INTERACTIONS OF ROOT ROT PATHOGENS AND PEA LEAF WEEVIL IN FIELD PEA

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DEDICATION

For my parents, who always believed in me; my brothers, who always inspired me; and for Willy, whose love and patience has kept me going.

"Science is not meant to cure us of mystery, but to reinvent and reinvigorate it."

- Robert Sapolsky

ABSTRACT

Additive or synergistic interactions between *Aphanomyces euteiches, Fusarium* spp., and the pea leaf weevil *Sitona lineatus* have the potential to increase yield loss in commercial pea crops. Greenhouse studies indicated that *A. euteiches* and *S. lineatus* are significant risk factors for increased severity of fusarium root rot. Sensitive, species-specific primers and probes for *A. euteiches* and field pea were developed, and multiplex qPCR indicated significant changes in colonization dynamics when multiple pathogens were present. qPCR assays also demonstrated that *A. euteiches* and *Fusarium* spp. co-infect pea roots under field conditions. The fungicides ethaboxam and fludioxonil did not reduce disease severity in pea or faba bean during a two-year field study. Thiamethoxam reduced nodule and foliar herbivory, whereas lambda-cyhalothrin and nitrogen fertilizer had no impact. No single or combined treatment improved yield or seed size, therefore an effective method of managing these constraints to pea production is still required.

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°C – degree Celsius
μ - micro
ai – active ingredient
ANOVA – analysis of variance
ARR – Aphanomyces root rot
BCA – biocontrol agent
BLAST – basic local alignment tool
bp – base pairs
Bt – *Bacillus thuringiensis*
CMC – carboxymethylcellulose
COX1 – cytochrome c oxidase subunit 1
C_t – cycle threshold
dNTP – deoxynucleotide triphosphate
DNA – deoxyribonucleic acid
fg – femto
FHB – Fusarium head blight
FRR – Fusarium root rot
Fsp – *Fusarium solani* f. sp. *pisi*
g – gram
ha - hectare
IPM – integrated pest management
ITS – internal transcribed spacer
L - litre
LSU rDNA - large subunit ribosomal deoxyribonucleic acid
m - metre
m – milli
M – molar
n – nano
N - nitrogen
NCBI - National Centre for Biotechnology Information
PDA – potato dextrose agar
PLW – pea leaf weevil
PRRC – pea root rot complex
qPCR – quantitative polymerase chain reaction
rDNA – ribosomal deoxyribonucleic acid
rRNA – ribosomal ribonucleic acid
s – second
SDW – sterile distilled water
SNA – synthetic nutrient agar (Spezieller Nährstoffarmer agar)
T_m – melting temperature

CHAPTER 1: LITERATURE REVIEW

1.1 PATHOGENS AND INSECT HERBIVORY AS CONSTRAINTS TO CANADIAN FIELD PEA PRODUCTION

International demand for pulses, the dried edible seed of several legume species, has led to a steady increase in the production of crops like dry peas, dry beans, lentils, and chickpeas in western Canada (Statistics Canada, 2015). Large expansions in production over the last four decades have put Canada at the forefront of the global market for a variety of pulse crops. Field pea (*Pisum sativum* L.) is grown in particularly high volume: 4.8 million tonnes of pea was produced in the 2016-2017 crop year, 3.9 million tonnes of which was exported (Agriculture and Agri-Food Canada, 2018). Like most legumes, *P. sativum* seed is high in protein, amino acids, fibre and several micronutrients, making it an important food source for humans and livestock (Jacob *et al*., 2016). Additionally, the ability of legumes to increase soil fertility through symbiosis with nitrogen-fixing bacteria has made them an important addition to crop rotation, as reduced fertilizer use lowers both production costs and the risk of water and atmospheric pollution (Jacob *et al*., 2016). However, vast increases in production area and short cropping intervals have facilitated the spread of pathogens and the range expansion of insect herbivores (Xue, 2003a; Olfert *et al*., 2012). Phytopathogens and herbivorous insects can cause extensive crop damage and yield loss, thereby posing a significant threat to the continued production of field pea and other susceptible pulse crops (Olfert *et al*., 2012; Gossen *et al*., 2016).

Root rot disease has become particularly problematic for pea producers in the Canadian Prairies (Xue, 2003a). Collectively known as the pea root rot complex (PRRC), several fungal and oomycete pathogens are associated with root decay in *P. sativum* (Xue, 2003b; Xue, 2003a). The primary PRRC pathogens include *Fusarium* Link spp., *Sclerotina sclerotiorum* (Lib.) DeBary and *Rhizotonia solani* Kühn, and the oomycetes *Aphanomyces euteiches* Drechs. and *Pythium* Pringsheim spp. (Xue, 2003a; Xue, 2003b; Esmaeili Taheri *et al*., 2016). Reduced germination, damping off, root rot, wilt and

premature death may occur following infection with one or more of these pathogens (Feng *et al*., 2010). Reductions in plant stand density related to root rot disease can increase competition by allowing weeds to become established, further impacting crop development (Chang *et al*., 2013). Root rot pathogens may also have indirect effects on long-term crop resilience, as plants weakened by disease will have a reduced capacity to deal with abiotic stressors such as drought or high temperatures (Hagerty *et al*., 2015). Root rot pathogens have rapidly become pervasive across the Canadian Prairies, and have significantly reduced yields of field pea in these regions (Feng *et al*., 2010; Gossen *et al*., 2016).

A number of factors can influence the community structure of the PRRC. These include crop rotation, crop sequence, soil type, soil compaction, soil moisture content, host growth stage, and crop management practices (Esmaeili Taheri *et al*., 2016). However, *Fusarium* spp. are the pathogens isolated most frequently from commercial pea fields in Alberta, Saskatchewan and Manitoba (Chang *et al*., 2005; Esmaeili Taheri *et al*., 2016). Of the several *Fusarium* spp. associated with pea, *F. solani* f. sp. *pisi* (Fsp) and *F. avenaceum* have been indicated as the primary causal agents of root rot disease (Feng *et al*., 2010; Esmaeili Taheri *et al*., 2016). *Fusarium* spp. persist in soil by overwintering in crop residues or, in many species, by producing highly resilient chlamydospores (Hargreaves & Fox, 1977; Kraft *et al*., 1993). When spores or mycelia contact a host plant, initial infection typically occurs in the cotyledonary attachment area (Kraft *et al*., 1993). The first symptom of Fusarium root rot (FRR) is the appearance of reddish-brown streaks on lateral and tap roots, which later coalesce to form black lesions most prevalent in the epi- and hypocotyl regions. Red staining of vascular tissue, visible root disintegration, and complete destruction of lateral roots and nodules may also occur (Kraft *et al*., 1993; Hwang *et al*., 1994). Above ground, plants may appear stunted, with leaves that become chlorotic, wilted, and eventually necrotic as vascular tissues become increasingly blocked (Kraft *et al*., 1993). *Fusarium* spp. are typically regarded as

opportunistic pathogens, though a variety of environmental stressors such as soil compaction, high temperature, excessive soil moisture or drought can increase the incidence and severity of disease (Chang *et al*., 2005; Harveson *et al*., 2005; Esmaeili Taheri *et al*., 2016). Yield losses related to FRR of up to 60% have been reported in field pea (Chang *et al*., 2005).

Aphanomyces euteiches has recently been identified as another pathogen of significant concern for Canadian pea producers (Banniza *et al*., 2013; Chatterton *et al*., 2015). Like other protist species within the class Oomycota, *A. euteiches* physiologically and morphologically resembles fungi but are phylogenetically distinct (Gaulin *et al*., 2007). *Aphanomyces euteiches* is soil-borne and has a broad host range, infecting the roots of multiple legume species including pea, alfalfa, lentil, dry and faba bean, vetch, clover, and several related wild legume species (Gaulin *et al*., 2007; Hughes & Grau, 2013). Pea and alfalfa are particularly susceptible to *A. euteiches* infection (Gaulin *et al*., 2007). The distribution of this pathogen is equally broad: *A. euteiches* is widespread in Europe, Japan, Australia, New Zealand and the United States, but was not reported in Canada until it was detected in Manitoba in 1980 (Papavizas, 1974; Lamari & Bernier, 1985; Hughes & Grau, 2013). Detection of *A. euteiches* in Saskatchewan and Alberta occurred even more recently, in 2012 and 2013, respectively (Banniza *et al*., 2013; Chatterton *et al*., 2015). Its high prevalence in both provinces, however, indicates that it was likely present for many years prior to detection. *Aphanomyces euteiches* is an aggressive pathogen that is favoured by high soil moisture levels, thus poor drainage and soil compaction are risk factors (Papavizas, 1974; Conner *et al*., 2013). Spread of *A. euteiches* between fields or over long distances occurs when contaminated soil or plant matter is transported from one field, typically on tools or machinery (Papavizas, 1974).

Oospores, a product of sexual reproduction, serve as the primary inoculum source for *A. euteiches* and can persist in soil for more than a decade (Papavizas, 1974; Hughes & Grau, 2013). In the presence of root exudates, oospores will either infect a host plant

directly by germinating into hyphae, or indirectly by producing sporangia. Sporangia give rise to motile zoospores, which locate their host chemotactically and will swim short distances to adhere to and subsequently encyst in root tissue (Gaulin *et al*., 2007; Hughes & Grau, 2013). Infection occurs in the cortex of lateral and taproots, with the first symptoms appearing as water-soaked lesions (Gaulin *et al*., 2007). These lesions will coalesce and become honey-coloured, and extensive cortical decay will follow (Papavizas, 1974; Conner *et al*., 2013; Chatterton *et al*., 2015). The cortex may eventually slough off entirely, exposing the vascular tissue. The epicotyl becomes pinched and necrotic, and plants will appear stunted, chlorotic, and may die prematurely (Lamari & Bernier, 1985). Yield loss is difficult to quantify due to the presence of other pathogens in soil, but reported losses of up to 80% suggest that this may be the most significant obstacle to pea producers in the Prairie provinces (Gaulin *et al*., 2007).

The pea leaf weevil (PLW) *Sitona lineatus* L. (Coleoptera: Curculionidae) is an invasive insect pest that feeds and reproduces on pea and faba bean (*Vicia faba* L.), often causing extensive damage (Jackson & Macdougall, 1920; Lohaus & Vidal, 2010). Already widespread in Europe and North Africa, *S. lineatus* was first observed in North America on Vancouver Island, British Columbia in 1936 (Jackson & Macdougall, 1920; Downes, 1938; Hoebeke & Wheeler, 1985). PLW was reported in southern Alberta in 1997, and has since rapidly expanded its range both northward and east into Saskatchewan (Olfert *et al*., 2012). This expansion can likely be attributed to the increased production area of pea and faba bean in these regions, and may further be facilitated by increasing annual temperatures related to climate change (Vankosky *et al*., 2009). The PLW is a highly mobile insect, with flight periods occurring in early spring and late summer (Hamon *et al*., 1987). During the spring migration, weevils disperse from the shelterbelts and perennial legumes that serve as overwintering sites to pea and faba bean fields to feed and reproduce (Fisher & O'Keeffe, 1979; Landon *et al*., 1995). The high nutritional quality of either crop maximizes adult fertility, and developing larvae

feed on root nodules containing the bacterial symbiont *Rhizobium leguminosarum* Frank (George, 1962; Johnson & O'Keeffe, 1981; Schotzko & O'Keeffe, 1986). Mating and oviposition continues into July, with female weevils laying up to 3000 eggs in a season (Schotzko & O'Keeffe, 1986). The duration of incubation is highly dependent on temperature. At 8°C eggs incubate for 70 ± 2.5 days before hatching, but will hatch in 6.3 ± 0.5 days at 29°C (Lerin, 2004). At 33°C, 100% egg mortality occurs (Lerin, 2004). Eggs are scattered on the soil surface near host plants, and upon hatching larvae immediately burrow beneath the soil and tunnel into root nodules to feed on *R. leguminosarum* (Jackson & Macdougall, 1920; Johnson & O'Keeffe, 1981). Feeding continues over one to two months as larvae develop over five instars, with resulting damage ranging from partial to complete excavation of nodules (Jackson & Macdougall, 1920; Landon *et al*., 1995). Mature larvae will then pupate in the soil for two to three weeks before emerging as adults (Jackson & Macdougall, 1920). New adults will continue to feed on pea and faba bean until foliage is either depleted or has senesced, at which point they disperse widely to feed on a variety of other legumes before returning to their overwintering sites (Jackson & Macdougall, 1920; Fisher & O'Keeffe, 1979; Hamon *et al*., 1987).

Yield losses in pea and faba bean crops are typically attributed to larval feeding. Defoliation by adult weevils does not typically reduce photosynthesis enough to compromise plant development (George *et al*., 1962; Cárcamo *et al*., 2015). Seedlings can withstand 50% defoliation with no significant impact on yield, thus only intense feeding on newly emerged plants and/or severe feeding that continues throughout the growing season are likely to reduce seed production (George *et al*., 1962; Vankosky, Cárcamo, McKenzie, *et al*., 2011). Population density of larvae reaches a maximum at the early flowering stage of the plant, coinciding with an increase in nodule production that continues until the onset of seed formation (Doré & Meynard, 1995; Lohaus & Vidal, 2010). When infestation is severe larvae may damage up to 98% of the root nodules on an

individual plant, depriving it of nitrogen fixed by *R. leguminosarum* and forcing it to rely on nitrogen reserves in the soil (El-Dessouki, 1971; Cárcamo *et al*., 2015). Losses in yield and seed quality are thus anticipated if soil is nitrogen deficient, and low accumulation in plant tissues may reduce soil nitrogen reserves for subsequent crops (Doré & Meynard, 1995; Lohaus & Vidal, 2010; Cárcamo *et al*., 2015). Yield losses due to larvae are not easily quantified due to the difficulty of eliminating adult feeding in field studies. Regardless, losses of up to 28% in faba bean (Nielsen, 1990) and 27% in pea have been reported (El-Dessouki, 1971).

1.2 The Potential for Interspecific Interactions

In both a natural and agricultural context, plants associate with neutral, beneficial, and parasitic microbes and invertebrates (Pieterse & Dicke, 2007; Pal & Gardener, 2011). These organisms may independently modify their host; in the case of agricultural pests, it is these independent effects that are typically investigated (Jesus Junior *et al*., 2014). However, the sharing of a mutual host can facilitate interspecific interactions that may alter individual effects, complicating diagnoses and making infestation patterns and yield loss difficult to predict (Hatcher, 1995; Jesus Junior *et al*., 2014). Despite this, the effect of pathogen complexes on plant development remains largely unknown, and the interface between studies of plant-pathogen and plant-insect interactions tends to be limited (Hatcher, 1995; Jesus Junior *et al*., 2014). This has left a substantial gap in our understanding of the combined effects of the most significant threats to pea production in Canada: root rot pathogens and pea leaf weevil herbivory.

Interspecific pathogen-pathogen and insect-pathogen interactions may have multiple consequences, including modifications to microbe and herbivore fitness, community structure, and host plant performance (Fournier *et al*., 2006). In order to understand and predict these consequences, it is first necessary to understand the manner in which interactions are taking place. If a plant has multiple exploiters that do not

interact, the combined effect of these exploiters will be equal to the sum of their individual resource consumption; the resulting damage is therefore defined as additive (Hatcher, 1995; Fournier *et al*., 2006). In the case that interactions are occurring, the abundance and fitness of plant exploiters may be altered, resulting in a combined impact to the host that is either less or greater than their individual effects. In this case, the damage to the host is non-additive. If plant exploiters interact in a mutualistic or commensalistic manner, they will synergistically increase damage to the plant (Fournier *et al*., 2006). Conversely, antagonistic interactions will occur when parasites compete for plant resources. Interspecific interactions can also be described as occurring either directly or indirectly (Hatcher, 1995). A parasite may directly alter the ability of another to establish on or otherwise access host resources. For example, an insect may consume fungal mycelia or spores, or a phytopathogen may infect a plant more efficiently when colonizing insect feeding wounds (Hatcher, 1995). Indirect interactions, also referred to as plant-mediated interactions, are more complex (Stout *et al*., 2006). Insects and microorganisms often induce physical and physiological changes in their plant hosts, resulting in a modified response to other organisms. Indirect interactions can therefore be far more difficult to detect, especially when exploiters are temporally or spatially separated (Stout *et al*., 2006). Plant compensatory responses are a particularly important example of an indirect effect as they have the potential to markedly change how parasites and herbivores impact overall host performance (Fournier *et al*., 2006; Pal & Gardener, 2011). Such responses may include the induction of plant defense pathways or compensatory regrowth of roots, foliage or other organs. Plants can cope with pathogens, insects, or both to a certain degree, but rapid degeneration may occur once the threshold of compensation is surpassed (Fournier *et al*., 2006). In this way parasites that interact additively or competitively may still synergistically influence plant performance under certain circumstances (Fournier *et al*., 2006). Accurate identification and effective

management of root rot disease will therefore benefit from the consideration of interspecific interactions between soil-borne pathogens and pea leaf weevil herbivory.

1.2.1 Quantification of Interspecific Interactions

The impacts of interactions involving plants, phytopathogens and insects are often quantified by estimating changes in microbe and insect population dynamics (e.g. Kerr, 1963; Leath & Hower, 1993; Kalb *et al*., 1994; Malvick *et al*., 1994; Friedli & Bacher, 2001; Bacher *et al*., 2002; Peters & Grau, 2002). Conventional methods of identifying and quantifying host-pathogen interactions include microbial isolations from diseased plant tissue or soil, bioassays to make semi-quantitative estimates, or assessment of disease symptoms using various indices of disease severity (Malvick *et al*., 1994; Bock *et al*., 2010). Pathogen culture on artificial media can be useful for morphology-based identification and approximate quantification through dilution plating. Limitations of this method are the exclusion or underestimation of fastidious and slow-growing microorganisms, difficulty in distinguishing between closely related fungal species or isolates, and the considerable skill, time, and resources required to achieve accurate identification (Paulitz, 2000; Gossen *et al*., 2016). Bioassays, such as soil indexing, most probable number and rolled-towel assays, can be used to estimate the quantity of soil-borne phytopathogens in a sample (Malvick *et al*., 1994; Paulitz, 2000). Bioassays are more inclusive of pathogens that are difficult or impossible to isolate using artificial media and can differentiate between pathogenic and non-pathogenic isolates (Paulitz, 2000). Interspecific interactions can be detected by comparing single versus multiple pathogen inoculations, or through simultaneous exposure of plants to pathogens and herbivores. Extrapolating the results of laboratory bioassays to the field must be done with caution, however, as pathogen growth is strongly influenced by biotic and abiotic environmental conditions that may not be consistently replicated under artificial growth conditions (Lamichhane & Venturi, 2015).

Real-time quantitative PCR (qPCR) is a fast and reliable alternative to conventional methods of microbial quantification. Using species-specific primers, qPCR can detect and quantify phytopathogens in a range of substrates that include soil, water, air, and plant tissue (Paulitz, 2000; Schena *et al*., 2004; Li *et al*., 2008). qPCR differs from endpoint PCR in that it uses fluorogenic probes to measure the amplified PCR product at the end of each thermal cycle (Lievens *et al*., 2006; Li *et al*., 2008). A major advantage to qPCR is that assays can be multiplexed to amplify and detect multiple DNA templates within a single reaction (Schena *et al*., 2004). This increases the efficiency of qPCR as a large-scale detection method, and allows for the use of host DNA as an endogenous control for pathogen quantification (Schena *et al*., 2004). qPCR has been used to detect and quantify *A. euteiches* in soil (Sauvage *et al*., 2007; Gangneux *et al*., 2014), and alfalfa roots (Vandemark *et al*., 2002; Vandemark & Grünwald, 2005), allowing for correlations between observed disease severity and pathogen DNA content in root tissue. The presence of *Fusarium* has been quantified in cereals (Strausbaugh *et al*., 2005), common bean, and soil (Filion *et al*., 2003), and multiplex qPCR has been used to detect and quantify multiple *Fusarium* spp. in field pea (Zitnick-Anderson *et al*., 2018), and maize (Scauflaire *et al*., 2012; Preiser *et al*., 2015).

When plants are subject to insect herbivory and microbial infections, conventional quantification methods similar to those described above may be used. Changes in disease severity and feeding intensity can be measured visually using appropriate indices, and the association of phytopathogens with feeding injury can be determined by culturing affected plant tissues (Leath & Hower, 1993; Kalb *et al*., 1994). The potential for insects to serve as mechanical vectors of phytopathogens can be measured by culturing insect tissues on selective media (Leath & Hower, 1993). The effect of interspecific interactions on insect fitness can be measured by rates of mortality, oviposition, egg quality, and egg size (Friedli & Bacher, 2001; Bacher *et al*., 2002). However, studies of interactions between root rot pathogens and root-feeding insects tend to focus on changes in disease

severity rather than insect fitness, despite the potential impact of both factors on crop yield. Molecular methods are often employed to quantify bacterial and viral phytopathogens that have accumulated in the tissues of herbivorous insects (Atzmon *et al*., 1998; Marzachi *et al*., 1998; e.g. Martini *et al*., 2015), but a similar application to studies of phytopathogenic fungi and oomycetes may shed light on the role of insects in spreading these microorganisms between plants or agricultural sites.

1.2.2 Pathogen-Pathogen Interactions

Research regarding root rot pathogens is dominated by single-pathogen studies while investigations of interactions between root rot pathogens are rare. Furthermore, many studies use conventional quantification methods that are less robust than modern molecular techniques. Regardless, there is evidence that interspecific interactions do occur between root rot pathogens. Kerr (1963) found that *F. oxysporum* f. sp. *pisi* and *Pythium ultimum* Trow interacted strongly in *P. sativum* when co-inoculated. *Fusarium oxysporum* was only mildly pathogenic to a wilt-susceptible pea cultivar when inoculated in isolation, inducing symptoms in only 3 out of 25 plants. In contrast, all plants exhibited symptoms of fusarium wilt when *P. ultimum* was also present. While the mechanism behind this interaction was not explored, these findings may indicate that *P. ultimum* can indirectly facilitate *Fusarium* infection by altering pea defense response. Similarly, a non-pathogenic isolate of *F. solani* interacted synergistically with two virulent isolates of *A. euteiches* when co-infecting pea (Peters & Grau, 2002). It was postulated that co-inoculation of *F. solani* and *A. euteiches* stimulates an increase in pisatin production, a phytoalexin that pathogenic strains of *F. solani* can rapidly degrade via demethylation but that non-virulent strains often cannot (Delserone *et al*., 1999). Pisatin would therefore become concentrated in plant tissues, and it was hypothesized that these conditions favour pathogenicity of *A. euteiches*. *In planta* experiments have shown that *A. euteiches* can tolerate high pisatin concentrations (Pueppke & VanEtten, 1976), but it has not been

demonstrated that the presence of pisatin will increase the severity of aphanomyces root rot (ARR). The mechanism behind this interaction, therefore, remains unclear. Real-time PCR has been used to indicate that an antagonistic interaction occurs between *A. euteiches* and *Phytophthora medicaginis* when co-infecting alfalfa (Vandemark *et al*., 2010). A lower accumulation of *P. medicaginis* DNA in root tissue was observed when both pathogens were present in comparison to plants inoculated with *P. medicaginis* only, whereas the amount of *A. euteiches* DNA did not change regardless of whether or not *P. medicaginis* was present. Multiplex qPCR has also been used to demonstrate that competitive interactions occur between seven *Fusarium* spp. isolated from pea roots (Zitnick-Anderson *et al*., 2018). As molecular methods of quantifying microorganisms in plant tissues become more refined and accessible, the use of real-time PCR and related techniques will become standard in diagnostic and quantitative studies.

Similar to the PRRC, the ascochyta blight disease complex describes a group of fungal phytopathogens that share a mutual host and contribute to significant yield losses (Gossen *et al*., 2011). Symptoms of ascochyta blight include lesions on the leaves, stems, flowers, and pods that will eventually coalesce and become necrotic as the disease progresses (Gossen *et al*., 2011). Several pulse crops are susceptible to ascochyta blight, and pathogen population dynamics vary based on the species of plant host. *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr., *Phoma medicaginis* var. *pinodella* (Jones) Boerema and *Ascochyta pisi* Lib. are the dominant species infecting pea (Le May *et al*., 2009; Gossen *et al*., 2011). Le May *et al*. (2009) investigated direct and indirect interactions between *M. pinodes* and *P. medicaginis* to determine the effects of simultaneous and sequential infection on disease progression. Simultaneous inoculation of both pathogens reduced disease severity and suppressed the development of fungal reproductive structures, whereas disease severity increased when pea plants were first inoculated with *M. pinodes* followed by *P. medicaginis* six days later. The antagonistic effect observed following simultaneous inoculation is likely a result of direct competition

for plant resources during pathogen establishment, or other factors such as antibiosis (Le May *et al*., 2009). When *M. pinodes* infection occurred first, the observed synergistic effect may have occurred as an indirect result of pisatin production. *Mycosphaerella pinodes* is a highly virulent pathogen capable of rapidly demethylating pisatin (Delserone *et al*., 1999), and this may have facilitated infection by the less aggressive *P. medicaginis* (Le May *et al*., 2009). This study therefore underscores the importance of both direct and indirect effects in pathogen establishment and disease progression, and suggests that the timing of infection may similarly impact disease severity related to soil-borne pathogen complexes.

1.2.3 Pathogen-Insect Interactions

Agricultural crops tend to concentrate populations of phytopathogens and herbivorous insects, with individual plants acting as the site of interspecific interactions (Hatcher, 1995). As in the case of pathogen-pathogen interactions, the detrimental effects of insect herbivores and phytopathogens tend to be studied independently. It is, however, becoming increasingly recognized that interactions between plant hosts and their exploiters have an essential role in shaping the community structures of both phytopathogens and herbivores. Defining these interactions can shed light on the ability of plants to cope with taxonomically diverse enemies (Fournier *et al*., 2006; Stout *et al*., 2006). Interspecific interactions therefore have important implications in both an ecological and agricultural context (Hatcher, 1995; Stout *et al*., 2006). Despite their frequent co-occurrence, interactions between root rot pathogens and pea leaf weevil have not been investigated, though each has been researched extensively in isolation. Given the close association of both PRRC pathogens and *S. lineatus* with the root system of their mutual host, the identification of tripartite interactions will be important in the development of effective pest and disease management strategies.

Root rot pathogens may directly interact with *Sitona* spp. by colonizing the feeding wounds inflicted by larvae. For example, larvae of the clover root curculio *S. hispidulus* F. are pests of alfalfa and other forage legumes (*Medicago sativa* L.) (Kalb *et al*., 1994). First-instar larvae feed on *Rhizobium* root nodules, whereas later developmental stages target the lateral and tap roots. Feeding wounds are often severe enough to penetrate both the cortex and vascular cylinder, leaving the surrounding tissues exposed (Kalb *et al*., 1994). *Fusarium* spp. are also closely associated with alfalfa roots, and are responsible for diseases such as wilt, root rot, and crown rot (Leath & Hower, 1993; Kalb *et al*., 1994). Isolations from diseased root tissue have indicated that *Fusarium* spp., particularly *F. oxysporum* and *F. solani,* make up 60 to 75% of the microbial population found in root lesions (Godfrey & Yeargan, 1987; Kalb *et al*., 1994). Furthermore, the severity of disease has been positively correlated with feeding injury inflicted by larval *S. hispidulus* as a result of fungal colonization of feeding wounds (Kalb *et al*., 1994). *Fusarium* spp. have been isolated from the head capsules of *S. hispidulus* larvae, suggesting that these insects have an additional role as a vector (Leath & Hower, 1993).

Given that *S. lineatus* is similar to *S. hispidulus* with regard to life history and proximity to root-rotting pathogens, comparable synergistic interactions may occur in *P. sativum*. Conversely, root rot pathogens may compete with *S. lineatus* larvae for resources. Whereas *S. hispidulus* larvae feed on *Rhizobium* root nodules only during early developmental stages, nodules serve as the sole food source of *S. lineatus* larvae until the onset of pupation (Johnson & O'Keeffe, 1981; Kalb *et al*., 1994). Root rot pathogens such as *Fusarium* spp. and *A. euteiches* reduce the abundance of root nodules through direct infection of nodule tissue or through the destruction of lateral roots (Hwang *et al*., 1994; Chang *et al*., 2013). Legumes are able to compensate for nodule loss by increasing both the number and size of root nodules (Quinn & Hall, 1992). Low to moderate levels of root rot disease may therefore indirectly benefit *S. lineatus* larvae by increasing the quality of

rhizobia root nodules. This presumably would increase feeding activity, providing more colonization sites for soil-borne pathogens. However, low nodulation has been observed when root rot severity is high (O'Rourke *et al*., 2012). Consequently, a crop exhibiting more severe symptoms of root rot would presumably serve as a dead-end host for *S. lineatus* larvae, in which case PRRC pathogens would directly influence larval fitness and mortality.

Indirect interactions between phytopathogens and insect herbivores can be difficult to detect, as there can be a large degree of separation between plant exploiters and extensive overlap in plant response to either threat (Stout *et al*., 2006; Pieterse & Dicke, 2007). For example, FRR in alfalfa was more severe when plants were subjected to above-ground feeding by pea aphids (Homoptera: Aphididae), and winter mortality rates increased when plants were exposed to both *Fusarium* spp. and feeding by either pea aphids or potato leafhoppers (Homoptera: Cicadellidae) during the previous growing season (Leath & Byers, 1977). Similarly, FRR severity in red clover was positively correlated with artificial defoliation (Fulton & Hanson, 1960). The impact of foliar feeding by adult PLW on root rot severity has not been investigated in field pea or faba bean, but has the potential to reduce vigour and increase susceptibility to phytopathogens.

Herbivory is often linked with higher levels of microbial colonization, but phytophagous insects can also benefit from interactions with plant pathogens. The stem-boring weevil *Apion onopordi* Kirby (Coleoptera: Apionidae) has a mutualistic relationship with the rust fungus *Puccinia punctiformis* (Str.) Röhl., both of which attack Canada thistle, *Cirsium arvense* (L.) Scop. (Friedli & Bacher, 2001; Bacher *et al*., 2002). Female *A. onopordi* vector *P. punctiformis* during oviposition, depositing fungal spores into the base of the stem (Friedli & Bacher, 2001). *Puccinia punctiformis* is a slow-growing fungus that requires establishment within the roots of its host to survive the winter. Transfer during oviposition by *A. onopordi* is therefore beneficial to the fungus as it increases the likelihood of mycelia reaching the root system of a newly-infected plant

before winter (Friedli & Bacher, 2001). Interestingly, larval *A. onopordi* developing in thistle shoots colonized by *P. punctiformis* are larger, survive longer, and produce more eggs than those that develop in healthy shoots (Friedli & Bacher, 2001; Bacher *et al.*, 2002). While the mechanism behind this interaction is unclear, infection by *P. punctiformis* may alter the nutritional quality of plant tissues to the benefit of *A. onopordi* (Bacher *et al.*, 2002). *C. arvense* is a highly destructive and invasive weed; therefore this interaction may have important implications for biocontrol (Friedli & Bacher, 2001; Bacher *et al.*, 2002; Ziska *et al.*, 2004). In an important agricultural crop such as *P. sativum*, however, the effects of a mutualistic interaction such as this one could be devastating.

1.3 Management Methods

Managing phytopathogens and insect herbivores in agricultural crops is often extremely challenging. Synthetic pesticides have been successful in some systems, and are advantageous in that they are typically convenient, cost-effective, and easily applied (Pal & Gardener, 2011). Chemical treatments can be problematic when target organisms develop resistance, and toxicity to non-target organisms and environmental contamination are common side-effects (Xue, 2003a; Ehler, 2006; Oerke, 2006). Biocontrol agents (BCAs) can be effective and have advantages over chemical treatments in that they are less ecologically harmful, often have longer periods of efficacy, and may be self-perpetuating (Oerke, 2006; Pal & Gardener, 2011). However, BCAs can be costly, have reduced reliability when environmental conditions are variable, and often require lengthy testing periods before becoming commercially available (Pal & Gardener, 2011). Host-plant resistance, acquired via selective breeding or transgenic methods, can be extremely successful in managing a variety of agricultural pests by providing long-term protection that usually has a low environmental impact (Groot & Dicke, 2002; Quenouille *et al.*, 2014). The wheat gene *Lr34,* for example, confers resistance to the rust species *Puccinia*

triticina and *P. striiformis* in addition to powdery mildew (*Blumeria graminis*) (Krattinger *et al*., 2009). Wheat cultivars with this gene have been used successfully to manage rust in multiple countries for over 50 years. Transgenic crops producing toxins from the bacterial entomopathogen *Bacillus thuringiensis* (Bt) are used in 20 countries and are effective in controlling a broad range of insect herbivores (Carrière *et al*., 2010). Bt crops are associated with increased yield and are considered to be less harmful to non-target insects than chemical pesticides (Carrière *et al*., 2010). However, adaptation of phytopathogens and insects to mechanisms of host-plant resistance is a well-documented phenomenon (McDonald & Linde, 2002; Carrière *et al*., 2010). Furthermore, cultivars with broad resistance are required for multi-pathogen systems such as PRRC (Esmaeili Taheri *et al*., 2016; Gossen *et al*., 2016). Integrated pest management (IPM) strategies, including chemical applications, cultural practices, host resistance, and biocontrol are utilized, offer a more holistic approach to crop protection (Ehler, 2006; Oerke, 2006). In addition to diverse suppressive strategies, the principles of IPM include the simultaneous management of multiple pest species, frequent monitoring of pest species and natural enemies, and abiding by economic thresholds when applying pesticides (Ehler, 2006). Ideally, the practice of IPM will reduce the cost and use of environmentally harmful chemicals while increasing the durability of crop protection measures (Ehler, 2006). In practice, the extensive monitoring, extensive biological information required, and the cost and complexity of simultaneously utilizing multiple suppressive strategies can often be deterring to producers (Ehler, 2006). In short, the management of agricultural pests is rarely straightforward. Currently, there are no satisfactory methods of managing root rot pathogens and PLW herbivory in field pea. An understanding of interspecific interactions may provide additional insights into the development of an effective management strategy.

1.3.1 Management of Fusarium Spp.

Fungicidal seed treatments are the most common method of protecting crops from FRR, in Canada and elsewhere (Chang *et al*., 2013; Gossen *et al*., 2016). The commercially available fungicide Apron Maxx (Syngenta Canada, 2017), containing the active ingredients metalaxyl and fludioxonil, significantly reduced root rot caused by *F. avenaceum* in *P. sativum* (Chang *et al*., 2013) and *V. faba* (Chang *et al*., 2014) in greenhouse and field trials. Similarly, fludioxonil completely suppressed mycelial growth of *F. graminearum* in corn and soybean seedlings grown in a greenhouse, while other fungicides such as captan, azoxystrobin and trifloxystrobin suppressed growth to a lesser extent (Broders *et al*., 2007). It was notable that 12 out of the 28 isolates of *F. graminearum* used by Broders *et al*. exhibited resistance to fludioxonil. Fungicidal efficacy is often limited to early season suppression of soil-borne plant pathogens (Oyarzun *et al*., 1994; Xue, 2003b; Gossen *et al*., 2016). This may reduce the occurrence of seedling blight, but protection extending past the seedling stage is required as *Fusarium* spp. can infect their host at any stage of development.

While fungicides are the standard method of crop protection against FRR, a number of BCAs with activity against *Fusarium* spp. have been identified. Strain ACM941 of the fungus *Chlonostachys rosea* (Link:Fr) Schroers suppressed growth of *F. solani* and *F. oxysporum,* in addition to *M. pinodes, R. solani* and *S. sclerotiorum,* in two *P. sativum* cultivars at a level comparable to the synthetic fungicides thiram and metalaxyl (Xue, 2003a; Xue, 2003b). ACM941 is antagonistic to soil-borne pathogens via mycoparastism, and endophytically colonizes the seed coat, primary root, secondary roots and hypocotyl of a plant host (Xue, 2003a). This suggests that the suppressive effect of ACM941 will persist longer than that of available fungicides. ACM941 also significantly reduced symptoms of fusarium head blight caused by *F. graminearum* in wheat (Xue *et al*., 2009). In the latter case ACM941 was less effective than the fungicide tebuconazole, but nonetheless has the potential to be an important component of an IPM strategy. Non-

pathogenic isolates of *F. oxysporum* similarly inhibited colonization of *P. sativum* roots by *F. solani* in both artificially inoculated and naturally infested soils (Oyarzun *et al.*, 1994). Likewise, inoculation with *R. leguminosarum* or the mycorrhizal endophyte *Glomus mosseae* reduced root rot severity caused by *F. solani* in common bean, and the strength of this effect increased when both symbionts were present (Hassan Dar *et al.*, 1997). Biocontrol has yet to surpass the use of synthetic fungicides for the control of FRR, despite significant progress in the identification of potential BCAs. Further research in this area would improve sustainable management of FRR.

Genetic resistance to *Fusarium* spp. and other components of the PRRC is an ongoing area of research. While no complete sources of resistance to FRR have been identified, partial resistance has been reported in field pea (Hwang *et al.*, 1995; Grünwald *et al.*, 2003; Li *et al.*, 2012) and dry bean (Bilgi *et al.*, 2008; Nicoli *et al.*, 2012; Conner *et al.*, 2014). Five quantitative trait loci (QTL) controlling resistance to Fsp have been located in a pea recombinant inbred line (RIL) population (Coyne *et al.*, 2015), and four QTL associated with resistance to *F. avenaceum* were similarly identified (Li *et al.*, 2012). Microsatellite markers linked to QTL controlling partial resistance to *F. solani* were identified in *P. sativum*, which have the potential to be used in marker-assisted selection for resistance (Feng *et al.*, 2011). In addition, high-yielding *P. sativum* breeding lines with partial resistance to root rot caused by Fsp and complete resistance to wilt caused by *F. oxysporum* have been released (Porter *et al.*, 2014). Genetic resistance likely represents the most sustainable method of managing FRR, and significant progress in locating sources of resistance in pea indicates that the development of cultivars with high levels of resistance may be possible.

Certain cultural practices may reduce crop damage caused by *Fusarium* spp., particularly when used in combination with the aforementioned management strategies. Soil compaction may induce plant stress by decreasing drainage and restricting root growth, and is considered a risk factor for the development of FRR (Harveson *et al.*,

2005). Modified tillage practices, in combination with strategic crop rotation, have been implicated as potential options to manage root rot in field pea (Abawi & Widmer, 2000). However, reports of the efficacy of these practices are often conflicting. Conservation tillage, which retains crop residues on a minimum of 30% of the soil surface post-planting, has been recommended as a method to maintain soil productivity, prevent erosion, and suppress pathogen proliferation (Abawi & Widmer, 2000; Larkin, 2015). Bailey *et al*. (2001) demonstrated that there were no significant differences in FRR severity in field pea sown in plots under zero, minimum or conventional tillage regimes, and that environmental conditions had the greatest overall influence on disease incidence and severity. In contrast, subsoiling decreased root rot severity in soybean caused by *F. solani* f. sp. *phaseoli,* presumably by increasing rooting depth and reducing contact with pathogens concentrated in the upper layer of soil (Burke *et al*., 1972). While disease symptoms were present early in the growing season, subsoiling facilitated the growth of larger, more vigorous roots that were able to regenerate diseased tissues. Similarly, zone tillage decreased disease severity in dry bean caused by *F. solani* f. sp. *phaseoli*, and increased plant vigour, yield, and seed size (Harveson *et al*., 2005). It should be noted, however, that over time conservation tillage may increase the inoculum load of *F. avenaceum* and other *Fusarium* spp. that overwinter in crop residues (Hargreaves & Fox, 1977; Fernandez *et al*., 2008). Crop rotation has the potential to reduce disease incidence and severity by breaking host-pathogen disease cycles through the introduction of non-host crops (Larkin, 2015), but this approach is impractical for *Fusarium* spp. as they can persist in soil well beyond the length of realistic rotations (Hargreaves & Fox, 1977; Hughes & Grau, 2013). Additionally, the broad host range of many *Fusarium* species makes the design of rotations that do not include susceptible plant species exceedingly difficult.

1.3.2 Management of A. euteiches

No effective fungicidal treatments are available for the control of *A. euteiches* (Gaulin *et al*., 2007; Gossen *et al*., 2016). Fungicides registered against other phytopathogenic oomycetes, such as metalaxyl (*Pythium*) and mefenoxam (*Phytophthora*), do not have activity against *Aphanomyces* (Hughes & Grau, 2013). Ethaboxam is registered in Canada against seedling diseases caused by oomycetes in several legume species, and has recently been granted registration for use against ARR in field pea (Nufarm, 2015, 2017). Ethaboxam is currently available as a seed treatment that also includes fludioxonil and metalaxyl for control of *Fusarium, Pythium, Rhizoctonia,* and *Ascochyta* spp. (Syngenta Canada, 2017). Ethaboxam has been shown to suppress colonization of the oomycetes *Pseudoperonospora cubensis*, *Phytophthora infestans,* and *P. capsici* in cucumber, potato and pepper, respectively (Kim *et al*., 1999), but activity against *A. euteiches* has not yet been demonstrated. In greenhouse trials, hymexazol and captan significantly reduced ARR in green bean (*Phaseolus vulgaris*) when used as seed treatments, and hymexazol, propamocarb, and azoxystrobin were effective against ARR when applied as a soil drench (Watson *et al*., 2013). Similar, but more variable effects were found in field trials. Plants were not grown to maturity in either setting, so the ability of these treatments to protect yield was not investigated (Watson *et al*., 2013). Fosetyl-Al reduced symptoms of ARR in *P. sativum* during early developmental stages, but this effect was less pronounced later in the season (Oyarzun *et al*., 1990).

While no methods of biological control are commercially available, there are several BCAs that show promise in mitigating the effects of ARR. The arbuscular mycorrhizal fungi *Glomus intraradices* and *G. claroideum* induced greater tolerance of *A. euteiches* infection in *P. sativum* (Thygesen *et al*., 2004). *Aphanomyces euteiches* colonized pea roots to the same extent regardless of mycorrhizal presence, but plants with symbiotic mycorrhizae had markedly higher biomass after 33 days than those without. Induced resistance, physiological changes, competition for resources, or a combination

thereof are possible mechanisms of this interaction. *Glomus fasciculatum* was shown to significantly reduce both oospore production and ARR in pea, though this antagonistic interaction was only observed when *G. fasciculatum* had colonized roots prior to *A. euteiches* exposure (Rosendahl, 1985). Both studies, however, were conducted in the greenhouse and require further testing in a field setting. An isolate of the spore-forming bacteria *Bacillus mycoides* slightly decreased ARR severity in the field, but these results were confounded by the presence of *Fusarium* spp. (Wakelin *et al*., 2002). However, both plot stand and yield increased significantly when *B. mycoides* was applied as a seed or granular treatment, respectively. While there is much work to be done before introducing a commercially available biocontrol to manage ARR, the urgent need for a low-impact, economical management method that provides season-long protection certainly warrants further investigation.

Breeding for resistance to *A. euteiches* in *P. sativum* has received considerable attention. The development of resistant cultivars has proven to be complex, however, and none that combine high levels of resistance with satisfactory agronomic traits are available (McGee *et al*., 2012; Hamon *et al*., 2013). Following the identification of resistant lines, resistance was found to be weak, partial, polygenically inherited, and often associated with traits undesirable for commercial production (Hamon *et al*., 2013). Further constraints to improving resistance in *P. sativum* included low heritability of resistance genes, high levels of genetic variability within *A. euteiches* populations, association with multiple root pathogens in field conditions, and the strong influence of environmental conditions on ARR severity (Gaulin *et al*., 2007). These factors have complicated breeding efforts as they often contributed to inconsistent performance and reduced durability of resistance within a breeding line (Gaulin *et al*., 2007). Despite these limitations, three decades of breeding programs taking place largely in the United States and France have resulted in the release of *P. sativum* lines with improved levels of resistance or tolerance to *A. euteiches* and traits that are more agronomically acceptable

(Roux-Duparque *et al*., 2004; McGee *et al*., 2012; Conner *et al*., 2013; Hamon *et al*., 2013). QTL associated with partial resistance have been identified (Pilet-Nayel *et al*., 2005; Hamon *et al*., 2013; Desgroux *et al*., 2016), and the confidence intervals of many of these QTL have subsequently been refined using genome-wide association mapping (Desgroux *et al*., 2016). The latter study also identified marker haplotypes at loci for *A. euteiches* resistance, and demonstrated that pyramiding of favourable haplotypes was an important means of accumulating resistance alleles in *P. sativum* lines. This strategy is expected to be essential in the ongoing development of cultivars with strong resistance, as the incorporation of multiple alleles with activity against different stages in the life cycle of *A. euteiches* will be more effective at inhibiting disease development and more durable to pathogen adaptation (Lavaud *et al*., 2016).

In the absence of a fully effective chemical, biological, or genetic defense against ARR, cultural crop management practices are the most viable method of yield protection. The production and mobility of zoospores are dependent on high soil moisture levels, so careful monitoring of irrigation and soil compaction may reduce infection rate (Hossain *et al*., 2012). Crop rotation has been recommended to limit pathogen accumulation in soil, but is likely ineffective due to the long-term resilience of oospores and the broad host range of *A. euteiches,* which includes pasture legumes and several weed species in addition to commercially produced pulse crops (Gaulin *et al*., 2007; Hossain *et al*., 2012; Hughes & Grau, 2013). Long-term avoidance of planting susceptible crops in fields known to have a high pathogen density is therefore advised, which requires testing soil of unknown inoculum potential (Malvick *et al*., 1994; Hughes & Grau, 2013). Inoculum potential refers to an index of potential disease activity that takes into account factors such as pathogen density, virulence, and soil characteristics that either inhibit or promote infection (Malvick *et al*., 1994). Soil indexing is commonly used to predict the inoculum potential of soil (Malvick *et al*., 1994). Using this method, plants are grown in soil collected from a field and any disease symptoms that develop are visually assessed

(Malvick *et al*., 1994; Paulitz, 2000). Soil indexing can provide information about the potential for ARR to develop, but is slow, labour intensive, and does not provide sufficient quantification of pathogen populations (Malvick *et al*., 1994; Gossen *et al*., 2016). Rolled towel and most probable number bioassays are commonly used to estimate propagule density in soil, but these methods are imprecise and must be interpreted with caution (Malvick *et al*., 1994). Molecular methods are a far more reliable way of quantifying *A. euteiches* oospores in soil. A qPCR assay capable of detecting less than 10 oospores per gram of soil has been developed, and the number of oospores in soil was found to be correlated to inoculum potential (Gangneux *et al*., 2014). In addition, a number of seed testing labs utilize PCR for detection, but not quantification, of *A. euteiches* in seed, soil, and plant tissue (20/20 Seed Labs, 2017; BioVision Seed Labs, 2017; Discovery Seed Labs, 2017).

The incorporation of certain green manures may reduce the incidence and severity of ARR. The use of non-host cultivars of oat, corn and cruciferous crops as green manures can reduce biomass loss and disease severity in *P. sativum,* particularly when soil compaction is relieved (Fritz *et al*., 1995; Williams-Woodward *et al*., 1997). *In vitro* experiments have demonstrated that zoospores produced by *Pythium*, *Aphanomyces*, and other oomycetes are initially attracted to rapidly-diffusing compounds produced by oat roots, but are subsequently immobilized and lysed by saponins that also exude from the roots (Deacon & Mitchell, 1985). This suggests that the use of oats as a pre-crop or a green manure may reduce the inoculum density and the infection rate of phytopathogenic oomycetes in soil (Deacon & Mitchell, 1985). Volatiles produced by cruciferous crops have similar fungitoxic properties. Glucosinolates are compounds produced by brassicas that hydrolyze during plant decomposition to produce volatile compounds such as isothiocyanates and nitriles (Smolinska *et al*., 1997). The distribution and concentration of glucosinolates varies among the Brassicaceae, but seeds and flower buds typically have the highest levels while roots, leaves, and stems contain lower concentrations (Hossain *et*

al., 2012). Seed meal from *Brassica napus* L. suppressed both mycelial growth and zoospore germination *in vitro* (Smolinska *et al*., 1997). In the same study, *P. sativum* seed inoculated with *A. euteiches* zoospores and incubated with *B. napus* volatiles had significantly lower disease ratings than control groups. The incorporation of cabbage leaves (Papavizas, 1966) and white mustard (Muehlchen *et al*., 1990) into soil reduced ARR, and in the latter case, increased yield of field pea in field trials. Using cover crops as green manures have the potential to simultaneously reduce moisture loss, weed encroachment, and oomycete inoculum potential in soil, and therefore represent an environmentally friendly and economical approach to crop management.

1.3.3 Management of S. lineatus

Several chemical pesticides, applied as seed and foliar treatments, have been investigated for use in the control of pea leaf weevil herbivory (reviewed by Vankosky *et al*., 2009). Foliar sprays may increase adult mortality and decrease fecundity, thereby indirectly reducing larval density (Vankosky *et al*., 2009). However, foliar applications do not provide consistent control of pea leaf weevil herbivory, likely due to the very short interval between adult arrival to reproductive crops and the onset of oviposition or to repeated weevil immigration (Doré & Meynard, 1995; Seidenglanz *et al*., 2010; Cárcamo *et al*., 2012). Immediate detection and timely application of foliar sprays is therefore a prerequisite for successful use. Additionally, high toxicity to the environment and non-target organisms has resulted in the deregistration of many formerly available pesticides (Cárcamo & Vankosky, 2011). Spraying of registered pesticides is recommended only when greater than 30% of seedlings between the second and fifth node stages exhibit terminal leaf damage (Cárcamo & Vankosky, 2011). Lower levels of herbivory or feeding occurring at later plant growth stages are unlikely to decrease yield, making foliar sprays unnecessary.

Systemic insecticides applied to seed appear to be the most effective chemical treatment (Vankosky *et al*., 2009; Cárcamo & Vankosky, 2011). To minimize both the cost and toxicity associated with insecticides, seed treatment is recommended only when susceptible crops are grown in areas of high infestation risk and low soil nitrogen levels (Cárcamo & Vankosky, 2011). The effect of seed treatment on *S. lineatus* larvae has also been reported as somewhat variable (Cárcamo *et al*., 2012). It has been demonstrated that broad-spectrum neonicotinoids such as thiamethoxam and clothianidin increase total nodulation and prolong the period of active nitrogen fixation (Seidenglanz *et al*., 2010; Vankosky, Cárcamo, McKenzie*, et al*., 2011), though the effect on yield remains unclear (Cárcamo *et al*., 2012). Thiamethoxam is used for the control of a range of agricultural pests, including aphids, thrips, leafhoppers, whiteflies, flea beetles, wireworms, and some lepidopterans, and is registered against *S. lineatus* in field pea in Canada (Maienfisch *et al*., 2001; Health Canada, 2016). Greenhouse assays have indicated that sub-lethal effects of this chemical may be most important in reducing pea leaf weevil herbivory during vulnerable plant stages (Cárcamo *et al*., 2012). Mortality rates of adult *S. lineatus* feeding on *P. sativum* grown from thiamethoxam-treated seed are low (15-30%), with death occurring up to a week following exposure. However, egg-laying is reduced up to 92% during the vulnerable 2-5 node stage. In comparison to untreated controls, oviposition occurred up to one week later and the average number of eggs laid per female was three times lower in weevils fed foliage from thiamethoxam-treated seedlings. Additionally, thiamethoxam increased larval mortality up to 70%. This suggests that reductions in adult fecundity and larval survival may protect yield by reducing larval feeding pressure during early growth stages. However, a field study reported that thiamethoxam seed treatments did not improve yield over untreated controls, and nodule damage remained high with 50-80% of nodules showing visible damage (Vankosky, Cárcamo, McKenzie*, et al*., 2011). Interestingly, inoculation with *R. leguminosarum* did increase yield and reduced foliar feeding even in the absence of thiamethoxam. The potential yield benefit of treating *P.*

sativum with thiamethoxam remains inconclusive, however, and further investigation in the field is necessary.

There are several known natural enemies of *S. lineatus* with potential for use as BCAs; the most significant examples include several species of hymenopteran parasitoids, carabid beetles, and entomopathogenic fungi (Aeschlimann, 1980; Vankosky *et al*., 2009; Vankosky, Cárcamo, & Dosdall, 2011; Cárcamo & Vankosky, 2013). *Perilitus rutilus* Nees, *Pygostolus falcatus* Nees (Hymenoptera: Braconidae), and *Campogaster exigua* Meigen (Diptera: Tachinidae) have been identified as parasitoids of adult *S. lineatus* in the Mediterranean (Aeschlimann, 1980), however, attempts to use these species for the biocontrol of the sweet clover weevil (*S. cylindricollis* Fahr) in Manitoba in the 1950s failed (Loan, 1961; De Clerck-Floate & Cárcamo, 2011). Climatic incompatibility may have been a factor in the failure of these species to become established (Aeschlimann, 1980). Predation of *S. lineatus* eggs by several species of carabid beetles has been observed in the laboratory (Vankosky, Cárcamo, & Dosdall, 2011). *Bembidion quadrimaculatum* L., one of the most common species found in southern Alberta, consumed 94.6% of *S. lineatus* eggs. The use of *B. quadrimaculatum*, in addition to less voracious but equally common carabid species, as BCAs could be an important component of an integrated strategy to supress *S. lineatus* populations (Vankosky, Cárcamo, & Dosdall, 2011; Douglas & Tooker, 2016). However, broad-spectrum insecticides must be avoided in order for the administration of natural enemies as biocontrols to be successful (Cárcamo & Vankosky, 2011). A recent meta-analysis of field studies indicated that seed-applied neonicotinoid and pyrethroid insecticides reduced the abundance of populations of insect natural enemies by approximately 16%, which could have consequences for biological control function (Douglas & Tooker, 2016).

Several fungal entomopathogens have been investigated for their efficacy in managing PLW, but none are commercially available (Vankosky *et al*., 2009). Laboratory experiments found *Beauveria bassiana* Vuilleman, *Metarhizium flavoviride* Metschn., *M.*

anisopliae Metschn., *Paecilomyces farinosus* Holmsk., and *P. fumosoroseus* Wize, will fatally infect *S. lineatus* larvae (Poprawski *et al*., 1985). Of these, *B. bassiana* and *M. flavoviride* were most virulent, and only the latter species also infected *S. lineatus* eggs. Both fungi were able to infect larvae hatching several weeks following the application of inoculum, indicating that consistent control during vulnerable plant growth stages may be possible. The efficacy of entomopathogens as a BCA for *S. lineatus* does not appear to have been well explored in the field, however, and the trials in this environment have yielded unsatisfactory results. *Metarhizium anisopliae* did not increase larval mortality of *S. lineatus* infesting *V. faba* in greenhouse or field trials conducted by Verkleij *et al*. (1992), possibly due to a non-virulent strain or excessive dilution of pathogen propagules in soil. Finally, the effect of these entomopathogens on non-target insects is not known. *Beauveria bassiana* and *M. anisopliae* are both widely present in soil and have a host range spanning several orders, with strains of either pathogen varying greatly with respect to host specificity (Zimmermann, 1993; Meyling *et al*., 2009). The careful selection of strains that minimize detrimental effects to beneficial insects will thus be critical when investigating entomopathogens as BCAs (Vankosky *et al*., 2009).

Phytophagous insects can adapt to overcome plant resistance factors (Clement *et al*., 1993). Resistance breeding for *S. lineatus* herbivory in *P. sativum* and other plant hosts will likely need to combine multiple types of partial resistance or tolerance to be successful. Pyramiding genes for both antixenotic and antibiotic traits will provide more durable resistance in the field, and partial resistance or tolerance, as opposed to complete resistance, may reduce selection for adaptive insect genotypes (Clement *et al*., 1993). This approach necessarily involves trade-offs, as agricultural plants are often attacked by multiple insect species. For example, pea cultivars with reduced leaf wax and semi-leafless architecture are more resistant to pea aphid feeding, but have greater susceptibility to pea weevils (*Bruchus pisorum* L.; Coleoptera: Chrysomelidae) (Chang *et al*., 2006). Low levels of resistance have been found in pea lines, but cultivars resistant to

larval or adult feeding by *S. lineatus* are still lacking (Tulisalo & Markkula, 1970; Nouri-Ghanbalani *et al*., 1978). However, biochemical and morphological traits have been identified that may serve as a focus for future attempts. Pea cultivars high in saccharose are more attractive to *S. lineatus,* whereas those high in tyrosine are repellent (Havlickova, 1980). Thick, waxy leaves also inhibit adult feeding (Havlickova, 1980; Chang *et al*., 2006). Transgenic modification of *P. sativum* to increase resistance to herbivory has also been attempted. By inhibiting the production of insect digestive enzymes, expression of α-amylase inhibitor 1 from *P. vulgaris* in transgenic peas resulted in strong resistance to *B. pisorum* in both greenhouse and field trials (Schroeder *et al*., 1995; Morton *et al*., 2000). Additionally, the close association between *P. sativum,* rhizobia, and *S. lineatus* larvae presented a unique opportunity for transgenic modification. Genes expressing a toxin lethal to beetle larvae were transferred from Bt subsp. *tenebrionis* to *R. leguminosarum* (Skøt *et al*., 1990). Clover root weevil larvae (*S. lepidus*) experienced higher mortality when feeding on roots and nodules of pea and white clover inoculated with transgenic rhizobia, and feeding injury was significantly reduced (Skøt *et al*., 1990). Similarly, symbiosis of pea with modified rhizobia decreased the development and survival of larval *S. lineatus* (Quinn & Bezdicek, 1996). While promising, these results have not been verified in field trials. Competition with indigenous populations of rhizobia and potential toxicity to non-target invertebrates are potential obstacles to the use of transgenic rhizobia as a biocontrol in commercial fields.

Field pea is most vulnerable to weevil attack during early developmental stages, therefore strategic planting to prevent the emergence of seedlings and *S. lineatus* from coinciding may reduce herbivory during this period. Pea plants grown in plots under conservation tillage emerged later than those grown under conventional tillage and were concealed by crop residues during early stages, resulting in decreased abundance of adult PLW (Hanavan *et al*., 2008). A similar impact on PLW abundance was observed when plots were covered with cereal residue to mimic a no-tillage system, though no impact on

foliar herbivory was observed (Hanavan & Bosque-Pérez, 2017). Delaying planting reduces foliar herbivory by PLW in field pea (Doré & Meynard, 1995; Hanavan & Bosque-Pérez, 2017), as seedlings are not available during peak dispersal periods. There is also evidence that increasing plant diversity by intercropping with non-host crops may reduce pea leaf weevil herbivory. A diculture of *V. faba* and spring oat (*Avena sativa* L.) reduced PLW population density and feeding injury, presumably because intercropping with *A. sativa* obscured both visual and olfactory detection of *V. faba* (Baliddawa, 1984). However, this practice is more suited to small-scale or organic farming due to the difficulty and expense of harvesting intercropped commercial fields (Vankosky *et al*., 2009). The use of high-nitrogen fertilizer has been proposed as a potential method of mitigating the damage caused by pea leaf weevil. Vankosky *et al*. (2011) investigated the effect of nitrogen fertilizer in the form of urea on feeding damage and yield in pea. It was found that fertilized plants were attractive to adult weevils, but had significantly fewer root nodules than unfertilized plants. Fertilized plants would therefore be unable to support large populations of pea leaf weevil larvae, in addition to be less reliant on nitrogen fixed by rhizobia. However, yield increases were observed in only one year of a three year study, and nitrogen fertilizer was not recommended for use in managing pea leaf weevil (Vankosky, Cárcamo, McKenzie*, et al*., 2011).

1.3.4 Integrated Management of Root Rot Pathogens and S. lineatus

Despite the frequency with which PRRC pathogens and pea leaf weevils co-occur, there has been surprisingly little progress in developing an integrated management strategy. Rather, each constraint to pea production is typically targeted individually. The efficacy of multi-targeted chemical treatments has been demonstrated in other systems, suggesting the potential for similar applications in Canadian crops. In India, treating mungbeans with a combination of thiamethoxam, the fungicide carboxin, and the bioagent *Trichoderma virens* provided simultaneous control of root rot (*R. solani*),

cercospora leaf spots (*Cercospora canescens* and *Pseudocercospora cruenta*) and white fly, the vector of yellow mosaic virus (Dubey & Singh, 2013). Cruiser Maxx Vibrance, a mix containing metalaxyl, fludioxonil, sedaxane, and thiamethoxam is currently the only seed treatment registered for pulse crops in Alberta that is designed to target both insect herbivores and soil-borne pathogens (Government of Alberta, 2017). While there is evidence that each chemical individually affords some protection to seedlings, the capacity of this combined treatment to protect yield does not appear to have been investigated in a field setting. Given that synthetic pesticides are the most widely-used management strategy for disease and insect control, there is certainly an identified need for an effective, multi-targeted treatment.

There is evidence of entomopathogenic fungi that have additional antagonistic effects on phytopathogens. Through a variety of mechanisms, fungal species within the genera *Beauveria* and *Lecanicillium* can suppress plant disease and colonize insect tissues (Ownley *et al*., 2010). Several *Lecanicillium* spp. are commercially available as biopesticides for arthropods such as mites, aphids, thrips and whiteflies (Goettel *et al*., 2008). *Lecanicillium* spp. have also shown potential for control of powdery mildews, *Penicillium* spp., *Pythium* spp., and *F. oxysporum* through mycoparasitism, antibiosis, induction of plant defense response, or a combination thereof (Goettel *et al*., 2008). *Lecanicillium* spp. simultaneously controlled powdery mildew and cotton aphids on cucumber plants, demonstrating the potential for a dual-action biopesticide (Goettel *et al*., 2008). Similarly, *B. bassiana* suppressed the development of root rot disease in tomato and cotton caused by *P. myriotylum* and *R. solani,* and inhibited mycelial growth of *F. oxysporum, R. solani, P. ultimum* and *Botrytis cinerea* during *in vitro* bioassays (Ownley *et al*., 2010). The use of fungi as biopesticides has some clear advantages over available synthetic counterparts, including low toxicity, lower application rates due to fungal establishment, and the potential for season-long or even multi-season protection. While

progress has been made, it appears that much work is still to be done before biopesticides with dual activity against insects and phytopathogens become widely available.

Broad-spectrum resistance to multiple species of soil-borne pathogens and insect herbivores would be a highly economical and sustainable method of managing multiple constraints to pea production. Resistance to the pathogens *Erwinia carotovora, Pythium aphanidermatum,* and the cotton bollworm (*Helicoverpa zea* Hardwick; Lepidoptera: Noctuidae) has been achieved by pyramiding protease-inhibitor defense genes in transgenic tobacco (Senthilkumar *et al*., 2010), but similar advances have not been achieved in legumes. Inoculation with rhizobia has been shown to reduce the effects of FRR in bean, in addition to reducing foliar *S. lineatus* feeding in pea. The use of transgenic Bt rhizobia may have the potential to reduce PLW herbivory and suppress FRR, though it does not appear that this interaction has been investigated.

Parallels in cultural control methods suggest certain practices may inhibit the establishment of both *S. lineatus* and PRRC pathogens. Relieving soil compaction minimizes ARR and FRR in wet conditions, and low-impact methods such as zone tillage reduce the severity of FRR (Harveson *et al*., 2005). Crop residues conceal seedlings from PLW during early stages of plant development (Hanavan *et al*., 2008; Hanavan & Bosque-Pérez, 2017), therefore conservation tillage practices may be useful in managing either threat. However, the potential for *Fusarium* spp. to accumulate in crop residues means that soil inoculum potential must be closely monitored. Where the length of growing season permits, delayed planting may allow growers to simultaneously avoid both the peak of *S. lineatus* emergence and wet spring conditions (Doré & Meynard, 1995; Hanavan & Bosque-Pérez, 2017). Intercropping with oats may be a suitable management method for smaller-scale or organic producers, as oats protect seedlings from PLW colonization and have antibiotic effects on the zoospores of *Aphanomyces* and *Pythium* spp. (Baliddawa, 1984; Deacon & Mitchell, 1985). Intercropping with brassicas

may have a similar effect, though there is evidence that this combination may result in small yield decreases (Fernandez *et al*., 2015).

Currently, there are no satisfactory methods of managing ARR, FRR, or *S. lineatus* in pea crops. Synthetic pesticides will remain the most viable option for producers until biopesticides are proven to be effective and durable in a field setting, or cultivars with acceptable levels of resistance become available. While the protection offered by synthetic seed treatments may be significant only during the seedling stage, efficacy may be increased when combined with partially resistant cultivars and inoculation with rhizobia and mycorrhiza. Intercropping and the use of green manures may also be more effective when applied in combination with pesticides, and both have the added benefit of weed suppression, moisture retention, and soil conservation. Avoidance of fields infested with PRRC pathogens will continue to be an essential component of efforts to reduce disease incidence and pathogen population density. Advances in molecular methods of pathogen detection and quantification will be essential to the management of root rot disease in the Prairie Provinces.

1.4 Conclusion and Research Objectives

Root rot pathogens and pea leaf weevil herbivory represent major constraints to field pea production in Canada. Furthermore, the close association of these organisms in the root system of their mutual host may facilitate interspecific interactions, with the potential to synergistically increase disease severity. There are no fully effective management strategies to suppress root rot disease or pea leaf weevil herbivory. The identification of interspecific interactions may prove to be critical to the design of an effective, integrated management plan to address both threats simultaneously. This thesis is focused on identifying and quantifying interactions between *A. euteiches, Fusarium* spp., and *S. lineatus* using both conventional and molecular tools. Additionally, the

efficacies of available fungicidal and insecticidal treatments are assessed in field trials taking place over two years. The following research objectives will address these goals:

1) Identify and quantify interactions between *A. euteiches* and *Fusarium* spp., and describe the impact of multi-pathogen infections on disease severity in field pea.

2) Identity interactions between root rot pathogens and *S. lineatus* when co-infesting field pea roots.

3) Evaluate the efficacy of integrated management techniques for the control of root rot pathogens and *S. lineatus* in pea and faba bean grown under field conditions.

CHAPTER 2: DETECTION OF INTERACTIONS BETWEEN THE ROOT ROT PATHOGENS *APHANOMYCES EUTEICHES* AND *FUSARIUM* SPP. USING A MULTIPLEX QPCR ASSAY

2.1 INTRODUCTION

The oomycete *Aphanomyces euteiches* Drechs. and several species in the fungal genus *Fusarium* (Link) are primary pathogens in the pea root rot complex (PRRC) (Xue, 2003a). In Canada, a substantial increase in pea production area combined with relatively short cropping intervals has facilitated the spread of *A. euteiches*, *Fusarium* spp., and other pathogens associated with the PRRC across the Prairie provinces (Xue, 2003a; Banniza *et al*., 2013; Chatterton *et al*., 2015; Esmaeili Taheri *et al*., 2016). *Fusarium* spp., particularly *F. avenaceum* and *F. solani* f. sp. *pisi*, are the primary pathogens isolated from diseased roots in commercial pea (*Pisum sativum* L.) fields in Alberta, Saskatchewan and Manitoba (Chang *et al*., 2005; Feng *et al*., 2010; Esmaeili Taheri *et al*., 2016). Fusarium root rot (FRR) presents as brown to black lesions occurring primarily in the epi- and hypocotyl regions and red staining of the vascular tissue, and can result in extensive root decay and wilting of aerial parts (Kraft *et al*., 1993; Figure 2-1). *Aphanomyces euteiches* was first detected in Manitoba in 1980 (Lamari & Bernier, 1985), and has since been reported in Saskatchewan in 2012 (Banniza *et al*., 2013) and in Alberta in 2013 (Chatterton *et al*., 2015). Its high prevalence in the latter two provinces, however, indicates that it was likely present for many years prior to detection. Aphanomyces root rot (ARR) is characterized by honey-coloured lesions on the root cortical tissues that can progress to cause complete destruction of the cortex, resulting in severe stunting, chlorosis, and premature death (Gaulin *et al*., 2007; Conner *et al*., 2013; Figure 2-1). Yield losses of up to 60% and 80% have been reported in field pea as a result of FRR and ARR, respectively (Chang *et al*., 2005; Gaulin *et al*., 2007), and complete loss due to root rot disease has been observed in severely affected fields in Alberta and Saskatchewan (Chatterton, personal communication).

Aphanomyces euteiches is the most damaging root pathogen of pea and alfalfa, but will also infect a number of other cultivated and wild legume species (Vandemark *et al*., 2002; Gaulin *et al*., 2007). This oomycete has a broad geographical range that includes Europe, Japan, Australia, New Zealand, and North America (Gangneux *et al*., 2014). Oospores are highly resilient to drought and temperature fluctuations, and can remain viable in soil for up to a decade. (Gaulin *et al*., 2007). In the presence of root exudates, oospores germinate, form sporangia, and release motile zoospores that encyst on root tissue. Infection can occur at any stage of plant growth (Gaulin *et al*., 2007; Gangneux *et al*., 2014). *Fusarium* is a large genus of fungi with a global distribution (Ma *et al*., 2013). Many species are agriculturally important phytopathogens, among which host specificity varies significantly (Ma *et al*., 2013). *Fusarium avenaceum, F. solani, F. redolens,* and *F. oxysporum* are frequently isolated from pea roots in North America (Esmaeili Taheri *et al*., 2016). *Fusarium* spp. can persist saprophytically in crop residues or in soil as chlamydospores, and can attack plants at multiple growth stages (Hargreaves & Fox, 1977; Kraft *et al*., 1993).

Fusarium spp. and *A. euteiches* are known to frequently co-occur in commercial pea crops: surveys conducted in 2016 indicated that 73% of tested fields with root rot in Alberta, Saskatchewan, and Manitoba were positive for *A. euteiches* and at least one species of *Fusarium*, while the remaining 27% were positive for either *A. euteiches* or *Fusarium* spp. (Chatterton, personal communication). Despite this, little is known about how these pathogens interact when simultaneously infecting the roots of their host. Antagonistic interactions may occur if PRRC pathogens compete for the same resources (Jesus Junior *et al*., 2014). For example, interspecific competition has been observed between some *Fusarium* spp. colonizing pea roots in greenhouse experiments (Zitnick-Anderson *et al*., 2018). Alternatively, pathogens may avoid competition by occupying different spatial or temporal niches within the root system, resulting in additive or neutral interactions. Finally, mutualistic and commensalistic interactions can synergistically

increase the severity of disease symptoms (Jesus Junior *et al*., 2014). Interspecific interactions between plant pathogens may increase disease pressure, complicate diagnoses, and make yield loss difficult to predict (Jesus Junior *et al*., 2014).

In order to have a clear picture of how PRRC pathogens interact when co-infecting a host, a rapid and sensitive method of quantifying microbial populations is required. Real-time quantitative PCR (qPCR) is an effective alternative to conventional methods of phytopathogen quantification and identification, such as plating techniques or microscopy. Assays can be multiplexed in order to detect multiple pathogen species within a single reaction, increasing efficiency and allowing for the use of host DNA as an endogenous control (Schena *et al*., 2004). qPCR assays have been developed to quantify *A. euteiches* in alfalfa roots (Vandemark *et al*., 2002) and soil (Sauvage *et al*., 2007; Gangneux *et al*., 2014). Multiplex qPCR has been used to quantify seven *Fusarium* spp. in *P. sativum* roots (Zitnick-Anderson *et al*., 2018), and several *Fusarium* spp. in maize (Scauflaire *et al*., 2012; Preiser *et al*., 2015). However, a multiplex qPCR assay designed to quantify the DNA of multiple *Fusarium* spp. and *A. euteiches* relative to that of *P. sativum* is not currently available. Such an assay would not only allow for simultaneous detection of the most damaging root pathogens to Canadian pea crops, but may also aid in identifying changes in colonization rates when multiple pathogens are present.

The objectives of this research were to determine if additive, competitive, or synergistic interactions occur between *Fusarium* spp. and *A. euteiches* when colonizing *P. sativum* roots, and to design a multiplex qPCR assay to detect and quantify these pathogens from infected root tissue. *Aphanomyces euteiches* was co-inoculated with three species of *Fusarium* to assess the impact of multiple root pathogens on disease severity and plant development. Disease severity was assessed by visually rating symptoms and taking measurements of root and shoot growth parameters. Finally, two triplex qPCR assays were designed to quantify the DNA of all three *Fusarium* spp. and *A. euteiches*

relative to that of *P. sativum* in order to detect changes in pathogen composition within the roots.

2.2 Materials and Methods

2.2.1 Fungal Cultures

Three species of *Fusarium* were used; the virulence of each to *P. sativum* had previously been determined in greenhouse tests (Chatterton, personal communication). *Fusarium avenaceum* isolate 1306.08 was the most virulent, *F. solani* f. sp. *pisi* isolate 1225.07 had moderate to high virulence, and *F. redolens* isolate 1306.03 had low virulence to *P. sativum*. This range of virulence was selected to determine if the strength or direction of interactions was altered by pathogenicity. All three *Fusarium* isolates were obtained from pea roots collected during field surveys near Lethbridge, Alberta, Canada, and were stored at 4°C on synthetic nutrient-deficient medium 'Spezieller Nährstoffarmer agar (SNA)' (Leslie & Summerell, 2006). Isolates were grown at room temperature on potato dextrose agar (PDA) amended with penicillin and streptomycin, each at a concentration of 150 mg L^{-1}. After 6 days of growth, sporulation was induced by transferring disks (8 mm diameter) taken from the outer edge of the PDA cultures to liquid carboxymethylcellulose (CMC) medium (Cappellini & Peterson, 1965). The inoculated CMC medium was incubated on a shaker at 170 rpm for 4-7 days, or until the culture was cloudy with visible clumps of mycelia. Cultures were agitated aggressively by hand to release conidia before filtration through four layers of cheesecloth to remove mycelia and agar. Conidia were collected by centrifugation at 4000 rpm for 10 minutes. The supernatant was poured off and the conidia were washed three times with sterile distilled water (SDW), centrifuging for ten minutes after each addition of water. Macro- and micro-conidia were resuspended in SDW and counted with the aid of a hemocytometer.

Aphanomyces euteiches isolate 1 was obtained from pea roots collected during field surveys near Saskatoon, Saskatchewan, Canada, and was maintained at room temperature on PDA amended with penicillin and streptomycin at a concentration of 150 mg L^{-1}. Oospores were produced by transferring 3 agar disks (8 mm diameter) from 5 day old PDA cultures to flasks containing 40 mL of 0.5% homogenized oatmeal broth (Schneider, 1978). The inoculated oatmeal broth was incubated at room temperature in the dark for 30 days. The mycelial mats and remaining broth were homogenized for 5 minutes in a stainless steel blender (Waring, Torrington, CT, USA) at 18,000 rpm, and the solution was then filtered through four layers of cheesecloth. The oospore concentration of the inoculant was counted using a hemocytometer.

2.2.2 Inoculation and Growth Conditions

'CDC Meadow', a *P. sativum* cultivar with susceptibility to both ARR and FRR, was used as a host for all trials. Seeds were inoculated with individual *Fusarium* isolates using the method described by (Porter & Pasche, 2015), wherein seeds were soaked overnight in a solution of conidia and SDW at a concentration of 4 x 10^4 conidia mL^{-1}. Inoculum combining all three species of *Fusarium* had 1.3 x 10^4 conidia mL^{-1} per isolate, for a final concentration of 4 x 10^4 conidia mL^{-1}. Seeds for treatments excluding *Fusarium* were soaked for an equal amount of time in SDW. Seeds were planted into soil that was inoculated with *A. euteiches* by adding the quantity of oospore solution required to obtain a final concentration of 500 oospores g^{-1} of soil, and mixing thoroughly by hand. Soil for treatments without *A. euteiches* was similarly mixed with an equal volume of SDW. Preliminary tests indicated that the described inoculum concentrations used for *A. euteiches* and *Fusarium* spp. would produce disease symptoms without killing the host plants. The soil used for all treatments was composed of equal parts peat, vermiculite, and topsoil. Seeds were sown into 10 cm diameter pots containing approximately 250 grams of soil. Five seeds were planted per individual pot, and each pot was contained within a

tray to prevent cross contamination of pathogens between treatments. Plants were grown for four weeks in a greenhouse with a 16:8 cycle of light at 22°C and dark at 18°C. Plants were watered daily without fertilizer for the duration of the growth period.

2.2.3 Experimental Design, Sample Processing, and DNA Extraction

Ten experimental treatments were used to evaluate interactions between *Fusarium* spp. and *A. euteiches* (Table 2-1). A control group was kept free of all pathogens. One treatment each was exposed to *F. avenaceum, F. solani, F. redolens,* or *A. euteiches*. Each *Fusarium* isolate was individually combined with *A. euteiches* in an additional three treatments. In the final two treatments, all three *Fusarium* spp. were combined with and without *A. euteiches*. All treatments had four replicates with five plants per replicate, and were arranged in a randomized complete block design. Two independent trials were conducted. At the end of the growth period, plants were removed from pots and roots were gently washed under running water to remove residual soil and vermiculite. Disease severity was visually assessed using a disease severity index that ranged from 0 to 5, with 0 indicating that no disease symptoms were present and 5 indicating a dead plant with a completely decayed root system (Table 2-2). ARR and FRR severity were not assessed individually as the symptoms of ARR and FRR can be difficult to distinguish, particularly in severe cases. Clean roots were severed from the stem at the epicotyl, and the length of the shoot from the epicotyl to the terminal node was measured. The roots were patted dry and the fresh weight was recorded. Roots were cut into small pieces approximately 3-5 mm in length, with care to take an equal amount of material from the taproot, cotelydonary attachment area, and randomly selected lateral roots for each sample. Scissors used to cut roots were sterilized with a 70% ethanol solution between samples to prevent cross-contamination of pathogens. One sample was taken per replicate for all treatments, and consisted of material from all five plants within the replicate. Root

sections were immediately frozen at -80°C before being lyophilized in a Harvest Right scientific freeze dryer (North Salt Lake, UT, USA) for 48 hours. For each sample, 25 mg of lyophilized root tissue was ground using one 3 mm tungsten carbide bead shaken at 1800 rpm with a TissueLyser II (Qiagen, Toronto, ON, CA) for 2 minutes. DNA was extracted from each sample in duplicate using the Biosprint-96 DNA plant kit in a Biosprint instrument (Qiagen) according to the manufacturer's instructions.

2.2.4 Design and Specificity of Primers and Probes

2.2.4.1 Aphanomyces euteiches

Primers and probes designed in this study are listed in Table 2-3. The primer pair Ae1.2_ITS1_Fwd/Ae1.2_ITS1_Rev was designed from the internal transcribed spacer (ITS) region of the rRNA for specific amplification of *A. euteiches*. All available sequences were collected using NCBI and the Barcode of Life Database (BOLD), and were aligned using MAFFT version 7.31 (Katoh, 2013). A database was generated using the aligned *A. euteiches* sequences in addition to several related non-target species. A nucleotide breakdown analysis was conducted to identify consensus sequences that were non-homologous to related species. Specificity of the consensus sequences was verified using a BLASTn search of the GenBank database. Primer Express 3.0.1 (Applied Biosystems, Foster City, CA, USA) was used to generate candidate primer and probe sets from the consensus sequences using the default parameters. The resulting list of primers and probes were checked against the database described above for regions of the sequence that had high sequence conservation for *A. euteiches* and low conservation for all non-targets.

Endpoint PCR was used to screen the primers against a panel of samples positive and negative for *A. euteiches,* as well as several related or commonly occurring non-target species (Table 2-4). Each 50 μL reaction contained 5 μL of 10x PCR buffer (ThermoFisher, Waltham, MA, USA), 3 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP, 500

nM of each forward and reverse primer, and 0.2 μL of *Taq* DNA polymerase (recombinant; Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were as follows: 94°C for 3 min; 40 cycles of 94°C for 30 s; 60°C for 20 s; extension at 72°C for 20 s extension, followed by a final 10 min extension and 4°C hold. Reactions were performed in an Eppendorf Mastercycler Pro S thermocycler (Hamburg, DE). Generated amplicons were run on a 2% agarose gel (Invitrogen) for one hour and imaged using the BioRad Gel Doc system (Hercules, CA, USA). A final primer pair was selected based on successful amplification of *A. euteiches* targets and minimal amplification of non-targets.

The selected primers and probe underwent a final screening using a singleplex qPCR assay with the same panel of samples used in endpoint PCR. Each 20 μL reaction contained 10 μL of PrimeTime Gene Expression 2X Master Mix (IDT, Skokie, IL, USA); 500 nM of each forward and reverse primer; 250 nM probe; 2 μL of template; and 7.1 μL of ultrapure H_2O. Reactions were run in MicroAmp Fast Optical 96-well plates (Applied Biosystems) using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Cycling conditions were set at 95°C for 3 min; 40 cycles of 95°C for 15 s and 60 s of annealing/extension at 72°C. Resulting threshold cycle (Ct) values were analyzed for specificity and sensitivity.

2.2.4.2 Pisum sativum

Stable expression is a critical characteristic of an endogenous reference gene, as fluctuation in expression levels can lead to inaccurate quantification when normalizing a target gene to an internal control (Die *et al*., 2010). Genomic β-tubulin was therefore chosen as the target gene for the design of the primer pair Psat_TUB1_3Fwd/Psat_TUB1_3Rev for specific amplification of *P. sativum,* as it was found to have high expression stability by Die et al. (2010). Candidate forward and reverse primers were generated using the NCBI Primer-BLAST tool with the target sequence set to this gene.

The primer sets and probes were evaluated for specificity to the target organism and gene using a BLASTn search of the GenBank database. Primer sets were commercially synthesized by IDT. Specificity of the primer sets was tested against the *P. sativum* genome, as well as *F. avenaceum*, *F. redolens*, *F. solani*, and *A. euteiches* using endpoint PCR (Table 2-4). Each reaction contained 12.5 μL of 2X Qiagen Multiplex MasterMix, 0.5 μL of each 0.2 uM primer, 5 ng of DNA and ultra-pure H_2O to a final volume of 20 μL. PCR conditions were as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s, 90 s at 57°C annealing, 60 s at 72°C elongation, followed by the final elongation of 10 minutes at 72°C. PCR product was visualized on a 2% agarose gel following electrophoresis as described above. The primer set that best amplified *P. sativum* DNA without non-target amplification was chosen. The 105 bp amplicon sequence produced by chosen primer set was input into the PrimerQuest Tool (IDT, 2017) using the default settings to generate a TaqMan probe, which was ordered from ThermoFisher.

2.2.5 MULTIPLEXING

Following screening, the selected primer and probe sets for *A. euteiches* (Ae1.2_ITS1) and *P. sativum* (Psat_TUB1) were optimized for multiplexing with previously developed primer and probe sets for *F. avenaceum* (Zitnick-Anderson *et al.*, 2018), *F. solani* (Zitnick-Anderson *et al.*, 2018), and *F. redolens* (Safarieskandari, personal communication) (Table 2-3). Standard curves were generated using ten-fold serial dilutions of DNA from each organism that ranged from 10^7 to 10^3 fg. Each DNA sample was run in singleplex and triplex reactions to compare reaction efficiency and sensitivity. A primer concentration of 500 nM was used for *Fusarium* standards run in singleplex, whereas a higher concentration of 900 nM was used to maintain acceptable efficiency in multiplex. The following groupings were used for the triplex reactions: *F. avenaceum* + *F. solani* + *P. sativum* and *F. redolens* + *A. euteiches* + *P. sativum*. Thermocycler conditions were 10 min. at 95°C, 40 cycles of 95°C for 15 s, and 60 s of

annealing/extension at 60°C, except the annealing temperature was increased to 63°C for singleplex and multiplex assays targeting *A. euteiches* to correct for a false positive signal from the *A. euteiches* probe. Each reaction contained 12.5 μL of 2x PrimeTime Gene Expression Master Mix, 2 μL of template DNA, appropriate primer and probe as shown in Table 2-3, and ultra-pure H_2O to make a 25 μL reaction. Experimental DNA samples were subject to qPCR analysis using the same conditions. All samples were analyzed in technical duplicate, and controls containing ultra-pure H_2O rather than DNA template were included on each plate.

2.2.6 Relative Quantification of DNA

Relative quantification of pathogen DNA from *P. sativum* roots was performed using the comparative C_t method ($\Delta\Delta C_t$) (Livak & Schmittgen, 2001). Here, *P. sativum* DNA served as an endogenous reference to which the quantity of target DNA in each sample was normalized ($\Delta C_{t\,=} C_{tREFERENCE} - C_{tTARGET}$). The ΔC_t value was then calibrated to the ΔC_t of the target pathogen in the non-treated control group ($\Delta\Delta C_t = C_{tTREATED} - C_{tCONTROL}$). Validation experiments to demonstrate that the amplification efficiencies of the targets were approximately equal to the endogenous control were performed. Standard curves using ten-fold serial dilutions ranging from 10^7 to 10^3 fg were generated using target and reference DNA. The ΔC_t for each dilution was calculated and plotted against the logarithmic value of the input DNA concentrations. Amplification efficiencies were determined to be equal if the slope of the log input amount versus ΔC_t was < 0.1.

2.2.7 Statistical Analyses

All statistical analyses were performed using JMP version 13.1 (SAS Institute Inc., 2016). Disease ratings, shoot height, root weight, and $\Delta\Delta C_t$ data were analyzed in separate mixed-effect analysis of variance models for a randomized complete block experimental design. Inoculum treatment was specified as a fixed effect, whereas trial and

replicate were considered random factors. Single degree of freedom contrast statements were used to test the hypothesis that disease severity and plant growth are negatively impacted when roots are inoculated with multiple versus single pathogens. To determine how individual pathogen populations were impacted by each treatment, means of $\Delta\Delta C_t$ data were separated using the Tukey-Kramer HSD test. Disease ratings and *F. solani* $\Delta\Delta C_t$ data were log transformed, whereas shoot height and *F. avenaceum* $\Delta\Delta C_t$ data were square root transformed to improve normality prior to analysis. Normality of all dependent variables was assessed using the Shapiro-Wilk test. Outlier detection was performed using standardized scores. Data from repeated experiments were pooled following F-tests for equality of variance. The Type 1 error rate (α) was set at 0.05 for all statistical tests.

2.3 RESULTS

2.3.1 DISEASE RATINGS

Disease severity increased significantly when multiple pathogens were inoculated, relative to single pathogen inoculations ($F_{(9, 30)} = 19.81$, $p < 0.0001$) (Figure 2-1; Table 2-5). Single degree of freedom contrast statements indicated that pea roots infected with both *F. redolens* and *A. euteiches* produced more severe symptoms than single inoculations ($p < 0.0001$). *Fusarium solani* and *A. euteiches* also induced greater disease severity when co-infecting pea roots, in comparison to isolated infections ($p = 0.047$). Symptoms related to *F. avenaceum* and *A. euteiches* did not differ significantly in single versus combined inoculations ($p = 0.20$). Relative to single pathogen inoculations, roots inoculated with *Fusarium* spp. had more severe symptoms when all three species were present ($p = 0.044$). The combination of all four pathogens also increased disease severity relative to single inoculations ($p < 0.0001$). Finally, roots were more diseased when inoculated with *A. euteiches* and *Fusarium* spp. relative to roots inoculted only with the three *Fusarium* spp. ($p = 0.027$).

2.3.2 Plant Biomass

Combined inoculations of *F. redolens* and *A. euteiches* significantly decreased shoot growth relative to single inoculations ($p = 0.0046$), whereas growth did not differ between single and combined inoculations of *F. solani* and *A. euteiches* ($p = 0.11$) or *F. avenaceum* and *A. euteiches* ($p = 0.64$) (Table 2-5). Shoot growth was similar between single and combined inoculations of the three *Fusarium* spp., but decreased significantly when all four pathogens were co-inoculated relative to single inoculations of *A. euteiches, F. avenaceum, F. solani,* and *F. redolens* ($p = 0.0007$), and to combined inoculations of *Fusarium* spp. ($p = 0.0006$).

Inoculation with root rot pathogens had a significant impact on pea root weight that closely mirrored the effect on shoot height ($F_{(9, 30)} = 8.11$, $p < 0.0001$) (Table 2-5). In comparison to single inoculations, *A. euteiches* significantly reduced root weight when combined with *F. redolens* ($p = 0.0052$), while root weight was similar between single and combined inoculations of *A. euteiches* and *F. solani* ($p = 0.16$), or *F. avenaceum* ($p = 0.64$). Differences in root weight were not observed between single and combined inoculations of *Fusarium* spp. ($p = 0.21$). The combination of all four pathogen species significantly increased disease severity relative to single inoculations ($p = 0.0016$) and combined inoculations of *Fusarium* spp. ($p < 0.0001$).

2.3.3 Design and Specificity of Primers and Probes

All available sequences for *A. euteiches* within the target gene were collected from NCBI and BOLD. Eleven candidate primer and probe sets were generated based on the aligned consensus sequences. The primer pair Ae1.2_ITS1, binding between base pairs 32 and 146, was chosen for qPCR screening based on successful amplification of *A. euteiches* DNA during endpoint PCR. The corresponding probe binds in the 81-103 region. Faint amplification of *Pythium ultimum* and *P. irregulare,* and strong

amplification of *Aphanomyces cochlioides* occurred during endpoint PCR (Table 2-4). All positive samples were amplified during qPCR screening of the Ae1.2_ITS1 primer set and FAM-labelled fluorophore probe, whereas no amplification was observed in negative samples (Table 2-4). *Aphanomyces cochlioides* was the only non-target species amplified during qPCR, with amplification occurring at approximately 31 cycles. Given that pea is a non-host of this species, the Ae1.2_ITS1 primer and probe set were used for multiplexing. Of the 10 *P. sativum* primer sets generated by the NCBI Primer-BLAST tool, three were chosen based on an amplicon size of 90-150 bp that allowed for future insertion of a TaqMan probe. Of these, the Psat_TUB1_3 primer set was chosen as it most strongly amplified *P. sativum* DNA and showed no non-target amplification in endpoint PCR screening (Table 2-4). Five potential probes were generated, of which one was chosen based on melting temperature. The Psat_TUB1_3 primer set binds between the 2290 and 2330 region of the β-tubulin gene, whereas the probe binds between the 1996 and 2024 region.

2.3.4 Multiplexing

No loss in sensitivity or reduction in R^2 values was detected in triplex reactions compared to single reactions (Table 2-6). All primer sets had similar sensitivity. Primer pairs for *A. euteiches, F. avenaceum, F. solani,* and *F. redolens* amplified 1 pg of DNA in ≤ 35 cycles in multiplex, whereas primers for *P. sativum* amplified down to 10 pg DNA (Figure 2-2). R^2 values of standard curves were > 0.99 and efficiencies were between 90 and 100% for all primer and probe sets in triplex reactions.

2.3.5 Relative Quantification of Pathogen DNA

Validation experiments determined that the amplification efficiency of each target was approximately equal to that of the endogenous control, based on an absolute value of < 0.1 for the slope of log input amount versus ΔC_t (Figure 2-3a-d). In comparison to

single inoculations, the relative quantity of *A. euteiches* DNA did not change when combined with *F. redolens* ($p = 0.85$), *F. solani* ($p = 1.00$), *F. avenaceum* ($p = 0.23$) or all three *Fusarium* spp. ($p = 1.00$) (Figure 2-4a). A low background level of *A. euteiches* DNA of unknown origin was present in the *F. redolens* ($p = 1.00$), *F. solani* ($p = 0.99$), *F. avenaceum* ($p = 0.98$), and the combined inoculation of all three *Fusarium* spp. ($p = 1.00$), but none of these treatments were significantly different from the non-treated control. *Fusarium redolens* DNA was also present in non-inoculated treatments (Figure 2-4b). The relative quantity of *F. redolens* DNA in roots inoculated singly was not significantly different than roots inoculated with *A. euteiches* ($p = 0.81$) and *A. euteiches* + *F. avenaceum* ($p = 0.089$). Small quantities of *F. redolens* DNA was also detected in the *F. solani* + *A. euteiches* and *F. avenaceum* treatments, but neither was significantly different from the non-inoculated control ($p = 0.33$ for each comparison). The relative quantity of *F. redolens* DNA increased when co-inoculated with *A. euteiches* in comparison to single inoculations ($p = 0.034$). A higher accumulation of *F. redolens* DNA was also detected when co-inoculated with other *Fusarium* spp. and *A. euteiches*, as opposed to co-inoculation only with *F. solani* and *F. avenaceum* ($p = 0.037$). Compared to single inoculations, the relative quantity of *F. solani* DNA did not change when co-inoculated with *A. euteiches* ($p = 1.00$), *F. redolens* and *F. avenaceum* ($p = 0.84$), or all pathogen species ($p = 0.69$) (Figure 2-4c). However, there was significantly more *F. solani* DNA when co-inoculated with *F. avenaceum, F. redolens* and *A. euteiches*, in comparison to the combined inoculation of all three *Fusarium* spp. ($p = 0.047$). *F. solani* DNA was detected in all treatments with the exception of roots inoculated with *F. redolens*. The quantity of *F. solani* DNA in these treatments was comparable to the non-inoculated control in all treatments but the combined inoculation of *F. avenaceum* and *A. euteiches* ($p = 0.0078$). *F. avenaceum* was detectable only in inoculated treatments, and the presence of *A. euteiches* did not change the relative quantity of DNA regardless of whether other *Fusarium* spp. were also present (*A. euteiches* + *F. avenaceum:* $p = 0.92$;

A. euteiches + *F. redolens* + *F.solani* + *F.avenaceum:* $p = 0.45$) (Figure 2-4d). However, the quantity of *F. avenaceum* DNA was significantly reduced when co-inoculated with *F. redolens* and *F. solani*, relative to all other treatments that included *F. avenaceum* (*F. avenaceum:* $p = 0.0019$; *A. euteiches* + *F. avenaceum:* $p = 0.0086$; *A. euteiches* + *F. redolens* + *F.solani* + *F.avenaceum:* $p = 0.044$).

2.4 DISCUSSION

Interactions between PRRC pathogens are poorly understood, despite their mutual association with *P. sativum*, but have the potential to interfere with diagnoses and disease management. Conventional tools such as indices of disease severity are widely used in plant pathology but cannot reliably identify the causal agents of disease, particularly when multiple pathogen species are present. Furthermore, changes in visible symptoms do not necessarily reflect corresponding shifts in microbial community structure. Measurements of disease severity and other plant health parameters were therefore supplemented by two triplex qPCR assays to quantify colonization dynamics between *A. euteiches* and *Fusarium* spp. in field pea. These assays are the first to allow for simultaneous screening of *A. euteiches, F. redolens, F. avenaceum,* and *F. solani,* in their respective groupings, while using *P. sativum* DNA as an endogenous control. The primer and probe sets designed here for the detection of *A. euteiches*, in addition to the previously developed primer and probe sets for *F. redolens, F. solani* and *F. avenaceum,* demonstrated detection limits of 1 pg of DNA. Primers for *P. sativum* amplified down to 10 pg of DNA. Standard curves showed slopes of -3.4 to -3.5 under triplex conditions, with R^2 values > 0.99. Amplification of non-target species by the Ae1.2_ITS primer set observed during endpoint PCR did not occur during qPCR, with the exception of *A. cochlioides,* indicating that the addition of a probe improves the specificity of this primer set. Pea is not a host of *A. cochlioides* (Islam & Tahara, 2001), so this is not anticipated to be problematic when screening pea roots. Therefore, the triplex assays described here

represent a sensitive and efficient method of detecting and quantifying four commonly occurring PRRC pathogens relative to the DNA of their host.

Based on visual ratings of single inoculations, the three species of *Fusarium* used here ranged from very mildly to highly virulent. This allowed for an analysis of changes in disease severity and fungal colonization rates when *A. euteiches* is co-inoculated with both weak and aggressive strains of *Fusarium*. Disease severity was altered when *P. sativum* seedlings were challenged with multiple PRRC pathogens in comparison to single inoculations. *Fusarium redolens* did not induce visible symptoms in isolation, but high levels of decay and reduced shoot and root growth were observed when *A. euteiches* was also present. An increase in the quantity of *F. redolens* DNA in the combined treatment indicates that *A. euteiches* facilitates *F. redolens* colonization of pea roots when both pathogens are present. qPCR results further indicated that the interaction between *A. euteiches* and *F. redolens* also occurred when other *Fusarium* spp. were present. A similar interaction was observed by Kerr *et al*. (1963), wherein a weakly pathogenic isolate of *Fusarium oxysporum* f. sp. *pisi* Snyd. & Hans. interacted synergistically with *Pythium ultimum* Trow to increase the severity of Fusarium wilt in pea. An increase in the severity of ARR symptoms has also been observed following the inoculation of pea seedlings with *A. euteiches* and a non-pathogenic isolate of *F. solani* (Peters & Grau, 2002). The present study indicates that co-inoculating *F. solani* with *A. euteiches* increases disease severity relative to single inoculations, though shoot and root growth were not affected. No changes were observed in the accumulation of *F. solani* or *A. euteiches* DNA when roots were inoculated singly or in combination, which may indicate that interactions between these species are additive rather than synergistic. *Aphanomyces euteiches* and *F. avenaceum* impacted the development of *P. sativum* seedlings by severely reducing root biomass, and to a lesser extent, shoot height. This effect was similar between single and combined inoculations, as was disease severity. When plants were challenged with all four pathogens, disease symptoms and plant growth were

severely impacted. Additionally, an increase in the DNA of all three *Fusarium* spp. was observed relative to treatments where *F. redolens, F. solani,* and *F. avenaceum* co-occurred in the absence of *A. euteiches*. This indicates that infection by *A. euteiches* is a significant risk factor for increased severity of symptoms related to FRR, particularly when multiple species are present.

While the mechanism behind the observed interactions was not investigated here, *A. euteiches* and *Fusarium* spp. may avoid direct competition for plant resources by colonizing different components of the root system. The composition of root pathogen populations can differ between the root, stem, and crown tissues of *P. sativum* (Esmaeili Taheri *et al*., 2016). *Aphanomyces euteiches* invades the cortical cells of its host, eventually leading to sloughing of the cortex while the vascular cylinder remains intact (Conner *et al*., 2013). Decay and 'pinching' of the epicotyl is a diagnostic feature of ARR, but cortical degradation is typically present throughout the entire root system. FRR primarily occurs in the cotyledonary attachment area, girdling the taproot near the base of the stem; *Fusarium* spp. will also invade the vascular tissue (Kraft *et al*., 1993). Colonization of different parts of the root system may explain the potentially additive interaction between *F. solani* and *A. euteiches*. It is also likely that less aggressive pathogens such as *F. redolens* will opportunistically invade roots already weakened by highly virulent pathogens like *A. euteiches*. *Fusarium redolens,* like most *Fusarium* spp., is hemibiotrophic and can infect necrotic tissue (Ma *et al*., 2013). Secondary infection of ARR-damaged tissue may drive the synergistic interaction observed between *A. euteiches* and *F. redolens*, and may also explain the overall increase in *Fusarium* DNA when all four pathogen species were present. This synergistic effect did not occur when *A. euteiches* was absent: the relative quantity of *F. avenaceum* DNA decreased when co-inoculated with other *Fusarium* spp., whereas the relative quantity of *F. redolens* and *F. solani* did not change between combined and single inoculations. This suggests that *F. solani* and *F. redolens* may avoid competition when co-infecting pea roots, whereas the

presence of congeners may inhibit the growth of *F. avenaceum*. Further study is required to confirm the mechanism of these effects, though antagonistic interactions between *Fusarium* spp. have been observed elsewhere. Differences in the colonization rates of several *Fusarium* spp. infecting pea roots were observed between single and combined inoculations, suggesting interspecific competition (Zitnick-Anderson *et al*., 2018). Additionally, the growth of *F. moniliforme* and *F. proliferatum* are inhibited by *F. graminearum* in maize kernels (Velluti *et al*., 2000).

To ensure that disease symptoms were consistent across inoculated treatments, peas were inoculated with *A. euteiches* and *Fusarium* spp. using different methods. Soil inoculation with oospores was used for *A. euteiches,* as oospores are easy to produce and serve as the primary inoculum source in the field (Gaulin *et al*., 2007). Seed inoculation is a method commonly used for *Fusarium* spp., and is favoured as it produces highly consistent disease symptoms (Grünwald *et al*., 2003; Feng *et al*., 2010). It is possible that seed inoculation may facilitate rapid infection of germinating seedlings by *Fusarium* spp., but it was not anticipated that using two inoculation methods would produce appreciable differences in disease dynamics after four weeks of growth. This was supported by the overall consistency in the quantity of *A. euteiches* DNA between single and combined inoculations. However, the impact of seed versus soil inoculation on the timing of infection during early growth stages was not investigated here. Further study of interactions between soil-borne pathogens at the seedling stage would therefore benefit from evaluation of the influence of inoculation methods on disease development.

The presence of *F. solani* and *F. redolens* DNA in non-inoculated treatments can most likely be attributed to seedborne infections or the presence of naturally-occurring *Fusarium* in the soil. This natural occurrence of *Fusarium* spp. on seed or in the soil was assumed to be equal for each treatment. Therefore, although a baseline contamination was present in all treatments, the resulting differences between treatments were over-and-above the level of natural inoculum and the observed treatment differences were still

recognizable and significant. The relative quantity of *F. redolens* DNA when inoculated singly was approximately equal to the quantity of *F. redolens* DNA detected in single inoculations of *A. euteiches*, and combined inoculations of *A. euteiches* and *F. avenaceum*, suggesting that *A. euteiches* may interact synergistically with *F. redolens* even when the inoculum level of the latter pathogen is very low. Field surveys suggest that *F. redolens* is ubiquitous across the Prairie provinces (Chatterton, personal communication), therefore the observed interaction may contribute to increased disease severity where *A. euteiches* is also present. A low level of *A. euteiches* DNA was also present in non-inoculated treatments, but none of these treatments were significantly different from the control group. Detection of *A. euteiches* in these treatments is likely the result of motile zoospores travelling between treatment groups during the four-week growth period. However, the late stage at which zoospore release would have occurred would likely allow qPCR detection but would not be sufficient to induce significant levels of root rot. Therefore, it was not expected that this would negate the treatment differences observed.

Root-associated phytopathogens are heavily influenced by the abiotic and biotic conditions of the rhizosphere (Dixon & Tilston, 2010). In particular, soil moisture conditions can have a profound impact on the PRRC community structure (Esmaeili Taheri *et al*., 2016). *Aphanomyces euteiches* and *F. avenaceum* are favoured by wet conditions, and are the predominant pathogens infecting pea roots in years of high rainfall (Feng *et al*., 2010; Esmaeili Taheri *et al*., 2016). Conversely, dryer conditions facilitate the germination of *F. solani* conidia (Palmero Llamas *et al*., 2008; Esmaeili Taheri *et al*., 2016). Additional factors such as soil temperature and composition, nutrient availability, and interactions with plant symbionts have roles in defining the community structure of the rhizosphere (Tu, 1994; Dixon & Tilston, 2010). It is important, therefore, to note that the controlled conditions of a greenhouse do not reflect the highly variable field environment. In the present study, greenhouse trials were used to identify changes in

disease severity and fungal colonization when single versus multiple pathogens infect pea roots under baseline conditions. Determining how PRRC population dynamics vary based on soil and annual weather conditions will be an invaluable contribution to disease forecasting, and the observed interactions will be useful in guiding future study in a field setting.

In summary, two triplex qPCR assays were used to detect and quantify *A. euteiches, F. avenaceum, F. solani,* and *F. redolens* DNA relative to that of their mutual host. *Aphanomyces euteiches* appears to synergistically facilitate infection by the otherwise weak pathogen *F. redolens,* and may interact additively with *F. solani*. The ubiquity of *F. redolens* in commercial pea crops suggests that this pathogen species may contribute to increased disease severity when co-occurring with *A. euteiches*. Therefore, further study using varying inoculum doses will be necessary to clarify their relationship. *Aphanomyces euteiches* predisposes pea roots to FRR when multiple *Fusarium* spp. are present, resulting in more severe symptoms and increased fungal colonization. The interactions observed here warrant further study in the field, where molecular quantification of pathogen DNA from infected roots will be an essential tool in defining interspecific interactions within the pea root rot complex.

Table 2-1. Experimental Treatments. Abbreviations are shown in parentheses.	
1	Untreated control
2	*A. euteiches* (Ae)
3	*F. redolens* (Fr)
4	*F. redolens* + *A. euteiches* (Fr + Ae)
5	*F. solani* (Fs)
6	*F. solani* + *A. euteiches* (Fs + Ae)
7	*F. avenaceum* (Fa)
8	*F. avenaceum* + *A. euteiches* (Fa + Ae)
9	*F. redolens* + *F. solani* + *F.avenaceum* (Fr + Fs + Fa)
10	*F. redolens* + *F. solani* + *F.avenaceum A. euteiches* (Fr + Fs + Fa + Ae)

Table 2-2. Disease severity index for the assessment of FRR in *P. sativum*.

Rating	Symptoms
0	No symptoms
1	Discolouration on 1-25% of roots
2	Discolouration on 26-50% of roots; some reduction in root mass
3	Discolouration on 51-75% of root mass; moderate to severe reduction in root mass
4	Discolouration on 76-100% of root mass; severe reduction in root mass
5	100% of tissues necrotic; plant is dead

Table 2-3. Sequences and concentrations of primers and probes for multiplex qPCR.

Organism	Target	Reference[a]	Amplicon Size (bp)	Oligo Name[b]	T_m[c] (°C)	Sequence 5' → 3'	Concentration
P. sativum	*β-TUB1*	X54844	105	Psat_TUB1_3F	66.1	TGGTTCTTGACA ATGAAGCCTTG	200 nM
				Psat_TUB1_3R	60.5	AGGGTCAACATA GGAGAGTGAA	200 nM
				Psat_TUB1_3Pr	67.1	**ABY-** CTGAAGCTTAGT AACCCAAGCTGT AAGCT**-AQY**	200 nM
A. euteiches	*ITS*	KY593270	134	Ae1.2_ITS1F	63.7	CCTGCGGAAGGA TCATTACC	500 nM
				Ae1.2_ITS1R	63.6	AAAATTACATCG GTTCCTTGCG	500 nM
				Ae1.2_ITS1Pr	64.3	**56-FAM-** TTCTTTATGAGG CTTGTGCTCTT**- BHQ_1**	250 nM
F. redolens	*EF-1α*	KX094910	85	RedF	63.3	CCCTCTTCCCAC ACAATCAC	900 nM
				RedR	61.8	AGCTCAGCGGCT TCCTATTA	900 nM
				RedPr	71.8	**HEX-** TGAGCGCGGATC ATCACGTG- **BHQ_1**	200 nM
F. solani[d]	*EF-1α*	AB674290	90	SolF	62.3	GCGCCTTACTAT CCCACATC	900 nM
				SolR	63.2	TTTTGTGACTCG GGAGAAGC	900 nM
				SolPr	72.1	**FAM** CCTCCTCCGCGA CACGCTCT-**BHQ-1**	200 nM
F. avenaceum[d]	*TEF-1α*	KU981027	100	AveF	60.0	GCTTATCTGCAC TCGGAACC	900 nM
				AveR	60.8	CGCGTAATCGAA GGGATATT	900 nM
				AvePr	65.5	**HEX-** CGACAAGCGAA CCATCGAGA- **BHQ-1**	200 nM

[a]GenBank accession number.
[b]F denotes the forward primer, R denotes the reverse primer, Pr denotes the probe.
[c]Melting temperature.
[d]Zitnick-Anderson *et al*. (2018).

Table 2-4. Specificity tests of primers designed to target the β-tubulin or ITS regions of *P. sativum* and *A. euteiches*, respectively. Tests were run on endpoint PCR for both target species, and on qPCR for *A. euteiches*.

	Primer sets		
	Endpoint PCR[a]		qPCR[b] (C_t value)
Species	Psat_TUB1_3	Ae1.2_ITS1	Ae1.2_ITS1
Fusarium avenaceum	-	-	-
Fusarium solani	-	-	-
Fusarium redolens	-	-	-
Aphanomyces euteiches	-	+	25.8
Aphanomyces cochlioides		-	31.2
Pisum sativum	+	-	-
Fusarium acuminatum		-	-
Fusarium culmorum		-	-
Fusarium graminearum		-	-
Fusarium equiseti		-	-
Fusarium oxysporum		-	-
Fusarium sporotrichiodes		-	-
Fusarium poae		-	-
Pythium irregulare		*	-
Pythium ultimum		*	-
Rhizoctonia solani		-	-
Puccinia heliantha		-	-
Botrytis cinerea		-	-
Sclerotinia sclerotiorum		-	-

[a]'-' indicates no amplification, '*' denotes faint amplification, and '+' indicates strong amplification.
[b]'-' indicates no amplification in > 35 cycles.

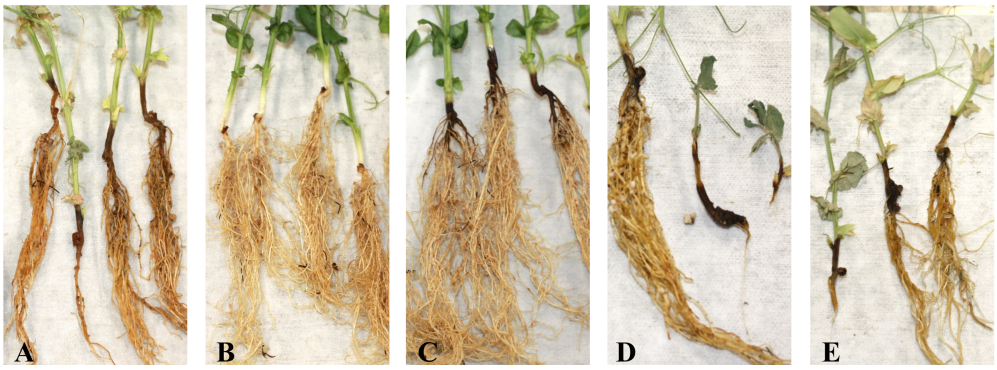

Figure 2-1. Disease symptoms induced in *P. sativum* by *A. euteiches* (a), *F. redolens* (b), *F. solani* (c), *F. avenaceum* (d), and a combination of all four pathogens (e).

Table 2-5. Disease severity, shoot height, and root weight (± SE) of pea seedlings inoculated with *A. euteiches, F. redolens, F. solani,* and *F. avenaceum*, singly or in combination. Differences between single and combined inoculations were evaluated using single degree of freedom contrast statements. The means and standard error were obtained from 4 replicates per treatment, and represent the combined values of two independent trials.

Treatment	Disease	Shoot height (cm)	Root weight (g)
Non-inoculated	1.05±0.22[a]	33.85±2.78	1.02±0.08
Ae	3.52±0.22	26.99±2.78	0.56±0.08
Fr	1.23±0.22	38.97±2.78	1.22±0.08
Fr + Ae	3.93±0.22	26.71±2.78	0.60±0.08
Fs	3.5±0.22	33.52±2.78	1.00±0.08
Fs + Ae	4.04±0.22	26.04±2.81	0.64±0.08
Fa	3.81±0.22	22.33±2.79	0.65±0.08
Fa + Ae	4.02±0.22	25.46±2.82	0.65±0.08
Fr + Fs + Fa	3.59±0.22	33.43±2.78	1.07±0.08
Fr + Fs + Fa + Ae	4.22±0.22	22.47±2.83	0.55±0.08
Contrasts			
Ae, Fr vs Fr + Ae	<0.0001[b]	0.0046	0.0052
Ae, Fs vs Fs + Ae	0.047	0.11	0.16
Ae, Fa vs Fa + Ae	0.20	0.64	0.64
Fs, Fa, Fr vs Fs+Fr+Fa	0.044	0.67	0.21
Ae, Fs, Fr, Fa vs Fa+Fr+Fs+Ae	<0.0001	0.0007	0.0016
Fs+Fr+Fa vs Fa+Fr+Fs+Ae	0.027	0.0006	<0.0001

[a]Least square means of dependent variables for each treatment.
[b]*p*-values of single degree of freedom contrast comparisons.

Table 2-6. Equations and R^2 values of standard curves for *P. sativum, A.euteiches, F. redolens, F. solani,* and *F. avenaceum* in singleplex and triplex. Standard curves were generated using five ten-fold serial dilutions that ranged from 10^7 to 10^3 fg.

Target	Singleplex		Triplex	
	Equation[a]	**R^2**	**Equation**	**R^2**
P. sativum	y = -3.685x + 49.947	0.999	y = -3.524x + 48.392	0.999
A. euteiches	y = -3.493x + 45.852	0.997	y = -3.435x + 47.812	0.994
F. redolens	y = -3.246x + 26.491	0.997	y = -3.463x + 46.358	0.996
F. solani	y = -3.344x + 26.911	0.990	y = -3.376x + 44.953	0.999
F. avenaceum	y = -3.450x + 26.936	0.973	y = -3.429x + 24.991	0.999

[a]x = mass of DNA; y = C_t value

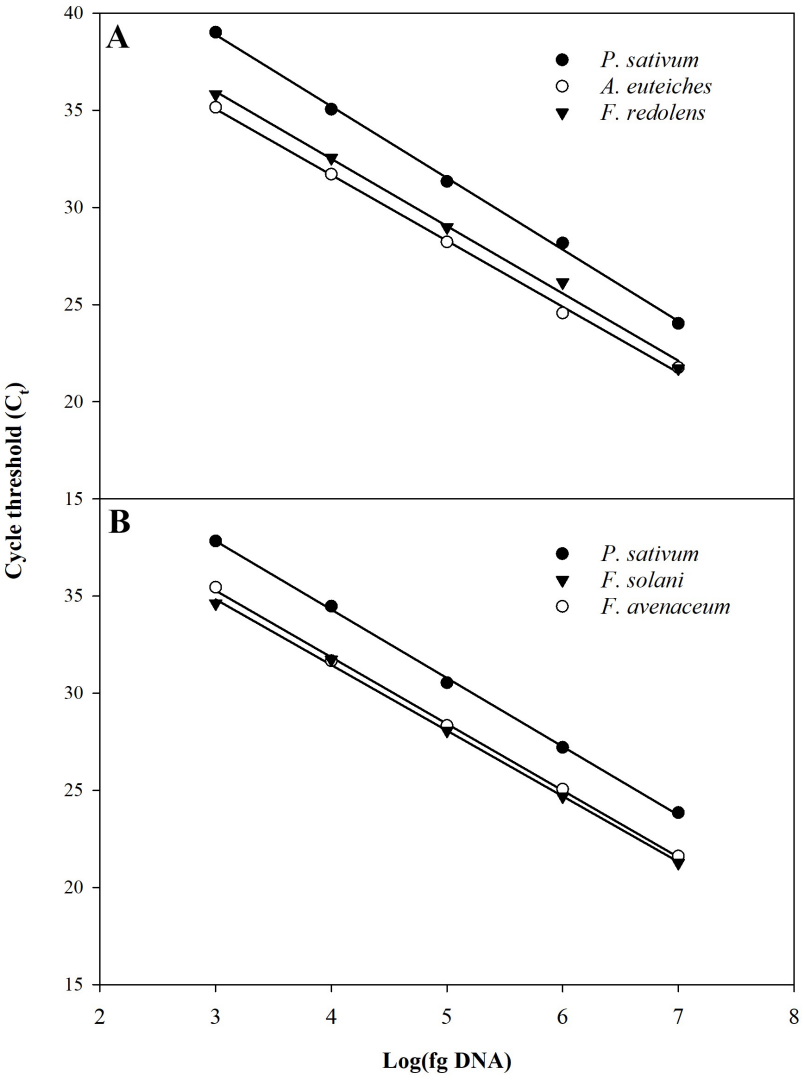

Figure 2-2. Standard curves of five ten-fold serial dilutions for multiplex qPCR. Assay 1 (A) quantified DNA of *P. sativum* (black circles), *A. euteiches* (open circles), and *F. redolens* (black triangles), and assay 2 (B) quantified DNA of *P. sativum* (black circles), *F. avenaceum* (open circles), and *F. solani* (black triangles).

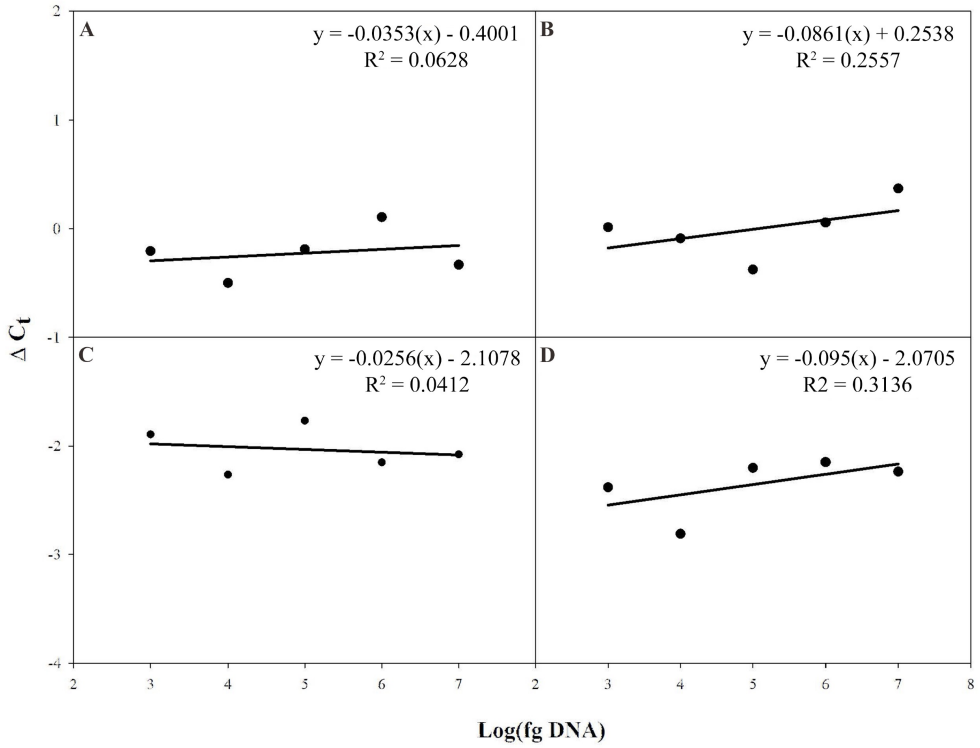

Figure 2-3. Log input amount of DNA versus ΔC_t for *A. euteiches* (a), *F. redolens* (b), *F. solani* (c), and *F. avenaceum* (d). Amplification efficiencies of the target and reference genes are considered equal when the absolute value of the slope is < 0.1. Mean C_t values were obtained from two replicates per dilution.

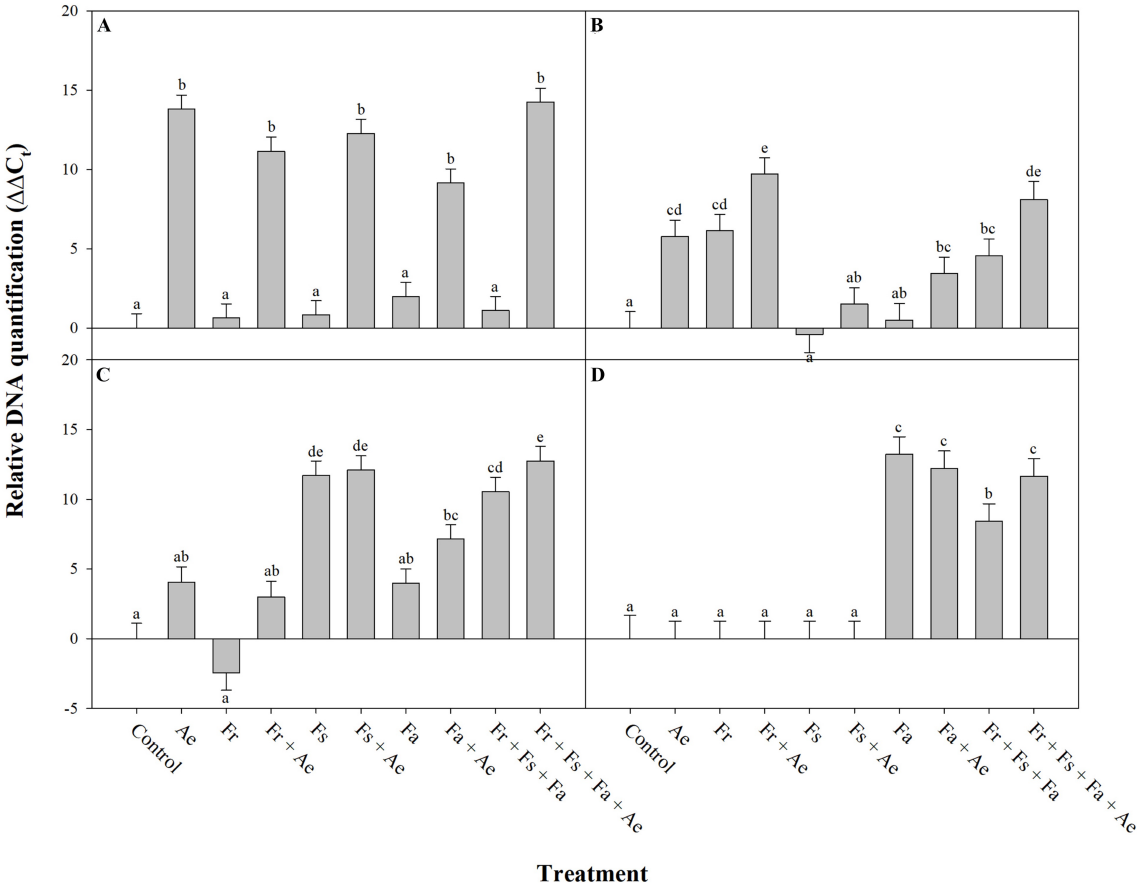

Figure 2-4. Quantification of *A. euteiches* (a), *F. redolens* (b), *F. solani* (c), and *F. avenaceum* (d) DNA relative to *P. sativum* using the comparative C_t ($\Delta\Delta C_t$) method. Columns marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 4 biological and 2 technical replicates per treatment, and represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.

CHAPTER 3: INTERACTIONS BETWEEN THE ROOT ROT PATHOGEN FUSARIUM AVENACEUM AND THE PEA LEAF WEEVIL (SITONA LINEATUS) IN FIELD PEA

3.1 INTRODUCTION

Dry pea (*Pisum sativum* L.) is a pulse crop with global economic and nutritional value (Jacob *et al*., 2016). Production of pea and other pulse crops has increased significantly since the 1980s: more than 11 million tonnes of dry pea were produced in 2014, primarily by Canada, Russia, and China (FAO, 2017). As land area dedicated to pea cultivation continues to increase, soil-borne pathogens and herbivorous insects are becoming serious obstacles to production (Xue, 2003a; Vankosky *et al*., 2009). The impacts of each threat are well understood in isolation, but interactions between herbivorous insects, phytopathogens, and their shared host are only beginning to be recognized as important determinants of plant performance (Fournier *et al*., 2006). Pathogens and insects often attack pea simultaneously during early stages of growth, and interspecific interactions between these organisms have the potential to increase plant damage and economic loss.

Fusarium Link spp. are the primary pathogens in the pea root rot complex (PRRC), a group of fungi and oomycetes that cause cortical and vascular decay in *P. sativum* roots (Xue, 2003a; Xue, 2003b; Esmaeili Taheri *et al*., 2016). *Fusarium* spp. have a cosmopolitan distribution and can persist in soil for many years as chlamydospores or by overwintering in crop residues (Hargreaves & Fox, 1977; Kraft *et al*., 1993; Ma *et al*., 2013). Infection typically initiates in the epi- and hypocotyl regions of the root and lower stem, with later stages of fusarium root rot (FRR) characterized by black root lesions, red staining of the vascular tissue, disintegration of the lateral and tap roots, and wilting and chlorosis of aerial parts (Kraft *et al*., 1993; Hwang *et al*., 1994). FRR can result in yield losses of up to 60% in field pea (Chang *et al*., 2005; Gossen *et al*., 2016). There are currently no satisfactory methods of managing FRR in pea or other pulse crops.

The pea leaf weevil (PLW) *Sitona lineatus* L. (Coleoptera: Curculionidae) is an agricultural pest in Europe, Africa, Asia, and North America (Vankosky *et al*., 2009). Adult weevils disperse from overwintering sites to pea and faba bean (*Vicia faba* L.) fields in the spring to feed on foliage and reproduce (Fisher & O'Keeffe, 1979; Landon *et al*., 1995). Immediately after hatching, larvae invade root nodules to feed on *Rhizobium* bacteria (Jackson & Macdougall, 1920; Johnson & O'Keeffe, 1981). Defoliation by adult weevils is not expected to reduce photosynthesis to the point where yield is affected unless feeding intensity is unusually severe (George *et al*., 1962). However, consumption of rhizobium root nodules by larvae may reduce yield and seed quality if soil nitrogen is deficient (El-Dessouki, 1971; Nielsen, 1990; Lohaus & Vidal, 2010). Pea leaf weevils are typically managed with synthetic insecticides applied to seed or foliage, though the success of these applications is highly variable (Seidenglanz *et al*., 2010; Vankosky, Cárcamo, McKenzie, *et al*., 2011; Cárcamo *et al*., 2012).

Interspecific interactions between PLW and root rot pathogens have not been investigated, despite the potential for frequent co-occurrence in pea crops: preliminary data from 2017 field surveys indicate that approximately one third of pea fields in Alberta had evidence of both root rot disease and PLW herbivory (Chatterton, personal communication). Synergistic interactions between *Sitona* and *Fusarium* spp. have been well documented. The clover root curculio (*S. hispidulus* F.) and the vascular wilt pathogen *F. oxysporum* Schlecht. f. sp. *medicaginis* (Weimer) Snyd. & Hans are frequently associated with the roots of alfalfa (*Medicago sativa* L.): *F. oxysporum* is known to colonize *S. hispidulus* feeding wounds, resulting in more severe disease symptoms and reductions in yield (Dickason *et al*., 1968; Godfrey & Yeargan, 1987; Kalb *et al*., 1994). Additionally, *S. hispidulus* larvae may locally vector *F. oxysporum* by transporting fungal propagules to uninfected plants (Leath & Hower, 1993). Similarities in disease cycle and insect life history suggest that comparable interactions may occur between *S. lineatus* and *Fusarium* spp. in pea. Both organisms are primary factors

constraining pea production, and additional increases in the severity of FRR instigated by PLW herbivory will have important consequences for producers. The identification of interspecific interactions is therefore required to inform management strategies aimed at minimizing yield loss. The objective of this research was to evaluate the relationship between larval PLW and *F. avenaceum* when co-occurring in *P. sativum* roots under controlled conditions, to serve as a basis for further experiments in the field. Greenhouse experiments were used to assess the impact of insect feeding injury on disease severity, the effects of root rot on patterns of herbivory, and changes in insect mortality in relation to plant infection status.

3.2 Materials and Methods

3.2.1 Fungal Culture and Conidia Isolation

Fusarium avenaceum isolate 1306.08 was chosen based on its high level of virulence, which was determined in greenhouse pathogenicity tests (Chatterton, personal communication). Isolate 1306.08 was obtained from pea roots collected during field surveys near Lethbridge, Alberta, Canada in 2014, and was stored at 4°C on synthetic nutrient-deficient medium 'Spezieller Nährstoffarmer agar' (Leslie & Summerell, 2006). 1306.08 was grown at room temperature on potato dextrose agar (PDA) amended with penicillin and streptomycin, each at a concentration of 150 mg/L. After 6 days of growth, sporulation was induced by transferring disks (8 mm diameter) taken from the outer edge of the PDA cultures to liquid carboxymethylcellulose (CMC) medium (Cappellini & Peterson, 1965). The inoculated CMC medium was incubated on a shaker at 170 rpm for 4-7 days, or until the culture was cloudy with visible clumps of mycelia. Cultures were agitated aggressively by hand to release conidia before filtration through four layers of cheesecloth to remove mycelia and agar. Conidia were collected by centrifugation at 4000 rpm for 10 minutes. The supernatant was poured off and the conidia were washed three times with sterile distilled water (SDW), centrifuging for ten minutes after each addition

of water. Conidia were resuspended in SDW and counted with the aid of a hemocytometer.

3.2.2 Seed Inoculation and Plant Growth Conditions

'CDC Meadow', a *P. sativum* cultivar with susceptibility to FRR, was used as a host for all trials. Pea seeds were inoculated with *F. avenaceum* by soaking them overnight in a solution of conidia and SDW containing 7500 conidia/mL. Seeds for disease-free treatments were soaked for an equal amount of time in SDW. Seeds were sown in 3 litre plastic pots containing a soil mix composed of equal parts sand, peat, and top soil. Three seeds were planted per individual pot. To facilitate the production of rhizobium root nodules, nitrogen fertilizer was excluded and TagTeam granular *Rhizobium leguminosarum* inoculant (containing 1.3 x 10^8 viable cells per gram from Monsanto BioAg, Winnipeg, MB, CA) was thoroughly combined with soil at the label rate. Plants were grown for four weeks in a greenhouse with a 16:8 cycle of light at 22°C and dark at 18°C. All pots were enclosed within a mesh sleeve constructed with Dacron Chiffon (Bioquip, Rancho Dominguez, CA, USA) immediately following seedling emergence. Drip irrigation lines were installed in each pot and plants were given 500 mL of water daily.

3.2.3 Insect Collection and Rearing Conditions

Adult PLWs were collected by sweep net in late August 2015 from alfalfa fields near Lethbridge, Alberta, Canada. Weevils were fed alfalfa for two weeks prior to overwintering at 4°C in 4 litre plastic buckets lined with moistened top soil and leaf litter. On February 3, 2016 approximately 1200 weevils were removed from cold storage and transferred to mesh rearing cages (MegaView Science Co., Taichung, TW) kept at 20°C with a 16:8 light and dark cycle. Weevils were fed alfalfa seedlings for 28 days. Following this, pea and faba bean seedlings were also provided and cages were checked

daily for mating pairs. Mating was first observed on April 4, and a total of eight mating pairs were identified over the next three weeks. Each pair was immediately removed from the rearing cages and isolated in a plastic Petri dish lined with wet filter paper and fresh pea leaves. If a pair did not produce eggs, the male was replaced with another from the colony. Eggs were collected daily from the Petri dishes using a fine-tipped paint brush moistened with SDW and were stored at 4°C until needed, but for no longer than 10 days. When sufficient eggs had been collected, they were transferred to the soil surface near pea plants between the first and second node growth stages. A total of 40 eggs per pot were added to treatments subject to insect exposure, whereas no eggs were added to control treatments. This egg density was selected based on field observations of 2,000 – 20,000 eggs per square metre in pea plots (Cárcamo, personal communication), and on the observations of Cantot (1989) that indicate a minimum density of 10 eggs per individual plant or 20 eggs per group of three plants is required to induce a negative impact on plant development. Eggs from each collection day were distributed among pots to control for variation in egg quality, and an equal number of eggs were distributed around each plant within a pot.

3.2.4 Experimental design and sample processing

Four experimental treatments were used to evaluate interactions between *S. lineatus* and *F. avenaceum* in field pea. A control group of plants was isolated from both weevils and *F. avenaceum*. One treatment each was exposed to either *F. avenaceum* or *S. lineatus* larvae, and the fourth treatment included both *F. avenaceum* and larvae. All treatments had six replicates with three plants per replicate, and were arranged in a randomized complete block design. Two independent trials were conducted in 2016, from April 8 to May 25 and April 22 to June 7, respectively. Plants were grown until the onset of flowering, which occurred approximately 6.5 weeks after planting. Flowering coincides with peak nodule activity in *P. sativum* (Depret & Laguerre, 2008), and this growth

period allowed two to three weeks for PLW eggs to hatch followed by four weeks of larval feeding (Jackson & Macdougall, 1920). At the end of the growth period, the contents of each pot were carefully removed and individually placed into a plastic tub filled with water. Once the mass of soil around the roots had softened, plants were removed gently to avoid dislodging nodules. Any larvae or pupae floating on the surface of the water were collected with forceps and recorded (Figure 3-1). The soil was rinsed through a 2 mm metal sieve to capture any remaining insects or nodules. Dislodged nodules with signs of feeding injury were dissected under a stereoscopic microscope, and larvae were counted. The roots were cleaned of residual soil by rinsing them over the sieve, and any nodules that detached were dissected and recorded.

3.2.5 Evaluation of Disease Severity, Nodulation Efficiency and Herbivory

FRR severity was visually assessed using a disease severity index that ranged from 0 to 5, with 0 indicating that no disease symptoms were present and 5 indicating a dead plant with a completely decayed root system (Table 2-2, Figure 3-2). Clean roots were severed from the stem at the epicotyl, and the length of the shoot from the epicotyl to the terminal node was measured. The roots were patted dry and the fresh weight was recorded. Nodulation efficiency was assessed using the protocol provided by 20/20 Seed Labs (2006) to measure the ability of the pea plants to fix nitrogen (Table 3-1). This consisted of a score derived from an evaluation of plant vigour, nodule abundance, leghemoglobin production, and the location of nodule clusters on the roots. Plants were considered vigorous when leaves and shoots were green with no signs of stunting or chlorosis. Finally, the total number of nodules and the number of nodules injured by larval feeding per plant were counted. Any nodule with signs of feeding injury was dissected under a stereoscopic microscope to recover PLW larvae.

3.2.6 Statistical Analyses

All statistical analyses were performed using JMP version 13.1 (SAS Institute Inc., Cary, NC, USA). The Kruskal-Wallis test was used to analyze all nodule efficiency data, as an ordinal scale was used for ranking. For nonparametric tests, pairwise comparisons between treatment means were conducted using the Wilcoxon matched pair test with a Bonferroni adjustment. Disease severity, shoot height, root weight, and nodule counts in separate mixed-effect analysis of variance models for a randomized complete block experimental design. Treatment was specified as a fixed effect, whereas trial and replicate were considered random factors. For significant treatment effects, means were separated using the Tukey-Kramer HSD test. Shoot height, root weight, and nodule count data were log transformed to improve normality prior to analysis. To evaluate treatment effects on the abundance of PLWs and nodules damaged by herbivory, treatments without insects were removed to avoid values of zero. Two-tailed independent sample t-tests were used to assess differences between the latter two dependent variables based on treatment. Normality of all dependent variables was assessed using the Shapiro-Wilk test. Outlier detection was performed using standardized scores. Data from repeated experiments were pooled following F-tests for equality of variance. The Type 1 error rate (α) was set at 0.05 for all statistical tests.

3.3 Results

3.3.1 Disease Severity

Inoculation of *P. sativum* seed with *F. avenaceum* resulted in moderate disease symptoms after 6.5 weeks of growth (Figure 3-3A). Plants that were not inoculated did not show evidence of root rot disease, regardless of whether or not *S. lineatus* larvae were present. Disease ratings above 0 in the control and PLW treatments reflect trace root discolouration of unknown origin. Disease severity increased significantly when both *F. avenaceum* and *S. lineatus* were present, relative to plants exposed only to *F. avenaceum*

($p = 0.023$). Neither shoot height ($F_{(3, 40)} = 2.53$; $p = 0.085$), nor root weight ($F_{(3, 40)} = 2.37$; $p = 0.10$) differed by treatment (Figure 3-3B, C).

3.3.2 Nodulation Efficiency

Herbivory was the only factor that had an impact on nodulation efficiency. The presence of PLW larvae significantly reduced overall nodulation efficiency in pea relative to the control group ($p = 0.0012$), and this effect was stronger when *F. avenaceum* was also present ($p < 0.0001$) (Table 3-2). Feeding injury had the greatest effect on nodule abundance and leghemoglobin expression ($p < 0.0001$), and this score decreased nearly two-fold when herbivory and root rot co-occurred ($p < 0.0001$). When PLW herbivory and FRR were present at the same time, the position of nodules within the root system was significantly modified ($\chi^2_{(3, N = 47)} = 33.58$, $p < 0.0001$). Whereas plants in the control group exhibited crown and lateral nodulation, plants exposed to PLW larvae and FRR tended more towards lateral nodulation ($p = 0.0018$). A similar trend was observed in comparison to the FRR treatment ($p = 0.021$). Plants in the FRR treatment were not significantly different from either the control ($p = 0.89$) or PLW treatments ($p = 0.67$). *F. avenaceum* alone did not have a significant effect on plant vigour, nodule abundance and leghemoglobin expression, or nodule position within the root system. Significant decreases in plant vigour relative to the control were only observed when plants had been exposed to both PLW and *F. avenaceum* ($p = 0.0054$).

3.3.3 Herbivory

Significant differences in the mean number of nodules between treatments were detected ($F_{(3, 40)} = 57.11$, $p < 0.0001$). Exposure to *S. lineatus* larvae resulted in significantly fewer nodules than the control group ($p < 0.0001$) (Figure 3-4). Nodulation was further limited when both FRR and PLW larvae were present, in comparison to the

control ($p < 0.0001$) and PLW treatments ($p = 0.018$). FRR alone did not have a significant impact on nodule abundance in comparison to the control ($p = 0.73$). The mean number of damaged nodules was comparable between plants exposed only to *S. lineatus* and those exposed to both *S. lineatus* and *F. avenaceum* ($p = 0.54$). Root nodules were small across treatments and those with damage had been completely consumed with only a small portion of the outer membrane remaining. No larvae were located inside of any nodules, as all damaged nodules had been completely excavated.

3.3.4 PLW Abundance

The survival rate of PLW larvae was significantly higher when insects were associated with pea plants infected by *F. avenaceum* in comparison to healthy plants ($p = 0.031$) (Figure 3-5). Few larvae were recovered from soil in either treatment, as most had developed into pupae by the early flowering stage of the host plants. The number of larvae recovered from the PLW treatment did not differ from the treatment with both PLW and FRR ($p = 0.84$). Significantly more pupae were recovered from soil when both the pathogen and insect were present, relative to plants exposed only to PLW herbivory ($p = 0.0035$).

3.4 Discussion

Exposure of pea plants to both *F. avenaceum* and PLW larvae resulted in more severe root rot disease relative to plants that were exposed only to *F. avenaceum*. Additionally, more larvae survived to pupation when weevils consumed the root nodules of infected versus healthy plants. This indicates that *S. lineatus* and *F. avenaceum* interact in a mutualistic manner when colonizing pea roots under controlled conditions, providing a basis for further investigation of this interaction in the field. Mutualistic interactions can synergistically increase the damage caused by multiple organisms, thus increasing the risk

of yield loss. Accordingly, the identification of interspecific interactions between insects and pathogens is a critical step in the management of agroecosystems, as the individual effects of either organism may be altered in a way that makes yield loss difficult to predict (Hatcher, 1995; Jesus Junior *et al*., 2014).

Interactions between insects and plant pathogens can occur both directly and indirectly. The former occurs when one plant exploiter directly alters access to plant resources for another; for example, microbes may colonize sites of insect feeding injury or an insect may serve as a pathogen vector (Hatcher, 1995; Stout *et al*., 2006). *Fusarium* spp. have been directly associated with the feeding wounds of insects spanning several orders. *Fusarium oxysporum* colonizes sites of *S. hispidulus* feeding injury on alfalfa roots, increasing both the incidence and severity of root rot disease (Dickason *et al*., 1968; Leath & Hower, 1993). The larvae of fungus gnats (*Bradysia* spp.; Diptera: Sciaridae) facilitate infection by *F. oxyporum* f. sp. *medicaginis* and *F. roseum* in the roots of alfalfa and red clover (Leath & Newton, 1969). Adult gnats predispose tomato and pinto bean to root and hypocotyl infections by *F. oxysporum* f. sp. *radices-lycopersici* (Gillespie & Menzies, 1993). Larvae of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), increase the incidence of Fusarium kernel rot and symptomless infection in maize (Sobek & Munkvold, 1999). Each of these insects has been found to vector *Fusarium* spp. by depositing conidia or mycelia on damaged plant tissue. PLW larvae feed exclusively on *R. leguminosarum* bacteroids found within the cells of root nodules (Sobek & Munkvold, 1999). In doing so, the cortical and vascular tissues of the root nodules are often damaged extensively, as was the case in the current study. It is likely that these damaged tissues served as infection loci for *F. avenaceum,* predisposing pea plants to higher infection rates and more severe disease symptoms.

In addition to direct interactions between *F. avenaceum* and PLW larvae, indirect interactions mediated by the host plant likely had a role in increasing disease severity.

Physical and physiological changes induced in plants following infection or injury can result in a modified response to other exploiters (Stout *et al*., 2006). *Fusarium* spp. are opportunistic pathogens, causing minimal to moderate damage when host plants are grown in conditions optimal for root development (Harveson *et al*., 2005). This was confirmed in the present experiment, as moderate levels of FRR did not negatively impact the growth of roots or shoots, nodule abundance, or nodulation efficiency under greenhouse conditions. Disease can become more severe when plants are stressed by environmental factors such as soil compaction, high temperature, excessive moisture or drought (Harveson *et al*., 2005; Esmaeili Taheri *et al*., 2016). The destruction of rhizobium root nodules by PLW larvae may similarly predispose plants to more severe root rot, particularly when soil is low in nitrogen. Inoculation with *R. leguminosarum* has reduced the severity of root rot caused by *F. solani* in pea (Chakraborty & Chakraborty, 1989) and common bean (*Phaseolus vulgaris* L.) (Hassan Dar *et al*., 1997), indicating that the availability of nitrogen is an important factor in the susceptibility of legumes to FRR. This is consistent with research showing that amendment of soil with nitrogen can reduce root rot disease in clover by up to 33% (O'Rourke *et al*., 2012). In the present study, pea that was simultaneously exposed to both *S. lineatus* larvae and *F. avenaceum* showed a marked decrease in nodulation efficiency brought about by a reduction in both the number of nodule clusters and leghemoglobin production within nodules. PLW herbivory also reduced the total number of nodules per plant; further reductions in nodulation were observed when *F. avenaceum* and PLW co-occurred. Additionally, the pattern of nodulation shifted from crown and lateral nodulation in healthy plants, to nodulation occurring almost solely in the crown region when PLW herbivory was present. Nodulation occurring throughout the root system is typical of plants inoculated with granular *Rhizobium* inoculant, and results in higher nitrogen accumulation than pea plants with only crown nodulation (Clayton *et al*., 2004). All of these factors likely decreased the nitrogen-fixing ability of the host plants, and were met by a significant increase in

disease severity. PLW herbivory may therefore have increased the susceptibility of pea to FRR by preventing plants from accessing sufficient nitrogen.

The interaction between *F. avenaceum, S. lineatus* and *P. sativum* appeared to decrease insect mortality rates, suggesting that FRR alters the quality of food resources for PLW larvae. More pupae were present in soil when plants were infected with *F. avenaceum*, but it was not clear why this occurred. Plant roots produce secondary metabolites in response to both pathogen infection and root herbivory, some of which confer resistance to a range of organisms (Cooper *et al*., 2005; Erb *et al*., 2013). Phytoalexins are a diverse group of compounds with antimicrobial properties that are produced by plants in response to abiotic or biotic stress (Purkayastha, 1995). Pisatin is the primary phytoalexin produced by *P. sativum*, and is considered to be an important component of disease resistance (Delserone *et al*., 1999). Many species of fungi pathogenic to pea can rapidly demethylate pisatin, rendering the compound non-toxic (Cruickshank, 1962; Delserone *et al*., 1999). There is increasing evidence that herbivorous insects also induce the production of phytoalexins, including pisatin, and that these compounds deter feeding in some cases (Sutherland *et al*., 1980; Berenbaum, 1988; Erb *et al*., 2013). Larvae of the root feeding beetles *Costelytra zealandica* White and *Heteronychus arator* F. (Coleoptera: Scarabaeidae) reduced feeding when given an artificial diet containing pisatin and a range of other phytoalexins (Sutherland *et al*., 1980). Glyceoline, a phytoalexin of soybean, similarly reduced food utilization of the soybean looper (*Pseudoplusia includens* Walker; Lepidoptera: Noctuidae) and strongly deterred feeding of the Mexican bean beetle (*Epilachna varivestis* Mulsant; Coleoptera: Coccinellidae) (Hart *et al*., 1983). Several *Fusarium* spp., including *F. solani, F. avenaceum, F. oxysporum* and *F. graminearum* are capable of demethylating pisatin (Cruickshank, 1962; Lappe & Barz, 1978; Delserone *et al*., 1999). If pisatin has toxic effects on PLW larvae, demethylation of this phytoalexin by pathogenic *Fusarium* spp. would explain the observed increase in insect survival. This was not demonstrated here,

but could be investigated as a potential mechanism for the observed interaction. Additionally, further investigation of aspects of PLW fitness such as larval weight, development time, and adult egg-laying potential when exposed to *Fusarium*-infected roots would reveal more about the relationship between these organisms.

In summary, a mutualistic interaction occurred between *F. avenaceum* and *S. lineatus* larvae when both organisms colonized the roots of *P. sativum* in a greenhouse experiment. The mechanism behind this interaction was not investigated, but the observed increase in disease severity likely occurred as a result of fungal colonization of injured root nodules, and more indirectly through a reduction in nitrogen fixation that increased plant susceptibility to infection. Pea leaf weevil larvae appeared to benefit from the interaction through decreases in mortality; pisatin detoxification by *F. avenaceum* presents an intriguing topic for future study of a potential mechanism for this interaction. These preliminary results suggest the potential for increased disease pressure in pea-producing regions where both *F. avenaceum* and *S. lineatus* are present. It is likely that environmental conditions such as soil compaction, moisture level, and nutrient availability have a considerable impact on the ability of pea to cope with insects and pathogens. Moreover, pea is often simultaneously infected with multiple PRRC pathogens, which may further exacerbate root damage and yield losses. Field experiments will therefore be required to determine how PLWs interact with root rot pathogens under diverse environmental conditions, and how these interactions impact yield. These results emphasize that the effects of insects and pathogens on plant performance should not be viewed as independent, and will be useful in the design of management plans that incorporate the monitoring of both insect and fungal populations in pea crops.

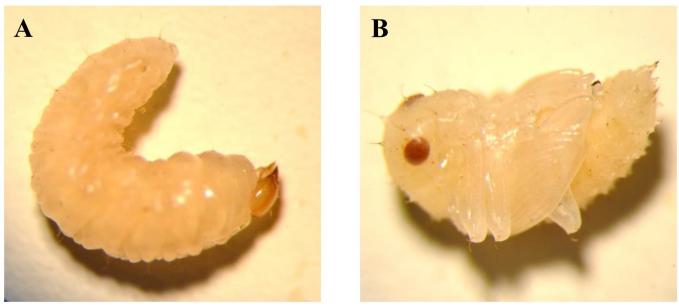

Figure 3-1. **PLW larvae (A) and pupae (B) recovered from rhizosphere soil of pea plants at the flowering stage.**

Figure 3-2. Symptoms of FRR in field pea. Disease severity has been rated using an index ranging from 0 (no symptoms) to 5 (dead plant) (A). Vascular tissue has been stained red by *F. avenaceum* (B).

Table 3-1. Nodulation efficiency index (20/20 Seed Labs, 2006).	
Plant vigour	
5	Plants green and vigorous
3	Plants green and relatively small
2	Plants slightly chlorotic
1	Plants very chlorotic
Nodule number/colour	
5	Greater than 5 clusters of pink nodules (expressing leghemoglobin)
3	3-5 clusters of predominately pink nodules (expressing leghemoglobin)
1	Less than 3 clusters of nodules or nodules white/green in colour (not expressing leghemoglobin)
0	No nodules or nodules white/green in colour (not expressing leghemoglobin)
Nodule Position	
3	Crown and lateral nodulation
2	Generally crown nodulation
1	Generally lateral nodulation
Total Score	
11-13	Effective nodulation. Good nitrogen fixation potential
7-10	Nodulation less effective. Fixation potential reduced.
1-6	Generally unsatisfactory nodulation.

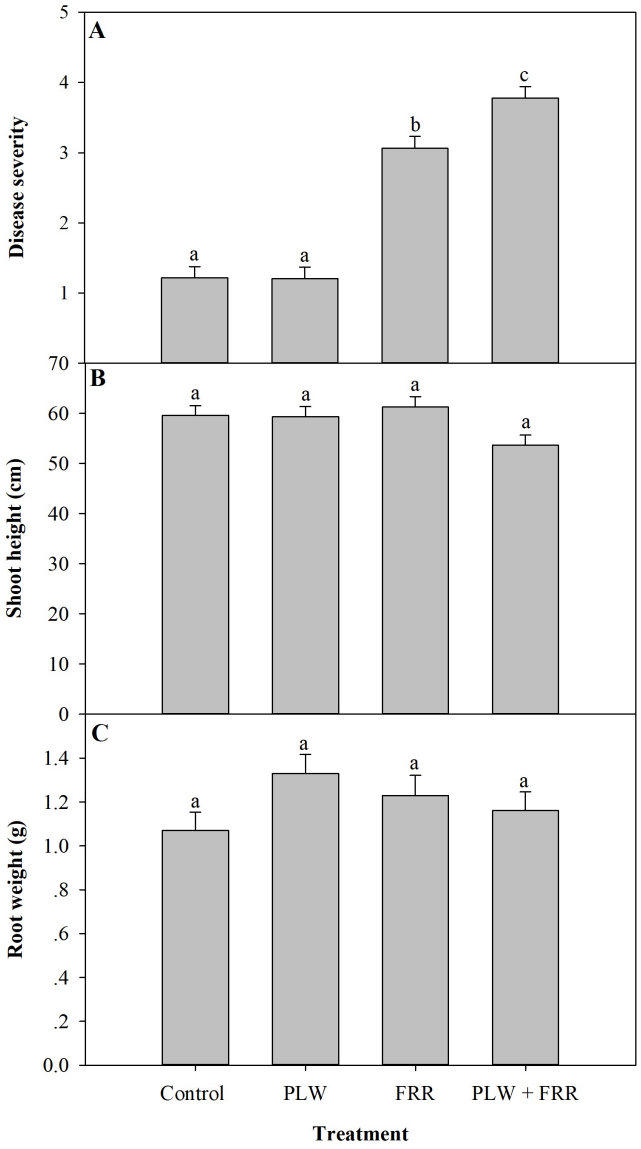

Figure 3-3. Disease severity (A), shoot height (B), and root weight (C) of *P. sativum* exposed to *S. lineatus* larvae (PLW), *F. avenaceum* (FRR) or both (PLW + FRR). Columns marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 replicates per treatment, and represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.

Table 3-2. Nodulation efficiency of *P. sativum* (± SE) based on exposure to *S. lineatus* larvae (PLW), *F. avenaceum* (FRR) or both (PLW + FRR). Means followed by the same letter are not significantly different based on the Wilcoxon matched pair test with a Bonferroni correction. The means and standard error were obtained from 6 replicates per treatment, and represent the combined values from two independent trials.

Treatment	Plant Vigour	Nodule Number/Colour	Nodule Position	Total Score
Control	4.72±0.21*a*	4.53±0.21*a*	2.29±0.068*a*	11.54±0.31*a*
PLW	4.38±0.21*ab*	2.95±0.21*b*	1.93±0.068*ab*	9.26±0.31*b*
FRR	4.48±0.22*ab*	4.59±0.22*a*	2.14±0.071*a*	11.28±0.33*a*
PLW + FRR	3.53±0.21*b*	1.78±0.21*c*	1.89±0.068*b*	7.19±0.31*c*

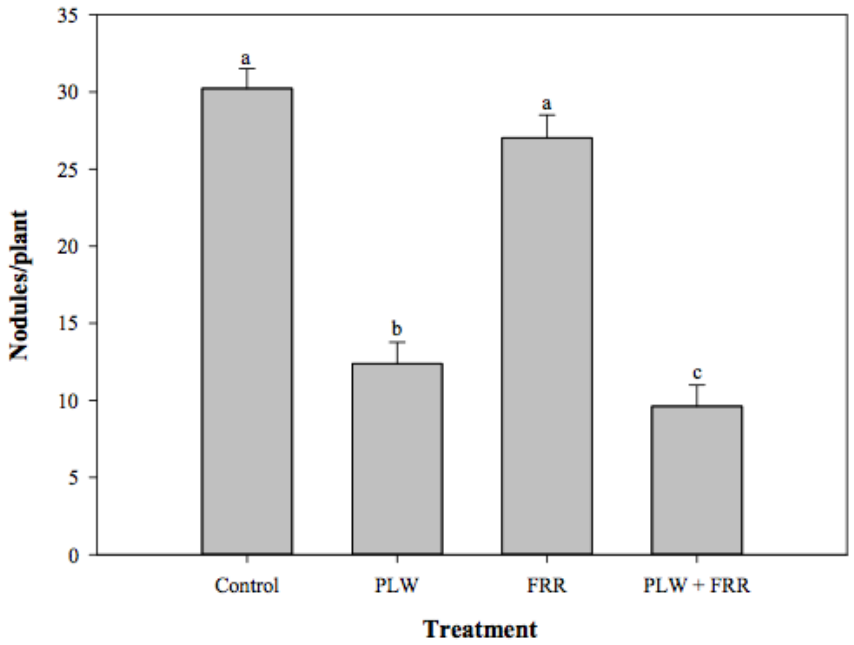

Figure 3-4. Nodules present in *P. sativum* roots at early flower following exposure to *S. lineatus* larvae (PLW), *F. avenaceum* (FRR) or both (PLW + FRR). Columns marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 replicates per treatment, and represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.

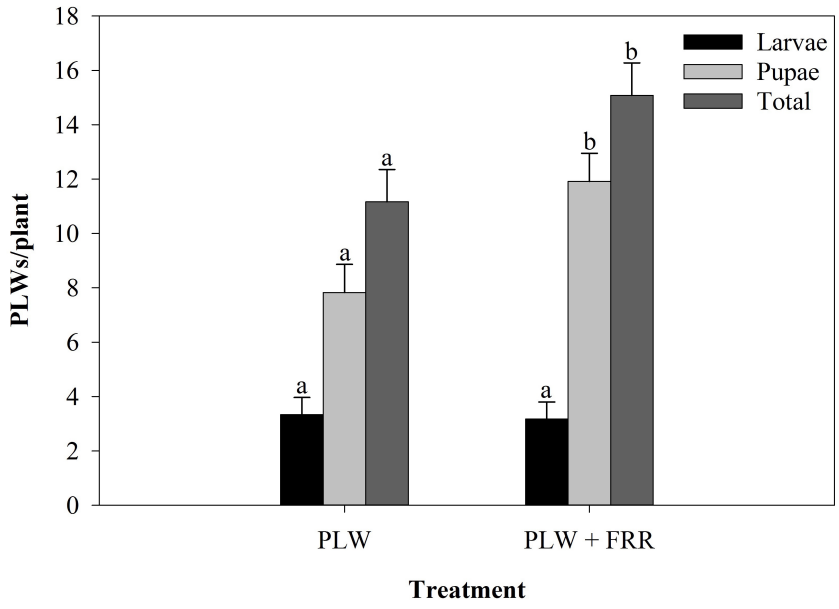

Figure 3-5. PLW larvae, pupae or total insects recovered from the rhizosphere soil of plants exposed to *S. lineatus* larvae (PLW) or a combination of larvae and *F. avenaceum* (PLW + FRR). For each developmental stage, columns marked with the same letter are not significantly different based on a two-tailed independent sample t-test. The means and standard error were obtained from 6 replicates per treatment, and represent the combined values from two independent trials. Vertical bars indicate standard error of the mean.

CHAPTER 4: EVALUATION OF INTEGRATED MANAGEMENT TECHNIQUES FOR THE CONTROL OF ROOT ROT DISEASE AND PEA LEAF WEEVIL HERBIVORY IN FIELD PEA AND FABA BEAN

4.1 INTRODUCTION

In natural and agricultural systems, plants are continuously challenged with a diversity of pathogens and herbivores (Fournier *et al*., 2006). The highly virulent root rot pathogens *Aphanomyces euteiches* Drechs. and *Fusarium* Link spp. frequently infect field pea (*Pisum sativum* L.) crops in Canada, along with several other fungal and oomycete pathogens that are collectively referred to as the pea root rot complex (PRRC) (Xue, 2003a; Esmaeili Taheri *et al*., 2016). These pathogens frequently co-occur in pea crops: field surveys conducted in 2016 indicated that 73% of pea fields tested in Alberta, Saskatchewan, and Manitoba were positive for *A. euteiches* and at least one species of *Fusarium* (Chatterton, personal communication). The range of the pea leaf weevil (PLW) *Sitona lineatus* L. (Coleoptera: Curculionidae), a pest of pea and faba bean (*Vicia faba* L.), is also rapidly spreading across the Canadian Prairies (Vankosky *et al*., 2009; Olfert *et al*., 2012). Approximately 30% of pea fields surveyed in Alberta in 2017 had root rot disease and PLW herbivory (Chatterton, personal communication). It was notable that the incidence of root rot disease in these surveys was relatively low, possibly due to hot and dry weather conditions across the Prairie provinces (Environment Canada, 2017). The overlap in the ranges of PRRC pathogens and PLW may be more extensive in wetter years. Given the observed overlap in the geographical range of *A. euteiches*, *Fusarium* spp., and *S. lineatus*, these species have the potential to frequently co-occur in pea crops. Faba bean, the other reproductive host of PLW, will also likely experience co-infestation by these species. While resistance to *A. euteiches* has been reported in many *V. faba* cultivars, *Fusarium* spp. are the pathogens most frequently isolated from faba bean roots (Sillero *et al*., 2010). Furthermore, chapters 2 and 3 of this thesis demonstrate that additive and synergistic interactions occur between *A. euteiches* and some *Fusarium* spp.,

and that *S. lineatus* interacts mutualistically with *F. avenaceum*. These interactions are likely to have significant impacts on insect and microbe population dynamics in the soil, and may have important consequences for yield protection in both pea and faba bean. An effective method of integrated management is therefore required to minimize damage caused by root rot pathogens and PLW in regions where they are likely to co-occur.

Synthetic pesticides are the most common method of managing PRRC pathogens (Chang *et al*., 2013) and PLW herbivory (Cárcamo & Vankosky, 2011). However, the efficacy of currently available treatments under field conditions is largely unclear. Moreover, an integrated treatment targeting *A. euteiches, Fusarium* spp., and PLW simultaneously is not currently available. At present, INTEGO Solo (Nufarm, Calgary, AB, CA; active ingredient: ethaboxam) is the only product registered for use against *A. euteiches* in pea and other pulse crops (Government of Alberta, 2017). The ability of ethaboxam to effectively suppress aphanomyces root rot (ARR) in the field has not been determined. Fludioxonil is frequently used as a seed treatment to prevent rot of seeds and roots associated with soilborne species of *Fusarium* (Government of Alberta, 2017). Fludioxonil can improve emergence and yield in pea (Chang *et al*., 2013) and faba bean (Chang *et al*., 2014) exposed to *F. avenaceum*. Fludioxonil and ethaboxam are available as a combined seed treatment (Apron Maxx with INTEGO from Syngenta Canada, Guelph, ON, CA), but the impact of this treatment on yield protection has not been determined.

Foliar and seed treatments are available to reduce PLW herbivory, targeting adult and larval weevils, respectively. Lambda-cyhalothrin is registered for use as a foliar spray against adult PLWs, though application must be carefully timed to prevent oviposition (Vankosky *et al*., 2009; Government of Alberta, 2017). The broad-spectrum neonicotinoid insecticide thiamethoxam has replaced several other compounds that have been deregistered due to significant environmental concerns (Vankosky *et al*., 2009; Cárcamo & Vankosky, 2011). Thiamethoxam suppressed PLW herbivory by reducing

adult fecundity and increasing larval mortality in greenhouse trials (Cárcamo *et al*., 2012), but has demonstrated variable efficacy in field trials (Seidenglanz *et al*., 2010; Vankosky, Cárcamo, McKenzie*, et al*., 2011). Finally, nodule availability has been identified as a limiting factor on the abundance of larval *Sitona* spp. (Quinn & Hower, 1986; Lohaus & Vidal, 2010). High soil nitrogen levels inhibit nodule production (George, 1962), thereby reducing the food supply for developing PLW larvae. The use of high N fertilizer has been investigated as a potential alternative to synthetic insecticides, but the benefit to yield protection requires further investigation (Vankosky, Cárcamo, McKenzie*, et al*., 2011).

Targeting root rot pathogens and insects individually increases the cost and time that producers must spend applying pesticides, thus reducing the feasibility of applying multiple products. An integrated treatment will therefore be an important component of crop protection in regions at risk of root rot disease and PLW herbivory. The efficacy of foliar, seed, and soil treatments can be measured by assessing visual signs of disease and insect infestation, nodulation, and leghemoglobin expression as an indicator of nitrogen fixation potential. Molecular quantification techniques such as real-time quantitative PCR (qPCR) are becoming increasingly accessible and sensitive, and are an important tool to measure the impact of pest management techniques on pathogen population dynamics in soil and roots. The objective of this research was to evaluate the efficacy of the fungicides ethaboxam and fludioxonil, the insecticides lambda-cyhalothrin and thiamethoxam, and a high N fertilizer when used singly or as combined treatments when used under field conditions. All field treatments were tested with pea and faba bean as hosts. Disease ratings, plant growth and nodulation parameters, and herbivory were measured at multiple stages of crop development. Additionally, multiplex qPCR was used to detect changes in the community structure of pathogens colonizing pea roots across treatments.

4.2 Materials and Methods

4.2.1 Field Locations

Three field locations in southern Alberta were used in 2016. Peas were grown in Lethbridge (49°41'48" N, 112°45'27" W) and Taber (49°39'43" N, 112°00'52" W) and faba beans were grown in Vauxhall (50°04'08'' N, 112°05'51" W) and Taber. In 2017, pea and faba bean were grown side-by-side in Lethbridge and Taber. These locations are within the Moist Mixed Grassland and Mixed Grassland Ecoregions of the Prairies Ecozone of western Canada (Ecological Stratification Working Group, 1995). Soils in this region are of the brown or dark brown chernozemic types, and annual precipitation is low (<450 mm). Average temperature and precipitation from May to August at each location and year are summarized in Table 4-1. Sites at Lethbridge and Vauxhall had no known history of *A. euteiches* infestation, whereas the Taber site had a history of both ARR and FRR (Chatterton, personal communication). Lethbridge and Vauxhall sites were tilled and irrigated, whereas Taber was a no-till, dryland site.

4.2.2 Fungal Cultures

Fusarium avenaceum isolate 1306.08 (Fa1306.08) was obtained from pea roots collected during field surveys near Lethbridge, Alberta, Canada, and was stored at 4°C on synthetic nutrient-deficient medium 'Spezieller Nährstoffarmer agar (SNA)' (Leslie & Summerell, 2006). Fa1306.08 was grown at room temperature on potato dextrose agar (PDA) amended with penicillin and streptomycin, each at a concentration of 150 mg/L. After 6 days of growth, PDA cultures were cut into small squares and distributed into trays of wheat grain that had been sterilized by autoclaving. The grain was then moistened with SDW, enclosed in sterile plastic bags, and grown at room temperature in a growth chamber for two weeks or until mycelia was clearly visible. The trays of

inoculated grain were then removed from the bags and allowed to dry for 2-3 days. Once dry, the fungus-grain mixture was crumbled by hand into fine pieces.

4.2.3 Experimental Design

Plots were 1.6 by 8.0 m with 8 rows in Lethbridge and 1.2 by 6.0 m with 6 rows in Taber in 2016 and 2017, and 1.6 by 8.0 m (8 rows) in Vauxhall in 2016. Plots were inoculated with *F. avenaceum* in Lethbridge in 2016 and 2017, and in Vauxhall in 2016 by broadcasting the wheat grain inoculum shortly after seeding. A randomized complete block design with six replicates of ten treatments was used at each site (Table 4-2). Treatments were: (1) untreated control, (2) fludioxonil applied as 325 mL Apron Maxx RTA (Syngenta Canada, Guelph, ON, CA) per 100 kg seed, (3) ethaboxam applied as 19.6 mL INTEGO Solo (NuFarm, Calgary, AB, CA) per 100 kg of seed, (4) fludioxonil + ethaboxam, (5) thiamethoxam applied as 50 mL Cruiser (Syngenta Canada, Guelph, ON, CA) per 100 kg of seed, (6) lambda-cyhalothrin applied as Matador (Syngenta Canada) at 10 g active ingredient (ai) ha^{-1} between the two- and five-node growth stages, (7) thiamethoxam + lambda-cyhalothrin, (8) fludioxonil + ethaboxam + thiamethoxam, (9) fludioxonil + ethaboxam + thiamethoxam + lambda-cyhalothrin, and (10) 46 kg N ha^{-1} applied as granular urea (46-0-0) at 100 kg ha^{-1}. To avoid conversion to ammonia, urea was either side-banded with seed or incorporated into soil post-seeding using a hand-pushed garden seeder (Earthway, Bristol, IN, USA).

Metalaxyl, applied as 110 mL Allegiance FL (Bayer CropScience Canada, Calgary, AB, CA) per 100 kg of seed, was added to seed treatments for suppression of *Pythium*. Metalaxyl is a component of Apron Maxx, and therefore was excluded from any treatment using this product. Prior to planting, 17 kg ha^{-1} ethalfluralin was applied to all sites to control weeds. Additionally, post-emergent applications of 20 kg imazamox ha^{-1} + 428 kg bentazon ha^{-1} were applied in Taber and Vauxhall in 2016, and in Taber and Lethbridge in 2017. *Rhizobium leguminosarum* inoculant was applied to all sites as 2.47

kg ha^{-1} TagTeam (containing 1.3 x 10^8 viable cells per gram from Monsanto BioAg, Winnipeg, MB, CA). In both years, *P. sativum* cultivar 'CDC Meadow' and *V. faba* cultivar Snowbird were used as hosts. Seeding dates for all site years are listed in Table 4-3. Target field pea density was 80 plants m^{-2} for field pea (15.8 g seed m^{-2}) and 45 plants m^{-2} faba bean (38.8 g seed m^{-2}), and rows were spaced at 18 cm.

4.2.4 Field Emergence and Foliar Damage Assessments

Emergence and foliar damage caused by adult PLW was assessed at the three-node growth stage for both pea and faba bean between late May and early June (Table 4-3), using the method described by Vankosky *et al* (2011). Two 1 m^2 quadrats were randomly placed within each plot, with one quadrat in each half of the plot to account for in-plot variability. All seedlings within the boundary of the quadrats were counted to calculate emergence m^{-2}. Feeding by adult PLWs results in characteristic U-shaped notches along leaf margins (Jackson & Macdougall, 1920; Nielsen, 1990), therefore feeding activity was assessed by counting the number of notches at each node and terminal leaf of ten seedlings randomly selected within each quadrat. Mean feeding damage per plant per plot, and the proportion of seedlings with damage on the terminal leaves were calculated for comparison with the economic threshold of 30% established by El-Lafi (1977).

4.2.5 Field Disease, Nodule Assessments, and DNA Extraction

Whole plants were destructively sampled at the seven node and early flowering growth stages from each site in both years (Table 4-3). The earlier sampling period was chosen to assess early-season suppression of pathogens and insects, whereas the latter sampling period would coincide with maximal nodulation and would allow for damage assessments at plant maturity (Vankosky, Cárcamo, McKenzie, *et al*., 2011). Five plants were randomly selected from each plot, with plants taken from both halves and the

middle of plots to account for in-plot variability. Plants were sampled as described by Vankosky *et al*. (2011), wherein the soil surrounding the roots of each plant from a distance of at least 10 cm from the stem to a depth of 20 – 30 cm was also collected. The roots and soil of each plant were contained within a plastic bag, and plants were immediately stored at 4°C to prevent degradation of leaves, roots, and nodules. In the lab, the contents of each bag were carefully removed and individually placed into a plastic tub filled with water. Once the mass of soil around the roots had softened, plants were removed gently to avoid dislodging nodules. Any larvae or pupae floating on the surface of the water were collected with forceps and recorded. The soil was rinsed through a 2 mm metal sieve to capture any remaining insects or nodules. The roots were cleaned of residual soil by rinsing them over the sieve, and any nodules that detached were dissected and scored for damage.

Disease severity and growth parameters were assessed as described in chapters 2 and 3. Each nodule on every individual plant was then removed, counted, and assessed for larval damage and the presence of larvae. In 2017, nodules were also characterized by type: single lobed or tumescent with multiple lobes. Disease data were collected from all samples in both years. In 2016, nodule assessments were conducted for all plants collected from all three locations at the seven node crop stage, but were excluded from the Lethbridge and Taber sites at flowering. This was due to suppression of nodule production in Lethbridge as a result of high soil N content, and an absence of adult or larval PLW damage in Taber. In 2017, nodule assessments were only conducted for four of the six replicates for each treatment due to time constraints. Larval damage assessments were excluded from roots collected during both sample collections at the Taber site due to a lack of adult or larval PLW damage.

Processed roots from the 5 plant samples were pooled by replicate and were immediately frozen at -80°C before being lyophilized in a Harvest Right scientific freeze dryer (North Salt Lake, UT, USA) for 48 hours. For each sample, 25 mg of lyophilized

root tissue was removed randomly and ground using one 3 mm tungsten carbide bead shaken at 1800 rpm with a TissueLyser II (Qiagen, Toronto, ON, CA) for 2 minutes. DNA was extracted from each sample in duplicate using the Biosprint-96 DNA plant kit in a Biosprint instrument (Qiagen) according to the manufacturer's instructions.

4.2.6 Quantification of Pathogen DNA from Roots

Fusarium avenaceum, F. solani f. sp. *pisi*, and *A. euteiches* are the most frequently occurring and damaging pathogens isolated from pea roots in western Canada (Xue, 2003a; Esmaeili Taheri *et al*., 2016), therefore the quantity of DNA from all three pathogens was determined by analyzing DNA extracted from pea roots collected from field sites in 2016 and 2017. Two multiplex qPCR assays were used to quantify pathogen DNA relative to the quantity of *P. sativum* DNA in each sample. The following groupings were used for the multiplex assays: (1) *F. avenaceum* + *F. solani* + *P. sativum* and (2) *A. euteiches* + *P. sativum*. The primers and probes used for each species and their concentrations are listed in Table 2-3. Each reaction contained 12.5 uL of 2x PrimeTime Gene Expression Master Mix (IDT, Coralville, IA, USA), 2 uL of template DNA, appropriate primer and probe as shown in Table 2-3, and ultra-pure H_2O to make a 25 uL reaction. Reactions were run in MicroAmp™ Fast Optical 96-well plates (Applied Biosystems, Waltham, MA, USA) using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Cycling conditions were set at 95°C for 10 min; 40 cycles of 95°C for 15 s; and 1 min of annealing/extension at 60°C, except the annealing temperature was increased to 63°C for the duplex assay to account for a false positive signal from the *A. euteiches* probe. Resulting threshold cycle (C_t) values were analyzed for specificity and sensitivity. Relative quantification of pathogen DNA from *P. sativum* roots was performed using the comparative C_t method ($\Delta\Delta C_t$) (Livak & Schmittgen, 2001), as described in section 2.2.6.

4.2.7 Statistical Analyses

All statistical analyses were performed using JMP version 13.1 (SAS Institute Inc., 2016). Disease parameters, emergence, nodule and foliar herbivory, nodulation and nodule characteristics, larval abundance, yield, and $\Delta\Delta C_t$ data were analyzed in separate mixed-effect analysis of variance models for a randomized complete block experimental design. Field data was analyzed on a yearly basis, and by location. Treatments were considered fixed effects, and replicate (block) was considered random. For significant treatment effects, means were separated using the Tukey-Kramer HSD test for mean overall disease severity by field location, yield, and $\Delta\Delta C_t$ data. Single degree of freedom contrast statements were used to test the hypothesis that single vs. combined fungicidal and insecticidal treatments impact emergence, disease severity, growth parameters, herbivory, nodulation and nodule characteristics, and larval abundance in root nodules. Normality of all dependent variables was assessed using the Shapiro-Wilk test. Variables that did not conform to a normal distribution were log transformed to improve normality. Outlier detection was performed using standardized scores. The Type 1 error rate (α) was set at 0.05 to be considered significantly different or $0.05 > p < 0.1$ to be marginally significant for all statistical tests.

4.3 Results

4.3.1 Emergence and Foliar Herbivory

Emergence (plants m^{-2}) at the three node stage was low at all sites relative to target densities of 80 seeds m^{-2} for field pea and 45 seeds m^{-2} for faba bean (Table 4-4). High weed density at Taber in 2017 contributed to exceptionally low emergence at this site. Emergence of pea or faba bean seedlings was not influenced by treatment at any location in 2016. At the Lethbridge site in 2017, field pea treated with Apron Maxx had the

highest overall emergence, but was only significantly higher than the control group (p = 0.025) and the ThiSdAE treatment (p = 0.038). No differences in emergence were found in field pea grown in Taber, or in faba bean from either site in 2017. Leaf notches from adult PLW herbivory were significantly reduced by thiamethoxam in both pea and faba bean in both years, except for the Lethbridge site in 2016 (p = 0.60) (Table 4-5). At all other sites, foliar herbivory was significantly reduced relative to the control by thiamethoxam in pea and faba bean (p < 0.0001 for all comparisons), while lambda-cyhalothrin had no effect. The combined effect of both insecticides also reduced herbivory more effectively than single applications in Lethbridge (p = 0.011) and Vauxhall (p = 0.0010). In Vauxhall the combination of both insecticides and fungicides reduced herbivory more effectively than single applications of thiamethoxam and lambda-cyhalothrin (p = 0.0002). The level of PLW herbivory in all plots at the Taber site in both years was either very low or did not occur at all, and therefore this data was not included.

4.3.3 Disease Severity and Growth Parameters

Disease pressure differed by location and year for pea ($F_{(7, 40)} = 85.05$, $p < 0.0001$) and faba bean ($F_{(7, 40)} = 91.79$, $p < 0.0001$). The overall severity of root rot symptoms at each site was generally higher in 2017 than 2016 (Figure 4-1A, B). In field pea, contrast statements indicated that fludioxonil did not reduce disease severity relative to the control at any location or crop stage (Table 4-6A). In 2016, ethaboxam reduced root rot severity in pea relative to the control at flowering in Lethbridge (p = 0.033), but not at seven nodes (p = 0.53). This effect was not observed at any other location. Disease severity was lower at the seven node stage in Taber 2016 (p = 0.019) and Lethbridge 2017 (p = 0.029) when peas were treated with both ethaboxam and fludioxonil, relative to single applications of these fungicides. This effect was not observed at seven nodes in Lethbridge in 2016 (p = 0.40) or Taber 2017 (p = 0.35), and did not occur at flowering in

any location or year. Neither ethaboxam nor fludioxonil improved disease symptoms relative to the control in faba bean at any location or crop stage (Table 4-6B). The effect of single versus combined fungicidal treatments also did not differ significantly in faba bean in any location or year. However, at flowering disease severity was reduced in faba bean treated with thiamethoxam, fludioxonil, and ethaboxam relative to seeds treated only with thiamethoxam in Taber (2017; $p = 0.035$). A marginal decrease was also observed in this treatment at seven nodes in Vauxhall ($p = 0.063$), in addition to a slight reduction in disease when thiamethoxam and lambda-cyhalothrin were both used relative to single treatments ($p = 0.051$).

Pea shoot height was generally unaffected by seed, foliar or N treatments (Table 4-7A). Relative to the control, single applications of fludioxonil, ethaboxam, thiamethoxam, lambda-cyhalothrin, and urea did not consistently impact shoot growth at any location or crop stage. Marginal increases in height were observed in peas treated with ethaboxam at flowering in Lethbridge in 2016 ($p = 0.094$), and Taber in 2017 ($p = 0.083$). In 2016, the combination of ethaboxam and fludioxonil slightly improved shoot growth at flowering in Lethbridge ($p = 0.097$) and at seven nodes in Taber ($p = 0.059$), but this was did not occur at any other location or crop stage. Similarly, combining thiamethoxam and lambda-cyhalothrin improved shoot growth at seven nodes ($p = 0.0013$) and, to a lesser extent, at flowering ($p = 0.095$) in comparison to individual treatments. However, this only occurred in pea grown in Lethbridge in 2017. Urea had a variable impact on shoot growth. A marginal increase in the height of fertilized peas was observed at seven nodes in Lethbridge in 2017 ($p = 0.085$), and a significant increase at flowering in Taber ($p = 0.020$), relative to the control. Urea did not impact shoot height in pea at any other location or crop stage. Faba bean shoot growth was comparable between treatments (Table 4-7B). In Lethbridge in 2017, shoots were significantly smaller at seven nodes in faba bean treated with thiamethoxam, ethaboxam and

fludioxonil than those treated only with ethaboxam or fludioxonil ($p = 0.033$). No other differences were observed in 2016 or 2017.

The impact of treatments on root weight was equally variable. In Lethbridge, thiamethoxam increased pea root growth at seven nodes relative to the control. This effect was marginal in 2016 ($p = 0.074$) and significant in 2017 ($p = 0.0022$) (Table 4-8A). In 2017, lambda-cyhalothrin slightly improved root growth relative to the control, but this only occurred at seven nodes ($p = 0.073$). Additionally, root mass was significantly greater in peas treated with combinations of both fungicides ($p = 0.001$), fungicides and thiamethoxam ($p = 0.0058$), and fungicides with thiamethoxam and lambda-cyhalothrin ($p = 0.023$) relative to single applications of ethaboxam or fludioxonil. However, this trend was not observed at any other location or growth stage. At seven nodes, application of urea slightly increased root mass in Lethbridge in 2017 relative to untreated plants ($p = 0.052$), however, the opposite trend was observed in Taber in 2016 ($p = 0.0069$). Urea did not impact root mass at any other location or crop stage. No other treatment effects were observed.

In faba bean the combined treatment of thiamethoxam, ethaboxam and fludioxonil slightly increased root mass relative to single applications of ethaboxam and fludioxonil ($p = 0.049$), but this was only observed in Vauxhall (Table 4-8B). At the Taber site in 2016, root mass was greater in peas treated singly with ethaboxam and fludioxonil in comparison to those treated with both fungicides at the seven node stage ($p = 0.0065$). At the same location and growth stage, faba bean roots were significantly larger when treated with urea relative to the control group ($p = 0.0069$). In Taber in 2017, faba bean treated with both fungicides, thiamethoxam, and lambda-cyhalothrin had increased root mass relative to plants treated only with ethaboxam or fludioxonil at the seven node stage ($p = 0.019$).

4.3.4 Nodule Assessments

4.3.4.1 Nodule Abundance

Nodulation was not observed in pea plots at the Lethbridge site in 2016. In 2017, no significant differences in the number of root nodules were observed between single and combined seed, foliar, and N treatments at seven nodes (Table 4-9A). At flowering, contrast statements indicated that thiamethoxam improved nodulation relative to untreated plants ($p = 0.046$). Additionally, there were significantly more nodules when ethaboxam and fludioxonil were combined with thiamethoxam ($p = 0.023$) or thiamethoxam and lambda-cyhalothrin ($p = 0.0012$) relative to either fungicide used singly. At the same crop stage, peas treated with urea had fewer nodules than those treated with insecticides ($p = 0.025$), but were comparable to the control group ($p = 0.60$). No differences between treatments were found in the number of nodules on peas grown in Taber in 2016 or 2017, except nodulation was reduced in peas fertilized with urea relative to the control ($p = 0.0003$) and insecticidal treatments ($p < 0.0001$) at the seven node stage in 2016. Urea also slightly reduced nodulation relative to insecticidal treatments at the same site and growth stage in 2017 ($p = 0.071$).

The mean number of nodules per plant did not differ between single and combined treatments in faba beans grown in Vauxhall at either sampling period (Table 4-9B). In 2016, faba bean grown in Taber had significantly fewer nodules when treated with urea than those treated with insecticides ($p = 0.013$), and slightly fewer than the control group ($p = 0.090$). In 2017, the number of nodules per faba bean plant was increased at seven nodes when ethaboxam, fludioxonil, and thiamethoxam were used in combination, relative to fungicides applied singly ($p = 0.010$). No differences in nodulation between single and combined treatments occurred in Lethbridge at either crop stage in 2017, except marginally significant increases were observed in plants treated with lambda-cyhalothrin relative to the control at seven nodes ($p = 0.054$) and flowering ($p = 0.087$).

4.3.4.2 Nodule Herbivory

Relative to the untreated control, thiamethoxam reduced nodule damage in seven-node field pea by approximately 68% at the Lethbridge site in 2017 ($p = 0.0002$) (Table 4-10). This effect was no longer evident at flowering ($p = 0.83$). Thiamethoxam was more effective at preventing damage than urea at the seven node stage ($p = 0.0002$), when nodulation between these treatments was comparable, but not at flowering ($p = 0.82$), where a significant reduction in the number of nodules was observed. Lambda-cyhalothrin did not reduce herbivory relative to the control group at seven nodes ($p = 0.15$), and herbivory in sprayed plants was slightly higher than the control at flowering ($p = 0.09$). Urea was more effective than lambda-cyhalothrin at flowering ($p = 0.037$), but these treatments were comparable at the seven node growth stage ($p = 0.18$). Nodule herbivory did not occur in Taber in either year.

In faba bean from Vauxhall, thiamethoxam reduced nodule damage by approximately 98% at seven nodes ($p < 0.0001$) and 79% at flowering ($p < 0.0001$), in comparison to the untreated control (Table 4-10). Similarly, thiamethoxam reduced herbivory by 55% at seven nodes ($p = 0.0085$) and 68% at flowering ($p = 0.035$) in Lethbridge. In contrast, lambda-cyhalothrin was comparable to the control at seven nodes and flowering in Vauxhall ($p = 0.98$ and $p = 0.14$, respectively) and Lethbridge ($p = 0.99$ and $p = 0.66$, respectively). The combination of both insecticides reduced damage at seven nodes ($p = 0.040$) and flowering ($p = 0.002$) in Vauxhall, relative to single applications. A similar but marginally significant decrease in damage was observed at seven nodes in Lethbridge ($p = 0.076$). Nodule damage was more severe in plants treated with urea than those treated with thiamethoxam. This was observed at both Vauxhall and Lethbridge at seven nodes ($p < 0.0001$ and $p = 0.016$, respectively) and flowering ($p < 0.0001$ and $p = 0.082$, respectively). Damage between these treatments can be compared directly, as urea did not significantly reduce nodulation relative to insecticidal treatments

at either site. The efficacy of thiamethoxam was not affected by the addition of fungicides at either site, except damage was significantly higher in the combined treatment of thiamethoxam, ethaboxam and fludioxonil in Lethbridge at flowering ($p = 0.036$). The combined treatment of ethaboxam, fludioxonil, thiamethoxam and lambda-cyhalothrin reduced herbivory to a greater extent than single applications of lambda-cyhalothrin at seven nodes ($p < 0.0001$) and flowering ($p < 0.0001$) in Vauxhall, and at seven nodes in Lethbridge ($p = 0.021$).

Relative to untreated pea plants, thiamethoxam reduced larval abundance in root nodules by 68% at seven nodes ($p = 0.0072$) (Table 4-11). This effect was not observed at flowering ($p = 0.33$). Lambda-cyhalothrin did not impact larval abundance relative to the control at seven nodes ($p = 0.65$) or flowering ($p = 0.47$). Thiamethoxam was more effective in reducing larval abundance when used singly than in combination with fungicides ($p = 0.022$). Thiamethoxam was also more effective than urea at reducing larval abundance in root nodules ($p = 0.043$). At flowering, thiamethoxam and lambda-cyhalothrin were more effective when used singly in comparison to combined applications ($p = 0.0003$).

In 2016, no larvae were found in the nodules of faba bean plants treated with thiamethoxam at seven nodes, in comparison to 6.33 ± 1.47 in untreated plants ($p = 0.0002$) and 3.83 ± 1.47 in plants fertilized with urea ($p = 0.0040$) (Table 4-11). In contrast, plants treated with lambda-cyhalothrin had a similar quantity of larvae in root nodules as the control ($p = 0.88$) and those fertilized with urea ($p = 0.28$). The combination of ethaboxam, fludioxonil, thiamethoxam, and lambda-cyhalothrin reduced larval abundance to a greater extent than single applications of lambda-cyhalothrin ($p = 0.0047$). At flowering, faba bean treated with both thiamethoxam and lambda-cyhalothrin had no larvae present in root nodules, which was a significant reduction relative to single applications of thiamethoxam and lambda-cyhalothrin ($p = 0.0083$). In 2017, thiamethoxam reduced larval abundance by 78% relative to the control ($p = 0.043$) and by

85% relative to high N treatments ($p = 0.017$) at seven nodes (Table 4-11). Conversely, more larvae were found in plants treated with lambda-cyhalothrin relative to the control ($p = 0.0084$), urea ($p = 0.021$), and combined treatment of ethaboxam, fludioxonil, thiamethoxam, and lambda-cyhalothrin ($p < 0.0001$). At flowering, thiamethoxam was slightly more effective at reducing larval abundance when used singly than in combination with ethaboxam and fludioxonil ($p = 0.047$). No other differences were observed at this crop stage.

4.3.4.3 Nodule Characteristics

In peas grown at the Lethbridge site in 2017, the mean number of tumescent nodules per plant did not differ in any treatment relative to the control at seven nodes (Table 4-12A). Peas treated with combinations of thiamethoxam, ethaboxam, and fludioxonil ($p = 0.033$), or thiamethoxam, lambda-cyhalothrin, and both fungicides ($p = 0.016$) had more tumescent nodules at seven nodes than plants treated with single applications of ethaboxam and fludioxonil. At flowering, more tumescent nodules were observed in plants treated with thiamethoxam ($p = 0.0008$) and lambda-cyhalothrin ($p = 0.045$) in comparison to untreated plants. Thiamethoxam slightly increased tumescence when used singly, relative to combined treatments of thiamethoxam plus fungicides ($p = 0.079$). Additionally, peas treated with thiamethoxam, lambda-cyhalothrin, ethaboxam, and fludioxonil had more tumescent nodules than those treated singly with fungicides ($p = 0.0003$). The combination of thiamethoxam and lambda-cyhalothrin increased the number of tumescent nodules relative to single applications of either insecticide ($p = 0.0018$), whereas fewer tumescent nodules were found in peas fertilized with urea ($p < 0.0001$). At the same site, no differences in leghemoglobin were observed between treatments at seven nodes, whereas fludioxonil decreased ($p = 0.027$) and thiamethoxam improved ($p = 0.0005$) leghemoglobin expression at flowering relative to the control. Combining ethaboxam and fludioxonil with thiamethoxam ($p = 0.0017$), or with

thiamethoxam and lambda-cyhalothrin ($p < 0.0001$), increased leghemoglobin expression in comparison to single applications of fungicidal seed treatments. However, thiamethoxam had a stronger impact on leghemoglobin when used singly than in combination with fungicides ($p = 0.0095$). More nodules expressed leghemoglobin at flowering when thiamethoxam and lambda-cyhalothrin were combined, as opposed to single applications of either insecticide ($p = 0.0082$). Urea significantly decreased leghemoglobin expression relative to thiamethoxam and lambda-cyhalothrin ($p = 0.0081$). At the Taber site, urea increased both tumescence ($p = 0.021$) and leghemoglobin expression ($p = 0.047$) at flowering, relative to the control. No other treatments differed in the number of tumescent nodules or the number of nodules expressing leghemoglobin at seven nodes or flowering.

Lambda-cyhalothrin increased the number of tumescent nodules in faba bean at seven nodes in Lethbridge, in comparison to untreated plants ($p = 0.047$) (Table 4-12B). The combined treatment of thiamethoxam and lambda-cyhalothrin resulted in a slight, but significant increase in tumescent nodules over single applications of either insecticide at flowering in Lethbridge ($p = 0.043$), and at seven nodes in Taber ($p = 0.045$). In comparison to untreated plants, ethaboxam reduced the number of tumescent nodules at seven nodes in Taber ($p = 0.027$). Soil amendment with urea also slightly reduced tumescence relative to treatment with insecticides at seven nodes ($p = 0.049$). Leghemoglobin expression did not vary between treatments in Lethbridge at seven nodes ($F_{(9, 50)} = 1.11$; $p = 0.39$) or flowering ($F_{(9, 50)} = 1.30$; $p = 0.28$). In Taber, leghemoglobin expression was similar between treatments at seven nodes ($F_{(9, 50)} = 1.52$; $p = 0.19$), while more pink nodules were observed in plants treated with lambda-cyhalothrin at flowering relative to untreated plants ($p = 0.024$).

4.3.5 Yield

Yield did not vary among treatments at any location in 2016 or 2017 (Table 4-13A, B). Overall yield, measured as the mean total weight of cleaned seed from each plot, and seed size as measured by 1000 kernel weight (KWT) was uniform across all treatments in both pea and faba bean. Pea and faba bean grown in Taber had the lowest overall yield, whereas kernel weight was similar between locations.

4.3.6 Relative Quantification of Pathogen DNA

Colonization patterns of root rot pathogens varied by location and year. Detectable levels of *A. euteiches* DNA were not present in peas grown in Lethbridge in 2016 or 2017, or in Taber in 2016 (Tables 4-14A-C). In 2016, *F. avenaceum* was also not detected in plants collected at seven nodes or at flowering in Lethbridge (Table 4-14A), but was detected at the Lethbridge site in 2017 (Table 4-14B), and in Taber in both years (Tables 4-14C, D). *Fusarium solani* was present in Lethbridge in both years, and in Taber in 2017 but not in 2016 (Tables 4-14A-D).

Fusarium solani was the only pathogen detected in Lethbridge in 2016 (Table 14-4A). ΔC_t values indicated that colonization was slightly higher at seven nodes than at flowering, though no treatment differed from control either stage. In 2017, lower levels of both *F. solani* and *F. avenaceum* were present (Table 14-4B). At seven nodes, *F. avenaceum* was only detected in peas treated with fludioxonil, ethaboxam and fludioxonil, thiamethoxam plus ethaboxam and fludioxonil, and thiamethoxam plus lambda-cyhalothrin, ethaboxam and fludioxonil. No differences were observed between these treatments. *Fusarium avenaceum* was not present at detectable levels at the flowering stage. *Fusarium solani* DNA was detected in pea plants from Lethbridge in both 2016 and 2017. The relative quantity of *F. solani* DNA did not differ from the control in any treatment.

In Taber in 2016, *F. avenaceum* was the only pathogen detected at either growth stage (Table 4-14C). The quantity of *F. avenaceum* DNA was low relative to the reference gene in each treatment, with the exception of peas treated with thiamethoxam, ethaboxam, and fludioxonil at seven nodes. Here, the quantity of *F. avenaceum* DNA was seven approximately seven-fold higher than the control group ($p = 0.0037$). At flowering, however, no treatment differed significantly from the untreated control group. ΔC_t values indicated that *A. euteiches* had colonized pea roots extensively at both growth stages in Taber in 2017 (Table 14-4D). At seven nodes, no differences in the relative quantity of *A. euteiches* were observed in any treatment relative to the control group ($F_{(9, 110)} = 1.28$; $p = 0.26$). At flowering, the relative quantity of *A. euteiches* DNA was significantly lower in plants treated with thiamethoxam, ethaboxam, and fludioxonil in comparison to the untreated control ($p = 0.016$). The quantity of *A. euteiches* DNA in this treatment was also lower than in peas treated singly with fludioxonil ($p < 0.0001$), thiamethoxam ($p < 0.0001$), or ethaboxam ($p = 0.024$). No differences in the quantity of *F. avenaceum* DNA were detected at seven nodes, but colonization was significantly lower in peas treated with thiamethoxam, ethaboxam and fludioxonil relative to the control ($p < 0.0001$) and all other treatments except for high N fertilizer at flowering ($p = 0.089$). In comparison to the control, peas treated with thiamethoxam and lambda-cyhalothrin had a reduced level of *F. solani* DNA relative to the untreated control at seven nodes ($p = 0.0206$), while no other differences between treatments were detected. At flowering, the combined treatment of thiamethoxam, ethaboxam, and fludioxonil had a lower relative quantity of *F. solani* DNA than the control ($p < 0.0001$) and all other treatments. Roots treated with both insecticides also had a lower level of *F. solani* colonization than thiamethoxam ($p = 0.0002$) or lambda-cyhalothrin ($p = 0.0052$) applied singly.

4.4 DISCUSSION

Root rot disease is a serious obstacle impacting the production of field pea and other pulse crops in Canada (Xue, 2003a; Esmaeili Taheri *et al*., 2016). The rapid range expansion of *S. lineatus* across the Prairie provinces poses an additional threat to susceptible crops such as pea and faba bean (Vankosky *et al*., 2009; Olfert *et al*., 2012). Based on evidence that *A. euteiches*, *Fusarium* spp., and *S. lineatus* do co-occur in pulse crops (Chatterton, personal communication), there is a risk that additive or synergistic interactions may further contribute to yield loss. As such, there is an urgent need for a reliable method of simultaneously managing root rot pathogens and PLW in pea and faba bean crops. Here, the ability of the fungicides ethaboxam and fludioxonil, the insecticides thiamethoxam and lambda-cyhalothrin, and a high N fertilizer to reduce damage related to root rot disease and PLW herbivory were tested, both singly and in combination. While these pesticides are currently registered for use in pulse crops in Canada, there is a lack of clear evidence that they are effective in a field setting. Therefore, the efficacy of fungicidal and insecticidal treatments was assessed in a two-year field study in southern Alberta.

Disease severity was low to moderate in 2016 at field sites in Lethbridge, Taber, and Vauxhall. In 2017, moderate to severe root rot symptoms were observed in pea and faba bean at both the Lethbridge and Taber sites, despite dryer, hotter weather conditions in 2017 (Environment Canada, 2017). qPCR results indicated that pea roots from Lethbridge were colonized by *F. solani* in 2016, and by both *F. solani* and *F. avenaceum* in 2017. *Fusarium avenaceum* was the only pathogen detected in pea roots from Taber in 2016, but *F. avenaceum, F. solani,* and *A. euteiches* were all present in 2017. PLW herbivory occurred at the Vauxhall and Lethbridge sites, but did not occur in Taber in either year. In Lethbridge, foliar herbivory was high in pea in 2016 with an average of 38 leaf notches per plant in the untreated control group, and moderate to high in 2017 with

an average of 26 notches per plant. Herbivory was high in faba bean at the Lethbridge site, with approximately 50 notches per plant in the untreated control in 2017. Foliar herbivory was considerably lower in faba bean from Vauxhall, with an average of 9 notches per plant. Nodulation was not observed in pea from Lethbridge in 2016, but in 2017 up to 18 nodules per plant were observed in the untreated control group at seven nodes and early flowering. PLW larvae damaged half of these nodules at seven nodes, while damage ranged from 40 to 75% at flowering. Faba bean grown at the same site had up to 13 and 27 nodules per plant at seven nodes and flowering, respectively. At seven nodes, 75% of nodules in the untreated control group were damaged, and 40% at flowering. In Vauxhall, there were an average of 12 nodules per plant at seven nodes in the untreated control group, 27% of which were damaged by PLW larvae. By early flower, there were approximately 20 nodules per plant, 28% of which were damaged.

Neither fungicide was consistently effective in managing root rot in pea or faba bean grown in the field in 2016 or 2017, even in early growth stages. Emergence was generally unaffected by any of the experimental treatments relative to the control: a slight increase was observed in pea treated with fludioxonil at the Lethbridge site in 2017, but did not occur at any other site in either year. Fungicidal efficacy was not improved under the lower disease pressure observed in 2016, relative to 2017. Quantification of pathogen DNA from each site indicated that neither fludioxonil nor ethaboxam consistently reduced populations of *F. avenaceum, F. solani,* or *A. euteiches,* and in some cases more pathogen DNA was found when fungicides were present. Additionally, no consistent effects on shoot height or root weight were observed in any treatment in the field. Ethaboxam was granted emergency registration for early-season suppression of *A. euteiches* in field pea in 2015, and Apron Maxx is registered for use against seed rot, pre-emergence, and post-emergence damping off caused by *Fusarium* spp., *Pythium* spp., and *Rhizoctonia solani* in field pea and faba bean (Government of Alberta, 2017). In the current study, roots were first sampled at seven nodes as both disease symptoms and root

nodules would be present at this point, allowing for simultaneous assessment of root rot severity and PLW herbivory. However, if ethaboxam and fludioxonil are primarily effective only during and very shortly after germination, the effects of these treatments may already have diminished by the seven-node growth stage. Further testing will therefore be useful in establishing when ethaboxam and fludioxonil are most effective in reducing fungal colonization under field conditions.

Fungicidal resistance may also influence the efficacy of seed treatments. Fludioxonil is registered for use in Canada in multiple legume species and a broad range of other crops, such as potatoes, corn, wheat, barley, and canola (Government of Alberta, 2017). Fludioxonil has activity against a range of pathogens including phytopathogenic species of *Fusarium, Rhizoctonia, Alternaria,* and *Botrytis*. Resistance to fludioxonil has been observed in *Fusarium* spp. isolated from corn and potato (Broders *et al*., 2007; Peters *et al*., 2007), and has been observed in other fungal pathogens such as *Botrytis cinerea* Pers. (Fernández-Ortuño *et al*., 2013) and *Alternaria brassicicola* (Schwein.) Wiltshire (Iacomi-Vasilescu *et al*., 2004). Testing of *F. avenaceum* isolates from pea is therefore recommended to determine if efficacy is related to concentration and potential resistance.

qPCR was used to quantify changes in the colonization dynamics of *A. euteiches, F. avenaceum,* and *F. solani* in pea roots at the seven node and early flowering growth stages in 2016 and 2017. The Lethbridge site had no prior history of legumes, therefore *A. euteiches* was not expected to be present. This site was artificially inoculated with *F. avenaceum*, but it was not known if other *Fusarium* spp. were already present in the soil. The Taber site had a history of legumes, and was known to support populations of both *A. euteiches* and *Fusarium* spp. (Chatterton, personal communication). As expected, *A. euteiches* was not detected in Lethbridge in either year. Interestingly, *F. avenaceum* was not detected at all in 2016, and had a patchy distribution in 2017. This suggests that

broadcasting inoculated grain on the soil surface following seeding does not provide sufficient contact of the fungus with roots during plant development. This method of inoculation may also increase exposure to hot, dry conditions and solar radiation. Incorporating the fungus into soil before or concurrently with seeding may provide a more effective alternative. *Fusarium solani* colonized roots from the Lethbridge site in both years; ΔC_t values indicated that colonization had increased at early flowering relative to the seven node stage in 2016 and 2017. This increase in colonization appears to correspond with the increase in disease severity that also occurred between these growth stages. However, more *F. solani* DNA was present in 2016 than 2017, though disease severity was higher in 2017. This suggests that other pathogens that were not screened for in the present study contributed to the increase in disease symptoms.

Fusarium avenaceum was the only pathogen detected in Taber in 2016. Disease severity was considerably higher in 2017, where all three pathogens were detected at seven nodes and at early flower. ΔC_t values indicate that *A. euteiches* was the dominant pathogen at this site in 2017, and that the rate of colonization remained fairly constant between the seven node and early flower growth stages. In contrast, the relative quantity of *F. avenaceum* and *F. solani* increased as the plants matured. This pattern was also observed in Lethbridge, where roots were infected with *F. solani* in the absence of *A. euteiches,* suggesting that these species do not compete for resources when co-occurring in pea roots. This is consistent with greenhouse experiments that indicate *A. euteiches* and *F. solani* interact additively when simultaneously infecting pea roots (Willsey *et al*., 2018).

The relative quantities of *A. euteiches, F. avenaceum* and *F. solani* DNA were variable between treatments, and neither fungicides nor insecticides had a consistent effect on colonization rates. For example, in 2016 a seven-fold increase in the quantity of *F. avenaceum* DNA was detected in pea treated with thiamethoxam, ethaboxam, and

fludioxonil relative to the control group at seven nodes. At the same growth stage in the following year, the quantity of *F. avenaceum* was two times lower in this treatment in comparison to the control group. The spatial distribution of *A. euteiches* and *Fusarium* spp. in soil has been described as patchy, with discrete foci of inoculum of varying intensity distributed throughout an infected field (Rekah *et al*., 1999; Moussart *et al*., 2009). A similar pattern has been observed with other soil-borne pathogens such as *Phytophthora* spp. (Larkin *et al*., 1995). The concentration of water in low-lying areas and direct plant-to-plant infection are factors contributing to the aggregation of soil-borne pathogens in soil (Larkin *et al*., 1995; Rekah *et al*., 1999; Moussart *et al*., 2009). Ethaboxam and fludioxonil were not effective in managing ARR and FRR, respectively, therefore the variability in root colonization observed here may be a result of an irregular distribution of pathogens across each field site.

Thiamethoxam was highly effective in reducing foliar herbivory by adult weevils and nodule herbivory by PLW larvae in both pea and faba bean. At the Lethbridge site in 2017, treatment with thiamethoxam reduced nodule damage at seven nodes by up to 68% in pea and 55% in faba bean. At flowering, peas treated with thiamethoxam were similar to the control group, whereas treated faba bean had 70% fewer damaged nodules than the control group. A corresponding reduction in larval abundance in root nodules was observed. In pea, up to 78% fewer larvae were found in nodules at seven nodes, whereas larvae were generally not present in any treatment at flowering. In faba bean, reductions in larval abundance of up to 88% occurred at seven nodes, and 53% at flowering. Faba bean grown in Vauxhall experienced a lower level of herbivory, and nodule herbivory was almost completely eliminated by thiamethoxam. Relative to the control group, a 97% reduction in nodule damage occurred at seven nodes and a 79% reduction at flowering. No larvae were found in nodules in plants treated with thiamethoxam, or with thiamethoxam, fludioxonil, and ethaboxam at seven nodes, relative to 6-8 larvae per nodule in the control, ethaboxam, and fludioxonil treatments. At flowering, thiamethoxam

reduced larval abundance by 48% relative to the control group. These results indicate that thiamethoxam protects pea and faba bean from nodule damage during the most vulnerable 2-5 node growth stages, and that this protection can persist into the flowering stage.

Similar reductions in foliar herbivory were observed in the thiamethoxam treatments at all sites, except in pea grown in Lethbridge in 2016, where thiamethoxam was not effective in reducing foliar herbivory. These results are consistent with those of (Vankosky, Cárcamo, McKenzie*, et al*., 2011) and (Cárcamo *et al*., 2012), who also observed reductions in foliar and root damage when thiamethoxam was used as a seed treatment. Investigation into the specific effects of thiamethoxam on adult and larval PLWs in a greenhouse study indicated that in addition to reducing foliar herbivory, thiamethoxam has a strong negative effect on both adult fecundity and egg viability (Cárcamo *et al*., 2012). Larval survivorship and nodule herbivory were also reduced. The present study confirms that thiamethoxam will reduce both above- and below-ground PLW herbivory in the field under both high and low infestation levels in both pea and faba bean.

Conversely, lambda-cyhalothrin had little effect on foliar and nodule herbivory. The use of foliar insecticides is only recommended when greater than 30% of plants are estimated to have terminal leaf damage (El-Lafi, 1977; Cárcamo & Vankosky, 2011). When this economic threshold has been surpassed, foliar sprays must immediately be applied in order to prevent oviposition (van de Steene *et al*., 1999; Vankosky *et al*., 2009). Field trials have indicated that lambda-cyhalothrin can reduce foliar herbivory by up to 56%, but is ineffective past the onset of oviposition (van de Steene *et al*., 1999). The latter appears to be the case in the current study. Monitoring the dispersal of adult weevils from overwintering sites to pea and faba bean fields is time-consuming and inefficient, therefore foliar sprays are likely often applied too late to be effective in managing PLW populations. Thiamethoxam applied as a seed treatment appears to

provide more reliable protection during vulnerable stages of plant development, and should therefore be used in preference over foliar insecticidal sprays.

In addition to reducing PLW herbivory, thiamethoxam has been associated with an increase in nodulation. A two-fold increase in the total number of nodules and a five-fold increase in the number of older, tumescent nodules relative to untreated controls was reported in pea plants treated with thiamethoxam in a field study (Vankosky, Cárcamo, McKenzie, *et al*., 2011). Elsewhere an increase in nodulation of up to 368% as a result of thiamethoxam use was observed (Seidenglanz *et al*., 2010). In greenhouse studies, thiamethoxam did not impact the number of nodules, but did increase the proportion of tumescent nodules (Cárcamo *et al*., 2012; Cárcamo *et al*., 2015). Here, thiamethoxam did not impact the number of nodules per plant at the seven node stage in pea, but did increase nodulation at flowering in some treatments. In Lethbridge, most treatments containing thiamethoxam did increase the number of tumescent nodules as well as the number of pink nodules in pea at the flowering stage, but not at seven nodes. This was not observed at the Taber site, likely because PLW herbivory was not a factor at this location. No consistent treatment effects on nodule characteristics were observed in faba bean. Lambda-cyhalothrin increased the number of tumescent nodules at seven nodes in Lethbridge and leghemoglobin expression at flowering in Taber. Additionally, the combination of both insecticides increased the number of older, tumescent nodules at flowering in Lethbridge and at seven nodes in Taber. The increase in the number of older, tumescent nodules and nodules actively expressing leghemoglobin in field pea and faba bean is likely due to the lower levels of herbivory observed in these treatments, allowing nodules to mature normally. However, this was not consistently observed in all treatments containing thiamethoxam. Root rot disease can reduce both the number and weight of root nodules on affected plants (Hwang *et al*., 1994; Gossen *et al*., 2016), and root rot pathogens were detected in both Lethbridge and Taber. The inconsistent effect of

thiamethoxam on nodule development may have been impacted by infection of roots by *A. euteiches*, *Fusarium* spp., and potentially other soil-borne pathogens.

The greenhouse study outlined in chapter 3 of this thesis indicated that pea leaf weevil increased the severity of root rot caused by *F. avenaceum*, whereas the pathogen appeared to have a positive impact on larval development. Direct interactions between other root and nodule-feeding *Sitona* weevils and *Fusarium* spp. have been observed elsewhere: for example, *Sitona hispidulus* F. (Coleoptera: Curculionidae) has a well-established association with root rot disease caused by *Fusarium* spp. in alfalfa and red clover (Dickason *et al*., 1968; Leath & Hower, 1993). Based on these results, it was expected that disease severity would be higher when root rot pathogens and pea leaf weevil co-occurred in the field. Treatment of the forage legume sainfoin with carbofuran reduced herbivory by *Sitona scissifrons* Say (Coleoptera: Curculionidae), and was met with a 50% decrease in tap-root disease (Morrill *et al*., 1998). However, a reduction in disease severity was not consistently observed in treatments that included thiamethoxam in the present field study, despite a notable decrease in nodule herbivory. Root rot disease is usually caused by a complex of fungi and oomycetes, and interactions between larval PLW and other pathogens within this complex have not been investigated. Furthermore, it is not clear how additive or synergistic interactions occurring between PRRC pathogens impact the individual effects of root herbivory on disease development, and vice versa. Further investigation will be required to determine the consequences of multiple root rot pathogens and PLW herbivory when they co-occur in pulse crops.

Yield losses related to PRRC are difficult to measure, as the composition of the microbial community, and other factors like climate and cultural practices, are variable by region (Xue, 2003a; Gaulin *et al*., 2007; Gossen *et al*., 2016). However, yield losses of up to 80% have been reported as a result of root rot disease (Xue, 2003a; Gaulin *et al*., 2007). Losses of approximately 25-30% have also been reported in pea and faba bean as a

result of PLW infestation (Nielsen, 1990; Lohaus & Vidal, 2010). Yield protection, therefore, is a primary concern for producers in regions where PRRC pathogens and PLW are problematic. In the current study, neither total yield per plot nor mean seed weight varied by treatment. This was expected in fungicidal treatments, as fludioxonil and ethaboxam were ineffective in reducing FRR and ARR, respectively. Furthermore, yield could not be compared to non-diseased or non-inoculated plots because plots were either located in producer's fields with uniform disease pressure, or in inoculated plot locations with natural background levels of *Fusarium*. Thiamethoxam was effective in managing PLW herbivory, however, even when infestation levels were observed to be high. (Vankosky, Cárcamo, McKenzie*, et al*., 2011) similarly did not observe a yield increase when seeds were treated with thiamethoxam, but had a much higher proportion of damaged nodules (50-80%) on treated pea plants. Here, thiamethoxam did not impact yield in locations with high or low levels of PLW infestation, despite nearly complete control of nodule herbivory in Vauxhall during early plant development. No other studies appear to have examined the role of thiamethoxam in yield protection. It is therefore unclear why yield was not improved in treatments with reduced levels of herbivory, but this may be related to the incidence of root rot disease. Even at relatively low inoculum levels, large increases in yield loss have been observed as a result of both ARR and FRR (Pfender & Hagedorn, 1983; Hwang *et al*., 2000). By the time plants had reached flowering, root disease ratings in pea ranged from 2 – 2.4 in 2016, and 3.8 – 4 in 2017. In faba bean, disease ranged from 1.9 – 2 in 2016 and 3.2 – 3.7 in 2017. Because root rot disease was not excluded by the use of fungicides at any location, it was not possible to determine the impact of PLW herbivory alone on yield. Therefore, root rot disease may have counteracted any beneficial effects to yield related to thiamethoxam use.

Treatment with high N fertilizer in the form of urea did not have an effect on yield in either year. Urea generally did not affect nodulation: the number of nodules per pea plant decreased by 57% at seven nodes at Taber in 2016, but this effect was not observed

at any other site. As such, there was no observed effect on nodule herbivory or yield in either pea or faba bean. (Vankosky, Cárcamo, McKenzie*, et al*., 2011) also found that urea did not improve yield in two years of a three year field study. Much higher concentrations of nitrogen are likely required to replace the N requirements of pea and faba bean and completely eliminate nodulation. Fertilizing with such high quantities of nitrogen is neither an environmentally or economically viable strategy to mitigate PLW damage, therefore soil amendment with nitrogen fertilizer should not be recommended as a means to control PLW populations in pulse crops.

In summary, fludioxonil and ethaboxam were not effective in managing root rot disease in pea and faba bean in field trials. Further study is required to determine the efficacy of these fungicides in suppressing early-season infection by *Fusarium* spp. and *A. euteiches,* respectively. Resistance to fungicides and interspecific interactions with other soil microbes are other potential factors that may impact the efficacy of fludioxonil and ethaboxam. Colonization of roots by *F. avenaceum* and *F. solani* tended to increase over the course of the growing season, whereas the quantity of *A. euteiches* DNA in roots remained relatively constant. While direct comparisons between *A. euteiches* and *Fusarium* spp. were only possible at one site in 2017, no evidence of antagonistic interactions were observed. Thiamethoxam is effective in reducing both foliar and nodule damage but did not have a positive effect on yield. The latter may be due to the detrimental effects of root rot pathogens on plant development. Yield protection may be improved if used with more effective fungicides, should they become available. Lambda-cyhalothrin similarly did not impact yield, and it is difficult to time application to effectively prevent oviposition. Lambda-cyhalothrin is therefore not an effective means of mitigating PLW damage in pea and faba bean crops. Finally, N fertilizer is not an effective means of reducing nodulation, herbivory, or increasing yield, and is not recommended for use in mitigating PLW damage.

Table 4-1. Mean temperature and total precipitation from May-August for Lethbridge and Taber, AB in 2016 and 2017 and in Vauxhall, AB in 2016 (Environment Canada, 2017).

Site/Year	Mean Temperature May-August (°C)	Precipitation (mm)
Lethbridge 2016	15.7	43.8
Lethbridge 2017	16.9	21.8
Taber 2016	16.1	64
Taber 2017	18.5	33.4
Vaux 2016	16.2	58.8

Table 4-2. Experimental treatments to assess foliar, seed, and soil treatments under field conditions.

	Treatment Name	Active ingredients
1	Control	None
2	Apron	Fludioxonil
3	Ethaboxam	Ethaboxam
4	EthAp	Fludioxonil + Ethaboxam
5	ThiSd	Thiamethoxam
6	Matador	Lambda-cyhalothrin
7	ThiMat	Thiamethoxam + lambda-cyhalothrin
8	ThiSdAE	Thiamethoxam + fludioxonil + ethaboxam
9	ThiMatAE	Thiamethoxam + fludioxonil + ethaboxam + lambda-cyhalothrin
10	Urea	Urea (46% N)

Table 4-3. Dates of plot seeding, emergence counts, damage assessments and harvest at sites in Lethbridge, Vauxhall and Taber in 2016 and 2017. Interim dates between planting and assessments or harvest are shown.

Site/Year	Crop	Activity	Date	Interim from seeding (days)
Lethbridge 2016	Pea	Plots seeded	3 May 2016	
		Emergence/foliar damage	3 June 2016	31
		Root sample collection 1	20 June 2016	48
		Root sample collection 2	28 June 2016	56
		Plot harvest	31 August 2016	120
Taber 2016	Pea + faba bean	Plots seeded	18 May 2016	
		Emergence/foliar damage	15 June 2016	28
		Root sample collection 1	29 June 2016	42
		Root sample collection 2	18 July 2016	61
		Plot harvest	8 September 2016	113
Vauxhall 2016	Faba bean	Plots seeded	12 May 2016	
		Emergence/foliar damage	1 June 2016	20
		Root sample collection 1	22 June 2016	41
		Root sample collection 2	6 July 2016	55
		Plot harvest	20 September 2016	132
Lethbridge 2017	Pea + faba bean	Plots seeded	3 May 2017	
		Emergence/foliar damage	23 May 2017	20
		Root sample collection 1	8 June 2017	36
		Root sample collection 2	29 June 2017	57
		Plot harvest	11 August 2017	100
Taber 2017	Pea + faba bean	Plots seeded	10 May 2017	
		Emergence/foliar damage	31 May 2017	21
		Root sample collection 1	12 June 2017	33
		Root sample collection 2	4 July 2017	55
		Plot harvest	16 August 2017	98

Table 4-4. Mixed model ANOVA results and treatment means (± SE) for emergence of pea (top) and faba bean (bottom) in 2016 and 2017. Means and standard error were obtained from 6 replicates per treatment. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test.

Treatment[a]	**Pea emergence (plants m^{-2}) by location**			
	2016		**2017**	
	Lethbridge	**Taber**	**Lethbridge**	**Taber**
Control	26.00 ± 2.57	16.83 ± 1.30	14.00 ± 1.71*b*	9.83 ± 1.15
Apron	31.00 ± 2.57	17.00 ± 1.30	22.67 ± 1.71*a*	11.33 ± 1.15
Ethaboxam	32.33 ± 2.57	17.33 ± 1.30	16.17 ± 1.71*ab*	10.33 ± 1.15
EthAp	33.20 ± 2.57	18.00 ± 1.30	20.33 ± 1.71*ab*	14.33 ± 1.15
Thiamethoxam	32.50 ± 2.57	19.00 ± 1.30	20.67 ± 1.71*ab*	10.67 ± 1.15
Matador	34.33 ± 2.57	18.17 ± 1.30	17.33 ± 1.71*ab*	9.83 ± 1.15
ThiMat	31.67 ± 2.57	18.50 ± 1.30	15.50 ± 1.71*ab*	9.33 ± 1.15
ThiMatAE	29.00 ± 2.57	18.33 ± 1.30	19.33 ± 1.71*ab*	12.00 ± 1.15
ThiSdAE	32.33 ± 2.57	15.67 ± 1.30	14.00 ± 1.87*b*	10.83 ± 1.15
Urea	36.00 ± 2.57	17.00 ± 1.30	14.67 ± 1.71*ab*	10.33 ± 1.15
$F_{(9, 50)}$	1.16	0.59	3.20	1.56
p	0.34	0.80	0.004	0.15
Treatment	**Faba bean emergence (plants m^{-2}) by location**			
	2016		**2017**	
	Vauxhall	**Taber**	**Lethbridge**	**Taber**
Control	29.67 ± 3.09	15.00 ± 1.29	16.67 ± 1.85	9.83 ± 1.13
Apron	28.17 ± 3.09	13.83 ± 1.29	19.00 ± 1.85	10.17 ± 1.13
Ethaboxam	27.67 ± 3.09	13.50 ± 1.29	15.33 ± 1.85	10.67 ± 1.13
EthAp	27.83 ± 3.09	14.17 ± 1.29	17.17 ± 1.85	9.67 ± 1.13
Thiamethoxam	27.33 ± 3.09	14.17 ± 1.29	14.67 ± 1.85	10.33 ± 1.13
Matador	28.33 ± 3.09	16.33 ± 1.29	15.33 ± 1.85	12.17 ± 1.13
ThiMat	31.00 ± 3.09	16.33 ± 1.29	14.00 ± 1.85	9.33 ± 1.13
ThiMatAE	30.50 ± 3.09	16.67 ± 1.29	15.33 ± 1.85	10.50 ± 1.13
ThiSdAE	26.17 ± 3.09	16.33 ± 1.29	17.33 ± 1.85	11.33 ± 1.13
Urea	22.83 ± 3.09	14.67 ± 1.29	14.00 ± 1.85	10.00 ± 1.13
$F_{(9, 50)}$	0.57	0.87	0.77	0.55
p	0.82	0.55	0.65	0.83

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

Table 4-5. Treatment means (± SE) for leaf notches from PLW herbivory in pea and faba bean measured in 2016 and 2017. The means and standard error were obtained from 6 replicates per treatment. Treatment means marked with an asterisk indicate that the proportion of seedlings with damage to terminal leaves was below the economic threshold value of 30%. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	**Leaf notches per plant**			
	Lethbridge pea		**Vauxhall faba**	**Lethbridge faba**
	2016	**2017**	**2016**	**2017**
Control	38.52 ± 5.34[b]*	26.22 ± 2.86	9.39 ± 0.88	50.80 ± 6.59
Apron	38.18 ± 5.34*	25.18 ± 2.86	8.27 ± 0.88	44.95 ± 6.59
Ethaboxam	42.85 ± 5.34	22.39 ± 2.86	6.72 ± 0.88	47.51 ± 6.59
EthAp	43.38 ± 5.34	29.50 ± 2.86	8.38 ± 0.88	62.13 ± 6.59
Thiamethoxam	35.64 ± 5.34	9.82 ± 2.86	3.55 ± 0.88	16.84 ± 6.59
Matador	34.79 ± 5.34	25.03 ± 2.86	8.68 ± 0.88	49.98 ± 6.59
ThiMat	30.40 ± 5.34	9.54 ± 2.86*	3.15 ± 0.88	15.63 ± 6.59
ThiMatAE	33.03 ± 5.34	14.97 ± 2.86	2.83 ± 0.88	23.40 ± 6.59
ThiSdAE	28.44 ± 5.34*	9.25 ± 2.86	3.34 ± 0.88	24.11 ± 6.59
Urea	32.63 ± 5.34	27.53 ± 2.86	7.93 ± 0.88	50.73 ± 6.59
Contrasts				
ThiSd vs Control	0.598[c]	**<0.0001**	**<0.0001**	**<0.0001**
Matador vs Control	0.57	0.83	0.77	0.94
ThiSd, Matador vs ThiMat	0.48	**0.011**	**0.0010**	**0.011**
ThiSd vs ThiSdAE	0.35	0.83	0.80	0.31
ThiSd, Matador vs ThiMatAE	0.77	0.47	**0.0002**	0.087
ThiMat vs ThiMatAE	0.71	0.11	0.67	0.45

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

[b]Least square means of dependent variables for each treatment.

[c]*p*-values of single degree of freedom contrast comparisons.

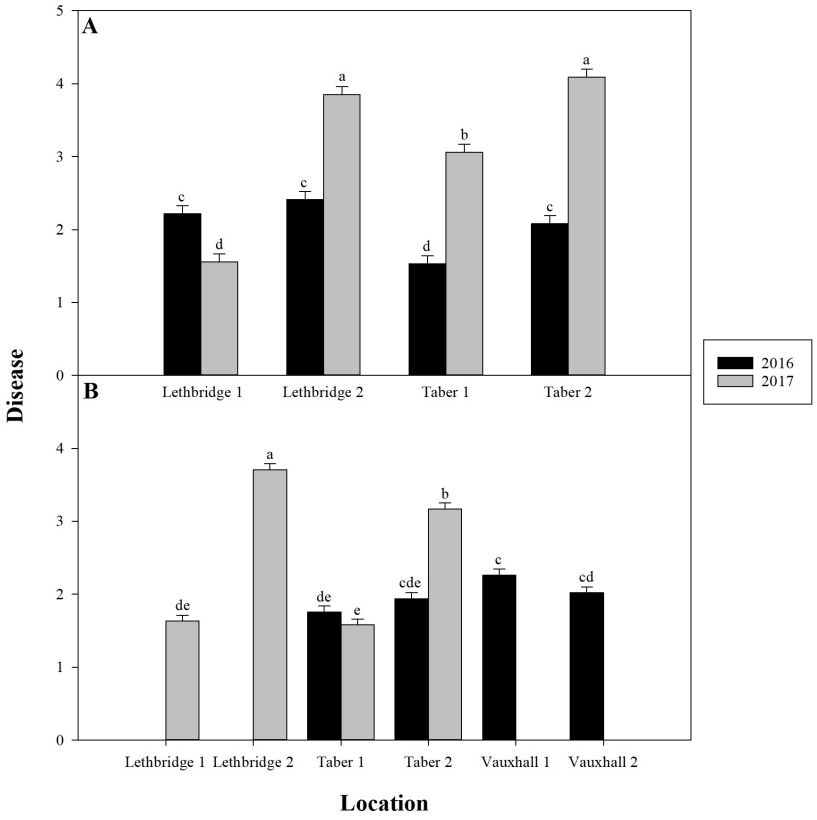

Figure 4-1. Mean disease severity of pea (A) and faba bean (B) seedlings collected from field locations in 2016 and 2017 at the seven node (1) and flowering (2) stages. Columns marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. Vertical bars indicate standard error of the mean.

Table 4-6A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on disease severity (0-5) in field pea. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Disease severity							
	Lethbridge pea				Taber pea			
	2016		2017		2016		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	2.24 ± 0.22[b]	2.68 ± 0.27	1.65 ± 0.14	3.83 ± 0.10	1.43 ± 0.28	2.20 ± 0.27	2.73 ± 0.42	4.03 ± 0.17
Apron	2.30 ± 0.22	2.49 ± 0.28	1.60 ± 0.14	3.90 ± 0.11	1.85 ± 0.28	2.03 ± 0.27	3.36 ± 0.42	3.95± 0.17
Ethaboxam	2.04 ± 0.22	1.83 ± 0.27	1.77 ± 0.14	3.92 ± 0.10	1.93 ± 0.28	1.77 ± 0.27	3.30 ± 0.42	4.11± 0.17
EthAp	2.40 ± 0.22	2.66 ± 0.27	1.38 ± 0.14	3.92 ± 0.10	1.07 ± 0.28	2.07 ± 0.27	2.88 ± 0.42	4.28 ± 0.17
ThiSd	2.05 ± 0.23	2.49± 0.27	1.46 ± 0.14	3.93 ± 0.10	1.58 ± 0.28	1.83 ± 0.27	3.07 ± 0.42	4.25 ± 0.17
Matador	2.40 ± 0.23	2.27 ± 0.27	1.70 ± 0.14	3.72 ± 0.10	1.80 ± 0.28	2.13 ± 0.27	3.58 ± 0.42	4.27 ± 0.17
ThiMat	1.77 ± 0.22	2.07 ± 0.27	1.57 ± 0.14	3.67 ± 0.10	1.17 ± 0.28	2.33 ± 0.27	2.73 ± 0.42	3.88 ± 0.17
ThiMatAE	2.43 ± 0.22	2.31 ± 0.27	1.43 ± 0.14	3.78 ± 0.10	1.57 ± 0.28	2.27 ± 0.27	3.28 ± 0.42	4.08 ± 0.17
ThiSdAE	2.40 ± 0.22	2.57 ± 0.27	1.55 ± 0.14	3.82 ± 0.10	1.39 ± 0.28	2.33 ± 0.27	2.87 ± 0.42	4.10 ± 0.17
Urea	2.10 ± 0.22	2.83 ± 0.27	1.45 ± 0.14	4.05 ± 0.11	1.57 ± 0.28	1.87 ± 0.27	2.80 ± 0.42	3.85 ± 0.17
Contrasts								
Apron vs. Control	0.83[c]	0.63	0.38	0.68	0.25	0.66	0.29	0.84
Ethaboxam vs. Control	0.53	**0.033**	0.59	0.58	0.26	0.26	0.31	0.74
Urea vs. Control	0.66	0.68	0.36	0.34	0.75	0.38	0.92	0.58
Apron, Ethaboxam vs. EthAp	0.40	0.14	**0.029**	0.97	**0.019**	0.61	0.35	0.24
Apron, Ethaboxam vs. ThiSdAE	0.40	0.23	0.36	0.62	0.15	0.19	0.40	0.97
Apron, Ethaboxam vs. ThiMatAE	0.34	0.67	0.049	0.34	0.29	0.27	0.88	0.90
ThiSd, Matador vs. ThiMat	0.10	0.37	0.71	0.26	0.14	0.28	0.30	0.11
ThiSd vs. ThiSdAE	0.11	0.84	0.81	0.47	0.52	0.19	0.89	0.43
ThiSd, Matador vs. ThiMatAE	0.44	0.83	0.15	0.56	0.55	0.39	0.96	0.37

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-6B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on disease severity (0-5) in faba bean. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Disease severity							
	Vauxhall faba		Lethbridge faba		Taber faba			
	2016		2017		2016		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	2.50 ± 0.20[b]	2.30 ± 0.29	1.65 ± 0.15	3.72 ± 0.19	1.90 ± 0.22	2.00 ± 0.22	1.95 ± 0.16	3.37 ± 0.19
Apron	2.10 ± 0.20	1.80 ± 0.30	1.55 ± 0.15	3.76 ± 0.19	1.85 ± 0.22	1.83 ± 0.22	1.54 ± 0.16	3.03 ± 0.19
Ethaboxam	2.37 ± 0.20	1.75 ± 0.29	1.52 ± 0.15	3.43 ± 0.19	1.63 ± 0.22	1.73 ± 0.22	1.63 ± 0.16	3.20 ± 0.19
EthAp	2.03 ± 0.20	1.45± 0.29	1.78 ± 0.15	3.97 ± 0.19	1.70 ± 0.22	1.60 ± 0.22	1.40 ± 0.16	3.30 ± 0.19
ThiSd	2.43 ± 0.20	1.96 ± 0.29	1.71 ± 0.15	3.68 ± 0.19	1.67 ± 0.22	1.93 ± 0.22	1.34 ± 0.16	3.35 ± 0.19
Matador	2.61 ± 0.20	1.88 ± 0.29	1.72 ± 0.15	3.61 ± 0.19	1.47 ± 0.22	2.23 ± 0.22	1.65 ± 0.16	3.18 ± 0.19
ThiMat	2.03 ± 0.20	2.29 ± 0.29	1.43 ± 0.15	3.76 ± 0.19	1.47 ± 0.22	2.07 ± 0.22	1.50 ± 0.16	3.12 ± 0.19
ThiMatAE	2.10 ± 0.20	2.32 ± 0.29	1.63 ± 0.15	3.62 ± 0.19	2.00 ± 0.22	2.00 ± 0.22	1.82 ± 0.16	3.35 ± 0.19
ThiSdAE	1.90 ± 0.20	2.33 ± 0.29	1.41 ± 0.15	3.79 ± 0.19	1.73 ± 0.22	1.73 ± 0.22	1.37 ± 0.16	2.75 ± 0.19
Urea	2.57 ± 0.20	1.79 ± 0.29	1.97 ± 0.17	3.68 ± 0.21	2.04 ± 0.22	2.30 ± 0.22	1.62 ± 0.17	3.02 ± 0.19
Contrasts								
Apron vs. Control	0.16[c]	0.30	0.38	0.95	0.83	0.60	0.43	0.83
Ethaboxam vs. Control	0.64	0.26	0.59	1.00	0.39	0.37	0.44	0.30
Urea vs. Control	0.81	0.31	0.35	0.88	0.75	0.38	0.25	0.16
Apron, Ethaboxam vs. EthAp	0.42	0.35	0.22	0.14	0.85	0.50	0.33	0.41
Apron, Ethaboxam vs. ThiSdAE	0.18	0.12	0.47	0.49	0.97	0.85	0.22	0.15
Apron, Ethaboxam vs. ThiMatAE	0.59	0.13	0.58	0.86	0.29	0.43	0.32	0.32
ThiSd, Matador vs. ThiMat	0.051	0.40	0.11	0.66	0.85	0.95	0.96	0.47
ThiSd vs. ThiSdAE	0.063	0.41	0.15	0.74	0.52	0.19	0.82	**0.035**
ThiSd, Matador vs. ThiMatAE	0.089	0.29	0.60	0.98	0.10	0.76	0.14	0.72

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-7A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on shoot height (cm) in field pea. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Shoot height (cm)							
	Lethbridge pea				Taber pea			
	2016		2017		2016		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	33.17 ± 2.09[b]	44.68 ± 3.22	16.40 ± 0.95	48.42 ± 2.37	34.75 ± 1.13	76.28 ± 3.05	16.31 ± 1.13	38.23 ± 3.03
Apron	32.28 ± 2.10	42.18 ± 3.25	17.45 ± 1.01	50.45 ± 2.42	32.48 ± 1.14	76.84 ± 3.01	15.04 ± 1.13	37.26 ± 3.05
Ethaboxam	37.22 ± 2.10	52.06 ± 3.20	16.68 ± 0.96	48.84 ± 2.38	33.76 ± 1.13	81.03 ± 3.01	15.87 ± 1.12	45.83 ± 3.05
EthAp	37.62 ± 2.09	53.69 ± 3.24	17.87 ± 0.95	48.31 ± 2.37	35.74 ± 1.13	79.67 ± 2.99	17.19 ± 1.12	39.48 ± 3.03
ThiSd	35.26 ± 2.12	49.27 ± 3.22	16.62 ± 0.95	47.63 ± 2.37	31.29 ± 1.14	80.30 ± 3.03	16.01 ± 1.12	36.60 ± 3.03
Matador	35.83 ± 2.11	44.00 ± 3.20	17.67 ± 0.95	49.16 ± 2.37	33.96 ± 1.13	78.98 ± 3.05	15.06 ± 1.12	39.24 ± 3.03
ThiMat	37.61 ± 2.09	49.01 ± 3.24	19.67 ± 0.96	53.37 ± 2.37	33.96 ± 1.14	78.48 ± 3.01	16.65 ± 1.23	42.43 ± 3.03
ThiMatAE	35.32 ± 2.09	48.80 ± 3.24	17.75 ± 0.96	50.34 ± 2.37	33.44 ± 1.13	80.89 ± 2.99	17.54 ± 1.12	40.51 ± 3.03
ThiSdAE	37.98 ± 2.09	50.44 ± 3.20	18.52 ± 0.95	53.23 ± 2.37	34.22 ± 1.14	79.44 ± 3.01	16.70 ± 1.12	42.48 ± 3.03
Urea	29.78 ± 2.09	45.07 ± 3.20	19.50 ± 0.96	51.18 ± 2.38	35.51 ± 1.13	76.69 ± 3.01	16.94 ± 1.13	48.57 ± 3.03
Contrasts								
Apron vs. Control	0.77[c]	0.74	0.27	0.52	0.15	0.81	0.43	0.82
Ethaboxam vs. Control	0.18	0.094	0.75	0.93	0.54	0.23	0.79	0.083
ThiSd vs. Control	0.49	0.33	0.81	0.74	**0.043**	0.31	0.85	0.71
Matador vs. Control	0.37	1.00	0.15	0.96	0.78	0.55	0.44	0.82
Urea vs. Control	0.26	0.90	0.085	0.46	0.58	0.70	0.69	**0.020**
Apron, Ethaboxam vs. EthAp	0.27	0.097	0.31	0.73	0.059	0.80	0.21	0.58
Apron, Ethaboxam vs. ThiSdAE	0.21	0.40	0.067	0.24	0.45	0.94	0.37	0.80
Apron, Ethaboxam vs. ThiMatAE	0.83	0.75	0.39	0.75	0.81	0.69	0.14	0.78
ThiSd, Matador vs. ThiMat	0.43	0.56	**0.0013**	0.095	0.23	0.81	0.45	0.23
ThiSd vs. ThiSdAE	0.37	0.67	0.28	0.091	0.096	0.82	0.67	0.18
ThiSd, Matador vs. ThiMatAE	0.93	0.59	0.44	0.37	0.69	0.77	0.15	0.49

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-7B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on shoot height (cm) in faba bean. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Shoot height (cm)							
	Vauxhall faba		Lethbridge faba		Taber faba			
	2016		2017		2016		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	23.48 ± 1.60[b]	44.50 ± 2.69	12.67 ± 0.85	34.56 ± 2.45	21.11 ± 0.98	41.95 ± 1.87	11.77 ± 1.05	38.11 ± 1.24
Apron	23.55 ± 1.60	47.70 ± 2.73	13.20 ± 0.85	32.83 ± 2.46	22.86 ± 0.99	42.85 ± 1.88	11.72 ± 1.06	38.29 ± 1.24
Ethaboxam	22.42 ± 1.60	42.31 ± 2.69	14.02 ± 0.85	37.86 ± 2.46	22.00 ± 0.98	42.85 ± 1.88	14.12 ± 1.05	39.80 ± 1.24
EthAp	22.70 ± 1.60	42.47 ± 2.70	13.13 ± 0.85	37.51 ± 2.45	20.48 ± 0.98	44.62 ± 1.88	12.54 ± 1.05	39.13 ± 1.24
ThiSd	21.22 ± 1.60	43.35 ± 2.70	13.33 ± 0.85	37.51 ± 2.46	20.31 ± 0.98	43.57 ± 1.87	12.62 ± 1.06	38.18 ± 1.24
Matador	22.35 ± 1.60	41.45 ± 2.69	13.06 ± 0.86	37.50 ± 2.46	20.43 ± 0.98	42.64 ± 1.87	12.85 ± 1.05	40.08 ± 1.24
ThiMat	22.20 ± 1.60	40.25 ± 2.70	14.08 ± 0.85	39.47 ± 2.46	20.39 ± 0.98	40.92 ± 1.87	11.83 ± 1.05	37.56 ± 1.24
ThiMatAE	23.50 ± 1.60	43.15 ± 2.70	12.89 ± 0.85	36.52 ± 2.45	21.22 ± 0.98	43.37 ± 1.88	13.40 ± 1.05	37.71 ± 1.24
ThiSdAE	20.21 ± 1.60	41.19 ± 2.68	11.31 ± 0.86	32.82 ± 2.46	21.08 ± 0.98	41.14 ± 1.87	13.46 ± 1.05	39.87 ± 1.24
Urea	21.25 ± 1.60	42.29 ± 2.70	12.61 ± 0.94	38.69 ± 2.68	22.59 ± 0.99	55.70 ± 1.87	11.88 ± 1.07	40.80 ± 1.24
Contrasts								
Apron vs. Control	0.96[c]	0.41	0.66	0.61	0.22	0.81	0.96	0.92
Ethaboxam vs. Control	0.68	0.57	0.27	0.37	0.52	0.74	0.16	0.34
ThiSd vs. Control	0.28	0.77	0.59	0.28	0.57	0.54	0.58	0.97
Matador vs. Control	0.61	0.43	0.75	0.42	0.63	0.80	0.45	0.27
Urea vs. Control	0.29	0.57	0.96	0.27	0.58	0.70	0.94	0.13
Apron, Ethaboxam vs. EthAp	0.80	0.45	0.65	0.48	0.11	0.41	0.88	0.96
Apron, Ethaboxam vs. ThiSdAE	0.15	0.25	**0.033**	0.59	0.27	0.49	0.73	0.59
Apron, Ethaboxam vs. ThiMatAE	0.91	0.58	0.50	0.69	0.32	0.78	0.78	0.38
ThiSd, Matador vs. ThiMat	0.77	0.52	0.40	0.47	0.99	0.34	0.52	0.36
ThiSd vs. ThiSdAE	0.68	0.57	0.10	0.18	0.096	0.82	0.62	0.34
ThiSd, Matador vs. ThiMatAE	0.44	0.82	0.78	0.64	0.48	0.91	0.71	0.36

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-8A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root weight (g) in field pea. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Root weight (g)							
	Lethbridge pea				Taber pea			
	2016		2017		2016		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	0.18 ± 0.042[b]	0.50 ± 0.058	0.39 ± 0.051	0.53 ± 0.053	1.09 ± 0.11	2.11 ± 0.24	0.47 ± 0.10	0.17 ± 0.12
Apron	0.22 ± 0.042	0.54 ± 0.059	0.47 ± 0.056	0.60 ± 0.062	0.99 ± 0.11	2.15 ± 0.24	0.48 ± 0.10	0.20 ± 0.12
Ethaboxam	0.27 ± 0.042	0.57 ± 0.058	0.36 ± 0.053	0.55 ± 0.060	0.87 ± 0.11	1.93 ± 0.24	0.57 ± 0.10	0.30 ± 0.12
EthAp	0.21 ± 0.042	0.51 ± 0.058	0.59 ± 0.051	0.49 ± 0.061	0.95 ± 0.11	1.97 ± 0.24	0.58 ± 0.10	0.33 ± 0.12
ThiSd	0.27 ± 0.042	0.60 ± 0.058	0.56 ± 0.051	0.50 ± 0.060	1.18 ± 0.11	2.18 ± 0.24	0.49 ± 0.10	0.30 ± 0.12
Matador	0.17 ± 0.042	0.52 ± 0.058	0.48 ± 0.051	0.62 ± 0.060	0.95 ± 0.11	2.05 ± 0.24	0.45 ± 0.10	0.15 ± 0.12
ThiMat	0.34 ± 0.042	0.61 ± 0.058	0.56 ± 0.052	0.55 ± 0.060	0.91 ± 0.11	2.14 ± 0.24	0.35 ± 0.12	0.41 ± 0.12
ThiMatAE	0.20 ± 0.042	0.48 ± 0.058	0.55 ± 0.052	0.59 ± 0.060	1.02 ± 0.11	2.08 ± 0.24	0.53 ± 0.10	0.36 ± 0.12
ThiSdAE	0.24 ± 0.042	0.51 ± 0.058	0.56 ± 0.051	0.50 ± 0.060	0.88 ± 0.11	2.04 ± 0.24	0.59 ± 0.10	0.35 ± 0.12
Urea	0.23 ± 0.042	0.48 ± 0.058	0.59 ± 0.052	0.65 ± 0.062	0.68 ± 0.11	2.21 ± 0.24	0.56 ± 0.10	0.44 ± 0.12
Contrasts								
Apron vs. Control	0.55[c]	0.62	0.11	0.34	0.55	0.92	0.95	0.88
Ethaboxam vs. Control	0.11	0.39	0.73	0.77	0.14	0.91	0.54	0.42
ThiSd vs. Control	0.074	0.29	**0.0022**	0.54	0.63	0.84	0.98	0.45
Matador vs. Control	0.93	0.80	0.073	0.28	0.34	0.85	0.85	0.89
Urea vs. Control	0.19	0.64	0.052	0.14	**0.0069**	0.76	0.63	0.11
Apron, Ethaboxam vs. EthAp	0.68	0.52	**0.001**	0.21	0.86	0.81	0.72	0.57
Apron, Ethaboxam vs. ThiSdAE	0.95	0.52	**0.0058**	0.31	0.70	0.99	0.62	0.41
Apron, Ethaboxam vs. ThiMatAE	0.35	0.28	**0.023**	0.84	0.57	0.88	0.91	0.49
ThiSd, Matador vs. ThiMat	0.08	0.50	0.36	0.87	0.30	0.94	0.39	0.22
ThiSd vs. ThiSdAE	0.51	0.35	0.97	0.72	0.067	0.70	0.50	0.66
ThiSd, Matador vs. ThiMatAE	0.45	0.28	0.74	0.51	0.70	0.93	0.60	0.38

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-8B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root weight (g) in faba bean. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	**Root weight (g)**							
	Vauxhall faba bean		**Lethbridge faba bean**		**Taber faba bean**			
	2016		**2017**		**2016**		**2017**	
	7 nodes	**Flowering**	**7 nodes**	**Flowering**	**7 nodes**	**Flowering**	**7 nodes**	**Flowering**
Control	2.43 ± 0.22[b]	4.34 ± 0.53	2.35 ± 0.29	3.50 ± 0.53	3.23 ± 0.22	7.99 ± 0.69	1.71 ± 0.24	5.82 ± 0.64
Apron	2.35 ± 0.22	4.24 ± 0.54	1.60 ± 0.29	3.26 ± 0.54	3.73 ± 0.22	7.98 ± 0.69	1.65 ± 0.24	6.56 ± 0.70
Ethaboxam	2.99 ± 0.22	3.86 ± 0.53	2.28 ± 0.29	4.99 ± 0.54	0.64 ± 0.22	7.89 ± 0.69	1.67 ± 0.24	5.61 ± 0.64
EthAp	2.82 ± 0.22	4.61 ± 0.53	0.12 ± 0.29	3.96 ± 0.53	2.93 ± 0.22	8.33 ± 0.69	2.11 ± 0.24	5.25 ± 0.64
ThiSd	2.67 ± 0.22	3.83 ± 0.53	2.16 ± 0.29	4.05 ± 0.53	3.29 ± 0.22	8.39 ± 0.69	2.18 ± 0.24	5.92 ± 0.64
Matador	2.65 ± 0.22	3.80 ± 0.53	2.33 ± 0.29	4.11 ± 0.54	3.34 ± 0.22	7.73 ± 0.69	2.08 ± 0.24	6.58 ± 0.64
ThiMat	2.47 ± 0.22	4.16 ± 0.53	2.43 ± 0.29	4.08 ± 0.59	3.42 ± 0.22	8.10 ± 0.69	2.22 ± 0.24	6.44 ± 0.64
ThiMatAE	3.06 ± 0.22	3.28 ± 0.53	2.09 ± 0.29	4.86 ± 0.53	3.40 ± 0.22	8.88 ± 0.69	2.37 ± 0.24	5.78 ± 0.64
ThiSdAE	3.18 ± 0.22	3.46 ± 0.52	2.00 ± 0.29	3.17 ± 0.54	3.22 ± 0.22	7.97 ± 0.69	1.86 ± 0.24	5.47 ± 0.64
Urea	2.51 ± 0.22	4.20 ± 0.53	1.75 ± 0.31	4.28 ± 0.58	3.87 ± 0.22	9.29 ± 0.69	2.09 ± 0.25	5.77 ± 0.64
Contrasts								
Apron vs. Control	0.69[c]	0.85	0.073	0.71	0.11	0.99	0.94	0.37
Ethaboxam vs. Control	0.078	0.55	0.84	0.072	0.19	0.92	0.87	0.92
ThiSd vs. Control	0.43	0.53	0.67	0.57	0.86	0.68	0.14	0.80
Matador vs. Control	0.51	0.49	1.00	0.50	0.73	0.79	0.27	0.36
Urea vs. Control	0.81	0.82	0.84	0.36	**0.0069**	0.76	0.25	0.88
Apron, Ethaboxam vs. EthAp	0.58	0.35	0.49	0.87	**0.0065**	0.64	0.12	0.19
Apron, Ethaboxam vs. ThiSdAE	**0.049**	0.33	0.86	0.13	0.090	0.96	0.41	0.44
Apron, Ethaboxam vs. ThiMatAE	0.15	0.23	0.70	0.33	0.29	0.27	**0.019**	0.69
ThiSd, Matador vs. ThiMat	0.54	0.87	0.73	0.99	0.69	0.96	0.90	0.78
ThiSd vs. ThiSdAE	0.10	0.54	0.67	0.24	0.067	0.70	0.38	0.68
ThiSd, Matador vs. ThiMatAE	0.16	0.36	0.59	0.27	0.75	0.33	0.43	0.63

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-9A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root nodule production in field pea. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment in 2016 and 4 replicates per treatment in 2017. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	**Root nodules per plant**				
	Lethbridge pea		**Taber pea**		
	2017		**2016**	**2017**	
	7 nodes	**Flowering**	**7 nodes**	**7 nodes**	**Flowering**
Control	12.35 ± 2.99[b]	5.50 ± 1.57	41.43 ± 5.19	26.25 ± 7.65	8.55 ± 5.52
Apron	8.25 ± 3.03	5.35 ± 1.67	34.19 ± 5.23	31.20 ± 7.65	19.20 ± 5.52
Ethaboxam	18.62 ± 3.11	3.15 ± 1.57	38.97 ± 5.19	42.14 ± 7.72	16.10 ± 5.57
EthAp	12.25 ± 3.00	4.20 ± 1.57	37.83 ± 5.19	40.10 ± 7.65	13.37 ± 5.61
ThiSd	14.10 ± 3.00	10.60 ± 1.57	48.35 ± 5.23	31.05 ± 7.65	14.17 ± 5.57
Matador	14.07 ± 3.03	7.10 ± 1.57	40.97 ± 5.19	44.55 ± 7.65	17.85 ± 5.52
ThiMat	10.00 ± 3.00	17.25 ± 1.57	38.05 ± 5.23	49.40 ± 7.72	25.60 ± 5.52
ThiMatAE	18.66 ± 3.03	8.20 ± 1.57	38.13 ± 5.19	30.80 ± 7.65	23.89 ± 6.12
ThiSdAE	18.35 ± 3.00	11.40 ± 1.57	36.61 ± 5.23	35.30 ± 7.65	21.58 ± 5.57
Urea	9.81 ± 3.03	4.30 ± 1.60	17.47 ± 5.19	19.84 ± 7.91	12.55 ± 5.52
Contrasts					
Apron vs. Control	0.21[c]	0.61	0.34	0.49	0.14
Ethaboxam vs. Control	0.33	0.25	0.61	0.098	0.33
ThiSd vs. Control	0.70	**0.046**	0.38	0.54	0.57
Matador vs. Control	0.76	0.34	0.96	0.064	0.21
Urea vs. Control	0.47	0.60	**0.0003**	0.71	0.74
Apron, Ethaboxam vs. EthAp	0.97	0.93	0.71	0.69	0.50
Apron, Ethaboxam vs. ThiSdAE	0.18	**0.023**	0.81	0.95	0.54
Apron, Ethaboxam vs. ThiMatAE	0.10	**0.0012**	0.78	0.67	0.86
ThiSd, Matador vs. ThiMat	0.17	0.08	0.35	0.37	0.13
ThiSd vs. ThiSdAE	0.66	0.40	0.16	0.60	0.18
ThiSd, Matador vs. ThiMatAE	0.27	0.40	0.30	0.62	0.61
ThiSd, Matador vs. Urea	0.18	**0.025**	**< 0.0001**	0.071	0.52

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-9B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root nodule production in faba bean. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment in 2016 and 4 replicates per treatment in 2017. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Root nodules per plant						
	Vauxhall faba bean		Taber faba bean			Lethbridge faba bean	
	2016		2016	2017		2017	
	7 nodes	Flowering	7 nodes	7 nodes	Flowering	7 nodes	Flowering
Control	11.70 ± 2.80[b]	19.19 ± 4.06	25.57 ± 4.63	27.73 ± 3.90	57.69 ± 9.31	4.85 ± 2.93	14.21 ± 5.23
Apron	11.10 ± 2.80	15.31 ± 4.13	35.08 ± 4.66	20.30 ± 3.82	57.60 ± 10.50	9.85 ± 2.97	12.30 ± 5.20
Ethaboxam	12.00 ± 2.80	11.21 ± 4.05	33.27 ± 4.63	20.25 ± 3.82	59.76 ± 9.20	4.12 ± 2.96	14.91 ± 5.26
EthAp	13.26 ± 2.81	19.96 ± 4.07	32.93 ± 4.63	24.50 ± 3.82	42.55 ± 9.09	10.55 ± 2.93	12.86 ± 5.23
ThiSd	9.37 ± 2.80	12.22 ± 4.07	30.37 ± 4.63	24.04 ± 3.97	56.35 ± 9.09	7.46 ± 2.94	22.21 ± 5.23
Matador	11.92 ± 2.80	14.40 ± 4.06	33.93 ± 4.63	25.80 ± 3.82	52.45 ± 9.09	12.96 ± 2.94	27.48 ± 5.30
ThiMat	11.24 ± 2.80	14.42 ± 4.08	34.83 ± 4.63	29.20 ± 3.82	45.55 ± 9.09	14.25 ± 2.94	20.29 ± 5.23
ThiMatAE	14.77 ± 2.80	19.40 ± 4.07	27.90 ± 4.63	19.84 ± 4.34	62.87 ± 9.35	8.10 ± 2.93	14.03 ± 5.30
ThiSdAE	7.73 ± 2.80	13.03 ± 4.03	34.47 ± 4.63	33.19 ± 3.99	52.20 ± 9.20	8.10 ± 2.93	18.55 ± 5.20
Urea	8.53 ± 2.80	15.40 ± 4.09	20.07 ± 4.63	24.73 ± 4.07	45.55 ± 9.31	6.68 ± 2.94	23.85 ± 5.20
Contrasts							
Apron vs. Control	0.73[c]	0.64	0.11	0.14	1.00	0.15	0.86
Ethaboxam vs. Control	0.94	0.096	0.18	0.12	0.90	0.81	0.84
ThiSd vs. Control	0.54	0.26	0.37	0.52	0.90	0.59	0.20
Matador vs. Control	0.99	0.49	0.17	0.68	0.72	0.054	0.087
Urea vs. Control	0.28	0.73	0.09	0.57	0.40	0.56	0.14
Apron, Ethaboxam vs. EthAp	0.54	0.11	0.58	0.19	0.091	0.50	0.39
Apron, Ethaboxam vs. ThiSdAE	0.15	0.74	0.83	**0.010**	0.58	0.088	0.79
Apron, Ethaboxam vs. ThiMatAE	0.41	0.21	0.21	0.89	0.74	0.80	0.98
ThiSd, Matador vs. ThiMat	0.79	0.38	0.77	0.64	0.29	0.34	0.69
ThiSd vs. ThiSdAE	0.51	0.81	0.48	0.15	0.76	0.13	0.70
ThiSd, Matador vs. ThiMatAE	0.36	0.28	0.38	0.23	0.49	0.61	0.13
ThiSd, Matador vs. Urea	0.25	0.52	**0.013**	0.95	0.48	0.44	0.94

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-10. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the percentage of nodules damaged by larval PLW herbivory in faba bean and field pea. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment in 2016 and 4 replicates per treatment in 2017. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	% of nodules damaged					
	Vauxhall faba bean		Lethbridge faba bean		Lethbridge pea	
	2016		2017		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	27.61 ± 4.40[b]	28.29 ± 4.10	75.36 ± 10.39	39.16 ± 8.36	50.20 ± 5.49	39.10 ± 12.81
Apron	30.14 ± 4.50	41.89 ± 4.37	73.56 ± 10.39	53.64 ± 7.94	54.05 ± 5.61	64.75 ± 13.61
Ethaboxam	37.67 ± 4.40	27.15 ± 4.10	71.13 ± 10.61	17.79 ± 8.36	48.72 ± 5.90	75.91 ± 12.96
EthAp	22.34 ± 4.49	21.88 ± 4.15	81.89 ± 10.21	48.65 ± 8.12	39.19 ± 5.61	72.21 ± 13.08
ThiSd	0.74 ± 4.40	5.82 ± 4.15	33.69 ± 10.64	12.79 ± 8.01	16.06 ± 5.49	35.11 ± 12.62
Matador	27.47 ± 4.44	25.54 ± 4.10	75.20 ± 10.28	22.55 ± 8.19	38.70 ± 5.61	70.61 ± 12.62
ThiMat	2.63 ± 4.44	3.67 ± 4.16	31.20 ± 10.28	29.40 ± 8.01	23.67 ± 5.61	39.32 ± 12.62
ThiMatAE	0.71 ± 4.44	5.85 ± 4.10	39.64 ± 10.28	29.39 ± 8.19	27.15 ± 5.61	43.49 ± 12.62
ThiSdAE	0.21 ± 4.67	7.88 ± 4.00	36.50 ± 10.28	37.17 ± 7.94	24.76 ± 5.49	51.97 ± 12.62
Urea	29.08 ± 4.88	42.69 ± 4.21	71.42 ± 10.28	31.37 ± 7.94	49.84 ± 5.74	30.93 ± 13.00
Contrasts						
ThiSd vs. Control	**<0.0001**[c]	**<0.0001**	**0.0085**	**0.035**	**0.0002**	0.83
Matador vs. Control	0.98	0.66	0.99	0.14	0.15	0.09
Urea vs. Control	0.35	0.052	0.79	0.66	0.53	0.92
ThiSd, Matador vs. ThiMat	**0.040**	**0.002**	0.076	0.20	0.59	0.39
ThiSd vs. ThiSdAE	0.93	0.73	0.85	**0.036**	0.27	0.35
ThiSd vs. ThiMatAE	1.00	0.79	0.69	0.23	0.17	0.64
ThiSd vs. Urea	**<0.0001**	**<0.0001**	**0.016**	0.082	**0.0002**	0.82
Matador vs. ThiMatAE	**<0.0001**	**<0.0001**	**0.021**	0.59	0.16	0.14
Matador vs. Urea	0.81	**0.019**	0.80	0.28	0.18	**0.037**

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-11. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the abundance of PLW larvae present in field pea and faba bean root nodules. Trials were conducted in 2016 and 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 6 replicates per treatment in 2016 and 4 replicates per treatment in 2017. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	PLW larvae in root nodules					
	Vauxhall faba bean		Lethbridge faba bean		Lethbridge pea	
	2016		2017		2017	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	6.33 ± 1.47[b]	3.50 ± 1.02	3.19 ± 1.81	0.81 ± 0.49	2.85 ± 0.97	0.16 ± 0.28
Apron	7.00 ± 1.47	2.67 ± 1.02	5.48 ± 1.81	1.15 ± 0.45	2.01 ± 0.98	0.00 ± 0.32
Ethaboxam	8.17 ± 1.47	4.50 ± 1.02	3.56 ± 1.86	0.71 ± 0.49	3.62 ± 1.00	0.32 ± 0.29
EthAp	5.83 ± 1.47	1.50 ± 1.02	9.25 ± 1.77	1.14 ± 0.47	4.00 ± 0.98	0.00 ± 0.30
ThiSd	0.00 ± 1.47	1.83 ± 1.02	0.69 ± 1.84	0.57 ± 0.46	0.90 ± 0.97	0.55 ± 0.27
Matador	5.33 ± 1.47	1.67 ± 1.02	11.04 ± 1.79	1.43 ± 0.46	3.30 ± 0.98	0.45 ± 0.27
ThiMat	0.50 ± 1.47	0.00 ± 1.02	2.70 ± 1.77	1.10 ± 0.46	2.09 ± 0.98	1.85 ± 0.27
ThiMatAE	2.50 ± 1.47	0.33 ± 1.02	0.78 ± 1.79	1.88 ± 0.48	2.90 ± 0.98	0.85 ± 0.27
ThiSdAE	0.00 ± 1.47	0.83 ± 1.02	1.80 ± 1.79	1.90 ± 0.45	3.90 ± 0.97	0.20 ± 0.27
Urea	3.83 ± 1.47	2.67 ± 1.02	4.75 ± 1.81	1.20 ± 0.45	2.90± 0.99	0.057 ± 0.29
Contrasts						
ThiSd vs. Control	**0.0002**[c]	0.22	**0.043**	0.38	**0.0072**	0.33
Matador vs. Control	0.88	0.44	**0.0084**	0.49	0.65	0.47
Urea vs. Control	0.35	0.99	0.69	0.42	0.79	0.80
ThiSd, Matador vs. ThiMat	**0.0096**	**0.0083**	0.23	0.86	0.73	**0.0003**
ThiSd vs. ThiSdAE	1.00	0.30	0.31	**0.047**	**0.022**	0.37
ThiSd vs. ThiMatAE	0.25	0.16	0.84	0.16	0.066	0.44
ThiSd vs. Urea	**0.0040**	0.21	**0.017**	0.084	**0.043**	0.22
Matador vs. ThiMatAE	**0.0047**	0.066	**<0.0001**	0.87	0.69	0.31
Matador vs. Urea	0.28	0.43	**0.021**	0.90	0.85	0.33

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-12A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root nodule tumescence and leghemoglobin expression in field pea. Nodules characteristics were measured in 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 4 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Lethbridge pea				Taber pea			
	Tumescent nodules		Expressing leghemoglobin		Tumescent nodules		Expressing leghemoglobin	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	4.90 ± 1.52[b]	1.39 ± 0.95	11.90 ± 3.07	1.11 ± 1.19	5.04 ± 1.83	0.051 ± 1.69	21.55 ± 7.53	0.67 ± 4.83
Apron	2.12 ± 1.53	2.50 ± 1.08	7.69 ± 3.10	0.075 ± 1.34	3.55 ± 1.78	1.77 ± 1.67	21.60 ± 7.16	8.53 ± 4.78
Ethaboxam	5.27 ± 1.57	1.69 ± 1.01	18.03 ± 3.18	0.63 ± 1.25	3.24 ± 1.79	1.63 ± 1.69	25.24 ± 7.22	7.33 ± 4.83
EthAp	5.50 ± 1.53	1.50 ± 1.01	12.49 ± 3.10	0.80 ± 1.25	4.15 ± 1.78	5.30 ± 1.73	31.75 ± 7.16	6.33 ± 4.92
ThiSd	5.25 ± 1.52	5.85 ± 0.90	14.00 ± 3.07	6.80 ± 1.13	5.36 ± 1.79	3.23 ± 1.67	25.68 ± 7.22	6.52 ± 4.78
Matador	5.71 ± 1.53	3.30 ± 0.90	13.69 ± 3.10	1.70 ± 1.13	3.15 ± 1.78	3.01 ± 1.68	31.55 ± 7.16	7.09 ± 4.80
ThiMat	5.41 ± 1.53	10.85 ± 0.90	10.58 ± 3.10	12.00 ± 1.13	7.31 ± 1.79	5.10 ± 1.67	39.43 ± 7.22	12.00 ± 4.77
ThiMatAE	8.52 ± 1.53	6.80 ± 0.90	18.19 ± 3.10	6.65 ± 1.13	3.95 ± 1.78	2.08 ± 1.90	14.55 ± 7.16	15.34 ± 5.32
ThiSdAE	8.65 ± 1.52	3.50 ± 0.90	18.35 ± 3.07	2.50 ± 1.13	5.40 ± 1.78	3.04 ± 1.69	30.85 ± 7.16	7.44 ± 4.83
Urea	4.62 ± 1.55	0.59 ± 0.98	9.62 ± 3.13	1.05 ± 1.22	3.15 ± 1.78	4.61 ± 1.74	17.35 ± 7.22	8.22 ± 4.93
Contrasts								
Apron vs. Control	0.14[c]	0.34	0.14	**0.027**	0.78	0.56	0.91	0.25
Ethaboxam vs. Control	0.83	0.82	0.48	0.27	0.60	0.30	0.66	0.36
ThiSd vs. Control	0.71	**0.0008**	0.62	**0.0005**	0.71	0.19	0.53	0.30
Matador vs. Control	0.86	**0.045**	0.72	0.84	0.61	0.11	0.28	0.099
Urea vs. Control	0.58	**0.28**	0.78	0.67	0.62	**0.021**	0.74	**0.047**
Apron, Ethaboxam vs. EthAp	0.46	0.47	0.58	0.32	0.90	0.071	0.41	0.97
Apron, Ethaboxam vs. ThiSdAE	**0.033**	0.14	0.13	**0.0017**	0.32	0.36	0.32	0.55
Apron, Ethaboxam vs. ThiMatAE	**0.016**	**0.0003**	0.072	**<0.0001**	0.75	0.61	0.32	0.37
ThiSd, Matador vs. ThiMat	0.79	**0.0018**	0.24	**0.0082**	0.41	0.14	0.38	0.53
ThiSd vs. ThiSdAE	0.37	0.079	0.65	**0.0095**	0.93	0.55	0.61	0.35
ThiSd, Matador vs. ThiMatAE	0.14	0.11	0.37	0.35	0.94	0.90	0.10	0.58
ThiSd, Matador vs. Urea	0.59	**<0.0001**	0.17	**0.0081**	0.62	0.41	0.22	0.77

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-12B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on root nodule tumescence and leghemoglobin expression in faba bean. Nodules characteristics were measured in 2017 in southern Alberta, and samples were collected at the seven node and early flower growth stages. The means and standard error were obtained from 4 replicates per treatment. Differences between single and combined seed and foliar treatments were evaluated using single degree of freedom contrast statements.

Treatment[a]	Lethbridge faba bean				Taber faba bean			
	Tumescent nodules		Expressing leghemoglobin		Tumescent nodules		Expressing leghemoglobin	
	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering	7 nodes	Flowering
Control	1.31 ± 1.39[b]	2.47 ± 1.64	3.39 ± 2.92	7.17 ± 3.50	3.75 ± 0.90	0.05 ± 1.69	13.95 ± 2.42	0.67 ± 4.83
Apron	4.09 ± 1.39	4.50 ± 1.57	8.49 ± 2.92	4.15 ± 3.38	3.65 ± 0.90	1.77 ± 1.67	11.80 ± 2.42	8.53 ± 4.78
Ethaboxam	1.67 ± 1.42	1.96 ± 1.64	3.59 ± 2.97	4.07 ± 3.50	0.95 ± 0.90	0.63 ± 1.69	7.65 ± 2.42	7.33 ± 4.83
EthAp	3.35 ± 1.37	3.56 ± 1.60	8.30 ± 2.88	4.77 ± 3.42	2.80 ± 0.90	5.30 ± 1.73	11.40 ± 2.42	6.33 ± 4.92
ThiSd	2.33 ± 1.42	3.52 ± 1.58	6.27 ± 2.96	11.26 ± 3.40	3.96 ± 0.91	3.23 ± 1.67	12.96 ± 2.46	6.52 ± 4.78
Matador	4.86 ± 1.38	4.60 ± 1.58	11.31 ± 2.90	8.14 ± 3.40	3.05 ± 0.90	3.01 ± 1.68	13.55 ± 2.42	7.09 ± 4.80
ThiMat	5.15 ± 1.37	8.78 ± 1.58	12.65 ± 2.88	16.84 ± 3.40	5.87 ± 0.91	5.10 ± 1.67	19.88 ± 2.46	12.00 ± 4.77
ThiMatAE	3.44 ± 1.38	4.68 ± 1.61	6.69 ± 2.90	9.61 ± 3.44	3.74 ± 0.91	2.08 ± 1.90	10.30 ± 2.46	15.34 ± 5.32
ThiSdAE	5.48 ± 1.38	7.40 ± 1.57	11.72 ± 2.90	12.50 ± 3.38	3.80 ± 0.90	3.04 ± 1.69	11.30 ± 2.42	7.44 ± 4.83
Urea	2.00 ± 1.38	3.55 ± 1.57	5.43 ± 2.90	7.50 ± 3.38	1.76 ± 0.94	4.61 ± 1.74	10.32 ± 2.55	8.22 ± 4.93
Contrasts								
Apron vs. Control	0.12[c]	0.15	0.14	0.58	0.55	0.42	0.77	0.28
Ethaboxam vs. Control	0.71	0.73	0.91	0.40	**0.027**	0.20	0.082	0.38
ThiSd vs. Control	0.50	0.43	0.40	0.50	0.46	0.17	0.89	0.24
Matador vs. Control	**0.047**	0.38	0.055	0.90	0.82	0.071	0.98	**0.024**
Urea vs. Control	0.70	0.33	0.59	0.79	0.20	0.38	0.41	0.89
Apron, Ethaboxam vs. EthAp	0.97	0.12	1.00	0.94	0.38	0.077	0.51	0.73
Apron, Ethaboxam vs. ThiSdAE	0.30	0.17	0.28	0.075	0.051	0.28	0.42	0.19
Apron, Ethaboxam vs. ThiMatAE	0.88	0.80	0.94	0.31	0.14	0.50	0.84	0.52
ThiSd, Matador vs. ThiMat	0.54	**0.043**	0.50	0.11	**0.045**	0.085	0.059	0.71
ThiSd vs. ThiSdAE	0.24	0.16	0.37	0.86	0.89	0.89	0.85	0.94
ThiSd, Matador vs. ThiMatAE	0.76	0.64	0.44	0.11	0.96	0.95	0.36	0.82
ThiSd, Matador vs. Urea	0.26	0.86	0.31	1.00	**0.049**	0.35	0.38	0.73

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil
[b]Least square means of dependent variables for each treatment.
[c]*p*-values of single degree of freedom contrast comparisons.

Table 4-13A. Mixed model ANOVA results and treatment means (± SE) for total clean weight of seed (g) harvested from field pea (top) and faba bean (bottom) in 2016 and 2017. Means and standard error were obtained from 6 replicates per treatment. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test.

Treatment[a]	Field pea clean seed weight (g)			
	2016		2017	
	Lethbridge	Taber	Lethbridge	Taber
Control	2135.87 ± 245.80	2456.99 ± 189.23	3801.67 ± 568.89	1023.63 ± 183.43
Apron	1973.78 ± 224.38	2326.03 ± 189.23	5657.67 ± 568.89	913.87 ± 183.43
Ethaboxam	2139.01 ± 224.38	2150.56 ± 189.23	3938.17 ± 568.89	1076.92 ± 183.43
EthAp	2084.75 ± 224.38	2255.99 ± 189.23	4574.50 ± 568.89	946.82 ± 183.43
Thiamethoxam	2207.62 ± 224.38	2353.96 ± 189.23	3989.17 ± 568.89	680.78 ± 183.43
Matador	2192.05 ± 245.80	2340.37± 189.23	4103.67 ± 568.89	855.39 ± 183.43
ThiMat	2437.51 ± 245.80	2448.30 ± 189.23	4906.83 ± 568.89	982.55 ± 183.43
ThiMatAE	2304.13 ± 224.38	2119.10 ± 207.29	4421.17 ± 568.89	884.62 ± 183.43
ThiSdAE	2112.03 ± 245.80	2191.75 ± 189.23	4118.67 ± 568.89	1204.48 ± 183.43
Urea	2231.34 ± 274.81	2598.02 ± 189.23	4206.17 ± 568.89	1099.70 ± 183.43
Treatment	**Faba clean seed weight**			
	2016		2017	
	Vauxhall	Taber	Lethbridge	Taber
Control	5115.73 ± 475.39	1506.24 ± 166.00	3549.67 ± 420.03	983.11 ± 126.34
Apron	5049.04 ± 433.97	1276.96 ± 166.00	3110.83 ± 420.03	1099.57 ± 126.34
Ethaboxam	4750.20 ± 433.97	1471.75 ± 166.00	3360.17 ± 420.03	823.34 ± 126.34
EthAp	4769.22 ± 433.97	1596.52 ± 166.00	3701.50 ± 420.03	1076.75 ± 126.34
Thiamethoxam	5151.08 ± 433.97	1412.13 ± 166.00	3204.33 ± 420.03	988.28 ± 126.34
Matador	4838.96 ± 433.97	1456.57 ± 166.00	3188.17 ± 420.03	968.75 ± 126.34
ThiMat	5053.29 ± 433.97	1592.32 ± 166.00	3543.00 ± 420.03	1038.31 ± 126.34
ThiMatAE	5400.29 ± 433.97	1344.64 ± 166.00	2800.00 ± 420.03	891.29 ± 126.34
ThiSdAE	5478.92 ± 433.97	1670.76 ± 166.00	3101.67 ± 420.03	989.49 ± 126.34
Urea	4432.98 ± 433.97	1785.65 ± 166.00	3106.17 ± 420.03	1092.25 ± 126.34

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

Table 4-13B. Mixed model ANOVA results and treatment means (± SE) for 1000 kernel weight (g) of seed harvested from field pea (top) and faba bean (bottom) in 2016 and 2017. Means and standard error were obtained from 6 replicates per treatment. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test.

Treatment[a]	Field pea 1000 kernel weight			
	2016		2017	
	Lethbridge	Taber	Lethbridge	Taber
Control	207.38 ± 4.86	184.33 ± 2.62	192.61 ± 4.33	176.59 ± 5.59
Apron	208.11 ± 4.43	183.14 ± 2.62	200.52 ± 4.33	185.22 ± 5.59
Ethaboxam	206.58 ± 4.43	180.37 ± 2.62	194.77 ± 4.33	197.14 ± 5.59
EthAp	211.76 ± 4.43	182.69 ± 2.62	188.61 ± 4.33	182.87 ± 5.59
Thiamethoxam	213.57 ± 4.43	184.26 ± 2.62	190.77 ± 4.33	190.27 ± 5.59
Matador	205.48 ± 4.86	181.53 ± 2.62	194.85 ± 4.33	189.94 ± 5.59
ThiMat	217.29 ± 4.86	187.94 ± 2.62	196.94 ± 4.33	178.53 ± 5.59
ThiMatAE	214.58 ± 4.43	184.42 ± 2.62	189.70 ± 4.33	193.13 ± 5.59
ThiSdAE	216.38 ± 4.86	187.93 ± 2.62	198.66 ± 4.33	186.95 ± 5.59
Urea	210.70 ± 5.43	185.47 ± 2.62	193.61 ± 4.33	181.61 ± 5.59
Treatment	**Faba bean 1000 kernel weight**			
	2016		2017	
	Vauxhall	Taber	Lethbridge	Taber
Control	312.75 ± 13.65	296.67 ± 15.22	350.49 ± 7.54	308.18 ± 9.04
Apron	303.77 ± 12.46	292.07 ± 15.22	352.72 ± 7.54	315.99 ± 9.04
Ethaboxam	325.48 ± 12.46	279.00 ± 15.22	349.07 ± 7.54	301.62 ± 9.04
EthAp	295.37 ± 12.46	292.02 ± 15.22	349.51 ± 7.54	314.16 ± 9.04
Thiamethoxam	346.34 ± 12.46	325.44 ± 15.22	358.17 ± 7.54	309.92 ± 9.04
Matador	310.38 ± 12.46	299.35 ± 15.22	357.03 ± 7.54	302.53 ± 9.04
ThiMat	339.99 ± 12.46	266.03 ± 15.22	365.82 ± 7.54	310.03 ± 9.04
ThiMatAE	337.43 ± 12.46	281.27 ± 15.22	354.41 ± 8.26	290.52 ± 9.04
ThiSdAE	332.14 ± 12.46	307.50 ± 15.22	360.48 ± 7.54	303.04 ± 9.04
Urea	299.05 ± 12.46	289.09 ± 15.22	356.16 ± 8.26	313.69 ± 9.04

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

Table 4-14A. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the relative quantity of *A. euteiches*, *F. solani*, and *F. avenaceum* DNA in pea roots from Lethbridge in 2016. ΔC_t values are normalized to the quantity of *P. sativum* DNA in each sample, and $\Delta\Delta C_t$ values are further normalized to the untreated control group. Samples were collected at seven nodes and flowering. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 biological and 2 technical replicates per treatment.

Treatment[a]	ΔC_t					
	7 nodes			Flowering		
	A. euteiches	*F. avenaceum*	*F. solani*	*A. euteiches*	*F. avenaceum*	*F. solani*
Control	-[b]	-	-3.87 ± 0.73*ab*	-	-	-2.65 ± 0.75*abc*
Apron	-	-	-4.23 ± 0.73*ab*	-	-	-3.70 ± 0.75*bc*
Ethaboxam	-	-	-5.53 ± 0.73*b*	-	-	-4.05 ± 0.61*c*
EthAp	-	-	-4.58 ± 0.73*ab*	-	-	-3.73 ± 0.61*bc*
ThiSd	-	-	-6.50 ± 0.73*b*	-	-	-0.22 ± 0.61*a*
Matador	-	-	-5.54 ± 0.73*b*	-	-	-3.48 ± 0.75*bc*
ThiMat	-	-	-7.07 ± 0.73*b*	-	-	-2.66 ± 0.61*abc*
ThiMatAE	-	-	-2.21 ± 0.73*a*	-	-	-2.66 ± 0.61*abc*
ThiSdAE	-	-	-1.70 ± 0.73*a*	-	-	-1.12 ± 0.61*ab*
Urea	-	-	-4.79 ± 0.73*ab*	-	-	-1.10 ± 0.75*abc*
	$\Delta\Delta C_t$					
Control	-	-	0.00 ± 0.73*ab*	-	-	0.00 ± 0.75*abc*
Apron	-	-	-0.36 ± 0.73*ab*	-	-	-1.06 ± 0.75*bc*
Ethaboxam	-	-	-1.66 ± 0.73*b*	-	-	-1.41 ± 0.61*c*
EthAp	-	-	-0.72 ± 0.73*ab*	-	-	-1.09 ± 0.61*bc*
ThiSd	-	-	-2.63 ± 0.73*b*	-	-	2.42 ± 0.61*a*
Matador	-	-	-1.67 ± 0.73*b*	-	-	-0.83 ± 0.75*bc*
ThiMat	-	-	-3.20 ± 0.73*b*	-	-	-0.014 ± 0.61*abc*
ThiMatAE	-	-	1.66 ± 0.73*a*	-	-	-0.020 ± 0.61*abc*
ThiSdAE	-	-	2.17 ± 0.73*a*	-	-	1.53 ± 0.61*ab*
Urea	-	-	-0.93 ± 0.73*ab*	-	-	1.55 ± 0.75*abc*

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

[b]Least square means of dependent variables for each treatment. '-' Indicates no amplification in ≤ 35 cycles.

Table 4-14B. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the relative quantity of *A. euteiches, F. solani,* and *F. avenaceum* DNA in pea roots from Lethbridge in 2017. ΔC_t values are normalized to the quantity of *P. sativum* DNA in each sample, and $\Delta\Delta C_t$ values are further normalized to the untreated control group. Samples were collected at seven nodes and flowering. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 biological and 2 technical replicates per treatment.

Treatment[a]	ΔC_t					
	7 nodes			Flowering		
	A. euteiches	*F. avenaceum*	*F. solani*	*A. euteiches*	*F. avenaceum*	*F. solani*
Control	-[b]	-	-7.62 ± 0.47*abcd*	-	-	-4.22 ± 0.47
Apron	-	-7.41 ± 1.37	-8.03 ± 0.47*bcd*	-	-	-4.59 ± 0.47
Ethaboxam	-	-	-8.59 ± 0.47*d*	-	-	-3.29 ± 0.47
EthAp	-	-5.94 ± 1.37	-7.10 ± 0.47*abcd*	-	-	-3.65 ± 0.47
ThiSd	-	-	-6.35 ± 0.47*abc*	-	-	-3.53 ± 0.47
Matador	-	-	-7.03 ± 0.47*abcd*	-	-	-4.33 ± 0.47
ThiMat	-	-	-5.57 ± 0.47*a*	-	-	-4.50 ± 0.47
ThiMatAE	-	-3.66 ± 1.37	-5.71 ± 0.47*a*	-	-	-4.01 ± 0.47
ThiSdAE	-	-5.08 ± 1.50	-5.94 ± 0.47*ab*	-	-	-4.56 ± 0.47
Urea	-	-	-8.37 ± 0.47*cd*	-	-	-3.63 ± 0.47
	$\Delta\Delta C_t$					
Control	-	-	0.00 ± 0.47*abcd*	-	-	0.00 ± 0.47
Apron	-	5.00 ± 1.37	-0.41 ± 0.47*bcd*	-	-	-0.37 ± 0.47
Ethaboxam	-	-	-0.97± 0.47*d*	-	-	0.92 ± 0.47
EthAp	-	6.47 ± 1.37	0.52 ± 0.47*abcd*	-	-	0.57 ± 0.47
ThiSd	-	-	1.28 ± 0.47*abc*	-	-	0.68 ± 0.47
Matador	-	-	0.59 ± 0.47*abcd*	-	-	-0.12 ± 0.47
ThiMat	-	-	2.05 ± 0.47*a*	-	-	-0.29 ± 0.47
ThiMatAE	-	7.33 ± 1.37	1.91 ± 0.47*a*	-	-	0.20 ± 0.47
ThiSdAE	-	8.75 ± 1.37	1.68 ± 0.47*ab*	-	-	-0.34 ± 0.47
Urea	-	-	-0.75 ± 0.47*cd*	-	-	0.58 ± 0.47

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

[b]Least square means of dependent variables for each treatment. '-' Indicates no amplification in ≤ 35 cycles.

Table 4-14C. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the relative quantity of *A. euteiches, F. solani,* and *F. avenaceum* DNA in pea roots from Taber in 2016. ΔC_t values are normalized to the quantity of *P. sativum* DNA in each sample, and $\Delta\Delta C_t$ values are further normalized to the untreated control group. Samples were collected at seven nodes and flowering. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 biological and 2 technical replicates per treatment.

Treatment [a]	ΔC_t					
	7 nodes			Flowering		
	A. euteiches	*F. avenaceum*	*F. solani*	*A. euteiches*	*F. avenaceum*	*F. solani*
Control	- [b]	-9.58 ± 1.01*b*	-	-	-6.19 ± 0.69*ab*	-
Apron	-	-	-	-	-8.93 ± 0.76*ab*	-
Ethaboxam	-	-7.74 ± 1.05*ab*	-	-	-7.04 ± 0.69*ab*	-
EthAp	-	-7.26 ± 1.01*ab*	-	-	-8.07 ± 0.69*ab*	-
ThiSd	-	-8.90 ± 1.42*ab*	-	-	-5.69 ± 0.69*a*	-
Matador	-	-5.31 ± 1.10*ab*	-	-	-6.14 ± 0.69*ab*	-
ThiMat	-	-8.52 ± 1.01*b*	-	-	-9.23± 0.69*b*	-
ThiMatAE	-	-	-	-	-8.94 ± 0.76*ab*	-
ThiSdAE	-	-2.62 ± 1.42*a*	-	-	-7.63 ± 0.69*ab*	-
Urea	-	-7.55 ± 1.10*ab*	-	-	-5.70 ± 0.69*a*	-
	$\Delta\Delta C_t$					
Control	-	0.00 ± 1.01*b*	-	-	0.00 ± 0.69*ab*	-
Apron	-	_	-	-	-2.74 ± 0.76*ab*	-
Ethaboxam	-	1.85 ± 1.05*ab*	-	-	-0.85 ± 0.69*ab*	-
EthAp	-	2.33 ± 1.01*ab*	-	-	-1.89 ± 0.69*ab*	-
ThiSd	-	0.69 ± 1.42*ab*	-	-	0.49 ± 0.69*a*	-
Matador	-	4.27 ± 1.10*ab*	-	-	0.048 ± 0.69*ab*	-
ThiMat	-	1.07 ± 1.01*b*	-	-	-3.04 ± 0.69*b*	-
ThiMatAE	-	-	-	-	-2.75 ± 0.76*ab*	-
ThiSdAE	-	6.97 ± 1.42*a*	-	-	-1.44 ± 0.69*ab*	-
Urea	-	2.04 ± 1.10*ab*	-	-	-0.48 ± 0.69	-

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

[b]Least square means of dependent variables for each treatment. '-' Indicates no amplification in ≤ 35 cycles.

Table 4-14D. Treatment means (± SE) for the effect of seed, N, and foliar treatments on the relative quantity of *A. euteiches, F. solani,* and *F. avenaceum* DNA in pea roots from Taber in 2017. ΔC_t values are normalized to the quantity of *P. sativum* DNA in each sample, and $\Delta\Delta C_t$ values are further normalized to the untreated control group. Samples were collected at seven nodes and flowering. In columns where a significant treatment effect was observed, means marked with the same letter are not significantly different based on a Tukey-Kramer HSD test. The means and standard error were obtained from 6 biological and 2 technical replicates per treatment.

Treatment[a]	**ΔC_t**					
	7 nodes			**Flowering**		
	A. euteiches	***F. avenaceum***	***F. solani***	***A. euteiches***	***F. avenaceum***	***F. solani***
Control	1.66 ± 0.99[b]	-6.89 ± 0.97	-5.09 ± 0.71*a*	-1.49 ± 0.32*ab*	-3.30 ± 0.72*bc*	-3.82 ± 0.39*abc*
Apron	-1.64 ± 0.99	-6.28 ± 1.32	-6.67 ± 0.71*ab*	-0.53 ± 0.32*a*	-4.27 ± 0.72*bc*	-5.19 ± 0.39*c*
Ethaboxam	0.083± 0.99	-7.64 ± 1.08	-7.63 ± 0.71*ab*	-1.54 ± 0.32*ab*	-5.67 ± 0.72*b*	-3.40 ± 0.39*abc*
EthAp	1.91 ± 0.99	-6.08 ± 0.93	-7.72 ± 0.74*ab*	-1.80 ± 0.32*abc*	-5.47 ± 0.72*b*	-3.72 ± 0.39*abc*
ThiSd	0.59 ± 0.99	-	-6.24 ± 0.74*ab*	-0.82 ± 0.32*a*	-4.13 ± 0.79*bc*	-2.24 ± 0.39*a*
Matador	1.48 ± 0.99	-8.26 ± 1.22	-6.49 ± 0.71*ab*	-1.69 ± 0.32*abc*	-4.51 ± 0.79*b*	-2.72 ± 0.39*ab*
ThiMat	0.37 ± 0.99	-10.38 ± 1.02	-8.65 ± 0.71*b*	-2.92 ± 0.32*bc*	-3.69 ± 0.88*bc*	-4.93 ± 0.39*c*
ThiMatAE	1.63 ± 0.99	-8.82 ± 1.02	-5.25 ± 0.71*a*	-1.91 ± 0.32*abc*	-2.27 ± 0.72*c*	-4.04 ± 0.39*abc*
ThiSdAE	0.63 ± 0.99	-8.48 ± 1.22	-5.82 ± 0.71*ab*	-3.12 ± 0.32*c*	-9.56 ± 0.72*a*	-7.08 ± 0.39*d*
Urea	-0.57 ± 0.99	-7.63 ± 0.93	-5.96 ± 0.71*ab*	-2.57 ± 0.32*bc*	-6.33 ± 0.79*ab*	-4.23 ± 0.39*bc*
	$\Delta\Delta C_t$					
Control	0.00 ± 0.99	0.00 ± 0.97	0.00 ± 0.71*a*	0.00 ± 0.32*ab*	0.00 ± 0.72*bc*	0.00 ± 0.39*abc*
Apron	-3.30 ± 0.99	0.61 ± 1.32	-1.58 ± 0.71*ab*	0.96 ± 0.32*a*	-0.97 ± 0.72*bc*	-1.37 ± 0.39*c*
Ethaboxam	-1.58 ± 0.99	-0.75 ± 1.08	-2.54 ± 0.71*ab*	-0.055 ± 0.32*ab*	-2.37 ± 0.72*b*	0.42 ± 0.39*abc*
EthAp	0.25 ± 0.99	0.81 ± 0.93	-2.64 ± 0.74*ab*	-0.31 ± 0.32*abc*	-2.17 ± 0.72*b*	0.097 ± 0.39*abc*
ThiSd	-1.08 ± 0.99	-	-1.15 ± 0.74*ab*	0.67 ± 0.32*a*	-0.83 ± 0.79*bc*	1.58 ± 0.39*a*
Matador	-0.19 ± 0.99	-1.36 ± 1.22	-1.40 ± 0.71*ab*	-0.20 ± 0.32*abc*	-1.21 ± 0.79*b*	1.10 ± 0.39*ab*
ThiMat	-1.30 ± 0.99	-3.49 ± 1.02	-3.56 ± 0.71*b*	-1.43 ± 0.32*bc*	-0.39 ± 0.88*bc*	-1.11 ± 0.39*c*
ThiMatAE	-0.04 ± 0.99	-1.93 ± 1.02	-0.16 ± 0.71*a*	-0.42 ± 0.32*abc*	-1.03 ± 0.72*c*	-0.22 ± 0.39*abc*
ThiSdAE	-1.04 ± 0.99	-1.59 ± 1.22	-0.73 ± 0.71*ab*	-1.63 ± 0.32*c*	-6.26 ± 0.72*a*	-3.26 ± 0.39*d*
Urea	-2.23 ± 0.99	-0.74 ± 0.93	-0.87 ± 0.71*ab*	-1.08± 0.32*bc*	-3.03 ± 0.79*ab*	-0.41 ± 0.39*bc*

[a]**Control** = untreated; **Apron** = fludioxonil; **EthAp** = ethaboxam + fludioxonil; **ThiSd** = thiamethoxam; **Matador** = λ-cyhalothrin; **ThiMat** = thiamethoxam + λ-cyhalothrin; **ThiMatAE** = thiamethoxam + λ-cyhalothrin + ethaboxam + fludioxonil; **ThiSdAE** = thiamethoxam + ethaboxam + fludioxonil

[b]Least square means of dependent variables for each treatment. '-' Indicates no amplification in ≤ 35 cycles.

CHAPTER 5: GENERAL CONCLUSIONS

This thesis addressed several research questions regarding interspecific interactions between several soil-borne pathogens and pea leaf weevil in field pea crops. Due to extensive overlap in geographical range, *Aphanomyces euteiches, Fusarium* spp., and *Sitona lineatus* are likely to co-occur in pea crops across the Canadian Prairies (Olfert *et al*., 2012; Chatterton *et al*., 2015; Esmaeili Taheri *et al*., 2016). However, little is known about the impact of a multi-specific infestation on disease progression, as antagonistic, additive, or synergistic interactions between these organisms have not previously been investigated. Furthermore, a fast and reliable method of detecting and quantifying *A. euteiches* and *Fusarium* spp. in pea roots was not available. The objectives of this research were therefore to 1) identify and quantify interactions between *A. euteiches* and *Fusarium* spp. using multiplex qPCR, 2) characterize interactions between *S. lineatus* larvae and *F. avenaceum*, and 3) evaluate the efficacy of an integrated approach to managing root rot pathogens and nodule herbivory in a field setting.

A greenhouse study investigating interactions between commonly occurring root pathogens of field pea showed that roots infected with *A. euteiches* were more susceptible to fusarium root rot, and that disease severity was generally higher when more than one pathogen species were present (Chapter 2). Here, visual disease ratings were supplemented by two multiplex qPCR assays to quantify changes in colonization dynamics when multiple pathogens were present. Primer and probe pairs targeting the internal transcribed spacer region of *A. euteiches* rRNA and the β-tubulin gene of *P. sativum* were developed, and were combined with previously developed primer and probe sets for *F. avenaceum, F. solani*, and *F. redolens* (Zitnick-Anderson *et al*., 2018). β-tubulin was used as an internal control for both assays, against which the quantity of pathogen DNA in each sample was normalized. Primers were grouped to form two triplex assays, making this a highly efficient method of simultaneously quantifying multiple

pathogens from root tissue. qPCR results indicate that co-inoculation with *A. euteiches* synergistically increases root colonization by *F. redolens*, the latter of which is only weakly pathogenic when occurring in isolation. In contrast, *A. euteiches* appears to interact additively with *F. avenaceum* and *F. solani*, both of which are aggressive pathogens of field pea. *Aphanomyces euteiches* is a highly virulent pathogen that poses a serious threat to pea production in several countries (Vandemark *et al*., 2002; Gaulin *et al*., 2007). Based on the results presented here, *A. euteiches* also appears to be a significant risk factor for increased severity of fusarium root rot (FRR) in regions where these species co-occur.

Nodule herbivory by *S. lineatus* larvae also predisposes pea roots to increased damage related to FRR. A greenhouse experiment indicated that *S. lineatus* and *F. avenaceum* interact mutualistically when co-occurring in pea roots, increasing symptom severity and decreasing larval mortality (Chapter 3). *Fusarium* spp. opportunistically invade the feeding wounds of other *Sitona* weevils, such as *S. hispidulus* in alfalfa (Dickason *et al*., 1968) and *S. scissifrons* in sainfoin (Morrill *et al*., 1998), increasing both the incidence and severity of disease. It is likely that a similar mechanism underlies the interaction observed in this thesis, wherein feeding damage to root nodules caused by *S. lineatus* serve as entry points for *F. avenaceum*. It was not determined why *S. lineatus* mortality decreased when larvae were reared on infected roots, in comparison to healthy plants. Demethylation of pisatin by *F. avenaceum* may have increased the quality of food resources for *S. lineatus* larvae: this hypothesis presents an intriguing topic of further research regarding the interactions between *S. lineatus* and *F. avenaceum* observed in this study.

The results of the greenhouse experiments described in Chapters 2 and 3 suggest the potential for increased disease severity in regions where *A. euteiches, Fusarium* spp., and *S. lineatus* co-occur. Effective, integrated management strategies are therefore

required to manage these threats and prevent additional losses in yield. A two-year field study was conducted to test the efficacy of fungicides, insecticides, and high N fertilizer in managing root rot disease and herbivory in pea and faba bean when applied singly or in various combinations (Chapter 4). The fungicides ethaboxam and fludioxonil, registered for use against *A. euteiches* and *Fusarium* spp., respectively, were not effective in reducing root rot severity in either crop. Both fungicides are registered for early-season suppression of root rot disease, and the efficacy of these treatments may have already diminished when plants were first sampled at the seven-node growth stage. The systemic neonicotinoid insecticide thiamethoxam was effective in reducing foliar and nodule damage associated with adult and larval pea leaf weevil. In contrast, application of lambda-cyhalothrin as a foliar spray did not reduce damage to leaves or root nodules. Foliar insecticides must be applied after adult insects have returned to pea and faba bean crops in the spring, but before oviposition occurs (Cárcamo & Vankosky, 2011). Extensive and time-consuming monitoring is required to apply lambda-cyhalothrin within this brief time period; therefore, thiamethoxam is a more efficient and reliable method of reducing damage caused by adult and larval pea leaf weevil. Finally, application of 46 kg N ha^{-1} did not consistently reduce nodulation in pea or faba bean. Consequently, there was no reduction in feeding intensity in fertilized versus untreated plants. Much higher concentrations of fertilizer are likely required to meet the N requirements of the plant, therefore the use of urea to prevent larval feeding by eliminating nodule production is not recommended.

qPCR was used to quantify the colonization dynamics of *A. euteiches, F. avenaceum,* and *F. solani* in pea roots from field sites in Lethbridge and Taber in 2016 and 2017 (Chapter 4). Overall, ethaboxam and fludioxonil did not significantly impact colonization of roots by *A. euteiches* or *Fusarium* spp. This was consistent with the lack of impact on root rot symptoms in treatments where these fungicides were applied. Similarly, thiamethoxam did not impact colonization patterns in a predictable manner at

either location. The variability in rates of colonization between treatments may be due to the spatial distribution of *A. euteiches* and *Fusarium* spp. at each site, as these pathogens typically occur in a patchwork of inoculum foci within an infected field (Rekah *et al.*, 1999; Moussart *et al.*, 2009). All three pathogens were detected in pea roots from Taber in 2017, demonstrating that *A. euteiches, F. avenaceum,* and *F. solani* can co-infect pea roots at both early and late stages of plant growth. *Aphanomyces euteiches* did not appear to impact the colonization rates of *F. avenaceum* or *F. solani*, which is consistent with the potentially additive interactions observed in the greenhouse experiments described in Chapter 2.

Neither yield nor seed size was impacted by the use of fungicides, insecticides, or nitrogen fertilizer. Yield protection was not anticipated in the fungicidal treatments due to the inefficacy of both ethaboxam and fludioxonil in reducing root colonization. Thiamethoxam was effective in reducing foliar and nodule herbivory by *S. lineatus,* but did not have a positive impact on yield. This is consistent with a previous field study that found no yield improvement in field pea treated with thiamethoxam, relative to untreated seed (Vankosky, Cárcamo, McKenzie*, et al.*, 2011). It is not known, however, if root rot disease was a factor in this case. In this thesis, root rot pathogens were not eliminated by the use of fungicides in any site or year. Consequently, it was not possible to quantify the individual impact of herbivory or root rot disease on yield. Applying insecticides is costly, and neonicotinoids are harmful to many non-target arthropods (Douglas & Tooker, 2016). Before thiamethoxam can be recommended for use, further research is needed to determine the utility of thiamethoxam in yield protection, and to identify factors that may decrease its efficacy in the field.

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