Functional Analysis of Proteins in the Conifer Ovular Secretion

by

Andrea Elizabeth Coulter
B.Sc. Hons. University of Victoria, 2005

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Abstract

Almost all conifer ovules produce a liquid secretion as part of reproduction. This secretion, termed an ovular secretion, is produced during ovule receptivity and is involved in pollen capture and transport. Historically, examinations of the ovular secretion have focused on how they are part of pollination mechanisms. As a result, the chemical composition of the ovular secretion has not been examined systematically. Investigations into the constituents of the ovular secretion were limited to analyses for simple water soluble compounds such as sugars, minerals, amino acids and organic acids. More recently, the protein component of the secretion has been investigated using mass spectrometry-based proteomics. Proteins involved in processes such as carbohydrate modification, proteolysis, and defence have been identified in conifer ovular secretions. This biochemical complexity suggests a broader view of the function of the ovular secretion is warranted. However, protein identifications only provide putative information on function. Functional characterization of these proteins is needed in order to fully understand how they contribute to ovular secretion function. The research outlined in this dissertation describes the first functional characterizations of proteins found in conifer ovular secretions. Three proteins - invertase, chitinase, and thaumatin-like protein - were characterized in the ovular secretions of Douglas-fir (*Pseudotsuga menziesii*) and hybrid yew (*Taxus* × *media*).

The Douglas-fir ovular secretion is capable of converting sucrose to glucose and fructose, confirming that invertases present in the secretion are functional. The invertase activity was maximal at pH 4.0. Activity was 77% of maximal at pH 4.5, the physiological pH. This indicates that post-secretory hydrolysis of sucrose occurs *in situ* in the Douglas-fir ovular secretion. Invertases in the ovular secretion are likely involved in controlling the movement of carbohydrates to developing pollen and could facilitate pollen selection.

Chitinases present in the Douglas-fir ovular secretion are functional at physiological conditions. All three modes of chitinolytic activity, i.e. endochitinase, chitobiosidase and β-N-acetylglucosaminidase, were detected at physiological pH. β-N-acetylglucosaminidase activity was 80 % of maximal at physiological pH. Chitinases are pathogenesis-related proteins capable of hydrolysing chitin in fungal cell walls. These results suggest the ovular secretion is capable of defending the ovule against infection by phytopathogens.

Thaumatin-like protein was immunolocalized to the cell wall and amyloplasts in Douglas-fir and yew nucellar tissue in a pattern consistent with a defensive role. It was also localized to the cell wall of fungal spores and germinating hyphae that were present in the micropyle of a yew ovule. These results provide additional evidence for an antifungal role for the ovular secretion.

Functioning enzymes involved in pollen-ovule interactions and ovule defence are present in the conifer ovular secretion. The ovular secretion has

functions beyond pollen capture. A revised functional model for the conifer ovular secretion is proposed.

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Chapter 1

Conifer Reproduction and the Ovular Secretion

Conifers are wind pollinated (anemophily). Pollen grains, containing the male gametes, are released into the air from the microsporangiate (male) strobili. They are released in a desiccated and dormant state and are carried by air currents, sometimes for long distances, to the megasporangiate (female) strobili (O'Connell *et al*. 2007). Once at the ovule (Appendix A), pollen grains hydrate and germinate a pollen tube. The pollen tube penetrates the nucellus and grows through the nucellus and into an archegonium, which contains an egg. The pollen tube releases two male gametes into the egg. Fertilization occurs when one of the male gametes fuses with the egg nucleus.

Conifer ovules produce a liquid ovular secretion during reproduction (Figure 1). Only the Araucariaceae, *Saxegothaea*, *Abies*, and *Tsuga* do not have a secretion (Owens *et al*. 1998; Gelbart and von Aderkas 2002). Two forms of the secretion have been observed: the pollination drop, which is secreted simultaneous to pollination, and the post-pollination prefertilization drop, which is secreted after pollination but before fertilization. The former is more common than the latter, which is restricted to two genera in the Pinaceae, *Pseudotsuga* and *Larix*.

Pollination drops are secreted during ovule receptivity. The secreted liquid fills the micropyle and then exudes beyond the micropylar opening to form an

Figure 1. Ovular secretions in conifers. **(A)** *Chamaecyparis lawsoniana* cone with multiple ovules bearing pollination drops. **(B)** *Taxus* × *media* ovule with pollination drop. **(C)** *Pseudotsuga menziesii* ovule with post-pollination prefertilization drop. Scale bars = 1 mm. Adapted from Coulter *et al*. (2012).

exposed drop. Pollen is captured by the drop and is brought into the ovule as the drop retracts. A relationship between ovule (and therefore pollination drop) orientation and the presence of sacci on pollen has been observed (Tomlinson 1994; Owens *et al*. 1998). Upright, oblique or horizontal ovules are associated with non-saccate pollen. The exuded drop increases the area of the receptive surface of the ovule. The non-saccate pollen sinks in the drop and enters the micropyle. This pollen capture mechanism is found in the Cephalotaxaceae, the Cupressaceae, the Sciadopityaceae, and the Taxaceae (von Aderkas *et al*. 2018). In contrast, inverted ovules are associated with saccate pollen. In this case, the pollination drop is not the primary mechanism for pollen capture. Instead, pollen is either captured by the integument (Pinaceae) or by extra-ovular surfaces (Podocarpaceae) and the exuded pollination drop scavenges the pollen from these surfaces. The air-filled sacci buoy the pollen grains, which float into the ovule. Two exceptions to this association have been observed. *Picea orientalis* (Pinaceae) and *Phyllocladus* (Podocarpaceae) have saccate pollen and upright ovules. The pollen of *P. orientalis* has a porous ektexine. It briefly floats in the pollination drop, but then sinks when the air is forced out of the porous sacci as the hydrated pollen grains swell (Runions *et al*. 1999). The sacci of *Phyllocladus* are vestigial and the pollen sinks in the pollination drop (Tomlinson *et al*. 1997; Möller *et al*. 2000). Because the pollen of both *P. orientalis* and *Phyllocladus* are functionally non-saccate, pollen capture is effected by the same mechanism as conifers with upright ovules and non-saccate pollen.

Post-pollination prefertilization drops are neither involved in pollen capture nor in transport into the ovule. *Pseudotsuga* and *Larix* ovules are inverted and the pollen is non-saccate. The integument develops sticky hairs to which pollen adheres. At the end of the receptive period, the hairs collapse into the micropyle, carrying the attached pollen into the ovule with them (Owens *et al*. 1998; von Aderkas *et al*. 2018). Weeks later, the micropyle fills with the post-pollination prefertilization drop (Barner and Christiansen 1960; von Aderkas and Leary 1999a; von Aderkas and Leary 1999b). The receding drop transports the pollen to the nucellus (Barner and Christiansen 1960; Takaso and Owens 1997). The drop also hydrates the pollen, initiating germination (Said *et al*. 1991).

A liquid secretion during reproduction has not been observed in conifers that have extra-ovular pollen germination and extended siphonogamy. Non-saccate pollen lands and germinates on extra-ovular structures such as cone scales or bracts. Pollen tubes grow towards the nucellus, which is generally exposed (Tomlinson and Takaso 2002; Williams 2009; Little *et al*. 2014). This pollination mechanism is found in the Araucariaceae, *Saxegothaea* and the *Micropeuce* species of *Tsuga* (Owens *et al*. 1998). A liquid secretion during reproduction has also not been observed in the *Hesperopeuce* species of *Tsuga* and in *Abies* (Owens *et al*. 1998). In these conifers, the integument develops splayed flaps (*Tsuga*) or a funnel (*Abies*) around the micropyle. Pollen adheres to microdroplets that are secreted on the inner surface of the flaps or funnel. These integument extensions then fold inwards, bringing the pollen to the nucellus. The nucellus

also elongates down the micropylar canal towards the micropylar opening (Singh and Owens 1981; Singh and Owens 1982; Owens and Blake 1983). It is possible that an ovular secretion is present in the *Hesperopeuce* species of *Tsuga* and in *Abies* but has been overlooked. Both have saccate pollen. The primary function of pollen sacci is to float pollen grains up to the nucellus through an ovular secretion (Tomlinson 1994; Leslie 2008). Both also have inverted ovules (Takaso and Owens 1995a; Owens *et al*. 1998). The combination of saccate pollen and inverted ovules is normally associated with the presence of a pollination drop. Ovular secretions have been overlooked before. For example, it was previously thought that *Cedrus*, which has a pollination mechanism that is very similar to that of *Abies*, did not have an ovular secretion (Doyle and O'Leary 1935; Singh 1978; Owens and Blake 1983). It is now known that *Cedrus* has a pollination drop (Takaso and Owens 1995a; Takaso and Owens 1995b). It has been argued that the onus should be to prove the absence of an ovular secretion, rather than its presence, given its ubiquity in conifers (Gelbart and von Aderkas 2002).

The term pollination mechanism refers to the suite of characteristics found in both pollen and ovules that facilitate pollination. Because ovular secretions assist with pollen capture, transport and hydration, they are a key part of conifer pollination mechanisms. Since first being observed by Vaucher in 1841, ovular secretions have been examined largely in the context of morphological aspects of pollination mechanisms (see, for example, Tison 1911; Doyle 1945; Dogra 1964; Singh 1978; Tomlinson 1994; Owens *et al*. 1998). However, this focus may have

restricted our understanding of conifer ovular secretions. The chemical composition of these secretions was not studied systematically (Singh 1978; Gelbart and von Aderkas 2002; Nepi *et al*. 2009; Nepi *et al*. 2017). Between the years 1900 and 2000, only five studies can be found in the literature that report on the composition of conifer ovular secretions (Table 1). From these studies, we know that glucose, fructose, and sucrose have been identified in conifer ovular secretions. The *Pinus nigra* pollination drop, for example, contains little sucrose (2.5 mM), but much more glucose (33 mM) and fructose (40 mM) (McWilliam 1958). The *Picea engelmanii* pollination drop contains glucose (4.3 %) and fructose (3.8 %) but lacks sucrose entirely (Owens *et al*. 1987). Fructose is often the dominant sugar. Sucrose, the primary transport sugar in plants, is not always present (Table 1). Total sugar concentration was reported to range between 1.25 % and 10 % (McWilliam 1958; Ziegler 1959; Owens *et al*. 1987). A limited number of other simple, water-soluble compounds such as sugars, minerals, organic acids and free amino acids were also identified in conifer ovular secretions (Table 1).

Analysis of ovular secretion constituents was likely limited by sample availability. Ovular secretions are produced once a year for a short period of time (approximately two weeks). They are also very small. On average, just 60 nL is secreted from an ovule (Seridi-Benkaddour and Chesnoy 1988), although it can be as low as 10 nL (Nepi *et al*. 2012a). It would have been challenging and costly to collect a volume large enough to perform extensive analyses using classical

Table 1. Conifer ovular secretion constituents discovered between the years 1900 and 2000. Adapted from Nepi *et al*. (2009).

Species	Compound[1,2]	Reference
Cephalotaxus drupacea	**Fructose**, glucose	Seridi-Benkaddour and Chesnoy 1988
	Two unidentified sugars	
	Unidentified polymer comprised of galactose, arabinose, glucose, rhamnose, mannose and a phenolic compound	
	Galacturonic acid	
	Pro, Asp, Glu, Ala, Ser, Leu, Iso, Thr, Gln, Asp	
Picea engelmannii	Glucose, fructose	Owens *et al*. 1987
Pinus elliottii	Fructose, glucose, sucrose	McWilliam 1958
Pinus nigra	**Fructose**, glucose, sucrose	McWilliam 1958
Taxus baccata	**Fructose**, glucose, sucrose	Ziegler 1959; Seridi-Benkaddour and Chesnoy 1988
	Unidentified sugar	Seridi-Benkaddour and Chesnoy 1988
	Unidentified polymer	
	Galacturonic acid	
	Calcium	Fujii 1903
	Inorganic phosphate	Ziegler 1959
	Malic acid, citric acid	
	Glu, Pro, Ala, Gln, Asp, Lys, Trp, Val, Leu, Ser	
	Unidentified peptide	
Thuja orientalis	Fructose	Seridi-Benkaddour and Chesnoy 1988
	Two unidentified sugars	
	Unidentified polymer	
	Galacturonic acid	
	Ser, Gly, Ala, Glu, Phe, Tyr, Leu, Iso, Thr, Asp	

1. The dominant sugar, when identified, is shown in bold
2. Amino acids are listed in order of relative concentration

analytical chemistry techniques. Limitations in the analytical sensitivity of the available tests would also have restricted discovery. However, improvements in laboratory technologies have increased analytical sensitivity, resulting in decreased sample volume requirements. More recently, high performance liquid chromatography has been used in three studies to assay for sugars and in one study to assay for free amino acids (Table 2). The sugar analyses doubled the number of conifer species that have been investigated, and the results confirm the findings of earlier investigations: fructose is often the dominant sugar and sucrose is frequently absent (Nepi *et al*. 2017). Contemporary amino acid analysis has tripled the number of conifer species investigated. Previously, only three conifer species had been studied (Table 1), making it difficult to ascertain trends. The more recent analysis reveals that proline is often the most abundant amino acid present. Serine, glutamic acid, histidine, alanine and glutamine are also commonly abundant (Table 2). Advances in mass spectrometry have also enabled the detection of proteins in conifer ovular secretions (Prior *et al*. 2013) (Table 3). The proteins that have been identified in six of the eight conifer species that have been analyzed to date are exclusively secretory, i.e., a secretome. Ovular secretions of the remaining two species, *Cephalotaxus koreana* and *C*. *sinensis*, contain intracellular proteins in addition to the secretome. Intracellular proteins are present because a pollen chamber develops in *Cephalotaxus*. The pollen chamber serves as a receptive surface for pollen and is formed by the degeneration of the uppermost apical cells of the nucellus. Intracellular proteins

Table 2. Sugars and free amino acids found in conifer ovular secretion constituents after the year 2000 using high performance liquid chromatography.

Species	Compound[1,2]	Reference
Cephalotaxus koreana	**Glucose**, fructose	Nepi *et al*. 2017
	Ser, Glu, Pro, Ala, Gly, His, Thr, Val, Ile, Leu	
Chamaecyparis lawsoniana	Glucose, fructose	Nepi *et al*. 2017
	Pro, Gln, Ser, *HPro*, Ala, Leu, His, Ile, Val, Gly, Thr, *β-Ala*	
Juniperus communis	**Fructose**, glucose	Mugnaini *et al*. 2007; Nepi *et al*. 2017
	Pro, Thr, Ser, Gly, Ala, Ile, Glu, Leu	Nepi *et al*. 2017
Juniperus oxycedrus	**Fructose**, glucose	Nepi *et al*. 2017
	Pro, Ser, Glu, Leu, Thr, Gly, Ile, Ala	
Larix × *marschlinsii*	**Sucrose**, fructose, glucose	von Aderkas *et al*. 2012; Nepi *et al*. 2017
	Glu, His, Ser, Ala, Pro, Asp, Thr, Arg, Gly, Leu, Lys	Nepi *et al*. 2017
Pseudotsuga menziesii	Glucose, fructose	von Aderkas *et al*. 2012; Nepi *et al*. 2017
	Pro, Glu, Ser, Ala, Asp, His, Thr, Ile, Val, Lys, Arg	Nepi *et al*. 2017
Taxus baccata	**Fructose**, glucose, sucrose	Nepi *et al*. 2017
	Pro, Gln, Ala, Ser, His, Asp, Val, Gly, *GABA*, Arg, Thr, Ile, Leu, *β-Ala*, *HPro*	

1. The dominant sugar is shown in bold
2. Amino acids are listed in order of relative concentration; non-protein amino acids are italicized; tryptophan was not assayed for.

Table 3. Proteins identified in conifer ovular secretions. Adapted from Nepi *et al*. (2009).

Species	Protein	Reference
Cepahlotaxus koreana	α-galactosidase	Pirone-Davies *et al*. 2016
	β-galactosidase	
	Glucan 1,3-β-glucosidase	
	Glucan endo-1,3-β-glucosidase	
	Three chitinases	
	Two thaumatin-like proteins	
	Peroxidase	
	PR5 allergen Jun r	
	Cup a 3 protein	
	Pollen allergen CJP38	
	Intracellular proteins	
Cepahlotaxus sinensis	α-galactosidase	Pirone-Davies *et al*. 2016
	β-galactosidase	
	Two chitinases	
	Thaumatin-like protein	
	Peroxidase	
	PR5 allergen Jun r	
	Cup a 3 protein	
	Pollen allergen CJP38	
	Intracellular proteins	
Chamaecyparis lawsoniana	Subtilisin-like proteinase	Wagner *et al*. 2007
	β-D-glucan exohydrolase	
	Glucan 1,3-β-glucosidase	
	Two thaumatin-like proteins	
Juniperus communis	Subtilisin-like proteinase	Wagner *et al*. 2007
	Glycosyl hydrolase	
	Glucan 1,3-β-glucosidase precursor	
	Chitinase	
	Thaumatin-like protein	
Juniperus oxycedrus	Chitinase	Wagner *et al*. 2007
	Thaumatin-like protein	
	Glucanase-like protein	
Larix × marschlinsii	Xyloglucan endotransglycosylase	O'Leary 2004
	Lipid transfer protein	
	Thaumatin-like protein	
	Arabinogalactan proteins	
Pseudotsuga menziesii	Two invertases	Poulis *et al*. 2005
	Two xylosidases	
	Two galactosidases	
	Aspartyl protease	
	Serine carboxypeptidase-like protein	
	Peroxidase	
	Thaumatin-like protein	Poulis 2004
	Eight chitinases	Coulter *et al*. 2012
Taxus × media	Eight thaumatin-like proteins	O'Leary *et al*. 2007; Prior 2014
	β-1,3-glucanase	O'Leary 2004
	Arabinogalactan proteins	O'Leary *et al*. 2004

are released when these nucellar cells degenerate, resulting in the production of a degradome in *C. koreana* and *C. sinensis* ovular secretions (Pirone-Davies *et al*. 2016).

The ovular secretion is biochemically complex. Historically, the examination of conifer ovular secretions has focused on how they are part of pollination mechanisms. The expanding list of constituents in the conifer ovular secretion suggests a broader view of the function of the ovular secretion is warranted. The ovular secretions of all conifer species analyzed to date contain secretory proteins. This begs the question, why would conifers secrete metabolically costly proteins into a solution that functions solely to capture and hydrate pollen when a watery solution containing simpler solutes would suffice? Conifer ovular secretions are released into the apoplast, so the answer to this question may be found by looking to secretions into the apoplast in the better-studied angiosperms.

Chapter 2

Secretions into the Angiosperm Apoplast

Portions of this chapter have been published as Coulter A, Poulis BAD, von Aderkas P. 2012. Pollination drops as dynamic apoplastic secretions. Flora. 207:482-490.

From roots to shoots, plants release a number of secretions into the apoplast to interact with and respond to biotic and abiotic elements of the surrounding environment. In the literature review that follows, four secretions into the angiosperm plant apoplast will be reviewed. The overview of floral and extrafloral nectar, stigma exudates and micropylar exudates will reveal that much of our understanding of these exudates has come from identification and functional analysis of their constituents.

Floral Nectar

Floral nectar is a sugary fluid produced to attract pollinators. Nectar is secreted into the nectary, a ring of cells located at the base of the ovary. During visits to flowers by nectar-consuming pollinators, pollen attached to the pollinator's body is transferred between flowers, thereby increasing both out-crossing and seed set.

Floral nectar is an aqueous solution with a high concentration of sugars, ranging from 10 % to 30 % sugar solutes by fresh mass (De la Barrera and Nobel 2004). The principal sugars are sucrose, glucose and fructose. Other simple

carbohydrates may also be present including arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehaliose, melezitose, raffinose and stachyose (Baker and Baker 1981). In addition, amino acids (Baker and Baker 1973), proteins (Scogin 1979), organic acids, terpenes, flavonoids, glycosides, vitamins, phenolics, oils and metal ions have been found in various nectars (Baker and Baker 1983). The major cation has been identified as K^+ (Heinrich 1989, cited in Thornburg *et al*. 2003).

The constitution of floral nectar varies between plant species. Just as plants have evolved flowers of different colours, shapes, and scents to attract different types of pollinators, floral nectar has also become specialized according to plant-pollinator relationships (Proctor and Yeo 1973; Baker and Baker 1979). Carbohydrates are the major food requirement of pollinators, so most research on the relationship between pollinators and nectar type has focused on the three principal sugars: sucrose, fructose, and glucose (Proctor and Yeo 1973). There is a significant relationship between pollinator type and the ratio of sucrose to glucose and fructose (Baker and Baker 1990). Sucrose-rich nectars are associated with butterflies, moths, hummingbirds, and large bees, whereas hexose-rich nectars are associated with small bees, passerine birds, and neotropical bats (Baker and Baker 1990). Flowers pollinated by birds, bats, and butterflies tend to produce dilute nectars (15-25 % sugars), whereas bee-pollinated flowers often contain nectar with more than 50 % sugar (Proctor *et al*. 1996).

A relationship has also been demonstrated between amino acid composition of floral nectar and pollinator type (Baker and Baker 1990). Nectars richest in amino acids typify flowers pollinated by carrion or dung flies. This appears to be an extremely specialized relationship as there is an approximate ten-fold decrease in amino acid content to the next richest nectar, that of butterfly-pollinated flowers. That nectar in turn has more than twice the amino acid content of nectar of bee-pollinated flowers. This difference is of particular importance given that the sugar profiles of nectar for butterfly- and bee-pollinated flowers do not always differ. Nectar of flowers pollinated by birds and nectar-seeking flies is generally low in amino acids, averaging about half the concentration of nectar of bee-pollinated flowers (Baker and Baker 1983). The correlation between amino acid content and pollinator type is thought to be based on the metabolic needs of the pollinator. While immediate energy requirements of insects (the most common biotic pollinator) must be satisfied, a long-lived adult insect also requires nitrogenous material for body maintenance. For insects that do not feed on pollen or other protein-rich materials, nectar is likely the only source of nitrogen (Proctor *et al*. 1996; Hall and Willmott 2000).

Which amino acids are present in nectar may be as important to plant-pollinator interactions as total amino acid content. Insects have chemoreceptors that allow them to detect amino acids with specificity and sensitivity (Schoonhoven 1969; Van Loon and Van Eeuwijk 1989) and they can demonstrate a preference for particular amino acids. Proline, for example, is preferred by bees,

and it is one of the most abundant amino acids in the nectar of some melittophilous plants (Nepi 2017). Interspecific variability in amino acid preference is observed in nectar-feeding ants and contributes to resource partitioning in the ant community (Blüthgen and Fiedler 2004). When present, phagostimulatory amino acids, such as proline, phenylalanine, and γ-aminobutyric acid, encourage sustained nectar feeding, which in turn can increase pollen loading and transfer (Nepi *et al*. 2012b; Nepi 2014, Nepi 2017). There is evidence that non-protein amino acids, including taurine, β-alanine, and γ-aminobutyric acid, improve muscle performance during flight in foraging insects. This enhanced locomotion could increase pollen movement, improving plant fitness and gene flow (Nepi 2017).

Floral nectar is, of course, nutritionally rich precisely to attract pollinators. However, this richness leaves it vulnerable to microbial infection. Microbial metabolism can change floral nectar chemistry and affect the ability to attract pollinators, particularly those with specific nectar needs. Proteins the provide microbial defence have been identified in the nectar of a number of plants (Roy *et al*. 2017).

A complex nectar defence system comprised of four proteins, termed nectarins, is present in ornamental tobacco (*Nicotiana langsdorfii* × *N. sanderae*). Three of the proteins, Nectarin I, V, and III, are involved in a redox cycle that accumulates antimicrobial levels of hydrogen peroxide. Nectarin I is a manganese-containing germin-like protein with superoxide dismutase activity. It

generates hydrogen peroxide from superoxide (Carter and Thornburg 2000; Thornburg *et al*. 2003; Carter and Thornburg 2004a). Nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. It catalyzes the oxidation of glucose, a readily available substrate in nectar, to gluconic acid and hydrogen peroxide (Carter and Thornburg 2004b). Nectarin III is a dioscorin-like protein with carbonic anhydrase and monodehydroascorbate reductase activity. The carbonic anhydrase activity of Nectarin III functions to buffer the pH of the nectar. Two chemical reactions associated with the production of hydrogen peroxide would otherwise make the nectar basic, negatively affecting nectarin function and pollinator feeding. First, Nectarin I consumes protons to produce hydrogen peroxide. Second, hydrogen peroxide breaks down into hydroxyl free radicals in the presence of metal ions found in nectar; ascorbate, also present in nectar, reduces these damaging free radicals to hydroxide anions. The monodehydroascorbate reductase activity of Nectarin III regenerates the ascorbate (Carter and Thornburg 2004c). The fourth nectarin protein to be characterized, Nectarin IV, is a potent xyloglucan-specific endoglucanase inhibitor that protects plant cell walls from degradation by fungal pathogens (Naqvi *et al*. 2005). Nectarin IV also promotes the catalytic activity of Nectarin V when it complexes with a fungal endoglucanase (Harper *et al*. 2010).

A number of other defence proteins have been identified in floral nectar. Nepi and colleagues (2011; 2012b) identified four xylosidases in the nectar of

Cucurbita pepo. They proposed that these enzymes defend the nectary by degrading oligosaccharides that are released from nectary cell walls by invading micro-organisms. These oligosaccharides increase the pathogenic potential of invading pathogens, so degrading them may reduce the virulence of pathogens present in nectar. Peroxidases have been identified in *Petunia hybrida* nectar, and peroxidase activity has been detected (Hillwig *et al*. 2011; Silva *et al*. 2020). Peroxidases produce reactive oxygen species that could protect nectar from microbial infestation (Hillwig *et al*. 2011). Chitinases are another defence protein that has been identified in *P*. *hybrida* nectar (Hillwig *et al*. 2011; Silva *et al*. 2020) as well as in multiple *Nicotiana* spp. (Silva *et al*. 2020) and *Rhododendron irroratum* nectar (Zha *et al*. 2016). Chitinase activity has been confirmed in the nectar of *P*. *hybrida* and multiple *Nicotiana* spp. (Silva *et al*. 2020). Chitinases are pathogenesis-related proteins that provide antimicrobial protection to plants by hydrolyzing fungal cell wall chitin, causing fungal cell lysis (Edreva 2005). Endoglucanases, another type of pathogenesis-related protein, have been identified in the nectar of *Nicotiana* spp. Endoglucanases also hydrolyze fungal cell walls and can act synergistically with chitinase (Silva *et al*. 2020). Overall, proteins involved in defence represent a large portion of the nectar proteome in *P*. *hybrida* (66.7 %) and *Nicotiana* spp. (34 %), suggesting that defence is an important function of nectar (Silva *et al*. 2020).

Extrafloral Nectar

Nectaries are also found on vegetative plant parts. These extrafloral nectaries generally do not assist with pollination. The nectar is produced to attract carnivorous ants, parasitoids, and generalist predators to gain protection from phytophagous insects (Nepi *et al*. 2009; Heil 2011). Secretion of extrafloral nectar is inducible in response to herbivore damage, and some plant species are also able to increase the number of extrafloral nectaries in response to herbivory (Heil 2008). Extrafloral nectar is an important food resource for small arthropods and is thought to contribute to the ecological dominance of ants (Davidson 1997; Marazzi *et al*. 2013).

Extrafloral nectaries are found in 11 pteridophyte genera, in one gymnosperm genus, and in over 70 angiosperm families (Koptur 1992; Nepi *et al*. 2009; Heil 2008). Fructose, glucose and sucrose are the main sugars found in extrafloral nectar. Free amino acids and proteins are also present (Koptur 1994; Heil 2008; Nepi *et al*. 2009). As with floral nectar, variations in the ratio and amounts of these constituents determines which mutualists are attracted to the plant (Heil 2011). Amino acid preference, for example, varies by ant species (González-Teuber and Heil 2009).

The proteome of extrafloral nectar is even larger than that of floral nectar. Fifty-two proteins have been identified in the extrafloral nectar of *Acacia cornigera* (González-Teuber *et al*. 2009). Defence proteins form a significant proportion of the proteome. Chitinases and β-1,3-glucanases comprise more than

50 % of the *A. cornigera* proteome. High numbers of these two proteins were also found in the extrafloral nectar of other *Acacia* species. Another commonly found protein is thaumatin-like protein (González-Teuber *et al*. 2009; Heil 2011). These three proteins are pathogenesis-related proteins (van Loon and van Strien 1999). Pathogenesis-related proteins possess hydrolytic activity that protects plants by weakening or decomposing fungal cell wall components such as glucans, chitin and proteins (Edreva 2005). Chitinase and β-1,3-glucanase activity have been detected in the extrafloral nectar of multiple *Acacia* species and *Prosopis juliflora* (González-Teuber *et al*. 2009; González-Teuber *et al*. 2010). Functioning pathogenesis-related proteins help protect extrafloral nectar from fungal infection. It also prevents changes in extrafloral nectar chemistry as a result of fungal metabolism. Maintaining control over extrafloral nectar chemistry is important since the chemistry is tailored to attract mutualists (Heil 2011).

Proteins involved in carbohydrate metabolism form a smaller proportion of the proteome. Invertases and glycoside hydrolases, for example, have been identified in *Acacia* extrafloral nectar (González-Teuber *et al*. 2009), and invertase activity has been confirmed in *Acacia* extrafloral nectar (Heil *et al*. 2005). Interestingly, the invertase activity in myrmecophytic *Acacia* species was significantly higher than in nonmyrmecophytic *Acacia* species. This, at first, seems contradictory. Many ant species have been reported to strongly prefer sucrose-rich nectars (Blüthgen and Fiedler 2004), and invertase activity has been detected in their digestive systems (Heil *et al*. 2005). Invertase hydrolyses sucrose

into glucose and fructose, so it would be expected that nectar containing invertase would not contain enough sucrose to be attractive to ants. However, ants that live in obligate mutualism with *Acacia* have no invertase activity in their digestive tracts and are unable to digest sucrose-rich nectars. Post-secretory regulation of the carbohydrate composition of extrafloral nectar has allowed for adaptive specialization between ants and myrmecophytic *Acacia* species (Heil *et al*. 2005).

Stigma Exudates

The stigma is the receptive surface for pollen in the angiosperm flower. Pollen binds to the stigma, hydrates and then germinates (Sanchez *et al*. 2004; Nepi *et al*. 2012a). A stigma is classified as either wet or dry, depending on whether a secretion is present (Heslop-Harrison and Shivanna 1977). When a secretion is present, it covers the outermost surface of the stigma (Rejón *et al*. 2014) and is secreted at pistil maturity (Sanchez *et al*. 2004). Wet stigmas are more common than dry stigmas (Dickinson 1993).

Stigma exudates have been studied in a number of families, including Solanaceae, Leguminosae, Liliaceae, Orchidaceae and the Oleaceae (Lush *et al*. 2000; Zinkl and Preuss 2000; Rejón *et al*. 2013). The composition of the exudate varies by species and genus (Martin 1969; Knox 1984). Carbohydrates, free amino acids, proteins, glycoproteins, proteoglycans, lipids, phenols and calcium ions have been identified in stigma exudates (Knox 1984; Cresti *et al*. 1986; Edlund *et al*. 2004; Bednarska *et al*. 2005). Sucrose and hexose sugars are found

in the exudate of only a few species (Shivanna 2003). Depending on its composition, an exudate is either classified as hydrophilic or hydrophobic (Knox 1984). Hydrophilic exudates, such as those of the Liliaceae, are carbohydrate-rich. Hydrophobic exudates, such as those of the Solanaceae, are lipid-rich. The majority of exudates are hydrophobic (Nepi *et al*. 2012a).

The stigma exudate facilitates pollen capture and adhesion (Wottiez and Willemse 1979; Shivanna 2003). The exudate is sticky (Konar and Linskens 1966). Clarke and colleagues (1979) analyzed stigma exudates of *Gladiolus gandavensis* and found components that could make up an ideal adhesive. Arabinogalactan proteins are a major component of *Gladiolus* exudate. They likely act as an adhesive base. Free monosaccharides are also present in the exudate and likely act as a plasticizer that prevents the adhesive exudate from becoming brittle. Glycoproteins in the exudate may act as a thickener, while glycolipids in the exudate may be ideal wetting agents. Similar constituents have been found in the stigma exudates of other species (see Knox 1984), so the role of these constituents in pollen adhesion may be widely applicable (Heslop-Harrison and Heslop-Harrison 1985; Shivanna 2003).

Pollen hydration and germination depends on the stigma exudate. Goldman and colleagues (1994) developed transgenic *Nicotiana tabacum* with an ablated stigma secretory zone and no detectable stigma exudate. Pollen of *N. tabacum* applied to the stigmas of transformed plants failed to hydrate and germinate, resulting in female sterility (Goldman *et al*. 1994; Sanchez *et al*.

2004). Application of wild-type stigma exudate to the transformed stigmas restored the ability of pollen to germinate (Goldman *et al*. 1994). Building on the work of Goldman and colleagues (1994), Wolters-Arts and colleagues (1998; 2002) found that the ability of pollen to hydrate on the stigma of the exudate-free transgenic *N. tabacum* was restored when exogenous lipids were applied to the stigma. Lipids, however, are not directly involved in pollen hydration. Wolters-Arts and colleagues (2002) found that dehydrated pollen grains placed in the lipid-rich exudate or in lipids *in vitro* did not hydrate. Pollen hydration depends on water originating from the pistil (Wolters-Arts *et al*. 2002). The lipids in the exudate appear either to protect the stigma from desiccation (Konar and Linskens 1966; Martin and Brewbaker 1971; Shivanna 2003) or to modify cuticle permeability (Wolters-Arts *et al*. 2002).

Nicotiana tabacum has a hydrophobic exudate (Nepi *et al*. 2012a). The conclusions drawn about the role lipids in hydrophobic exudates play in pollen germination may not apply to hydrophilic exudates. Unfortunately, there has been minimal research into the interaction between pollen and hydrophilic exudates. It is known that hydrophilic stigma exudates are reservoirs of water (Lush *et al*. 2000). It can therefore be speculated that hydrophilic exudates may participate directly in pollen hydration and that lipids would not be necessary to control water relations at the stigma.

Calcium ions in the stigma exudate are also involved in pollen germination. Calcium is essential for pollen germination; however, most pollen

has a low internal calcium content (Brewbaker and Kwack 1963). Uptake of exogenous calcium is required for pollen germination (Bednarska 1989). Indeed, calcium in the exudate is taken up by germinating pollen grains (Bednarska 1991). Calcium ions in the stigma exudate are also involved in pollen tube growth. Exogenous calcium ions are taken up by growing pollen tubes (Jaffe *et al*. 1975). The influx of calcium is focused at the growing tip (Kühtreiber and Jaffe 1990). Pollen tube growth is the primary driver of the calcium influx. The calcium is utilized in pollen tube wall biosynthesis. The pollen tube wall is largely composed of pectin, which is synthesized and secreted as methoxylated esters and then deesterified, which exposes carboxyl residues. The negatively charged carboxyl residues become cross-linked by the positively charged calcium ions. These cross-links impart wall rigidity (Hepler *et al*. 2013).

Pollen tubes continually deposit new cell wall material as they grow (Hepler *et al*. 2013). Kroh and colleagues (1971) and Labarca and Loewus (1972) have experimentally demonstrated that carbohydrates in stigma exudates are a source of precursors for pollen tube wall biosynthesis. D-glucose-1-^{14}C was introduced into the vascular system of *L*. *longiflorum* pistils. Radiolabelled exudate was collected and pollen was exposed to it both *in vitro* and *in situ*. In both conditions, label was readily incorporated in pollen tube wall polysaccharides, primarily as pectic substances. Proteins present in the stigma exudate may facilitate the uptake of carbohydrates by pollen tubes. The most abundant proteins in *L*. *longiflorum* stigma exudate are glycosyl hydrolases

(Rejón *et al*. 2013). Their abundance suggests that polysaccharide catabolism is important in the exudate. Glycosyl hydrolases such as β-galactosidases and β-xylosidases present in the exudate may contribute to the degradation of large polysaccharides into smaller units that can be incorporated into the growing pollen tube (Rejón *et al*. 2013).

Pollen tube pectin is deesterified by apoplastic pectin methylesterase associated with the pollen tube wall (Bosch and Hepler 2005). This deesterification releases protons, causing a localized reduction in pH. Paradoxically, the reduction in pH can promote the activity of cell wall hydrolases that modify the pollen tube wall (Bosch *et al*. 2005). The interplay between the opposing effects of wall stiffening by pectin methylesterase and wall loosening by cell wall hydrolases may regulate pollen tube growth (Krichevsky *et al*. 2007). Both pectin methylesterase and cell wall modifying enzymes have been identified in the stigma exudates of *L*. *longiflorum* and *Olea europaea* (Rejón *et al*. 2013). Cell wall modifying enzymes are the second most abundant type of protein in *N*. *tabacum* stigma exudate (Sang *et al*. 2012). The presence and abundance of these enzymes suggests that the stigma exudate may be involved in the regulation of pollen tube growth (Sang *et al*. 2012; Rejón *et al*. 2013).

Stigma exudate is also necessary for pollen tube penetration of the stigma. Using transformed *N*. *tabacum* that lacks a stigma exudate, Goldman and colleagues (1994) demonstrated that pollen is unable to penetrate the stigma when exudate is not present. Application of exudate from wild-type *N*. *tabacum* restored

the ability of pollen to penetrate the stigma. Exudate from *P. hybrida* and *L. longiflorum* was also applied (Wolters-Arts *et al*. 1998). Only the *P. hybrida* exudate restored the ability of pollen tubes to penetrate the stigma. *Nicotiana tabacum* and *P. hybrida* have lipid-rich exudates, whereas *L. longiflorum* has a carbohydrate-rich exudate. When lipids similar to those found in lipid-rich exudate were combined with *L. longiflorum* exudate and applied exogenously to transformed *N. tabacum* stigmas, pollen tubes were able to penetrate the stigma. Pollen tubes were also able to penetrate the stigma of transformed *N. tabacum* when lipids alone were applied exogenously. Pollen tubes could even be manipulated to penetrate leaves in the presence of exogenously applied lipids (Wolters-Arts *et al*. 1998). Lipid-rich exudate contains saturated and unsaturated fatty acids (Cresti *et al*. 1986). Wolters-Arts and colleagues (1998) were able to demonstrate that *cis*-unsaturated triacylglycerides are the type of lipid that is sufficient and essential for pollen tube penetration.

The inability of the carbohydrate-rich *L. longiflorum* exudate to restore pollen tube penetration of the stigma in transformed *N. tabacum*, a species that has a lipid-rich exudate in the wild-type condition, suggests there may be a complementarity between pollen and the exudate. Since wet stigmas are considered to be the primitive condition (Heslop-Harrison and Shivanna 1977; Dickinson 1995), it has been suggested that the pollen coat assumed the role of the exudate in the evolution of dry stigmas (Elleman and Dickinson 1996). Lipids are required for pollen tube penetration of the stigma in plants with either a wet

stigma or a dry stigma (Wolters-Arts *et al*. 1998). In genera with a dry stigma, lipids are present in the pollen coat (Preuss *et al*. 1993; Wolters-Arts *et al*. 1998). The lipids required for pollen tube penetration may also be supplied by the pollen coat in genera with a carbohydrate-rich exudate such as *Lilium*.

Wet stigmas are indiscriminate. In addition to pollen, the stigma exudate can capture fungal spores (Dickinson 1995). The sugar-enriched nature of the exudate also makes it susceptible to microbial infection (Rejón *et al*. 2013). Defence proteins are found in the stigma exudate that protect the stigma from infection (Kuboyama *et al*. 1997; Sang *et al*. 2012; Rejón *et al*. 2013). In *N*. *tabacum*, defence proteins account for 40 % of the total proteins identified in the exudate, making them the most abundant type of protein (Sang *et al*. 2012). To date, the proteomes of the stigma exudate of three species have been analyzed: *N*. *tabacum* (Kuboyama *et al*. 1997; Sang *et al*. 2012), *L*. *longiflorum*, and *O*. *europaea* (Rejón *et al*. 2013). Pathogenesis-related proteins, including thaumatin-like protein, β-1,3-glucanase, and chitinase, have been identified in the exudate of all three species.

Micropylar Exudates

After pollination, pollen tubes grow through the stigma and style and then enter the ovary. Once in the ovary, pollen tubes enter an ovule through its micropyle. In some angiosperms, an ovular secretion is present in the micropyle

at the time of pollen tube entry (Herrero 2001). In the literature, this secretion is termed a micropylar exudate.

Micropylar exudates have been identified in the following genera and families: *Senecio* in the Asteraceae (Mottier 1893); *Paspalum*, *Pennisetum*, and *Zea* in the Poaceae (Chao 1971; Reger *et al*. 1992; Heslop-Harrison *et al*. 1985); *Ornithogalum* in the Liliaceae (Tilton 1980); *Rhododendron* in the Ericaceae (Kaul *et al*. 1986); *Beta* in the Chenopodiaceae (Bruun and Olesen 1989); *Gasteria* in the Aloaceae (Franssen-Verheijen and Willemse 1993); *Illicium* in the Schisandraceae (Sage *et al*. 1994); *Asclepias* in the Asclepiadaceae (Sage and Williams 1995); *Saururus* in the Saururaceae (Pontieri and Sage 1999); *Narcissus* in the Amaryllidaceae (Sage *et al*. 1999); and *Musa* in the Musaceae (Fortescue and Turner 2005). It is difficult to observe ovules directly. Exudates identified to date have been observed using light and electron microscopy. The exudate can be lost during sample processing for microscopy. It is therefore likely that this phenomenon is underreported. Carbohydrates, protein, RNA and calcium ions have been histologically identified in exudates. The composition of the exudate has not been found to be consistent across species. However, the techniques used to process samples for microscopy can affect constituent discovery (Sage *et al*. 1994). More comprehensive testing of the exudate is needed.

Micropylar exudates are involved in pollen tube guidance. Willemse and colleagues (1995) used an *in vitro* pollination system to investigate the influence of micropylar exudate on pollen tube penetration of the micropyle of *Gasteria*

verrucosa. They concluded that a pollen tube attractant was present in the exudate after observing that pollen tubes showed a preference for micropyles with micropylar exudate. Isolated ovules were washed with different solutions and then pollinated *in vitro* to investigate the nature of the attractant. Pollen tube penetration decreased when ovules were washed with solutions that affected proteins, suggesting a proteinaceous component to the attractant. Pollen tubes were also unable to penetrate isolated ovules with micropylar exudate that had been subjected to denaturing treatments, providing additional confirmation of a proteinaceous chemoattractant (Willemse *et al*. 1995). The micropylar exudate is not the only source of chemoattractants involved in pollen tube guidance. Pollen tube attractants are also expressed in the synergid cells, the egg cell and the central cell and they have been shown to play key roles in guidance of pollen tubes to the micropyle (Mizuta and Higashiyama 2018). It is likely that pollen tube guidance provided by the micropylar exudate is complementary to the guidance cues from the embryo sac.

The Apoplast

The term apoplast was first coined by the botanist Ernst Münch in 1930. He wanted to separate the plant into two principal compartments, the ‘dead’ apoplast and the ‘living’ symplast. Münch thought the apoplast functioned solely to transport water and solutes (Sattelmacher 2001). We now know that apoplastic functions are much more numerous, and we now define the apoplast in broader

terms than those originally introduced by Münch. Importantly, the research reviewed above shows that secretions into the apoplast confer function onto the apoplastic space. More importantly, the research shows that it is the constituents of those secretions that confer function. Plants can respond to and interact with biotic and abiotic elements of the surrounding environment through what they secrete into the apoplast.

Chapter 3

Examining the Constituents of the Conifer Ovular Secretion

Research into the conifer ovular secretion has focused on the morphological context of pollination mechanisms. The constituents of the ovular secretion have not received the same attention as constituents of the secretions into the angiosperm apoplast. Indeed, it has even been proposed that rainwater can supplement or even supplant the conifer ovular secretion (Greenwood 1986; Brown and Bridgewater 1987; Owens *et al*. 2001) and that pollen scavenging assisted by rainwater, and not by an ovular secretion, was the ancestral condition (Owens *et al*. 1998). However, this model does not take into account the presence of ovular secretions in more basal gymnosperms and pteridosperms (Rothwell 1977; Gelbart and von Aderkas 2002, Prior *et al*. 2018). It also discounts the metabolic cost of an ovular secretion flush with biomolecules. Constituents of secretions into the angiosperm apoplast have been examined in an effort to understand how those secretions function. Constituents of the conifer ovular secretion should be similarly examined.

Pollen-Ovule Interactions

Pollen germinates in the conifer ovular secretion (Little *et al*. 2014). Constituents of the ovular secretion likely support pollen germination and pollen tube growth. Calcium ions, for example, are present in the ovular secretion (Fujii

1903; von Aderkas *et al*. 2012). The essential role of calcium in pollen germination is widespread among the seed plants (Brewbaker and Kwack 1963; von Aderkas *et al*. 2012). However most pollen has a low internal calcium content (Brewbaker and Kwack 1963). It is likely that germinating pollen takes up calcium from the ovular secretion as it does from the stigmatic exudate. The calcium likely also supports pollen tube growth, as it does in the stigmatic exudate. Gymnosperm pollen grains are low in total carbohydrate reserves (Vasil 1987). When grown *in vitro*, pollen readily utilizes exogenous carbohydrates for tube growth and starch synthesis (Nygaard 1977). Glucose, fructose, and sucrose are found in the ovular secretion (e.g. McWilliam 1958, Nepi *et al*. 2017) and could be taken up by pollen. It has been postulated that glycosyl hydrolases present in the stigmatic exudate catabolize large polysaccharides into smaller units that can be incorporated into the growing pollen tube (Rejón *et al*. 2013). Similar enzymes have been identified in the ovular secretion (Poulis *et al*. 2005; Wagner *et al*. 2007; Pirone-Davies *et al*. 2016). Free amino acids present in the ovular secretion may also support pollen development. Proline, for example, is often the most abundant amino acid in the ovular secretion. Proline is used directly by germinating pollen grains, and it is used to synthesize hydroxyproline-rich proteins in the pollen tube wall (Shivanna 2003).

Constituents of the ovular secretion may also support pollen selection. Nepi and colleagues (2017) found species-specific amino acid profiles in the ovular secretions of all conifer species analyzed. Duhoux and Pham Thi (1980,

cited in Gelbart and von Aderkas 2002) demonstrated that *Juniperus communis* pollen tubes had improved growth *in vitro* when the amino acids present in the culture medium were the same as those found in ovular tissues. Such improved growth would provide an advantage to homospecific pollen, suggesting that species-specific amino acid profiles may contribute to pollen selection. Non-protein amino acids have been identified in the ovular secretions of two conifer species (Nepi *et al*. 2017). Non-protein amino acids have been found to affect pollen germination and tube growth in five species of Fabaceae *in vitro*. Both promotion and inhibition of growth were observed. The effect on growth varied by species, which would suggest that the amino acids may contribute to hybridization barriers (Simola 1967).

Ovule Defence

Constituents of the conifer ovular secretion may protect the ovule from biotic and abiotic threats. Antimicrobial proteins are present in the conifer ovular secretion (O'Leary *et al*. 2007; Wagner *et al*. 2007; Coulter *et al*. 2012; Pirone-Davies *et al*. 2016). The ovular secretion is an interface between the ovule and the surrounding environment, so it is likely that these proteins defend the ovule from infection, as they do in angiosperm floral nectar, extrafloral nectar, and stigmatic exudates. Chitinases, β-1,3-glucanases, and thaumatin-like proteins are among the antimicrobial proteins identified (O'Leary 2004; O'Leary *et al*. 2007; Wagner *et al*. 2007; Coulter *et al*. 2012; Pirone-Davies *et al*. 2016). These

pathogenesis-related proteins can also function as antifreeze proteins (Griffith and Yaish 2004). Antifreeze activity has been detected in the apoplastic fluid collected from Douglas-fir needles and is attributed to an extracellular chitinase (Zamani *et al*. 2003). It is possible that these proteins also demonstrate antifreeze activity in the ovular secretion. Many species of conifers produce an ovular secretion during cold weather. Antifreeze activity in the ovular secretion would protect the ovule from freezing injury.

Research Objective

The constituents of the conifer ovular secretion need to be examined more closely. Of particular interest is the proteinaceous component of the ovular secretion. The secretory proteins found in the ovular secretion are costly to produce and are evolutionarily conserved (Wagner *et al*. 2007). It is unlikely that they are secreted superfluously. Proteomic identification of the proteinaceous component of the ovular secretion has yielded a list of proteins that may be involved in pollen-ovule interactions and ovule defence (Nepi *et al*. 2009). However, protein identifications only provide putative information on function. Functional characterization of these proteins is needed in order to fully understand how they contribute to ovular secretion function.

The experimental work contained in this dissertation focuses on the proteinaceous component of the ovular secretion. Three proteins were selected for

functional characterization: invertase, chitinase, and thaumatin-like protein.

Investigations into these proteins were guided by the following hypotheses:

H_0: The constituents of the ovular secretion confer function on the ovular secretion.

H_1: Proteins in the ovular secretion are functional.

H_3: Proteins in the ovular secretion are involved in pollen-ovule interactions.

H_4: Proteins in the ovular secretion are involved in ovule defence.

Chapter 4

Invertase Activity in the *Pseudotsuga menziesii* Post-Pollination Prefertilization Drop

Portions of this chapter have been published as von Aderkas P, Nepi M, Rise M, Buffi F, Guarnieri M, Coulter A, Gill K, Lan P, Rzemieniak S, Pacini E. 2012. Post-pollination prefertilization drops affect germination rates of heterospecific pollen in larch and Douglas-fir. Sex Plant Reprod. 25: 215-225.

Introduction

Two extracellular invertases are present in the Douglas-fir (*Pseudotsuga menziesii*) post-pollination prefertilization drop (Poulis *et al*. 2005). Invertase catalyzes the hydrolysis of the disaccharide sucrose into the monosaccharides fructose and glucose (Roitsch *et al*. 2003). Sucrose is the major transport sugar in plants. Catabolism of sucrose must occur before plants can utilize the component hexoses (Kingston-Smith *et al*. 1999). This catabolism is carried out by either invertase or sucrose synthase, which cleaves sucrose in the presence of UDP into UDP-glucose and fructose. Only invertase irreversibly catalyzes the cleavage of sucrose. It is also the only sucrose-catabolizing enzyme found in the apoplast (Kingston-Smith *et al*. 1999; Sturm 1999).

Autotrophic tissues such as mature leaves produce a surplus of photosynthate. This surplus is transported as sucrose via the phloem to non-photosynthetic tissues such as roots, reproductive structures, developing organs and storage tissues (Truernit 2001; Hammond and White 2008). Sucrose can move from the phloem to the sink (i.e. non-photosynthetic) tissues through the

symplast and/or the apoplast. Sucrose moves through the symplast via plasmodesmata. Sucrose can also move across the cell wall, entering the apoplast. Sucrose is unloaded into the apoplast by a sucrose transporter (Roitsch *et al*. 2003; Roitsch and González 2004). Extracellular invertase then cleaves the sucrose. The resulting glucose and fructose are transported into the sink cell by monosaccharide transporters (Roitsch *et al*. 2003). Catalysis by invertase is the rate-limiting step. It is also the only irreversible step in the phloem unloading pathway (Roitsch *et al*. 2003). Thus, invertase activity establishes a sucrose gradient that drives apoplastic phloem unloading.

Apoplastic invertase is also involved in the mobilization of carbohydrates during active secretion of nectar. The extracellular hydrolysis of sucrose allows nectaries to maintain a constant sink status (Kram and Carter 2009), which facilitates secretion of sugars into the extracellular space (Heil 2011). Invertase activity has also been observed in extrafloral nectar. Such activity allows for the post-secretory regulation of the carbohydrate composition of nectar (Heil *et al*. 2005).

The aim of this research was to determine if the two invertases found in the Douglas-fir drop are functional at physiological conditions. Functional invertases in the Douglas-fir drop would not be involved in phloem unloading; the ovule is not vascularized (Singh 1978). Functional invertases in the drop are likely involved in processes similar to those observed in floral and extrafloral nectar, namely the mobilization of carbohydrates and post-secretory hydrolysis of

sucrose. If the invertases are found to be functional, this will be the first published evidence that drop proteins are functional. It will also be the first evidence that post-secretory regulation of drop composition occurs.

Methods and Materials

Plant Material

Pseudotsuga menziesii (Mirbel) Franco (Douglas-fir) and *Larix* × *marschlinsii* Coaz (hybrid larch) cones were collected from trees on the campus of the University of Victoria (Victoria, British Columbia, Canada). Cone collection was timed to coincide with post-pollination prefertilization drop formation, which occurs 6-10 weeks after pollination. The post-pollination prefertilization drop of both species can only be accessed by pulling cones apart. Ovuliferous-bract complexes were laid individually in closed Petri dishes lined with Whatman No. 1 filter paper moistened with distilled water. Drops appeared at the tip of the micropyle after 5-10 min. They were then collected with micropipette tips which, once full, were voided into Eppendorf vials that were kept on ice. The Eppendorf vials were subsequently flash-frozen in liquid nitrogen and stored at -20 °C until analysis.

Invertase Activity

Isocratic high-performance liquid chromatography was used to test for invertase activity in the post-pollination prefertilization drops of Douglas-fir.

Invertase is not present in hybrid larch post-pollination prefertilization drops, so these were used as a control. Drops were diluted 1:30 in a sucrose solution (1 mg/mL in distilled water). Sucrose, glucose, and fructose concentrations were measured at 0, 15, and 30 min after dilution to test for sucrose hydrolysis by invertase. Sugar concentrations were measured by injecting 20 µL of diluted drop solution into a Waters LC Module 1 HPLC (Waters Corporation, Milford, MA, USA). Sugars were separated in a Waters Sugar-Pak I column (300 x 6.5 mm) and identified with a Waters 2410 refractive index detector. The mobile phase was deionized water, the flow rate was 0.5 mL/min, and the column temperature was 85 - 90 °C. Standard solutions were injected in 20 µL aliquots. The analysis was performed in triplicate.

A colorimetric assay was then used to analyze the pH dependence of the invertase activity. Post-pollination prefertilization drops were diluted 1:2 with deionized water. A 1.0 µL aliquot of this diluted post-pollination prefertilization drop was combined with 100 µL of 100 mM buffered sucrose solution. As a reference, 1.0 µL of deionized water was combined with 100 µL of 100 mM buffered sucrose solution. The 100 mM sucrose solutions were buffered at 12 different pH values using the following buffers: 50 mM glycine pH 2.5, 2.7, 3.0, 3.3, 3.5; 50 mM sodium acetate pH 3.7, 4.0, 4.3, 4.5, 4.7, 5.0; 50 mM sodium phosphate pH 6.0. The buffered drop/sucrose and reference/sucrose solutions were incubated for 45 min at 30 °C. A 5.0 µL aliquot of the incubated drop/sucrose and reference/sucrose solutions was combined with 250 µL Glucose

(HK) Assay reagent (Glucose (HK) Assay Kit, Sigma-Aldrich, St. Louis, MO, USA) in a 96-well plate (Microtest, Sarstedt, Nümbrecht, Germany) and incubated at room temperature for 20 min. The amount of glucose released from sucrose by invertase was quantified by reading the absorbance at 340 nm (Sunrise microplate reader, Tecan, Salzburg, Austria). The assay was performed in triplicate. A smoothed curve was fit to the data using KaleidaGraph 4.0 (Synergy Software, Reading PA, USA).

Invertase activity was then quantified at the optimal pH for invertase activity and at the *in situ* pH of the drop, pH 4.5. A 1.0 µL aliquot of the same diluted post-pollination prefertilization drop as was used in the pH dependence assay was combined with 100 µL of 100 mM sucrose solution buffered to pH 4.0 and 4.5 (as described above). A 1.0 µL aliquot of deionized water combined with 100 µL of 100 mM sucrose solution buffered to pH 4.0 and 4.5 (as described above) served as a reference. The four solutions were incubated at 30 ˚C. A 2.5 µL aliquot was withdrawn from each of the four solutions every 10 min for 50 min and combined with 125 µL Glucose (HK) Assay Reagent in a 96-well plate (Microtest, Sarstedt, Nümbrecht, Germany). The plate was incubated at room temperature for 20 min and the absorbance was read at 340 nm. The assay was performed in triplicate. Standardization was accomplished by measuring the absorbance of known amounts of glucose. Aliquots of glucose solution (2.5 µL) of known concentration (0 ng/µL to 5 ng/µL) were incubated for 20 min at room

temperature with 125 µL Glucose (HK) Assay Reagent and the absorbance was read at 340 nm. Three replicates were analysed for each glucose standard.

Results and Discussion

Sucrose, glucose and fructose concentrations remained constant in the post-pollination prefertilization drop of the control species, hybrid larch. Glucose and fructose concentrations rose as sucrose concentrations declined in the Douglas-fir post-pollination prefertilization drop (Figure 2). These results confirm that there is invertase activity in the Douglas-fir post-pollination prefertilization drop. A pH dependency was observed for the invertase activity in the Douglas-fir post-pollination prefertilization drop (Figure 3). The invertase activity was maximal at pH 4.0. Invertase activity was found to be 17.6 ± 0.877 ng glucose min^{-1} L $drop^{-1}$ at pH 4.0. Invertase activity was found to be 13.5 ± 0.499 ng glucose min^{-1} L $drop^{-1}$ at pH 4.5. The activity at pH 4.5 is 77 % of the maximum activity observed at pH 4.0. The pH of the Douglas-fir drop is 4.5 ± 0.33 (von Aderkas *et al*. 2012). These results indicate that post-secretory hydrolysis of sucrose occurs *in situ* in the Douglas-fir drop.

Apoplastic invertases control the movement of sucrose through the plant by establishing sucrose concentration gradients (Sturm and Tang 1999; Tang *et al*. 1999; Roitsch *et al.* 2003). In the angiosperms, pollen is symplastically isolated very early in development in the anther (Truernit *et al*. 1999). Extracellular invertase is required for the movement of carbohydrates to

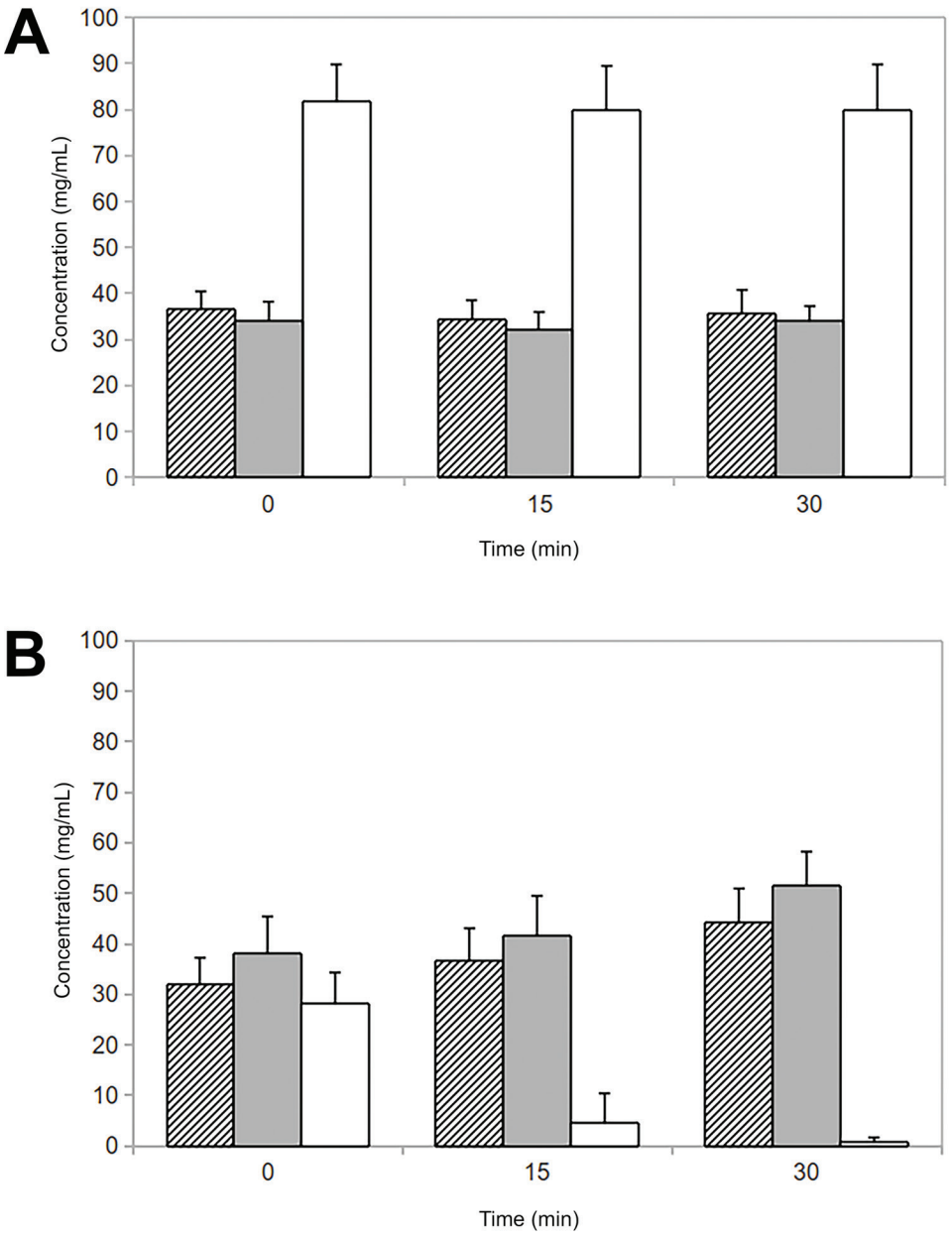

Figure 2. Sucrose hydrolysis in the post-pollination prefertilization drops of **(A)** *Larix* × *marschlinsii* and **(B)** *Pseudotsuga menziesii* (average ± SE). Isocratic high-performance liquid chromatography was used to measure the concentration of fructose (hatched), glucose (solid) and sucrose (empty) immediately after the drops were diluted 1: 30 with sucrose (1 mg/mL in distilled water), and at 15 min and 30 min after dilution.

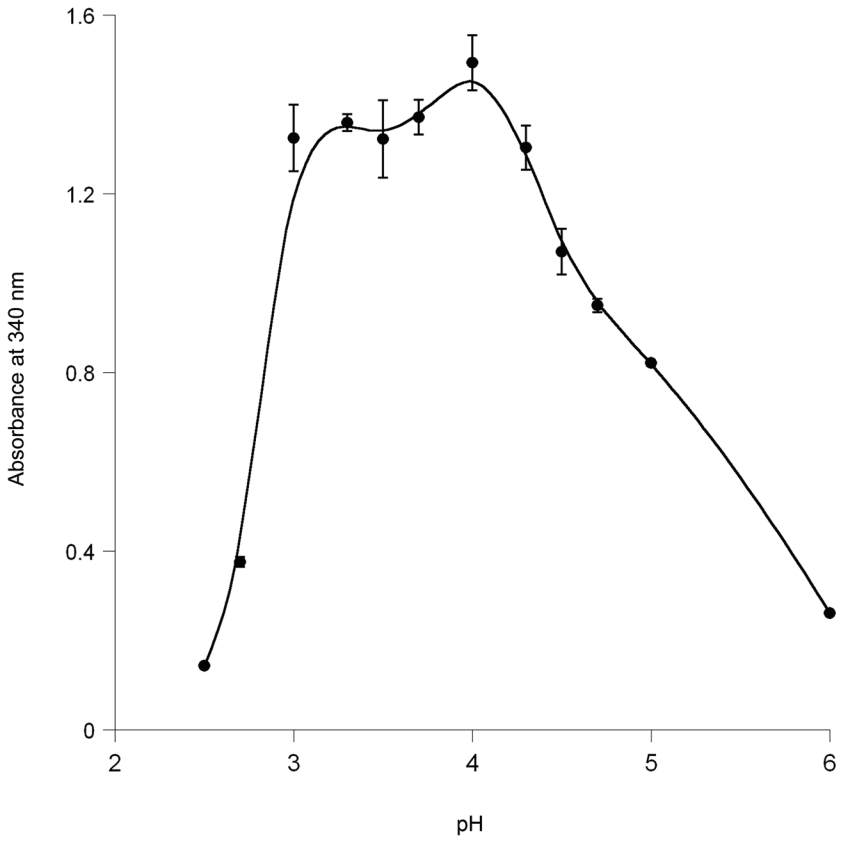

Figure 3. pH dependence of invertase activity in the *Pseudotsuga menziesii* post-pollination prefertilization drop (average ± SD). Post-pollination prefertilization drops of *P. menziesii* were combined with buffered sucrose solutions. The amount of glucose released was measured colorimetrically.

developing pollen via the apoplast. Anther-specific interference with apoplastic invertase activity negatively affects pollen development and results in male sterility in *Nicotiana tabacum*, *Arabidopsis thaliana*, *Brassica napus* and *Solanum lycopersicum* (Goetz *et al*. 2001; Proels *et al*. 2006; Hirsche *et al*. 2009; Engelke *et al*. 2010; Engelke *et al*. 2011). Angiosperm pollen remains symplastically isolated after pollination. The ensuing pollen tube growth is supported by uptake of carbohydrates from the apoplast (Kroh *et al*. 1970; Labarca and Loewus 1972; Labarca and Loewus 1973). Gymnosperm pollen is also symplastically isolated after pollination. The two invertases found in the Douglas-fir drop may facilitate the movement of carbohydrates from maternal tissue to germinating pollen via the apoplast. Such metabolic cooperation between male and female tissues occurs in *N*. *tabacum*, where extracellular invertase in the pistil provides nutritional support to the growing pollen tube (Goetz *et al*. 2017).

Controlling the movement of carbohydrates is one way in which apoplastic invertase plays a critical role in pollen development. There is also evidence that apoplastic invertase is involved in metabolic signalling that is required for pollen development. Sucrose can act as a signalling molecule, as can glucose and fructose (Eveland and Jackson 2012; Ruan 2014). Sugar signalling can therefore be mediated by invertase activity (Ruan *et al*. 2010; Ruan 2014). Anther-specific antisense repression of cell wall invertase in *N*. *tabacum* results in a block during early stages of pollen development. The application of exogenous sucrose and glucose only partially restores pollen development. The

developmental block is therefore not solely due to a perturbation of the carbohydrate supply, but is likely also due to interference with metabolic signals required for pollen development (Goetz *et al*. 2001). Sugar signalling is also involved in the regulation of pollen germination and pollen tube growth. Glucose, for example, serves as a signalling molecule during pollen germination and tube growth in *Arabidopsis* (Rottman *et al*. 2016; Hirsche *et al*. 2017; Rottman *et al*. 2018). The two invertases found in the Douglas-fir drop (Poulis *et al*. 2005) may provide post-secretory regulation of sugar signalling molecules in the ovular secretion that mediate pollen germination and tube growth. This requires further investigation.

Sucrose is absent from the Douglas-fir post-pollination prefertilization drop due to its conversion to glucose and fructose by the two invertases found in the drop. The conversion of sucrose to glucose and fructose lowers the osmotic potential of the drop, opening up the possibility that invertase activity may be involved in drop formation. Currently, there are only suggestions in the literature as to how pollination drops form. No mechanism has been elucidated. Apoplastic invertase activity has been shown to be essential for nectar production in *Arabidopsis*. It has been suggested that the lower osmotic potential of hexose-rich nectar may facilitate the movement of water to nectar (Ruhlmann *et al*. 2010). Even though nectar and pollination drops are analogous but not homologous (Nepi *et al*. 2009), this further suggests that apoplastic invertases may be involved in the formation of the Douglas-fir drop.

Ovular secretions of eight conifer species have been proteomically analyzed (O'Leary 2004; O'Leary *et al*. 2004; Poulis *et al*. 2005; O'Leary *et al*. 2007; Wagner *et al*. 2007; Pirone-Davies *et al*. 2016). To date, invertases have only been identified in the Douglas-fir drop (Poulis *et al*. 2005). It is possible that proteomic analysis of additional conifer species will reveal that invertase activity is more widespread than this limited sample size would suggest. However, invertase activity is not conserved at the family level. Douglas-fir and hybrid larch are both members of the Pinaceae, but only the Douglas-fir drop contains invertase (O'Leary 2004; Poulis *et al*. 2005; von Aderkas *et al*. 2012). Proteomic studies of ovular secretions have indicated that the proteins are highly conserved (Wagner *et al*. 2007), so the presence of invertase in the post-pollination prefertilization drop of only one of a pair of closely related genera is interesting. It has been demonstrated that pollen selection occurs at the stage of pollen germination in the post-pollination prefertilization drops of Douglas-fir and hybrid larch (von Aderkas *et al*. 2012). This suggests that the composition of the post-pollination prefertilization drop is important for pollen selection. Invertase activity will affect the composition of the post-pollination prefertilization drop. Thus, it is possible that invertase activity uniquely present in the Douglas-fir drop serves as a mechanism for selection of homospecific pollen.

Successful pollen germination and tube growth *in vitro* requires the correct composition and concentration of carbohydrates and is species-specific (Shivanna 2003; Rottman *et al*. 2018). Glucose, for example, supports pollen

germination in *N. tabacum* pollen but inhibits both germination and tube growth in *Arabidopsis* (Goetz *et al*. 2017; Hirsche *et al*. 2017; Rottman *et al*. 2018). *Nicotiana tabacum* pollen germination is also supported by sucrose (Goetz *et al*. 2017), whereas *Arabidopsis* pollen requires sucrose to germinate (Stadler *et al*. 1999). Sucrose is widely used in pollen germination media (Brewbaker and Kwack 1963; Read *et al*. 1993), and it is known that the pollen of some conifers, e.g. *Pinus roxhburghii*, requires sucrose to germinate (Dhawan and Malik 1981). Douglas-fir pollen, however, does not require sucrose to germinate *in vitro* (Webber and Bonnet-Masimbert 1993), and sucrose negatively affects pollen tube growth and morphology *in vitro* (Dumont-BéBoux and von Aderkas 1997). This implies that by removing sucrose from the ovular secretion, the two invertases found in the Douglas-fir drop create a medium that is supportive to homospecific pollen germination and tube growth.

Germinating pollen is also sensitive to osmolarity (Webber and Bonnet-Masimbert 1993). Douglas-fir pollen is difficult to germinate *in vitro* (Dumont-BéBoux *et al*. 1999). The osmotic effect of the media has a more significant effect on germination response than the metabolic effect of the media (Webber and Bonnet-Masimbert 1993). Invertase activity in the Douglas-fir drop must alter the osmolarity of the drop. This ability may be yet another way in which invertase activity in the Douglas-fir drop contributes to selection of homospecific pollen.

Currently, it is not possible to state with certainty that the invertases found in the Douglas-fir post-pollination prefertilization drop contribute to the movement of carbohydrates to pollen tubes, drop formation, or selection of homospecific pollen. More research is necessary. However, it can be stated with certainty that there is invertase activity in the Douglas-fir post-pollination prefertilization drop, and that this activity is responsible for post-secretory regulation of the carbohydrate composition of the drop. This research is the first to confirm that extracellular proteins found in conifer ovular secretions are functional. It is also the first to demonstrate that post-secretory regulation of the composition of conifer pollination drops occurs. The results of this research open the door for a re-evaluation of the role the ovular secretion plays in conifer reproduction.

Chapter 5

Chitinase Activity in the *Pseudotsuga menziesii* Post-Pollination Prefertilization Drop

Portions of this chapter have been published as Coulter A, Poulis BAD, von Aderkas P. 2012. Pollination drops as dynamic apoplastic secretions. Flora. 207:482-490.

Introduction

Eight chitinases have been identified in the post-pollination prefertilization drops of Douglas-fir (*Pseudotsuga menziesii*) (Coulter *et al*. 2012). Chitinases hydrolyze the β-(1,4)-glycoside bonds of biopolymers of N-acetylglucosamine (GlcNAc) (Collinge *et al*. 1993). The main substrate of chitinase is chitin, a natural homopolymer of unbranched chains of β-1,4-linked N-acetylglucosamine residues (Patil *et al*. 2000; Kasprzewska 2003). There are two classes of chitinases: endochitinases and exochitinases. Endochitinases catalyze the random cleavage at internal points in the chitin chain releasing low molecular mass multimers of N-acetylglucosamine such as chitotetraose, chitotriose and di-acetylchitobiose (Sahai and Manocha 1993). Exochitinases cleave chitin at the non-reducing end of the chain and are divided into two categories: chitobiosidases and β-N-acetylglucosaminidases. Chitobiosidases catalyze the progressive release of acetylchitobiose from the non-reducing end of chitin while β-N-acetylglucosaminidases catalyze the progressive release of N-acetylglucosamine monomers from the non-reducing end of chitin and the

oligomeric products of endochitinase and chitobiosidase (Sahai and Manocha 1993; Cohen-Kupiec and Chet 1998).

Chitin is a major constituent of fungal cell walls. Fungi have significant internal turgor pressure, so even slight perturbations of the cell wall results in lysis (Selitrennikoff 2001). Because they can digest the chitin in fungal cell walls, plant chitinases are antifungal. *In vitro* studies have demonstrated the inhibitory effect that chitinases have on spore germination and fungal growth (Schlumbaum *et al*. 1986; Broekaert *et al*. 1988; Roberts and Selitrennikoff 1988; Markovich and Kononova 2003). It has also been observed that the enzyme accumulates around fungal cell walls *in planta* (Wubben *et al*. 1992). Chitinases are members of the pathogenesis-related group of proteins that are strongly upregulated in response to infection (Grover 2012).

Conifer ovules are vulnerable to infection during the period of pollen receptivity. Ovules are exposed and lack a structural barrier to pathogens: the integument has a protective cuticle, but the nucellus does not (Singh 1978). Additionally, pollination mechanisms are indiscriminate. Fungal spores and bacteria can be captured along with windborne pollen. Moreover, the ovular secretion is metabolically rich and contains compounds that can support microbial growth (Whitaker *et al*. 2008). Yet, despite these vulnerabilities, infection of the ovule is rarely observed (O'Leary *et al*. 2007). Proteomic studies have identified putative defence proteins, including chitinases, in conifer ovular secretions. It is proposed that these defence proteins are responsible for keeping the ovule aseptic

(O'Leary 2004, Poulis 2004; O'Leary *et al*. 2007; Wagner *et al*. 2007; Coulter *et al*. 2012; Pirone-Davies *et al*. 2016).

It is known that the Douglas-fir drop is capable of digesting chitin (Coulter *et al*. 2012). However, the mode of chitinolytic activity is unknown. It is also unknown if the chitinases are functional at the physiological pH of 4.5 ± 0.33 (von Aderkas *et al*. 2012). The aim of this research is to further characterize the chitinase activity found in the Douglas-fir drop. All three modes of chitinolytic activity will be investigated: chitobiosidase, endochitinase, and β-N-acetylglucosaminidase activity. This research also aims to determine if the chitinases found in the Douglas-fir drop are functional at physiological pH. Confirmation will provide evidence for a defensive role for the conifer ovular secretion.

Methods and Materials

Plant Material

Pseudotsuga menziesii (Mirbel) Franco (Douglas-fir) cones were collected from trees on the campus of the University of Victoria (Victoria, British Columbia, Canada). Cone collection was timed to coincide with post-pollination prefertilization drop formation, which occurs 6-10 weeks after pollination. The post-pollination prefertilization drop can only be accessed by pulling cones apart. Ovuliferous-bract complexes were laid individually in closed Petri dishes lined with Whatman No. 1 filter paper moistened with distilled water. Drops appeared

at the tip of the micropyle after 5-10 min. They were then collected with micropipette tips which, once full, were voided into Eppendorf vials that were kept on ice. The Eppendorf vials were subsequently flash-frozen in liquid nitrogen and stored at -20 °C.

β-N-acetylglucosaminidase Activity

A colorimetric assay was used to analyze the pH dependence of β-N-acetylglucosaminidase activity. β-N-acetylglucosaminidase activity was tested for using the substrate 4-nitrophenyl N-acetyl-β-D-glucosaminide (Chitinase Assay Kit, Sigma-Aldrich, St. Louis, MO, USA). Hydrolysis of 4-nitrophenyl N-acetyl-β-D-glucosaminide by β-N-acetylglucosaminidase releases *p*-nitrophenol. Sodium carbonate is then added to cause ionization of colourless *p*-nitrophenol to the pale yellow *p*-nitrophenylate ion.

The substrate was buffered at a concentration of 1 mg/mL using the following buffers: 50 mM glycine pH 2.5, 3.0, 3.3, 3.5; 50 mM sodium acetate pH 3.7, 4.0, 4.3, 4.5, 4.7, 5.0; 50 mM sodium phosphate pH 6.0 and 7.0. Post-pollination prefertilization drops were diluted 1:2 with deionized water. A 1.0 µL aliquot of the diluted drop was combined with 100 µL of the buffered substrate for each pH. As a reference, 1.0 µL of deionized water was combined with 100 µL of the buffered substrate for each pH. The buffered substrate/drop and buffered substrate/reference solutions were incubated at 30 °C for 2 hours. The buffered substrate/drop and buffered substrate/reference solutions were then

combined with 200 µL of 0.39 M sodium carbonate in a 96-well plate (Microtest, Sarstedt, Nümbrecht, Germany) and incubated at room temperature for 20 min. The absorbance was read at 405 nm (Sunrise microplate reader, Tecan, Salzburg, Austria). The assay was performed in triplicate. A smoothed curve was fit to the data using KaleidaGraph 4.0 (Synergy Software, Reading PA, USA).

Chitobiosidase and Endochitinase Activity

A colorimetric assay was used to test for chitobiosidase and endochitinase activity. Chitobiosidase activity was tested using the substrate 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside (Chitinase Assay Kit, Sigma-Aldrich, St. Louis, MO, USA). Endochitinase activity was tested using the substrate 4-nitrophenyl β-D-N,N',N''-triacetylchitotriose (Chitinase Assay Kit, Sigma-Aldrich, St. Louis, MO, USA). Hydrolysis of 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside and 4-nitrophenyl β-D-N,N',N''-triacetylchitotriose by chitobiosidase and endochitinase activity, respectively, releases *p*-nitrophenol. Sodium carbonate is then added to cause ionization of colourless *p*-nitrophenol to the pale yellow *p*-nitrophenylate ion.

Both substrates were buffered at pH 4.8 in a 50 mM sodium acetate buffer at a concentration of 1 mg/mL. A 1.0 µL aliquot of the post-pollination pre-fertilization drop was combined with 99 µL of buffered substrate. As a positive control, 1.0 µL of a 0.01 mg/mL solution of chitinase was combined with 99 µL of buffered substrate. The 0.01 mg/mL chitinase solution was prepared by

dissolving chitinase from *Trichoderma viride* (Chitinase Assay Kit, Sigma-Aldrich, St. Louis, MO, USA) in a modified Dulbecco's Phosphate Buffered Saline (containing no calcium chloride or magnesium chloride). As a negative control, 1.0 µL of deionized water was combined with 99 µL of buffered substrate. The drop/positive control/negative control with buffered substrate solutions were incubated for two hours at 37 °C. The drop/positive control/negative control with buffered substrate solutions were then transferred to a 96-well plate (Microtest, Sarstedt, Nümbrecht, Germany) and 200 µL of 0.39 M sodium carbonate (Chitinase Assay Kit, Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The plate was incubated for 10 minutes at room temperature and then the absorbance was read at 405 nm (Sunrise microplate reader, Tecan, Salzburg, Austria). The assay was performed in triplicate.

Results and Discussion

β-N-acetylglucosaminidase activity was observed in the Douglas-fir post-pollination prefertilization drop over a broad pH range, from pH 3.0 to 7.0, the last pH tested (Figure 4). Activity increased from pH 2.5 until reaching a maxima at pH 3.5, dropped slightly, and then increased again from pH 3.7 to a second maxima at pH 5.0. Activity was at least 70 % of maximal between pH 3.0 and 7.0. Activity was more than 80 % of maximal at pH 4.5. The pH of the Douglas-fir drop is 4.5 ± 0.33 (von Aderkas *et al*. 2012). Therefore, these results confirm that

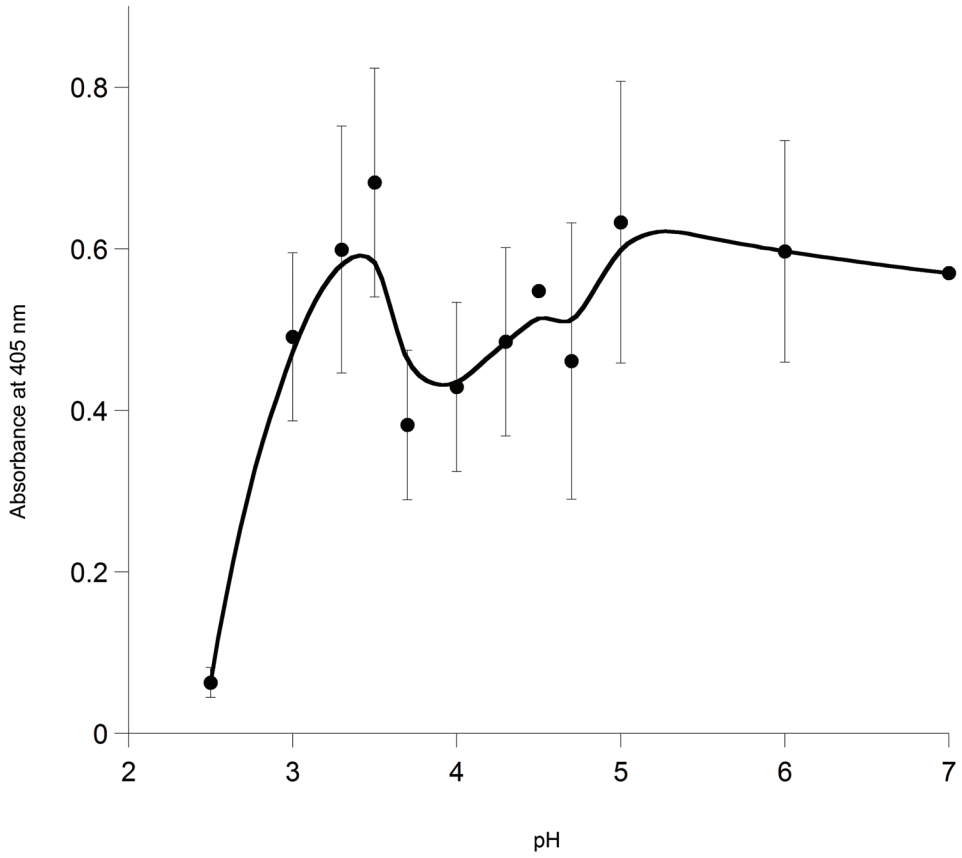

Figure 4. pH dependence of β-N-acetylglucosaminidase activity in the post-pollination prefertilization drop of *Pseudotsuga menziesii* (average ± SD). Post-pollination prefertilization drops of *P. menziesii* were diluted 1:2 with deionized water and combined with buffered 1 mg/mL 4-nitrophenyl N-acetyl-β-D-glucosaminide solutions. Hydrolysis of this substrate was measured colorimetrically.

there is β-N-acetylglucosaminidase activity in the Douglas-fir post-pollination prefertilization drop in physiological conditions.

Hydrolysis of the substrates 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside and 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose, a test for chitobiosidase and endochitinase activity, respectively, was observed in the Douglas-fir post-pollination prefertilization drop. Hydrolysis was not observed in the negative control (Figure 5). These results confirms that there is chitobiosidase and endochitinase activity in the Douglas-fir drop. Hydrolysis occurred at pH 4.8. The pH of the Douglas-fir drop is 4.5 ± 0.33 (von Aderkas *et al*. 2012). Therefore, these results confirm that there is chitobiosidase and endochitinase activity in the Douglas-fir drop in physiological conditions.

Plant chitinases are localized in the apoplast and in vacuoles. The apoplastic chitinases are considered to be part of an early defence response. They inhibit the growth of fungal hyphae and they may also release fungal elicitors that trigger downstream defence pathways. Vacuolar chitinases play a role later in the infection process. They are released from the vacuole if fungal hyphae penetrate plant tissue and lyse the protoplast (Collinge *et al*. 1993; Grover 2012). The chitinases present in the Douglas-fir ovular secretion are apoplastic and likely function as a first line of defence against fungal infection. Conifer ovules are vulnerable during pollen receptivity. They are exposed, they lack a structural barrier to pathogens, and pollen capture is indiscriminate (Singh 1978). Furthermore, the gymnosperm ovular secretion is nutrient-rich and can support

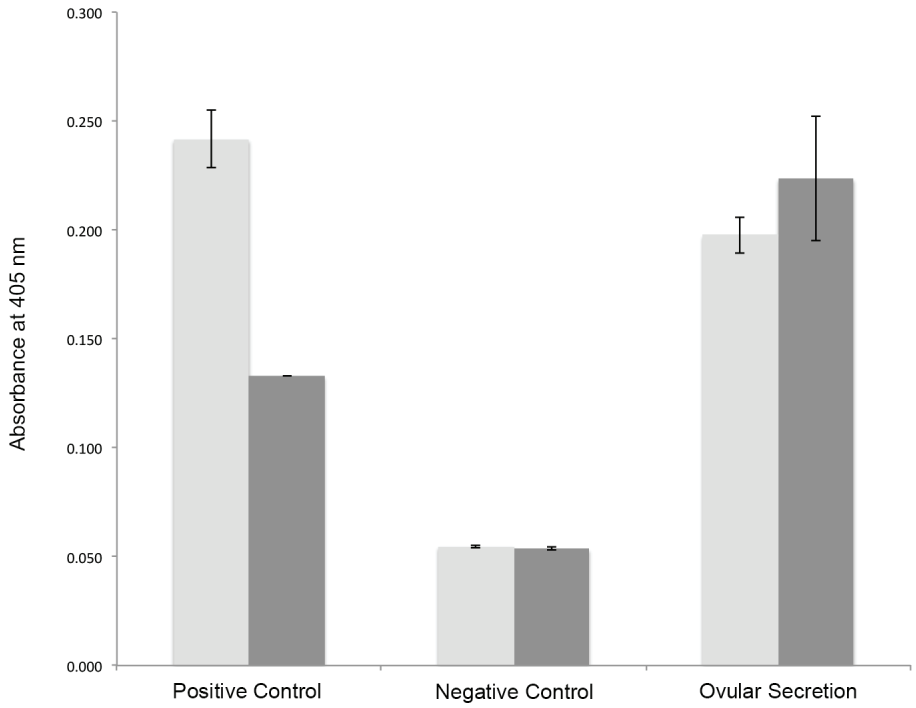

Figure 5. Chitobiosidase and endochitinase activity in the post-pollination prefertilization drop of *Pseudotsuga menziesii* (average ± SD). Post-pollination prefertilization drops of *P. menziesii* were combined with buffered 1 mg/mL substrate. The substrate 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside (light grey) was used to detect chitobiosidase activity. The substrate 4-nitrophenyl β-D-N,N',N''-triacetylchitotriose (dark grey) was used to detect endochitinase activity. Chitinase from *Trichoderma viride* was used as a positive control. Deionized water was used as a negative control.

microbial growth (Whitaker *et al*. 2008). Despite this apparent vulnerability, infection of the ovule is rarely observed (O'Leary *et al*. 2007).

There is precedence for a defensive role for chitinase in plant reproductive secretions. Chitinase has been found in stigma exudates of *Lilium longiflorum* and *Nicotiana tabacum* (Sang *et al*. 2012; Rejón *et al*. 2013). It is proposed that chitinase protects the sugar-enriched exudate from infection by phytopathogens. The stigma exudate is analogous to the ovular secretion. As with the ovular secretion, the stigma exudate is involved in pollen capture and is vulnerable to infection. Chitinase is also present in nectar. One or more chitinases have been identified in the floral nectar of *Petunia hybrida* (Hillwig *et al*. 2011; Silva *et al*. 2020), *Rhododendron irroratum* (Zha *et al*. 2016) and numerous *Nicotiana* species (Silva *et al*. 2020). Activity assays have confirmed chitinase activity, and a defensive role has been proposed (Hillwig *et al*. 2011; Zha *et al*. 2016; Silva *et al*. 2020). Chitinases have also been identified in the extrafloral nectar of multiple *Acacia* species (González-Teuber *et al*. 2009; González-Teuber *et al*. 2010). Activity assays have confirmed chitinase activity in the extrafloral nectar of multiple *Acacia* species and *Prosopis juliaflora* (González-Teuber *et al*. 2009). Bioassays have confirmed the extrafloral nectar of multiple *Acacia* species are capable of inhibiting microbial growth (González-Teuber *et al*. 2009; González-Teuber *et al*. 2010). Nectar and the ovular secretion are also analogous. The basic chemical composition of nectar and the ovular secretion is similar and both are vulnerable to microbial infection (Nepi *et al*. 2009). Chitinase is also found in

non-reproductive tissues of Douglas-fir and is implicated in defence. Both synthesis and activity in response to infection by the fungal pathogens *Armillaria ostoyae* and *Phellinus weirii* have been observed (Robinson *et al*. 2000; Zamani *et al*. 2003).

All three types of chitinolytic activity, i.e. endochitinase, chitobiosidase and β-N-acetylglucosaminidase, were present in the Douglas-fir ovular secretion at physiological pH. Endochitinases randomly cleave chitin at internal points in the chain. Chitobiosidases and β-N-acetylglucosaminidases cleave chitin at the non-reducing end of the chain and are classified as exochitinases. Endochitinases and exochitinases act synergistically to inhibit fungal growth (Harman *et al*. 1993; Lorito *et al*. 1993; Bolar *et al*. 2001). When endochitinases digest chitin, they increase the availability of non-reducing ends of the chitin chain for exochitinases to act on (Bolar *et al*. 2001). The presence of all three types of chitinolytic activity in the Douglas-fir drop, therefore, maximizes the efficiency of chitin degradation. This, in turn, optimizes antifungal activities of the drop. The use of a synergistic combination of chitinases may also benefit ovule development. Endochitinase expression is negatively correlated with plant growth in transgenic *Malus* × *domestica*, while exochitinase expression has no effect. High levels of disease resistance with adequate plant vigor were achieved when endochitinase expression was low and exochitinase expression was high (Bolar *et al*. 2001). If endochitinase is similarly vigor-reducing in Douglas-fir, it would be advantageous

to have both endo- and exochitinase activity in the drop so that endochitinase activity could be minimized without compromising antifungal activity.

The chitinases found in the Douglas-fir ovular secretion may have additional functions. Plant cells can perceive chitooligosaccharides released by chitinases (Wan *et al*. 2008). The chitooligosaccharides serve as signal molecules that activate defence responses, but they can also elicit other plant cell responses, e.g. oxidative burst, phosphorylation of specific proteins, phytoalexin biosynthesis and cell division activation. Receptors responsible for detection of the chitooligosaccharides are located in the plasmalemma (Kasprzewska 2003). These receptors share similarities to Nod factor receptors, suggesting an evolutionary relationship between chitin and Nod factor signalling pathways (Wan *et al*. 2008). The chitinases in the Douglas-fir ovular secretion may generate signal molecules that are involved in pollen-ovule interactions. It has been similarly proposed that chitinases in the analogous stigma exudate in angiosperms may generate signals involved in pollen tube growth (Rejón *et al*. 2014). In support of this possibility, it has been observed that chitinase activity in the stigma increases after anther dehiscence (Leung 1992). In addition to the chitin in fungal cell walls, extracellular chitinases can also act on arabinogalactan proteins (Rejón *et al*. 2013). *In vitro* studies of *Pinus caribaea* suggest that signal molecules are generated by chitinase-mediated catabolism of arabinogalactan proteins (Domon *et al*. 2000). To date, arabinogalactan proteins have not been identified in the Douglas-fir ovular secretion. However, they have been identified

in conifer ovular secretions, e.g. *Taxus* × *media* and the closely related *Larix* × *marschlinsii* (O'Leary 2004). Further research is necessary to determine if there is a signalling role for chitinases in the conifer ovular secretion.

Apoplastic chitinases are also known to have antifreeze activity. Freezing-tolerant plants survive subzero temperatures by forming ice within their tissues in a controlled manner. No ice forms within the cells; intracellular ice is lethal. Instead, ice formation is initiated in the apoplast by heterogeneous ice nucleators. Freezing injury can arise from cellular dehydration if intracellular water is lost to the growing extracellular ice. In many cold-tolerant plants, growth of extracellular ice is modulated by antifreeze proteins. These proteins adsorb onto the growing ice crystals and inhibit the binding of additional water molecules to the ice crystal lattice. At low antifreeze protein concentrations, inhibition of ice recrystallization is observed. During recrystallization, large ice crystals grow at the expense of smaller ones. Larger ice crystals are problematic because they are more likely to cause tissue damage. At higher antifreeze protein concentrations, thermal hysteresis is observed and the freezing point is depressed beyond what would be expected from colligative effects. Antifreeze proteins are also able to interact with ice nucleators, either inhibiting or enhancing ice nucleation (Hon *et al*. 1995; Griffith *et al*. 1997; Griffith and Yaish 2004). Most antifreeze proteins are homologous to pathogenesis-related proteins, including chitinases (Hon *et al*. 1995; Griffith and Yaish 2004). An apoplastic chitinase with antifreeze protein activity has been isolated from Douglas-fir needle tissue (Zamani *et al*. 2003). It

is possible that the apoplastic chitinases found in the Douglas-fir ovular secretion also have antifreeze activity. Frost can occur during the pollination period, leaving young ovular and pollen tube tissue vulnerable to freezing injury. Further investigation is required.

The results of this research confirm that there is chitinase activity in the Douglas-fir post-pollination prefertilization drop. It is widely accepted that plant chitinases are antifungal (Grover 2012). The presence of functioning chitinases in the Douglas-fir drop therefore provides evidence for a defensive role for the conifer ovular secretion. All three modes of chitinolytic activity, i.e. endochitinase, chitobiosidase, and β-N-acetylglucosaminidase, were detected in the drop. The three modes act synergistically, suggesting that the drop is optimized to defend the ovule against phytopathogens. Chitinase is the second enzyme in the Douglas-fir post-pollination prefertilization drop to be confirmed functional. This second confirmation provides additional evidence that the role the ovular secretion plays in conifer reproduction needs to be re-evaluated.

Chapter 6

Immunolocalization of Thaumatin-like Proteins in the Nucellus of *Pseudotsuga menziesii* and *Taxus* × *media*

Introduction

Members of the PR-5 family of pathogenesis-related proteins have been identified in the pollination drops of conifers (Poulis 2004; O'Leary *et al.* 2007; Wagner *et al.* 2007; Pirone-Davis *et al.* 2016). The PR-5 family of proteins includes, among others, osmotins, permatins, allergenic proteins and thaumatin-like proteins (Liu *et al*. 2010a). Proteins in this family are grouped together because they share significant sequence homology with thaumatin, a sweet-tasting protein found in the fruit of *Thaumatococcus daniellii* (Selitrennikoff 2001). Because of their sequence similarity to thaumatin, PR-5 proteins are often referred to as thaumatin-like proteins, even though none have been found to have a sweet taste (Velazhahan *et al*. 1999). In this chapter, the PR-5 family will be referred to as thaumatin-like proteins.

Thaumatin-like proteins have been found in bryophytes (Lehtonen *et al*. 2014), lycophytes (Petre *et al*. 2011), gymnosperms (e.g. O'Leary *et al*. 2007; Wagner *et al*. 2007; Liu *et al*. 2010b), and angiosperms (e.g. Abad *et al*. 1996; Misra *et al*. 2016). They have been detected in tissues of both roots and shoots. All plant tissues are capable of synthesizing thaumatin-like proteins, depending on factors such as a plant's age, its exposure to elicitors, and a range of

developmental cues (Velazhahan *et al*. 1999). Thaumatin-like proteins can accumulate to high levels (up to 12 %) in plant tissue (Velazhahan *et al*. 1999). They are generally highly soluble proteins and can be found in both the symplast and the apoplast (Velazhahan *et al*. 1999).

Thaumatin-like proteins are low molecular-weight proteins that are divided into two size classes. Members of the larger size class range from 201 to 229 amino acids with molecular masses from 22 to 26 kDa. They have 16 conserved cysteine residues. The smaller size class of thaumatin-like proteins have an internal deletion of 58 amino acids. These smaller thaumatin-like proteins range in size from 148 to 151 amino acids and have a molecular mass of around 16 kDa. Ten cysteine residues are found at conserved positions (Velazhahan *et al*. 1999; Liu *et al*. 2010a). The conserved cysteines mediate intra-molecular disulfide bonds that impart a stability to the folded protein, thereby conferring resistance both to proteolytic degradation and to denaturation at extreme pH and temperature (Liu *et al*. 2010a).

Antifungal activity for thaumatin-like proteins has been well documented. Zeamatin, isolated from maize seeds, was the first thaumatin-like protein found to have antifungal activity (Roberts and Selitrennikoff 1990). Since then, antifungal activity has been demonstrated *in vitro* for thaumatin-like proteins found in numerous plant species (e.g. Hejgaard *et al*. 1991; Vigers *et al*. 1992; Hu and Reddy 1997; Jayaraj *et al*. 2004). Enhanced resistance to phytopathogens has also been experimentally confirmed in transgenic plants expressing thaumatin-like

proteins (e.g. Velazahan *et al*. 1999; Singh *et al*. 2013; Misra *et al*. 2016). As a result of thaumatin-like protein activity, fungal spore and hyphal lysis as well as inhibition of spore germination and hyphal extension have been observed (Batalia *et al*. 1996; Velazhahan *et al*. 1999). Thaumatin-like proteins are known to be upregulated in response to fungal infection (van Loon *et al*. 2006). Expression of thaumatin-like protein can vary. For example, expression can be constitutive, it can occur in an organ-specific manner, or it can be developmentally regulated (Liu *et al*. 2010a).

The mechanism of antifungal activity of thaumatin-like proteins has not been elucidated. Some studies have demonstrated that thaumatin-like proteins permeabilize fungal membranes (Batalia *et al*. 1996; de Freitas *et al*. 2011). Other studies have demonstrated that thaumatin-like proteins bind to β-1,3-glucan and can display β-1,3-glucanase activity (Trudel *et al*. 1998; Grenier *et al*. 1999; Osmond *et al*. 2001). β-1,3-glucans are a major component of fungal cell walls. However, hydrolysis of β-1,3-glucans is not, in itself, sufficient for antifungal activity (Barre *et al*. 2000; Menu-Bouaouiche *et al*. 2003). Instead, it has been suggested that thaumatin-like protein interaction with β-1,3-glucans may act as a precursor to membrane permeabilization (Abad *et al*. 1996; Trudel *et al*. 1998; Ibeas *et al*. 2000; Ramos *et al*. 2015). It may also contribute to fungal target specificity (Ibeas *et al*. 2000). An acidic cleft is present in thaumatin-like proteins with antifungal activity. The cleft contains a conserved region, suggesting this area is involved with function (Koiwa *et al*. 1999; Osmond *et al*. 2001).

Molecular models indicate that the cleft could bind β-1,3-glucans (Osmond *et al*. 2001), but this possibility has not been confirmed. Alternatively, residues in the cleft may interact electrostatically with membrane proteins such as ion or water channels, altering the flow of water or ions across the membrane (Batalia *et al*. 1996; Ramos *et al*. 2015).

Thaumatin-like proteins can also play a role in a plant's response to abiotic stress. Expression of some members of the family is induced by osmotic stress (Velazahan *et al*. 1999; Liu *et al*. 2010a). One of the earliest thaumatin-like proteins to be discovered was named osmotin because it accumulates in cells undergoing gradual osmotic adjustment to either salt or desiccation stress (Singh *et al*. 1987). Transgenic plants expressing thaumatin-like proteins such as osmotin display tolerance to drought and salt stress (Velazahan *et al*. 1999; Anil Kumar *et al*. 2015). Thaumatin-like proteins also accumulate in response to cold stress (Liu *et al*. 2010a; Anil Kumar *et al*. 2015). They have been shown to have a cryoprotective effect *in vitro* (Newton and Duman 2000) and to enhance cold tolerance in transgenic plants (Anil Kumar *et al*. 2015). Additionally, two apoplastic thaumatin-like proteins have been shown to function as antifreeze proteins in cold-acclimated winter rye (Hon *et al*. 1995).

Thaumatin-like proteins are present in the ovular secretions of Douglas-fir (*Pseudotsuga menziesii*) and hybrid yew (*Taxus* × *media*): one protein has been identified in the Douglas-fir post-pollination prefertilization drop (Poulis 2004) and eight have been identified in the yew pollination drop (O'Leary *et al*. 2007;

Prior 2014). Immunolocalization studies have shown that the thaumatin-like proteins found in the ovular secretion originated in the nucellus (Poulis 2004). The aim of this research was to characterize the subcellular localization of thaumatin-like protein within the nucellus of Douglas-fir and yew.

Methods and Materials

Plant Material

Pseudotsuga menziesii (Mirbel) Franco (Douglas-fir) cones were collected from trees on the campus of the University of Victoria (Victoria, British Columbia, Canada). Cone collection was timed to coincide with post-pollination prefertilization drop formation, which occurs 6-10 weeks after pollination. Ovules exuding drops were excised and immediately fixed in sodium cacodylate buffer containing 3 % (v/v) glutaraldehyde and 3 % (v/v) formaldehyde (Karnovsky 1965) and stored at 4 °C.

Taxus × *media* Rehder (hybrid yew) branches were removed from trees on the campus of the University of Victoria (Victoria, British Columbia, Canada). Branch removal was timed to coincide with pollination drop formation. Ovules exuding drops were excised and immediately fixed as described above.

Sample Preparation

Douglas-fir and yew ovules in fixative were warmed to room temperature and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer for 60 min.

They were then washed three times for 10 min each with 0.1 M cacodylate buffer before being placed in 50 % ethanol for 60 min followed by block-staining in 5 % uranyl acetate in 50 % ethanol for 60 min. Ovules were next dehydrated in a graded ethanol series and washed twice with propylene oxide for 10 min each. Ovules were infiltrated first with a mixture of propylene oxide and Epon plastic and then twice with pure Epon. Ovules were then embedded in Epon. Ultrathin sections were cut and mounted on formvar/carbon-coated nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA).

Immunolocalization of Thaumatin-like Protein in Douglas-fir and Yew

Sections were incubated with 1 % (w/v) ovalbumin (Sigma-Aldrich, Oakville, ON, CAN) in phosphate buffered saline (PBS) for 10 min and then incubated with the primary antibody, TLP1, diluted 1:200 in 1 % (w/v) ovalbumin in PBS for 60 min. TLP1 was produced in rabbit against recombinant thaumatin-like protein (Dafoe *et al*. 2009) and was kindly supplied by C. Peter Constabel (University of Victoria, BC, CAN). Sections were then washed with 1 % (w/v) ovalbumin in PBS three times (10 min each for Douglas-fir, 5 min each for yew) and incubated with the secondary antibody, 12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., PA, USA), diluted 1:50 in 1 % (w/v) ovalbumin in PBS for 60 min. Sections were washed with 1 % (w/v) ovalbumin in PBS three times (10 min each for Douglas-fir, 5 min each for yew) and then washed four times with deionized

water for one min. Sections were stained with 5 % (w/v) uranyl acetate in 50 % (v/v) ethanol for 10 min, washed four times with deionized water for 1 min, stained with 5 % (w/v) lead citrate for 4 min, and washed four times with deionized water for 1 min. Controls included omitting the primary antibody or incubating the sections with preimmune serum instead of primary antibody. Sections were viewed with a Hitachi H-7000 transmission electron microscope and images were taken with a digital camera (Advanced Microscopy Techniques, Corp., MA, USA).

Immunolocalization of Callose in Yew

The procedure was as described for the immunolocalization of thaumatin-like protein, but with the following exceptions. The primary antibody used was an anti-(1,3)-β-glucan mouse monoclonal IgG (Biosupplies Australia Pty. Ltd., VI, AUS) and was diluted 1:600 in 1 % (w/v) ovalbumin in PBS. The secondary antibody was 18 nm Colloidal Gold AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., PA, USA) and was diluted 1:100 in 1 % (w/v) ovalbumin in PBS. Washes with 1 % (w/v) ovalbumin in PBS were for 10 min each. The primary antibody was omitted as a control.

Results and Discussion

Immunogold label corresponding to thaumatin-like protein was observed in the apoplast and symplast of Douglas-fir and yew nucellar tissue. In

Douglas-fir, the label was associated with the cell wall and with starch granules (Figure 6). Label was similarly associated with the cell wall and starch granules in yew. Additionally, in yew, label was associated with ingrowths of the cell wall (Figure 7).

Yew ovules were collected from plants growing outdoors and were therefore in an unsterile environment. By chance, fungal spores were observed in the micropyle at the apical end of the nucellus of one yew ovule. Immunogold label corresponding to thaumatin-like protein antiserum was observed in the cell walls of the spores and germinating hyphae (Figure 8).

Immunolocalization with an anti-1,3-β-glucan antibody was carried out to investigate the composition of the cell wall ingrowths observed in yew nucellar tissue. Immunogold label was observed in the ingrowths. It was not observed in the cell wall. Sections from the ovule with observed fungal spores were included in the analysis. Fungal cell walls contain β-1,3-glucans (Ruiz-Herrera and Ortiz-Castellanos 2019). As expected, label was observed in the cell walls of the spores and geminating hyphae (Figure 9).

The ovular secretion originates in the nucellus (Nepi *et al*. 2009). The thaumatin-like proteins localized to the cell walls of the nucellus are, therefore, most likely part of the secretory pathway for the thaumatin-like proteins found in the ovular secretion. Antifungal activity for thaumatin-like proteins has been well documented (e.g. Roberts and Selitrennikoff 1990; Hu and Reddy 1997; Misra *et al*. 2016). It has been proposed that the thaumatin-like proteins found in the

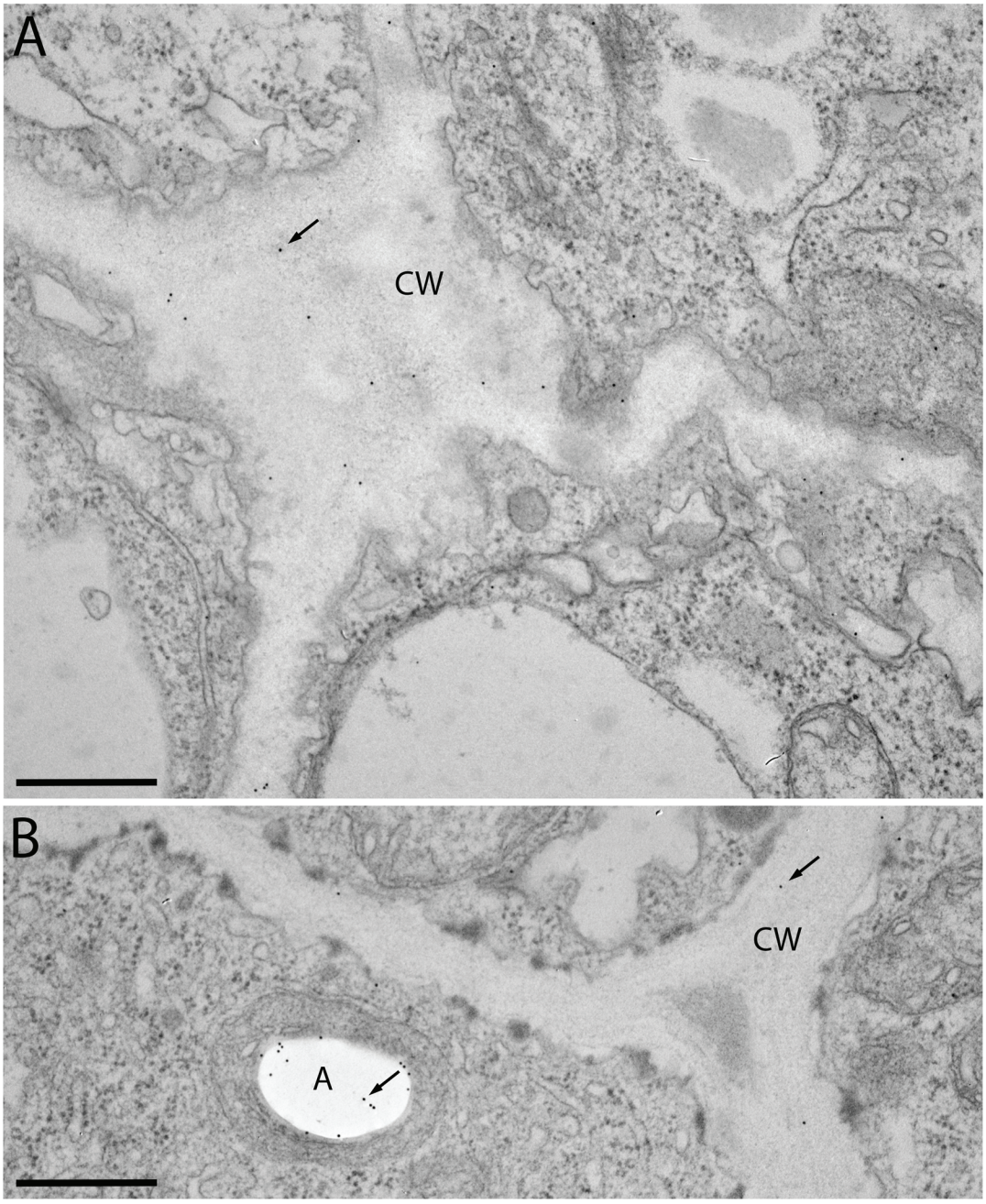

Figure 6. Transmission electron micrographs showing immunolocalization of thaumatin-like protein in the *Pseudotsuga menziesii* nucellus at the time of post-pollination prefertilization drop production. Immunogold label (arrow) was observed in the cell walls **(A, B)** and in amyloplasts **(B)**. Scale bars = 500 nm. CW, cell wall; A, amyloplast.

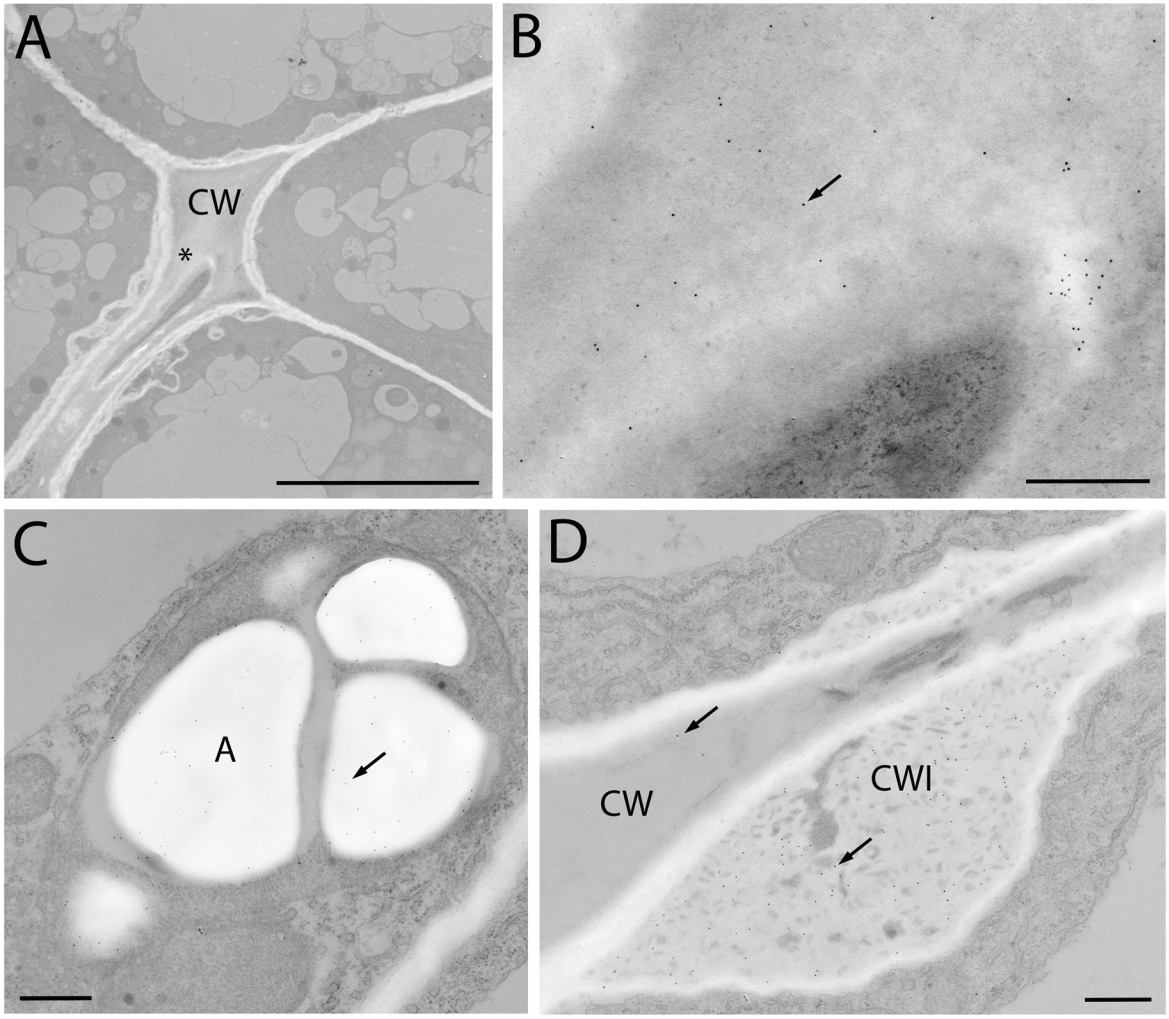

Figure 7. Transmission electron micrographs showing the *Taxus* × *media* nucellus at the time of pollination drop production. **(A)** Nucellus cells at lower magnification. Asterisk indicates area magnified in (B). Scale bar = 10 µm. **(B-D)** Immunolocalization of thaumatin-like protein. Scale bars = 500 nm. Immunogold label (arrow) was observed in the cell walls (B, D), in amyloplasts (C), and in ingrowths of the cell wall (D). CW, cell wall; A, amyloplast; CWI, cell wall ingrowth.

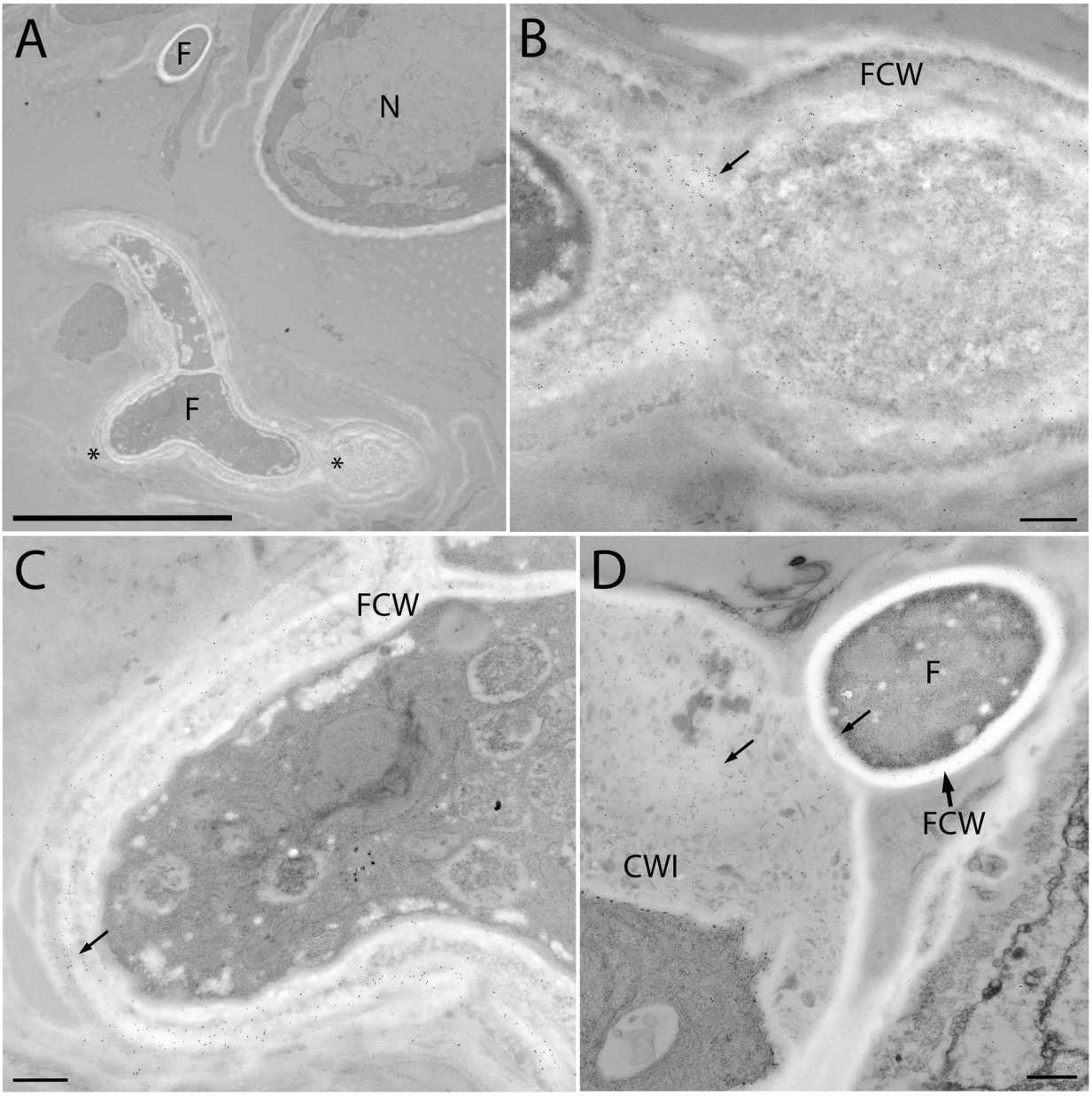

Figure 8. Transmission electron micrographs showing fungal spores in the micropyle of *Taxus* × *media* near the apical end of the nucellus at the time of pollination drop production. **(A)** Fungal spores at lower magnification. Asterisks indicate areas magnified in (B) and (C). Scale bar = 10 µm. **(B-D)** Immunolocalization of thaumatin-like protein. Scale bars = 500 nm. Immunogold label (thin arrow) was observed in fungal cell walls (B, C, D) and in cell wall ingrowths in the *T.* × *media* nucellus (D). F, fungal spore; N, nucellus cell; FCW, fungal cell wall; CWI, cell wall ingrowth.

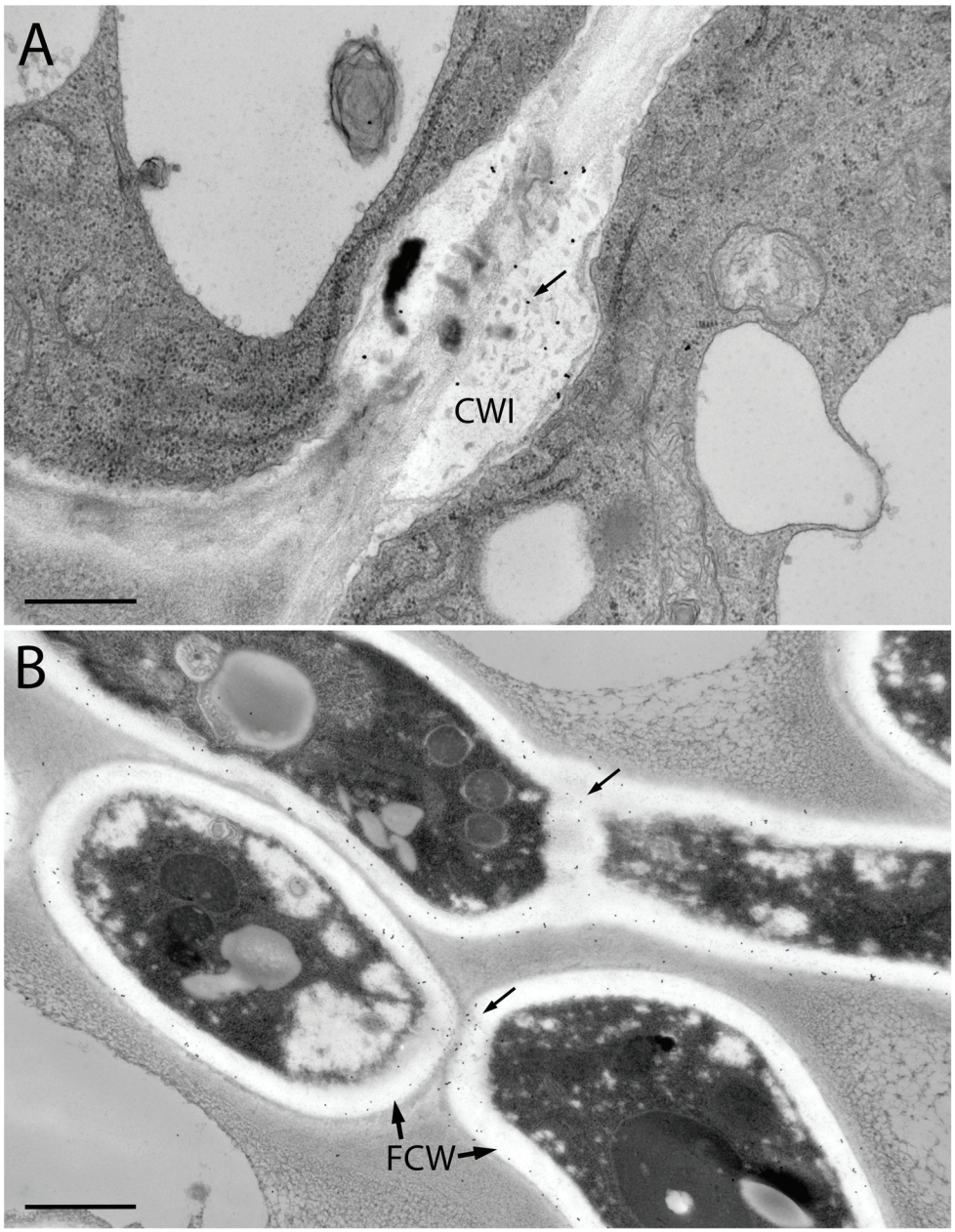

Figure 9. Transmission electron micrographs showing imunolocalization of callose in the *Taxus* × *media* ovule at the time of pollination drop production. **(A)** Immunogold label (thin arrow) was observed in cell wall ingrowths in the nucellus. Scale bar = 0.5 μm. **(B)** Fungal spores were observed in the micropyle near the apical end of the nucellus. Immunogold label (thin arrow) was observed in fungal cell walls. Scale bar = 1.0 μm. CWI, cell wall ingrowth; FCW, fungal cell wall.

ovular secretion protect the ovule from infection by phytopathogens (Poulis 2004; O'Leary *et al*. 2007; Prior 2014). The cell wall plays a role in plant immunity (Malinovsky *et al*. 2014). Thaumatin-like protein found in the cell walls of nucellar tissue likely also protects the ovule from infection. The presence of these proteins in the cell walls would create a continuous line of defence throughout the apoplast of the nucellus. Fungal pathogens not neutralized by the initial line of protection, the ovular secretion, would encounter this second line of protection when attempting to penetrate nucellar tissue. Conifer ovules are exposed, leaving them vulnerable to infection during pollen receptivity. This vulnerability is highlighted by the observation of fungal spores in the micropyle of one yew ovule. Yet the micropyle and nucellus of most ovules are very clean and infection of the ovule is rarely observed. A system to maintain asepsis must be in place (Gelbart and von Aderkas 2002; O'Leary *et al*. 2007). Localization of thaumatin-like protein to the cell walls of fungal spores and germinating hyphae that were serendipitously found in one yew ovule supports a defensive role for thaumatin-like protein in the ovular secretion and nucellar tissue.

Thaumatin-like proteins contain an N-terminal signal peptide targeting them into the secretory pathway. They are most commonly found in the cell wall and in apoplastic fluids (van Loon *et al*. 2006; Liu *et al*. 2010a). Localization of thaumatin-like protein to amyloplasts was therefore not anticipated. However, there is precedence for the localization of thaumatin-like proteins in this organelle (Jeun and Buchenauer 2001; Dafoe *et al*. 2010). In tomato, the thaumatin-like

protein AP24 was found to accumulate in starch granules. Accumulation was observed both after systemic acquired resistance was induced and after plants were inoculated with *Phytophthora infestans*, suggesting a role in defence (Jeun and Buchenauer 2001). A similar role in defence was also hypothesized for thaumatin-like proteins localized in poplar amyloplasts (Dafoe *et al*. 2010).

Some specialization of cell wall morphology appears to occur in yew. Thaumatin-like protein was localized to cell wall ingrowths. Such wall ingrowths are known to occur in transfer cells and to develop in response to fungal infection (Offler *et al*. 2003; Chowdhury *et al*. 2014). In transfer cells, the wall ingrowths enhance plasma membrane transport capacity and develop where a high rate of transport across the apo-/symplast boundary occurs (Offler *et al*. 2003; McCurdy *et al*. 2008). In response to fungal infection, dome-shaped appositions, often referred to as papillae, form to reinforce the secondary cell wall as a way to prevent fungal penetration (Underwood 2012; Chowdhury *et al*. 2014). The β-1,3-glucan callose is found in both transfer cell ingrowths and in papillae (Offler *et al*. 2003; Underwood 2012; Chowdhury *et al*. 2014). Immunogold labelling revealed that callose occurs in the yew nucellar tissue wall ingrowths, suggesting that these ingrowths could be part of a transfer cell or they could be papillae. However, transfer cells in the nucellus are only known in wheat, barley, and Japanese millet, all monocots and members of the Poaceae. Additionally, the morphology of the ingrowths observed in yew does not match the branching morphology of the ingrowths found in nucellar transfer cells in these monocots

(Zee and O'Brien 1971; Cochrane and Duffus 1980; Wang *et al*. 1994). It is more probable that these ingrowths are papillae. Accumulation of thaumatin-like protein has been observed in papillae (Jeun and Buchenauer 2001), and the dome-shaped morphology of the ingrowths observed in yew more closely matches that of papillae (Jeun and Buchenauer 2001; Chowdhury *et al*. 2014). Papillae form at the site of attempted fungal penetration, and they are usually confined to an infected cell and its immediate neighbours (Jeun and Buchenauer 2001; Bolwell *et al*. 2001). Fungal spores were observed adjacent to cell wall ingrowths (Figure 8D). However, further, controlled experimentation is required before a conclusion can be drawn.

Thaumatin-like proteins have been shown to provide multi-stress tolerance to plants (Singh *et al*. 2013; Chowdhury *et al*. 2017). In addition to being antifungal, they can provide protection from abiotic stress. They accumulate in response to osmotic and cold stress and can function as antifreeze proteins (Hon *et al*. 1995; Velazahan *et al*. 1999; Liu *et al*. 2010a; Anil Kumar *et al*. 2015). Cold stress, in particular, is a threat to young ovular and pollen tube tissue. These tissues develop in the spring when male and female buds have lost any cold acclimation. Frost can occur during the pollination period of both Douglas-fir and yew, leaving these tissues vulnerable to freezing injury. Sugars and amino acids present in the ovular secretion of these species will depress the freezing point (Coulter 2005; Nepi *et al*. 2017). However, further investigation to see if

thaumatin-like proteins in the ovular secretion and nucellar tissue confer cold tolerance or function as antifreeze proteins is warranted.

Thaumatin-like protein was immunolocalized to the cell wall and amyoplasts in Douglas-fir and yew nucellar tissue in a pattern consistent with a defensive role. It was also localized to the cell wall of fungal spores and germinating hyphae that were present in the micropyle of a yew ovule. These results provide evidence for an antifungal role for thaumatin-like protein in the ovule and ovular secretion of Douglas-fir and yew. Thaumatin-like proteins have been found in the ovular secretion of other conifer species (O'Leary *et al*. 2007; Wagner *et al.* 2007; Pirone-Davis *et al.* 2016), suggesting a more widespread role in conifer ovule defence for these proteins.

Chapter 7

Re-examining the Ovular Secretion

Portions of this chapter have been published as Coulter A, Poulis BAD, von Aderkas P. 2012. Pollination drops as dynamic apoplastic secretions. Flora. 207:482-490.

The ovular secretion has historically been viewed through the lens of pollination mechanisms. There has been a lack of curiosity about the biochemical composition of the drop. Logistical challenges are certainly among the reasons why: the secreted volume is very small, the secretion window is of a short duration, and ovules are often difficult to access. However, it may also be due, in part, to the widely held view that selection in conifers is postzygotic (Williams 2009). Prefertilization mechanisms known from angiosperms, such as the self-incompatibility system, are not thought to be present in conifers. Instead, an embryo-lethal system is hypothesized to promote outcrossing, following the observation of a high rate of rapid embryo death in self-fertilized zygotes (Williams *et al*. 2001). The focus on post-zygotic selection has, it seems, resulted in a lack of exploration into conifer pollen-ovule interactions. However, the presence of robust postzygotic selection does not mean the absence of prezygotic selection. In fact, it has been demonstrated that selection occurs at the stage of pollen germination in Douglas-fir (*Pseudotsuga menziesii*) and hybrid larch (*Larix* × *marschlinsii*). More importantly, this selection occurs when the pollen is immersed in the post-pollination prefertilization drop (von Aderkas *et al*. 2012).

A closer look at prezygotic events and the role the ovular secretion plays in conifer reproduction is warranted.

Modern technology has broadened our ability to explore the prezygotic reproductive biochemistry of conifers. Mass-spectrometry based proteomics has enabled investigation of the ovular secretion proteome, and has revealed that the conifer ovular secretion is rich in proteins (Prior *et al*. 2013). It is only possible, however, to attach a putative function to an identified protein. Functional studies are required to confirm that a protein confers function on the ovular secretion as anticipated. The research outlined in this dissertation describes the first functional characterizations of proteins found in conifer ovular secretions. Three proteins - invertase, chitinase, and thaumatin-like protein - were characterized. Each was found to be functional. The presence of functional invertase, an enzyme that cleaves sucrose into fructose and glucose, demonstrates that drop composition is not static. Post-secretory regulation of the ovular secretion has not been previously described. Functioning invertases likely facilitate the movement of carbohydrates to developing pollen and could facilitate pollen selection. In other words, invertases present in the ovular secretion are likely involved in pollen-ovule interactions. Chitinases and thaumatin-like proteins are pathogenesis-related proteins that protect plants from infection by phytopathogens. Functioning pathogenesis-related proteins in the ovular secretion suggests the secretion is involved in ovule defence.

It is anticipated that more proteins present in conifer ovular secretions will be found to be functional. The putative functions associated with proteins identified to date are numerous, but, upon closer examination, they can also be grouped into the two categories of pollen-ovule interactions and ovule defence (Table 4). A functional model for the conifer ovular secretion is proposed here (Figure 10). The model is based on the findings outlined in this dissertation, on the putative functions of proteins identified in conifer ovular secretions to date, and on the functions seen in analogous and homologous secretions into the angiosperm apoplast. It proposes that the conifer ovular secretion is involved in pollen-ovule interactions and in ovule defence.

A Model for Ovular Secretion Function

Pollen-Ovule Interactions

The ovular secretion provides a medium for pollen germination (Gelbart and von Aderkas 2002). Pollen selection has been demonstrated at the stage of pollen germination, when the pollen is immersed in the ovular secretion (von Aderkas *et al*. 2012), suggesting the secretion may also support pollen selection. Species-specific profiles of sugars and amino acids in the secretion (Nepi *et al*. 2017) may contribute to pollen selection by providing nutrients and/or an osmolarity best suited to homospecific pollen. Catabolic proteins, such as invertase, that provide post-secretory regulation of secretion composition could contribute to profile maintenance. Peroxidases are present in conifer ovular

Table 4. Putative functions for proteins identified in conifer ovular secretions can be grouped into two functional categories. Adapted from Coulter *et al*. (2012).

Functional category	Putative function	Protein	Reference
Pollen-ovule interactions	Pollen selection	Invertase	von Aderkas *et al*. 2012
		Peroxidase	Poulis *et al*. 2005; McInnis *et al*. 2006a
	Pollen tube growth	β-D-glucan exohydrolase	Wagner *et al*. 2007
		Glucan 1,3-β-glucosidase	Wagner *et al*. 2007
		Glucan 1,3-β-glucosidase precursor	Wagner *et al*. 2007
		Glucan endo-1,3-β-glucosidase	Pirone-Davies *et al*. 2016
		Glycosyl hydrolase	Wagner *et al*. 2007
		Glucanase-like protein	Wagner *et al*. 2007
		Galactosidase	Poulis *et al*. 2005
		Xylosidase	Poulis *et al*. 2005
		Lipid-transfer protein	O'Leary 2004; Nieuwland *et al*. 2005
		Peroxidase	Passardi *et al*. 2005; McInnis *et al*. 2006b; Pirone-Davies *et al*. 2016
		Subtilisin-like proteinase	Wagner *et al*. 2007
		Aspartyl protease	Poulis *et al*. 2005
		Serine carboxypeptidase-like protein	Poulis *et al*. 2005
		Invertase	Poulis *et al*. 2005
	Pollen tube guidance	Arabinogalactan proteins	O'Leary *et al*. 2004
	Pollen tube penetration	Peroxidase	McInnis *et al*. 2006a
Ovule defence	Antimicrobial defence	Glucan endo-1,3-β-glucosidase	Pirone-Davies *et al*. 2016
		Chitinase	Wagner *et al*. 2007
		Thaumatin-like protein	O'Leary *et al*. 2007
		Peroxidase	Poulis *et al*. 2005; Pirone-Davies *et al*. 2016
		Xylosidase	Nepi *et al*. 2011; Nepi *et al*. 2012b
	Defence signalling	Subtilisin-like proteinase	Balakireva and Zamyatnin 2018
		Aspartyl protease	Balakireva and Zamyatnin 2018
		Serine carboxypeptidase-like protein	Balakireva and Zamyatnin 2018
		Peroxidase	McInnis *et al*. 2006b
	Antifreeze activity	Glucan endo-1,3-β-glucosidase	Griffith and Yaish 2004
		Chitinase	Griffith and Yaish 2004
		Thaumatin-like protein	Griffith and Yaish 2004

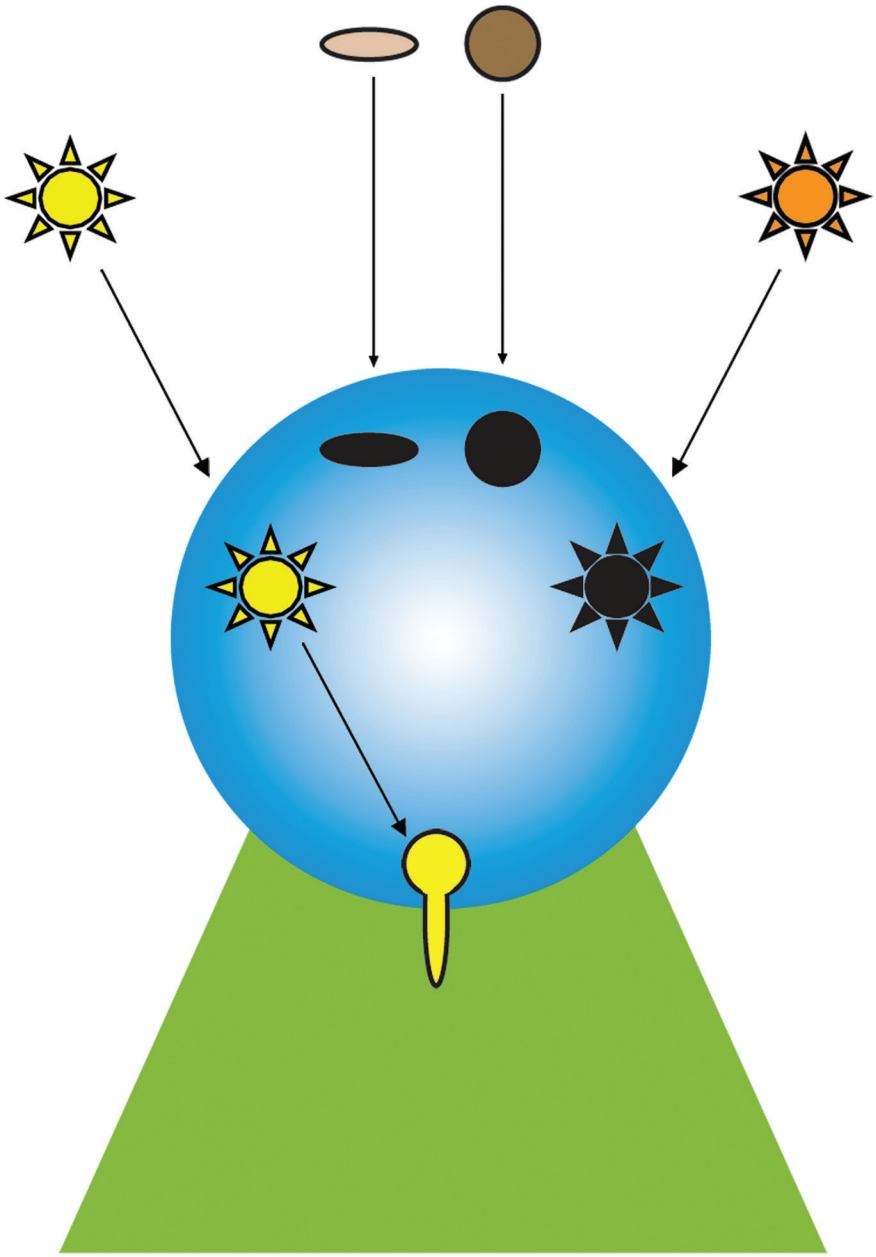

Figure 10. A model for conifer ovular secretion function. The ovular secretion (blue) supports the development of homospecific pollen (yellow) while selecting against heterospecific pollen (orange). Constituents of the secretion protect the ovule (green) and seed from infection by fungus (circle) and/or bacteria (ellipse). Adapted from Coulter et al. (2012).

secretions. It is proposed that the reactive oxygen species generated by these enzymes are involved in pollen selection at the stigma (McInnis *et al*. 2006a). They may act similarly in the ovular secretion.

The ovular secretion is required for pollen germination (Villar *et al*. 1984; Seridi-Benkaddour and Chesnoy 1988; Takaso and Owens 1996), just as the angiosperm stigma exudate is required for germination (Cheung 1996; Wolters-Arts *et al*. 1998; Lush *et al*. 2000). The ovular secretion and the stigmatic exudate are analogous. External calcium is required for pollen germination (Brewbaker and Kwack 1963; Picton and Steer 1983). Angiosperm pollen takes up calcium from the stigma exudate (Bednarska 1991). Conifer pollen may similarly take up calcium present in the ovular secretion (von Aderkas *et al*. 2012). The wide range of carbohydrates and amino acids found in the ovular secretion (Nepi *et al*. 2009; Nepi *et al*. 2017) may serve as a nutritive source for the growing pollen tube (Nygaard 1977). Nutrients may also be mobilized by enzymes that cleave carbohydrates and proteins (Table 4).

Cell wall elongation is required for pollen tube growth. The canonical proteins involved in this process are glycoside hydrolases and expansins. Glycoside hydrolases, such as galactosidases, xylosidases, and glucosidases, are present in ovular secretions (Table 4). Four of the most abundant proteins in the Douglas-fir ovular secretion are glycoside hydrolases (Poulis *et al*. 2005). To date, expansins have not been found in the ovular secretion. Proteins involved in cell wall elongation are similarly found in the analogous stigma exudate, and a

role in pollen tube elongation has been proposed (Pezzotti *et al*. 2002; Nieuwland *et al*. 2005; Rejón *et al*. 2013). One of the cell wall loosening proteins found in the stigma exudate, a lipid transfer protein, has also been identified in the ovular secretion (Table 4). Cell wall rigidity can be altered by reactive oxygen species generated by peroxidases (Passardi *et al*. 2005). Peroxidases in the ovular secretion (Table 4) could also facilitate pollen tube growth.

In common with most pollen, that of many conifers can germinate and grow *in vitro* without the addition of wall metabolizing enzymes (Vasil 1987). It is therefore unlikely that these enzymes are necessary for pollen tube development. It may be that the enzymes improve the growth rate, but this will need to be shown.

Developing angiosperm pollen tubes grow towards the eggs with the help of guidance cues from within the pistil (Cheung and Wu 2001). The chemotropic arabinogalactan proteins and calcium present in the stylar extracellular matrix are also found in the conifer ovular secretion (Fujii 1903; O'Leary *et al*. 2004; von Aderkas *et al*. 2012). Arabinogalactan proteins have been immunolocalized to the micropylar region of the nucellus (O'Leary *et al*. 2004). It is unknown if gradients of either arabinogalactan proteins or calcium exist within the ovule. Once at the nucellus, pollen tubes must penetrate nucellar tissue to reach the eggs. Reactive oxygen species generated by peroxidases at the angiosperm stigma may loosen cell wall components, allowing pollen tubes to penetrate (McInnis *et al*.

2006a). Peroxidases present in the ovular secretion may similarly assist with pollen tube penetration (Table 4).

Ovule Defence

A suite of defence proteins are found in the ovular secretion. Many of these proteins are pathogenesis-related proteins (Table 4). It is proposed that these proteins protect the ovule from microbial infection. Defence proteins keep the similarly vulnerable nectar, extrafloral nectar, and stigma exudates aseptic (Carter and Thornburg 2004a; Heil 2011; Rejón *et al*. 2013). The conifer ovule is vulnerable during the entire period of receptivity. There is no change in protein profile in the ovular secretion over the course of the secretory period (O'Leary 2004), suggesting a constitutive defence system is in place.

The ovular secretion appears to be optimized for defence. Pathogenesis-related proteins known to act synergistically (Mauch *et al*. 1988; Hejgaard *et al*. 1991; Broekaert *et al*. 2000) are simultaneously present in conifer ovular secretions (e.g. Poulis 2004; Wagner *et al*. 2007; Pirone-Davies *et al*. 2016). There are also proteins in the ovular secretion associated with defence signalling (Table 4). The elicitors produced by these enzymes may help to amplify the defence response during pathogen invasion.

Future Research

The ovular secretion is integral to conifer reproduction, yet much remains unknown. Future work needs to tease out the physical-chemical basis of pollen-ovule interactions and ovule defence in conifer ovular secretions. Cataloguing proteins in more conifer species is a first step. As we learn more about the constituents of the secretion, we will learn more about how the secretion may contribute to prezygotic events. We will also be able to see if there are any phylogenetic relationships in the secretion composition. Ovular secretions are observed in all extant gymnosperms (Prior *et al*. 2018), so there is an opportunity to consider secretion constituents from an evolutionary perspective. To date, proteomic analysis of ovular secretions has been directed towards protein discovery. Protein quantification is an obvious next step.

Systematic characterization of the small molecular weight compounds such as minerals, amino acids, and carbohydrates, would also be beneficial. Phosphate compounds, for example, are found to be in high concentrations in the *Taxus baccata* pollination drop (Ziegler 1959). Preliminary evidence indicates that extracellular ATP is present in the ovular secretions of all species tested by our lab (unpublished data). Calcium acts as a signalling molecule in many cellular processes (Aldon *et al*. 2018) and is essential for pollen germination (Brewbaker and Kwack 1963; Picton and Steer 1983). How common is it in ovular secretions? Peroxidases have been identified in ovular secretions. Reactive oxygen species generated by these proteins have been implicated in a number of pollen-ovule

interactions and in plant defence (Passardi *et al*. 2005). An investigation of the reactive oxygen species content of the secretion is warranted. Are lipids, phenolics, or volatile organic compounds present in the drop? There has been no attempt to characterize these to date.

Measuring the osmolarity and pH of ovular secretions is fundamentally important if we are to understand how the secretion functions. Are the conditions of the secretion amenable to biochemical reactions? Is there regulation of osmolarity and pH? Is a buffer system present? We do not know how the ovular secretion forms or recedes. An understanding of the osmotic potential of the drop is needed to expand our understanding of secretion formation and recession. Conifers in the Northern hemisphere frequently experience night temperatures below freezing during the pollination period. Knowledge about the osmotic potential of the drop will also help us understand if the ovular secretion leaves the ovule vulnerable to freezing injury. Some of the pathogenesis-related proteins found in the ovular secretion are known to also function as antifreeze proteins (Table 4; Griffith and Yaish 2004). The ovular secretion should be assayed for antifreeze protein activity.

Functional characterization of the ovular secretion constituents is needed in order to fully understand the role of the secretion in prezygotic events. However, drop volume remains a significant limitation. Towards that end, we have developed a system to culture the nucellus. The tissue readily secretes proteins into the culture medium, and the protein profile is consistent across

genotype (A. Coulter, unpublished data). The next step is to compare sequence data of the secreted proteins and the ovular secretion proteins to see if this system is a way to scale-up the amount of protein available for experimentation. Assays such as fungal growth inhibition trials become feasible when more protein is available.

Concluding Remarks

Research into the function of ovular secretions has historically focused on pollination mechanisms. This focus has limited the interpretation of ovular secretion function and ignored the chemical complexity of the secretion. When the ovular secretion is compared to secretions into the angiosperm apoplast, it becomes clear that a revised model for ovular secretion function is appropriate. The experimental results presented in this dissertation show that the ovular secretion contains functioning enzymes. This discovery further supports a revised model. The ovular secretion has been demonstrated to be a dynamic secretion, capable of responding to the surrounding environment. Conifers may lack the showy flowers and the biotic pollinators of the angiosperms, but their reproductive systems are by no means more passive or less intriguing.

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Appendix A

The Conifer Ovule

The conifer ovule is comprised of three parts: the integument, the nucellus, and the megagametophyte (Figure 11). The integument and nucellus are diploid whereas the megagametophyte is haploid. The integument forms the outer, protective layer of the ovule. An opening in the integument at the apex of the ovule, termed a micropyle, allows the male gametes to enter the ovule. The nucellus produces the megagametophyte. A cell within the nucellus, the megasporocyte (or megaspore mother cell), undergoes meiosis. Three of the four resulting haploid cells degenerate. The surviving cell, the megaspore, undergoes mitosis to produce the megagametophyte. Multiple archegonia develop within the megagametophyte. Each archegonium contains one egg cell.

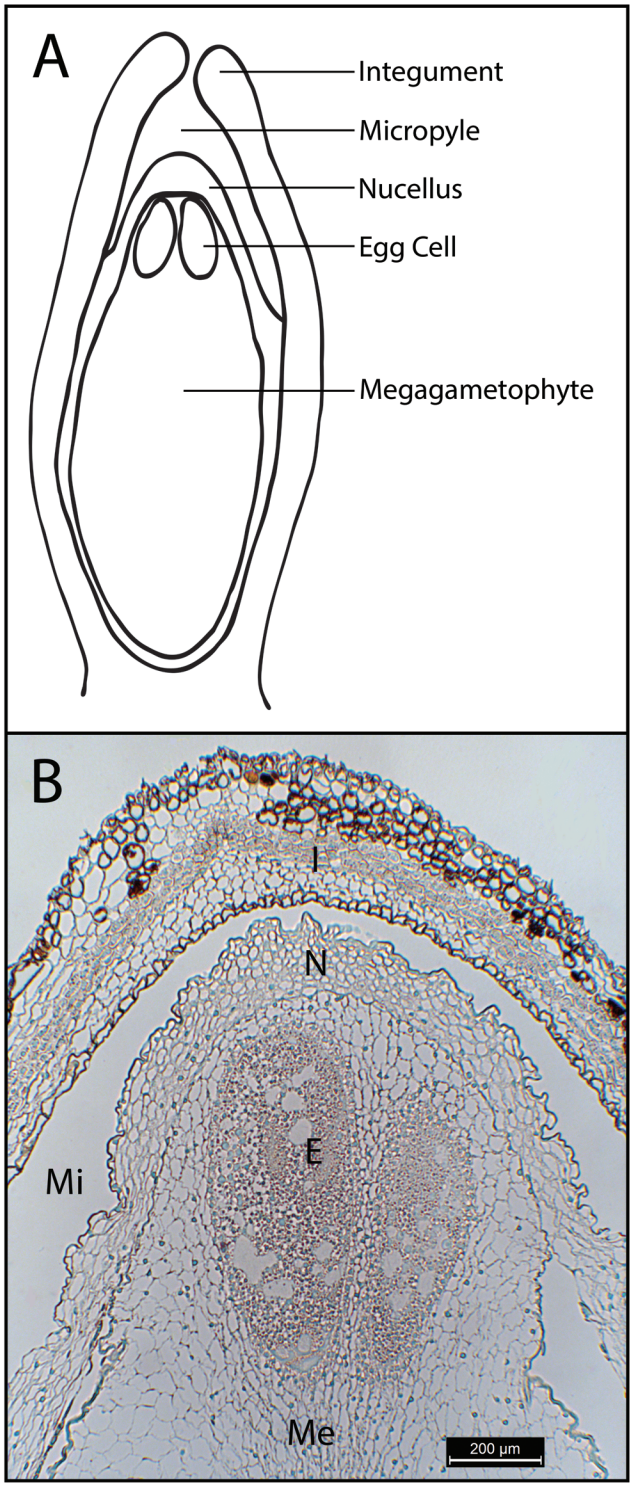

Figure 11. The conifer ovule. **(A)** Schematic of a longitudinal section of an ovule. **(B)** Slightly oblique longitudinal section of a *Pseudotsuga menziesii* ovule. I, integument; Mi, micropyle; N, nucellus; E, egg cell, Me, megametophyte.