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SEED LIFE EXPECTANCY:

THE SPERMOSPHERE, DEFENSE CHEMISTRY, AND WEED RECRUITMENT

A Thesis in

Agronomy

by

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ABSTRACT

Plant seeds may remain dormant in the soil for extended periods of time, creating soil seed banks that contribute to the perpetuation of plant species. Weed seed banks can create weed management challenges for farmers, and reduction of these seed banks could become an important piece of an effective weed management strategy. A better understanding of the seed-soil-microbe interactions in the soil could help develop effective seed bank management tools. This study focuses on phenolic acid, short aliphatic organic acid, and long-chain fatty acid changes within dormant wild oat (*Avena fatua* L.) seeds and the surrounding soil (spermosphere) as the seed imbibes, and on seed defense mechanisms used to protect against harmful soil microorganisms. It is hypothesized that during imbibition, the seed will experience a loss of compounds from soluble chemical fractions, and this loss will be quantifiable in the seed and surrounding soil. In addition, it is hypothesized that during fungal attack, the seed will elevate the levels of defense compounds, especially those bound to the cell wall. During imbibition, wild oat hull chemical composition and short aliphatic concentration were most affected, with soluble short aliphatic concentration decreasing 69% in the hulls and 62% in the caryopses. Free phenolic concentration decreased 77% in concentration in the hulls. The spermosphere is likely composed of phenolics and short aliphatics, which may have varying effects on the surrounding soil ecology. When exposed for 3d to *Fusarium avenaceum* and *F. culmorum*, the greatest defense response observed in wild oat seeds was a substantial accumulation (40%-650%) of soluble-fraction phenolics in the caryopses. Soluble-fraction short aliphatics also increased substantially (up to 1800%) in caryopses. Some increase in phenolic concentration occurred in the seed hulls. Insoluble cell wall-bound fractions exhibited no change, and treatments at little effect on long-chain fatty acid concentration. Implications for seed survival in the soil and potential management options are considered.

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Chapter 1: INTRODUCTION

Weed Seed Banks and the Spermosphere

Wild oat (*Avena fatua* L.) is a prevalent weed globally, with the potential to cause significant losses in the yield of many crops. Cultivated oat (*Avena sativa* L.), one of the most important cereal grains worldwide (USDA, 2003), is particularly susceptible to yield reduction by wild oat due to the difficulty in controlling wild oat alone. Oat crop yield may be reduced by up to 70% when wild oat density is high (Willenborg *et al*., 2005). Other small grain crops, such as wheat and barely, are also highly susceptible to yield reductions by wild oat. Kirkland (1993) found that spring wheat yields were reduced by 28% and 39% at wild oat densities of 64 and 118 plants m^{-2}, respectively. Barley yields may also be significantly reduced by the weed (Scursoni and Satorre, 2005; O'Donovan *et al*., 2000).

Herbicides are commonly used to control wild oat. Triallate, a thiocarbamate pre-emergence herbicide, was one of the initial chemical controls used against the weed. However, as triallate resistance became a prevalent issue, acetyl-CoA carboxylase (ACCase) and acetolacetate synthase (ALS) inhibitors became more widely used (Beckie *et al*., 1999; Beckie *et al*., 2008), as well as some other alternatives (Blackshaw *et al*., 1996). More recently, ACCase inhibitor-resistant populations are beginning to become more widespread (Legere *et al*., 2000; Beckie *et al*., 2008).

In light of these emerging problems, an increasing number of studies have investigated non-chemical management options. Harker *et al*. (2003) showed that early harvest of silage barley reduces wild oat density over time. Similarly, Shirtliffe *et al*. (2000) suggested changing the timing of wheat harvest to reduce the drop and spread of wild oat seeds. Seeding rate may

also have a considerable impact on wild oat densities. At high barley seeding rates, wild oat density and biomass declined, and crop biomass was not affected by higher wild oat densities (Scursoni and Satorre, 2005). In addition, high barley seeding rates can have a detrimental effect on wild oat seed production (O'Donovan *et al*., 2000). Similar trends were shown in winter wheat crops, with higher crop density lowering wild oat head and seed production (Wilson *et al*., 1990). Beckie *et al*. (2008) completed an extensive survey of farms in the Canadian prairies and concluded that practices such as tillage, forage crop rotation, fallow periods, and equipment sanitation reduce the risk of herbicide resistance in wild oat.

Although above-ground management can be effective for weed control, managing the seed population present in the soil should also be considered (Wagner and Mitschunas, 2008; Boyetchko, 1996). The soil seed bank is crucial for the maintenance of populations of plants. Variation in the length of seed dormancy within species, due to physiological and environmental factors (Baskin and Baskin, 2001), allows for the storage of viable seeds in the soil for germination in subsequent years. This acts as an insurance mechanism; should a high percentage, or perhaps all, of the plant crop fail to seed in one growing season due to adverse conditions, the species will be sustained by germination from the soil seed bank when conditions improve. Plant species with longer lived seeds have a lower rate of local extinction (Stocklin and Fischer, 1999), illustrating the significance of the seed bank in population dynamics and disturbance survival.

Although the seed bank is an ecologically important mechanism for plant populations, it is a management challenge for farmers. A seed longevity database constructed by Thompson *et al*. (1997) shows that wild oat seeds may remain viable in the soil for up to 9 years; thus, wild oat has the ability to form persistent populations in crop fields. Reducing the number of seeds that enter the soil by preventing weeds from reaching maturity is one way to reduce the weed seed

bank and future weed populations (Harker *et al*., 2003; Shirtliffe *et al*., 2000). Conservation tillage and no-till practices can also reduce seed dormancy and limit the soil seed bank (Beckie *et al*., 2008). However, practices aimed at reducing herbicide resistance or other goals unrelated to weed control, such as fallow seasons or crop rotations, may enhance the seed bank by favoring weed populations with higher seed dormancy (Jana and Thai, 1987). Further complicating the matter is the recent finding by Owen *et al.* (2010) of a potential positive correlation between seed dormancy and weed herbicide resistance. Seed bank management can be an effective weed control tool, but more research is needed to understand the mechanisms behind seed bank dynamics.

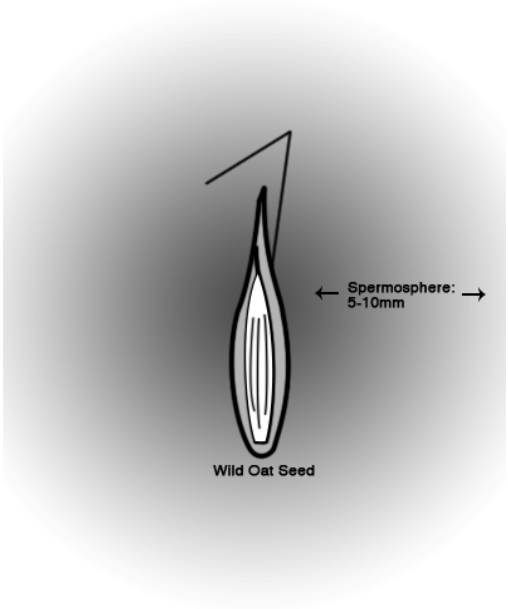

Fig 1.1. Conceptual diagram of the spermosphere around a wild oat seed. Seed shown enlarged appx. 4x.

Seeds interact with their surrounding soil via the spermosphere, the region of soil surrounding and affected by a seed (Figure 1.1). When seeds imbibe, a variety of compounds are passively exuded from the seed into the spermosphere. These compounds may alter nutrient availability around the seed, and they may also change the local environment to induce a microbial community response (reviewed in Nelson, 2004). Compared to the analogous

rhizosphere around roots, relatively little research has been done on the spermosphere, but this is an important stage in the plant-soil-microbe relationship. Increased concentrations of organic compounds in the rhizosphere may change soil nutrient availability through more than one mechanism. The release of organic acids changes the surrounding soil pH and alters the solubility of inorganic phosphorous compounds, increasing P availability (Hinsinger, 2001). Organic acids also form metal-chelate complexes, which, in the soil, can increase iron solubility in low-pH (Fe-limiting) conditions and can reduce aluminum toxicity (Jones, 1998). It has been hypothesized that other micronutrients, such as zinc and copper, may be solubilized and mobilized by organic acids, but little research has been done to investigate (Jones *et al*, 2003). Should these and similar compounds be released into the spermosphere, the effects could be very beneficial to the young plant, especially in nutrient-limiting soils.

Increased microbial activity has been well documented in the rhizosphere (El-Shatnawi and Makhadmeh, 2001), and to a lesser extent in the spermosphere (Nelson, 2004). Microbial community response to exudates may be beneficial or harmful to the seed. The plant pathogenic oomycete *Pythium ultimum* can cause seedling damping-off diseases in many crops, and responds to seed-exudate cues in the spermosphere (Nelson, 2004). Linoleic, oleic, and palmitic acids, all long-chain fatty acids, diffuse from seeds through the soil and stimulate the germination of *P. ultimum* sporangia (Ruttledge and Nelson, 1997). *Fusarium solani* chlamydospores are also stimulated in the spermosphere, though it is not clear which compounds are responsible (Short and Lacy, 1974). *Rhizoctonia* fungal growth is stimulated by the presence of root exudates (Hietala, 1997), which are often similar in composition to seed exudates. In contrast, root exudates from some species may inhibit growth of fungal hyphae (Stevenson *et al.*, 1995; Oba *et al*., 2002). Numerous non-pathogenic microorganisms are also stimulated in the

spermosphere and rhizosphere. According to Fischer *et al*. (2010), microbial utilization of low molecular weight compounds is far greater than sorption in the soil, leading to the compounds having a very short-lived presence; however, this trend may vary with differing soil types and conditions. Increased microbial activity in the soil can create favorable conditions for the germinating plant, including elevated nutrient cycling and potentially increased nutrient availability to the plant (Dakora and Phillips, 2002), and increased organic matter and soil moisture. In addition, numerous mycorrhizal relationships are initiated through fungal stimulation by root exudates (Dakora and Phillips, 2002). Biocontrol agents may also be stimulated in the spermosphere, reducing the effects of seed pathogens (Simon *et al*., 2001). However, microbial stimulation may also create competition between soil microorganisms and plant, especially in nutrient-poor soils. For example, in low-N environments, microbes may incorporate N into their tissues at a rate exceeding N-mineralization, rendering that N unavailable to plants (Kastovska and Santruckova, 2011). Finally, numerous organic compounds promote microbial synthesis of ethylene (Arshad and Frankenberger, 1990), which accumulates in the soil and can stimulate the germination of dormant seeds (Gallagher and Fuerst, 2006). The interactions between seeds and microbes in the spermosphere may be very complex, and a better understanding of seed dynamics in the soil will require a better understanding of the spermosphere.

Not only are there varying interactions depending on the microbial community and the soil conditions, but seed species also behave differently from one another and, in turn, have different effects on the surrounding microbes (Roberts *et al*., 2009). Current research on spermosphere composition and interactions is limited, and thus we are just beginning to understand the ecology of the seed-soil interface. What is the complete chemical composition of

the spermosphere and how might this affect the surrounding microorganisms? In addition, the implications of exudation for the seed itself have rarely been studied. For example, during soil wetting and drying cycles, how do dormant seeds change chemically and what are the implications for the plant? Studying these questions is pivotal to understanding the spermosphere and potentially implementing more effective seed bank management.

Gallagher *et al.* (2010) characterized the phenolic and short-chained aliphatic organic acid constituents of dormant, stored wild oat caryopses and hulls. Majority of the phenolics were found in the hull, with only 9% of the total phenolics found in the caryopsis. Ferulic and *p*-coumaric acids were the most dominant phenolic acids, with vanillic and *p*-hydroxybenzoic (benzenecarboxylic) acids also present in notable amounts. Four aliphatic organic acids, malic, succinic, fumaric, and azelaic, were found in significant quantities, with malic being the most abundant. The fraction of phenolic or organic acid bound to cell components compared to the free, soluble fraction differed between compounds. There were slight differences in phenolic and organic acid concentrations between non-dormant and dormant isolines, possibly giving some indication of the compounds with roles in seed dormancy or protection.

The goal of this project in regards to the spermosphere was to determine the organic acid composition of the region around wild oat seeds. In addition, the changes in chemical composition of the wild oat seeds during imbibition were characterized. By pursuing these goals we hope to begin to postulate how wild oat seeds may interact with and be affected by their surroundings, and discuss how this information may be extrapolated to other plant species and other environments.

Seed Pathogen Defense

The seed is a potentially vulnerable stage in a plant's life. Though germination is often a significant contributor of the loss of seeds from the seed bank (Russi *et al*., 1992; Zorner *et al*., 1984), viable seeds in the soil may lose viability through aging processes or through seed predation or decay. It has been hypothesized that seeds may lose viability due to aging through food reserve exhaustion or alteration, DNA alteration, or the loss of functional membranes (Baskin and Baskin, 2001). Most research of seed aging has focused on the storage of agricultural seeds in controlled settings (Hull, 1973; Steiner and Ruckenbauer, 1995), and it is unlikely that aging is a significant contributor to the loss of seed viability in field soil relative to predation and decay. In a study of woodland shrubs, Campbell and Clarke (2006) found little germination of seeds from the seed bank due to the nature of the study species, but also found no seed bank accumulation, which they attributed to high rates of seed predation, mainly by invertebrates. There has been a fair amount of research focusing on seed predators due to the potential for seed predation to control weed density in agricultural fields (Gallandt, 2006). Ground beetles (Carabidae) have been shown to consume weed seeds in both the lab and field setting, with preferences for certain weed seeds and variability in consumption between fields of different crops (Honek *et al*., 2003).

There are also many genera of soil microorganisms that can cause seed death or plant disease. A number of species in the fungal genus *Fusarium* are saprophytic or pathogenic to seeds and plants (Leslie and Summerell, 2006), as are some *Aspergillus* species, notably *Aspergillus niger*, which causes black mold of onion (Ozer, 2011). In addition, the aforementioned *Pythium ultimum* causes damping-off in many plant species (Van Dijk and Nelson, 2000). These and other microorganisms impact the seed bank and, consequently, weed

establishment. In one study, 80% of initially dormant and 70% of initially non-dormant wild oat seeds in shallow seed bags lost viability after 6 months in field soil, with this figure decreasing with increasing depth. A high percentage of seeds were also lost from the seed bank due to germination, leading to little seed bank persistence (Zorner *et al*., 1984). In contrast, a separate study found that only 16% of buried seed were dead after 10-month incubation periods (Gallandt *et al*., 2004). This study also noted high variability between years, with seed mortality occurring for 28% of buried seeds in a conservation tillage field in 1999, but less than 3% of buried seeds in the same field in 2000. Kropac *et al.* (1986) found that 11-25% of wild oat seeds buried between 0cm and 20cm were still viable after 4 years of burial, while seeds buried deeper remained viable for longer periods of time. With variability between years and sites, it is difficult to quantify the true impact on the seed bank of seed mortality in the soil.

To remain persistent in the soil, seeds must have mechanisms to defend themselves against attack by pathogenic and saprophytic organisms. Both chemical and physical defenses are important to seed persistence. Davis *et al*. (2008) suggested that water soluble chemical defenses are important to short-term persistence, whereas physical defenses become more important in the long-term. There is fairly widespread evidence for the importance of phenolic compounds in chemical defense against pathogens (Cvikrova *et al*., 1995; Nicholson and Hammerschmidt, 1992). Accumulation of phenolic compounds is often seen in plant response to pathogens (Matern and Kneusel, 1988). Fernandez and Heath (1989) detected elevated phenolic concentrations in bean leaves in response to pathogenic and saprophytic fungal spore infection, with saprophytic fungi stimulating the formation of extra-cellular phenolic deposits. Compared to controls, the periderm of disease-resistant potatoes had elevated concentrations of caffeic, *p*-hydroxybenzoic, ferulic, vanillic, and *p*-coumaric acids (Cvikrova *et al*., 1995), and the

concentrations of *p*-hydroxybenzoic, ferulic, vanillic, and *p*-coumaric acids all increased in tomato roots in response to fungal elicitors (Mandal and Mitra, 2007). Anti-fungal compounds were found at higher concentrations in onion plants whose seeds were treated with microbes antagonistic to *Aspergillus niger*, the causal agent of onion black mold (Ozer, 2011), suggesting that interactions at the seed stage could help protect the germinating plant from soil-borne diseases.

More specifically than a simple increase or decrease in phenolics in plant tissues as a response to pathogen attack, the distribution and form of phenolics may also be altered. The primary forms that the compounds may take are free (unbound), esterified (linked to other phenolics through an ester linkage), and wall-bound (linked to the cell wall through an ester linkage) (Figure 1.2). A study by de Ascensao and Dubery (2003) investigated the response of these fractions in banana roots during exposure to elicitors from the fungal pathogen *Fusarium oxysporum*. The greatest accumulation of phenolics over controls was found to be in the wall-bound fraction, where a 6.3-fold increase in concentration was noted over 36h. The esterified fraction also increased in the treatment plants, but to a lesser extent. While the free fraction increased the least, there was still substantial elevation in concentration over the control (de Ascensao and Dubery, 2003). Previous work also supports phenolic fraction shifts as a plant defense response (de Ascensao and Dubery, 2000; Hammerschmidt, 1984; Grand *et al*., 1987).

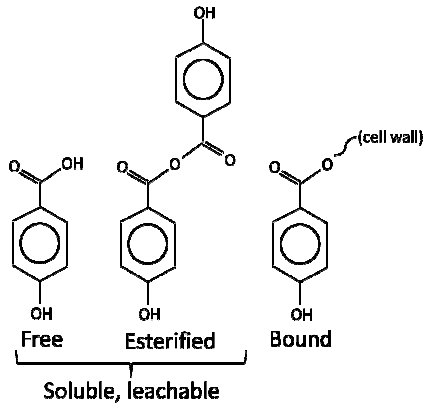

Fig 1.2. The three compound fractions considered. Free and esterified fractions are soluble and potentially leachable.

Many of these compounds may have direct inhibitory effects on microorganisms through mechanisms such as growth inhibition, plant resistance, and suppression of spore germination. As early as the 1930s, catechol and protocatechuic acid, as well as some other phenols, were recognized as having anti-microbial properties (Walker and Link, 1935). Caffeic and *p*-coumaric acids were among the phenolics that inhibited the activities of lignin peroxidase, manganese peroxidase, and laccase in a number of fungal pathogens (del Rio *et al*., 2004), three enzymes that aid in plant infection (Lozovaya *et al*., 2006). In addition, del Rio *et al*. (2004) found that caffeic and *p*-coumaric acids substantially inhibited the growth of the same fungi on potato dextrose agar. Chlorogenic, vanillic, and caffeic acids have been shown to inhibit spore germination of the tomato pathogen *Alternaria alternata* to some extent individually and a greater extent in combination (Ruelas *et al*., 2006). While the above studies on plant phenolic response and phenolic effects on pathogens cover a range of conditions, plants, and pathogens, a number of common compounds, namely caffeic, *p*-hydroxybenzoic, ferulic, vanillic, and *p*-coumaric acids, are highlighted. Thus, these are likely to be important in pathogen resistance throughout nature.

It is widely accepted that lignin is a primary physical plant defense as it is very difficult for pathogens to penetrate. In addition to promoting increases in phenolic concentrations in plant

tissues, fungal elicitors stimulate gene expression and production of the lignin-synthesis enzyme cinnamyl-alcohol dehydrogenase in beans (Walter *et al*., 1988; Grand *et al*., 1987) and lignin synthesis in cells of tomato roots (Mandal and Mitra, 2007). Cinnamic, *p*-coumaric, caffeic, ferulic, and sinapic acids are all involved in the biosynthesis of lignin (Vance *et al*., 1980). Though there are pathogens that possess enzymes capable of degrading lignin (*Fusarium solani* f. sp. *glycines,* Lozovaya *et al*., 2006), many are incapable and even those that can may be slowed by the physical barrier. Increases in these phenolic compounds, coupled with elevated activity of enzymes involved in lignin formation, could thus provide indirect plant defense through increased formation of lignin and lignin-like compounds.

Plants also employ anti-microbial enzymes to protect against attack by soil microbes. A large group of proteins called pathogenesis-related proteins (PR-proteins) are integral in plant defense. Fourteen families of PR-proteins, proteins that are induced in plant tissues as a result of microbial attack or similar stress, are now recognized, including chitinases, β-1,3-glucanases, proteinase inhibitors, and peroxidases. While some are known to have anti-bacterial or even anti-viral properties, anti-fungal properties have been observed in most of these proteins (Edreva, 2005), which may directly or indirectly affect fungal growth. Chitinases break down chitin, the major component of fungal cell walls, and thus directly inhibit fungal growth. While chitinases are effective in inhibiting fungal growth and may have other minor functions in the plant, they are ineffective defense enzymes against bacteria (varying cell walls) or oomycetes (walls composed of cellulose) (Gomez *et al*., 2002). Chitinases are present in a wide variety of plants, and increased presence and activity are induced by fungal invasion (Punja and Zhang, 1993; Caruso *et al*., 1999). Enzymes may also play an indirect role in plant defense by controlling the distribution of chemical defenses in a plant. Similar to the significance of increased cinnamyl-

alcohol dehydrogenase activity and lignin formation, the increased activity of polyphenol oxidase (PPO) enzymes can bolster plant defenses. PPOs convert phenolic monomers into polymers, which, as with lignin, are more difficult for pathogens to break down. Catechol is one of the main PPO substrates, and a darkening of seeds during PPO activity could indicate polymerization of phenolics and elevated defense (Anderson and Morris, 2001). Activity of these enzymes in wild oat seeds increases during attack by the fungal pathogen *Fusarium avenaceum* (Anderson *et al*., 2010), and thus it is expected that the distribution of phenolic compounds in the free, soluble ester, and cell wall-bound fractions would change accordingly as PPO polymerizes individual subunits.

The goal of this project in regards to pathogen defense was to characterize the changes in phenolic, short aliphatic, and long-chain fatty acid concentration and distribution in wild oat seeds in response to pathogen attack. This will help us to gain a better understanding of how wild oat seeds remain viable in the soil over long periods of time. In addition, we hope to further describe the defense mechanisms of plants against fungal pathogens, and begin to more fully understand these mechanisms at the seed stage.

Chapter 2: THE SPERMOSPHERE

INTRODUCTION

The objective of this chapter was to characterize the chemical composition of the spermosphere region around wild oat seeds. An additional goal was to quantify the changes in the chemical composition of the wild oat seeds during imbibition. Compounds may move within the seed, or between the seed and the spermosphere (Figure 2.1). Our intent was to further our understanding of how seeds interact with the surrounding soil environment, and how dormant seeds might be altered over time in the soil due to wetting and drying cycles. This could give us a better idea of the potential mechanisms regulating seed bank dynamics and how weed seed banks might be more effectively managed.

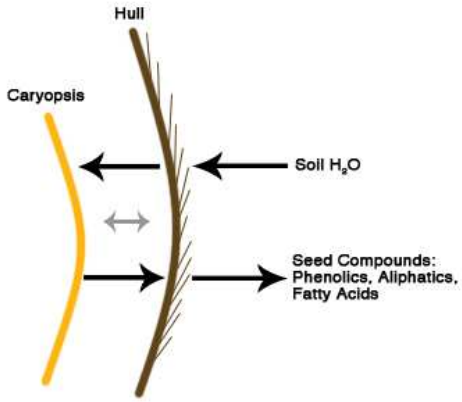

Fig 2.1. Conceptual model of the flow of water and chemical compounds between the seed and the spermosphere, and within the seed.

General study of the chemical composition of the spermosphere has been completed in the past. Nelson (2004) completed a quite comprehensive review of microbial interactions in the spermosphere from a pathogen infection standpoint, and found that a number of compounds may

be present in the spermosphere. Sugars, amino acids, aliphatic and aromatic organic acids, flavonoids and other phenolics, and some other categories of compounds have all been detected at various levels in the spermosphere of various species. However, the specific compounds in these groups are not typically quantified, and thus a thorough characterization of the spermosphere is still lacking. In addition, the seeds themselves also may be altered during imbibition and seed exudation, potentially affecting their viability, vigor, and ability to defend against pathogens.

The present study focuses on the phenolic and aliphatic organic acid and long-chain fatty acid composition of wild oat (*Avena fatua* L.) seeds. Phenolic organic acids are commonly involved in plant chemical and physical defenses, as well as some signaling roles (Nicholson and Hammerschmidt, 1992). Aliphatic organic acids have metabolic roles in the plant, with a few being involved in the tricarboxylic acid (TCA) cycle (Goldberg *et al*., 2006), and may also be high quality substrates for microbes. Long-chain fatty acids are metabolically important, but also may be readily consumed by microbes and stimulate microbial activity in the spermosphere (Ruttledge and Nelson, 1997). Wild oat seeds were imbibed in sand, and the seed and sand were analyzed via gas chromatography-mass spectrometry to investigate movement of these compounds.

METHODS

Experimental Setup

Seeds used were wild oat (*Avena fatua* L.) cv. M73, a highly dormant line of wild oat (Adkins et al. 1986). Seeds from two different growing seasons were used, 2005 and 2006, grown under greenhouse conditions. To determine the movement of compounds between

caryopses and hulls and out of the seeds, seeds were placed into 60g sand with gravimetric moisture content of 23% in plastic specimen cups (150mL, 5.5cm base diameter). Prior to set up, sand was washed with 50% commercial bleach solution, rinsed 3 times with filtered water (Barnstead Nanopure, Thermo Scientific, Palm Beach, FL), and oven dried at 70°C overnight. Forty seeds were evenly spaced into each of 4 replicate specimen cups for both seed sources. Four cups containing only sand with no seeds were used as a sand control, whereas 4 cups containing only 40 seeds from each of the seed sources were also incubated as a seed control. Seeds were surface sterilized for 30s in 50% bleach solution, rinsed in filtered water (2x20s), and dried prior to placement in sand. Surface sterilization was shorter here than in many studies to prevent the excessive leakage of compounds from the seeds that may quickly occur when the seeds are placed in water (Nelson, 2004). The hydroscopic awns were removed to keep seeds in place when exposed to wet soil. Seeds were placed straight down in the sand so that just the tip and remaining awn were visible. The specimen cups were capped to reduce evaporation and incubated for 7 days in the dark with a 25°/15°C 12-hour rotation.

After incubation, the seeds were removed and excess sand removed. Seeds were then air-dried and hulls and caryopses were separated. Seed fractions were ground in liquid nitrogen with a mortar and pestle, freeze-dried, and capped and placed in a freezer for storage until extraction. Sand was air dried in a fume hood and distributed into scintillation vials for extraction.

Extraction and GC-MS Quantification

Extraction methods for sand and seeds were adapted from Krygier *et al.* (1982) and Gallagher *et al*. (2010). Chemical fractions refer to those noted in Figure 1.2 (p. 10). Sand was extracted 4 times by hand-shaking with methanol:acetone:water solution (1:1:1) (first extractions

used 10mL solvent, subsequent extractions used 5mL solvent) and centrifuging briefly to remove particles from solution. The solvent layer was removed and concentrated to approximately 10mL in a SpeedVac system (Savant AES2020, Thermo Fisher Scientific, West Palm Beach, FL). The total resulting solution was then acidified (pH < 3.0) in Corex high-speed centrifuge tubes and centrifuged (~10000g) to precipitate the esterified chemical fraction (Krygier *et al*., 1982). The solvent layer was removed and extracted 6 times with 4mL diethyl ether:ethyl acetate solution (1:1). The diethyl ether:ethyl acetate solution layers were combined (containing the "free" chemical fraction) while the aqueous layers were mixed back into the centrifuge tubes containing the esterified fraction. The free chemical fraction solution was dried through anhydrous $MgSO_4$ and evaporated to dryness. The samples were then reconstituted in 0.5mL HPLC-grade acetone. Esterified fraction samples were hydrolyzed by adding 5mL 4M NaOH and shaken in a 60°C water bath overnight. They were then re-acidified and extracted with diethyl ether:ethyl acetate solution, evaporated, and reconstituted using the same procedure as the free fraction samples.

Three fractions of compounds (free, soluble ester, and cell wall-bound) were extracted from seed hulls and caryposes (Krygier *et al*., 1982; Gallagher *et al*., 2010). The freeze dried seed fractions were weighed prior to extraction. These samples were placed into 15mL Corex centrifuge tubes, where they were combined with 4mL methanol:acetone:water solution (1:1:1) and homogenized for ~15s using a Powergen 125 homogenizer with a 10mm generator attachment (Fischer Scientific, Pittsburgh, PA). Between samples, the generator was rinsed in a test tube containing 1mL solvent mixture, which was then added to the corresponding sample. Samples were then centrifuged at ~10000g to precipitate pellet. The solvent layer was then removed and saved. This extraction was repeated 6 times, after which the pellets were transferred to scintillation vials and solvent layers were combined and concentrated to approximately 10mL

in the SpeedVac system. Using the same procedure as the sand extraction, the solvent layers were acidified with concentrated HCl and centrifuged to precipitate esterified fraction, extracted with diethyl ether:ethyl acetate solution (1:1), filtered through $MgSO_4$ to remove any remaining water, evaporated to dryness, and reconstituted in 1.5mL HPLC-grade acetone. Esterified fractions were also treated in an identical manner as those in the sand extraction. The pellets from the original homogenization and extraction, representing the "bound" chemical fraction (present in the seed due to binding to cell constituents), were combined with 4mL methanol:acetone:water solution (1:1:1) and 5mL 4M NaOH and shaken in a 60°C water bath overnight to hydrolyze ester bonds to cell constituents. They were then re-acidified and extracted with diethyl ether:ethyl acetate solution in an identical manner as the free and esterified chemical fractions.

All samples (free and esterified chemical fractions from the sand samples, and free, esterified, and bound chemical fractions from hull and caryopsis seed samples) were transferred to 2 ml GC vials, to which 2 to 3 molecular sieves (Type 3A, EM Science, Darmstadt, Germany) were added to maintain anhydrous conditions. Samples were stored under argon gas at -20°C until analysis via gas chromatography-mass spectrometry (GC-MS).

For GC-MS analysis, subsamples were taken from each stored vial and derivatized with 5.4µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) (Supelco Analytical, Bellefonte, PA) to reduce boiling point of the compound as well as reduce the polarity of active functional groups. For the free and esterified fraction samples, 44.6µL of sample was used, 20µL for the bound caryopsis samples, and 2µL for the bound hull samples. HPLC-grade acetone was added to each sample to bring the total solution of each subsample to 50µL. Linoleic and oleic acids in bound caryopsis samples were at levels beyond the upper

quantification limit of GC-MS analysis. To quantify those fatty acids in the bound caryopsis samples, a second dilution was performed using the same amounts as the bound hull samples.

Analysis of samples was completed using a Thermo Scientific Trace Ultra GC with a PTV inlet, DSQ MS and a TriPlus autosampler (Thermo Fisher Scientific, West Palm Beach, FL). Samples were injected at a volume of 1μL, with 1μL internal standard (butylated hydroxytoluene, 20mg/L acetone) through a 30m x 0.25mm i.d. Rxi-5 ms 30 column (Restek, Bellefonte, PA). Inlet was held at 60°C for 12 s, ramped to 280°C at a rate of $10°C \cdot s^{-1}$, and cleaned for 30 s at 350°C. Helium carrier gas was set at a constant flow of $1.5mL \cdot min^{-1}$. An initial oven temperature of 80°C was held for 30 s, followed by a ramp to 275°C at a rate of $10°C \cdot min^{-1}$. Post-run, the oven was held at 300°C for 30 s to clean the column. The transfer line to the MS was held at 300°C, and the ion source temperature held at 290°C. Xcalibur 1.4 (Thermo Fisher Scientific, West Palm Beach, FL) was used as interface software for GC-MS and for data processing. Processing and quantification were performed in the same manner as Gallagher *et al*. (2010), with the exception of using 7 calibration standards to construct a standard curve, dilutions ranging from 0.5 to 20 $mg \cdot L^{-1}$ acetone and 1 to 40 $mg \cdot L^{-1}$ acetone for ferulic acid. Additionally, with this study, extraction methods allowed for the quantification of fatty acids palmitic, linoleic, oleic, and stearic. Thus, these were also included in standard curves, with dilutions ranging from 2 to 80 $mg \cdot L^{-1}$ acetone for linoleic and oleic acids, and dilutions ranging from 1 to 40 $mg \cdot L^{-1}$ acetone for palmitic acid. All compounds quantified via GC-MS can be found in Table 2.1. Though long-chain fatty acids have an aliphatic component, the term "aliphatic organic acids" will thus forth indicate the four compounds listed in that category in Table 2.1.

Chemical sources were as follows. ACS-grade methanol and acetone were from Alfa Aesar (Ward Hill, MA). Hydrochloric acid and ethyl acetate were from EMD Chemicals (Darmstadt, Germany). Diethyl ether (stabilized with 2% ethanol) and HPLC-grade acetone were provided by Honeywell Burdick and Jackson (Muskegon, MI)

Table 2.1. Compounds quantified in seed and sand samples, based on Gallagher *et al*. (2010).

Phenolic acids	*p*-hydroxybenzoic, caffeic, catechol, cinnamic, ferulic, gallic, hydrocaffeic, hydroquinone, *p*-coumaric, phenyl proprionic, protocatechuic, salicylic, sinapic, syringic, vanillic.
Aliphatic organic acids	Azelaic, fumaric, malic, succinic.
Long-chain fatty acids	Linoleic, oleic, palmitic, stearic.

Statistical analysis was completed using R (v2.12.1, R Development Core Team, 2006). A significance level of $\alpha = 0.05$ was used for least squared means tests.

RESULTS

No significant difference in chemical composition was found between 2005 and 2006 seed grow-outs, so those years were pooled to bolster sample size. Regardless of treatment, the dominant phenolic acids detected in the seeds were ferulic, *p*-coumaric, and caffeic acids, with syringic and vanillic acids also detected at comparable levels in some samples (Table 2.2). Ferulic acid alone made up ~76% of all phenolics in the seeds, with *p*-coumaric and caffeic acids making up an additional 14% and 8%, respectively. All other phenolics made up <1% of the total. Seeds were found to contain, on average, ~164,000 $ng \cdot seed^{-1}$ phenolic acids in the free, esterified, and bound forms. The bound fraction accounted for 99% of the total seed phenolics, and the esterified and free fractions 1%. Of the two soluble fractions, free and esterified, 87% of the phenolics were in the esterified form. Within the seeds, 97% of the total phenolics were

located in the hull and 3% in the caryopsis. This trend is driven by the bound and esterified fractions, with 97% of the bound phenolics and 96% of the esterified phenolics located in the hull. Of the free fraction (the lowest in concentration), 83% were found in the hull, while 17% were found in the caryopsis. These results are similar to those reported by Gallagher *et al.* (2010).

The total aliphatic organic acid concentration was distributed much more evenly between the quantified compounds than was seen with the phenolics, but they otherwise behaved in a similar manner (Table 2.2). In the seed as a whole, succinic acid made up 40% of the total aliphatic content, and the other three compounds made up ~20% each. The seed content of aliphatic organic acids was substantially lower than that of phenolics, with an average of only ~2,000 $ng \cdot seed^{-1}$. As with the phenolic compounds but to a lesser extent, majority was found in the bound form (76%), followed by the esterified (16%) and free (8%) forms. Again, majority of the soluble fraction was found in the esterified form. Within the seed, 71% of the aliphatics were found in the hull and 29% in the caryopsis. The bound fraction was split between the two seed fractions, with the 64% in the hull and 36% in the caryopsis. A much higher percentage of the esterified and free fractions, 97% and 87%, respectively, were located in the hull.

Table 2.2. Mean compound concentration in wild oat seeds by chemical form and dominant compound.

	All chemical forms ng · seed^{-1}	Free form ng · seed^{-1}	Esterified form ng · seed^{-1}	Bound form ng · seed^{-1}
Total Phenolics	**164142**	**213**	**1404**	**162525**
Ferulic	124688	82	831	123775
p-coumaric	23217	38	215	22964
Caffeic	13384	7	18	13359
Total Aliphatics	**1941**	**153**	**302**	**1486**
Succinic	844	35	121	696
Fumaric	447	51	29	367
Azelaic	390	22	95	273
Malic	259	46	64	149
Total Fatty Acids	**1003696**	**1708**	**13706**	**988282**
Linoleic	436904	354	3966	432584
Oleic	394979	467	3150	381362
Palmitic	178996	832	6442	171722
Stearic	2817	55	148	2615

There were a few major differences between the long-chain fatty acid distribution and that of the previous two compound groups (Table 2.2). The fatty acids were by far the most abundant compounds in the seed, averaging ~1,000,000 ng · seed^{-1}. Linoleic and oleic acids accounted for 44% and 38% of the total fatty acids, respectively, followed by palmitic acid (18%) and stearic acid (<1%). The bound fraction again dominated, with 98% of the fatty acids in this form. However, the vast majority of total fatty acids, nearly 98%, were located in the caryopsis. Linoleic and oleic acids' dominance of the fatty acids was driven by the bound caryopsis fraction, as palmitic acid was actually found at the highest levels in the free and esterified fractions of both the caryopsis and hull. The majority (80%) of the esterified fraction was also found in the caryopsis, while the free fraction was split evenly between the hull and caryopsis.

Imbibition effects on seed chemistry

Due to the insolubility of the bound fraction, imbibition and leaching had no effect on the concentrations of bound phenolics, aliphatics, or fatty acids (Table 2.2). Since the bound fraction was the dominant chemical form of the compound groups, the effects of leaching on the total compound concentrations (free + esterified + bound) were masked. Nevertheless, the 30% reduction in total aliphatics in the hull was statistically significant. This was due to a 69% reduction ($P < 0.05$) in the total soluble (esterified + free fraction) hull aliphatics in the leached seeds. There was also a 62% reduction in total soluble caryopsis aliphatics ($P = 0.07$). Overall, aliphatics were affected most during seed imbibition, with significant reductions in both the free and esterified fractions in the hull, where the free fraction dropped 90% in leached seeds compared to controls and the esterified fraction 57%. The free fraction of phenolics was also significantly reduced after leaching, dropping 77% below the control seeds. While the chemical composition of the hulls was affected substantially during imbibition and leaching, the caryopses experienced very little change (Table 2.2). Similarly, the fatty acid concentration, regardless of chemical fraction or seed component, was not significantly different between treatments.

Table 2.3. Mean phenolic, aliphatic, and fatty acid concentrations in the caryopses and hulls of control and leached wild oat seeds. Asterisks mark significance within the seed fraction and row ($P < 0.05$).

	Caryopsis		Hull	
	Control Seeds mean, ng • seed^{-1}	Leached Seeds mean, ng • seed^{-1}	Control Seeds mean, ng • seed^{-1}	Leached Seeds mean, ng • seed^{-1}
Total Phenolics	**4170**	**4730**	**156970**	**162410**
Free	26	45	289 *	67 *
Esterified	56	51	1500	1200
Bound	4090	4640	155180	161140
Total Aliphatics	**527**	**585**	**1630 ***	**1140 ***
Free	31	10	243 *	23 *
Esterified	11	6	410 *	177 *
Bound	485	570	974	942
Total Fatty Acids	**987660**	**970790**	**27130**	**21810**
Free	645	970	712	1089
Esterified	13980	7910	2840	2680
Bound	973030	961910	23580	18040

The reduction in free phenolics in the hull was reflected in the concentration change in individual compounds. The dominant phenolics (ferulic, *p*-coumaric, caffeic, syringic, and vanillic) all experienced significant drops in free fraction hull concentration after imbibition, with ferulic and *p*-coumaric concentrations reduced by 68% and 65%, respectively (Figure 2.2). However, these same compounds in the esterified fraction were mostly found at similar concentrations in the control and leached seed hulls, with significant differences coming only in the lower-concentration syringic and vanillic acids. In the caryopses, no phenolic compounds were found at significantly different levels between control and leached seeds.

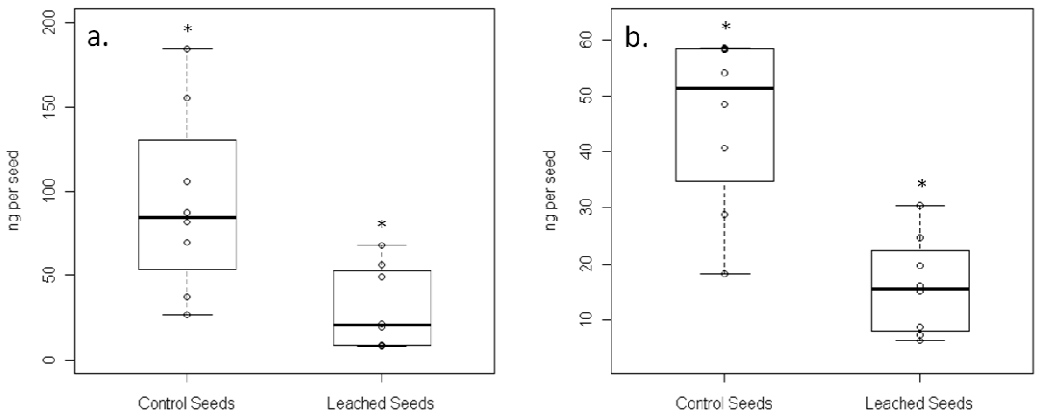

Fig 2.2. Concentrations of free ferulic (a) and *p*-coumaric (b) acids in the hulls of control and leached seeds. Note difference in scales. (* = $P < 0.05$)

The large differences in total hull aliphatic organic acid concentrations noted previously were reflected across all four quantified compounds. Each aliphatic acid's free fraction concentration was significantly lower in the leached hulls, with the two most abundant compounds, malic and fumaric, experiencing 99% and 98% concentration reductions, respectively. In the esterified fraction, concentrations of the two most abundant compounds, succinic and malic, were reduced 59% and 98%, respectively (Figure 2.3), with a significant reduction also occurring in fumaric acid concentration. In caryopses, no difference in aliphatic concentration was detected between treatments.

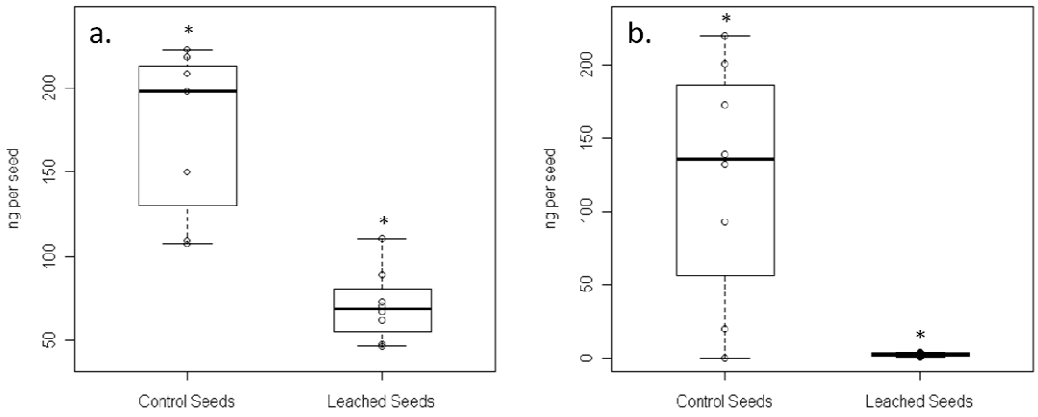

Fig 2.3. Concentrations of esterified succinic (a) and malic (b) acids in the hulls of control and leached seeds. Note difference in scales. (* = P < 0.05)

The concentrations of long-chain fatty acids in the caryopses and hulls remained largely unchanged between control and leached seeds, regardless of chemical fraction. Only esterified palmitic acid showed any significant change due to the treatment, dropping 42% in concentration in the caryopsis (Figure 2.4). Palmitic was, however, the most dominant fatty acid in the free and esterified forms in the caryopsis, so a significant reduction in the esterified concentration of this compound does have an impact on the chemical makeup of the caryopsis.

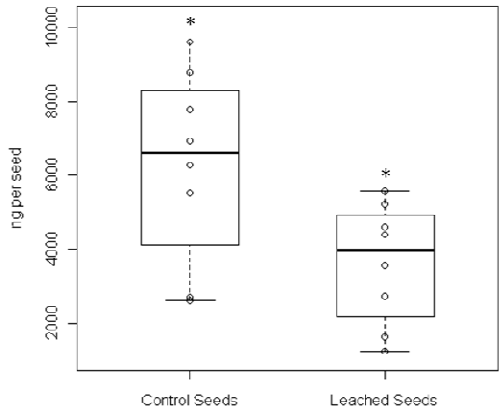

Fig 2.4. Concentration of palmitic acid in the caryopses of control and leached seeds. (* = P < 0.05)

Spermosphere composition

Concentrations of the compounds were much smaller in the spermosphere and thus characterization of this proved difficult. Further confounding analysis was the higher than expected background concentrations of compounds in the control sand, especially in the case of the long-chain fatty acids (though these background concentrations were still <0.1% of seed tissue concentrations).

Since the bound fraction is defined as compounds bound to cell wall components, it was assumed that no bound fraction would be present in the spermosphere and thus no quantification was conducted. Concentrations of compounds in the sand are reported as ng exudate · seed^{-1}, indicating the average amount of compound released into the soil per seed.

Table 2.4. Mean phenolic, aliphatic, and fatty acid concentrations in control and wild oat spermosphere sand.

	Sand	
	Control mean, ng exudate · seed^{-1}	Spermosphere mean, ng exudate · seed^{-1}
Total Phenolics	**5.3**	**4.7**
Free	2.5	2.4
Esterified	2.8	2.3
Total Aliphatics	**3.9**	**8.5**
Free	2.0	3.5
Esterified	1.9	5.0
Total Fatty Acids	**116.2**	**114.0**
Free	68.1	64.5
Esterified	48.1	49.5

Fatty acids were the most abundant compounds in the sand, averaging ~115ng exudate · seed^{-1}, followed by aliphatics at 8.5ng exudate · seed^{-1} and phenolics at ~5ng exudate · seed^{-1} (Table 2.4). There were no significant differences noted in the total phenolic concentration (free + esterified) or in the free or esterified total phenolic concentration between

spermosphere sand and control sand. The same was true for both total aliphatic organic acids and total long-chain fatty acids. While the differences were not statistically significant, there were increases in both fractions of aliphatics between the control sand and spermosphere sand (Table 2.4). The total aliphatics in the spermosphere sand were 218% higher than in the control sand. The lack of significance likely comes from the high variability in the spermosphere sand, with individual measurements ranging from 1.1 to 14.6 ng exudate·seed^{-1} (Figure 2.5).

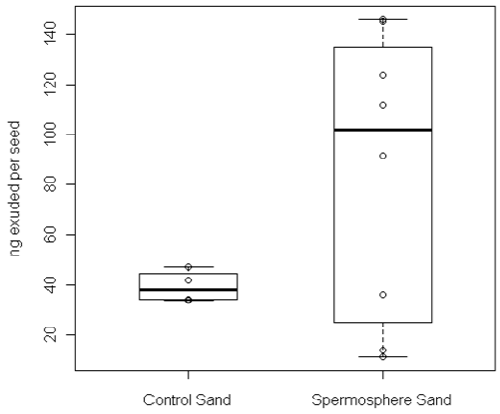

Fig 2.5. Total aliphatic organic acid concentration in control and spermosphere sand.

Of the individual compounds, only azelaic acid and vanillic acid showed noteworthy patterns in the sand. As with the total compound group concentrations, statistical significance with these two compounds was confounded by high variability in the spermosphere sand. Azelaic acid increased nearly 250% between the control and spermosphere sand (0.27 to 0.67 ng exudate·seed^{-1}), and vanillic acid increased over 400% (0.04 to 0.17 ng exudate·seed^{-1}). These concentrations are, however, very low, and it is debatable whether or not this increase would have any biological significance.

DISCUSSION

As expected, the wild oat hulls had much higher concentrations than caryopses of phenolics and aliphatics, and the majority of both of these groups were in the bound form (Gallagher *et al*., 2010). Similar results were previously reported by Gallagher *et al*. (2010). The high concentrations of phenolics likely provide a measure of defense against antagonistic soil microorganisms. As suggested by results from de Ascensao and Dubery (2003), a high concentration of bound phenolics is a particularly important initial plant defense mechanism. It is especially important to the seed to have these high concentrations in the hull, as it is the first seed component that potential pathogens encounter.

Relatively little previous research has documented the changes in seed chemistry during imbibition. Here, wild oat seeds were fairly significantly altered chemically, with the greatest losses coming in the aliphatic organic acids' concentrations. The studies that do look at seed chemistry typically study the seeds during the early stages of germination. One such study (Morohashi and Shimokoriyama, 1972) added C-14 labeled glucose and aspartic acid to seeds and found evidence of TCA cycle activity during the beginning stages of germination, and an increase after ~5.5 hours, at which time they also noted an accumulation of malic acid. Other studies, such as Krackhardt and Guerrier (1995), have also noted increases in organic acids during early germination of seeds under various treatments. However, no studies have investigated the imbibition of dormant seeds. With the importance of succinic, malic, and fumaric acids to the metabolic activity of the seed, the loss of a large proportion of these compounds during imbibition could suppress the activation of seed metabolism and allow the seed to remain dormant and in the soil seed bank. Alternatively, excessive loss of these organic

acids through a number of wetting and drying periods could potentially lead to the loss of vigor through reduced capacity for respiration (Siegenthaler and Douet-Orhant, 1994).

The phenolic compounds analyzed were found to be less mobile than the aliphatic organic acids, but there was still a reduction in concentration of the free fraction in the hull. Since phenolic compounds are often considered defense compounds, this reduction could render the seed more vulnerable to microbial attack. However, since the free fraction was present in relatively low concentration, and since the bound fraction likely plays the greatest role in seed defense, the concentration reduction is not likely to hinder the seeds defenses. The high concentration of bound phenolics in the hull coupled with the finding that the concentration is not affected during imbibition show that the one of the seed's primary defenses could remain in place for a number of seasons, contributing to the seed's ability to remain viable in the soil seed bank.

In general, both aliphatic and phenolic acids were found to be somewhat less mobile when in the esterified form than in the free form. While a number of studies have quantified the soluble ester-bound fraction (Shahidi and Naczk, 1992; Liyana-Pathirana and Shahidi, 2006; Dvorakova *et al*., 2008), the biological significance of this fraction, which is typically higher in concentration than the free fraction, remains unclear. It is possible that the lower mobility of the esterified fraction could be one biological reason for its abundance. In addition, there is some evidence that the esterified phenolic fraction has slightly higher free radical scavenging activity than the free fraction (Dvorakova *et al*., 2008), highlighting the potential importance of ester-bound phenolics to seed survival.

In Gallagher *et al*. (2010) it was speculated that, during imbibition, phenolics and other soluble compounds may be drawn from the hull into the caryopsis as a result of the movement of

water from the soil into the seed (as in Figure 3). The present study did not find evidence supporting that hypothesis. While there were increases in both the average free and total phenolic contents in the leached caryopses, neither was statistically significant. However, the length of incubation of the seeds in this study likely allowed equilibration between the seed and soil water potentials, perhaps causing the diffusion of compounds back out of the caryopsis down a concentration gradient (Gallagher *et al*., 2010).

Complicating this matter, however, was the lack of a significant increase in concentration of any compounds in the sand (spermosphere) in the present study. The reason for this is unclear, as other studies have readily detected seed exudation through the same or less sensitive analytical techniques (Ruttledge and Nelson, 1997; Simon and Harun, 1972; Nelson, 2004). Many previous studies investigate seed exudation through soaking in water or some other buffered solution. Thus, the use of sand in this study, while a closer simulation of field conditions, could lead to a higher difficulty of detection of exuded compounds. Our extraction techniques are relatively untested on sand, and it is possible that more method development is needed. Though the background levels of compounds in the sand were less than 0.1% of the concentrations found in the seed tissue, these were still higher than expected. This could have masked small signals from seed exudation. Thus, it is difficult to say where the aliphatic organic acids and phenolic acids lost from the hull went. The most likely scenarios are that either they were transported into the sand but not effectively extracted or detected, or that they were metabolized by the seed or some surviving microbes in the sand or on the seed surface. A shorter incubation time with pre-extracted, more thoroughly sterilized sand could help to answer these remaining questions about the spermosphere.

Chapter 3: SEED DEFENSE CHEMISTRY

INTRODUCTION

The objective of this chapter was to characterize the changes in seed chemical composition during attack by microbial pathogens. The responses of germinating and mature plants to soil pathogens has been relatively well characterized, but a number of plant diseases occur or are initiated at the seed level, including pre-emergence damping off (van Dijk and Nelson, 2000). In addition, dormant seeds in the soil could lose viability due to attack by soil microorganisms. Thus, it is important to understand the defenses employed by seeds to survive in the soil and remain viable.

Many compounds have been highlighted as potentially important in plant defense. Phenolics such as caffeic, *p*-hydroxybenzoic, ferulic, vanillic, and *p*-coumaric acids have all been implicated in plant responses to microbial attack and all possess some degree of anti-microbial activity (Cvikrova *et al*., 1995; Mandal and Mitra, 2007; del Rio *et al*., 2004; Ruelas *et al*., 2006; Vance *et al*., 1980). A number of studies have found increases in phenolic compounds during microbial attack of roots, as well as an increase in lignification, which involves the increase and polymerization of phenolics. In two of the few studies of seed response to pathogens, Anderson *et al*. (2010) and Fuerst *et al*. (2011) found elevated activity of polyphenol oxidase (PPO) in wild oat seed under attack from *Fusarium* species. This enzyme polymerizes phenolics, converting them to a form more effective in plant defense.

The present study investigates the concentrations of seed phenolics, aliphatic organic acids, and long-chain fatty acids, and how these concentrations change in wild oat seeds as an initial response to attack by two *Fusarium* species. These soil fungi have been shown to respond

to seed exudates in the spermosphere (Short and Lacy, 1974), and have the capability to decay seeds in the soil (Anderson *et al*., 2010). It is hypothesized that the amount of phenolics in the free and esterified soluble forms will be reduced as increased PPO activity (Fuerst *et al*., 2011) causes their polymerization into more complex forms. Alternatively, the seeds could produce greater concentrations of phenolics to boost chemical defenses. The results from this chapter will contribute to our knowledge of biochemical seed response to pathogens, which is integral to understanding the dynamics and potential control of the weed seed bank.

METHODS

Seeds used for this study were dormant wild oat seeds, isoline M73, from the same source as Chapter 2. Prior to treatment, seeds were surface sterilized as previously noted (Chapter 2). Two species of *Fusarium*, *F. culmorum* (isolate R-1127) and *F. avenaceum* (isolate R-6623), were obtained from the Fusarium Research Center at The Pennsylvania State University and initially grown on carnation leaf agar (Fisher *et al*., 1982). Sterile distilled water was added to these plates to produce a spore suspension. Spore suspensions in 15% sterile glycerol were then stored at -80°C until needed.

For infection of wild oat seeds, the fungal isolates were grown out on potato dextrose agar (PDA, Dot Scientific, Inc., Burton, MI) at 25°C. The six pathogen exposure treatments included whole seed (hull + caryposis) and de-hulled caryopsis exposure to *F. culmorum*, *F. avenaceum*, or uninoculated PDA (control). Seeds from the previously mentioned 2005 and 2006 grow-outs were used. De-hulled caryopses were used to gauge the importance of the hull to wild oat seed defense, as well as to simulate caryopsis response to fungi that have penetrated the hull. To expose seeds to *Fusarium* isolates, 10 seeds were placed on a 2% water agar plate around

6mm plugs taken from the fringe of 10d old *Fusarium* cultures. Two plugs of uncultured PDA, *F. avenaceum*, or *F. culmorum*, and 20 seeds (whole or de-hulled) of were placed on each water agar plate (Figure 3.1). Each treatment was replicated 4 times. All inoculation and experimental set-up was competed in a laminar flow hood with sterile utensils. All plates were incubated in the dark for 3d at 25°C.

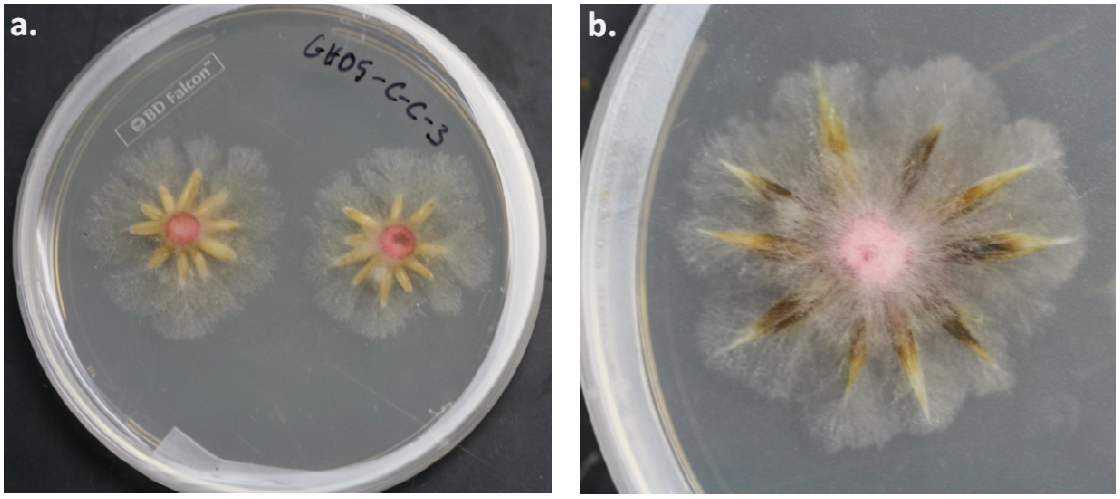

Fig 3.1. Experimental set-up of exposure of wild oat caryopses (a) and whole seeds (b) to *F. culmorum* (shown) and *F. avenaceum*.

After the 3d exposure period, seeds were removed from plates and their surfaces wiped to remove as much fungal hyphae as possible. Caryopses were placed directly into vials for extraction preparation, while hulls and caryopses of whole seeds were separated. In preparation for chemical extraction, samples were ground in liquid nitrogen in a Geno/Grinder 2000 (SPEX SamplePrep, Metuchen, NJ) (10 minutes at 1300 strokes · min^{-1}) and then freeze-dried to remove moisture. Freeze-dried samples were stored at -20°C until chemical extraction.

Extraction procedure for the seeds was identical to the procedure outlined in Chapter 2, with an initial methanol:acetone:water extraction, followed by acidification and a diethyl ether:ethyl acetate extraction. After drying through anhydrous $MgSO_4$, samples were evaporated to dryness on a hot plate and reconstituted in HLPC-grade acetone. All free and esterified

samples were reconstituted in 0.5mL acetone, and bound samples were reconstituted in 1.5mL as in Chapter 2. Samples were then analyzed via GC-MS using nearly the same instrument parameters as stated in Chapter 2. For this experiment, the post-run GC column clean was completed at 330°C, and the MS transfer line was also held constant at that temperature. In addition, a fluted inlet was used. Due to the lower number of seeds used for this experiment than with the spermosphere study, linoleic and oleic acids in the bound caryopsis samples were within the range of detection and thus samples were not re-run to quantify those compounds alone, and 4μL of sample was used for the bound hull samples. Samples were run one replicate at a time in a randomized order, with quality controls present to monitor instrument sensitivity. After noting major inconsistencies in the data after processing, two replicates were re-run using 11μL BSTFA + 1% TMCS rather than 5.4μL as noted in previous method. The need for this was most likely due to the high amounts of fatty acids present, which had been removed via a hexane extraction during method development in Gallagher *et al*. (2010). Volumes of samples were adjusted to keep the total subsample volume at 50μL. For the re-run replicates, 39μL sample was used for free and esterified samples, 10μL for bound caryopsis samples, and 4μL for bound hull samples. HPLC-grade acetone was added to bound samples to reach the desired subsample volume. Data processing and statistical analysis were completed in the same manner as in Chapter 2. Tukey's HSD (95% CI) was used for post-hoc tests between groups.

RESULTS

Overall, the concentrations of compounds were similar to those reported in Chapter 2. Likewise, the proportion of total compounds in the free, esterified, and bound fractions was also similar. The bound fraction dominated all compound groups, and the majority of phenolic and

aliphatic organic acids were found in the hull while the majority of fatty acids were found in the caryopsis. As noted previously, these seed concentrations of compounds agree with those reported in Gallagher *et al*. (2010). The only major difference in the present data were the concentrations of aliphatic organic acids, which tended to be approximately 4x higher in total than reported in Chapter 2, likely due to more complete derivitization by the higher amount of derivitizing agent used (BSTFA + 1% TMCS). One replicate exhibited much lower compound concentrations than the other three, and thus only the three with reasonable quantification were used for analysis. This discrepancy was likely due to error in the GC-MS analysis for that replicate.

Unlike the results observed in the spermosphere study, where seed grow-out years did not differ in their response to treatments, the two seed grow-outs differed substantially in their response to fungal attack in this study. This difference varied depending on the compound, compound group, and chemical fraction, but was especially prevalent in the seed phenolic and long-chain fatty acid concentrations. As a result, the two seed sources (2005 and 2006) were analyzed separately. Seeds from the 2006 grow-out averaged ~149,000 $ng \cdot seed^{-1}$ total phenolics, compared to ~140,000 $ng \cdot seed^{-1}$ total phenolics in 2005 grow-out seeds. This difference was more prevalent in the caryopses than in the hulls, where the total phenolic concentration was 41% higher in the 2006 grow-out seeds (Table 3.1). There was also a substantial difference in total long-chain fatty acid concentration, with the 2006 grow-out seeds averaging ~1,581,000 $ng \cdot seed^{-1}$, compared to ~1,000,000 $ng \cdot seed^{-1}$ in the 2005 grow-out seeds.

Fungal attack had the most significant effect on the de-hulled caryopses (Table 3.1), and the greatest seed response was an increase in phenolic compounds. In 2005 caryopses, the free phenolic concentration increased ~350% and ~500% in response to *F. avenaceum* and *F.*

culmorum, respectively. This response was even greater in the 2006 caryopses, with the concentration increasing ~650% and ~450% in response to *F. avenaceum* and *F. culmorum*, respectively (Figure 3.3A). The phenolic acids ferulic, *p*-coumaric, vanillic, and syringic had the greatest statistically significant contributions to these effects. Though the concentrations of esterified phenolics were greater, there was still an increase during fungal attack. However, this effect was greater in the 2005 caryopses than 2006. Caryopses from the 2005 grow-out responded to fungal attack by *F. avenaceum* and *F. culmorum* with 115% and 177% increases in esterified phenolic concentrations, respectively, compared to 40% and 73% increases in the 2006 seeds. No changes were observed in the bound phenolic concentrations in either seed year.

Table 3.1. Compound concentrations in de-hulled caryopses of 2005 and 2006 wild oat grow-outs exposed to *F. avenaceum* and *F. culmorum*. Free (F), esterified (E), bound (B), and total chemical compound fractions are shown. Significance is indicated within seed year columns ($\alpha = 0.05$).

	Total Phenolics, ng · seed^{-1}				Total Aliphatics, ng · seed^{-1}				Total Long-Chain Fatty Acids, ng · seed^{-1}			
	F	E	B	Total	F	E	B	Total	F	E	B	Total
2005												
Control	22^{a}	247^{a}	6265^{a}	6734^{a}	30^{a}	187^{a}	1526^{a}	1743^{a}	3425^{a}	198386^{a}	471338^{a}	673149^{a}
F. avenaceum	101^{ab}	531^{ab}	6142^{a}	6774^{a}	573^{c}	265^{a}	2232^{a}	3070^{a}	4698^{a}	257738^{a}	1140292^{b}	1402729^{a}
F. culmorum	131^{b}	684^{b}	6258^{a}	6844^{a}	330^{b}	538^{a}	2198^{a}	3150^{a}	10986^{a}	286406^{a}	523347^{ab}	725271^{a}
2006												
Control	35^{a}	446^{a}	9356^{a}	9837^{a}	180^{a}	508^{ab}	1953^{a}	2641^{a}	88005^{ab}	388131^{ab}	940010^{ab}	1416145^{a}
F. avenaceum	262^{b}	622^{ab}	7408^{a}	8293^{a}	77^{a}	246^{a}	2229^{a}	2552^{a}	6715^{a}	244142^{a}	1451364^{a}	1702221^{a}
F. culmorum	193^{b}	772^{b}	9627^{a}	10592^{a}	198^{a}	596^{b}	1408^{a}	2202^{a}	138294^{b}	410943^{b}	778273^{b}	1327511^{a}

There was a significant increase in free aliphatic concentration in the 2005 caryopses in the fungal-exposed seeds (Table 3.1, Figure 3.2), with *F. culmorum* causing a 1000% increase and *F. avenaceum* causing an 1800% increase. While increases in the esterified fraction of the 2005 seeds were also noted, they were not statistically significant. The same was true for the bound fraction. The 2006 caryopses responded in a very different way, perhaps due to the higher initial concentrations of aliphatics, as shown by controls. Caryopses exposed to *F. avenaceum*

had lower amounts of free and esterified aliphatics, on average, than controls, though the differences were not statistically significant. There was a significant difference between *F. avenaceum* and *F. culmorum* exposure in the 2006 seeds, with the seeds exposed to the former having significantly lower esterified aliphatic organic acid concentration than the seeds exposed to the latter. As with phenolics, the total aliphatic organic acid concentration did not differ between treatments, largely due to the lack of difference in the bound fraction, which dominates the total concentration.

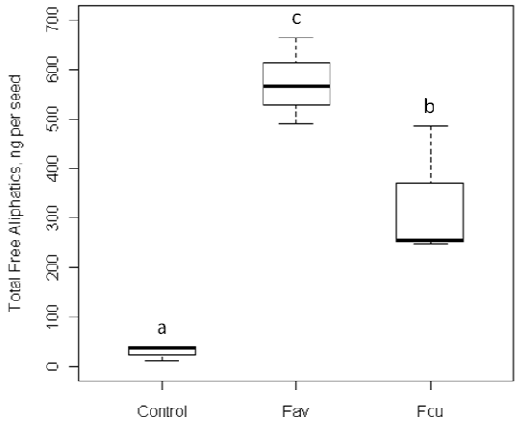

Fig 3.2. Total free aliphatic concentrations in 2005 de-hulled control, *F. avenaceum* (Fav), and *F. culmorum* (Fcu) caryopses. ($\alpha = 0.05$)

The long-chain fatty acid data were somewhat more variable, especially in the 2006 caryopses (Table 3.1). There were no significant differences between fungal exposure treatments in the 2005 seeds with the exception of the *F. avenaceum*-exposed caryopses having significantly higher bound fatty-acids compared to the control. This trend was not seen in 2005 caryopses exposed to *F. culmorum*. Significant differences in the three fatty-acid chemical fractions were seen between the two fungal exposures in the 2006 caryopses, but neither differed significantly from the control. In the free and esterified fractions, caryopses exposed to *F. avenaceum* had significantly lower fatty acid concentrations than those exposed to *F. culmorum*, and in the bound fraction *F. culmorum* had the higher fatty acid concentration.

Table 3.2. Total phenolic concentrations in whole-seed caryopses of 2005 and 2006 wild oat grow-outs exposed to *F. avenaceum* and *F. culmorum*. Free (F), esterified (E), bound (B), and total chemical compound fractions are shown. Significance is indicated within seed year columns ($\alpha = 0.05$).

	Total Phenolics, ng · seed^{-1}			
	F	E	B	Total
GH05				
Control	34.6[a]	392[a]	7058[a]	7485[a]
F. avenaceum	41.2[a]	537[a]	5339[a]	5917[a]
F. culmorum	93.7[b]	491[a]	8656[a]	9241[a]
GH06				
Control	67.1[a]	504[a]	7454[a]	8025[a]
F. avenaceum	37.1[a]	538[a]	8009[a]	8584[a]
F. culmorum	92.4[a]	465[a]	6321[a]	6787[a]

While there was significant reaction of de-hulled caryopses to fungal attack, the presence of a hull around those caryopses reduced this effect (Figure 3.3, Table 3.2). Aliphatic organic acid and long-chain fatty acid concentrations in whole-seed caryopses did not differ significantly between fungal treatments and controls. Total phenolics, the group of compounds that changed the most during fungal attack of the de-hulled caryopses, also changed little in whole-seed caryopses, but did display some patterns (Table 3.2). In both the seed years, there was an increase in free phenolics during *F. culmorum* attack, and this effect was significant in the 2005 seeds. The primary individual phenolics contributing to this were ferulic, vanillic, and *p*-coumaric acids (Figure 3.4). While they did not differ significantly between fungal treatments and controls, their contribution to the total was substantial. There was a drop in the total phenolic concentration in 2006 whole-seed caryopses, but the difference was not statistically significant.

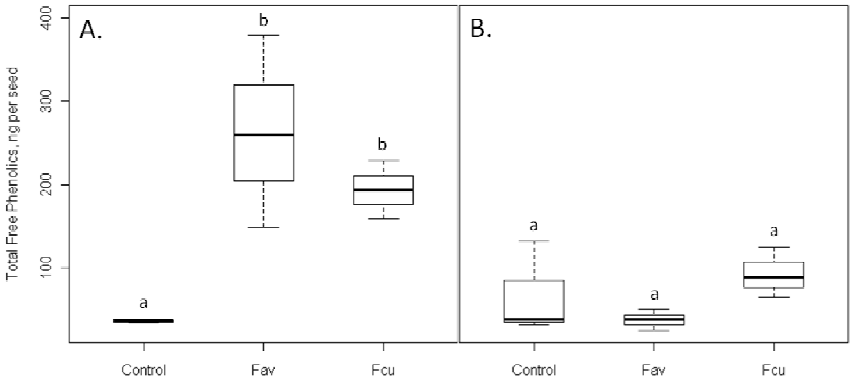

Fig 3.3. Total free phenolic concentrations in 2006 de-hulled caryopses (A) and whole-seed caryopses (B) in control seeds and *F. avenaceum* (Fav) and *F. culmorum* (Fcu) treatments. Significance applies within graph pane (α = 0.05).

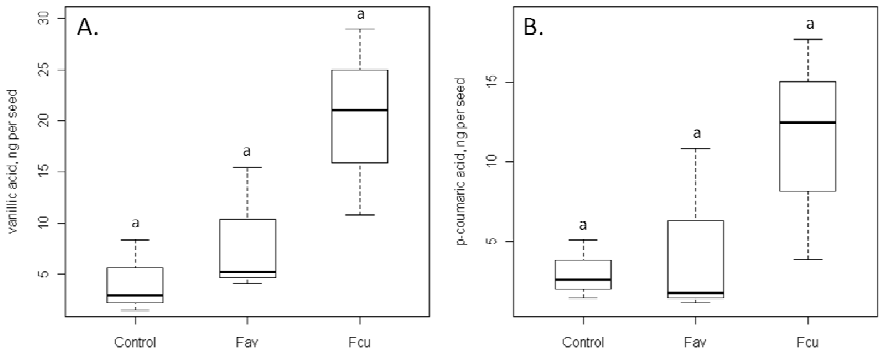

Fig 3.4. Free fraction vanillic (A) and *p*-coumaric (B) acid concentrations in 2005 whole-seed caryopses exposed to *F. avenaceum* (Fav) and *F. culmorum* (Fcu). Note difference in y-axis scales.

The hulls, as with the whole-seed caryopses, had less of a response than did the de-hulled caryopses and the response also depended on the seed year (Table 3.3). In the 2006 seed hulls, the average total free phenolics increased 300% (222 ng·seed^{-1} change) after exposure to *F. avenaceum* ($p = 0.07$), and the average total esterified phenolics increased 168% (526 ng·seed^{-1} change) with *F. avenaceum* attack ($p < 0.05$) (Table 3.3). A number of the major hull phenolic compounds contributed to changes in total free phenolics (Figure 3.5). Ferulic, *p*-coumaric, and syringic acids all increased significantly in concentration during *F. avenaceum* attack, and vanillic acid decreased substantially due to *F. culmorum* exposure ($p = 0.07$). Esterified ferulic, *p*-coumaric, and syringic acids also significantly increased in concentration, contributing to the

trend in total esterified phenolic compounds. There were no differences in bound phenolic concentration regardless of fungal treatment or seed year.

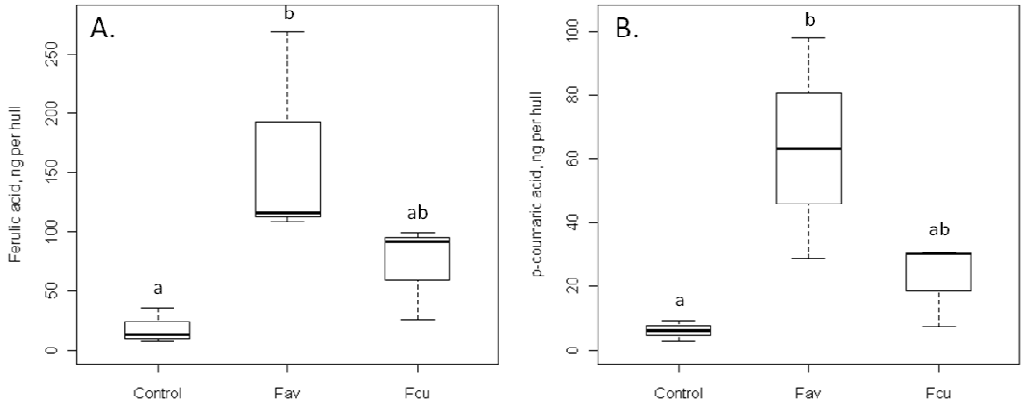

Fig 3.5. Free ferulic (A) and *p*-coumaric (B) acid concentrations in 2006 seed hulls exposed to *F. avenaceum* (Fav) and *F. culmorum* (Fcu) ($\alpha = 0.05$). Note difference in y-axis scales.

Table 3.3. Total phenolic concentrations in seed hulls of 2005 and 2006 wild oat grow-outs exposed to *F. avenaceum* and *F. culmorum*. Free (F), esterified (E), bound (B), and total chemical compound fractions are shown. Significance is indicated within seed year columns ($\alpha = 0.05$).

	Total Phenolics, ng · hull-1			
	F	E	B	Total
GH05				
Control	111[a]	655[a]	123782[a]	124548[a]
F. avenaceum	209[a]	625[a]	123183[a]	124017[a]
F. culmorum	294[a]	941[a]	149038[a]	150273[a]
GH06				
Control	72[a]	313[a]	142869[a]	143254[a]
F. avenaceum	295[a]	839[b]	143402[a]	144536[a]
F. culmorum	124[a]	269[a]	133213[a]	133606[a]

While there were no significant differences in aliphatic organic acid concentration due to fungal treatment, long-chain fatty acid concentrations in the hulls showed some response. Total fatty acid concentration was 80% higher (2608 ng · seed^{-1} change) in 2005 seed hulls exposed to *F. culmorum* ($p < 0.05$). This trend was caused largely by a 125% (692 ng · seed^{-1}) increase in linoleic acid ($p < 0.05$). Oleic acid also increased substantially, with the hulls of *F. culmorum*

exposed seeds averaging an 80% (750 ng·seed^{-1}) higher concentration. Linoleic acid also increased significantly in 2005 seed hulls due to attack by *F. avenaceum*, with an average concentration 100% (555 ng·seed^{-1}) higher than the control hulls.

DISCUSSION

The major overall response of seeds to fungal attack was an increase in phenolic compounds in the free and soluble-ester forms. In some seed and chemical fractions, this increase was greater than 6-fold. A number of previous studies have linked higher tissue phenolic content to greater disease resistance (Cvikrova *et al*., 1995; Nicholson and Hammerschmidt, 1992) and noted increases in tissue phenolic concentrations as a response to fungal elicitors or direct fungal infection (Mandal and Mitra, 2007; Fernandez and Heath, 1989; Matern and Kneusel, 1988). However, most studies focus on mature or actively developing plant tissue, and few study plants at the seed stage. Davis *et al.* (2008) found a negative correlation between seed mortality in the soil and phenolic content, possibly indicating that higher phenolic content helps protect the seed. To our knowledge, no other previous studies have investigated the phenolic content of physiologically dormant seeds that, in nature, would be major contributors to the soil seed bank. In addition, the study by Davis *et al*. (2008) studies a relatively narrow scope of phenolic compounds, and does not investigate varying phenolic content within species or as an active seed defense. Results from the present study indicate that dormant wild oat seeds in the soil have the ability to actively increase the concentration of defense compounds in their tissue, which would contribute to longer seed life-spans in the soil.

The origins of these increases are not completely clear. The seeds may be synthesizing these compounds *de novo* in the cells, but a redistribution of phenolics and aliphatics from sinks

that were not quantified in this study is also possible, such as more complex cell components or precursors not quantified via GC-MS. In addition, the possibility of these compounds originating from the fungi themselves must be taken into consideration. While majority of the hyphae were removed from the surfaces of the seeds, there was still some present that may have been extracted and contributed to these increases. However, due to the number of previous studies noting the increase of similar compounds in active tissue due to fungal elicitation, we believe that the chemical change did originate from the seeds.

The plant responses to pathogens noted in some studies include the increase in cell wall-bound phenolics (de Ascensao and Dubery, 2003). In the present study, there was no effect of fungal exposure on cell wall-bound phenolics, whereas de Ascensao and Dubery (2003) found a 6.5-fold increase in cell wall-bound phenolics in *Musa acuminata* roots exposed to fungal elicitors. In dormant seeds, the presence of wall-bound phenolics could act as more of a passive defense. In addition, tissue concentrations of bound phenolics in the caryopses and hulls of wild oat seeds found in this study were vastly higher than the concentrations found in *Musa acuminata* roots in de Ascensao and Dubery (2003), and thus it is understandable that the seed would not invest energy into increasing the already high bound phenolic concentrations. Mature, active plants may also have a greater ability to reinforce chemical defenses such as bound phenolics. In addition, another plant response to pathogen attack reported in a number of studies was an increase in lignification in plant tissues (Walter *et al*., 1988; Grand *et al*., 2007; Mandal and Mitra, 2007). A higher concentration of lignin would act as a physical barrier to pathogen attack, as many fungi and bacteria have difficulty breaking it down. In the wild oat seeds in this study, it is unlikely that the extraction procedures used allowed for the quantification of lignin, as it is difficult to degrade chemically. As a result, we cannot say whether the wild oat seeds

increased lignin concentration in response to *Fusarium* exposure. However, a number of compounds that are precursors to lignin were present in the seeds and changed in concentration during fungal exposure. Ferulic, *p*-coumaric, syringic, and vanillic acids were all found to increase in the caryopses of wild oat seeds exposed to pathogenic fungi, and all have roles in the formation and structure of lignin (Burges *et al.*, 1964; Hartley, 1972; Vance *et al.*, 1980). While confirming the actual formation of lignin would require enzyme activity assays, the increased presence of lignin precursors indicates to potential for increased lignification. In some wild oat hulls, free vanillic acid was found to decrease during fungal exposure, potentially indicating the production of lignin polymers that would not be quantified in this study. However, increases in the free fraction of other lignin precursors were found in the same hulls, confounding the vanillic acid speculation.

A number of differences were found in seed responses between the two *Fusarium* species used for wild oat exposure. In some seeds and fractions, there was a greater response to *F. avenaceum* than *F. culmorum*, and in other seeds and fractions the opposite was true. In many cases, *F. culmorum* exposure elicited a greater response in phenolic content. In the wild oat hulls, there was a greater phenolic response to *F. avenaceum*. This could also be related to the rate of infection, or it could be a result of slight differences in the growth and infection modes of the two fungal species.

Overall, the data from this study show that dormant wild oat seeds actively respond to fungal attack. This may contribute to wild oat seeds' persistence in the soil and this species' formation of substantial soil seed banks. While this is only one weed species, it is possible, even likely, that other weed seeds that form persistent seed banks also respond to soil microorganisms and pathogens in similar ways. In addition, plant seeds exposed to non-pathogenic

microorganisms may also exhibit similar responses (Ozer, 2011), and thus have a preemptive protection against pathogenic soil microbes. Other seed species should be studied in a similar way to determine their reaction to pathogens, and to gain a broader understanding of the mechanisms of seed persistence in the soil.

Chapter 4: CONCLUSIONS AND FUTURE DIRECTION

Wild oat (*Avena fatua* L.) seeds are known to form soil seed banks (Thompson *et al*., 1997), which help the species persist in the agricultural setting. The study of dormant seeds (Adkins *et al*., 1986) could be integral in determining effective methods for reducing the wild oat soil seed bank and thus controlling wild oat populations and reducing the economic effect this weed has on farmers (Beckie *et al*, 1999). In addition, many other weed species form persistent seed banks in the soil (Thompson *et al*., 1997) and may have similar interactions with their surroundings (Nelson, 2004). Thus, understanding seed-soil-microorganism interactions could become important in controlling many different weed populations.

The present study investigated two different but closely related interactions in the seed-soil-microorganism system. First, wild oat seed changes during imbibition in the soil were investigated, and the spermosphere region around the seeds was studied. During imbibition, the seed hulls were most affected, and the concentrations of a number of compounds were reduced, including both phenolic and aliphatic organic acids. While few conclusions could be made about the spermosphere concentrations of seed exudates, this was probably due to experimental methods rather than the lack of presence of the compounds. Judging by the decrease in the concentration of the seed compounds during imbibition, the spermosphere around wild oat seeds is likely composed of soluble phenolic and aliphatic compounds at relatively low concentrations. Soluble sugars may also be present (Nelson, 2004), but they were not quantified in this study. While the spermosphere concentration of compounds is relatively low compared to the seed concentrations, they may have an effect on microorganisms in the surrounding soil (Keeling, 1974; El-Shatnawi and Makhadmeh, 2001; Ruttledge and Nelson, 1997). Thus, the presence of antagonistic soil fungi (pathogens and saprophytes) could negatively impact the number of

dormant seeds in the soil, helping to control weed populations. However, stimulation of non-pathogenic organisms could create competition between microbes and reduce this effect. Microbe-microbe interactions in the spermosphere should be studied more thoroughly to gain a better understanding of the ecology of the spermosphere.

The second interaction studied was the reaction of wild oat seeds to direct exposure to two pathogenic fungal species. Here, a stronger reaction was seen in the caryopses, where the soluble forms of phenolics and aliphatics increased in concentration as a result of fungal attack. Phenolic compounds have been highlighted numerous times as being important in plant defense against microbes (Cvikrova *et al*., 1995; Nicholson and Hammerschmidt, 1992; de Ascensao and Dubery, 2003; del Rio *et al*., 2004), and the findings of this study further confirm these observations. It is important, however, that we now have a better understanding of these reactions at the seed level, as this is an important and vulnerable stage in the plant's life. In addition, the use of dormant seeds that are similar to those that would be found in a persistent seed bank shows that seeds employ induced chemical defenses to remain viable in the soil. The control of soil seed banks has been discussed previously as an effective way of controlling weed populations (Boyetchko, 1996; Dalling *et al*., 2011), and understanding how dormant seeds defend themselves in the soil is an early step in developing seed bank control methods.

Though studied separately here, seed exudation and the spermosphere and seed defense responses are obviously not independent processes. Both likely occur simultaneously in the soil. By combining the results of the two experiments performed in the present study, it is possible to speculate on what changes a wild oat seed might experience while dormant in the soil. During imbibition, a number of compounds would be lost from the hull, but the presence of the hull likely reduces this effect in the caryopsis. Thus, the caryopsis initially remains relatively

unchanged and viable, though over many seasons it may be affected in a similar way to the hull. The seed exudation may stimulate microbial activity in the spermosphere (Nelson, 2004), and the seed would come under attack by some soil microorganisms. As shown by the lower degree of defense reaction in whole-seed caryopses (Chapter 3), the hull provides a high degree of protection, but extended exposure would likely induce defense response in the caryopses similar to those seen in de-hulled caryopses in this study. This response would be an increase in the soluble fractions of phenolic compounds and potentially the increase in lignification. Overall, the seed has lost soluble compounds out of the hull due to imbibition, but gained phenolics and aliphatics in the caryopsis as a defense response. As a result, the net seed gain or loss of compounds is minimized, but the concentration of soluble phenolics and aliphatics may shift towards the caryopsis over time.

While this is purely speculation, it demonstrates the possibility of very complex interactions occurring in the seed-soil-microbe system. Though the present study begins to explore these interactions, the complexity of the system requires much more extensive study if effective seed bank weed control methods are to be developed. Results from this study indicate that creating soil conditions favorable to fungal growth could lead to pathogen stimulation not only through those conditions but also through seed exudation, and cause seeds to be more vulnerable through the loss of compounds important to defense and metabolism. However, these conditions could also have the same detrimental effects on crop plants as weed seeds, and thus further study is needed to determine the proper timing and degree of management to tip the competitive scale towards crops. Since seed bank management would likely be a long-term (multi-year) weed control strategy, it is possible that methods could be implemented during non-growing or fallow seasons, avoiding the direct effects they could have on crops. While we

believe the phenolic and aliphatic increases were seed responses to fungi and not merely compounds present in the hyphae in the seeds during attack, this would be further clarified by extracting fungal hyphae directly and analyzing chemical content. In addition, dead seeds, killed through irradiation or some other technique, could be exposed to fungi to elucidate whether the active, viable seeds were synthesizing compounds and reacting to fungi, or if phenolic and aliphatic responses were an artifact the chemicals present in fungi. Future studies should also explore the interactions of other weed species' seeds with their surroundings, how varying soil conditions affect these interactions, and how we might create soil conditions that are detrimental to weed seed banks while favoring the desired crops. The current research does suggest that manipulating the system by creating conditions favorable to microbes antagonistic to dormant weed seeds has potential in controlling weed populations, and also suggests mechanisms that crop seeds may use to resist disease while in the soil.

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