

Somatic embryogenesis in conifers was first reported by Hakman et al. (1985) in *Picea abies* from cultures initiated from immature zygotic embryos. Since then, the focus has shifted to optimizing culture conditions for a number of different explant types for several conifer species. Toward this goal, a number of researchers have attempted to initiate somatic embryogenesis from mature seeds as well as from cotyledons obtained from germinated embryos (See Becwar, 1993 for review). This technology is particularly attractive for conifers because it provides a way to shorten the long reproductive cycles, which range from 2 years in some species e.g. Douglas fir to 3 years in *Pinus*. The potential to commercialize somatic embryogenesis by using it together with tree improvement programs in conifers provides a means for mass propagating embryos resulting from controlled crosses of genetically superior parents (Adams et al., 1994; see Becwar, 1993 for review).

Somatic embryogenesis can be divided into four steps; 1) initiation 2) proliferation 3) maturation 4) germination (Becwar, 1993; von Arnold et al., 1996). Initiation of somatic embryos from cells that are not naturally embryogenic requires induction with plant hormones. The transition is mediated by auxins and cytokinins (Dodeman et al., 1997). Culture of immature zygotic embryos on media supplemented with auxins and cytokinin produced embryogenic tissue in *Picea abies* (Hakman et al., 1985; Simola and Santanen, 1990), larch (von Aderkas et al., 1990; Thompson and von Aderkas, 1992), *Picea glauca* (Dunstan et al., 1988), *Pinus taeda* (Becwar et al., 1990) and in Douglas fir and *Pinus taeda* (Gupta et al., 1988). It appears that auxin and cytokinin mediate embryogenic tissue initiation by affecting cell polarity and promoting asymmetric cell divisions in the primary explant (reviewed in Dodeman et al., 1997). Repeated cell divisions by some cells in the primary explant results in the formation of dense clusters of cells which give rise to somatic embryos (von Arnold et al., 1996). Although a majority of studies utilize immature zygotic embryos as the primary explant, Jalonen and von Arnold (1991) reported somatic embryo formation from mature embryos of *P. abies*.

The initiated somatic embryos continue to divide and give rise to embryogenic cell lines in the proliferation step (von Arnold et al., 1996). During maturation, proliferation stops and the embryos grow in size and accumulate storage products (von Arnold et al., 1996). Flinn et al. (1991) found that somatic and zygotic embryos accumulated the same storage proteins during development in interior spruce. Maturation requires removal of somatic embryos from auxin and cytokinin containing media and placement on media supplemented with ABA (Jalonen and von Arnold. 1991; Lelu et al., 1995; Dunstan et al., 1988; Attree et al., 1992; Becwar and Feirer, 1989; Becwar et al., 1990; Gutman et al., 1996). Attree et al. (1992) reported that high osmoticum together with ABA resulted in embryos that closely resembled mature zygotic embryos in lipid content for *P. glauca.* Lipid accumulation was also increased in loblolly pine somatic embryos cultured on media containing ABA (Becwar and Feirer, 1989). Culture of somatic embryos on ABA without osmoticum resulted in precocious germination during late maturation, and this was attributed to a decrease in sensitivity to ABA (Attree et al., 1992). Desiccation of the mature embryos improved plantlet recovery in hybrid larch (Lelu et al., 1995) as well as in white spruce (Attree et al., 1992). The first report of successful plant regeneration from haploid embryogenic cultures in conifers was made by von Aderkas and Bonga (1993) in *L. decidua*, which lends credence to the use of this system in alternative breeding programs for conifers.

2.4 Plant hormones during seed development

2.4.1 Background

Studies investigating hormone action in plants have been limited to either the effect of exogenous hormones or quantification of endogenous levels followed by attempts to correlate this with physiological processes. While these studies have contributed invaluable information on the effects of different classes of plant hormones, the signal transduction pathways have remained a mystery. Signal transmission to the nucleus may be through any one of several systems, including GTP binding proteins, protein kinase cascades and membrane ion channels. Hormones may change the activity of DNA-binding proteins which in turn may change the expression of genes directing developmental programs (Mulligan et al., 1997). The recent advent of molecular genetic studies with *Arabidopsis* mutants has resulted in the identification of genes involved in the signal transduction pathways of ethylene (see Lelièvre et al., 1997 for review) and ABA (Cutler et al., 1996; Pei et al., 1998; see McCourt, 1999; Leung and Giraudat, 1998 for reviews). Although these pathways have not been fully described, researchers now have a new tool for further elucidation of plant hormone signaling pathways. In addition, the major focus has been on angiosperm species and as a result, there is a dearth of information in the literature on the role of plant hormones during conifer seed development. The studies in angiosperms are reviewed in the section below.

2.4.2 Angiosperm seed development

Embryo development in an angiosperm starts with an asymmetric transverse division by the zygote which establishes polarity soon after fertilization (Mayer et al., 1993). The embryo develops from the distal cell and the suspensor from the proximal cell. Fertilization is followed by a period of rapid growth and differentiation by the embryo (Rock and Quatrano, 1995). Embryogenesis can be divided into four phases; the proembryo, globular, heart-shaped and finally the torpedo stages. Prior to suspensor formation, the embryo is in the proembryonic stage. Further divisions result in the formation of the globular stage embryo with an attached suspensor. The heart-shaped stage occurs following divisions by the meristematic cells of the cotyledons. Torpedo-stage embryos results from elongation of the cotyledons as the apical and root tips are formed (Raven et al., 1986).

Embryo development is under the influence of the surrounding seed and maternal environments as well as under genetic control (Xu and Bewley, 1991). Some of the various gene sets that are expressed at different stages during embryogenesis and seed development have been identified (Mayer et al., 1993; Meinke et al., 1994; reviewed in Goldberg et al., 1989; reviewed in Dodeman et al., 1997). In maize, 51 embryo-specific mutations of genes crucial to the morphogenesis of the embryo at various developmental stages have also been isolated and characterized (Clark and Sheridan, 1991). During the maturation phase, there is a marked increase in the accumulation of storage reserves, cessation of embryogenic development, acquisition of desiccation tolerance, and development of dormancy mechanisms (Rock and Quatrano, 1995).

Evidence for hormone involvement during seed development originates from studies employing two main approaches; 1)results from *in vitro* experiments, and 2)results from correlation studies. However, both these approaches have limitations. Because culture conditions are very different from the situation in the developing seed environment, results from *in vitro* studies do not completely describe events *in vivo*. Hormone concentrations used in culture are generally several orders of magnitude higher than those found in seeds developing in vivo. The second approach relies on the determination of endogenous hormone levels in developing seeds followed by an attempt to correlate any changes in hormone levels with events occurring at various developmental stages. The main problem with studies using this approach, apart from the limitations inherent in the methodologies used, is the assumption that detection of high hormone levels at a particular developmental stage means the hormone is responsible for the observed event. As Trewavas (1981) pointed out, the ability of the tissue to respond to the hormone (tissue sensitivity) should also be taken into account. A change in tissue sensitivity could be a reflection of a change in the level of hormone receptor expression or changes in affinity by the receptor (Davies, 1995; Firn, 1986). So, while both these approaches are an invaluable first step in investigating the role played by hormones during seed development, the attention is shifting towards the use of genetic and molecular studies to further understand the mechanisms involved in this process.

2.4.2.1 The role of ABA during angiosperm seed development

Studies investigating the role of plant hormones in the regulation of seed development have centered on the role played by abscisic acid (ABA) and these have been largely confined to angiosperm species with short reproductive cycles. The common pattern of ABA accumulation in a majority of angiosperm seeds starts with low levels early in embryo development, followed by an increases to peak levels in mid-to-late embryogenesis, and finally a decline to low levels in mature seeds (Ackerson, 1984; Neill et al., 1987; Finkelstein et al., 1985; Groot et al., 1991; Rock and Quatrano, 1995). A biphasic ABA accumulation pattern has been reported in a few species during embryo development, including *Phaseolus coccineus* (Perata et al., 1990).

ABA has both stimulatory and inhibitory roles at different times during seed development. The hormone has been implicated in the prevention of precocious germination, which allows the embryo to mature before germination can occur (Ackerson, 1984; Neill et al., 1987; Pence, 1991), in the acquisition of desiccation tolerance (Pence, 1991; 1992; Ooms et al., 1993), in promoting storage protein synthesis (Ooms et al., 1993; Farrant et al., 1996; Finkelstein et al., 1985; Barratt and Clark, 1991; Berge et al., 1989; Kermode et al., 1989), and in the development of dormancy mechanisms (Karssen et al., 1983; Hole et al., 1989; Ooms et al., 1993; Le Page-Degivry and Garello, 1992). Lipoxygenase activity during embryo development coincides with ABA synthesis and action and this has been suggested as a key enzyme catalyzing ABA synthesis from carotenoids (Belefant and Fong, 1991).

2.4.2.1.1 Precocious germination

Correlations between high endogenous ABA levels in immature embryos and their failure to germinate have been reported in several species. During early embryogenesis of soybean and *Brassica napus*, ABA stimulates growth and storage protein accumulation while inhibiting precocious germination (Ackerson, 1984; Finkelstein et al., 1985). *Phaseolus coccineus* embryos showed a biphasic pattern of ABA accumulation, the first peak coinciding with the time of rapid embryo growth at the early cotyledonary stage (Perata et al., 1990). In alfalfa, while isolated embryos at all stages of development germinate *in vitro* on culture media supplemented with 3% sucrose, germination is slower during mid-development, the time of highest endogenous ABA concentration (Xu and Bewley, 1991). Le Page-Degivry and Garello, (1992) reported that exposure of young non-dormant sunflower embryos to exogenous ABA in culture also prevented precocious germination.

Further evidence comes from experiments showing the occurrence of precocious germination in the presence of reduced endogenous ABA levels. Mid-development alfalfa embryos excised from seeds treated with the ABA biosynthetic inhibitor, fluridone, have much higher germination rates than untreated embryos (Xu et al., 1990). Premature drying, which also depletes endogenous ABA in embryos and endosperm, results in precocious germination of immature embryos of soybean (Ackerson, 1984) and castor bean (Kermode et al., 1989).

ABA may act by inhibiting gibberellin's effects in immature grass seeds e.g. barley. A switch from a developmental mode to a germinative mode is paralleled by an increase in gibberellin-responsiveness, which can be examined by measuring α-amylase activity. The antagonistic effects of ABA and GA on α-amylase have been reported during germination in barley aleurone (Wang et al., 1996). Treatments which lower ABA levels and permit precocious germination of immature seeds, such as premature drying, induce α-amylase production (Cornford et al., 1986).

Sensitivity to ABA appears to change with the developmental stage of the embryo. Kermode et al. (1989) reported that imposed drying of immature castor bean embryos causes a 10-fold decrease in sensitivity of isolated embryos to exogenous ABA in culture. Excised mature embryos that have undergone natural drying in the seed have a similar response to exogenous ABA (Kermode et al., 1989). In *Brassica napus* (Finkelstein et al., 1985) and alfalfa (Xu and Bewley, 1991), germination is suppressed by lower levels of exogenous hormone for embryos that have not begun to desiccate, while desiccating embryos require higher concentrations. Further support comes from the observation that immature embryos cultured prior to dehydration synthesize storage proteins in response to ABA while older immature embryos do not (Finkelstein et al, 1985). This evidence suggests that older embryos are less sensitive to ABA.

It has been suggested that a reduction of ABA in the embryo and surrounding tissue alone is insufficient to evoke a switch to germination (Kermode et al., 1989). Support for this statement comes from several studies. Endogenous ABA levels decline in desiccating *Brassica napus* embryos entering a period of developmental arrest, indicating that factors other than ABA are involved in blocking germination during the desiccating phase (Finkelstein et al, 1985). In addition, whole seeds from fluridone-treated alfalfa pods do not express vivipary i.e. do not precociously germinate before maturity (Xu et al., 1990). In alfalfa, mid-to-late stage embryos excised and placed on water germinate faster and with greater frequency than whole seeds at the same developmental stages (Xu and Bewley, 1991). These results and the observation that isolation of immature embryos from whole seeds often results in germination (Kermode et al., 1989; Xu and Bewley, 1991; Xu et al., 1990) suggest that the surrounding seed environment may have an inhibitory effect on precocious germination.

This raises the question of what additional factor present in the surrounding seed environment causes this inhibition of germination? Xu et al.(1990)and Xu and Bewley (1991) reported that precocious germination of excised immature alfalfa embryos can also be inhibited by application of osmoticum to the culture medium. Furthermore, synthesis of proteins unique to developing embryos *in situ* only occurred in excised immature embryos cultured in the presence of osmoticum, while ABA caused synthesis of proteins which arise during maturation (Xu et al., 1990). Although ABA and osmoticum both inhibit germination, the fact that fluridone-treated alfalfa seeds do not express vivipary suggests that osmoticum may be more important in maintaining embryos in a developmental mode in some species.

2.4.2.1.2 Storage reserve accumulation

ABA has been implicated in the expression of storage protein genes in developing seeds. Most of the evidence comes from studies which found a correlation between high

ABA concentrations and the expression of storage protein mRNAs in embryos developing either *in vitro* or *in vivo*. In *Phaseolus coccineus* embryos with a biphasic ABA accumulation pattern, the second ABA peak coincided with synthesis of vicilin storage protein in embryos at the late cotyledonary stage (Perata et al., 1990). Highest endogenous ABA levels in *Brassica napus* embryos coincide with the accumulation of storage protein reserves and their mRNAs just prior to embryo dehydration, and then ABA concentration declines to low levels until seed maturation (Finkelstein et al, 1985). Precocious germination was observed in immature embryos which had low endogenous ABA at the time of isolation when cultured on hormone-free medium. However, exogenous ABA inhibited germination and enhanced storage protein synthesis in immature embryos cultured at the same developmental stage (Finkelstein et al., 1985).

Storage protein genes that are expressed during the time of high endogenous ABA levels have been identified in several species (reviewed in Dodeman et al., 1997) including wheat (Berge at al., 1989) and pea (Barratt and Clark, 1991) embryos. Expression of an aspartate kinase homoserine dehydrogenase (AK/HSD) gene, which encodes a polypeptide consisting of two enzymes in the biosynthesis of aspartate-family amino acids (i.e. Lys, Thr, Met and Ile), also coincided with the initiation and beginning of storage protein synthesis during embryo development in tobacco (Zhu-Shimoni et al., 1997). In wheat, globulin protein and Em genes are only expressed in the presence of ABA (Berge et al., 1989). Immature embryos cultured prior to the increase in endogenous ABA accumulate the gene products only if exogenous ABA is added to the culture medium. In the absence of hormone, normal embryo maturation and developmental arrest do not occur, resulting in precocious germination. Thus, ABA is required for activation of these genes, whose products are important for supporting normal embryogenesis and maturation (Berge et al., 1989).

The response of globulin and Em genes to ABA does not appear to be embryonic-tissue specific. The mRNAs can still be detected in germinating embryos supplied with exogenous ABA as well as in mature leaves from water-stressed wheat plants in which endogenous ABA levels are elevated. Thus, although these genes are important during embryogenesis and maturation, they appear to have a more general function in response to water stress in developing seed as well as other tissues (Berge et al., 1989).

In pea, two ABA-responsive seed proteins (ABR 17 and ABR 18) have been identified which are synthesized during normal embryogenesis and have an accumulation pattern similar to that of LEA proteins (Barratt and Clark, 1991). Maximum accumulation of mRNAs coincides with high endogenous ABA levels in developing embryos while the proteins reach maximum levels during desiccation. Like the storage proteins in wheat, expression of these two genes is not embryonic-tissue specific. The ABR 18 protein is also present in the testa during early seed development, while the ABR 17 protein is synthesized during germination and in non-stressed leaves and roots (Barratt and Clark, 1991). Although these two genes are ABA-responsive during embryogenesis, their expression in other tissues, and the significant sequence homology of their translation products with pea disease-resistance response proteins suggests that they may be involved in pathogen resistance in the seeds (Barratt and Clark, 1991).

Although most studies have focused on the effect of ABA on storage protein synthesis in developing embryos, several researchers have investigated lipid and carbohydrate accumulation. In developing embryos of *Theobroma cacao*, peak ABA levels coincide with early anthocyanin and lipid accumulation (Pence, 1991). *In vitro* culture of immature cacao embryos on ABA-media increases endogenous ABA levels which in turn increases lipid accumulation. Treating embryos with fluridone decreases endogenous ABA and significantly reduces lipid accumulation, indicating ABA involvement in stimulating lipid accumulation during maturation of cacao embryos (Pence, 1992).

Accumulation of soluble carbohydrates, which occurs during the maturation phase of seed development, has been investigated in wild-type and ABA mutants of

Arabidopsis thaliana seeds (Ooms et al., 1993). The *aba-1,abi3-1* double mutant seeds contained higher total soluble sugars compared to wild-type, suggesting that ABA may not be required for carbohydrate accumulation in Arabidopsis (Ooms et al., 1993).

The effect of environmental conditions on seed growth rate and storage reserve accumulation appear to be regulated by ABA concentration. In *Zea mays*, translocation of ABA from maternal tissue (i.e. leaves) of plants subjected to water-stress prior to ABA accumulation in embryos raises endogenous levels in the immature kernels (Ober and Setter, 1990). The elevated ABA levels had no effect on either the timing or the establishment of starch and protein synthesis in maize endosperm. However, the maternal ABA in kernels of water-stressed plants inhibited endosperm cell division, thus decreasing cell number and final grain size (Ober et al., 1991). Short-day photoperiods increased ABA levels in soybean seeds and stimulated sucrose movement to the seeds, which in turn increased cotyledon cell number and subsequent seed growth for the rest of the storage reserve accumulation phase (Morandi et al., 1990)

2.4.2.1.3 Desiccation tolerance

Orthodox (desiccation tolerant) seeds acquire desiccation tolerance during the late stages of development while recalcitrant (desiccation sensitive) seeds do not (Pence, 1991; Farrant et al., 1996). This is usually followed by a period of seed dormancy, in which the embryo is dehydrated and developmentally arrested (Sussex, 1975; Goldberg et al., 1989). Late embryogenesis-abundant (LEA) proteins, including dehydrins, are a group of proteins that accumulate in developing embryos as they enter the desiccation phase. In peas, degradation of these proteins occurs during germination and correlates with the loss of desiccation tolerance (Baker et al., 1995). The appearance of LEA mRNA coincides with the time of high endogenous ABA levels in the embryo as the seeds approach maturity (Berge et al., 1989; Barratt and Clark, 1991). LEA proteins have been identified in several species, including *Brassica napus* (Finkelstein et al, 1985), wheat (Berge et al., 1989) and *Pisum savitum* (Barratt and Clark, 1991; Baker et al., 1995).

LEAs or dehydrin-like proteins have been implicated in the acquisition of desiccation tolerance in orthodox seed. These proteins are not found in the axes of mature, undried recalcitrant seeds of mangroves and *Barringtonia racemosa*, which are all tropical wetland species (Farrant et al., 1996). However, LEA proteins were identified in mature recalcitrant seeds of a number of temperate species (Farrant et al., 1996). The temperate species had high endogenous ABA levels in the axes of mature fresh seeds whereas the tropical wetland species had very low amounts (Farrant et al., 1996).

The presence of ABA and these proteins in mature recalcitrant seeds appears to be associated with the extent of dehydration or low temperatures to which the seeds are exposed. Imposed dehydration of fresh mature seeds of *Barringtonia racemosa* (a tropical wetland species) over silica gel resulted in increased ABA concentrations and induced the appearance of LEAs in the axes. Hence, this wetland species is genetically capable of expressing these proteins but requires imposed water loss to activate LEA gene expression (Farrant et al., 1996). Further support is provided by the presence of these proteins in the seeds of *Castanospermum australe*, a tropical species, which had been grown in a temperate climate.

The study by Farrant et al. (1996) is limited in that it only focuses on mature recalcitrant seeds. Pence (1991) followed ABA levels in developing embryos of *Theobroma cacao*, a tropical species which also produces recalcitrant seeds. As has been described for other angiosperm seeds, ABA levels peaked during early maturation. This period represents the early stages of lipid accumulation and moisture loss in cacao seeds. Moisture level did not reach below 30% fresh weight during late maturation, unlike the situation in orthodox seeds in which moisture content can be as low as 5-10% (Pence, 1991). Therefore, although ABA levels peak during embryo development in *T. cacao*, they do not stimulate desiccation tolerance. This could be because the embryos lack

ABA-responsive mechanisms for desiccation tolerance or that they have reduced sensitivity to the hormone. The author suggested that the high levels of ABA during embryo development may be essential for stimulation of maturation events, which include storage anthocyanin, lipid and fatty acid synthesis, since exogenous ABA has this effect on cultured immature *T. cacao* embryos (Pence, 1991; 1992).

To further clarify the role played by ABA in seed development, the focus has shifted to studying ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants, which have been isolated in a few species, including *Arabidopsis* (Koornneef et al., 1982; Ooms et al., 1993), tobacco (Marin et al., 1996), tomato (Groot et al., 1991) and viviparous mutants of maize (Neill et al., 1987). In *Arabidopsis thaliana*, *aba* and *abi* mutants produce seeds which are desiccation tolerant, probably due to leakiness of the mutations, while double mutants (*aba,abi*) produce viable seeds which never acquire desiccation tolerance (Ooms et al., 1993). In an effort to clarify the role of ABA in the acquisition of desiccation tolerance in *Arabidopsis*, the researchers compared soluble carbohydrate and protein accumulation in mutant seeds that differed in ABA sensitivity. They found that desiccation-intolerant *aba,abi* double mutants accumulated the most soluble carbohydrates compared to desiccation-tolerant wild-type seeds, indicating that carbohydrates do not play a major role in the acquisition of desiccation tolerance. However, the synthesis of a maturation related protein in wild type seed which was lacking in the double mutant suggests a correlation with desiccation tolerance (Ooms et al., 1993).

The ABA-deficient *sitiens* mutation in tomato results in low endogenous ABA levels in developing seeds (Groot et al., 1991). However, this has no effect on seed dry weight, embryo development or storage protein content and composition. It appears that these processes are not regulated by endogenous ABA in tomato. The contradictory results in Arabidopsis and tomato suggest that the role of ABA in the acquisition of desiccation tolerance is species specific.

2.4.2.1.4 Dormancy

Dormancy is defined as a temporary arrest of visible growth of any plant structure containing a meristem (Arteca, 1996). Seed development in most plants ends with dormancy, a state which allows the mature dehydrated seed to survive until environmental conditions are conducive to germination and early seedling development. However, viviparous plants bypass dormancy and there is uninterrupted progression from embryogenesis to germination. For example, in the mangrove *Rhizophora mangle* (Sussex, 1975) and the viviparous mutant of *Zea mays* (*vp-1*) (Neill et al., 1987), germination occurs while the seed is still attached to the parent plant.

A comparison between embryo development in wild-type and *vp-1 Zea mays* shows some profound differences. Unlike wild-type embryos, viviparous embryos do not desiccate during embryogenesis, suggesting either the absence of the signal for arrested development and inhibition of germination or an inability to respond to the signal (Neill et al., 1987). A similar result was obtained for *Rhizophora mangle* in which embryo water content was also found to be high during late embryogenesis (Sussex, 1975). Neill et al.(1987) found no differences in endogenous ABA content between wild-type and mutant *Zea mays* embryos at all stages of development. Precocious germination of excised young embryos of both genotypes in culture was prevented by exogenous ABA or high osmotic concentration, although mutant embryos were generally less sensitive to ABA than wild-type embryos (Neill et al., 1987). Therefore a decrease in sensitivity to ABA by the mutant could explain the observed expression of vivipary in maize.

Viviparous seeds are invaluable for understanding seed dormancy mechanisms. The observations that immature *Zea* kernels fail to germinate until late in development when endosperm moisture content is low, and the absence of embryo desiccation in viviparous seeds of mangrove imply the development of moisture stress as a trigger for the onset of dormancy (Sussex, 1975; Neill et al., 1987). In ABA-deficient *A. thaliana*, dehydration and growth cessation occur in the absence of ABA. In these seeds, limited access to water is the most important factor preventing precocious germination *in vivo* and promoting developmental arrest (Karssen et al., 1983).

However, several studies have provided evidence for ABA involvement in the induction of dormancy through the use of ABA biosynthetic inhibitors, such as fluridone, during seed development. Treatment of developing wild-type *Zea mays* kernels with fluridone *in vitro* or in the field causes vivipary (Hole et al., 1989). This correlates with reduced levels of endogenous ABA found in embryos cultured in the presence of the inhibitor. The effects of fluridone could be reversed by culturing fluridone-treated maize kernels between the ages of 13-15 DAP (days after pollination) on ABA-containing media. Older kernels still expressed vivipary on the ABA media (Hole et al., 1989). In sunflower, exogenous ABA is effective only if applied to embryos cultured just before natural induction of dormancy (Le Page-Degivry and Garello, 1992). These results indicate that the hormone is required at a critical point in development for the induction of dormancy in maize and sunflower, suggesting that sensitivity to ABA changes during embryo development in these species. An alternate explanation could be the requirement for a second factor along with ABA for the induction of dormancy. Smith et al. (1989) proposed that synthesis of a regulatory protein (VP) is required prior to the induction of dormancy by ABA. Binding of the VP protein to ABA during the critical period could then regulate the induction of dormancy. Using this model, the absence of the VP protein in young sunflower embryos could explain the inability of ABA to induce dormancy in these embryos (Smith et al., 1989 cited in Le Page-Degivry and Garello, 1992). However, it is difficult to evaluate the validity of this proposal as the signal transduction pathway for this hormone is still unknown.

The use of ABA-deficient mutants has also shed some light on the role of ABA on the onset of dormancy. The ABA-deficient mutant seeds of *Arabidopsis thaliana* have lower endogenous levels of the hormone than wild-type seeds during development

(Karssen et al., 1983). These researchers identified a dual origin of ABA, one was regulated by the maternal genotype and peaked half-way through seed development. The second was of embryonic origin and was present at earlier stages of development as well as during maturation. Induction of dormancy only occurred in seeds containing embryos with the dominant *Aba* allele, regardless of the genotype of the mother. Similarly, spraying plants with exogenous ABA raised hormone levels in the seeds, but dormancy was not induced in recessive *aba* seeds. It appears from these results that embryonic ABA is required for induction of dormancy as opposed to maternal ABA in Arabidopsis. Although ABA is necessary for the initiation of dormancy, it is not required for its maintenance since endogenous ABA levels decline during maturation (Karssen et al., 1983). This is contrary to results of Le Page-Degivry and Garello (1992). Application of fluridone to dormant sunflower embryos decreased ABA in the axes and resulted in germination. Exogenous ABA inhibited germination in the fluridone-treated embryos. However, transfer of the same embryos to control medium resulted in germination, suggesting that *in situ* ABA synthesis is required to impose and maintain embryo dormancy in sunflower (Le Page-Degivry and Garello, 1992).

Cytosolic class 1 small heat shock proteins (sHSPs) are thought to have a role in the acquisition of desiccation tolerance and dormancy due to their accumulation pattern in several species, with high levels present in maturing seeds followed by a decline during germination. Expression of class 1 sHSPs is first detected during mid-maturation in Arabidopsis seeds, and coincides with peak embryonic ABA levels and seed storage protein synthesis (Wehmeyer et al., 1996). Using several Arabidopsis seed mutants with reduced sensitivity to ABA, the authors found that seeds of *aba1*, *abi1*, *abi2* and *abi1,abi2* mutants (all of which have greatly reduced dormancy) had wild-type sHSHs levels, suggesting that wild-type levels are not sufficient for seed dormancy. Reduced levels of sHSPs in seeds of the desiccation-tolerant *abi3-1* mutants suggests that wild-type levels are not necessary for the acquisition of desiccation tolerance. However, absence of these proteins in the desiccation-intolerant *abi3-6* seeds supports a possible role in desiccation tolerance (Wehmeyer et al., 1996).

It appears from the above that ABA can have both inhibitory and stimulatory effects at different times during seed development. Although it is by far the most studied of all plant hormones, other classes of hormones have also been identified during embryogenesis.

2.4.2.2 Other hormones during fruit and seed development

Apart from ABA, gibberellins and auxins have been identified in developing angiosperm seeds. Gibberellins are known for their stimulatory effect on cell division and/or cell elongation while auxins stimulate growth by promoting cell elongation (Arteca, 1996). In tomato, determination of the auxin indole-3-Acetic acid (IAA) in whole seeds by ELISA showed two accumulation phases during seed development (Hocher et al., 1992). The first peak coincided with the transition from active cell division following fertilization to the growth phase, which is characterized by the commencement of cell enlargement in the developing embryo. The second peak occurred during the growth phase, and preceded the accumulation of storage reserves which occurs later in the maturation phase (Hocher et al. 1992). IAA was also identified in developing melon seeds, although levels only peaked once (Lee et al. 1997). Rodrigo et al. (1997) identified and quantified gibberellins in pea. Consistent with the known effect of GAs, peak levels of GA_1 and GA_3 coincided with the time of highest growth rate during pea seed development (Rodrigo et al., 1997). These latter researchers also detected GA_4 and GA_7 during early development, although they constituted only a minor proportion of GAs in the seeds.

The effects of GAs and auxins during fruit growth have also been studied. The effect of auxin on fruit growth was reported as early as 1950 from work with strawberry fruits (Nitsch, 1950). An analysis of IAA levels in fruit tissue (placenta and mesocarp) of tomato in the study by Hocher et al. (1992) showed a similar biphasic accumulation pattern as in the seeds, although levels were lower in fruit tissue. It has been hypothesized that IAA in fruit tissue is transported from the seeds, which are the site of IAA biosynthesis in tomato (Hocher et al., 1992) and melon (Lee et al., 1997). However, in parthenocarpic cucumber in which fruit growth occurs in the absence of seed development, high endogenous IAA levels in ovaries correlated with fruit set (Takeno and Ise, 1992), suggesting that ovaries may also synthesize auxin. In melon ovaries, exogenous IAA stimulated activity of cell wall-bound and soluble acid invertases (Lee et al., 1997). Hydrolysis of sucrose to glucose and fructose by these enzymes in ovaries may stimulate sucrose unloading and promote growth by creating a sink in fruit tissue (Lee et al., 1997).

Gibberellins have been identified in developing fruit of citrus (Ben-Cheikh et al., 1997), blueberry (Cano-Medrano and Darnell, 1997) and pea (Rodrigo et al., 1997). Ben-Cheikh et al. (1997) reported that pollination increased the levels of several GAs in developing ovaries of citrus. Ovary abscission was high in unpollinated ovaries and in pollinated ovaries treated with paclobutrazol, a GA biosynthetic inhibitor. The low levels of GAs in unpollinated ovaries could be reversed by exogenous GA_3 application which in turn reduced ovary abscission, suggesting that GAs may play a role in the completion of ovary development (Ben-Cheikh et al., 1997). In addition, exogenous GA_3 application to unpollinated ovaries caused parthenocarpic fruit set in blueberry (Cano-Medrano and Darnell, 1997) and GA_3 or GA_1 had the same effect in pea (Rodrigo et al., 1997).

Ethylene has well known effects on fruit ripening. Levels increase with the onset of fruit ripening, and this process can be delayed by the use of ethylene action inhibitors such as CO_2 and silver (Arteca, 1996). The sharp increase in ethylene production is believed to control a series of biochemical and physiological changes that result in fruit ripening. The ethylene biosynthetic pathway has been well studied and the two key enzymes involved, ACC synthase (ACS) and ACC oxidase (ACO) have been identified.

Results from several studies suggest that upregulation of ACO and ACC genes prior to fruit ripening is under developmental regulation and allows autocatalytic ethylene production during fruit ripening (reviewed in Lelièvre et al., 1997). The ETR1 and NR genes which encode ethylene receptors have been identified with the use of ethylene-receptor mutants in Arabidopsis (Chang et al., 1993) and tomato (Lanahan et al., 1994) respectively. NR transcription peaks in wild-type tomato at the time of high endogenous ethylene levels suggesting upregulation of receptors during fruit ripening. In contrast, ethylene synthesis is at a much lower level in the *Nr* mutants and expression of receptors is minimal (Lanahan et al., 1994). Exogenous ethylene stimulates NR gene expression in wild-type fruit at the mature-green stage suggesting that transcription of this gene is under developmental control and as well as under ethylene regulation (Wilkingson et al, 1995).

In summary, plant hormones appear to play a central role during seed development. The emergence of molecular genetic studies with hormone-deficient and hormone-insensitive mutants in *Arabidopsis*, particularly for ABA and ethylene, has allowed considerable progress to be made towards elucidating the role of plant hormones during this process.

2.4.3 Plant hormones during embryogenesis in conifers

2.4.3.1 Zygotic embryogenesis

Very few studies have investigated changes in hormone levels during seed development in conifers. Kapik et al. (1995) used ELISA to quantify ABA levels during zygotic embryogenesis in loblolly pine. They reported that highest concentrations occurred during early embryo development in both embryos and megagametophytes and this was followed by a decline to low levels during maturation. Carrier et al. (1999) obtained similar results in white spruce. The latter researchers also reported that highest ABA concentrations occurred in megagametophytes prior to fertilization. These results are unlike the accumulation pattern observed in a large number of angiosperms (Rock and Quatrano, 1995). However, the increase in hormone levels in embryos of both loblolly pine and white spruce preceded the accumulation of storage reserves, suggesting hormonal involvement in this process (Kapik et al., 1995, Carrier et al., 1999).

In another study with white spruce seeds, Kong et al (1997) reported similar results to those of angiosperms, in which ABA concentration increased from the precotyledonary stage through to the late maturation stage. These results are not in agreement with those by Carrier et al. (1999), who used a different method of analysis, although both studies utilized the same species.

Kong et al. (1997) also identified and quantified endogenous auxin and gibberellins during white spruce seed development. GA_4 levels were high during early embryo development while GA_9 peaked during later stages, suggesting GA involvement during white spruce embryogenesis (Kong et al., 1997). In this study, GA_7 levels remained low throughout the study period.

Kong et al., (1997) reported a biphasic auxin accumulation pattern. IAA levels peaked at pollination, declined during the proembryo stage and then increased from the club-shaped embryo until seed maturation. High levels of IAA in the megagametophyte during pollination preceded rapid embryo growth in the first week post-fertilization (Kong et al., 1997). They suggested that the second IAA peak may be essential for subsequent embryo growth from the club-shaped stage through to the cotyledonary stage (Kong et al., 1997). These results are consistent with those by Sandberg et al. (1987) who previously reported high auxin levels at the times of rapid cell division during embryogenesis and germination in *Pinus sylvestris*.

Expression of seed dormancy in Douglas fir seems to be under ABA regulation (Jarvis et al., 1997; Bianco et al., 1997). *In vitro* culture of dormant seeds resulted in poor germination and was accompanied by a dramatic decrease in ABA content of the seed coats while megagametophytic and embryonic ABA levels increased (Bianco et al., 1997). Dormant seeds cultured in the presence of fluridone germinated fully, suggesting that inhibition of ABA synthesis permitted germination to occur. Because of these results, Bianco et al. (1997) proposed that *de novo* ABA synthesis in embryos and megagametophytes is required for the expression of seed dormancy in Douglas fir.

Although there is an obvious requirement for studies investigating the role of plant hormones during conifer zygotic embryogenesis, the above studies indicate that plant hormones are involved in this process.

CHAPTER 3

Douglas fir megagametophyte development

3.1 Introduction

Descriptions of seed development in gymnosperms have an extensive literature at the light microscope level (see Singh, 1978 for review). The anatomical studies describing this process in conifers have provided invaluable fundamental information on events that occur at different stages of megagametophyte and embryo development (Schopf, 1943; Allen, 1946; 1947a; 1947b, Grob et al., 1999; Allen and Owens, 1972). More recently, studies focusing on megagametophyte development just prior to fertilization and during proembryo development were done at the ultrastructural level (Owens and Morris, 1990; 1991; Runions and Owens, 1999a; 1999b; Anderson and Owens, 1999). The ultrastructural studies were performed in an effort to identify the basis for low seed production in conifer seed orchards (Runions and Owens, 1999a), as well as to investigate cytoplasmic inheritance of chloroplasts and mitochondria in Douglas fir (Owens and Morris, 1990; 1991), interior spruce (Runions and Owens, 1999b) and *Taxus* (Anderson and Owens, 1999).

In addition to anatomical descriptions of zygotic embryogenesis in conifers, storage reserve accumulation, which begins during early embryo development (Schopf, 1943), has been the subject of several studies. Molecular and biochemical investigations of protein accumulation in conifer seeds determined that crystalloid proteins were a major constituent of the storage proteins in mature seeds (Gifford, 1988; Baker et al., 1996; Hakman et al., 1990; Misra and Green, 1990; 1991). The accumulation of lipids has been investigated during zygotic embryogenesis in several species including *Pinus resinosa* (Gates and Greenwood, 1991), *Pseudotsuga menziesii* (Owens et al., 1993) and *Picea glauca* (Carrier et al., 1999; Krasowski and Owens, 1993). Biochemical changes in soluble sugars have also been described during seed development in *Pinus resinosa* (Gates and Greenwood, 1991) and *Pseudotsuga menziesii* (Owens et al., 1993).

Although conifer zygotic embryogenesis and storage reserve accumulation have been described over the course of seed development, the possible involvement of plant hormones in regulating these processes has scarcely been addressed. However, in order for seed development to be fully described, it is imperative to study embryogenesis and megagametophyte development anatomically and histochemically, and then relate these changes to the endogenous levels of plant hormones. With this information, the question of whether morphological and physiological changes during seed development are independent of one another can then be addressed.

The objective of this study was to anatomically and histochemically analyze megagametophyte development, zygotic embryogenesis and nutrient reserve accumulation during seed development in Douglas fir. This study also provided information on the developmental stages of megagametophytes that were used for the megagametophyte culture and hormonal analysis experiments in Chapters four and five of this thesis, respectively.

3.2 Materials and Methods

3.2.1 Plant Materials

One clone of Douglas fir trees (clone 3265) at the Glyn Road Research Station in Victoria was kindly provided by Dr. Joe Webber of the BC Ministry of Forests Research Branch. Bagged seed cones were control pollinated with pollen obtained from trees growing at the University of Victoria campus. Cones were collected weekly for a period of 11 weeks, starting 7 weeks after pollination (WAP). Five megagametophytes were isolated weekly without the nucellus under a dissecting microscope. The samples were fixed in 2.5% glutaraldehyde in 0.075 phosphate buffer at pH 7.2 and stored at 4^0C until they were processed in glycol methacrylate for sectioning.

3.2.2 Anatomical analysis of megagametophyte development

The fixed weekly megagametophyte samples were washed three times over two days in 0.075 phosphate buffer (pH 7.2). They were left to wash overnight, were initially washed for 5 hr. on the second day, and then left in phosphate buffer overnight. The samples were then dehydrated in an ethanol series (30, 50 and 70 % for 1 hr each, 95% for 3 hr., and twice in 100% ethanol for 30 min. each). This was followed by pre-infiltration overnight in 1:1 (v/v) ethanol : Technovit 7100 base containing 1% (w/v) hardener I (Marivac, Canada). The megagametophytes were then left in Technovit 7100 base containing 1% (w/v) Technovit 7100 hardener I to infiltrate for 5 days. The infiltration solution was discarded and fresh solution added on days 6 and 7. Megagametophytes isolated 7-11 WAP were embedded in glycol methacrylate after 7 days of infiltration. Samples from weeks 12-17 were infiltrated for 8 days prior to embedding. The embedding solution used was composed of the Technovit 7100 base containing 1% (w/v) Technovit 7100 hardener I, supplemented with 6.25% (v/v) hardener II. The blocks were sectioned at 5 µm using a Leica SM 2400 sledge microtome with a 16 cm tungsten carbide knife. Each section was placed on a drop of distilled water on a glass microscope slide, flattened, and allowed to dry at room temperature. The developmental stages of the megagametophytes were determined by observing stained slides of the weekly samples under the light microscope.

3.2.3 Histochemical analysis of megagametophyte development

Sections were stained with periodic acid / Schiff's reagent (PAS) for insoluble carbohydrates according to O'Brien and McCully (1981), Aniline Blue Black for proteins (Jensen, 1962), and Sudan Black B for lipids (O'Brien and McCully, 1981).

3.3 Results

3.3.1 Anatomical analysis of megagametophyte development

During the 11 week period analyzed, the outward appearance of the megagametophytes changed from translucent during the first collection dates to a milky-white color with further development. In the first week studied (7 WAP), megagametophytes were at the early central cell stage. The primary neck cell had already divided to give rise to the neck cells at the micropylar end of the archegonium (Figure 2a). The central cell cytoplasm had numerous large and lightly stained vacuoles (Figure 2a). In the following week (8 WAP), the vacuoles within the central cell were smaller in size and the cytoplasm more intensely stained (Figure 2b). An asymmetric mitotic division by the central cell resulted in a smaller ventral canal cell and the much larger egg cell at 9 WAP (Figure 2c). By 10 WAP, megagametophytes were at the mature egg cell stage, with the nucleus more centrally located within the large egg cell (Figure 2d).

Embryogenesis was initiated 10 WAP, which is when fertilization occurred. By 11 WAP, the zygote had divided to give rise to the proembryo, which had migrated to the chalazal end of the archegonium within the megagametophyte (Figure 2e). Embryogenesis continued with the elongation of the suspensor forcing the apical tier of the proembryo out of the archegonium and into the megagametophyte during week 12 (Figure 2f). The prothallial cells in the central region of the megagametophyte had not degenerated to form the corrosion cavity at this stage. Early embryo development continued in the following four weeks (13-16 WAP). The archegonia collapsed and suspensor elongation forced the dominant early embryo deeper into the megagametophyte. By 15 WAP, the embryo had divided to give rise to the embryonal mass stage early embryo, located within the corrosion cavity (Figure 3a). Continued cell divisions by the embryos resulted in the club-shaped embryo, polarized into the root cap and the stele promeristem, during the following week. Histodifferentiation continued

Figure 2A. Light micrograph of a glycol methacrylate embedded Douglas fir megagametophyte isolated 7 WAP showing the early central cell (cc) with large lightly stained vacuoles in the cytoplasm, central cell nucleus (n), neck cells (nc) and prothallial cells (pc). Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 50 µm.

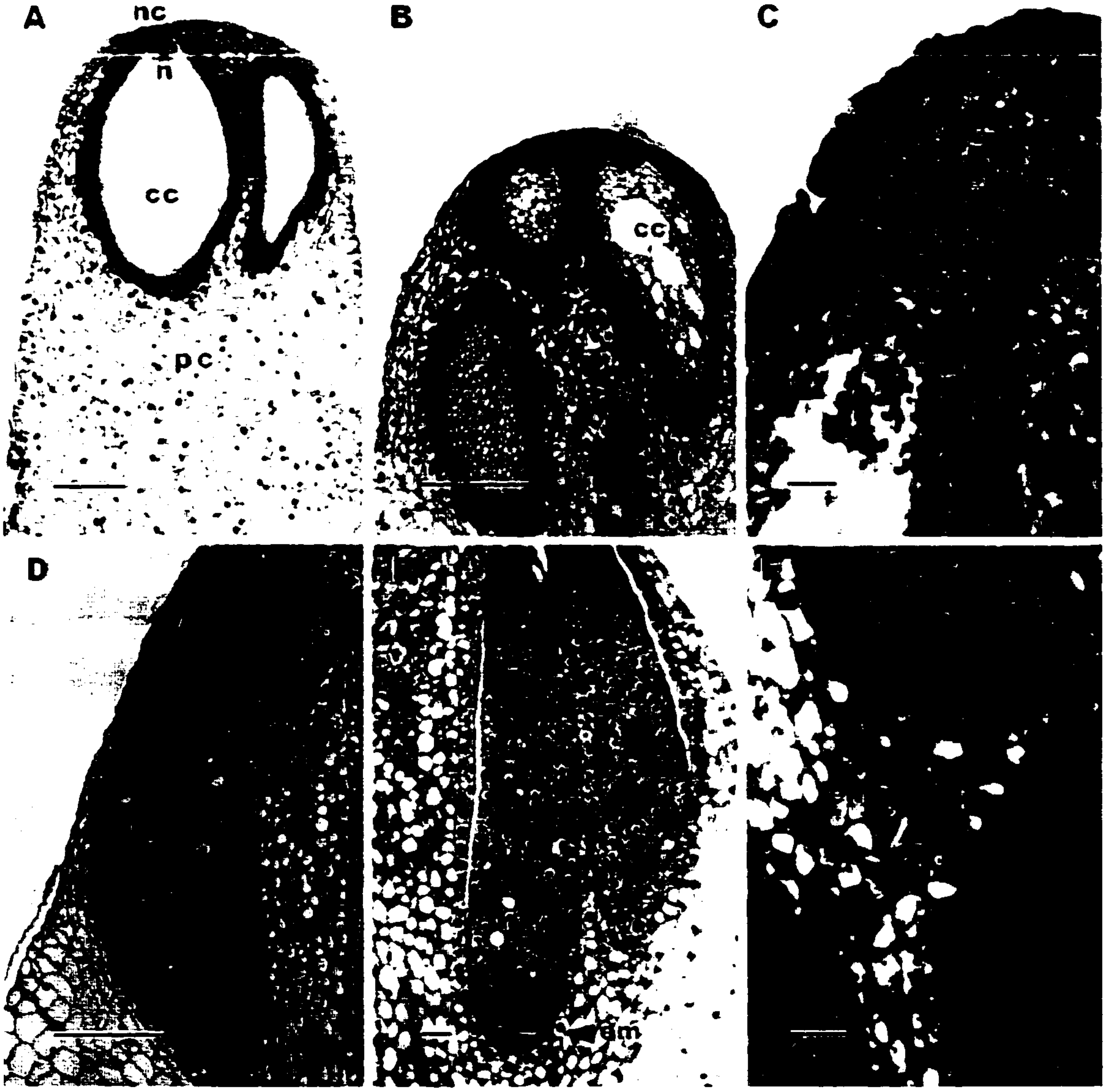
Figure 2B. Late central cell stage megagametophyte isolated 8 WAP. The cytoplasm is more intensely stained and contains numerous smaller vacuoles. Stained with Aniline Blue Black. Scale bar = 50 µm.

Figure 2C. Early egg cell (ec) stage megagametophyte isolated 9 WAP showing the ventral canal cell (vcc) and the neck cells. Stained with Aniline Blue Black and periodic acid/Schiff's reagent. Note starch in the neck cells. Scale bar = 10 µm.

Figure 2D. Mature egg cell stage megagametophyte isolated 10 WAP with the nucleus (n) centrally located within the egg cell (ec). Stained with Aniline Blue Black. Scale bar = 50 µm.

Figure 2E. An archegonium containing a proembryo (em) in a megagametophyte isolated 11 WAP. The proembryo has already migrated to the chalazal end of the archegonium. Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 µm.

Figure 2F. A megagametophyte isolated 12 WAP showing the elongation of the embryonal suspensor forcing the early embryo (em) into the prothallial cells. Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 µm.


A
nc
n
cc
pc
B
cc
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during week 17, which was the last date that material was collected in this study. The early maturation stage embryos had a well established root cap, and root and shoot apical meristems were differentiating (Figure 3b).

3.3.2. Histochemical analysis of megagametophyte development

The accumulation of storage products in megagametophytes and embryos was separated both temporally and spatially during the 11 week period studied. Prior to fertilization (7-9 WAP), histochemical staining of central cell and egg cell stage megagametophytes yielded negative results for starch, protein and lipid reserves within the prothallial cells (Figures 2a and 3c). However, small starch grains were observed in the neck cells of early egg cell stage megagametophytes (Figure 2c). Storage reserves in the prothallial cells were first visible at 11 WAP, when megagametophytes were at the proembryo stage. During this week, starch was the only nutrient reserve deposited, and was concentrated in the central region of the megagametophytes adjacent to the chalazal end of the archegonia (Figure 3d). With the beginning of early embryo development (12-13 WAP), starch accumulation continued toward the chalazal end of megagametophytes, but was restricted to prothallial cells in the corrosion cavity region, while cells in the periphery remained devoid of any storage products (Figure 3e).

Proteins were first detected in prothallial cells in the peripheral region of megagametophytes isolated 14 WAP (Figure 3f). Lipids were first detected histochemically in prothallial cells in the peripheral region of megagametophytes at 15 WAP (Figure 4a), when embryos were at the embryonal mass stage. During this week, protein accumulation continued in the peripheral cells (Figure 4c) while abundant starch was deposited in the prothallial cells surrounding the corrosion cavity (Figure 4b). No storage reserves were detected in the embryonal mass stage embryos (Figure 3a).

In the last two weeks analyzed, proteins (Figure 4d and 4e) and lipids (Figure 4f) were deposited in cells in the periphery of the megagametophytes, while starch was

Figure 3A. An embryonal mass stage embryo in the corrosion cavity of the megagametophyte isolated 15 WAP. Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 μm.

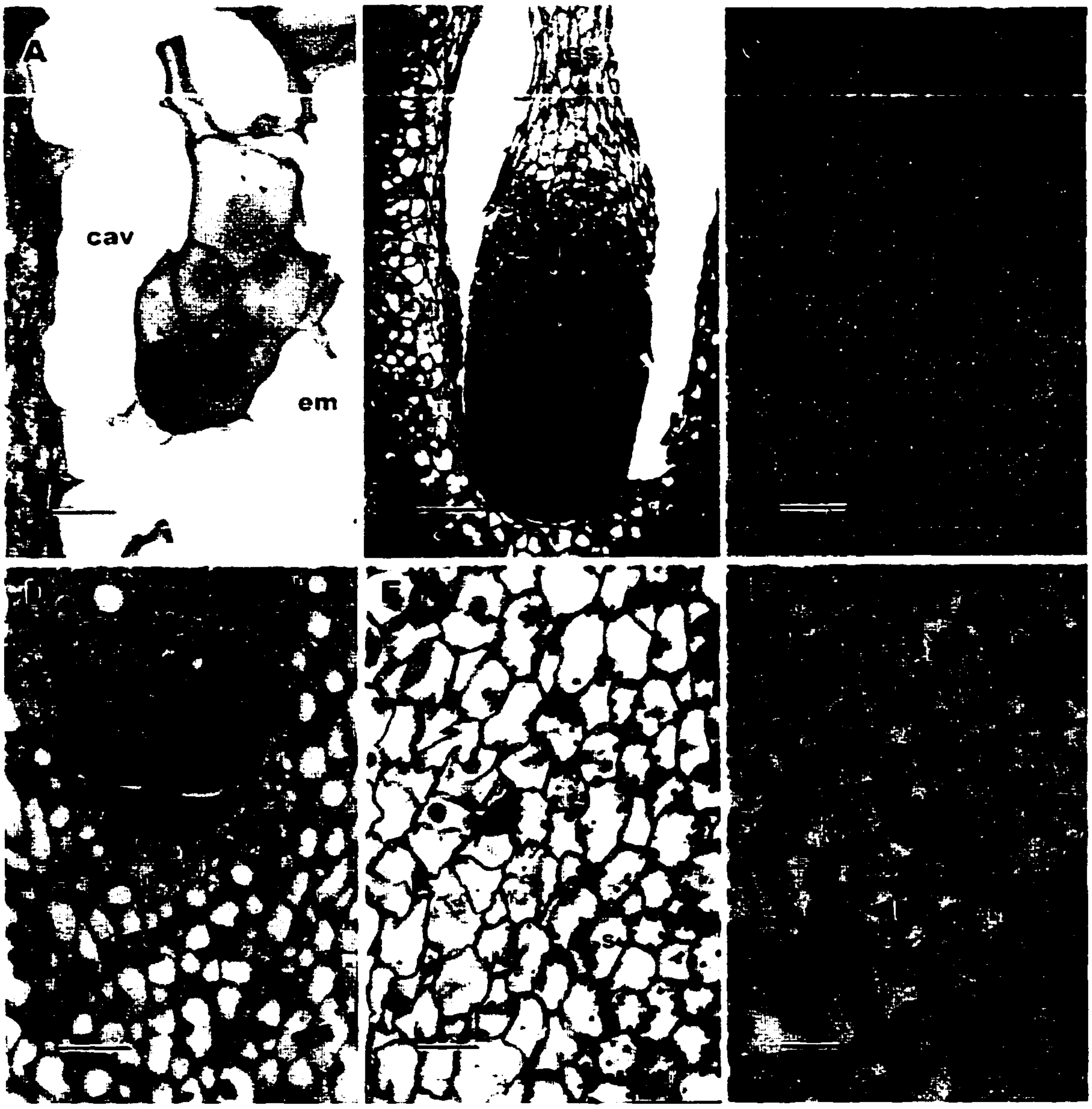
Figure 3B. Median longitudinal section of an embryo within the corrosion cavity (cav) at 17 WAP showing starch grains within the well developed root cap (r) and embryonal suspensor cells (es). Stained with periodic acid/Schiff's reagent and Aniline Blue Black. Scale bar = 25 μm

Figure 3C. The central region of the late central cell stage megagametophyte isolated 8 WAP. The prothallial cells within this region do not contain any storage reserves (arrow head). Stained with Aniline Blue Black. Scale bar = 10 μm.

Figure 3D. The chalazal half of the archegonium in a megagametophyte isolated 11 WAP showing the proembryo and starch grains (s) in the central region of the megagametophyte prior to formation of the corrosion cavity. Stained with periodic acid/Schiff's reagent and Aniline Blue Black. Scale bar = 10 μm.

Figure 3E. A portion of the megagametophyte isolated 13 WAP showing numerous starch grains (s) in the central region and the absence of storage reserves in the prothallial cells in the peripheral region. Stained with periodic acid/Schiff's reagent. Scale bar = 10 μm.

Figure 3F. A portion of the megagametophyte isolated 14 WAP showing the presence of proteins (p) in prothallial cells in the peripheral region and starch (s) accumulation in the central region. Stained with periodic acid/Schiff's reagent and Aniline Blue Black. Scale bar = 10 μm.


A
cav
em
D
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s

Figure 4A. A portion of the peripheral region of the megagametophyte isolated 15 WAP showing the megaspore membrane (mm) and lipids (l) in the prothallial cells. Stained with Sudan Black B and periodic-acid/Schiff's reagent. Scale bar = 10 µm.

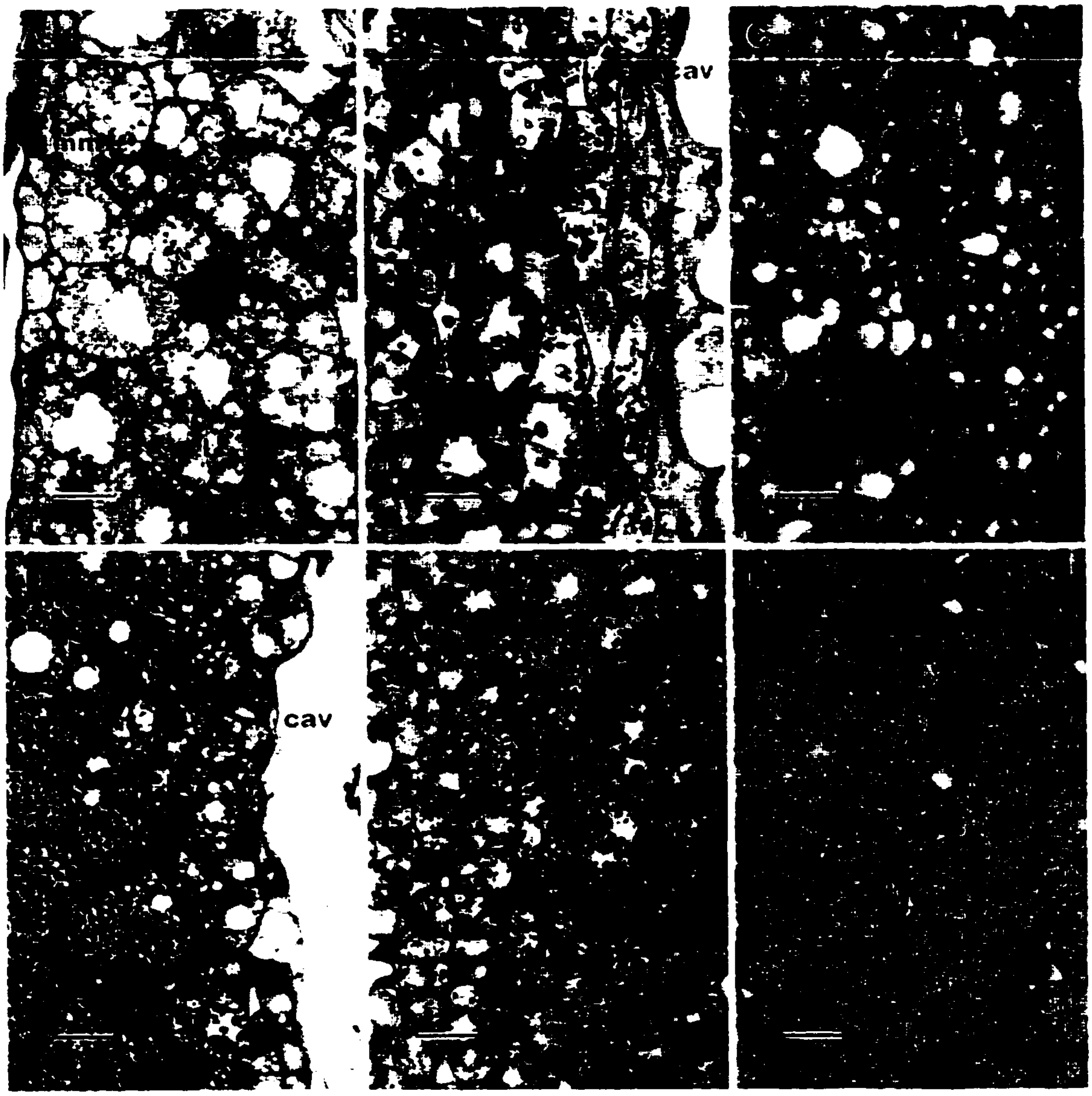
Figure 4B. A portion of the central region of the same megagametophyte shown in Figure 3a isolated 15 WAP showing numerous starch grains (s) in the prothallial cells surrounding the corrosion cavity (cav). Note the absence of starch in the degenerating cells right next to the corrosion cavity (arrow heads). Stained with periodic-acid/Schiff's reagent. Scale bar = 10 µm.

Figure 4C. A portion of the peripheral region of the same megagametophyte shown in Figures 3a and 4b isolated 15 WAP showing an increase in protein (p) content of megagametophyte cells. Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 µm.

Figure 4D. A portion of the micropylar end of the megagametophyte isolated 16 WAP showing abundant protein (p) in the prothallial cells and the absence of starch in cells next to the corrosion cavity (cav). Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 µm.

Figure 4E. A portion of the chalazal end of the same megagametophyte shown in Figure 4d isolated 16 WAP showing starch (s) accumulation in cells in the central region next to the corrosion cavity and abundant protein (p) in the peripheral cells. Stained with Aniline Blue Black and periodic-acid/Schiff's reagent. Scale bar = 10 µm.

Figure 4F. The peripheral region of the megagametophyte isolated 17 WAP showing an increase in lipid deposition (l). Stained with Sudan Black B and periodic-acid/Schiff's reagent. Scale bar = 10 µm.


av
cav

concentrated at the chalazal end in cells around the corrosion cavity (Figure 4e). The prothallial cells around the corrosion cavity at the micropylar end of the megagametophytes contained abundant protein whereas starch was no longer detected (Figure 4d). The only storage product present in embryos at 17 WAP was starch, which was localized in the root cap and the embryonal suspensor cells (Figure 3b).

3.4 Discussion

The stages of megagametophyte development and zygotic embryogenesis observed in this experiment were similar to the pattern outlined by Owens et al. (1993) and Allen and Owens (1972) for Douglas fir, and by Singh (1978) for members of the Pinaceae. The 11 week period analyzed in this study encompassed megagametophyte development from the early central cell stage prior to fertilization, through the proembryo stage, and terminated during early maturation (see Table 1).

Owens et al. (1993) suggested that the precursors for storage reserves already exist in soluble, mobile forms within the ovules at fertilization. Over the course of seed development, the less mobile proteins, lipids and carbohydrates are synthesized. Starch was the first storage reserve detected in the prothallial cells of megagametophytes at the proembryo stage in the present study. This is in agreement with the results of a similar histochemical study by Owens et al. (1993) in Douglas fir, who reported the localization of starch in the area of the megagametophyte that later collapsed to form the corrosion cavity. Suspensor elongation pushes the proembryo out of the archegonium, and starch is rapidly hydrolyzed as the prothallial cells break down to enlarge the corrosion cavity during early embryo development (Owens et al., 1993). The deposition of starch in prothallial cells surrounding the corrosion cavity continued toward the chalazal end during early embryogenesis. An increase in hexose sugars was noted in megagametophytes at a similar developmental stage in a study by Gates and Greenwood (1991) in *Pinus resinosa*.

Table 1. Time scale of developmental events in pre-fertilization and postfertilization megagametophytes as a function of weeks after pollination (WAP)

(WAP)	Developmental stage	Storage reserves in megagametophytes or embryos
7	early central cell	
8	late central cell	
9	early egg cell	starch (neck cells)
10	mature egg cell / fertilization	
11	proembryo	starch (central region of megagametophyte)
12	early embryo / no corrosion cavity	starch (central region of megagametophyte)
13	early embryo in corrosion cavity	starch (central region of megagametophyte)
14	early embryo	starch (central region of megagametophyte) proteins (peripheral cells of megagametophyte)
15	early embryo / embryonal mass stage	starch (central region of megagametophyte) lipids and proteins (peripheral cells of megagametophyte)
16	polarized embryo	starch (central region of megagametophyte and root cap and suspensor cells of embryo), lipids and proteins (peripheral cells of megagametophyte)
17	early maturation	starch (root cap of embryo), lipids and proteins in peripheral cells of megagametophyte whereas starch around corrosion cavity

In the present study, lipids and proteins began to accumulate in the peripheral cells of megagametophytes at the embryonal mass stage. This is similar to what was reported in white spruce by Krasowski and Owens (1993) and in Douglas fir (Owens et al., 1993). An increase in the lipid and protein content of megagametophytes containing embryos at the precotyledonary stage was also reported by Gates and Greenwood (1991) in *Pinus resinosa.*

The appearance of storage reserves in the zygotic embryo of white spruce occurs in a sequence, with starch being deposited first, followed by lipids and lastly proteins (Krasowski and Owens, 1993). However, in Douglas fir, proteins were detected ultrastucturally in club-shaped embryos prior to lipid deposition, which occurred during cotyledon initiation (Owens et al., 1993). Support for the results of Owens et al. (1993) for protein deposition comes from a molecular study by Flinn et al. (1991) in interior spruce, who also detected storage proteins in embryos as early as the club-shaped stage. In the present study, proteins and lipids were not detected in the embryos prior to and during cotyledon initiation by light microscopy, but starch was evident in the root cap and the embryonal suspensor cells. The spatially localized accumulation of starch in the root cap was also observed by Krasowski and Owens (1993) and Owens et al. (1993) in embryos at the club-shaped stage and during cotyledon initiation. It appears that the major deposition of proteins and lipids occurs in embryos at the maturation stage during cotyledon elongation (Owens et al., 1993; Krasowski and Owens, 1993). A molecular study by Flinn et al. (1991) yielded similar results for protein accumulation during embryogenesis in interior spruce.

The histochemical study of storage reserve accumulation in megagametophytes and embryos revealed that the deposition of starch, proteins and lipids was separated both temporally and spatially over the course of seed development. The precise manner in which these events are regulated *in situ* still remains to be elucidated. In this study, as well as that by Owens et al. (1993), the prothallial cells did not contain any storage products prior to fertilization, suggesting that the presence of an embryo is required to initiate reserve deposition. This in turn suggests that the megagametophyte must receive some sort of signal when embryogenesis is initiated. However, whether this signal is hormonal in nature still remains to be established. An analysis of the plant hormones present in megagametophytes at this developmental stage may be the first step in answering this question.

From the results of this study, as well as those by Krasowski and Owens (1993) and Owens et al. (1993), lipid and protein deposition in megagametophytes was limited to prothallial cells in the periphery, whereas starch was restricted to cells around the corrosion cavity. In the embryos, starch accumulation was also spatially confined to cells of the suspensor and the root cap. Krasowski and Owens (1993) suggested that starch may function as a temporary storage reserve form, which could be readily hydrolyzed in megagametophytes to fulfill the energy requirements of the developing embryo. Support for this statement appears to come from the study by Gates and Greenwood (1991), who reported an increase in hexose sugar content of gametophytic supernatant from the proembryo to the early cotyledonary stages during seed development in *Pinus resinosa.* Owens et al. (1993) also found that megagametophytes had a higher soluble sugar content during early embryogenesis compared to mature seeds in Douglas fir. At seed maturation, soluble sugars comprised only 2 % of the megagametophyte dry weight (Owens et al., 1993).

A recent study by Stone and Gifford (1999) in loblolly pine determined that lipids made up over half of the storage reserves at seed maturation. Similar results were obtained by Carrier et al. (1999) in white spruce and Owens et al. (1993) in Douglas fir. These results are in agreement with Krasowski and Owens (1993), who suggested that lipids may represent a more long-term storage reserve form in the developing conifer seed. It appears that proteins also represent a long-term storage reserve form. In Douglas fir, proteins made up 16 % of the megagametophyte dry weight in mature seeds (Owens et al., 1993). Crystalloid proteins represent a large proportion of the storage proteins (Gifford, 1988; Baker et al., 1996; Misra and Green, 1990; 1991), and they are rapidly mobilized to support embryo development at seed germination (King and Gifford, 1997).

CHAPTER 4

In vitro culture of Douglas fir megagametophytes

4.1 Introduction

The demand for genetically improved stock for reforestation of conifers has led to the establishment of tree improvement programs for a number of economically important species. However, the supply of seed from seed orchards is sometimes inadequate. This is due to the production of low calibre seed with limited viability resulting from developmental variations at maturity (Owens, 1995). In an effort to identify hindrances to seed production, studies investigating the reproductive biology of several species have been done. Conifer megagametophyte and embryo development have been investigated at the molecular, biochemical, and anatomical levels. Studies at the molecular level have centered on analyzing expression patterns for genes encoding seed storage proteins during development (Leal and Misra, 1993; Baker et al., 1996) and translation of the mRNAs into proteins (Misra and Green, 1991; Flinn et al., 1991). Biochemical studies characterizing the nature of these storage proteins in mature seeds have been done in *Pinus* (Gifford, 1988; Baker et al., 1996; Lammer and Gifford, 1989), *Picea* (Hakman et al., 1990; Flinn et al., 1991; Misra and Green, 1990; 1991) and *Pseudotsuga* (Green et al., 1991). Anatomical investigations of megagametophyte and embryo development include studies in *Taxus brevifolia* (Anderson and Owens, 1999), *Picea glauca* (Krasowski and Owens, 1993), *Pseudotsuga menziesii* (Grob et al., 1999; Owens et al., 1993; Owens and Morris, 1990; 1991; Takaso and Owens, 1994), interior spruce (Runions and Owens, 1999a; 1999b), *Larix* (Schopf, 1943) and *Podocarpus totara* (Wilson and Owens, 1999).

Factors limiting seed production have been identified and they include failure to initiate cones, ovule abortion, and insufficient pollination (Owens, 1995). Solutions to some of these problems have been proposed. The use of gibberellic acid to enhance flowering in conifers addresses the problem of inadequate cone initiation (Owens, 1991; Pharis and King, 1985). The main cause of ovule abortion in species such as western red cedar (Owens et al., 1990) and Douglas fir (Owens et al., 1991), is inadequate pollination. Supplemental mass pollination is used to overcome this problem in some seed orchards (Webber, 1991). Postzygotic incompatibility responses due to selfing lead to embryo abortion during early seed development (Owens, 1995). *In vitro* culture methods, such as embryo rescue, may be useful in overcoming this obstacle to seed production. Alternatively, selfing and inadequate pollination may be reduced in seed orchards by supplemental mass pollination with out-cross pollen (Webber, 1991).

Takaso and Owens (1994) suggested that prezygotic selection mechanisms, acting through secretions from the ovule, may hinder pollen from germinating, preventing fertilization in Douglas fir. In vitro fertilization (IVF), which requires the utilization of controlled conditions for pollen germination and pollen tube growth, may provide a means of bypassing these prezygotic incompatibility responses. Intraspecific crosses have been successfully done in vitro by Fernando et al. (1997; 1998) utilizing Douglas fir as the study species.

To use megagametophytes for IVF, an important developmental characteristic is exploited: megagametophyte development progresses normally not only in pollinated ovules, but in unpollinated ones as well. A number of genera show this developmental characteristic in the Cupressaceae and Pinaceae (Owens, 1995), including Douglas fir (Owens et al., 1991). It is this aspect of conifer species in the aforementioned families that makes them amenable to IVF experiments. Megagametophytes bearing egg cells can be dissected out of unfertilized ovules and co-cultured with germinated pollen to achieve fertilization. This method allows the investigation of events occurring just before and at fertilization under controlled conditions, and may be invaluable in clarifying prezygotic incompatibility response mechanisms in conifers (Dumont-BéBoux et al., 1998). Evidence that breeding barriers between genera can be bypassed in vitro was provided by

Dumont-BéBoux et al. (1998), who achieved gamete delivery between Sitka spruce (*Picea sitchensis*) pollen and *Larix* x *eurolepis* megagametophytes.

The major constraint to IVF in conifers is the rapid decline of eggs in cultured megagametophytes, which limits the time during which IVF experiments can be done. The number of archegonia in Douglas fir ranges from four to six per megagametophyte (Allen and Owens, 1972), with four archegonia being most common (Fernando et al., 1998). Fernando et al. (1998) reported that after ten days in culture, most (>95%)of the eggs had degenerated. These results suggest that culture conditions need to be optimized to prolong egg viability in vitro. In addition, establishing culture conditions that promote further development of immature megagametophytes to the egg cell stage may widen the window for IVF experiments.

Fernando et al. (1998) achieved four-nucleate proembryo development from IVF in Douglas fir, which marked the first report of successful employment of this technology in any gymnosperm. However, in order for this technique to realize its full potential and be incorporated into tree improvement programs for conifers, the embryos must be rescued and raised. Historically, all attempts to rescue embryos soon after fertilization have failed in conifers (Sterling, 1949; Gates and Greenwood, 1991). Embryo rescue has been more successful with isolated megagametophytes containing embryos at least at the early embryo stage in Douglas fir (von Aderkas et al., 1997), or fully mature in *Pinus* (Stone and Duffield, 1950; Sacher, 1956; Haddock, 1954).

In angiosperms, breeding plants have been regenerated from in vitro culture of fertilized embryo sacs of maize (Matthys-Rochon, 1998; Mòl et al., 1993; 1995; Campenot et al., 1992). These studies illustrated the importance of supplementing culture media with plant hormones and a carbon source to promote development of embryos into plants. In conifers, culture media containing plant hormones, organic nitrogen and carbon sources are generally used for somatic embryogenesis in several species. Because most of the embryogenic cultures are derived from immature zygotic embryos as the primary explants (Hakman et al., 1985; Simola and Santanen, 1990; von Aderkas et al., 1990; Thompson and von Aderkas, 1992; Dunstan et al., 1988; Becwar et al., 1990; Gupta et al., 1988), somatic embryogenesis in these studies is a form of embryo rescue. The carbon source is usually sucrose (see Bonga and von Aderkas, 1992). Organic nitrogen sources used in the media include casein hydrolyzate (see Bonga and von Aderkas, 1992 for review) and the amino acids arginine and glutamine (Simola and Santanen, 1990; for reviews, see Hohtola, 1995; Bonga and von Aderkas, 1992). Plant hormones also play a central role in somatic embryogenesis systems for conifers. The importance of auxin and cytokinin for initiation and maintenance of embryogenic cultures has been reviewed (von Arnold et al., 1995; Wilson and Thorpe, 1995; Klimaszewska, 1995; John et al., 1995; Afele and Saxena, 1995). Somatic embryo maturation requires the transfer of the embryos from media containing auxin and cytokinin onto media supplemented with abscisic acid (ABA) (Wilson and Thorpe, 1995; Jalonen and von Arnold, 1991; Lelu et al., 1995. Dunstan et al., 1988; Attree et al., 1992; Becwar and Feirer, 1989; Becwar et al., 1990; Gutman et al., 1996).

In contrast to their widespread use in somatic embryogenesis systems, plant hormones have been identified and quantified during seed development in only a few conifer species. Endogenous ABA levels have been investigated during zygotic embryogenesis in white spruce (Kong et al., 1997; Carrier et al., 1999) and loblolly pine (Kapik et al., 1995), and in dormant seeds of Douglas fir (Bianco et al., 1997). Auxin and gibberellic acid levels have been reported in developing white spruce seeds (Kong et al., 1997). Sandberg et al. (1987) reported high auxin levels at the time of rapid cell division during zygotic embryogenesis in *Pinus sylvestris*. The identification of plant hormones during seed development in conifers, coupled with their predominant use during somatic embryogenesis, suggests that plant hormones may be essential for embryo and megagametophyte development.

The objectives of this study were to investigate whether: 1) Douglas fir megagametophytes isolated prior to fertilization could be cultured, and 2) embryos could be rescued from pollinated megagametophytes isolated soon after fertilization. For the first experiment, various combinations of auxin and cytokinin supplements were tested to determine whether immature megagametophyte, ranging from the early central cell to the mature egg cell stages, would continue to grow in vitro. The objective of the second series of experiments was to determine if plant hormone supplements in these media would support proembryo development. The embryos, which were co-cultured within megagametophytes, were then transferred to media containing ABA to see if they would mature.

4.2 Materials and Methods

4.2.1 Plant Materials

4.2.1.1. Pre-fertilization megagametophytes

Douglas fir trees of one clone (clone 3265) that had been previously treated with gibberellic acid were provided by Dr. Joe Webber at the BC Ministry of Forests (BCMF) Research Branch at Glyn Road Research Station in Victoria. Seed cones were bagged just before they were receptive to pollen. Bagging prevents pollination, and consequently fertilization. Cones were collected weekly for four weeks, starting three weeks before fertilization i.e. 7 weeks after pollination (WAP).

The developmental stages of the megagametophytes at the time of isolation were monitored by fixing freshly dissected material in formalin acetic acid (FAA), followed by dehydration in an ethanol series, and clearing in a methylsalicylate series (Fernando and Cass, 1997). The prothallial cells were made transparent by this procedure, which allowed the archegonial contents and the developmental stages of the megagametophytes to be observed under the light microscope. Each week, five of the freshly isolated megagametophytes were fixed in FAA, dehydrated, embedded in paraffin, and sectioned

(Johansen, 1940). These slides served as confirmation of observations made on cleared material.

4.2.1.2 Post-fertilization megagametophytes

A few of the Douglas fir seed cones of clone 3465 that had been bagged at the Glyn Road Research Station were pollinated with pollen collected from trees growing at the University of Victoria. Cone collections began during the week of fertilization (10 WAP) and were carried out on a weekly basis for a period of three weeks. To keep track of development, freshly isolated megagametophytes were fixed in FAA and either cleared in methylsalicylate or sectioned after embedding in paraffin.

4.2.2 Media composition, experimental design and statistical analysis

4.2.2.1 Pre-fertilization megagametophytes

4.2.2.1.1 Media composition

The basic medium used was a modified half-strength Litvay's medium (1/2 LM) (Litvay et al., 1985) supplemented with 10% sucrose (Gates and Greenwood, 1991) and adjusted to pH 5.8 before autoclaving. The sterilized medium was further supplemented with 500 mg/L arginine, a cytokinin, benzylaminopurine (BAP), at a concentration of 2 mg/L, and one of three structurally different auxins: naphthylacetic acid (NAA), indole-3-butyric acid (IBA) or 2,4-dichlorophenoxyacetic acid (2,4-D). Each of the auxins was tested at one of three concentrations of 0.1, 1.0 or 10 mg/L. Solutions containing plant hormones and arginine were adjusted to pH 5.8 and filter-sterilized. There were two control treatments for the experiment, one consisting of basic 1/2 LM containing no plant hormones (control -) and the second was 1/2 LM supplemented with BAP only (control +). As a result, a total of 11 different treatments were undertaken.

4.2.2.1.2 Experimental design and statistical analysis

Seed cones were surface sterilized by flaming in 95% ethanol. Ovuliferous scales were removed in the laminar flow hood and the megagametophytes were isolated without the nucellus under a dissecting microscope. Four hundred and forty megagametophytes were dissected weekly, 40 of which were placed on each of the 11 media. Each treatment had four petri dishes with ten megagametophytes per plate. Megagametophyte length was measured on the day of dissection (0 days), and after 9 and 18 days. Since megagametophytes in situ elongate as they mature, these measurements gave an indication of whether the megagametophytes were growing in culture.

The absence of the nucellus allowed archegonia to be easily observed under the dissecting microscope. On days 0, 9 and 18, megagametophytes were classified as either dead or viable. Observations from preliminary work indicated that freshly dissected megagametophytes had archegonia with translucent cytoplasm. Cultured megagametophytes that had this appearance under the dissecting microscope were defined as viable. Dead megagametophytes were those that had archegonia with shrunken or opaque cytoplasm. Data were collected as percentages of viable megagametophytes on each of the 11 media treatments.

The experiment with pre-fertilization megagametophytes was designed to test for the effect of one factor (namely media type) at 11 levels (11 treatments), on two variables (namely length and % viability). Because the media were specifically chosen, the experiment represented a fixed effects model. The length or viability data were subjected to analysis of variance using the linear model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} is the length (or percent viability) of megagametophytes on the *i*th medium; μ is the experimental mean; T_i is the effect of the *i*th medium; e_{ij} is the experimental error term. Because percentages form a binomial rather than normal distribution, viability data were transformed using the arcsine transformation (Zar, 1984). Analysis of variance was performed on the length and transformed viability data collected from megagametophytes after 9 days of culture, using the general linear models (GLM) procedure. Data for the weekly samples were analyzed separately.

To test whether auxins and cytokinin had any effect on megagametophyte growth or viability, the average lengths or the percentages of viable megagametophytes after 9 days on the different treatments were compared to the average of material on the control treatment without any plant growth regulators (control -) using Dunnett's T test at $\alpha = 0.05$. In order to determine the effect of auxin concentration on megagametophyte growth, pairwise comparisons of the average lengths of megagametophytes on each of the three auxin concentrations were done using Scheffe's test at $\alpha = 0.05$ with data collected after 9 days in culture for each auxin type. Results of these analyses were used to select one concentration of each auxin type (NAA, IBA or 2,4-D) that was then used for part of the embryo rescue experiment.

In order to establish the overall effect of culture conditions on megagametophyte growth, the average lengths of material isolated 7, 8, 9 and 10 WAP were calculated. Lengths were averaged on the day of isolation (0 days) and after 9 and 18 days for the megagametophytes placed on media weekly.

4.2.2.2 Post-fertilization megagametophytes

4.2.2.2.1 Media composition

Megagametophytes isolated from pollinated seed cones were pretreated using only those media that allowed growth in the previous pre-fertilization experiment. The most optimal treatment for each auxin type was selected. After 21 days, the megagametophytes were transferred to 1/2 LM media supplemented with 10% sucrose and abscisic acid (ABA) at one of four concentrations (0, 5, 20 or 40 μM), in combination with BAP at 0.5 mg/L. The organic nitrogen sources used were L-glutamine and arginine, each at a concentration of 500 mg/L.

4.2.2.2.2 Experimental design

One hundred and twenty megagametophytes were isolated on a weekly basis for three weeks, 40 of which were placed on each of the three auxin-containing media selected from the pre-fertilization experiment. Each treatment had four plates with ten megagametophytes per plate. After 21 days, ten megagametophytes from each of the three auxin containing media were transferred to media supplemented with one of the four ABA concentrations tested. After eight weeks of culture on the ABA media, embryos were dissected from megagametophytes and maintained on the same medium for another four weeks. The lengths of pollinated megagametophytes while on the auxin treatments were measured at 0, 9 and 18 days. Because the focus of this experiment was to rescue embryos from megagametophytes, the length data collected during the auxin pretreatment period were used to determine whether these pollinated megagametophytes continued to grow in culture. The average lengths of megagametophytes that had been isolated 10, 11 or 12 WAP were calculated at 0, 9 and 18 days in culture.

The embryo rescue experiment was designed to test for the effects of two factors, ABA and auxin. The ABA factor had four levels (concentrations of 0, 5, 20 or 40 μM) and the auxin factor had three levels (the three auxin containing media selected from the pre-fertilization experiment). The variable that was being investigated was the number of mature embryos rescued from megagametophytes that had been cultured on the different ABA media after being pretreated with auxin. The experimental design was a 3 x 4 factorial, Model 1 ANOVA, with ABA and auxin as fixed effects. The intention was to subject the percentages of mature embryos rescued from megagametophytes placed on each of the ABA media to analysis of variance using the linear model:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where Y_{ijk} is the number of mature embryos rescued from the *j*th ABA medium subjected to the *i*th auxin pretreatment; μ is the experimental mean; A_i is the effect of the *i*th auxin pretreatment; B_j is the effect of the *j*th ABA medium; AB_{ij} is the interaction effect of the *i*th auxin pretreatment and the *j*th ABA medium; e_{ijk} is the experimental error component.

4.3 Results

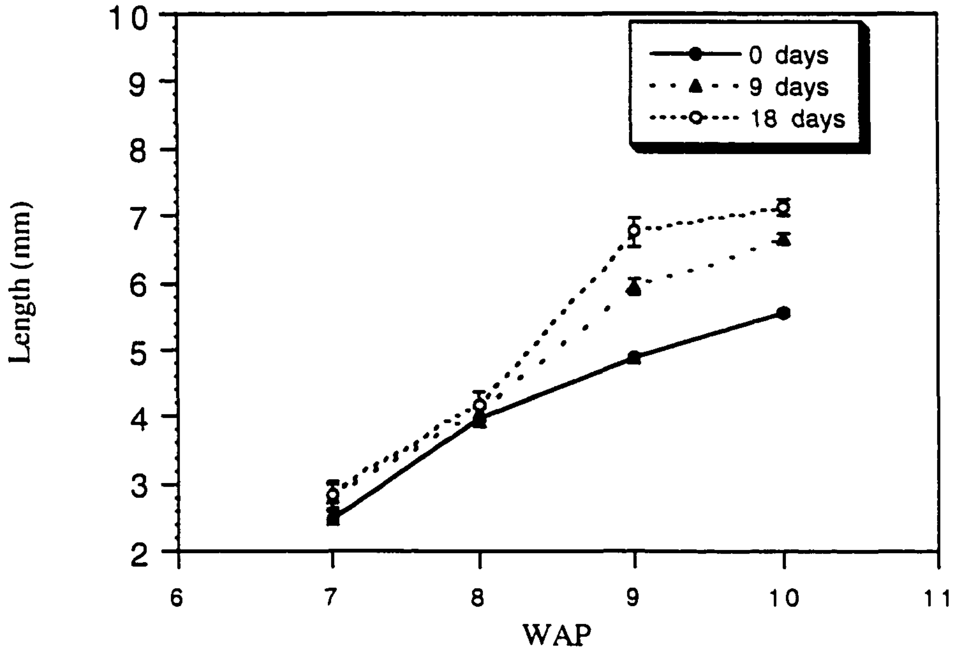
4.3.1 Pre-fertilization megagametophytes

Megagametophytes isolated prior to fertilization and cleared in methylsalicylate were at the early central cell, late central cell, early egg and mature egg cell stages of development at 7, 8, 9 and 10 WAP, respectively. The megagametophytes continued to grow in culture after 9 and 18 days (see Figure 5). The growth of cultured megagametophytes was dependent on the developmental stage at the time of isolation. After 9 and 18 days of culture, megagametophytes isolated from the early egg cell stage of development and beyond (i.e. 9 to 12 WAP) grew to greater lengths compared to lengths at 0 days (Figure 5). By 18 days, cultured megagametophytes achieved lengths that surpassed those normally reached by material in situ. For example, megagametophytes at the early egg cell stage that were collected 9 WAP (one week before fertilization) had an average length of 6.8 mm after 18 days in culture compared to material collected 12 WAP (i.e. two weeks past fertilization) which had an average length of 5.9 mm at 0 days (see Figure 5). In contrast, early and late central cell stage megagametophytes (7 and 8 WAP, respectively) did not show the same growth response after 9 or 18 days in culture.

4.3.1.1 Length responses on the different auxin concentrations

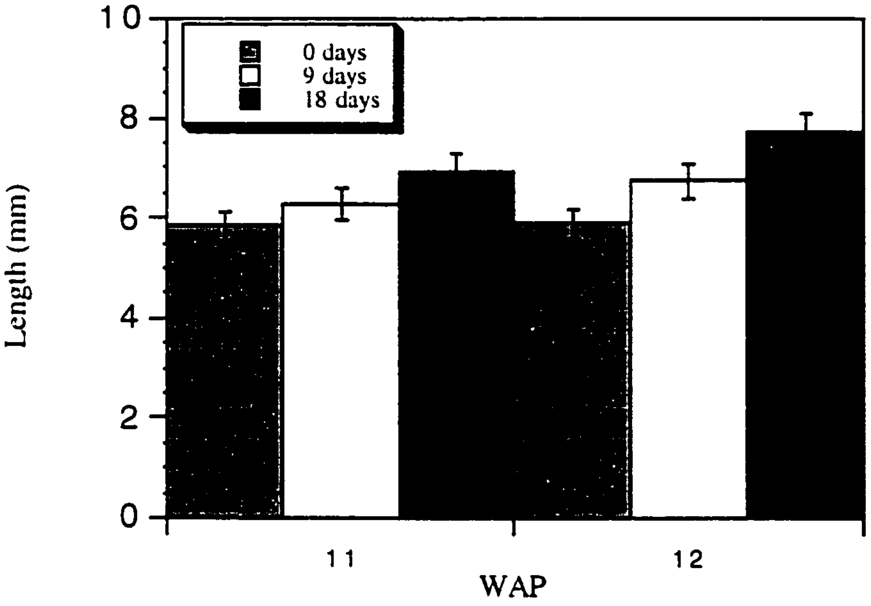
The addition of auxin in combination with cytokinin to the culture media promoted megagametophyte growth to varying degrees depending on the developmental stage of the megagametophytes at the time of isolation, the auxin type, as well as the auxin concentration. Figure 6 shows the average lengths of megagametophytes at 0 days, and after 9 and 18 days, that had been cultured 7 WAP (i.e. early central cell stage

Figure 5A. Average lengths of Douglas fir megagametophytes isolated prior to fertilization on the day of isolation (0 days) and after 9 and 18 days in culture. (mean ± SD).



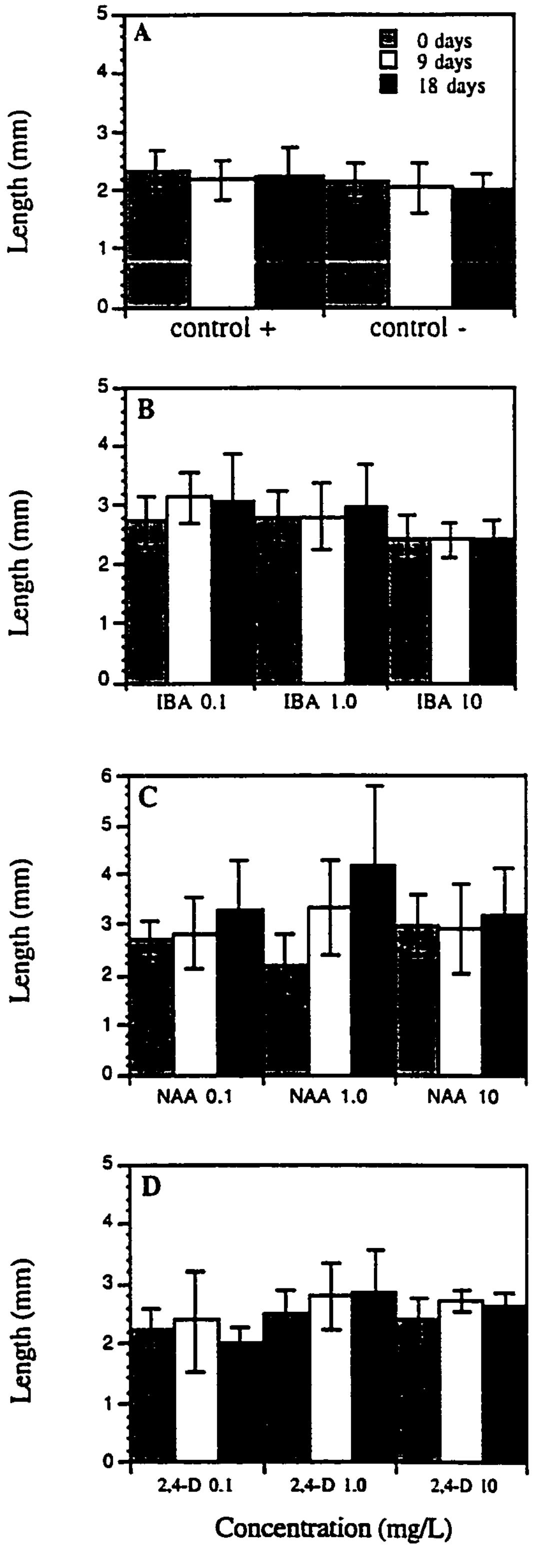
Note: Each data point for weeks 7 to ten past pollination represents the average lengths of megagametophytes cultured pre-fertilization on all 11 media tested.

Figure 5B. Average lengths of Douglas fir megagametophytes isolated after fertilization on the day of isolation (0 days) and after 9 and 18 days in culture. (mean ± SD).



Note: Each data point is the average length of measurements taken during the auxin pretreatment period from post-fertilization megagametophytes that were used in the embryo rescue experiment.

Figure 6. Length of megagametophytes isolated 7 WAP and culture on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -), or supplemented with 2 mg/L BAP only (control +) (**A**), or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Measurements were taken on the day of isolation (0 days) and after 9 and 18 days in culture.


A
0 days
9 days
18 days
Length (mm)
0
1
2
3
4
5
control +
control -
B
Length (mm)
IBA 0.1
IBA 1.0
IBA 10
C
Length (mm)
6
NAA 0.1
NAA 1.0
NAA 10
D
Length (mm)
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

megagametophytes collected three weeks before fertilization) and placed on the 11 media. Megagametophytes cultured on the control medium without any plant growth regulators (control -) did not show an increase in length after 9 days (2.0 mm) or 18 days (2.0 mm) compared to the length at isolation (2.1 mm) (Figure 6a). The addition of cytokinin (BAP) alone to the medium did not promote megagametophyte growth after 9 days (2.2 mm) or 18 days (2.3 mm) in culture (Figure 6a). After 9 days of culture, material placed on media containing BAP and all three concentrations of NAA (0.1, 1.0 or 10 mg/L) had average lengths of 2.8 mm, 3.4 mm and 2.9 mm respectively, which were significantly greater than the average length of material placed on the control treatment without any plant hormones (Table 2). BAP in combination with IBA at 0.1 or 1.0 mg/L, or 2,4-D at 1.0 mg/L, promoted megagametophyte growth to significantly greater lengths of 3.0 mm, 2.7 mm and 2.7 mm respectively, compared to the control (-) treatment, on which megagametophytes averaged 2.0 mm at 9 days (Table 2).

Although 2,4-D at 1.0 mg/L promoted megagametophyte growth when compared to the control (-) treatment, no significant differences in lengths were detected when compared

to megagametophytes placed on media containing 2,4-D concentrations of 0.1 or 10 mg/L (see Table 2 for results of Scheffe's pairwise comparisons for each auxin type). The effect of IBA concentrations of 0.1 or 1.0 mg/L on growth was significantly different from that of IBA at 10 mg/L, and NAA at 1.0 mg/L was the best medium compared to NAA concentration of 0.1 or 10 mg/L for promoting growth of material isolated 7 WAP (see Table 2).

Lengths of megagametophytes cultured at the late central cell stage (8 WAP), early egg cell stage (9 WAP) and the mature egg cell stage (10 WAP) are shown in Figures 7, 8 and 9, respectively. Material isolated 8 and 9 WAP continued to grow on all media after 18 days of culture (Figures 7 and 8, respectively). After 9 days, none of the megagametophytes placed on media containing either BAP alone (control +), or BAP and

Table 2. Analysis of lengths of pre-fertilization Douglas fir megagametophytes after 9 days on various culture media (mean ± SD) compared to the control (-) treatment using Dunnett's T test and Scheffe's pairwise comparison for each auxin type.

Treatment	Week 7	Week 8	Week 9	Week 10
Concentration (mg/L)		Length (mm)		
Control -	1.96 ± 0.43^{a}	3.18 ± 0.84^{a}	5.01 ± 0.78^{a}	5.26 ± 0.31^{a}
Control +	2.15 ± 0.33^{a}	3.41 ± 0.80^{a}	4.74 ± 0.67^{a}	5.28 ± 0.41^{a}
2,4-D 0.1	2.32 ± 0.79^{a}	3.15 ± 0.53^{a}	4.95 ± 0.50^{a}	5.33 ± 0.36^{a}
2,4-D 1.0	2.69 ± 0.53^{a*}	3.50 ± 0.91^{a}	4.84 ± 0.67^{a}	5.40 ± 0.27^{a}
2,4-D 10	2.54 ± 0.25^{a}	3.21 ± 0.83^{a}	4.73 ± 0.50^{a}	5.34 ± 0.35^{a}
IBA 0.1	2.98 ± 0.43^{a*}	3.45 ± 0.78^{a}	4.14 ± 0.69^{b}	5.65 ± 0.38^{a*}
IBA 1.0	2.67 ± 0.55^{a*}	3.63 ± 0.68^{a}	5.09 ± 0.37^{a}	5.37 ± 0.42^{b}
IBA 10	2.28 ± 0.28^{b}	3.21 ± 0.97^{a}	4.79 ± 0.55^{a}	5.47 ± 0.36^{ab}
NAA 0.1	2.83 ± 0.70^{b*}	3.14 ± 0.79^{a}	4.55 ± 0.99^{a}	5.55 ± 0.37^{a*}
NAA 1.0	3.35 ± 0.95^{a*}	2.85 ± 0.39^{a}	4.84 ± 1.09^{a}	5.15 ± 0.66^{b}
NAA 10	2.94 ± 0.89^{b*}	2.99 ± 0.66^{a}	4.25 ± 0.69^{a}	4.78 ± 0.25^{c}

Note:

* Length significantly greater than (control -) treatment at $\alpha = 0.05$ using Dunnett's T test.

^{a} Mean values followed by the same letter do not differ significantly for each auxin type based on Scheffe's test at $\alpha = 0.05$.

Figure 7. Length of megagametophytes isolated 8 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Measurements were taken on the day of isolation (0 days) and after 9 and 18 days in culture.

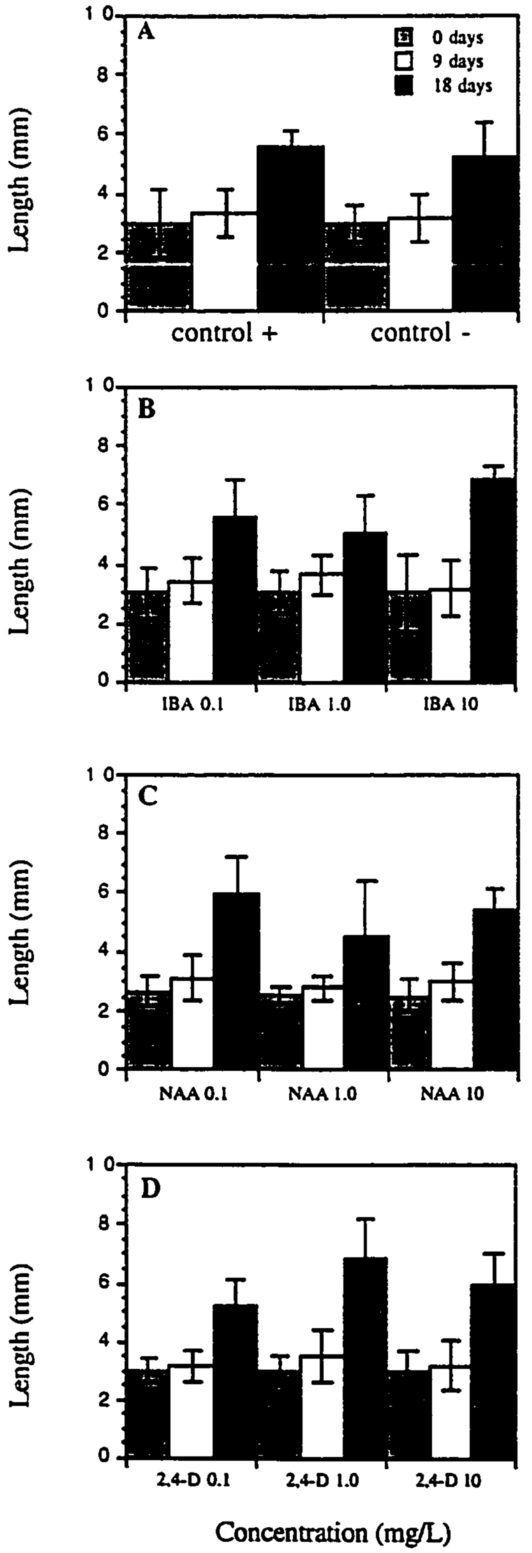

A
▨ 0 days
☐ 9 days
■ 18 days
Length (mm)
0
2
4
6
8
10
control +
control -
B
Length (mm)
IBA 0.1
IBA 1.0
IBA 10
C
Length (mm)
NAA 0.1
NAA 1.0
NAA 10
D
Length (mm)
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

Figure 8. Length of megagametophytes isolated 9 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Measurements were taken on the day of isolation (0 days) and after 9 and 18 days in culture.

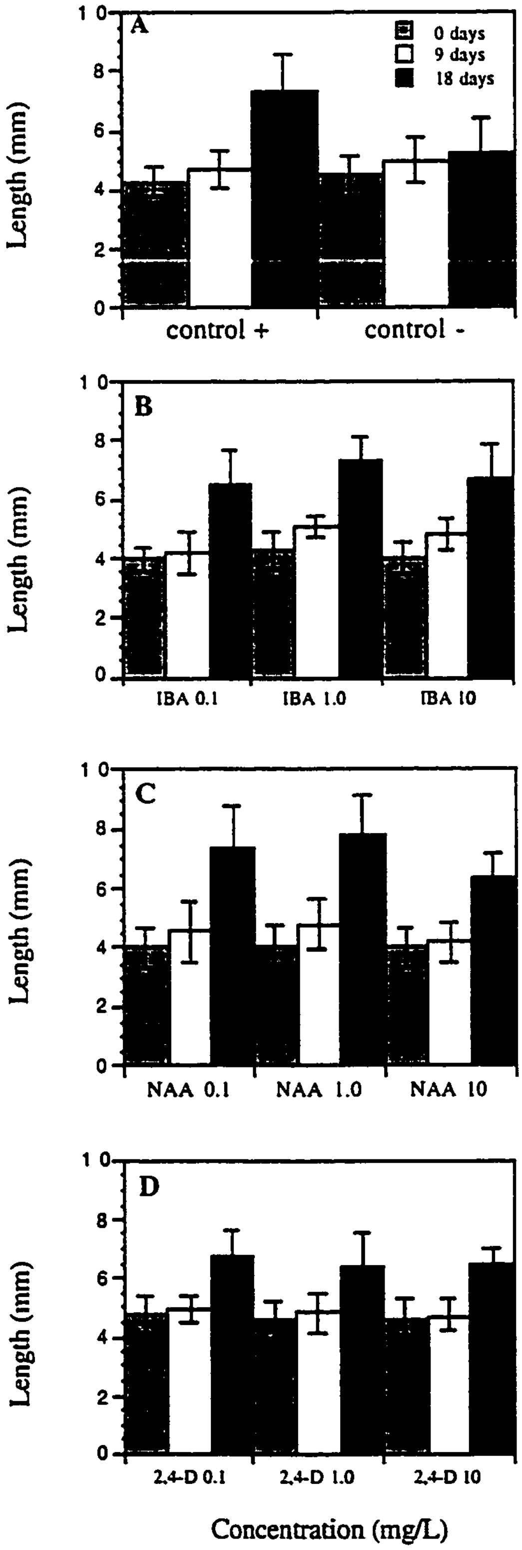
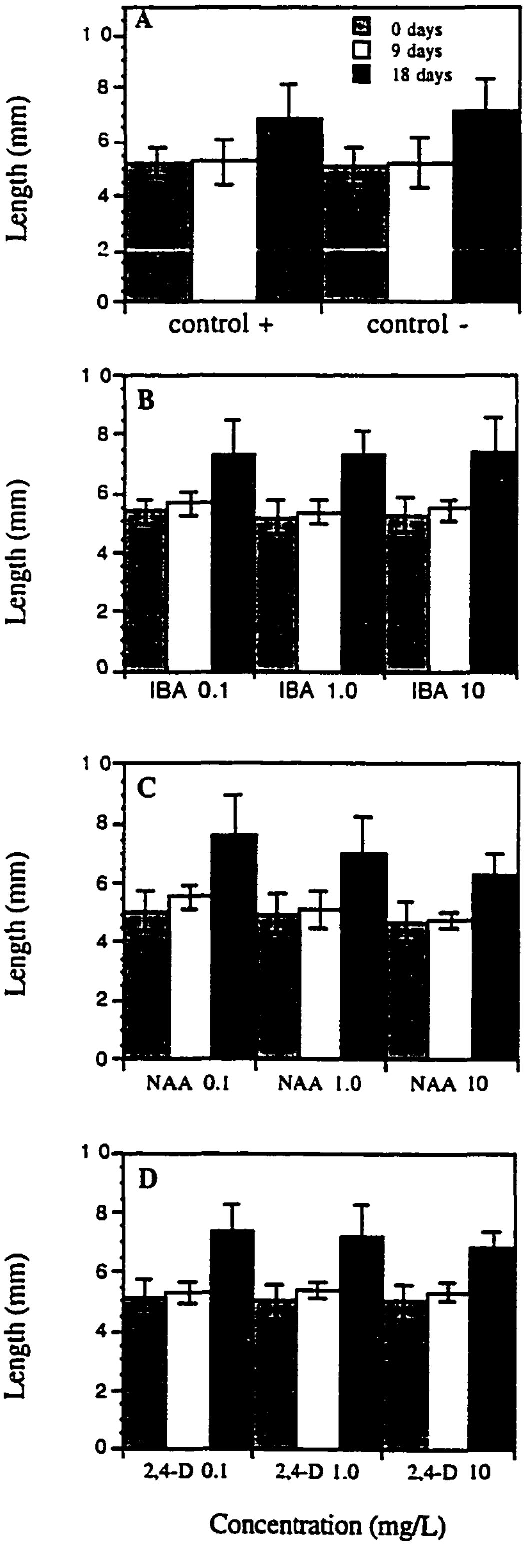

A
▨ 0 days
☐ 9 days
■ 18 days
Length (mm)
10
8
6
4
2
0
control +
control -
B
Length (mm)
IBA 0.1
IBA 1.0
IBA 10
C
Length (mm)
NAA 0.1
NAA 1.0
NAA 10
D
Length (mm)
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

Figure 9. Length of megagametophytes isolated 10 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Measurements were taken on the day of isolation (0 days) and after 9 and 18 days in culture.


A
0 days
9 days
18 days
Length (mm)
10
8
6
4
2
0
control +
control -
B
IBA 0.1
IBA 1.0
IBA 10
C
NAA 0.1
NAA 1.0
NAA 10
D
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

auxin, had lengths significantly greater than those of material on the control treatment without any plant hormones (Table 2). However, by the mature egg cell stage (Figure 9), megagametophytes on media containing BAP in combination with IBA or NAA at 0.1 mg/L grew to significantly greater lengths than those placed on the control treatment (see Table 2). Results from Scheffe's test revealed these to be the optimal concentrations for the two auxins (see Table 2).

In summary, the addition of auxin and cytokinin to media had a significant effect on growth for material isolated either 7 or 10 WAP (Table 2). The three media that were chosen for part of the embryo rescue experiment were those containing BAP in combination with either 0.1 mg/L NAA, 1.0 mg/L IBA or 1.0 mg/L 2,4-D.

4.3.1.2 Viability of megagametophytes on various media

Cultured megagametophytes that had archegonia with translucent cytoplasm under the dissecting microscope were classified as viable (Figure 10a) whereas those with opaque cytoplasm (Figure 10b) were classified as dead. Viability of archegonia decreased with time on all media tested (see figures 11, 12 and 13). For material isolated 7 WAP, addition of BAP to the medium did not maintain viable archegonia after 9 or 18 days in culture (see Figure 11a). Better megagametophyte viability was achieved on media containing 2,4-D or NAA concentrations of 1.0 mg/L (Figure 11d and 11c, respectively), or IBA at 1.0 or 10 mg/L (Figure 11b) after 18 days in culture when compared to the control treatment (Figure 11a). However, a comparison of viability of early central cell stage megagametophytes on the different treatments against the control (-) treatment using Dunnett's T test with data collected at 9 days showed that the only medium that was significantly different from the control treatment was IBA at 10 mg/L (See Table 3).

Viability data for material isolated 8 and 9 WAP (late central cell and early egg cell stages, respectively) on the different treatments are shown in Figures 12 and 13. Archegonia remained viable on all media including the control treatment at 9 days

Figure 10A. Appearance of cultured Douglas fir megagametophytes under the dissecting microscope that were isolated prior to fertilization and classified as viable on the day of isolation (0 days) or after 9 and 18 days. The megagametophyte is comprised of numerous prothalial cells (pc) and archegonia with translucent cytoplasm (tc). Bar equals 100 µm.

Figure 10b. Appearance of cultured Douglas fir megagametophytes under the dissecting microscope that were isolated prior to fertilization and classified as dead on the day of isolation (0 days) or after 9 and 18 days. The megagametophyte has archegonia with opaque cytoplasm (oc). Bar equals 100 µm.

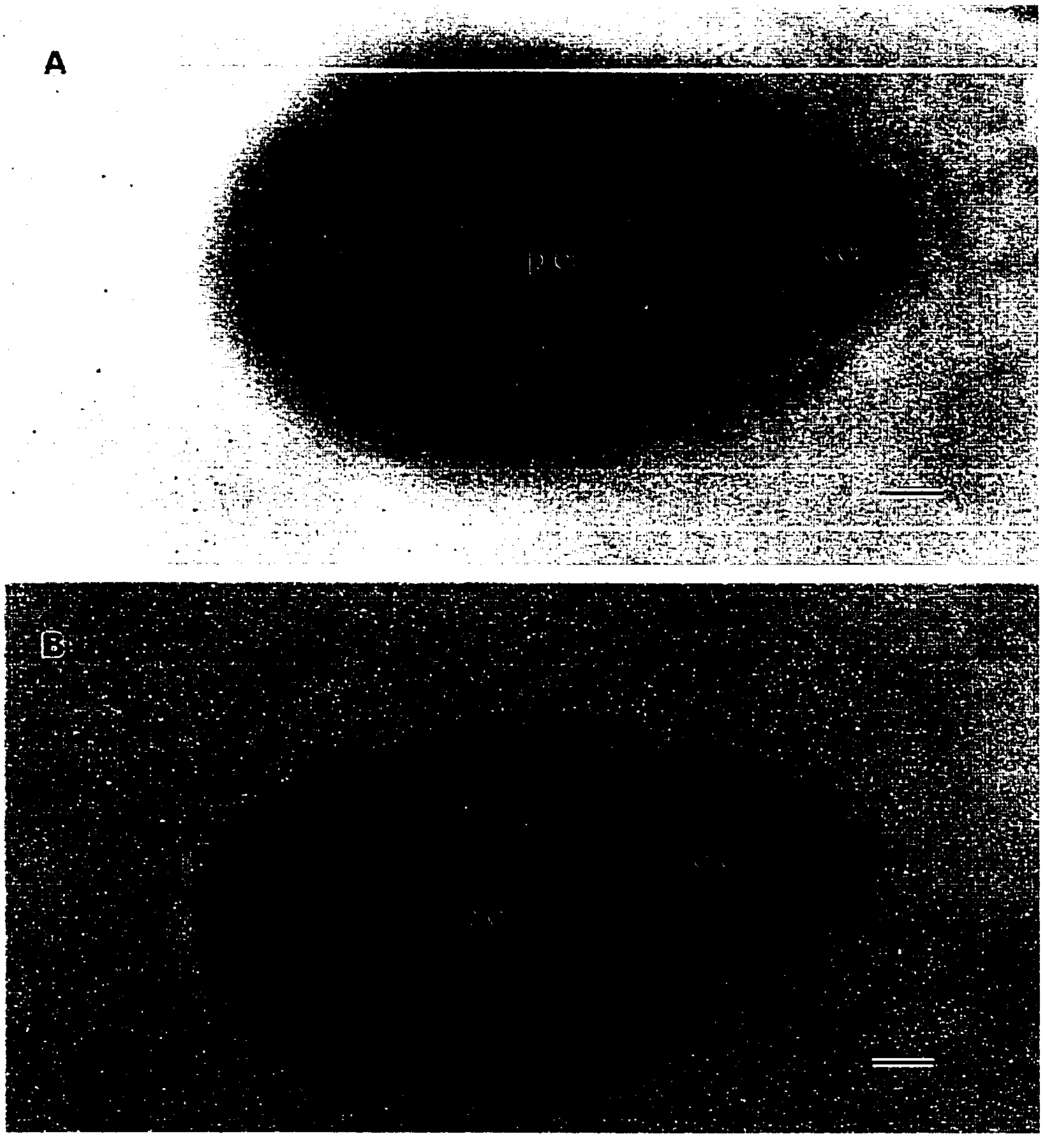

A
pc
cc
B

Figure 11. Viability of megagametophytes isolated 7 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Viability data were collected on the day of isolation (0 days) and after 9 and 18 days in culture.

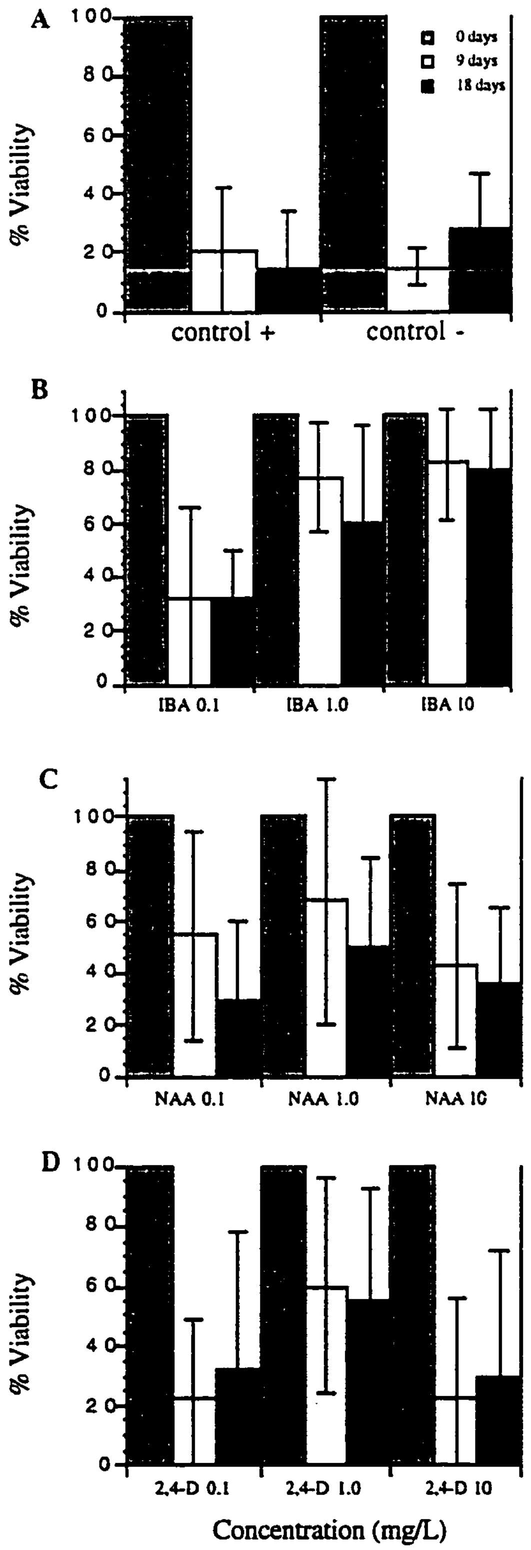

A
B
C
D
% Viability
1 0 0
8 0
6 0
4 0
2 0
0
0 days
9 days
18 days
control +
control -
IBA 0.1
IBA 1.0
IBA 10
NAA 0.1
NAA 1.0
NAA 10
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

Table 3. Viability of pre-fertilization Douglas fir megagametophytes after 9 days on various media (mean ± SD).

Treatment Concentration mg/L	% viability		
	week 7	week 8	week 9
Control -	15.0 ± 5.80	72.5 ± 34.0	70.0 ± 29.4
Control +	20.0 ± 21.6	80.0 ± 28.3	70.0 ± 34.6
2,4-D 0.1	22.5 ± 26.3	57.5 ± 31.0	27.5 ± 34.0
2,4-D 1.0	60.0 ± 35.6	90.0 ± 14.1	35.0 ± 40.4
2,4-D 10	22.5 ± 33.0	90.0 ± 14.1	40.0 ± 16.3
IBA 0.1	32.5 ± 33.0	82.5 ± 20.6	62.5 ± 27.5
IBA 1.0	77.5 ± 20.6	82.5 ± 17.1	67.5 ± 39.5
IBA 10	82.5 ± 20.6**	100 ± 0.0	60.0 ± 45.5
NAA 0.1	55.0 ± 40.4	97.5 ± 5.0	82.5 ± 23.6
NAA 1.0	67.5 ± 47.2	100 ± 0.0	75.0 ± 33.2
NAA 10	42.5 ± 32.0	85.0 ± 13.0	60.0 ± 42.4

** Viability greater than Control (-) treatment at alpha = 0.05 using Dunnett's T test.

Figure 12. Viability of megagametophytes isolated 8 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Viability data were collected on the day of isolation (0 days) and after 9 and 18 days in culture.

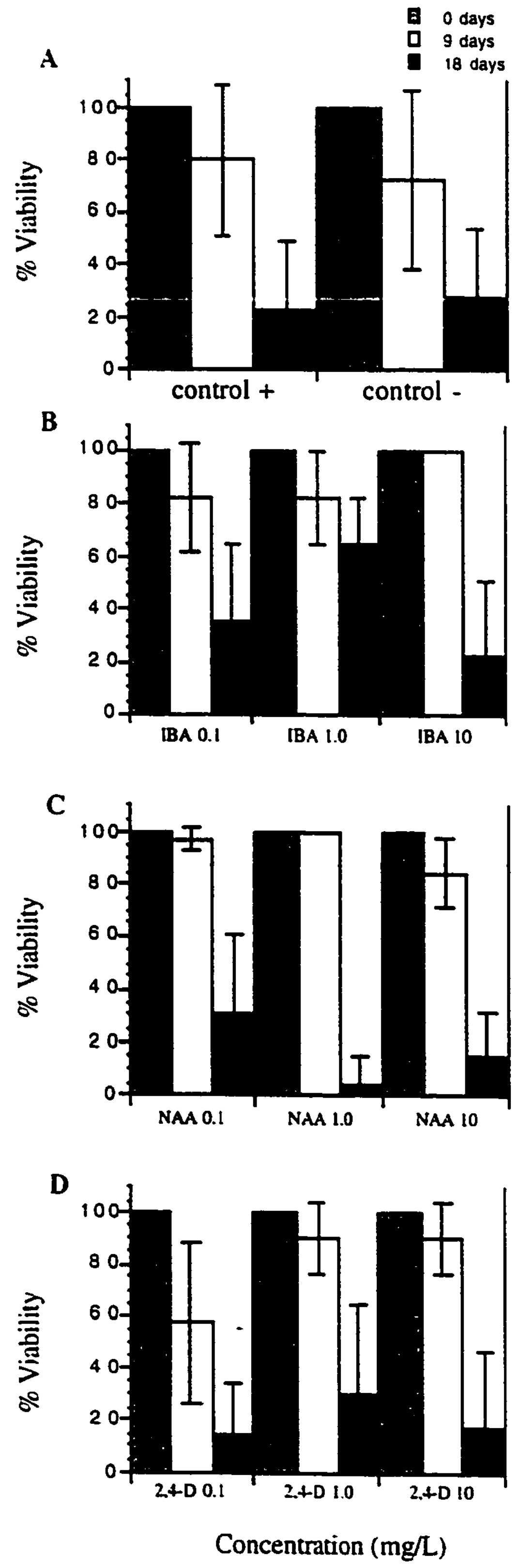
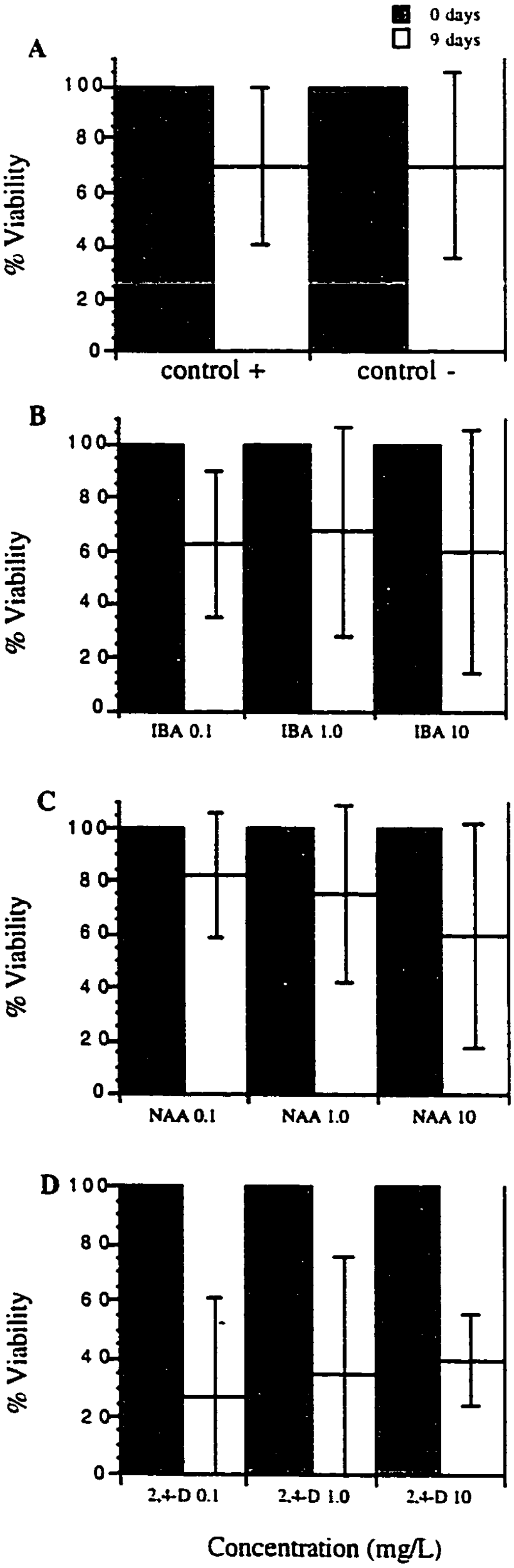

A
B
C
D
0 days
9 days
18 days
% Viability
0
2 0
4 0
6 0
8 0
1 0 0
control +
control -
IBA 0.1
IBA 1.0
IBA 10
NAA 0.1
NAA 1.0
NAA 10
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

Figure 13. Viability of megagametophytes isolated 9 WAP and cultured on media containing various combinations of auxins and cytokinin. The basic medium used was a modified 1/2 LM without any plant hormones (control -) or supplemented with 2 mg/L BAP only (control +) (**A**) or in combination with three different concentrations of IBA (**B**) or NAA (**C**) or 2,4-D (**D**). Viability data were collected on the day of isolation (0 days) and after 9 days in culture.


A
B
C
D
■ 0 days
□ 9 days
% Viability
0
2 0
4 0
6 0
8 0
1 0 0
control +
control -
IBA 0.1
IBA 1.0
IBA 10
NAA 0.1
NAA 1.0
NAA 10
2,4-D 0.1
2,4-D 1.0
2,4-D 10
Concentration (mg/L)

(Figures 12 and 13) and as a result, no significant differences were detected by Dunnett's test at alpha = 0.05 (Table 3). However, for material isolated 8 WAP, megagametophyte viability was 65% on medium containing IBA at 1.0 mg/L (Figure 12b) after 18 days, a value almost triple that for material on the control treatment without any plant growth regulators (Figure 12a).

4.3.2. Post-fertilization megagametophytes

Megagametophytes isolated after fertilization, which were used for the embryo rescue experiment, contained embryos at the proembryo and early embryo stages. The pollinated megagametophytes continued to grow after 9 and 18 days during the pretreatment period with auxin and cytokinin (11 and 12 WAP in Figure 5). Although the development of embryos, which were co-cultured within megagametophytes, could not be assessed until the end of the experiment, some of the material that had been placed on medium containing 2,4-D at 1.0 mg/L had the embryonal suspensors growing out of the micropylar end of the megagametophytes (Figure 14a). The growth of the embryonal suspensors forced the remnants of the archegonia, located at the micropylar tip of the suspensor, out of the megagametophytes (arrow head in Figure 14a).

When embryos were dissected from megagametophytes at the end of the experiment, a majority of the embryos were developmentally arrested at the globular stage, with clearly established embryonal masses and suspensors (Figure 14b; Table 4). Megagametophytes that had been dissected 10 WAP and contained proembryos at the time of isolation yielded embryos that had developed further to the globular stage. In two cases, the embryonal masses continued to grow within the megagametophytes and became enlarged compared to the suspensors (Figures 14c and 14d). Somatic embryogenic tissue was regenerating from one of the two embryos with the enlarged embryonal masses (arrow heads in Figure 14d).

Figure 14a. Embryonal suspensor (es) growing out of the micropylar end of a megagametophyte (mg) after 15 days in culture. The remnants of the archegonia (arrow head) can be seen at the tip of the embryonal suspensor. The megagametophyte was isolated 11 WAP (one week after fertilization) and cultured on medium containing 2,4-D at a concentration of 1.0 mg/L for the first 21 days. Bar equals 500 µm.

Figure 14b. An embryo arrested at the globular stage, with a clearly defined embryonal mass (em) and suspensor (es). The embryo was dissected out of a megagametophyte that had been cultured for eight weeks on medium containing ABA. Bar equals 100 µm.

Figure 14c. A globular stage embryo with an enlarged embryonal mass (em) dissected out of a megagametophyte that was isolated during the week of fertilization (10 WAP) and cultured on medium containing IBA at 1.0 mg/L and 2 mg/L BAP for the first 21 days. Bar equals 100 µm.

Figure 14d. A globular stage embryo with an enlarged embryonal mass dissected out of a megagametophyte that was isolated during the week of fertilization and cultured on medium containing 2,4-D at 1.0 mg/L and 2 mg/L BAP for the first 21 days. Somatic embryogenic tissue (arrow heads) is regenerating from the enlarged embryonal mass (em). Bar equals 100 µm.

Figure 14e. An ungerminated mature embryo with over 30 cotyledons (ct) that was dissected out of a megagametophyte isolated two weeks after fertilization (12 WAP) and cultured on medium containing 0.1 mg/L NAA and 2 mg/L BAP for the first 21 days, and then transferred to 20 µM ABA medium for eight weeks. The hypocotyl (h) and root cap (not shown) are normally developed. Bar equals 100 µm.

Figure 14f. The milky white (wm) appearance of megagametophytes isolated two weeks past fertilization that yielded mature embryos (Shown in Figure 15) after eight weeks of culture on 20µM ABA medium. Bar equals 500 µm.

Figure 14g. Plantlet produced when the embryo in Figure 15 was cultured on germination medium without any plant growth regulators. Although the shoot apex is not discernible, the root (r) and the shoot (st) are distinguishable. Bar equals 500 µm.

Figure 14h. Appearance of a normal seedling obtained from a mature embryo that grew in situ and was cultured on germination medium without any plant growth regulators. The cotyledons (ct), true leaves (tl) and shoot apex (sa) are normally arranged. Bar equals 500 µm.

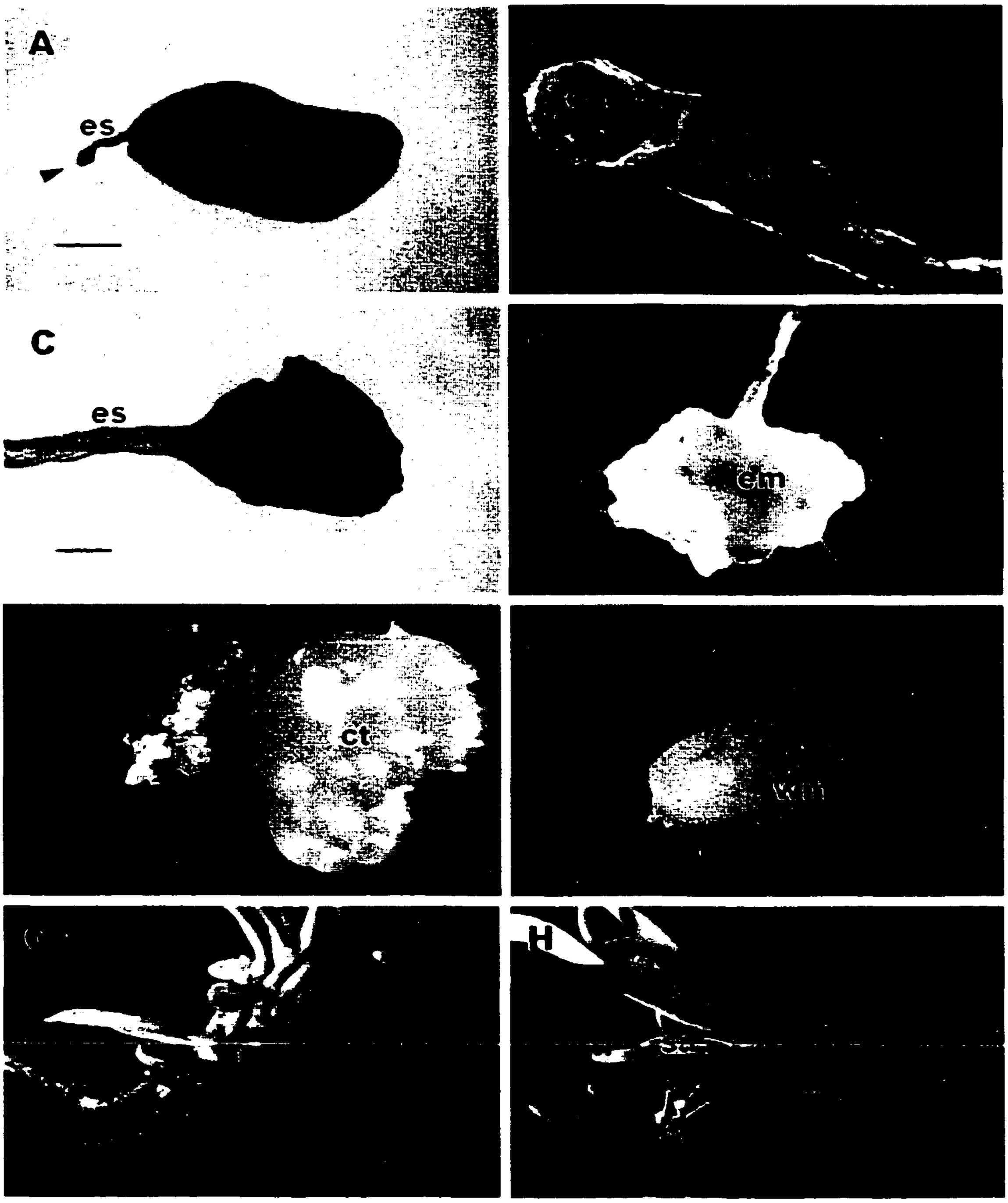

A
es
C
es
em
ct
wm
G
H

Table 4. The number of embryos rescued from Douglas fir megagametophytes isolated ten, 11 and 12 WAP. The embryos were co-cultured within megagametophytes on media containing auxin and cytokinin for the first 21 days and then transferred to media supplemented with ABA for eight weeks.

Isolation date (WAP)	Developmental stage of rescued embryos	
	Globular stage	Mature embryos
10	5	0
11	3	0
12	15	3

Note: In the table, the total number of embryos rescued each week were dissected out of the forty megagametophytes that were cultured weekly

Only three mature embryos were rescued (Table 4), but these embryos possessed an abnormally high number of cotyledons (over 30) (see Figure 14e). The three embryos were dissected out of megagametophytes that had been isolated 12 WAP (two weeks past fertilization) and had been initially cultured on media containing BAP in combination with NAA at 0.1 mg/L, and then transferred to ABA concentrations of 5, 20 or 40μM. The megagametophytes that yielded the mature embryos were a milky white color when the embryos were dissected at the end of the experiment (Figure 14f), indicating that they accumulated storage reserves during the culture period. The three mature embryos germinated when they were placed on germination medium without any plant growth regulators (Figure 14g), but their growth was quite different from that of normal embryos (Figure 14h).

In summary, the addition of auxin and cytokinin to culture media promoted proembryo development into globular stage embryos. However, the embryos did not continue to develop past this stage. Transferring megagametophytes from the auxin and cytokinin containing media onto media supplemented with ABA did not promote embryo maturation, although three mature embryos were rescued in the experiment. These embryos were dissected out of megagametophytes that had been isolated two weeks past fertilization (12 WAP) and cultured on media containing ABA concentrations of 5, 20 or 40μM.

4.4 Discussion

Megagametophytes isolated prior to fertilization from unpollinated seed cones continued to grow in culture. However, the length responses on the various auxin-cytokinin combinations were dependent on the developmental stage of the material at the time of isolation. The addition of BAP in combination with the auxins 2,4-D at 1.0 mg/L, IBA at 0.1 or 1.0 mg/L, or NAA at all three concentrations, promoted growth of central cell stage megagametophytes (Table 2). Late central cell and early egg cell stage megagametophytes grew well on all media, including the two control treatments. Only one medium containing BAP in combination with IBA at 0.1 mg/L promoted growth of mature egg cell stage megagametophytes above the control treatment without any plant hormones (Table 2).

In agreement with what has been previously reported by Allen and Owens (1972), length measurements taken from megagametophytes that were used in the pre-fertilization experiment at isolation (0 days) indicated that material in situ was growing from 7 to 10 WAP (Figure 5). The elongation of cultured megagametophytes past lengths normally achieved by material in situ after 18 days of culture (Figure 5) may be attributed to the combined effects of auxin and cytokinin. Auxins have well known effects on cell elongation whereas cytokinins stimulate growth by influencing cell division (Arteca, 1996). The multiplication of prothallial, neck and jacket cells in culture has also been reported for Douglas fir megagametophytes isolated at fertilization (Fernando et al., 1997; Ma et al., 1998).

Megagametophyte growth in culture was not dependent on the presence of viable archegonia. Unpollinated material isolated during fertilization week in the first experiment (10 WAP) did not degenerate in culture, but continued to increase in length after 9 and 18 days. This is unlike what occurs in situ, where unfertilized megagametophytes abort within two weeks past fertilization period (von Aderkas et al., 1997). The prothallial cells of megagametophytes continued to grow even as viability of archegonia decreased. The rapid degeneration of egg cells in cultured megagametophytes has also been reported by Fernando et al. (1997) and von Aderkas et al. (1997). The results of the pre-fertilization experiment showed that development of prothallial cells can be easily maintained for prolonged periods in vitro. However, these megagametophytes are not useful for IVF as the eggs rapidly abort under the same culture conditions. The fact that the unfertilized megagametophytes did not degenerate in culture implies that whatever causes abortion in situ is not within the embryo or the megagametophyte.

Attempts to rescue embryos soon after fertilization in *Pinus resinosa* (Gates and Greenwood, 1991) and larch (Sterling, 1949) yielded embryos, but these failed to complete embryogenesis. The media used by Gates and Greenwood (1991) did not contain any plant hormone supplements. Pretreatment with auxin and cytokinin for the first 21 days promoted elongation of fertilized Douglas fir megagametophytes isolated for the embryo rescue experiment (Figure 4). However, plant hormone supplements in the media did not promote embryogenesis for embryos co-cultured within the megagametophytes. The embryos did develop further than the proembryo and early embryo stages, but a majority were arrested at the globular stage when they were dissected from megagametophytes at the end of the experiment. These results suggest that the culture media used in this experiment promoted partial embryogenesis to the globular stage, but were not suitable for promoting further development into mature embryos.

In developing white spruce seeds, a biphasic auxin accumulation pattern was reported by Kong et al. (1997). Indole-3-acetic acid levels (IAA) levels peaked at pollination, declined during the proembryo stage and then increased from the club-shaped embryo stage until seed maturation. The high levels of auxin in the megagametophytes during pollination preceded the period of rapid embryo growth in the first week post-fertilization (Kong et al., 1997). They suggested that the second IAA peak may be essential for subsequent embryo growth from the club-shaped embryo stage through to the cotyledonary stage (Kong et al., 1997). If this biphasic accumulation pattern for auxin is common for conifers, then these results by Kong et al. (1997) may explain why embryos failed to complete embryogenesis in the embryo rescue experiment undertaken with Douglas fir megagametophytes. In this experiment, embryos were pretreated with auxin and cytokinin while at the proembryo and early embryo stages at isolation. The embryos did progress further to the globular stage but did not develop into mature embryos. This may be because they were transferred onto medium containing ABA without any auxin after 21 days. If the second IAA peak identified by Kong et al. (1997) is required for growth from the globular to the cotyledonary stages, then the absence of auxin in the ABA media may have prevented embryos from completing embryogenesis. The presence of auxin in the media does not appear to be a requirement for the maturation of somatic embryos in conifers. In terms of plant hormone supplements, a majority of the media used for maturation in these studies are supplemented with ABA alone. However, in *Picea abies*, somatic embryo maturation may also be improved by the addition of ABA and auxin or cytokinin to the medium (for review, see von Arnold et al., 1995).

Kong et al. (1997) also reported that ABA content was high at the early embryo and precotyledonary stages in developing white spruce seeds. The addition of ABA to the culture media did not promote maturation of Douglas fir embryos in this experiment. Only three embryos matured from megagametophytes isolated two weeks after fertilization. However, the culture media used in this experiment did not promote normal embryogenesis because the embryos possessed over 30 cotyledons each. It is not clear whether this abnormality was a result of the pretreatment with auxin and cytokinin or of the addition of 0.5 mg/L cytokinin in the ABA media. The megagametophytes containing these embryos did accumulate storage reserves in culture. Gates and Greenwood, (1991) also found that fertilized *Pinus resinosa* megagametophytes that had been maintained on media containing sucrose concentrations of up to 21% resembled those in situ in terms of storage reserves. However, while megagametophyte development was promoted by sucrose supplements in their media, embryo development was suspended on the same media.

CHAPTER 5

Endogenous levels of plant hormones during Douglas fir megagametophyte development

5.1 Introduction

Seed formation in higher plants is the result of a developmental program that is directed toward ensuring the establishment of the next generation of the plant through sexual means. The course of seed development represents a series of distinct physiological events of cell growth and differentiation that are regulated both temporally and spatially (Rock and Quatrano, 1995). The sequence of events that initiate the process of seed development in conifers is quite different from that in angiosperms. In angiosperms, double fertilization initiates two separate genetic programs, one directing embryogenesis and the other specific for development of the endosperm (Goldberg et al., 1989). Embryogenesis begins with the fusion of one male gamete with the egg cell of the embryo sac to form the zygote. Fusion of the second male gamete with the diploid central cell nucleus of the embryo sac initiates development of the triploid endosperm tissue. The endosperm is required for providing nourishment for the embryo during angiosperm seed development (Goldberg et al., 1989).

In conifers, two separate genetic programs control embryogenesis and megagametophyte development, but unlike angiosperms, these programs have very marked temporal separation. The process of seed development is initiated well before fertilization with the establishment of the haploid megagametophyte, a large structure made up of thousands of prothallial cells (Owens, 1991). The megagametophyte produces eggs (Singh, 1978), and in Douglas fir, each megagametophyte typically contains four egg cells (Fernando et al., 1998).

The further development of an ovule into a seed is dependent on the fertilization of at least one of the eggs. The megagametophyte degenerates if an egg is not fertilized, and in Douglas fir, this occurs within two weeks post fertilization (von Aderkas et al., 1997). Multiple embryos may be formed if more than one egg cell is fertilized, a phenomenon known as simple polyembryony (Owens, 1991). Due to postzygotic selection during early embryo development, only one of these embryos usually matures (for reviews, see Owens, 1991; Singh, 1978). The megagametophyte provides nourishment for the developing embryo and is the primary site for nutrient reserve accumulation during the maturation phase of seed development (Stone and Gifford, 1999; Owens et al., 1993; Misra and Green, 1994; Baker et al., 1996, Lammer and Gifford, 1989). The conifer megagametophyte is therefore functionally equivalent to the angiosperm endosperm (Owens, 1991).

According to physiological criteria, the series of events that occur during the course of seed development can be divided into three major stages; embryogenesis, maturation, and germination (Rock and Quatrano, 1995). Several classes of plant hormones have been identified in developing angiosperm seeds that have been implicated in the regulation of physiological processes that occur during the different phases. Embryogenesis begins with a period of increased cell division and growth during early development (Rock and Quatrano, 1995) to give rise to a globular embryo. The differentiation of two organ systems, the axis and the lateral organs, the cotyledons, result in a polarized embryo (Goldberg et al., 1989) in which the basic structure of the plant is defined (Mayer et al., 1993). The shoot and root meristems are contained within the embryonic axis and are required for postembryonic development (Mayer et al., 1993). The importance of gibberellins (GA), cytokinins (CK), and auxins (IAA) during this period of seed development has been suggested from several studies (for review, see Rock and Quatrano, 1995). Abscisic acid (ABA) is thought to play an important role in the maturation stage, which is characterized by the synthesis and accumulation of storage reserves (Ooms et al., 1993; Kermode et al., 1989; Berge et al., 1989; Farrant et al., 1996), the acquisition of desiccation tolerance (Pence, 1991; Ooms et al., 1993), the development of dormancy mechanisms (Karssen et al., 1983; Hole et al., 1989; Le Page-Degivry and Garello, 1992), and a reduction in seed moisture content (Rock and Quatrano, 1995). A switch from a maturation to a germinative program occurs at seed germination, and gibberellins are believed to be involved in this stage of embryo development (Wang et al., 1996). The storage reserves deposited during the maturation stage are hydrolyzed during germination to provide energy during early seedling growth (King and Gifford, 1997; Goldberg et al., 1989; Gifford et al., 1989; Lammer and Gifford, 1989).

Studies quantifying endogenous levels of biologically active forms of plant hormones during zygotic embryogenesis in conifers have been done for only a few species; Scots pine (Sandberg et al., 1987), white spruce (Carrier et al., 1999; Kong et al., 1997) and loblolly pine (Kapik et al., 1995). Studies on Douglas fir have been limited to investigating the role played by ABA in the establishment of dormancy in mature seeds (Jarvis et al., 1997; Bianco et al., 1997). Hormone levels during earlier stages of seed development have not been studied in this species. In addition, the levels of conjugated forms of the plant hormones identified to date in developing conifer seeds have not been investigated. Several studies in angiosperms have demonstrated the important role played by plant hormone conjugation in regulating the levels of the free forms of IAA, CK, GA, and ABA at a given developmental stage (for review, see Kleczkowski and Schell, 1995).

Hormone conjugation plays different roles depending on the hormone in question. In developing seeds, the synthesis of hormone conjugates may regulate the amount of a biologically active hormone by converting it to an inactive storage form, which may then be hydrolyzed in the presence of an appropriate developmental stimulus to release the free hormone (for reviews, see Kleczkowski and Schell, 1995; Bandurski et al., 1995; Walton and Li, 1995; McGaw and Burch, 1995). It appears that conjugates of the auxin indole-3-acetic acid (IAA), may function as a source of free IAA. Conjugated IAA was rapidly hydrolyzed to the free hormone during germination in *Pinus sylvestris* (Sandberg et al., 1987) and *Picea abies* (Sandberg and Ernstsen, 1987). The synthesis of cytokinin riboside conjugates does not result in complete inactivation, as the ribosides still show cytokinin activity, although to a lesser extent than the free cytokinins (for reviews, see McGaw and Burch, 1995; Kleczkowski and Schell, 1995). However, for the hormone ABA, the glucose ester conjugate (ABA-GE) is believed to be an end product of ABA metabolism, which cannot be hydrolyzed to the biologically active form in situ (for review, see Kleczkowski and Schell, 1995). Since the levels for the various plants hormones identified in developing conifer seeds change dramatically at different stages, it is conceivable that the levels of their conjugated forms may change at various developmental stages.

The objective of this study was to quantify the endogenous levels of the free and conjugated forms of three classes of plant hormones: the auxin IAA, the cytokinins zeatin (Z) and isopentenyladenine (iP), and ABA, in developing Douglas fir megagametophytes utilizing an enzyme-linked immunosorbent assay method. This study was done to address the question: Do the endogenous levels of the three major classes of plant hormones under investigation change during megagametophyte development and embryogenesis in Douglas fir?

5.2 Materials and Methods

5.2.1 Plant Materials

One clone of Douglas fir trees (clone 3265), which was provided by the BC Ministry of Forests (BCMF) Research Branch at Glyn Road Research Station in Victoria, was used for this experiment. Bagged seed cones were control pollinated with pollen collected from trees at the University of Victoria campus. Cones were collected weekly for 11 weeks, starting two weeks before fertilization i.e. eight weeks past pollination.

Megagametophytes were isolated without the nucellus under a dissecting microscope. In order to monitor the developmental stages at the time of isolation, five megagametophytes were fixed in 2.5% glutaraldehyde in 0.075M phosphate buffer at pH 7.2 and stored at 4^{o}C to be further processed and embedded in glycol methacrylate and sectioned. Megagametophytes for hormonal analysis were dissected a few at a time and placed on Whatman No. 1 filter paper moistened with double-distilled deionized water in a petri dish. They were then collected and frozen in a vial stored in liquid nitrogen, and this procedure was repeated until all the required material for each week had been dissected. The fresh weights and the number of megagametophytes collected each week were recorded. The vials were stored at -80^{0}C while dissections for the other weeks continued. When all collections were completed, the frozen samples were lyophilized for 48 hours and the dry weights for the weekly samples were recorded. The vials were stored at room temperature over silica gel until extracted.

5.2.2 Hormonal analysis

5.2.2.1 Extraction

The extraction procedure was as outlined in Jourdain et al. (1997). Approximately 25 mg (exact weight was recorded) of lyophilized samples from each week were placed in 5 ml of ice cold extraction buffer containing 100µl H^{3}-ABA (specific activity 1.59 Bq/mmol, 43.0 Ci/mmol, Amersham) and 100µl H^{3}-IAA (specific activity 999 Gbq/mmol, 27.0 Ci/mmol, Amersham) as internal standards. The extraction buffer was composed of 1:4 (v/v) acidified water : methanol supplemented with 2 mM butylhydroxytoluene (BHT), an antioxidant. The acidified water was made up with 2.3 mM glacial acetic acid and 0.29 mM triethylamine (pH 3.3) in a litre of double-distilled deionized water. Each sample was homogenized with a high speed tissue homogenizer (X520 CAT 500W M. Zipperer GmbH, courtesy of Dr. Will Hintz at the University of Victoria) and was immediately placed on ice until all samples had been ground. The homogenized samples were then placed on an orbital shaker at 350 rpm and left to extract for 72 hours at 4°C.

5.2.2.2 Purification of samples

Each extracted sample was purified through a 5 µm nitrocellulose filter (Sartorius) attached to a Sep-Pak C18 column (Waters) which had been equilibrated with methanol followed by extraction buffer. The Sep-Pak C18 column was in turn attached to a 0.2 µm teflon filter (Sartorius). The system was rinsed with 2 ml extraction buffer and this volume was combined with the eluted sample. The purified samples were concentrated to about 400 µl by rotary evaporation (Rotavapor-R, Büchi) at 40°C. Each flask was rinsed with 400 µl acidified water for a final volume of about 800 µl for each sample. These vials were stored at 4°C until fractionated.

5.2.2.3 Fractionation of samples

Concentrated samples were fractionated by reverse-phase high-performance liquid chromatography (rv-HPLC) using an octadecylsilane silica column (Lichrospher 100, RP-18, 5µm) at room temperature. In order to determine the retention times for the various plant hormones investigated, standard samples of the pure hormones were injected individually. Once the retention times had been determined, a sample composed of all the standards was run twice daily, one prior to fractionation of extracted megagametophyte samples and the other at the end of the day. Fractions were eluted for 1 hr at 1 ml / min. against an acetonitrile : acidified water gradient (see Jourdain et al., 1997) with a Beckman Gold HPLC System (Beckman, USA) connected to a Cygnet ISCO collector. The 60 fractions that were collected were stored at 4°C. Later these were dried on a Speed-Vac (Savant), methylated with 500 µl diazomethane in ether and then left to evaporate in a fumehood. The methylated fractions were reconstituted by adding 1.5 ml double-distilled deionized water supplemented with 3 mM sodium azide as a preservative and stored at 4°C until quantified.

5.2.2.4 Quantification of plant hormones

The endogenous levels of plant hormones investigated included the auxin indole-3-acetic acid (IAA), the cytokinins zeatin (Z) and isopentenyladenine (iP), abscisic acid (ABA) and their conjugates i.e. indole-3-aspartate (IAAsp), zeatin riboside (ZR), isopentenyladenosine (iPA) and abscisic acid-glucose ester (ABA-GE), respectively. An enzyme-linked immunosorbent assay (ELISA) method utilizing a streptavidin-biotin complex was used for quantification. Rabbit polyclonal antibodies against the various plant hormones were prepared by Dr. Godbillon at the University of Nancy I (France) and their working dilutions were provided by Dr. Phillippe Label at I.N.R.A. (France). The polyclonal antibodies were used according to the method described by Maldiney et al. (1986). The ELISA method used was as described by Jourdain et al. (1997). Polystyrene microtitration plates (Nunc-Immuno plates, MaxiSorp surface) were coated with ZR-, iPA-, ABA- or IAA-conjugated to ovalbumin and incubated at room temperature overnight. The plates were subjected to three fast washes followed by three ten-minute washes in buffer containing 0.1% Tween 20. A standard series of concentrations of the hormones under investigation (methylated forms for ABA and IAA) or megagametophyte sample and the appropriate rabbit polyclonal antibodies (primary antibodies) were then added. For methylated ABA and IAA standards, nine concentrations for the standard series ranging from 100 pM to 10 fM were used , and each concentration was replicated four times on each plate. For the cytokinins Z and iP, the nine concentrations used in the standard series ranged from 30 pM to 3 fM. Rabbit anti-IAA polyclonal antibodies were used to measure methylated IAA and IAAsp concentrations in the megagametophyte extracts. Anti-ABA antibodies were used for measuring methylated ABA and ABA-GE levels, anti-iPA antibodies were used for iP and iPA, and Z and ZR were measured using anti-ZR polyclonal antibodies. The plates were incubated at 4^{o}C in the dark for 2 hr. and then subjected to three fast washes followed by three ten-minute washes. A biotinylated secondary antibody (goat anti-rabbit

IgG) was added and the plates incubated at 40^0C for 1 hr. After washing as described above, an alkaline phosphatase conjugated to streptavidin in Tris-HCl buffer, pH 8, was added to the plates which were once more incubated at 40^0C for 1 hr. This was followed by addition of the substrate paranitrophenylphosphate (PNPP) after another washing step. The plates were incubated at 40^0C and the absorbance read at 405 nm using an MRX microplate reader (Dynatech Laboratories Inc.). The concentrations of the various hormones were calculated from the log transformed standard curves created on each plate for each hormone using a BioLinx program (Dynatech Laboratories Inc.). In only a few cases, the concentration could not be determined from the standard curve because the absorbance was below the detection limits of the curve, and the concentration for these samples was set at zero. Five replicates of each sample of megagametophyte extract were run for each hormone under investigation and confidence intervals were calculated ($\alpha = 0.05$).

5.2.2.5 Determination of correction factors for losses due to the procedures

H^3-ABA or H^3-IAA (100 µl each; obtained from the stock solution prepared by combining 2.5 µl of tritiated hormone with 4 ml extraction buffer) were fractionated separately by HPLC and eluted at 1 ml / min. against an acidified water : acetonitrile gradient for 1 hr as described above. 100 µl aliquots of each of the 60 fractions of the radiolabelled standard collected were mixed with 1000 µl scintillation fluid and counted on a liquid scintillation counter (Wallac 1410). Five vials containing only scintillation fluid (negative controls) were also counted to determine the background activity of the machine. The purity values for the tritiated ABA and IAA standards (which accounted for losses due to the fractionation procedure) were calculated separately using the formula:

$$\text{Purity value} = [(PV) - (N \times B)] / [(M) - (F \times B)]$$

where: PV is the sum of activity in DPM in tritiated fractions; N is the number of tritiated peaks; B is the average DPM of the five negative controls; M is the total DPM for all 60 fractions collected; F is the total number of fractions collected (60).

Five vials containing 100 µl tritiated standard in 1000 µl scintillation fluid (positive controls) were counted on the scintillation counter. These were used to calculate the reference values using the formula:

Reference value = average DPM x PV

where average DPM was calculated from the five positive controls and PV is the purity value calculated for the radiolabeled ABA or IAA standard.

The reference value represents the amount of radioactivity which should have been detected had there been no losses at various stages of purification, fractionation and methylation. This value was used to determine the correction factors for the megagametophyte samples. To determine losses due to the extraction, purification and fractionation procedures, the activity of 100 µl aliquots of the fractions containing the H^3-ABA internal standard in each sample were counted. The same procedure was repeated for the fractions containing the H^3-IAA. The correction factor for each of the extracts was calculated using the formula:

Correction factor = [TP x 15] / RV

where: TP is the sum of activity (DPM) of fractions containing tritiated ABA or IAA; 15 is the dilution factor for each fraction and RV is the reference value for ABA or IAA.

The correction factors calculated for ABA in each sample were used to correct the concentrations of ABA and ABA-GE determined by ELISA in that sample. IAA and IAAsp concentrations were corrected with the IAA correction factors. For each of the 11 weeks of samples analyzed, the average of the ABA and IAA correction factors for each sample was used to correct the concentrations of iP, iPA, Z and ZR determined by ELISA. Using these correction factors allowed the concentrations of the various hormones determined by ELISA to be adjusted for losses due to extraction, purification, fractionation and methylation procedures.

5.3 Results

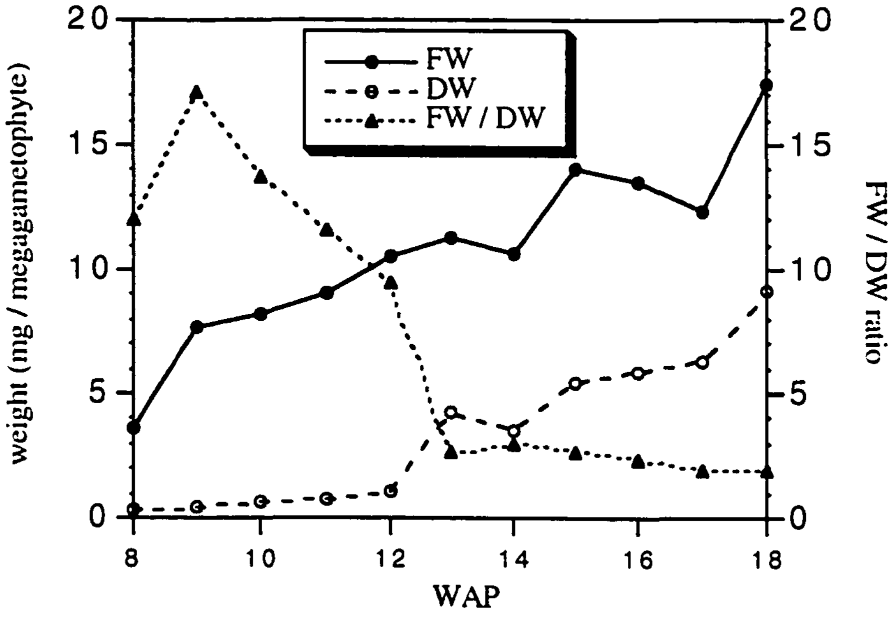
5.3.1 Megagametophyte fresh and dry weights

Megagametophytes dissected eight and nine weeks past pollination were at the late central cell and early egg cell stages, respectively. The period of embryogenesis studied spanned 9 weeks, from fertilization (10 WAP) until the early cotyledonary stage (18 WAP), during which time megagametophyte fresh weight increased continuously. Prior to fertilization, late central cell stage megagametophytes had an average fresh weight of 3.5 mg at 8 WAP and this value doubled to 7.6 mg at 9 WAP (Figure 15). The increase in megagametophyte fresh weight during this period was not accompanied by a change in the dry weight, and this was indicated by the high fresh weight to dry weight ratio in Figure 15. After fertilization, megagametophyte fresh weight increased from 8.2 mg to 17.4 mg between 10 and 18 WAP (Figure 15). The dry weight remained low for the first two weeks after fertilization, then increased dramatically between 12 and 13 WAP, and continued to increase until the early maturation stage during week 18 (Figure 15). The increase in dry weight between 12 and 13 WAP was accompanied by a sharp decrease in the fresh weight to dry weight ratio.

5.3.2 Hormonal analysis

The three classes of plant hormones investigated in this study were identified in Douglas fir seeds, and the levels of their free and conjugated forms varied during development. The concentrations of the free and conjugated forms of cytokinin, auxin and ABA at various times after pollination are presented separately below on a fresh weight as well as on a dry weight basis.

Figure 15. Changes in the fresh and dry weights of Douglas fir megagametophytes over time.



5.3.2.1 Endogenous cytokinin concentration

Cytokinin levels varied during development. On a dry weight basis, Z levels were highest in megagametophytes at the late central cell stage two weeks before fertilization (8 WAP), at a concentration of 24 ng / g DW. The concentration declined over two weeks to reach undetectable levels at 10 WAP (Figure 16). A sharp increase in Z levels occurred in the first three weeks of embryogenesis, and the concentration peaked during week 13 at 18 ng / g DW. Z concentration declined 2-fold between 13 and 14 WAP, and then remained constant until 18 WAP (Figure 16). ZR levels were low at 8 WAP and remained constant until 13 WAP, at which point they reached a peak level of about 300 ng / g DW (Figure 16). Although this peak coincided with the peak for the free form of the cytokinin, the concentration of the conjugate was 17 times greater than Z at 13 WAP. Between 13 and 14 WAP, ZR levels declined and remained constant until 16 WAP. A second ZR peak occurred at 17 WAP, during which the concentration increased to over 2000 ng / g DW. At 18 WAP, the ZR content of megagametophytes had decreased to undetectable levels (Figure 16).

On a fresh weight basis, ZR levels were low between 8 and 12 WAP (Figure 17). Prior to fertilization, free Z levels decreased from 2.0 ng / g FW in late central cell stage megagametophytes to undetectable levels at fertilization (10 WAP)(Figure 17). The endogenous Z content of fertilized megagametophytes increased between 10 and 13 WAP, with the major accumulation occurring between 12 and 13 WAP. On a fresh weight basis, megagametophytic Z content was highest 13 WAP, during which Z levels reached a peak of 6.5 ng / g FW (Figure 17). This was followed by a sharp decline to about 3.0 ng / g FW at 14 WAP, at which point the concentration remained constant until 18 WAP (Figure 17). ZR levels peaked twice in megagametophytes collected after fertilization, with concentrations of 100 and 1000 ng /g FW at 13 and 17 WAP, respectively (Figure 17).

Figure 16. Endogenous levels of Z and ZR, on a dry weight basis, during Douglas fir megagametophyte development.

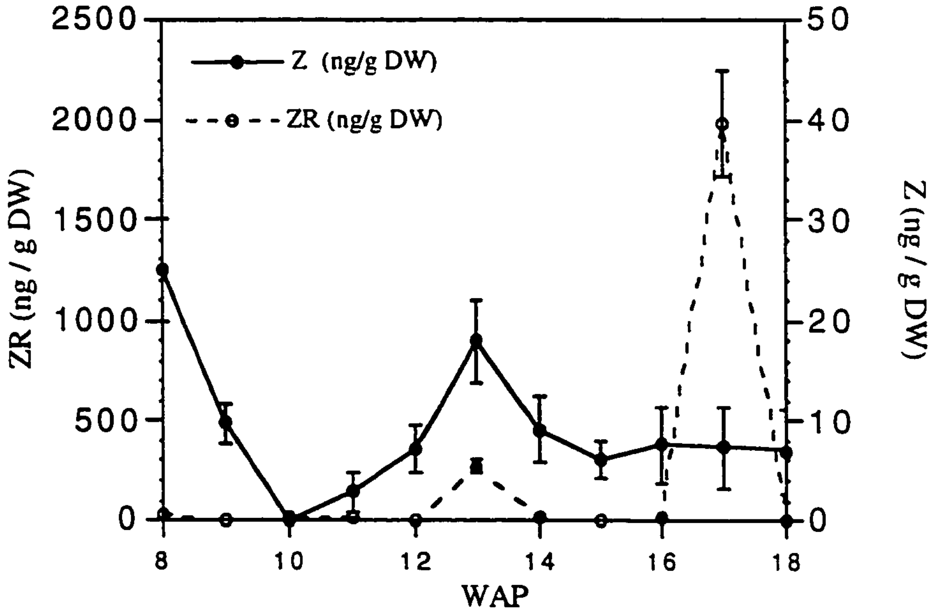
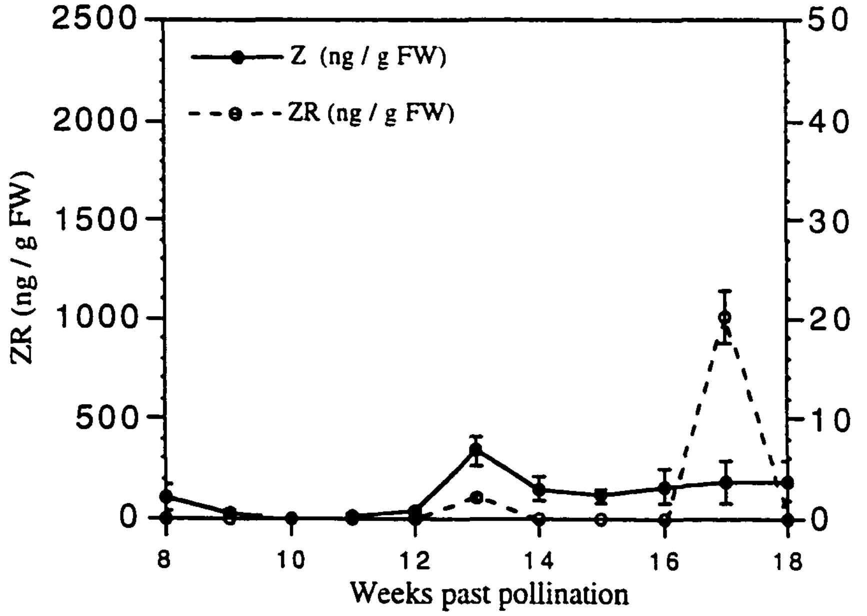


Figure 17. Endogenous levels of Z and ZR, on a fresh weight basis, during Douglas fir megagametophyte development.



The accumulation pattern for iP during megagametophyte development was different from that for Z. On a dry weight basis, endogenous iP concentrations were generally two orders of magnitude higher than Z concentrations. The free iP concentration was low in megagametophytes prior to fertilization at 8 and 9 WAP, increased 9-fold between 9 and 10 WAP to peak at a concentration of about 2250 ng / g DW during fertilization week, and then decreased over the next two weeks to reach low levels at 12 WAP (Figure 18). The concentration increased to 1250 ng / g DW in megagametophytes at 13 WAP, declined to low levels between 14 and 16 WAP, peaked at 1500 ng / g DW at 17 WAP and then declined during week 18.

The concentration of the iP conjugated form, iPA, remained consistently lower than the free form of the cytokinin between 8 and 12 WAP (Figure 18). Between 12 and 18 WAP, iPA concentration in megagametophytes increased dramatically during weeks 13 and 17. The highest iPA accumulation of about 5250 ng/g DW occurred at 17 WAP, after which iPA concentration decreased to undetectable levels at 18 WAP (Figure 18).

On a fresh weight basis, iP and iPA were present at low levels in late central cell and early egg cell stage megagametophytes (Figure 19). iP levels increased to 200 ng/g FW at 10 WAP, declined over the next two weeks, and then increased to 500 ng /g FW at 13 WAP. iPA levels increased notably between 13 and 17 WAP. The conjugated iPA levels increased to about 700 ng / g FW at 13 WAP. The major iPA accumulation occurred in megagametophytes at 17 WAP, when the concentration increased to 2700 ng / g FW. This iPA peak coincided with high levels of iP at 700 ng / g FW. The levels of both the free and conjugated forms of the cytokinin showed a sharp decrease in megagametophytes during week 18 (Figure 19).

5.3.2.2 Endogenous IAA and IAAsp concentrations

The endogenous auxin content of megagametophytes changed with time (Figure 20). Prior to fertilization, IAA reached its highest concentration of 175 ng / g DW in

Figure 18. Endogenous levels of iP and iPA, on a dry weight basis, during Douglas fir megagametophyte development.

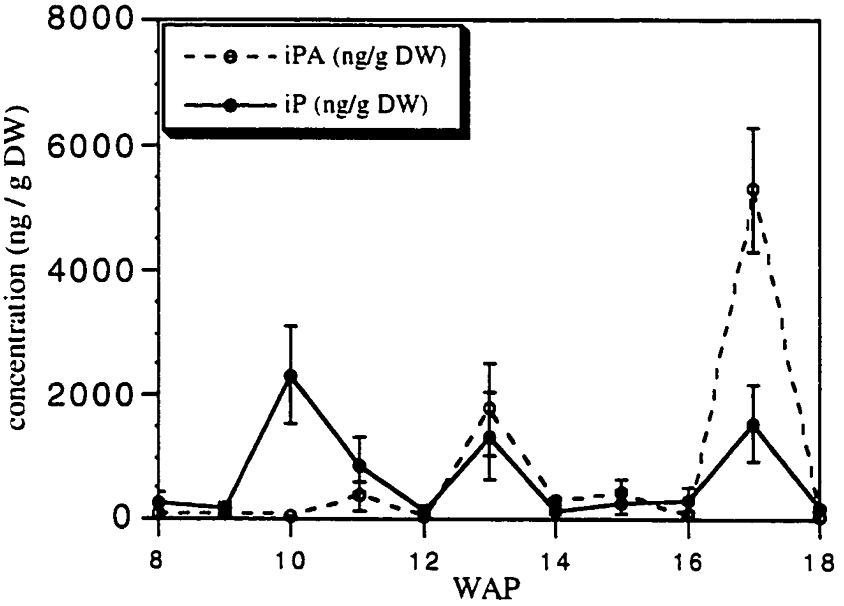


Figure 19. Endogenous levels of iP and iPA, on a fresh weight basis, during Douglas fir megagametophyte development.

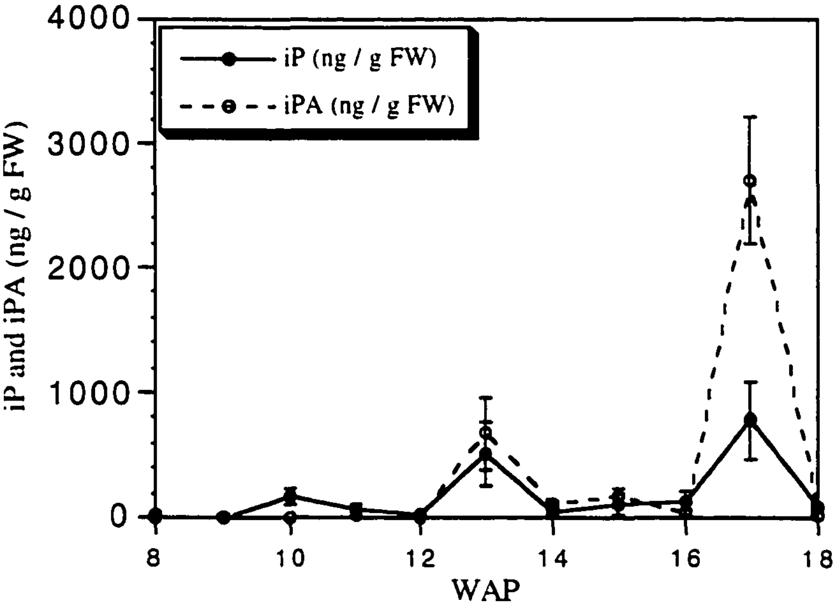
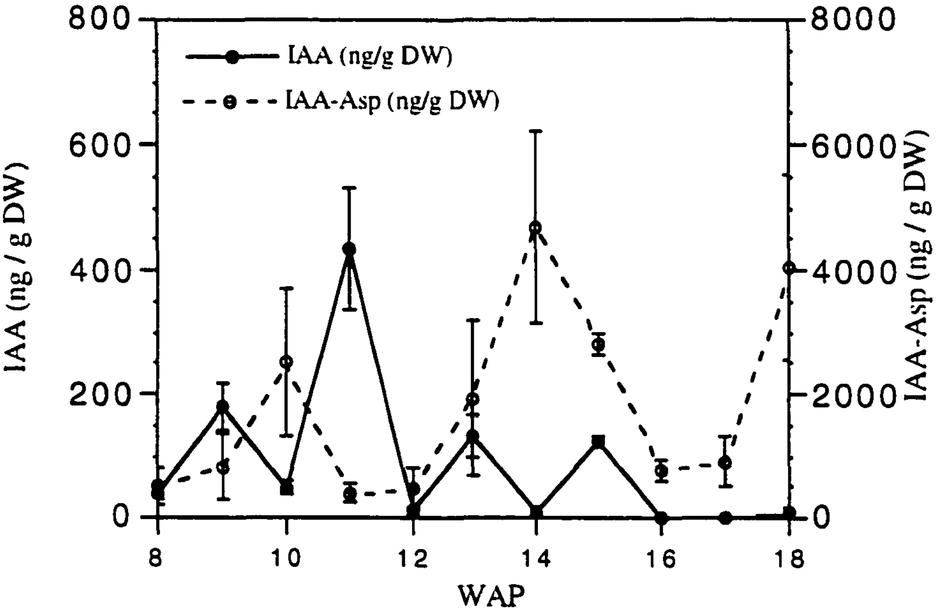


Figure 20. Endogenous levels of IAA and IAAsp, on a dry weight basis, during Douglas fir megagametophyte development.



megagametophytes a week before fertilization, at 9 WAP. The IAA content decreased at fertilization to about 50 ng / g DW and then increased almost 9-fold to reach its highest level 11 WAP (Figure 20). IAA levels decreased sharply to undetectable levels at 12 WAP, peaked again at 13 and 15 WAP and then remained low until 18 WAP (Figure 20). IAAsp concentrations reached their highest levels at 10, 14 and 18 WAP (Figure 20). During these times, IAAsp levels in megagametophytes were two orders of magnitude higher than free IAA concentrations, with the highest accumulation occurring at 14 and 18 WAP, when IAAsp concentrations were 4750 and 4000 ng / g DW, respectively.

On a fresh weight basis, IAAsp levels in megagametophytes were generally higher than free IAA (Figure 21). IAA content peaked at 9, 11, 13 and 15 WAP, with the major peaks occurring during embryogenesis (Figure 21). IAAsp concentration was low until 12 WAP, at which point the conjugated hormone levels increased and remained constant until week 15. IAAsp concentration declined between 15 and 17 WAP, and then increased dramatically at 18 WAP (Figure 21).

5.3.2.3 Endogenous ABA and ABA-GE concentrations

ABA and ABA-GE showed different accumulation patterns in megagametophytes at various times during development. In megagametophytes isolated prior to fertilization, ABA-GE concentrations were slightly higher than ABA concentrations (Figure 22). On a dry weight basis, megagametophytes isolated 8 WAP contained ABA at a concentration of 0.5 µg / g DW and ABA-GE at 1.5 µg /g DW. The ABA content declined in the following two weeks to reach low levels at 10 WAP whereas the ABA-GE content remained constant. Between 10 and 11 weeks, an increase in ABA concentration to 1.5 µg / g DW was accompanied by a decrease in ABA-GE concentration to 0.5 µg / g DW. At 12 WAP, ABA-GE levels increased as the ABA concentration declined. ABA-GE remained constant from 12 WAP until 18 WAP. ABA concentration peaked at 13 WAP and then declined over the next three weeks. The major ABA accumulation occurred at

Figure 21. Endogenous levels of IAA and IAAsp, on a fresh weight basis, during Douglas fir megagametophyte development.

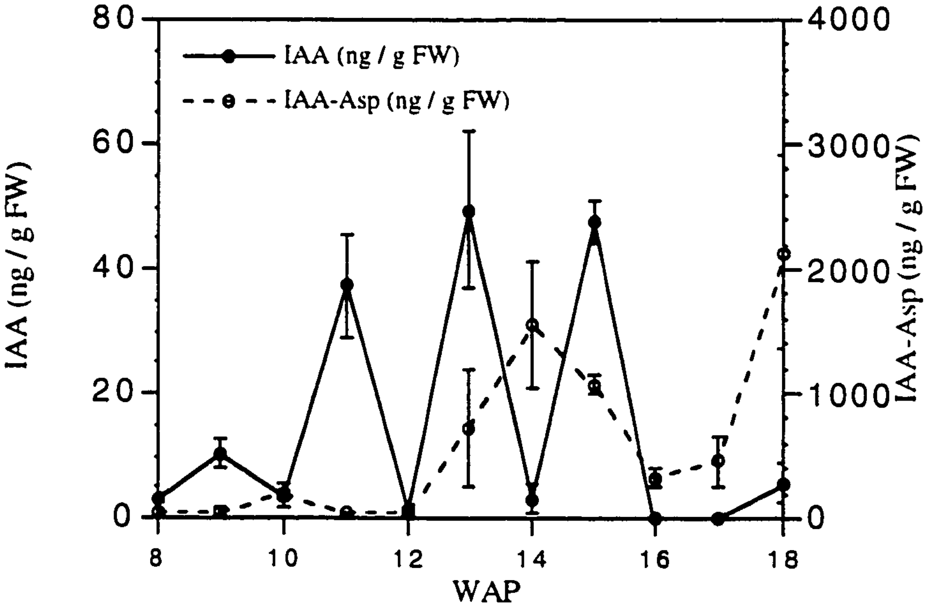


Figure 22. Endogenous levels of ABA and ABA-GE, on a fresh weight basis, during Douglas fir megagametophyte development.

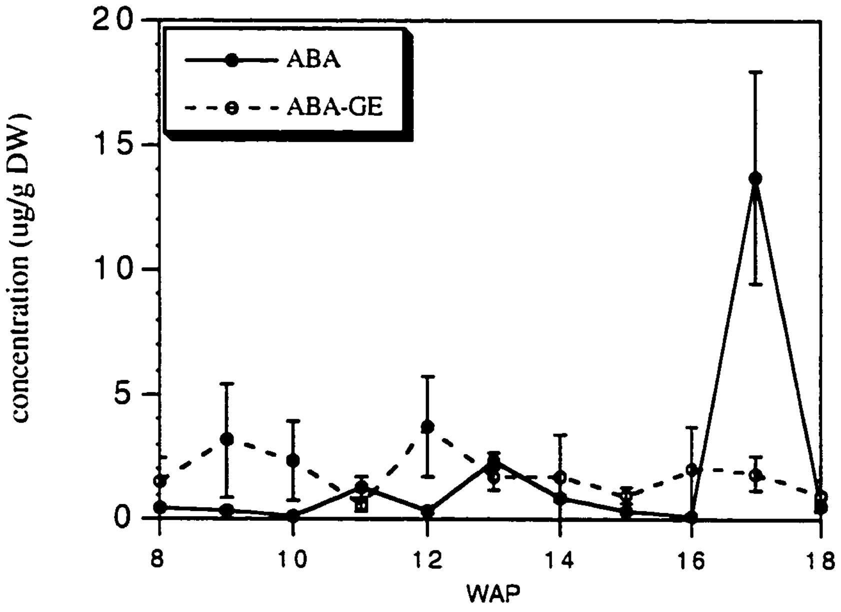
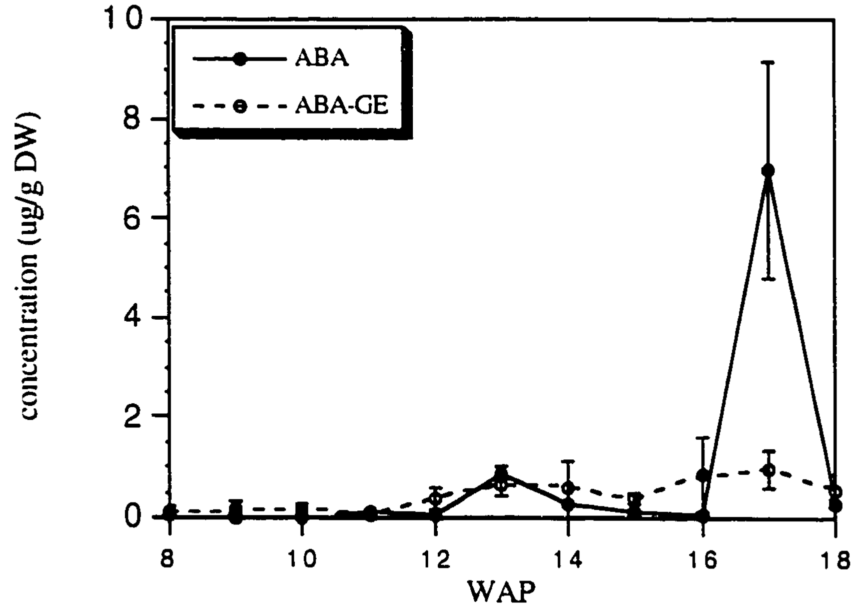


Figure 23. Endogenous levels of ABA and ABA-GE, on a fresh weight basis, during Douglas fir megagametophyte development.



17 WAP, during which ABA levels increased dramatically to almost 14 μg / g DW, which was followed by a sharp decline to 0.5 μg /g DW at 18 WAP (Figure 22).

On a fresh weight basis, ABA and ABA-GE levels were low between 8 and 11 WAP (Figure 23). At 12 WAP, ABA-GE concentration increased to 0.3 μg / g FW and remained constant until 18 WAP. ABA concentration increased to about 1 μg / g FW at 13 WAP, and then decreased over the next three weeks until the levels increased markedly between 16 and 17 WAP. The endogenous ABA and ABA-GE content of megagametophytes at 18 WAP was low.

5.4 Discussion

In this study, the endogenous levels of free and conjugated forms of three classes of plant hormones were investigated during Douglas fir megagametophyte development. Prior to fertilization, megagametophytes were at the late central cell and early egg cell stages at 8 and 9 WAP, respectively. Fertilization occurred at 10 WAP, and the total hormonal content of megagametophytes and embryos was quantified from fertilization and terminated a week after cotyledon initiation (18 WAP) during the early maturation stage. From 8 to 12 WAP, the fresh weight to dry weight ratio was high, indicating that megagametophytes were mostly aqueous. During later stages of development, the ratio declined and remained constant as the megagametophyte DW increased. A similar trend occurred during seed development in white spruce (Carrier et al., 1999; Kong et al., 1997). Because of the change in the fresh weight to dry weight ratio with development, there is a question as to whether the levels should be expressed on a fresh weight or a dry weight basis. During early stages, hormone concentrations calculated on a fresh weight basis are probably more significant than those on a dry weight basis. The concentrations reported in this study were calculated on a dry weight basis as well as on a fresh weight basis to account for these differences in megagametophyte structure.

The overall trends in the concentrations of the three classes of plant hormones investigated were different over the course of megagametophyte development. A slight increase in ABA was detected in megagametophytes a week after fertilization, when megagametophytes were at the proembryo stage. Storage reserve accumulation begins about two week after fertilization in Douglas fir (von Aderkas et al., 1997). The increase in ABA at 11 WAP coincided with the deposition of starch in prothallial cells in the central region of the megagametophyte prior to corrosion cavity formation, suggesting that the hormone may be involved in stimulating the synthesis of nutrient reserves in the developing seed. A second increase in ABA occurred at 13 WAP, during which there was a dramatic increase in megagametophyte dry weight. The highest ABA accumulation occurred at 17 WAP, during cotyledon initiation. Carrier et al. (1999) recently reported the deposition of lipids in developing white spruce seeds containing early embryos, with the major accumulation occurring in the megagametophytes. Their results are in agreement with what was published for *Pinus resinosa* by Gates and Greenwood (1991), who detected an increase in soluble protein and lipid in supernatant from megagametophytes containing early embryos. There was a dramatic increase in the megagametophytic content of protein and lipid at the early cotyledonary stage (Gates and Greenwood, 1991). Kong et al. (1997) reported peak levels of ABA during late maturation in white spruce seeds. However, the study by Kong et al. (1997) yielded different results from that by Carrier et al. (1999). The latter researchers reported high ABA levels in megagametophytes prior to fertilization and a decrease to low levels during maturation. The different results within the same species could be a reflection of the different methods used for hormone quantification in the two studies. ABA levels decreased during late maturation in loblolly pine (Kapik et al., 1995), suggesting that the pattern of ABA accumulation during seed development in conifers may be species dependent (Kong et al. (1997)

Prior to fertilization, Z levels were highest in megagametophytes at the late central cell. After fertilization, the cytokinin peaked at 13 WAP during early embryogenesis and then declined and remained constant with further development. iP levels increased during fertilization (10 WAP), and at 13 and 17 WAP. An increase in IAA levels occurred prior to fertilization in megagametophytes at the early egg cell stage at 9 WAP. Kong et al. (1997) obtained similar results for IAA in white spruce seeds analyzed prior to fertilization, and suggested that IAA may be required to support megagametophyte growth during this period. IAA concentration increased from the proembryo through to the late maturation stage in white spruce (on a fresh weight basis), indicating that auxins play a significant role during embryogenesis in this species (Kong et al., 1997). Sandberg et al. (1987) also reported high levels of free IAA in immature Scots pine seeds during periods when the embryos were actively growing. In the present study, IAA levels increased in megagametophytes at the proembryo stage (11 WAP) as well as during early embryo development (13 and 15 WAP), and then began to increase during early maturation at 18 WAP, suggesting that auxins may also be important during Douglas fir embryogenesis.

The relationship between the concentration of the free hormone and its conjugated form varied depending on the hormone in question. For the auxin investigated in this study, IAAsp levels were always higher than free IAA (on a dry weight basis) at all stages of megagametophyte development studied. The peak levels of the conjugate (on a dry weight basis) occurred at the stages when free IAA levels were lowest, suggesting that hormone conjugation may play a role in IAA metabolism during seed development in Douglas fir. The sharp increase in IAAsp that occurred during late embryo development at 18 WAP agrees with what has been published for *Pinus sylvestris* by Sandberg et al. (1987). These authors found that IAA occurred predominantly in the conjugated form during the later stages of seed development. In *Picea abies*, Sandberg and Ernstsen (1987) also reported higher levels of conjugated IAA in mature seeds. The conjugated IAA was rapidly hydrolyzed to the free hormone during germination (Sandberg et al., 1987; Sandberg and Ernstsen, 1987).

ABA-GE was identified at all stages of seed development studied, although the concentration remained relatively constant. Although it is generally believed that ABA-GE is an end product of ABA metabolism (for review, see Kleczkowski and Schell, 1995), Walton and Li (1995) suggested that ABA-GE may function as a storage form of ABA, which could be hydrolyzed by esterases to release the free hormone. It does not seem likely from the results of this study that the conjugate functioned as a source of the free hormone, since the major accumulations of ABA were not accompanied by a dramatic decrease in ABA-GE levels. However, between eight and 12 weeks past pollination, the conjugated ABA was present at a higher concentration at times when the free ABA was at low levels, suggesting that ABA metabolism during Douglas fir seed development may be through synthesis of ABA-GE. ABA-GE was also identified and quantified during tomato seed development by Hocher et al. (1991). They found that the conjugated ABA levels were lower than the free ABA, and suggested that ABA metabolism in tomato seeds was through its conversion to phaseic acid and hydrophaseic acid (Hocher et al., 1991).

The riboside conjugates of Zt and iP, i.e. ZR and iPA, were also identified in this study. Although cytokinin ribosides are generally less active than the free cytokinins (for review, see Kleczkowski and Schell, 1995), ZR and iPA are found as constituents of tRNA through which their activity is mediated, whereas activity of the free cytokinins is not dependent on tRNA (McGaw and Burch, 1995). The precise mechanism of cytokinin action has not been fully described, but it has been suggested that cytokinins may stimulate protein synthesis by increasing the rate of RNA synthesis. The tRNA cytokinins, including ZR and iPA, may exert their effect on protein synthesis at the translational level (McGaw and Burch, 1995). Both ZR and iPA showed their largest accumulations at 13 and 17 weeks past pollination which coincided with high levels of

ABA. ABA has been shown to stimulate the synthesis of storage proteins during seed maturation (Perata et al., 1990; Finkelstein et al., 1985; for review, see Dodeman et al., 1997) as well as proteins required for the acquisition of desiccation tolerance during the late maturation stage (Farrant et al., 1996; Berge et al., 1989; Barratt and Clark, 1991; Baker et al., 1995; Wehmeyer et al., 1996). The high levels of ZR and iPA during the early maturation stage of Douglas fir megagametophyte development suggests that these tRNA cytokinins may be required for promoting protein synthesis during this period.

In conclusion, the identification of the free forms of the auxin IAA, the cytokinins Zt and iP, and of ABA in developing seeds of Douglas fir suggests that these hormones play an important role during embryogenesis and megagametophyte development. The conjugated forms of these hormones were also identified at various stages, suggesting that synthesis of plant hormone conjugates plays an important role in regulating the levels of the biologically active hormones during the course of seed development in Douglas fir.

CHAPTER 6

GENERAL DISCUSSION

A review of the literature reveals that seed development in conifers has been studied at various levels. Consequently, the events occurring at different stages of megagametophyte and embryo development have been described. In contrast, the levels of plant hormones over the course of seed development in conifers have been investigated in only a few species. This chapter discusses the results of my research during Douglas fir megagametophyte development with respect to the four major questions as originally outlined in the introduction in Chapter 1. I have summarized the developmental stages of the pre-fertilization and post-fertilization megagametophytes that were used for experiments in this thesis in Table 1 of Chapter 3. Storage reserves that were detected at the various stages are also summarized in the table. The hormones that were present at high concentrations during the different stages are summarized in Table 5. The levels of the plant hormones reported in this study are then compared to the levels of plant hormones in other conifers in Table 6.

6.1. Question 1. Do Plant hormone levels change during megagametophyte development in Douglas fir?

Seed development in Douglas fir is characterized by a series of distinct morphological and physiological events that occur at different times during megagametophyte development and embryogenesis. The accumulation patterns for the three classes of plant hormones investigated varied with the developmental stage of the megagametophytes. High amounts of endogenous auxin and cytokinin were detected prior to fertilization, and may be required for development of megagametophytes from the central cell stage to the egg cell stage (see Table 5). von Aderkas et al. (1997) reported that Douglas fir megagametophytes in situ undergo considerable growth prior to fertilization, between 7 and 10 WAP.

During fertilization week, the only free hormone that was detected at a peak level in megagametophytes (on a dry weight basis) was iP (Table 5). Even though fertilization was not observed in the present study, proembryos were present at the chalazal end of the archegonia at 11 WAP, suggesting that fertilization occurred during 10 WAP. As reported by Allen and Owens (1972), the zygote stage in Douglas fir is very brief. Embryo development begins with a period of free nuclear divisions within the archegonium, followed by cell wall formation to give rise to a cellularized proembryo within a week after fertilization (Allen and Owens, 1972). During the proembryo stage, there was an increase in the concentrations of IAA and ABA, suggesting that auxin may be involved in promoting proembryo development. The high levels of ABA may be involved in the cellular changes that occur within the prothallial cells in the corrosion cavity region of the megagametophyte, which accumulate abundant starch (see Table 4). The increase in IAA content of Douglas fir megagametophytes during the proembryo stage is unlike what was reported by Kong et al. (1997) for white spruce, in which IAA levels decreased in seeds at a similar developmental stage.

The next phase of embryo development, the early embryo stage, begins with elongation of the embryonal suspensors, which pushes the embryo into the corrosion cavity within the megagametophyte tissue (Allen and Owens, 1972). This is followed by a period of increased cell division and growth, resulting in an embryonal-mass stage embryo (Krasowski and Owens, 1993) and eventually the formation of a club-shaped embryo (Allen and Owens, 1972). The club-shaped embryo is polarized into a stele promeristem and a root cap (Grob et al., 1999). At three weeks after fertilization (13 weeks past pollination), there was an increase in the levels of IAA, iP, Z, and ABA in

Table 5. Plant hormone detected at high levels during the different stages of megagametophyte development

(WAP)	Developmental stage	Plant hormones present at high concentrations (on a dry weight basis)
7	early central cell	
8	late central cell	Z
9	early egg cell	Z, IAA
10	mature egg cell / fertilization	iP, IAAsp.
11	proembryo	IAA, ABA
12	early embryo / no corrosion cavity	
13	early embryo in corrosion cavity	Z, ZR, iP, iPA, IAA, ABA
14	early embryo	IAAsp.
15	early embryo / embryonal mass stage	IAA
16	polarized embryo	
17	early maturation	iP, iPA, ZR, ABA

megagametophytes (Table 5). The high levels of plant hormones during this week coincided with the dramatic increase in megagametophyte dry weight. The high concentration of ABA at 13 weeks past pollination, coupled with the dramatic increase in megagametophyte dry weight, suggests that the hormone may be involved in stimulating the synthesis and accumulation of storage reserves in the developing seed.

When embryos were at the embryonal mass stage (15 WAP) a week prior to polarization, IAA concentration increased in megagametophytes (Table 5). An increase in auxin levels occurred in white spruce seeds (on a fresh weight basis) from the club-shaped embryo stage through to the late maturation stage (Kong et al., 1997). These results suggest that auxin and cytokinins may be required for promoting early embryo growth. It is unlikely that the auxins and cytokinins in Douglas fir seeds were required for further growth of the megagametophytes. von Aderkas et al. (1997) found that Douglas fir megagametophytes in situ had achieved their maximum lengths by fertilization.

During the early maturation stage of seed development (17 WAP) when cotyledons were being initiated, the ABA and cytokinin content of megagametophytes increased markedly (Table 5). IAA was not detected during this week, suggesting that cotyledon initiation may be regulated by cytokinins. It is during this stage of embryogenesis that the root and shoot apical meristem are established (Allen and Owens, 1972).

Although the precise mechanisms of auxin and cytokinin action are not fully understood, CK have been shown to play an important role in promoting growth by stimulating cell division (for review, see McGaw and Burch, 1995). Auxins, on the other hand, exerts their influence on growth by promoting cellular elongation. The acid-growth hypothesis has been suggested as an explanation for auxin induced cell growth (for review, see Cleland, 1995). According to this theory, auxin stimulates active excretion of protons from a cell into the surrounding cell wall. The low pH then activates cell-wall loosening enzymes, which break down non-covalent bonds between xyloglucans and cellulose in the cell wall, which in turn results in a decrease in cell wall tension. The decrease in cell turgor, resulting from the low tension in the cell wall, allows additional water uptake by the cell, which exerts an outward pressure on the plastic cell wall causing cellular elongation.

6.1.1 Comparison of plant hormone levels in this study with those from other conifer studies.

The determination of plant hormone levels requires the utilization of highly sensitive and specific methods. Physico-chemical methods currently available for hormone quantification include gas chromatography (GC), gas chromatography/mass spectroscopy (GC/MS), and gas chromatography-selected ion monitoring-mass spectroscopy (GC-MS) (Hock et al., 1992). The ELISA method which was used in this study is an indirect method, and there is always a question on the efficiency of the procedure. Ideally, the results should be validated using independent physico-chemical methods (Hock et al., 1992). In conifers, the results from two studies that used an ELISA method for investigating the levels of ABA in developing seeds of white spruce (Carrier et al., 1999) and loblolly pine (Kapik et al., 1995) were also validated with physico-chemical methods. The levels of ABA and IAA in developing Douglas fir seeds determined by ELISA in this study agree with the levels reported by Kong et al. (1997), who used GC-MS to quantify these hormones in developing white spruce seeds. For the cytokinin analysis in the present study, tritiated standards were not available and as a result, experimental losses due to extraction, purification and fractionation procedures could only be approximated. In addition, the results could not be validated by GC-MS because this method only became possible for cytokinins recently (Astot et al., 1998).

The levels of free IAA and ABA in Douglas fir megagametophytes in this study were within the same ranges as those published for other conifers studied to date. These results are summarized in table 6 below. Endogenous cytokinin levels were not available

Table 6. A comparison of the levels of plant hormones in seeds of different conifer species studied to date.

Authors	Species studied	Hormone studied	Method used	concentration range
Kong et al. (1997)	*Picea glauca*	ABA	GC-MS	1-25 μg/g DW
Carrier et al. (1999)	*Picea glauca*	ABA	ELISA	1-23 μg/g DW
Kapik et al. (1995)	*Pinus taeda*	ABA	ELISA	2-6.5 μg/g DW
This study	*Pseudotsuga menziesii*	ABA	ELISA	0.5-13 μg/g DW
Kong et al. (1997)	*Picea glauca*	IAA	GC-MS	0.5-5.5 μg/g DW
This study	*Pseudotsuga menziesii*	IAA	ELISA	0.05-0.45 μg/g DW
This study	*Pseudotsuga menziesii*	Z	ELISA	6-25 ng/g DW
This study	*Pseudotsuga menziesii*	iP	ELISA	0.25-2.3 μg/g DW

Note: In the table, the concentration range for ABA in the studies by Carrier et al. (1999) and Kapik et al. (1995) have been calculated in μg/g DW from those reported in the respective papers for easier comparison.

in the literature for conifer seeds, and as a result, only the levels of Z and iP from the present study are shown in Table 6. I have compared the levels of cytokinins in Douglas fir seeds from my research with those that have been published for angiosperms and somatic embryogenic masses for hybrid larch in Table 7. The levels for Z in Douglas fir agree with those reported in the review by McGaw and Burch (1995) in developing *L. luteus* seeds and for somatic embryogenic masses for hybrid larch (Jourdain et al., 1997), but are higher than those published by Astot et al. (1998) in leaves of *Arabidopsis thaliana* (Tables 6 and 7). iP and iPA levels from this study are higher than the concentrations reported in the other studies in Table 7. However, it is difficult to conclude whether this is a reflection of the levels in the tissues analyzed or the different methods used for quantification.

6.2. Question 2. Are morphological and physiological changes during development independent of one another?

Based on the results of my research, the levels of plant hormones investigated correlated with events occurring in Douglas fir megagametophytes and embryos at different developmental stages (see Tables 4 and 5). However, the absence of receptor data for any of these hormones makes it difficult to draw definite conclusions about their effects during seed development. The fact that the levels of hormones change does not necessarily mean that there cannot be a change in tissue sensitivity (Trewavas, 1981). Of the nine classes of plant hormones identified to date, considerable progress has been made in elucidating the signaling pathways for ethylene (reviewed by Lelièvre et al., 1997) and ABA (Cutler et al., 1996; Pei et al., 1998). Genes encoding proteins involved in ABA signaling during seed development were identified using genetic analysis experiments with response mutants in *Arabidopsis*. One of the genes encodes a protein with phosphatase activity, suggesting that protein phosphorylation and dephosphorylation plays a role in ABA signaling (see McCourt, 1999 for review).

Table 7. Comparison of cytokinin levels in developing *L. luteus* seeds (see McGaw and Burch, 1995), somatic embryogenic masses of hybrid larch (Jourdain et al., 1997), *Arabidopsis thaliana* leaves (Astot et al., 1998) and Douglas fir seeds in this study.

Authors	Species	Organ	Cytokinin	concentration
McGaw and Burch (1995)	*L. luteus*	seed	Z	18.4 ng/g FW
McGaw and Burch (1995)	*L. luteus*	seed	ZR	393.5 ng/g FW
Astot et al. (1998)	*A. thaliana*	leaves	Z	0.39 ng/g FW
Astot et al. (1998)	*A. thaliana*	leaves	ZR	0.77 ng/g FW
Astot et al. (1998)	*A. thaliana*	leaves	iP	0.24 ng/g FW
Astot et al. (1998)	*A. thaliana*	leaves	iPA	0.34 ng/g FW
This study	*P. menziesii*	seed	Z	1-7 ng/g FW
This study	*P. menziesii*	seed	ZR	0.1-1 µg/g FW
This study	*P. menziesii*	seed	iP	0.2-0.7 µg/g FW
This study	*P. menziesii*	seed	iPA	0.2-2.7 µg/g FW
Jourdain et al. (1997)	Hybrid larch	somatic embryos	Z	5-9 ng/g DW
Jourdain et al. (1997)	Hybrid larch	somatic embryos	iP	30-79 ng/g DW
Jourdain et al. (1997)	Hybrid larch	somatic embryos	iPA	71-87 ng/g DW

6.3. Question 3. Can megagametophytes be cultured prior to fertilization?

Allen and Owens (1972) reported that Douglas fir megagametophytes undergo considerable growth prior to fertilization. Similar to what occurs in situ, unpollinated megagametophytes isolated prior to fertilization continued to grow on media supplemented with auxin and cytokinin. However, the different cell types of the megagametophytes showed different responses to culture conditions. The prothallial cells could be maintained for long periods in culture whereas the viability of the archegonia rapidly declined under the same conditions. Fernando et al. (1997) and Ma et al. (1998) also found that the prothallial, jacket and neck cells continued to divide in vitro.

High levels of auxin and cytokinin were detected in megagametophytes isolated prior to fertilization in the hormone analysis experiment (see Table 5), suggesting that these hormones are required for megagametophyte development. The reason for the rapid decline of archegonia in cultured megagametophytes is unclear.

6.4. Question 4. Can embryos be rescued from megagametophytes isolated soon after fertilization?

In vitro fertilization and four nucleate proembryo formation was achieved in Douglas fir by Fernando et al. (1998). No embryos were rescued in their experiment and these authors pointed out the difficulty in maintaining proembryo development in vitro. In the embryo rescue experiment with Douglas fir megagametophytes in the present study, further development of proembryos and early embryos was promoted up to the globular stage on media supplemented with auxin and cytokinin. However, a majority of the embryos did not develop past this stage when cultured on media supplemented with ABA and cytokinin. Only three embryos matured on media containing ABA concentrations of 5, 20 or 40 μM. The low yield of mature embryos could have been because the hormone supplements used in the media did not closely mimic those of megagametophytes in situ. The megagametophytes were cultured on media supplemented with auxin and cytokinin for the first 21 days, and then transferred onto ABA containing media. The hormone analysis experiment detected high levels of auxin and cytokinin during the first two weeks of embryo development in situ, both of which were present in the culture media. However, ABA accumulated in megagametophytes at the proembryo stage, which coincided with the deposition of starch in prothallial cells in the central region prior to corrosion cavity formation in situ (see Tables 4 and 5). In the in vitro experiment, ABA was not added to the media for the first 21 days, which may have prevented the deposition of starch during this culture period. The absence of auxin in the ABA media may also have prevented the completion of embryogenesis. High levels of IAA were detected in megagametophytes developing in situ a week prior to polarization by the embryos. In future embryo rescue experiments, the use of media that closely resemble the natural megagametophyte environment in terms of plant hormone supplements may be useful in improving the yeild of mature embryos.

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APPENDIX

The concentrations of free and conjugated forms of (A) iP, (B) IAA, (C) ABA and (D) Z as determined by ELISA which were used to create the figures in Chapter 5 (mean ± Confidence Interval).

A

WAP	iP (ng/g DW)	CI	iPA (ng/g DW)	CI	iP (ng/g FW)	CI	iPA (ng/g FW)	CI
8	250.95	155.05	69.86	13.71	20.91	12.92	5.82	1.14
9	178.35	91.11	123.69	81.27	10.41	5.32	7.22	4.74
10	2302.10	784.80	24.38	3.84	167.52	57.11	1.77	0.28
11	873.55	429.61	367.82	243.06	75.12	36.94	31.63	20.90
12	108.13	116.90	40.47	20.05	11.39	12.31	4.26	2.11
13	1340.20	686.58	1784.90	742.25	505.65	259.04	673.42	280.04
14	141.68	59.23	294.02	66.29	46.97	19.64	97.48	21.98
15	276.85	205.85	445.35	174.79	105.81	78.68	170.22	66.81
16	320.55	191.43	90.42	52.77	138.51	82.72	39.07	22.80
17	1540.30	610.20	5301.50	990.20	784.25	310.68	2699.20	504.15
18	175.71	53.02	25.10	8.98	92.55	27.93	13.22	4.73

B

WAP	IAA (ng/g DW)	CI	IAAsp (ng/g DW)	CI	IAA (ng/g FW)	CI	IAAsp (ng/g FW)	CI
8	37.800	8.710	509.640	315.330	3.150	0.726	42.470	26.278
9	178.720	38.120	821.920	528.750	10.433	2.225	47.983	30.867
10	48.510	10.160	2514.000	1199.700	3.530	0.739	182.940	87.298
11	433.780	97.100	400.620	151.870	37.303	8.350	34.451	13.060
12	11.700	6.100	480.590	341.250	1.232	0.642	50.616	35.941
13	131.300	33.620	1929.600	1247.200	49.539	12.686	728.010	470.570
14	9.570	7.000	4687.400	1539.000	3.171	2.322	1554.100	510.240
15	124.370	9.080	2805.600	182.510	47.537	3.471	1072.300	69.757
16	0.000	0.000	759.630	178.150	0.000	0.000	328.230	76.978
17	0.000	0.000	913.810	395.740	0.000	0.000	465.260	201.490
18	10.640	6.120	4054.200	1480.200	5.607	3.225	2135.500	779.650

C

WAP	ABA (ug/g DW)	CI	ABA-GE (ug/g DW)	CI	ABA (ug/g FW)	CI	ABA-GE (ug/g FW)	CI
8	0.458	0.027	1.470	0.947	0.038	0.002	0.123	0.079
9	0.330	0.087	3.150	2.300	0.019	0.005	0.184	0.134
10	0.141	0.008	2.360	1.560	0.010	0.001	0.172	0.114
11	1.260	0.410	0.571	0.197	0.108	0.035	0.049	0.017
12	0.317	0.078	3.730	2.040	0.033	0.008	0.393	0.215
13	2.320	0.315	1.670	0.474	0.875	0.119	0.629	0.179
14	0.861	0.075	1.700	1.670	0.283	0.025	0.562	0.552
15	0.304	0.104	0.940	0.303	0.116	0.040	0.359	0.116
16	0.162	0.027	2.000	1.730	0.070	0.012	0.863	0.750
17	13.700	4.260	1.860	0.722	6.990	2.170	0.946	0.368
18	0.551	0.188	1.000	0.616	0.290	0.099	0.528	0.324

D

WAP	Z (ng/g DW)	CI	ZR (ng/g DW)	CI	Z (ng/g FW)	CI	ZR (ng/g FW)	CI
8	25.060	5.040	22.210	2.450	2.088	1.253	1.851	0.204
9	9.740	2.010	4.420	1.540	0.568	0.117	0.258	0.090
10	0.000	0.000	8.630	2.250	0.000	0.000	0.628	0.164
11	2.910	2.000	8.660	2.290	0.250	0.172	0.744	0.197
12	7.270	2.360	4.770	0.800	0.765	0.249	0.502	0.084
13	18.000	4.040	271.210	34.470	6.791	1.526	102.330	13.006
14	9.170	3.370	17.300	0.760	3.041	1.118	5.735	0.253
15	6.130	1.880	5.040	1.450	2.345	0.719	1.928	0.553
16	7.640	3.800	13.640	1.330	3.301	1.640	5.892	0.577
17	7.330	4.130	1979.100	265.220	3.731	2.101	1007.700	135.040
18	6.970	4.220	0.000	0.000	3.670	2.224	0.000	0.000