Towards Development of a Disease Risk Model for Pea Root Rot Disease

By

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Abstract

Pea root rot is one of the major diseases of field pea (*Pisum sativum*) in the Canadian prairies. Field avoidance is one possible approach for reducing yield loss caused by root rot. Current research aimed to investigate the possibility of developing a model to predict root rot. Surveys were conducted in Alberta pea fields and pathogens were isolated and identified by PCR from infected roots. The three main pathogens identified were *Fusarium avenaceum*, *F. solani* f. sp. *pisi* and *Aphanomyces euteiches.* Greenhouse tests showed that these were the most aggressive species, and *F. avenaceum* and *F. solani* f. sp. *pisi* had high varied from weakly to highly aggressive. A soil greenhouse bioassay and a quantitative PCR (qPCR) test of soil were compared for predicting root rot. First, the inoculum dose-disease response relationship was determined for *F. avenaceum* and *F. solani* f. sp. *pisi*, as this is the initial step to develop a predictive model. Analyses indicated a positive linear correlation between *Fusarium* spp. inoculum dose, disease severity and recovered DNA quantity. The ability of greenhouse bioassays to predict field disease severity was tested, and the results showed a significant positive correlation, which indicated the bioassay method is likely to provide reliable results for disease prediction. The incidence of pathogen DNA in soil was also a good predictor of the incidence of pathogens in roots, but was dependent on year, and the amount of pathogen DNA quantified in soils was low compared with observed disease severity. To develop a preliminary model, 260 samples were collected during the 2016 and 2017 growing seasons prior to planting pea in early April from commercial pea fields across Alberta. The DNA quantity, temperature and rainfall data were used to develop a regression model to assess the potential for disease prediction prior to planting. Temperature, rainfall and DNA quantity of *F. solani* f. sp. *pisi* and

A. euteiches had a significant effect on root rot development. DNA quantity of *F. solani* f. sp. *pisi*, *F. avenaceum* and *A. euteiches* explained 5.8, 5.1 and 6.0%, respectively of the disease severity. Improving the model requires finding improved DNA extraction methods for soil with high clay content and validation across a wide range of cultural practices and environments.

Preface

This thesis is an original work conducted by Samira Safarieskandari. Ms. Safarieskandari conducted all experiments and wrote the first draft of all chapters. Mr. Michael Heynen assisted in performing the experiments described in Chapter 3, and Ms. Safarieskandari analyzed the data and wrote the chapter. The chapters were then examined by Ms. Safarieskandari's supervisors, Dr. Linda Hall and Dr. Syama Chatterton. They provided editorial revisions and suggestions for each chapter, which were then incorporated by Ms. Safarieskandari.

Chapter 2 of the thesis has been submitted to the Canadian Journal of Plant Pathology, as: 'Pathogenicity and host range of *Fusarium* species associated with pea root rot in Alberta Canada', and has been provisionally accepted pending minor revisions.

Ms. Safarieskandari was responsible for the method development, data collection, analysis, and manuscript composition, while Dr. Hall and Dr. Chatterton developed the initial research concept and assisted with manuscript editing and interpretation of results.

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Dedicated to

to my parents Jaleh and Parviz, and my sister Sara for

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Table of contents

List of Tables

List of Figures

List of Abbreviations

Ae	*Aphanophyces euteiches*
C	Celsius
CFU	colony forming unit
Ct	quantification cycle
DNA	deoxyribonucleic acid
DS	disease severity
EC	soil electrical conductivity
f.sp	forma specialis
g	grams
ITS	internal transcribed spacer region
LSD	least significant difference
Ln	natural logarithm
PCR	polymerase chain reaction
spp.	species
qPCR	quantitative PCR

1 Introduction and literature review

1.1 General Introduction

Pulses are leguminous crops planted and harvested for dry grains. The Food and Agriculture Organization (FAO) recognizes 11 types of pulses grown worldwide (Miller et al., 2003). One of the most important is pea (*Pisum sativum* L.), which is a cool season crop belonging to the Leguminosae family (Hulse, 1994). The centres of origin of field pea is in central Asia and the Mediterranean (Vavilov, 1951) and field pea is grown in several parts of the world (Zohary & Hopf, 1973). The six main pea producing countries are Russia, China, Canada, France, Australia and the United States (McKay et al., 2003). Yellow cotyledon dry pea is the most widely grown pea and is used for animal and human consumption. Green cotyledon dry pea is mostly produced for human food, but in some regions is also used for forage production. Both types have a high amounts of protein and carbohydrate (Mcphee, 2003).

Pea is grown in a wide range of environmental conditions but prefers cool or semi-arid conditions. It is cultivated during the summer in mild regions and in cool seasons in warmer regions (Elzebroek, 2008). Pea is adapted to a wide range of soil types and able to grow in light sandy soils to heavy clay soils (McKay et al., 2003), but it is most productive in well-drained, light textured soils (Elzebroek, 2008). Pea plants are susceptible to soil salinity and water-logged conditions (Mckay et al., 2003). Extreme soil acidity is detrimental to pea production, and pea plants grow best at soil pH 5.5-7.0 (Hartman, 1988).

Pea is commonly planted in rotation with cereal and oilseed crops (Nayyar et al., 2009). Peas fixes atmospheric nitrogen by symbiotic relationship with rhizobacteria (Hardarson & Atkins, 2003), and biological nitrogen fixation has both economic and environmental advantages (Knight, 2012). In addition to enhancing soil nitrogen (N) availability, planting pulse crops in a rotation with other crops provides additional agronomic advantages to the subsequent crops, such as increasing soil water conservation. Planting cereals in rotation with a pulses decreased crown rot incidence and enhanced yield (Bailey et al., 1992). Crop sequence also has an impact on the soil microbial communities and soil structure (Kirkegaard et al., 2008). Soil microbes play a principal role in various ecosystem processes that drive the productivity of agricultural systems (Venter et al., 2017). Planting of two or more pulses in four year crop rotations compared with one pulse crop in four-year rotations considerably reduced fungal diversity (Bainard et al., 2017).

Field pea is susceptible to many pathogens including fungi, bacteria, viruses and nematodes. Under favorable conditions for these pathogens, may cause significant reduction in both yield and quality (Grünwald et al., 2004). Ascochyta blight, white mold, and powdery mildew are the main foliar diseases of field pea (Kraft & Pfleger, 2001). Pea root rot is caused by a disease complex (Kraft et al, 1988), consisting of pathogens are *Fusarium* spp.*, Pythium* spp.*, Rhizoctonia solani* and *Aphanomyces euteiches* (Oyarzun et al., 1993). Generally, any kind of biotic and abiotic stress can enhance disease damage (Kraft & Pfleger, 2001). Root rot development is associated with many components such as pathogen population, environmental conditions and soil physical, chemical and biological characteristics (Kraft & Pfleger, 2001).

1.2 Field pea production in Canada

Canada is among the major pea producing countries. Growing field pea began in a limited area 100 years ago and has dramatically increased during the last 20 years. Pea is now the largest pulse crop in Canada and accounts for most of our pulse exports in the multibillion-dollar industry (AAFC 2016). The main growing areas include Saskatchewan (49.1%), Alberta (47.5%) and Manitoba (3.4%) (Statistics Canada 2016). Pea production increased from 74,400 ha in 1985 to about 1.7 million hectares in 2016 (Statistics Canada 2016). This increase was influenced by the opening of the European feed pea market, increasing the value of pea. Pea production is subject to yearly fluctuations due to market forces and weather factors. In 2016 production reached 4.9 million tonnes compared to 3.2 million tonnes in 2015, a year when some growers experienced a drought (Statistics Canada 2016).

1.3 Pea root rot diseases in Canada

Seedling blight, damping off and root rot are among the main soil-borne diseases limiting pea production (Kraft & Pfleger, 2001). Seedling blight is identified by poor emergence, reduced vigor of seedlings and poor plant stand establishment. Primary pathogens associated with seedling blight are *Pythium* spp. (Hwang et al., 2000), *Fusarium* spp. (Chang et al., 2013), and *Rhizoctonia* spp. (Hwang et al., 2007). Post-emergent damping-off is described as the a reduction of seedling stand density and the associated pathogens are *R. solani* and *P. ultimum* (Xi et al., 1995). Root rot is identified by decay and brown discolouration of the roots, wilting and yellowing of older plants (Kraft & Pfleger, 2001; Xue et al., 2002). A recent survey in Alberta indicated that four *Fusarium* species including *F. avenaceum*, *F. solani, F. acuminatum*, and *F.*

redolens were the predominant fungi isolated from infected pea roots (Chatterton et al., 2014; Chatterton et al., 2015; Esmaeili Taheri et al., 2017). Among *Fusarium* species, *F. avenaceum* and *F. solani* f. sp. *pisi* are the major species that cause severe damage on pea (Kraft & Pfleger, 2001; Feng et al., 2010). A key pathogen associated with root rot in all pea growing areass of the world is *A. euteiches*, it was first reported in Alberta as one of the root rot agents in 2013 (Chatterton et al, 2015). Other root rot pathogens were also isolated from pea roots, but with less frequency, and thus they were less important.

1.4 Root rot pathogens

1.4.1 *Fusarium* spp.

Fusarium species are associated with various diseases such as root rot, stem rot, wilt and head blight on numerous plant species (Leslie et al., 2006). Generally fungal plant pathogens are categorized in to three groups based on their nutrient feeding habits: biotrophic, necrotrophic and hemibiotrophic (Horbach et al., 2011). Biotrophic pathogens obtain nutrients from living host cells, necrotrophs pathogens feed from dead cells, and hemibiotrophs infect living host cells followed by a necrotrophic phase (Glazebrook, 2005; Horbach et al., 2011). *Fusarium* spp. are considered necrotrophic or hemibiotrophic pathogens, since different species have a wide range of strategies for host plant infection (Vajna, 1985; Josefsen et al., 2012; Ma et al., 2013).

Several studies have focussed on the etiology of *Fusarium* species causing pea root rot. The primary *Fusarium* species in central Alberta was *F. avenaceum*, since 80% of species isolated from pea roots from this area were *F. avenaceum* (Feng et al., 2010). However, when a

larger area was surveyed in Alberta, Taheri et al. (2016) found that *F. avenaceum*, *F. solani*, and *F. redolens* were common.

Some *Fusarium* species are pathogenic on a particular host or a very narrow range of host species which are characterized as formae speciales (Ma et al., 2013). *Fusarium solani* f. sp. *pisi* which is pathogenic on pea, is distinct from other formae speciales, because it can infect plants other than pea and has been reported as a pathogen on pea, chickpea, mulberry and ginseng (Matuo & Synder, 1972). Root rot symptoms caused by *F. solani* f. sp. *pisi* are dark reddish brown lesions on primary and secondary roots. Above ground symptoms caused by this pathogen include yellowing and stunting. If soil has proper structure and appropriate water supply, above ground symptoms are not always observed, even if root rot severity is high (Kraft, 2001).

Fusarium avenaceum has a broad host range and has been isolated from multiple crops such as beans, pea, lupine, cereals and *Brassica* spp. (Nelson et al., 1972; Satyaprasad et al., 1997), is able to adapt to various ranges of environmental conditions (Lysoe et al., 2014). A wide range of variability was observed in *F. avenaceum* isolates from pea and they were not all equally aggressive (Feng et al., 2010). *Fusarium avenaceum* causes various disease symptoms in different crops including root rot of legumes (Kraft et al., 1988), dry rot of potato tubers (Peters et al., 2008), and stem rot and ear blight of cereals (Parry et al., 1995; Chelkowski, 1998). It can survive as mycelium in soil and crop residues (Dill-Macky et al., 2000; Fernandez et al., 2008). The symptoms of disease appear at the cotyledon attachment point with reddish brown streaking of the roots, then as dark lesions on the roots and epicotyls. The colour of infected tissue changes to chocolate brown. The vascular system is also affected and develops a red discolouration (Kraft & Pfleger, 2001). At an advanced stages of disease, roots become blackened and weak, and when

infection is sever stunting occurs (Tu, 1987). *Fusarium avenaceum* was not considered a major pathogen of concern for pea root rot diseases until recently reported in Canada (Chatterton et al., 2018).

Fusarium solani f. sp. *pisi* and *F. avenaceum* have different overwintering structures. *Fusarium solani* forms microconidia, macroconidia and chlamydospores. Chlamydospore formation is triggered by deficiency in carbon source or soil compounds produced from bacteria (Mondal et al., 1998, Goh et al., 2009). Chlamydospores are produced within or on hyphae or macroconidia and remain in the soil as resting spores and form initial inoculum in the next growing season (Leslie et al., 2006). *Fusarium avenaceum* does not produce chlamydospores but instead survives as mycelium in soil and in crop residues which are considered the primary inoculum source, and also produces micro- and macroconidia (Leslie et al., 2006). The initial inoculum is thus resting spores or the mycelium, which are stimulated to by compounds produced by germinating seeds or roots. Chlamydospores or mycelium then germinate to produce secondary inoculum, which can be macroconidia or mycelium that are the infective propagules (Leslie et al., 2006).

1.4.2 *Aphanomyces euteiches*

The oomycete species *A. euteiches* was reported as the most damaging root rot pathogen in many pea growing areas of the world. It can destroy an entire field or cause significant yield reduction (Oyarzun et al.,1989; Persson et al., 1997; Gaulin et al., 2007). It was reported in Alberta in 2013 as a primary pea root rot pathogen (Chatterton et al., 2015). Disease symptoms are most severe in growing seasons with a cool, wet spring, followed by a warm dry summer, or

where soil is saturated over long periods (Hagedorn, 1984). Host crops include alfalfa, snap bean, vetch, clover, and sweet clover (Gaulin et al., 2007). Field pea is susceptible to this pathogen at all growth stages. Aphanomyces root rot presents as water soaked, honey coloured lesions on the root and stem, in addition, stunting and yellowing are observed and the plant dies after severe damage (Hagedorn, 1976). Infected tissue might be colonized by other fungi turning it black colour (Papavizas & Ayers, 1974). *Aphanomyces euteiches* produces two types of spores: zoospores (asexual spore), which are the infective structure, and oospores (sexual spores), which are produced in infected plant tissue and are the resting spores. Oospores germinate in the presence of host signals from the roots to produce motile zoospores that infect the roots (Hardham et al., 1997; Heyman, 2008). Pathogen dispersal occurs between plants in the soil through movement of zoospores (Gossen et al., 2016).

1.5 Effect of root rot on field pea yield

Different pathogens infect and colonise specific sites of root tissue and affect root function in different ways, and root rot pathogens may damage roots both mechanically and physiologically (Emmett et al., 2014). *Aphanomyces euteiches* destroys the root cortex, which interferes with nutrient and water uptake (Emmett et al., 2014). *Fusarium* species can enter into the vascular system, colonize it and prevent water uptake, causing wilting and leaf discoloration in above ground parts of plant (Li et al., 2017).

Legumes are unique in the plant kingdom in that they develop a symbiotic association with *Rhizobium* bacteria to fix atmospheric nitrogen. The symbiotic bacteria form nodules on legume roots and then use nitrogenase enzymes to change nitrogen from N_2 to a plant accessible

form (Ferguson et al., 2010). Peoples et al. (2009) estimated that globally, each year 20-22 million tonnes of nitrogen are produced by the symbiotic relationship between rhizobacteria and legume crops. Weicht et al. (1994) investigated the impact of root rot disease on nodulation and reported that in chickpea, root rot severity was negatively correlated with nodule numbers and nodule weight was reduced as a result of damage to the root. *Rhizobium* spp. were reported to have an antagonist effect against *F. solani* f. sp. *phaseoli* and suppress pathogen colonization of dry beans (Buonassisi et al., 1986).

Establishing the relationship between yield loss and disease is challenging due to the many variables that impact plant growth and disease development, including agronomic practices, soil physical and chemical characteristics, the numerous crop cultivars grown and various geographic locations (Madden et al., 1995; Cooke, 2006). It is possible to model this association and estimate yield loss (Madden et al., 1995). Plant growth stage at the time of infection and weather conditions play a critical role in the extent of yield reduction. A previous research has indicated that pea root infection by *A. euteiches* at early growth stages causes more yield loss compared with late infection (Papavizas et al., 1974). Navas et al. (2000) reported the impact of Fusarium wilting in yield reduction of chickpea was due to a reduction in seed number, while a decrease of seed weight was less important. Early infection caused greater yield loss compared with late infection since the number of seeds produced decreased. Thus, yield losses seemed to be related to a the negative impact of root rot on pod set.

Commercial field and plot experiments have been used to determine the linkage between disease severity and yield loss, by comparing the yield of healthy plants and inoculated plants. Results of plot experiments indicated that Fusarium root rot of pea with moderate to high

disease severity caused approximately 35 to 57% yield loss (Basu et al., 1976; Basu, 1978). Kraft & Pfleger. (2001) reported 30% pea yield reduction by *F. solani* f. sp. *pisi,* while 80% yield reductions have been caused by *A. euteiches* in regions where this pathogen is prevalent (Gaulin et al., 2007). In commercial fields, pea plants with moderate root rot may not show above ground symptoms so a below-ground survey is essential to distinguish disease levels (Basu et al., 1973). Lack of correlation between visual disease assessment and pathogen biomass may result in incorrect conclusions about disease presence and yield loss (Cooke, 2006).

1.6 Root rot management strategies

Pea root rot is difficult to control since it is usually caused by a combination of pathogens that survive in the soil for up to 10 years, eradication is unlikely (Grünwald et al., 2004). In addition, there are no available pea cultivars with acceptable levels of resistance to these pathogen complexes (Gossen et al., 2016). There is no single approach for root rot management, but a combination of all available methods such as biological control, seed treatment and cultural practices are the best strategies for disease management. Most studies on root rot management focus on a single pathogen and there are a limited number of studies, which address the interactions of multiple pathogens. Increased knowledge of pathogen interactions and environmental conditions may assist in the development a decision support system that could be an effective method to avoid yield loss (Gossen et al., 2016).

1.6.1 Chemical control

Fungicide seed treatment is an effective management strategy for some seed-borne diseases. For Fusarium seedling blight of pulse crops, treating the seed with fungicide provided

good protection (Chang et al., 2013; Chang et al., 2014). For field pea, Chang et al (2013) reported an increase in seedling emergence under both greenhouse and field conditions when seeds were treated with Apron Maxx (fludioxonil plus metalaxyl). Treatments with fungicide such as metalaxyl, furakxyl, and benalaxyl provided protection against *Aphanomyces* and *Pythium* spp., but only metalaxyl is registered for use in Canada, and is ineffective against *A. euteiches* (Tu, 1987; McKay et al., 2003). Fungicide seed treatments can protect plants for up to four weeks so they can provide appropriate protection against seedling blight and damping-off. However, root rot pathogens are able to attack plants in any growth stage. Most of seed treatments have no, or minimal effects on rhizobia inoculants, but some are toxic so it is important to check the effect of the applied fungicide (McKay et al., 2003).

1.6.2 Cultural control

Agronomic practices influence both soil quality and crop health, and as a result have an impact on root rot incidence and severity directly and/or indirectly. Using cultural practices, such as cover crops, crop rotation, composts, and tillage systems may be considered disease management strategies. These practices affect the soil microbial communities associated with the breakdown of organic materials, mineralization of nutrients, nitrogen fixation, and suppression of plant diseases and pests (Abawi et al., 2000). Crop residue management can directly influence plant and soil health; by either increasing or reducing the disease depending on each pathogen (Bailey et al., 2003).

Depending on crop type and associated pathogens, tillage systems might have a positive, negative or neutral effect on disease severity and incidence (Ristaino et al., 1997; Bockus et al.,

1998; Bailey et al., 2003). Tillage practices might reduce root rot by reducing soil compaction and improving soil drainage (Abawi et al., 1992; Abawi et al, 2000). In a minimum tillage system, crop residues retained on the soil surface are colonised by many microorganisms. Most plant pathogens are weak saprophytes so these colonised materials may not provide suitable substrates for them. However some pathogens are able to overwinter in crop residue, and depending on the life cycle of pathogens, these crop residues may play an important role in disease incidence (Jackson et al., 2002; Stone et al., 2004). A reduced tillage system reduced diseases like Phytophthora blight of pepper (Ristaino et al., 1997), and black scurf and dry rot of potato (Peters et al., 2003). The reductions were attributed to improving soil physical properties, decrease in soil temperatures and higher levels of soil moisture and as a result, plant stress was reduced. However, reduced tillage systems can be challenging for managing those pathogens which survive in the previous crop residue (Sumner et al., 1981). Field pea is predominantly grown using no-till practices in Canada, which is likely to enhance the pathogen inoculum levels, particularly *F. avenaceum*, in the field.

1.6.3 Cover crops

There is an interest in enhancing soil suppressiveness by applying organic amendments and crop residue management (Bailey& Lazarovits., 2003). Crop residues and soil microorganisms produce substances, that may reduce or enhance the pathogen populations. For example, some species of cover crops have a direct negative effects on pathogen propagules and are able to reduce diseases severity (Sequeira, 1962; Candole et al., 1997; Stone et al., 2004). Brassica crop green manure or in crop rotation sequence can suppress soil-borne pathogens (Larkin et al., 2007). The mechanism of suppression is associated with sulfur compounds such as

isothiocyanates produced by these crops during tissue decomposition, which are toxic to soil-borne microorganism (Sarwar et al., 1998). The toxic compounds do not always eliminate the pathogen, but sometimes they damage pathogen propagule that become sensitive to unfavorable environmental condition (Stapleton et al., 1998). However, the reports of Brassica crops for use to control soil-borne disease are inconsistent, for instance previous reports suggested that these crops had a minor impact on *Fusarium* species (Smolinska, 2000; Larkin et al., 2007). Two Brassica species *Brassica juncea* and *Sinapis alba* were selected, and impact of combination of plant tissues with field soil on *A. euteiches* was investigated, but results did not show any suppression. However, in vitro tests indicated that volatiles produced by *B. juncea* prevent the growth of *A. euteiches* mycelium (Muehlchen et al., 1990; Hossain et al., 2012).

1.6.4 Crop rotation

Cultural methods of root rot management focus mostly on reducing the amount of inoculum and improving crop health. Crop rotation is one of the common cultural practices for disease control. The impact is dependent on the nature of the pathogen, since some amount of pathogens inoculum are able to survive in the absence of the host by producing long-lived resting structures, while other pathogens in rotation with non-host crops are unable to persist. Therefor planting non-host crops in rotation is suggested, to reduce the amount of inoculum,. However, it may not be a practical technique when the pathogen has a wide host range such as *F. avenaceum.* Selecting the appropriate crop in a rotation sequence is a key factor in reducing plant disease, since it could enhance or decrease the antagonist soil microbial community or act as a saprophytic host for the plant pathogen (Peters et al., 2003). For instance, continuous-pea production in a field had negative effects on the soil microbial community and also reduced pea

productivity (Niu et al., 2018). Using wheat in rotation with pea had positive impacts on soil microbial communities and nutrient availability (Nayyar et al., 2009). However, pathogens like *A. euteiches* and *F. solani* produce resting spores, that can survive in soil for many years even in the absence of the host. Due to the influence of cultural practices on soil-borne pathogens and soil microbial communities, expanding our knowledge of the impact of these practices would enhance disease management for root rot pathogens (Cook et al., 1978; Abawi et al., 1992; Abawi et al., 2000).

1.6.5 Biological control

One of the environmentally acceptable methods to control plant pathogens is biological control, which may replace for chemical management techniques (Baker et al, 1996). *Pseudomonas* spp. reduced *Pythium* spp. damping-off by an average of 40% (Parke et al., 1991; King et al., 1993). Treating pea seeds with *P. cepacia* and *P. fluorescens* was an effective method to reduce Aphanomyces root rot and increased the yield up to 17% (Xi et al., 1996). *Clonostachys rosea,* which is recognized as a mycoparasite of many root rot complex pathogens, was able to increase pea seed germination, seedling emergence and decrease disease severity by about 76%, which suggested that it is an efficient agent to reduce disease damage (Xue, 2003). Although some of these agents showed potential to reduce disease, none of them have been broadly adopted as a practical method to prevent root rot (Grünwald et al., 2004).

1.6.6 Breeding and development of resistant cultivars

Developing resistant cultivars is a key alternative in pea root rot disease management strategies, however, no pea cultivars currently have a reasonable level of resistance to root rot

pathogens (Pilet-Nayel et al., 2002). Identification and use of a resistance genes is an essential part of the process (Gururani et al., 2012). There are two types of genetic resistance to plant pathogens: qualitative where are one to a few genes confer resistance, or quantitative where several genes function together to provide partial resistance (Zhang et al., 2013). Plant resistance to pathogens could be associated with their physiological, morphological and biochemical features (Alexander et al., 1993). Using biotechnological tools like marker-assisted selection and genetic engineering for developing resistant cultivars to plant pathogens play a key role in disease resistance breeding programs. Breeding projects have made considerable progress in developing disease resistance screening techniques, identifying sources of resistance and also transferring resistance genes to high yielding varieties. One of the major goals in pea breeding is to enhance crop resistance to biotic and abiotic stresses (Tayeh et al., 2015). Generally, in pulse crops, genetic resistance to necrotrophic pathogens is partial and related to other factors like environmental conditions and plant growth stage (Tivoli et al., 2006). Resistance to root rot pathogens is under multigene control and environmental conditions also have a great influence on resistance. There are no commercial cultivars with acceptable levels of resistance to root rot (McPhee, 2003; Mukankusi et al., 2011).

Some pea germplasm lines have showed high level of partial resistance to *A. euteiches*, but no cultivars are commercially available (McGee et al., 2012). In the case of two important *Fusarium* species which are involved in pea root rot, partial resistance to *F. solani* f. sp. *pisi* was recognised in germplasm lines (Grünwald et al., 2004). Chittem et al. (2012) evaluated 21 field pea varieties for resistance against *F. avenaceum* and *F. solani* f. sp. *pisi* in growth chamber experiments. None of them showed complete resistance to these pathogen, only the commercial

cultivar Franklin showed partial resistance to both *F. avenaceum* and *F. solani* f. sp *pisi*. There were also partially resistant lines to both *P. ultimum* and *F. solani* f. sp. *pisi* described in 1994, but these are not commercially adapted (Ali et al., 1994). Bodah et al. (2016) assessed various pea cultivars for resistance to *F. solani* f. sp. *pisi* and concluded that some of the tested cultivars, most of which had pigmented flowers like Austrian winter pea, showed high level of resistance under greenhouse condition and significant decrease of plant growth was not observed. These genotypes may provide options for use in breeding programs, but their response to *F. avenaceum* should also be assessed. Pilet-Nayel et al (2002) identified seven genetic loci related to *A. euteiches* root rot resistance. For resistance to *F. avenaceum,* four quantitative trait loci linked with resistance to this pathogen were identified (Li et al., 2013). A three year experiment was conducted that identified five quantitative trait loci (QTL) associated with partial resistance to *F. solani* f. sp. *pisi* (Coyne et al., 2015). Six breeding lines with resistance to *F. oxysporum* and *F. solani* with high yield performance were recognized, though this experiment was conducted under greenhouse conditions (Porter et al, 2014). Variation between greenhouse and field conditions and considering the interaction of other pathogenic fungi is a challenge for developing resistant cultivars (Papavizas, 1974). In spite of all the difficulties, the goal to develop resistant cultivars is still in progress (Lavaud et al., 2015).

1.7 Factors affecting root rot development

1.7.1 Soil physical parameters effect on root rot disease

Soil quality, including physical, chemical and biological aspects, plays a principal role in disease severity. The maximum damage of soil-borne pathogens occurs when soil conditions are

poor. Generally, by improving the soil physical condition by cultural practices, soil health increases and as a result disease incidence may be reduced (Abawi et al., 2000). Some of the factors which cause poor soil conditions are high level of compaction, low fertility and organic matter, and/or poor drainage (Abawi et al., 2000). The effect of soil compaction, temperature, and moisture on pea root rot caused by *Fusarium* spp. was investigated under controlled conditions. Results from this study indicated that by increasing the soil compaction and temperature, disease severity increased. In the case of soil moisture, the minimum level of disease severity was observed at 75% of field capacity, and in both the higher and lower of ranges of over 75% moisture, disease severity increased (Tu, 1994). Soil compaction reduced root elongation and nutrient uptake in pea (Castillo et al., 1982). In addition, soil compaction decreased seed emergence, yield and dry weight of pea plants, but by reducing soil compaction, root growth and nutrient uptake improved (Hebblethwaite et al., 1980).

1.7.2 Effect of soil properties on root rot disease

One approach for predicting the risk of soil-borne disease is associating soil types and soil properties with disease severity. The level and availability of soil nutrients has an important role in plant disease suppression and reducing or increasing disease severity (Höper et al., 1996; Mazzola, 2004; Gatch, 2013). Nitrogen and the form of nitrogen, which could be ammonium or nitrate ions, might influence disease development (Jarvis et al., 1980). However, the impact is variable on different pathogens. There was no effect of form of applied nitrogen on tomato root rot development caused by *F. oxysporum* f. sp. *lycopersici* (Jarvis et al., 1980), although disease severity caused by *F. oxysporum* on lime was higher when nitrogen form was ammonium compared with nitrate form (Morgan et al., 1984). Applying ammonium fertilizer reduced the

soil pH which could enhance availability of micronutrients such as iron (Fe) and zinc (Zn), which are related to soil suppression against diseases, like Fusarium wilt (Jones et al., 1970; Gatch, 2013). Calcium is associated with plant functions like cellular signaling, and can affect plant-pathogen interactions (Dodd et al., 2010). For instance, Ca is involved in the biochemical pathway associated with phytoalexin production, which is involved in plant defense (Klüsener et al., 2002; Clapham, 2007). It can also influence pathogens directly. For example, excess Ca^{2+} inhibited the production of the enzyme of polygalacturonase by *F. oxysporum* which lead to a decrease in aggressiveness (Kim et al., 2015).

Correlation between some soil nutrient properties and soil receptivity to root rot pathogens of pea was examined. Results showed that the amount of soluble K, P, Mg and total C and N in soil had a negative correlation with suppressiveness to *F. solani* f. sp. *pisi*, but no correlation was found for *A. euteiches* (Oyarzun et al., 1998). Regardless of these correlations between soil chemical properties and disease severity, experiments conducted with sterilized soil indicated that soil microorganisms play a more important role in disease progress compared with soil chemical properties (Oyarzun et al., 1998). However another study conducted by Heyman et al. (2007) showed that calcium concentration in soil had a negative correlation with both disease severity and disease prevalence of *A. euteiches* under greenhouse condition. In addition, in vitro experiments showed calcium inhibits zoospore production. Bioassays and field experiments on pea root rot caused by *A. euteiches* indicated that Ca^{+} concentration, soil pH and clay content was correlated with disease suppression (Persson et al., 2000). However, results of soil properties associated with root rot severity are likely to be region specific (Lazarovits et al., 2007).

1.7.3 Environmental factors

Environmental factors can enhance or reduce disease progress; thus the environment is a key factor to consider in plant pathology studies (Colhoun, 1973). Depending on the predominant pathogen and plant growth stage, root rot severity can be related to soil temperature (Benedict, 1969). *Fusarium* spp. caused more damage in hot and dry seasons, because high soil temperatures were more suitable for progress and infection of this pathogen (Hagedorn, 1984). Pathogens associated with root rot have a range of optimum temperatures and soil moistures. Rhizoctonia and Fusarium caused severe disease at higher temperatures (24 ° to 30 °C) (Grünwald et al., 2004) and Pythium is more severe at cool temperatures and high soil moisture (Kraft et al., 1988). *Aphanomyces euteiches* can cause infection over a wide range of temperatures, but optimum temperature is 16 °C, and disease damage increases in wet soil (Hagedorn, 1984). The effect of environmental condition on root rot of wheat and lentil was investigated (Bailey et al., 2000). Results indicated that 75% of the variation in disease severity was related to changes in environmental condition, which showed the key role of these factors. However, this study also showed that tillage and rotation have little impact on disease severity (Bailey et al., 2000). A recent study in Alberta indicated that *F. avenaceum* was dominant in a wet and cool year, whereas *F. solani* had a highfrequency in dry and hot conditions (Esmaeili Taheri et al., 2017).

1.7.4 Role of crop residue as a source of inoculum for pea root rot pathogens

Plant pathogenic fungi survive and overwinter by producing resting spores or colonising dead plant tissue (Menzies, 1963). Under no or minimum tillage systems, crop residues play a

key role in survival of *Fusarium* species (Pereyra et al., 1999; Dill-Macky et al., 2000; Pereyra et al., 2008; Pereyra et al., 2013). Several studies confirmed the presence of *F. avenaceum* in crop residue (Sturz et al., 1985; Hofgaard et al., 2016), while fewer studies have identified *F. solani* in crop residue, although it was recovered from sunflower crop residues (Pineda et al., 1991). Research conducted on the Canadian prairies on fungi recovered from various crop residues indicted that plant pathogenic fungi have different survival potential and reproduction on each type of crop residue. A high level of *F. equiseti* and a low level of *F. culmorum* were recovered from barley stubble, *Gaeumannomyces graminis* was dominant in wheat stubble and *Fusarium* spp. were less frequent in canola and flax crop residue (Sturz et al, 1987). Hofgaard et al. (2016) reported that *F. avenaceum* is the predominant species isolated from oat residues. Similarly et al. (2007) described *F. avenaceum* as one of the dominant species in wheat residues. However, there is still more research needed to better understand of the survival time of *F. avenaceum* on pea and wheat residue.

1.8 Disease risk assessment

Disease management would be aided if fields at high risk for disease could be identified and avoided. Molecular techniques may be used to identify and quantify pathogens present (inoculum potential) and then used in combination with field history and environmental factors to assess the risk of disease. Disease risk assessments have been developed and used for some soil-borne plant diseases such as *F. oxysporum* f. sp. *lycopersici* on tomato (Stirling et al., 2004), *A. cochlioides* on sugar beet (Almquist et al., 2016), and root rot disease of cereals (Poole et al., 2015). In Canada, a precise assessment of inoculum potential could assist pea growers to make a

decisions to avoid high risk fields or plant pea more frequently in low risk areas. However, a prediction system may require testing and calibration for each region to account for differences in environmental parameters and soil types in the vast growing region of the Prairies.

There are several methods for determining inoculum potential. One method is plating dilutions of soil on selective medium, but this method requires that the pathogen can be cultured and that it can be easily identified in culture (Elad et al., 1981). A greenhouse bioassay uses soil samples taken from the field, a sensitive crop is sown and the disease severity assessed (Gatch et al., 2015). This method is labour intensive and time consuming (Oyarzun, 1993). In this method the impact of soil environment and host are considered as a key factor in disease severity (Gatch, 2013). This method has been used for two important root rot pathogens: *A. euteiches* (Malvick et al., 1994) and *F. solani* f. sp. *pisi* (Oyarzun et al ., 1994). Pfender et al (1983) described the concept of half-life for *A. euteiches* by using a greenhouse bioassay, which indicated that in the absence of host crop inoculum was reduced by 50% after one year. However this method is not likely to be an alternative for molecular methods, since it is not practical in large area and the results are not consistent.

Molecular techniques have been developed for plant pathogen detection including PCR, quantitative PCR (qPCR) and droplet digital PCR (ddPCR) (Okubara et al., 2005; Zijlstra et al., 2011; Zhao et al., 2016). Quantitative PCR offer new insights into studying phylogenetic, taxonomy and gene expression of various microorganisms in environmental samples like soil and water (Smith & Osborn, 2009; Rastogi & Sani, 2011). It is sensitive and reliable and could be used as a diagnostic test for estimation of pathogen quantity in soil samples (Okubara et al., 2005). For analysing data from quantitative PCR, a standard curve with a known amount of

DNA or pathogen propagules is generated and then used to define the unknown amount of target organism DNA (Arya et al., 2014). Quantitative PCR has the capability to detect target DNA in the primary exponential phase and PCR amplicon is measurable in each cycle (Smith & Oborn, 2009). There are different fluorescent chemistry formats available in qPCR including: SYBR green, TaqMan, molecular beacons and Scorpion (Schena et al., 2004). The SYBR green assay uses specific primers that target the pathogen of interest, but DNA quantification is not based on a specific sequence but rather on the detection of double stranded DNA, it is cheap and provides valid results (Schena et al., 2004). However, the TaqMan system was found to be more specific, sensitive, and reliable compared with SYBR green. In TaqMan assay hydrolysis probes use for measuring the amplification of a specific target of DNA sequence (Smith & Osborn, 2009). Probe is a sequence-specific oligonucleotide which fluorescently labelled, each probe has two parts, quencher and reporter which labelled with fluorescent dye (Navarro et al., 2015). In annealing stage of PCR probe binds to target sequence and during extension phase, by Taq polymerase activity, reporter's fluorescent released and produce fluorescent signal which uses for measuring PCR amplicons (Smith & Osborn, 2009). TaqMan assay is more expensive but it has the potential to be use for multiplexing and detecting more than one target in a qPCR assay (Schena et al., 2004; Okubara et al., 2005). Despite all the challenges of developing and applying molecular techniques for quantification of plant pathogens, there are several successful examples of applying TaqMan chemistry for quantification and detection of soil borne pathogens including: *A. cochliodes* (Almquist et al., 2016), *F. oxysporum* f. sp. *lycopersici* (Huang et al., 2016), *R. solani* and *R. oryzae* (Okubara et al., 2008).

Commercial testing services based on molecular assays for estimation of pathogen inoculum in soil samples are routinely used in Australia to provide risk assessments mainly for cereals and pulse crops (Ophel-Keller et al., 2008; Poole et al., 2015). Steps towards developing a risk assessment tool for soil-borne diseases include choosing appropriate sampling strategy to collect good representative samples, suitable DNA extraction method for large amount of soil, an understanding of the inoculum dose-disease response relationship, and a specific and sensitive assay for the target pathogen (Poole et al., 2015; Wei et al., 2015; Shishido et al., 2016). The grower can make the decision about planting pea or choose another crop, which may not be sensitive and thus reduce the pea root rot pathogen population in the field.

In Canada, several commercial seed-testing labs offer soil testing services for presence/absence of *A. euteiches* based on internal transcribed spacer (ITS) region by qPCR detection from soil, but testing for *Fusarium* spp. is not currently offered because i) DNA-based assays are not available for *F. avenaceum* or *F. solani* f. sp. *pisi*; and ii) the relationship between *Fusarium* spp. inoculum in soil and disease severity has not yet been established. Quantification results are also not offered for the same reason, lack of knowledge of the inoculum dose-response relationship for *Fusarium* spp. and pea root rot severity.

1.8.1 Development of species-specific qPCR assays for *Fusarium* spp.

Accurate detection and identification of plant pathogens is an essential step in disease control and developing management strategies. Early detection of pathogens in seeds, plants and propagative plant material may reduce introduction and spread of new pathogens. For that reason, the availability of fast, sensitive and accurate methods for detection and identification of

fungal pathogens is an essential part of disease control decision making (Lievens et al., 2005). Identification and distinguishing of *Fusarium* species based on morphological characters that fungi produce on selective medium requires an expert taxonomist (Leslie et al., 2008), while using DNA-based techniques has great accuracy (Summerell et al., 2003; Mishra et al., 2007). Real time PCR is a sensitive and reliable procedure, which can distinguish phylogenetically close species and quantify small amounts of DNA, and in addition it can be used for detection of non-culturable pathogens (Heid, et al., 1996; Schena et al., 2004).

Many species-specific assays have been developed for identification and quantification of *Fusarium* spp. in plant tissue (Filion et al., 2003; Gao et al., 2004; Reischer et al., 2004; Haegi et al., 2013) and soil samples (Filion et al., 2003; Zhang et al., 2005; Mbofung et al., 2011). A primer set for the detection of *F. avenaceum* was developed based on SYBR green chemistry by Nicolaisen et al. (2009). However, previous reports showed that using the SYBR green assay is not suitable for soil samples since it is highly affected by the humic acids existing in soil DNA and does not provide precise results (Alaeddini, 2012).

Due to the importance of *F. avenaceum* as a pathogen of several crops, it is essential to have a specific qPCR assay to detect and quantify this pathogen in plant tissue and soil samples. For identification of *Fusarium* species and phylogenetic studies, the translation elongation factor 1-a (TEF) gene is a functional and informative gene (Geiser et al., 2004). This gene is generally used for developing specific primers of *Fusarium* spp. (Geiser et al., 2004).

1.8.2 DNA extraction procedures from soil samples

There are several challenges with extracting DNA from soil samples. Due to the heterogeneity of soil, selecting a sampling strategy and sample size has a key role in accuracy of results (Ophel-Keller et al., 2008; Tsui et al., 2011). Processing samples for DNA analysis requires either drying or freezing (Rudi et al., 2006; Martí et al., 2012). Soil enzyme activity was less influenced by freezing than drying, while soil biomass was decreased by drying (Dadenko et al., 2009; Cui et al., 2014). Generally, there are two main procedures for DNA extraction from soil samples: direct and indirect, and DNA yield and quality provided by each procedure is different (Robe et al., 2003). In the indirect approach, cells are first removed from soil via a centrifugation followed by a lysis process using lysozymes and ionic detergent (Jacobsen et al., 1992; Delmont et al., 2011). Cell lysis in soil by mechanical or chemical methods are the basis of direct techniques, and generally used in commercial DNA extraction kits (Roose-Amsaleg et al., 2001).

Commercial extraction kits provide reliable results when the aim of the DNA analysis is to compare results in multiple time periods and environments (Morgan et al., 2010; Zielińska et al., 2017). Moreover the quantity of the DNA and procedure time is satisfactory compared with many traditional methods of DNA extraction (Robe et al., 2003). However due to great variation in soil characteristics, quality or quantity of extracted DNA may also vary. Therefore, to provide the best result from DNA analysis, it may be essential to have a procedure for each soil type with different physical and chemical features (Zielińska et al., 2017). The soil microbial community composition can also affect DNA extraction procedures (Carrigg et al., 2007).

A standardised procedure is required for DNA extraction from different soil types, and the extraction procedure may need to be optimised for several variables such as sample size and number (Elphinstone et al., 2018). Selecting appropriate internal controls can help with issues of consistency of DNA extraction efficiency and to verify the precision of DNA extraction and qPCR analysis (Ophel-Keller et al., 2008). To provide a routine system for soil testing the expense of molecular analysis also needs to be considered, thus the assay developed must have the capability to investigate multiple target pathogens with high accuracy and low cost (Elphinstone et al., 2018).

Previous research demonstrated that soil texture and predominant soil particles could play a critical role in recovery of DNA from soil samples since soil microorganism adhere to soil particles (Daniel, 2005). If the predominant soil particle is clay, the DNA extraction is more challenging (Cai et al., 2006). Yankson et al. (2009) developed a DNA extraction procedure, which has high performance for soil with a high clay content. Additionally, soil physiochemical features can affect DNA recovery and affect PCR. Zhou et al. (1996) examined an extraction method for eight different soil types and showed it is essential to select the proper cell lysis procedure and purification depending on soil features. Dineen et al. (2010) evaluated six different commercial kits to compare the yield and quality of the extracted DNA and found that the Power Soil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) had the highest performance for eliminating the PCR inhibitors in soil samples. Both the soil type and the extraction method can influence the yield and quality of the pathogen DNA.

1.8.3 Challenges for developing a prediction model for soil-borne pathogens

Developing a predictive model for assessing disease risk in pea may be challenging for several reasons. DNA based prediction system have been developed for several soil borne diseases such as root and stem rot of cereal and brassica crops in Sweden and Australia and are routinely used by growers (Ophel-Keller et al., 2008; Wallenhammar et al., 2012 ; Poole et al., 2015; Wallenhammar et al., 2016). The first step towards developing a prediction system is to investigate the role of inoculum quantity in disease incidence and disease severity (Ophel-Keller et al., 2008). There are some successful models that were solely developed based on pathogen levels in pre-sow samples, including Aphanomyces root rot of sugar beet (Almquist et al., 2016), clubroot of oilseed rape (Wallenhammar et al., 2012), and take-all of wheat (Herdina et al., 2000). However, inoculum potential alone is not always the best predictor in other pathosystems and estimated DNA quantity explained only a small portion of disease incidence and severity, such as Verticillium wilt of spinach (Okubara et al., 2013) and Rhizoctonia root rot of wheat (Poole et al., 2015). Environmental factors can play a critical role in development of soil borne diseases (Colhoun et al., 1973). Recent studies indicated a combination of DNA quantity with environmental factors improved the model accuracy for predicting cereal root rot diseases (Poole et al., 2015). However, it is currently unknown what environmental factors may increase or reduce the risk of pea root rot in Alberta. Furthermore, many environmental factors that may influence the incidence and severity of pea root rot cannot be known prior to planting and thus may be difficult to include in the model, including soil moisture, soil microbial community and temperature (Doohan et al., 2003).

Another challenge for developing a predictive model for pea root rot is the association of multiple pathogens with diverse epidemiology, favourable conditions for each pathogen may differ (Gossen et al., 2016). Chatterton et al. (2018) demonstrated numerous pathogenic and saprophytic fungi were associated with the root rot complex in the Canadian prairies and reported higher root rot severity in field seasons with higher precipitation which indicated the important role of environmental parameters on root rot progress. A recent study of root rot pathogens indicated that the interaction between *Fusarium* spp. and *A. euteiches* enhanced disease severity (Willsey et al., 2018). Despite the importance of these variables, they are insufficiently understood or quantifiable currently to be formulated into a model (Almquist et al., 2016).

There are a number of limitations to using qPCR analysis, such as it can not differentiate between pathogenic and non-pathogenic isolates, which is likely to be a source of error for estimation of disease severity (Okubara et al., 2013). In addition, qPCR assay cannot discriminate between live and dead fungal spore and therefore may provide false negative results necessitating an additional molecular viability analysis (Cangelosi et al., 2014). However there are some methods to distinguish between DNA from viable and dead cell in a qPCR assay, one method is treating samples by biological dyes prior to DNA extraction. Ethidium monoazide and propidium monoazide are two dyes which routinely use for this purpose (Yang et al., 2011; Chen et al., 2018). These compounds selectively entre live cells and membrane integrity consider as a sign of viability, by using these compounds in PCR reactions, results will be more accrue (Zeng et al., 2016).

The nature of the relationship between the quantity of pathogen DNA and disease severity and yield loss also needs to be differentiated. Moreover molecular methods need to be sufficiently sensitive to quantify a level of DNA that is below an acceptable disease threshold (Stirling et al., 2004). Finally, the model needs to be verified to determine if it is predictive of root rot levels under field conditions.

1.9 Summary and aims of research

Root rot is the outcome of the interaction between a pathogen and a susceptible host under environmental conditions favourable to disease development. An appropriate disease management strategy includes manipulating or changing one or more of these factors to reduce the disease damage. By having a predictive system, farmers can be advised not to plant pea in their fields when there will be a high risk yield reduction. Disease prediction models are effectively established for many soil-borne pathogens and using a similar approach for pea root rot disease management will be beneficial. The outcome from this project will allow growers to be aware of disease potential in the field prior to planting. Identifying the best disease predictors is the main goal of this project and will indicate if soil DNA level, environmental parameters and soil properties or a combination of these features can be used for this purpose. The pre-plant test will allow prediction of disease prior to planting: farmers can submit their soil and crop residue samples for analysis and results will indicate if the field is at risk of root rot. They may also choose to reduce disease pressure by treating seeds with fungicide, using an appropriate tillage system and removing crop residue from the fields, which may play a role in pathogen, survival.

Before a pre-plant model can be tested knowledge of the key pathogens in the root rot complex and their relationship to disease development needs to be better understood.

Hypothesis (I): *Fusarium* species will be pathogenic on pea and other crops and aggressiveness will vary both between and within species. To test this hypothesis we will evaluate the pathogenicity and host range of *Fusarium* species associated with pea root rot in Alberta in order to select species for further evaluation.

Hypothesis (II): DNA concentration of *F. avenaceum* and *F. solani* f. sp. *pisi* in soil and stubble is linearly associated to disease severity. To test this hypothesis we will determine the relationship between the inoculum levels of two key root rot pathogens *F. avenaceum* and *F. solani* f. sp. *pisi* (as determined from objective 1) to root rot severity under greenhouse condition to establish this relationship for a prediction model.

Hypothesis (III): Disease severity is correlated with pathogen population, by reducing the amount of inoculum in soil disease severity is reduced, and root rot rating from plants grown in greenhouse in field soils will be the same as disease ratings from plants collected from the field. To test this hypothesis we will determine whether a greenhouse bioassay is good predictor of pea root rot severity.

Hypothesis (IV): Root rot can be predicted by estimation of initial inoculum in soil and crop residues, and environmental parameters and soil properties. To test this hypothesis we will evaluate the significance of pathogen DNA quantity and selected environmental variables in developing a prediction model for pea root rot.

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2 Fusarium Root Rot of Pea in Alberta: Pathogenicity and Host Range

2.1 Introduction

Pea (*Pisum sativum*.) is an economically important crop in Canada, and the Canadian pulse industry plays a key role in global pulse production. Alberta, Manitoba and Saskatchewan are the major pea growing provinces in Canada (Statistics Canada, 2017). Symptoms of the pea root rot complex have been observed in almost all pea fields in western Canada (Hwang & Chang, 1989; Bailey et al., 2001; Foroud et al., 2014). Root rot disease causes by different pathogens such as *Fusarium* spp., *Aphanomyces euteiches*, *Rhizoctonia solani*, *Pythium* spp. In Alberta, *F. avenaceum* was identified as a predominant pathogen (Feng et al., 2010; Chatterton et al., 2014; Chatterton et al., 2015; Esmaeili Taheri et al., 2017; Chatterton et al., 2018), along with *F. solani* f. sp. *pisi* (Hwang & Chang, 1989; Esmaeili Taheri et al., 2017). *Aphanomyces euteiches* is another important pathogen, which was reported for the first time on field pea in Alberta in 2013 (Chatterton et al., 2015). *Fusarium avenaceum* is distributed in many regions worldwide and is considered to be a generalist pathogen with a wide host range of more than 80 plant species (Leach et al., 2013; Lysoe et al., 2014).

Fusarium avenaceum causes various diseases on host plants such as stem and root rot of legumes (Kollmorgen, 1974; Feng et al., 2010) and cereals (Backhouse et al., 2004), dry rot of potato tubers (Hanson et al., 1996; Peters et al., 2008), ear blights of cereals (Parry et al., 1995),

and is a member of the Fusarium head blight complex of cereals (Pancaldi et al., 2010; Nielsen et al., 2011). *Fusarium solani* f. sp. *pisi* is also frequently associated with pea root rot (Kraft & Pfleger, 2001) and causes disease symptoms such as foot and root rot, and wilting, chlorosis and stunting of the shoots (Coleman, 2016). In contrast with other forma specialis, *F. solani* f. sp. *pisi* has a broad host range (Sisic et al., 2018), and was pathogenic on ten different plant species (VanEtten, 1978). In addition, it was an asymptomatic colonizer of many more crops (Sisic et al., 2018).

Field surveys conducted in Alberta in 2013- 2017, and earlier, indicated *F. avenaceum* and *F. solani* f. sp. *pisi* were the predominant fungal species associated with pea root rot (Hwang & Chang, 1989; Feng et al., 2010; Chatterton et al., 2014; Chatterton et al., 2015; Chatterton et al., 2018). Although *Fusarium* species were collected from these surveys in 2013 and 2014, their potential role in root rot was not confirmed using pathogenicity tests. There is a need for more in depth information about the pathogenicity of isolated *Fusarium* spp. to pea and other host crops. Information about these pathogens, host range can clarify if crop rotation is an effective management strategy and also can help to identify other crops, which can be affected by these pathogens.

Two key plant pathology term which used in current study are pathogenicity and aggressiveness. Ability of a parasite to infect host define as pathogenicity and quantitative capacity of pathogenicity describes as aggressiveness (Van Baarlen et al., 2007; Pariaud et al., 2009). Aggressiveness can be measured by parameters like: infectious period, lesion size and disease severity (Pariaud et al., 2009). Percentage of the infected plant tissue by pathogen such as

root or leaf are examples of components for disease severity measurement (Cumagun & Miedaner, 2004; Pariaud et al., 2009).

This study was undertaken to: (i) assess the pathogenicity of the principal *Fusarium* species isolated from pea that cause root rot, and (ii) evaluate the host range of isolates of *F. solani* f. sp. *pisi* and *F. avenaceum*.

2.2 Materials and methods

2.2.1 Inoculum source and preparation

In order to identify *Fusarium* spp. associated with pea root rot in different regions of Alberta, surveys were conducted during 2013-2014 and the results reported previously (Chatterton et al., 2014; Chatterton et al., 2015; Chatterton et al., 2018). *Fusarium* isolates recovered from pea roots were purified using hyphal tip transfer and verified to be pure cultures by PCR with species specific primers and sequencing of the partial elongation factor α gene (Esmaeili Taheri et al., 2017), but pathogenicity tests were not conducted at the time.

To evaluate the aggressiveness of the predominant *Fusarium* spp. recovered from diseased pea roots from these surveys, pathogenicity tests were performed under greenhouse conditions. Isolates from six *Fusarium* species, *F. solani* f. sp. *pisi, F. avenaceum*, *F. culmorum, F. oxysporum, F. redolens* and *F. acuminatum* were tested, as these were commonly identified species from diseased pea roots. The number of isolates chosen from each species for pathogenicity testing was roughly equivalent to their frequency of isolation in culture. Isolates originated from a number of different counties in southern and central Alberta including:

Warner, Forty Mile, Taber, Lethbridge, Vulcan, Newell, Wheatland, Kneehill, Starland, Red Deer, Lacombe and Vermilion River Counties.

For each isolate, inoculum was prepared as described by Cappellini & Peterson (1965). Selected isolates were sub-cultured on to potato dextrose agar (PDA) medium and kept at 20 °C and 12 h light for five days. Two to three ten mm plugs were then transferred to flasks, which contained carboxymethylcellulose (CMC) medium (7.5 g of sodium carboxymethylcellulose, 0.5 g of NH_4NO_3, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$ and 0.5 g of yeast extract in one liter of deionized water). Flasks were agitated at 170-190 x g at 20 °C in the dark to induce sporulation. After seven days, the suspension was filtered through four layers of cheesecloth to collect macro- and micro-conidia, which were then transferred to a Falcon tube and centrifuged at 4000 x g for ten min, followed by washing three times with sterile distilled water. The conidia concentration of each isolate was measured to 2×10^6 conidia ml^{-1}, using a haemocytometer.

The pea cultivar 'CDC Meadow' was used in all experiments. Seeds were surface-sterilized for three minutes in 10% bleach and a drop of Tween 20 and then washed three times with sterile distilled water (SDW). Seeds were soaked in 50 mL of inoculum suspension overnight, and a control was soaked in sterile-distilled water (SDW) (Porter et al., 2015). Seeds were planted in root trainers (International Marketing Inc. Coquitlam, BC) (Hillson size: 32 cells/tray, cell volume = 160 cc; cell size = 3.8 x 3.8 x 12.7cm), containing vermiculite, with four seeds in each cell and four cells per each treatment. Plants were then grown in a greenhouse at 24:18 °C day $night^{-1}$ temperature with16 hours photoperiod . Plants were watered twice per week, but were not provided any fertilization. After 21 days, roots were washed under running tap water and rated for disease symptoms based on a 1-7 scale (Schneider et al., 2000) (Table 1).

For descriptive purposes, severity ratings of 2-3 were considered low, 4-5 as moderate and 6-7 as high. Non-emerged seeds that showed visible signs of Fusarium mycelial growth were rated as a seven. The experiment was arranged in a complete randomized design (CRD) with four replications and experiment was performed twice. Replicate trials were combined for analysis following tests for homogeneity of variance (Levene's test). Root rot ratings were analyzed using PROC GLM and means separated using the Tukey- Kramer test ($P < 0.05$) in SAS version 9.4.

2.2.2 Host range experiment

A greenhouse experiment was conducted to determine the host range of *F. avenaceum* and *F. solani* f. sp. *pisi* recovered from infected pea roots. Following the pathogenicity tests described above, three isolates of *F. avenaceum* and two isolates of *F. solani* f. sp. *pisi* were selected for host range assessment based on their pathogenicity on pea, and isolates that caused the highest disease severity on pea were chosen for testing on other crops.

Inoculum was prepared as described above and a mixture of isolates was used for seed inoculation. Ten crops, wheat (*Triticum aestivum*) 'Lillian'; barley (*Hordeum vulgare*) 'CDC Anderson' and 'AC Metcalf '; rye (*Secale cereale*)'Rogo', 'Gazelle', 'Prima', 'Hazlet'; canola (*Brassica napus*) an unknown cultivar; soybean (*Glycine max)* 'NSC Warren'; faba bean (*Vicia faba)* 'Snowbird'; dry bean (*Phaseolus vulgaris*) 'Maverick', and 'US1140'; chickpea (*Cicer arietinum)*'CDC Consul', 'CDC Limerick', 'CDC Leader', and 'CDC Orion'; and lentil (*Lens culinaris)* 'CDC Maxim', 'CDC Dazil', and 'CDC Impower', were tested.

Host seeds were surface-sterilized and inoculated with a mixture of all isolate for each species, as described above. Seeds of the control plants were incubated in SDW. Seeds were planted in root trainers (International Marketing Inc. Coquitlam, BC; Hillson size: 32 cells/tray),

containing vermiculite, three seeds were planted per individual cell. Plants were then grown in a greenhouse at 24/18 °C day/ night temperature with 16 hours photoperiod and were watered twice per week. Percentage emergence was determined by counting the number of normal and healthy seedlings that emerged in each cell after seven days, and dividing by the number of seeds planted in each cell (assuming 100% germination) and multiplied by 100.

Roots were subjected to visual disease assessment after 14 days based on the scale from 1-7. Data was analyzed using the mixed model procedure and repeats were considered as a random component. Fisher's least significant difference (LSD) test in SAS version 9.4 was applied to determine significant difference between inoculated plants and respective control plants.

2.3 Results

Forty-five isolates belonging to six species *F. avenaceum* (19 isolates), *F. solani* f. sp. *pisi* (three isolates), *F. redolens* (seven isolates), *F. culmorum* (six isolates), *F. oxysporum* (eight isolates) and *F. acuminatum* (two isolates) were selected to determine their aggressiveness under greenhouse conditions. The majority of the examined isolates were pathogenic and showed a range of aggressiveness from weakly (DS=1-3) to highly (DS=6-7) aggressive. *Fusarium avenaceum* and *F. solani* f. sp. *pisi* showed the highest level of disease severity followed by *F. oxysporum*, *F. culmorum*, *F. redolens* and *F. acuminatum* (Table 2.2). *Fusarium avenaceum* had a significantly higher disease severity (DS=6.0) than *F. oxysporum*, *F. culmorum*, *F. redolens,* and *F. acuminatum*, but was not significantly different from *F. solani* f. sp. *pisi* (DS = 5.4). *Fusarium culmorum* (DS=4.1), *F. redolens* (DS=4.0) and *F. acuminatum* (DS=3.4) caused lower

disease severities, but were not significantly different from *F. solani* f. sp. *pisi* and *F. oxysporum* (Table 2.2).

Analysis among isolates from each species indicated that there were significant difference among aggressiveness of isolates of *F. avenaceum*, *F. solani* f. sp. *pisi* and *F. culomorum* (Table 2.2). However, no significant differences were observed among isolates of *F. oxysporum, F. redolens*, and *F. acuminatum* (Table 2.2). The isolates of *F. solani* f. sp. *pisi* and *F. avenaceum* causing the highest disease severity on pea were further tested to determine their ability to cause disease on other crops. Seed emergence of the cereal crops, canola and soybean was not affected by inoculation with either *Fusarium* spp. (Table 2.3, 2.4). Significant reduction in seed emergence was observed in faba bean, dry bean 'Maverick', chickpea 'CDC *Leader*' and '*CDC* Orion', and red lentil 'CDC *Dazil*' following inoculation with *F. solani* f. sp. *pisi* (Table 2.3). None of the inoculated seed of the chickpea cultivar 'CDC Orion' emerged (Table 2.3). Seed emergence of faba bean, dry bean 'Maverick', chickpea '*CDC* Orion' and 'CDC *Leader*', and red lentil 'CDC *Maxim*' was significantly reduced by inoculation with *F. avenaceum* (Table 2.4). Inoculated seeds of the chickpea cultivar 'CDC Leader*'* did not emerge *(*Table 2.4).

Fusarium avenaceum and *F. solani* f. sp. *pisi* did not cause obvious disease symptoms on various cultivars of cereal crops including wheat, barley and rye and also canola plants compared with control plants of the same species; inoculated plants showed slight or no disease symptoms (DS< 1.5) (Table 2.3, 2.4). The lowest mean disease severity among all tested crops for *F. solani* f. sp. *pisi* was on wheat, barley, rye and canola (DS=1.4) (Table 2.3). For *F. avenaceum,* the lowest severity was observed on barley and spring rye (DS= 1.1) (Table 2.4).

Pulse crops showed variation in their susceptibility to *F. avenaceum* and *F. solani* f. sp. *pisi* (Table 2.3, 2.4). Generally, dry bean, pea, chickpea, faba bean, and lentil inoculated with *F. avenaceum* and *F. solani* f. sp. *pisi* had significantly greater disease severity compared to non-inoculated plants, with severity ranging from moderate (4- 5) to high (6- 7) (Table 2.3, 2.4). Pea and faba bean showed the highest level of disease followed by dry bean and chickpea.

Soybean inoculated with *F. solani* f. sp. *pisi* showed disease symptoms (DS=2.0) significantly different from control plants (Table 2.3) but not significant when inoculated with *F. avenaceum* (DS=1.3) (Table 2.4). Faba bean was highly susceptible to both pathogens and showed significant difference between inoculated and non-inoculated plants with an average disease severity rating of 4.5 when inoculated with *F. solani* f. sp. *pisi* (Table 2.3) and 5.0 when inoculated with *F. avenaceum* (Table 2.4). One cultivar of dry bean 'Maverick' inoculated with *F. solani* f. sp. *pisi* had a disease severity of 3.7 whereas the other dry bean cultivar, 'US1140', showed minor disease symptoms (DS=2.1) (Table 2.3). 'Maverick' inoculated with *F. avenaceum* showed low-moderate disease symptoms (DS=3.6) while US 1140 cultivar had moderate disease symptoms (DS=5.0) (Table 2.4). Disease severity in all inoculated dry bean cultivars with both pathogens were significantly higher than non-inoculated plants.

Four different cultivars of chickpea were tested. For chickpea plants inoculated with *F. solani* f. sp. *pisi*, CDC Orion had the highest disease level (DS=7.0) followed by CDC Limerick (DS=3.0), and *'*CDC *Consul'* (DS=2.4), while 'CDC Leader' (DS=1.5) was not significantly different from the control. (Table 2.3). For *F. avenaceum,* cultivars 'CDC Leader' (DS=7.0), '*CDC* Orion (DS=3.5), and 'CDC Limerick (DS=3.5), were the most susceptible and showed higher level of disease severity, while 'CDC Consul' showed minor disease symptoms (DS=2.3)

(Table 2.4). Red and green lentil inoculated with *F. solani* f. sp. *pisi* showed slight disease symptoms but were not significantly different than the non-inoculated plants. Results for *F. avenaceum* test on lentils indicated both cultivars of red lentil 'CDC Maxim' (DS=2.1) and 'CDC Dazil' (DS=2.0) were significantly different from the control plants while the disease severity on green lentil 'CDC Impower' was not significantly different (DS=1.5) (Table 2.4).

2.4 Discussion

The pathogenicity and aggressiveness of 45 isolates of *Fusarium* spp. isolated from root samples collected during surveys of commercial pea fields in 2013 and 2014 (Esmaeili Taheri et al., 2017) was examined to identify species causing root rot and to identify isolates and species for use in screening field pea breeding programs in Canada. *Fusarium avenaceum* was one of the most aggressive root pathogens of pea and other pulse crops (faba bean, chickpea and lentil) commonly grown in Canada. *Fusarium solani* f. sp. *pisi* was as equally aggressive on pea as *F. avenaceum*.

Considerable variability in range of aggressiveness was observed among isolates of *F. avenaceum* and *F. solani* f. sp. *pisi* which may be related to distinct geographical locations from which samples were collected. Previous studies indicated plant pathogen aggressiveness is affected by factors like environmental conditions and cropping system (Laloli et al., 2016; Suffert et al., 2018). For example, molecular analysis indicated significant variation among aggressiveness of isolates of *F. culmorum* which was attributed to the geographical scale of tested isolates (Mishra et al., 2003). Therefore it is likely that one of the reason for different levels of aggressiveness of isolates in the current study was related to the diverse sampling regions. Similarly *F. avenaceum* isolates recovered from field pea in North Dakota (Chittem et al., 2015) and central Alberta (Feng et al., 2010) also varied in aggressiveness.

Fusarium oxysporum can be a moderately to highly aggressive wilt pathogen on pea (Persson et al., 1997). However, wilt symptoms were not observed in any of the field surveys or plants from which *F. oxysporum* was isolated (Esmaeili Taheri et al., 2017). In the current study, *F. oxysporum* isolates caused low-moderate root rot severity, but often caused pre-emergent seed decay on pea (data not shown). Low aggressiveness of *F. redolens* and *F. culmorum* were previously reported on pea (Persson et al., 1997; Esmaeili Taheri et al., 2011), which is similar to the results of current study, which showed that this species caused low to moderate disease symptoms on pea. *Fusarium acuminatum* is a pathogenic fungus on cereal crops (Marín et al., 2012), but was recently reported as a pea pathogen (Zitnick et al., 2018), and in this study it was weakly to moderately aggressive on pea.

Fusarium species are among the most frequently occurring damping-off pathogens and cause seed emergence reduction or pre-emergence damping off in many crops such as soybean (Rizvi et al., 1996), wheat, rye (Kollmorgen, 1974; Arseniuk et al., 1993) and canola (Chen et al., 2014). However in the current study, *F. avenaceum* and *F. solani* f. sp. *pisi* did not reduce seed emergence of cereal species and canola, but had varying effects on the emergence of pulse crops. Furthermore, some pulse crops appeared to be more susceptible to pre-emergence damping off than to root rot or vice versa, when compared to their respective non-inoculated control. For example, *F. solani* f. sp. *pisi* caused root rot on soybean and chickpea ('CDC Consul' and 'CDC Limerick') without affecting emergence, but caused significant reductions on emergence of red lentil 'CDC Maxim' and chickpea 'CDC Leader' without causing root rot

symptoms. This could indicate differences in age-related susceptibility of pulse crops and cultivars to *Fusarium* pathogens.

While there is limited information available on differences in susceptibility of pulse seeds compared to root infection by *Fusarium* spp., differences in cultivar resistance to seedling blight and root rot have been reported previously on dry bean in western Canada (Conner et al., 2014). *Fusarium solan*i f. sp. *pisi* macroconidia and chlamydospore germination are stimulated by a number of seed exudates resulting in early seed colonization and seedling rot (Nelson et al., 2004). Highly vigorous seedlings can rapidly outgrow this susceptible growth stage, while seedlings with poor vigour will die off (Nelson et al., 2004; Lamichhane et al., 2017). One approach to enhance germination and seedling vigor is fungicide seed treatment (Lamichhane et al., 2017). Results from the current study indicated higher susceptibility of some pulse crops in the seedling stage, therefore it is important to take into account protection of seeds by treating with fungicide. Seedling emergence reduction caused by *Fusarium* spp. can be managed by use of seed treatments with chemical fungicides (Chang et al., 2013; 2014; Gossen et al., 2016), whereas late-season root rots are not effectively managed by seed treatments (Willsey, 2018).

Previous studies in Alberta showed that *F. avenaceum* causes severe disease on soybean (Zhou et al., 2018) and canola (Chen et al., 2014). *Fusarium avenaceum* has also been associated with wheat and barley crown rot (Backhouse et al., 2004; Smiley et al., 2005), wheat seedling blight (Fernandez et al., 2005) and is one of the causal agents of a Fusarium head blight (FHB) (Logrieco et al., 2002). However, *F. avenaceum* did not cause significant disease symptoms on

any of these crops in this study. In all of these previous studies, the *F. avenaceum* isolates that were tested on a specific host crop originated from that host crop, suggesting host specialization related to the origin host crop. Previous pathogenicity tests also used different inoculation methods, depending on the crop. For example, for canola and soybean, fungi mycelium was mixed with soil (Chen et al., 2014; Zhou et al., 2018); for wheat, colonized millet seed was used (Smiley et al., 2005); whereas the seed soak method was used for peas as a reliable method for screening resistant cultivars and pathogenicity test (Porter et al., 2015). Plant response to pathogens can be affected by inoculation methods and the best method for assessment of pathogen aggressiveness can differ depending on crop and pathogen (Jin et al., 1996; Scandiani et al., 2011). For example, in soybean sudden death syndrome, *Fusarium* aggressiveness was affected by inoculation method, and plants inoculated with infested soil showed a higher level of disease severity compared with plants inoculated using a toothpick method (Scandiani et al., 2011). One of the main reason for this variation is likely to due to interactions between host, pathogen and environment which varies depending on experimental set up (Scandiani et al., 2011; Shin et al., 2014).

Although *F. avenaceum* has not previously been reported as an important pathogen of chickpea (Nene et al., 1996), it caused severe root rot and reduced emergence of this crop. Similar results were also found for *F. solani* on chickpea. To our knowledge this is the first report indicating that *F. avenaceum* and *F. solani* are pathogens to chickpea in the Canadian prairies. Both *F. solani* and *F. avenaceum* are among *Fusarium* species involved in faba bean root rot in Alberta (Chang et al., 2014) and Manitoba (McKenzie et al., 1973). The results of

present study also showed that faba bean was highly susceptible to both *F. avenaceum* and *F. solani* f. sp. *pisi*. Both pathogens caused moderate disease symptoms on dry bean in this study, although they were previously reported as pathogens on bean (Reinking, 1950; Clarkson, 1978). *Fusarium avenaceum* caused significant disease symptoms on red lentil, but *F. solani* f. sp. *pisi* did not significantly affect lentils

Due to the apparent wide host range of *F. avenaceum* and *F. solani* f. sp. *pisi* on all species of pulse crops commonly grown in Canada, further field testing is recommended to determine the impact on inoculum potential when multiple pulse species are included in rotations.

Host specificity of *F. solani* f. sp. *pisi* to pea was not observed in the current study. Similarly, cross pathogenicity of *F. oxysporum* was recently reported between Solanaceae and Cucurbitaceae crops which was likely related to horizontal gene transfer of pathogenicity gene factors from other forma speciales of *F. oxysporum* (Lopez et al., 2019). Similar changes may likely be involved in the pathogenicity of *F. solani* f. sp. *pisi* to other pulse crops, however there is a major knowledge gap in this area. Previous studies indicated a degree of host specificity of *F. avenaceum* based on the origin host (Yli Mattila et al., 1996; Satyaprasad et al., 1997), which is in agreement with results from the current study. Our findings that isolates of *F. avenaceum* and *F. solani* f. sp. *pisi* from pea are aggressive on chickpea, faba bean, dry bean and lentil, but not cereals or canola, highlight the need for additional research to re-examine the host specificity of these pathogens based on the origin host.

2.5 Tables

Table 2-1. Severity rating scale used to rate roots from pathogenicity and host range trials (adapted from Schneider and Kelly 2000).

Rating	Lesions	% Root discolouration	Root mass reduction
1	0	0	0
2	0.1-0.2 cm, Small reddish brown lesions at seed attachment area	0	0
3	Coalescing of localized tap root lesions approximately 180 around the tap root, with lesions from 0.5 to 1 cm	10-20%	0
4	Lesions extending and completely encircling the tap root (1 – 2 cm)	95%	5-10%
5	Increasingly discolored and extended tap root lesions (2 – 4 cm)	100%	20-50%
6	Lesions encircling the tap root extending over 4 cm	100%	50-80%
7	Tap root completely brown/black	Dead	Dead

Table 2-2. Mean disease severity (DS) of pea plants in greenhouse pathogenicity tests and variation in root rot severity caused by isolates of *F. avenaceum, F. solani* f. sp. *pisi*, *F. oxysporum*, *F. culmorum, F. redolens* and *F. acuminatum* after inoculation with fungi isolated from infected roots during survey in Alberta

Species/Isolate number	Disease severity[a]	Letter Group[b]
F. avenaceum	6.0	A [b]
1306.04	7.0	a[c]
1315.06	7.0	a
1325.01	7.0	a
1329.1	7.0	a
1306.08	7.0	a
1317.09	7.0	a
1240.07	6.8	a
1204.09	6.7	a
1336.1	6.5	a
1217.06	6.1	a
1219.1	6.1	a
1241.05	5.8	ab
1239.05	5.6	ab
1220.09	5.1	ab
1302.06	5.0	ab
1329.07	4.8	ab

1319.06	4.7	ab
1332.1	4.7	ab
1338.05	3.5	b
F. solani f. sp. *pisi*	5.4	AB
1247	6.2	a
1333	5.0	ab
1325	4.8	b
F. oxysporum	4.7	B
1228.03	3.7	a
1235.01	4.6	a
1301.05	5.3	a
1320.03	4.1	a
1320.04	6.1	a
1330.70	4.4	a
1337.07	4.6	a
1350.09	5.3	a
F. culmorum	4.1	B
1310.03	6.8	a
1303.01	5.9	ab
1201.09	4.2	abc
1219.03	3.7	bc
1308.01	2.5	c

1237.03	2.4	c
F. redolens	4.0	B
1339.06	5.1	a
1220.06	4.8	a
1213.01	4.6	a
1329.08	4.4	a
1316.04	3.6	a
1208.01	3.4	a
1235.02	2.5	a
F. acuminatum	3.4	B
1203.05	3.5	a
1343.01	3.3	a

[a]The standard error of the mean (SEM) was 0.5 for *F. avenaceum*, 0.4 for *F. solani* f. sp. *pisi,* 0.7 *F. oxysporuym,* 0.6 for *F. culmorum*, 0.8 for *F. redolens*, 0.7 for *F. acuminatum* and the standard deviations were respectively 1.6, 1.2, 1.1, 2.2, 2.2, 1.8.
[b]Means with the same upper case letter are not significantly different between *Fusarium* species according to Tukey-Kramer test (P <0.05)
[c]Means with the same lower case letter are not significantly different between different isolates within each *Fusarium* species according to Tukey-Kramer test (P <0.05)

Table 2-3. Seedling emergence and disease severity of different crop species inoculated with *F. solani* f. sp. *pisi* under greenhouse conditions.

Crop (cultivar)	Emergence (%) [a]		Disease severity [b]	
	Inoculated	Non-inoculated	Inoculated	Non-inoculated
Spring wheat (Lillian)	83	91	1.4	1.1
Barley (CDC Anderson)	65	91	1.5	1.1
Barley (AC Metcalfe)	83	91	1.4	1.0
Spring Rye (Rogo)	88	91	1.5	1.0
Spring Rye (Gazelle)	88	100	1.5	1.1
Fall Rye (Prima)	91	100	1.4	1.1
Fall Rye (Hazlet)	83	100	1.5	1.2
Canola (unknown)	83	91	1.4	1.1
Soybean (NSC Warren)	83	91	2.0*	1.2
Faba bean (Snowbird)	22*	66	4.5*	1.7
Dry bean (Maverick)	16*	74	3.7*	1.2
Dry bean (US1140)	58	83	2.1*	1.4
Chickpea (CDC Consul)	77	91	2.4*	1.2
Chickpea (CDC Limerick)	33	49	3.0*	1.2
Chickpea (CDC Leader)	24*	66	1.5	1.2
Chickpea (CDC Orion)	0*	58	7.0*	1.1
Red lentil (CDC Maxim)	66	91	1.5	1.2
Red lentil (CDC Dazil)	44*	91	1.7	1.2
Green lentil (CDC Impower)	83	100	1.5	1.2
Pea (CDC Meadow)	66	71	4.1*	1.7

* = statistical difference between inoculated crop species and control plants ($P < 0.05$) according to the LSD test

[a]The standard error of mean (SEM) for seed emergence was 10.1

[b]The standard error of mean (SEM) for root rot severity was 0.2

Table 2.4. Seedling emergence and disease severity of different crop species inoculated with *F. avenaceum* under greenhouse conditions.

Crop	Emergence (%) [a]		Disease severity [b]	
	Inoculated	Non-inoculated	Inoculated	Non-inoculated
Spring wheat (Lillian)	75	87	1.2	1.0
Barley (CDC Anderson)	75	87	1.1	1.0
Barley (AC Metcalfe)	87	100	1.1	1.0
Spring Rye (Rogo)	87	100	1.2	1.0
Spring Rye (Gazelle)	87	87	1.1	1.0
Fall Rye (Prima)	87	100	1.3	1.0
Fall Rye (Hazlet)	87	100	1.1	1.0
Canola (unknown)	74	91	1.3	1.1
Soybean (NSC Warren)	74	91	1.3	1.0
Faba bean (Snowbird)	25*	75	5.0*	1.5
Dry bean (Maverick)	25*	91	3.6*	1.3
Dry bean (US1140)	49	91	5*	1.5
Chickpea (CDC Consul)	41	83	2.3*	1.1
Chickpea (CDC Limerick)	33	50	3.5*	1.0
Chickpea (CDC Leader)	0*	62	7.0*	1.1
Chickpea (CDC Orion)	25*	91	3.5*	1.3
Red lentil (CDC Maxim)	44*	91	2.1*	1.1
Red lentil (CDC Dazil)	50	87	2.0*	1.1
Green lentil (CDC Impower)	83	100	1.5	1.0
Pea (CDC Meadow)	75	71	4.5*	1.6

* = statistical difference between inoculated crop species and control plants ($P < 0.05$) according to the LSD test

[a]The standard error of mean (SEM) for seed emergence was 10.1

[b]The standard error of mean (SEM) for root rot severity was 0.2

2.6 References

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3 Relationship between inoculum level of *Fusarium avenaceum* and *F. solani* f. sp. *pisi* with pea root rot disease severity

3.1 Introduction

Pea (*Pisum sativum*) is an economically important crop in Canada, the main growing area is located in Alberta and Saskatchewan, with smaller acreage in Manitoba. Root rot is considered a serious threat for pea production in Canada (Gossen et al., 2016). Generally numerous pathogens are associated with pea root rot (Xue, 2003). Various studies indicated that *F. solani* f. sp *pisi, F. avenaceum* and *Aphanomyces euteiches* are the main pea root rot pathogens in the Canadian Prairies (Hwang et al., 1989; Feng et al., 2010; Chatterton et al., 2014; Chatterton et al., 2015; Esmaeili Taheri et al., 2016). Plants infected by *Fusarium* spp. show reddish-brown to blackish brown lesions on roots and vascular root system discoloration (Kraft et al., 1988), while *A. euteiches* causes water-soaked and honey-brown or blackish-brown lesions on roots (Gaulin et al., 2007). In the field it can be difficult to distinguish the symptoms caused by each member of the species complex, as these pathogens often co-occur. In surveys conducted in the Canadian prairies from 2014-2017, 55% of samples were infected by a combination of these three species (Chatterton et al., 2018).

There is no effective management strategy to control pea root rot, and disease management mostly relies on using a combination of strategies including increasing crop health

and reducing plant stress (Bruce et al., 2018), rotation with non-susceptible hosts, biological control, seed treatment, cultural practices, use of resistant cultivars (Gossen et al., 2016) and cultivation of Brassicaceae species as cover crops (Hossain et al., 2012; Hossain et al., 2015). One possible effective management strategy to minimise pea root rot damage is to identifying high risk fields prior to sowing to avoid planting susceptible hosts in high risk fields. Predictive models which are based on an estimation of the quantity of pathogen DNA in soil are considered an effective strategy to avoid yield loss from soil-borne pathogens (Ophel-Keller et al., 2008). Quantitative PCR (qPCR) is a sensitive and reliable technique applied for estimation of pathogen quantity (Okubara et al., 2005). It has been used for quantification of inoculum of soil borne fungi such as *F. oxysporum* f. sp. *lycopersici* (Huang et al., 2016), *Verticillium dahliae* (Bressan et al., 2016), *Rhizoctonia solani* and *R. cerealis* (James et al., 2013; Woodhall et al., 2016), and oomycetes like *Aphanomyces cochlioides* (Almquist et al., 2016) and *Phytophtora nicotianae* (Huang et al., 2010). Recently, a multiplex qPCR assay was developed for detection of *Fusarium* species associated with pea root rot diseases (Zitnick-Anderson et al., 2018).

A predictive system based on soil inoculum monitoring can be effective for monocyclic diseases where the inoculum propagules survive in soil or crop residue (Ophel-Keller et al., 2008). The pathogens causing pea root rot are all soil- or residue-borne, but have different types of initial inoculum. *Fusarium solani* produces chlamydospores that remain in the soil for 3-5 years (Smith, 2007; Leslie & Summerell, 2008), *F. avenaceum* survives saprophytically on crop residue (Dill-Macky et al., 2000; Fernandez, 2007; Kohl et al., 2007) or in soil as mycelium (Hargreaves et al., 1977), and *A. euteiches* produces oospores that can remain dormant in soil for

up to 10 years (Chan et al., 1987). *Fusarium* spp. generally have a saprophytic stage in which they overwinter in soil, crop residue or grass weeds as mycelia or chlamydospores. The process of plant tissue infection by *Fusarium* spp. can occur via two mechanisms: conidial germination or direct penetration by hyphae (Nelson et al., 1972; Nelson, 2012). *Fusarium* spp. penetrate root tissue through several branches of hyphae that arise after conidia germinate (Mendgen et al., 1996; Kang et al., 2005). Hyphae penetrate primarily directly through the epidermal cell wall followed by intra- and intercellular colonization by micro and macro conidia (Caradus, 1990; Mendgen et al., 1996; Wagacha et al., 2007). Both conidia and mycelium can infect host plants (Kazan et al., 2018), so it essential to investigate their potential contribution to disease progress.

The first step in developing predictive models is understanding the relationship between soil borne inoculum levels and disease development (Ophel-Keller et al., 2008). There are several examples of positive correlations between pathogen inoculum density and disease severity in various crops such as *Fusarium* spp. and cereal root rot (Ophel-Keller et al., 2008), black root rot of cucumber caused by *Phomopsis sclerotioides* (Shishido et al., 2016), Fusarium wilt in chickpea (Landa et al., 2001), and verticillium wilt of olive (López-Escudero et al., 2007). Ophel-Keller et al. (2008) developed standard curves that described the relationship between pathogen DNA quantity and disease severity, including curves for *F. oxysporum* and *F. pseudograminearum,* which followed a logarithmic linear relationship. Similarly, this relationship has been described for *A. euteiches*, where DNA quantity in roots was linearly related to disease severity (Vandemark et al., 2005). However, the relationship between levels of

F. avenaceum and *F. solani* f. sp. *pisi* inoculum and DNA in soil with root rot disease severity in pea has not yet been established.

There is limited research on the influence of inoculum type on pathogenicity of *Fusarium* spp. Couteaud et al. (1989) compared disease severity caused by *F. oxysporum* f. sp. *lini* when it was applied as chlamydospores or conidia to the plants and reported variation in disease potential. Therefore, it is important to test two kinds of inoculum sources on disease development for pathogenic *Fusarium* species on pea. Once disease severity has been related to inoculum concentration in soil and inoculum concentration related to DNA, a connection can then be made between disease severity and DNA quantity, and potentially used for prediction purposes. Therefore, objectives of this study were to: (1) assess the relationship between inoculum level of *F. solani* f. sp. *pisi* and *F. avenaceum* and disease severity, (2) evaluate the association between DNA quantity and disease severity, and (3) investigate the impact of two inoculation methods: conidia suspension and colonized wheat straw with disease severity.

3.2 Material and methods

3.2.1 Inoculum preparation

To determine the association between DNA quantity in soil and disease severity, a greenhouse experiment was conducted with *F. avenaceum* and *F. solani* f. sp. *pisi*. Two inoculation methods were used in this study: (i) conidial suspension applied as a soil drench, and (ii) colonized wheat straw ground and mixed with soil. In order to produce the spore suspension, a single three mm mycelial plug from *F. avenaceum* and *F. solani* f. sp. *pisi* from long-term

storage cultures on Spetzieller Nahrstoffarmer Agar (SNA) were subcultured onto potato dextrose agar with streptomycin and penicillin (PDAA) (Leslieet al, 2008). Plates were incubated for seven days at 20° C with approximately eight hours of fluorescent light each day. Two 10 mm mycelial plugs from each of these cultures were transferred to 100 mL of sterilized carboxymethylcellulose liquid medium (CMC) (Cappellini & Peterson, 1965). Inoculated medium was placed on a shaking incubator at 180 x g for seven days at room temperature in the dark. The suspension was then shaken vigorously and filtered through four layers of cheesecloth. The resulting flow-through was centrifuged at 4000 x g for 10 minutes and the supernatant discarded. The pelleted conidia were washed with sterile distilled water three times, each time centrifuged at 4000 x g for 10 minutes. The final washed pellet was suspended in 25 mL of sterile distilled water (SDW). The concentration of the conidia was determined by counting on a haemocytometer as conidia mL^{-1} .

The conidia from the shaken CMC cultures were diluted to the following concentrations at 10 mL volumes in four replicates: 1×10^7, 2×10^6, 10^6, 2×10^5, 10^5, 2×10^4, 1×10^4, and 1×10^3 conidia mL^{-1}. These suspensions were each used to inoculate 200 g of dry Cornell mix, resulting in concentrations of 500,000, 100,000, 50,000, 10,000, 5000, 1000, 500, and 50 conidia $gram^{-1}$ of dry soil, mixed and transferred into 200 mL pots. For the inoculated wheat straw experiment , 500 g of wheat stubble was collected from wheat research plots after harvest, and autoclaved twice at 121° C for 30 minutes with a 24 h interval in clear autoclave bags placed in tin foil trays. Fifty mL of conidia adjusted to 1×10^7conidia mL^{-1} , was added to the wheat straw. The inoculated stubble was placed in a growth chamber at 22 °C, under a 12 hour per day exposure to

fluorescent and incandescent light for 23 days. The inoculum was then dried in room temperature for two days, and ground in a plant tissue grinder into powder with a diameter of ≤1 mm. Inoculum concentration was determined by plating suspensions of inoculum on PDA in one-tenth serial dilutions from 10^{-2} g mL^{-1} to 10^{-7} g mL^{-1} in duplicate. For the wheat straw inoculum experiments, the assumption was that each colony forming unit (CFU) is equal to one conidia (Petrikkou et al., 2001). The ground wheat inoculum was mixed with Cornell mix to create 20, 10, 5, 2, 1 and 0.5% v/w mixtures in four replicates. Mixtures were homogenized and transferred to 200 mL pots. The initial concentration of fungi on colonized wheat stubble was determined by dilution plating on PDA+ antibiotic medium but results were not available until after soil was used in the experiments. Initial concentration was 6.3×10^5 CFU for *F. avenaceum* and 5.7×10^7 CFU per gram of stubble for *F. solani* f. sp. *pisi*. Therefore, the colony forming unit concentration of *F. avenaceum* was: 10000, 5000, 2000, 1000, 500, 300, and 150, and for *F. solani* f. sp. *pisi*, it was 200000, 100000, 50000, and 10000 CFU per gram dry soil.

3.2.2 Plant growth conditions

Seeds of cultivar CDC Meadow pea were surface sterilized by soaking in 70% ethanol for 30 seconds, rinsing with SDW, followed by soaking in 10% bleach with one drop of tween 20 for five minutes. Seeds were then washed three times with SDW. Five seeds were placed 2.5 cm deep into each pot containing 200 g of each inoculated soil treatment.

Control plants were planted into Cornell mix treated with sterile distilled water only. Plants were grown in the greenhouse at 22 ˚C with 16 hours photoperiod and watered manually

twice per week. Plants were rated for root rot severity at four weeks after seeding, using a 1 to 7 point based on scale adapted from Schneider (Schneider & Kelly, 2000). Trials were repeated twice, with four replicates per inoculum concentration. The ratings of the five plants in each pot were averaged before performing statistical analysis.

3.3.3 DNA extractions and qPCR assay

Immediately after adding inoculum to the soil but prior to seeding, a five g aliquot of soil was removed from each treatment. Soil was stored fresh in a Whirlpak bag at 4 ºC until ready for DNA extractions. DNA extractions of each sample were performed in duplicate with a Mo Bio Powerlyzer Powersoil DNA Isolation Kit from a 0.4 g aliquot of each sample (MoBio, Carlsbad, California, USA) according to the manufacturer's instructions. In order to evaluate DNA extraction efficiency, the gene sequence from the *Thynnus thynnus* NADH dehydrogenase subunit from Li et al. (2015), ligated into pIDTSMART-AMP (synthesized by IDT), was added to each sample at a final mass of 125 fg prior to DNA extraction. To prepare standards, DNA was extracted from 7 - 10 day old cultures of *F. avenaceum* and *F. solani* f. sp. *pisi* growing on PDA using the Plant DNeasy kit (Qiagen) according to manufacturer's directions. The DNA was quantified using a NanoDrop (NanoVue Plus Spectrophotometer) and serially diluted to produce a six fold dilution standard curve ranging from 10 ng μL^{-1} to 100 fg μL^{-1} *Fusarium* genomic DNA. Similarly for *T. thynnus,* the standard curve consist of six dilutions ranging from 100 pg to 1 fg. Real time PCR mixtures were made to a final volume of 25 µL containing: 2.5 µL template DNA, 12.5 µL Environmental Master Mix 2.0 (Thermo Fisher Scientific), 1.25 µL of each

primer (18 µM) and 1.25 µL of probes (5 µM), 0.5µL of each primer and probe (10 µM) for *T. thynnus,* and 4.75 µL ultrapure H_2O. Assays were run on a QuantStudio 6 Flex Real-Time PCR System (Thermofisher) with the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer and probe sequences for *F. avenaceum* (Nicolaisen et al., 2009), and *F. solani* f. sp. *pisi* were based on the partial elongation factor gene as described by Zitnick-Anderson et al.(2018), and internal control primer and probe sequences from Li et al. (2015).

The Ct values obtained from the qPCR analysis were converted to DNA quantity by comparison to the DNA standard curve for each pathogen. This quantity was then used to calculate ng DNA g soil^{-1} based on a starting soil volume of 250 mg of soil and elution volume of 100 µl.

Because DNA quantities are not normally distributed, all DNA quantity data were transformed to ln (x+1) for analysis and reporting. Regression analysis using the 'lm' function of R (version 3.4.1) was used to determine the association between inoculum density, DNA quantity and disease severity. A covariate test was used for comparing slopes of the two repeated trials, and when analysis indicated that there was no difference between slopes, trials were combined for analysis.

Analysis of covariance (ANCOVA) was also used to evaluate the statistical differences between regression lines (slope and intercept) of disease severity and DNA quantity for the two inoculation methods by using by 'aov' function in R. Based on the assumption that each colony

forming unit (CFU) is equivalent to one conidium, the equivalent inoculum doses from each inoculation method were selected and pairwise comparisons were conducted using Tukey HSD to compare disease severity and DNA quantity at inoculum concentrations of 10000, 5000, 1000, and 500 conidia or CFU for *F. avenaceum* and 100,000, 10,000, and 5,000 conidia or CFU for *F. solani* f. sp. *pisi*. All statistical analysis was performed with R version 3.4.1 (https://www.r-project.org).

3.3 Results

3.3.1 Relationship between inoculum level of *F. avenaceum* or *F. solani* f. sp. *pisi* and disease severity

For both pathogens there was a positive logarithmic relation between disease severity and inoculum concentration when inoculum was applied as conidia to the soil or on colonized wheat straw (13.1). Control plants did not show disease symptoms (DS = 1) and were not included in the regression analysis due to its impact on forcing lines through the origin and influencing the regression coefficient.

Regression analysis between disease severity and inoculum density indicated there was a linear relationship for both pathogens, but there was a stronger linear relationship for the conidia treatment. For *F .avenaceum* it was R^2= 0.86, P= 0.002 for conidia compared to colonized wheat straw R^2= 0.64, P = 0.005 (Figure 3.1, A), and for *F. solani* f. sp. *pisi* conidia treatment was R^2= 0.96, P = 0.005 compared to colonized wheat straw (R^2= 0.90, P = 0.05) (Figure 3.1, B). The

highest disease severity for conidia was observed at 500,000 conidia g^{-1} soil which was 6.8 for *F. avenaceum* and 4.9 for *F. solani* f. sp. *pisi*, respectively, the lowest disease severity was 2 and 1.7, observed in plants grown in 50 conidia g^{-1} soil (Figure 3.1). For *F. avenaceum,* inoculum concentrations were lower for wheat straw inoculum than conidia, but the highest inoculum concentration was 10, 000 cfu g^{-1} soil because it was the maximum levels that could be achieved by growing mycelia on wheat straw. However, the *F. solani* f. sp. *pisi* concentration on wheat straw was much higher than *F. avenaceum*, and the highest concentration was 200, 000 cfu g^{-1} soil.

The slope of the regression line for the *F. avenaceum* colonized wheat straw treatment was significantly higher than conidia soil drench treatment (P = 0.01), and there were significant differences between intercepts (P = 0.02) (Figure 3.1. A). For *F. solani* f. sp. *pisi*, the slopes of the regression lines for the two inoculation treatments did not differ (P = 0.6), but the intercepts did (P = 0.001) (Figure 3.1.B). Pairwise comparisons between the disease levels at equivalent inoculum doses for each treatment method and disease severity indicated no significant difference for *F. avenaceum* or *F. solani* f. sp. *pisi* (Table 3.1). 3.3.2 Relationship between DNA quantity, primary inoculum level and disease severity *F. avenaceum* and *F. solani* f. sp. *pisi*

The Ct value obtained from qPCR analysis was used for estimation of pathogen DNA quantity associated with each inoculum dose based on comparison to standard curves. There was a association between primary inoculum dose and recovered DNA from soil and stubble for *F. avenaceum* (Figure 3.2, A). The association between primary inoculum dose and recovered DNA

was stronger in colonized wheat straw treatment (R^2= 0.90, P = 0.001) compared with the conidia treatment (R^2= 0.77, P = 0.006) (Figure 3.2, A). The range of DNA quantity recovered from wheat straw inoculation was between 1.8-6.6 ng g^{-1}, while DNA recovered from conidia soil drench ranged between 0.05-6 ng g^{-1} soil (Figure 3.2, A). For *F. solani* f. sp. *pisi* there was linear association between primary inoculum dose and recovered DNA for conidia treatments (R^2= 0.86, P = 0.001) but for wheat straw treatments, recovered DNA quantity was not significantly associated with inoculum concentration dose (R^2= 0.7, P= 0.1) (Figure 3.2, B). Recovered DNA from wheat straw inoculation was between 3.5-9.5 ng g^{-1} soil and DNA quantity obtained from soil inoculated with conidia ranged between 0.8- 7 ng g^{-1} soil (Figure 3.2, B). Pairwise comparison between equivalent inoculum dose of each treatment method and quantity of recovered DNA of *F. avenaceum* was significantly (P <0.01) higher in wheat straw compared with soil, while for *F. solani* f. sp. *pisi* there was no significant difference (Table 3.1). ANCOVA analysis indicated that slope was not significantly different for wheat straw inoculum and conidia inoculation for both *F. avenaceum* (P = 0. 3) and *F. solani* f. sp. *pisi* (P = 0.9) (Figure 3.2). Intercepts for inoculum type were statistically different for *F. avenaceum* (P = 0.00001) and for *F. solani* f. sp. *pisi* (P = 0.0001) (Figure 3.2).

The relationship between disease severity and DNA quantity was then inferred from each inoculum density treatment. For *F. avenaceum,* an association between DNA quantity and disease severity was stronger in the colonized wheat treatment (R^2= 0.88, P = 0.006) compared with conidia treatment (R^2= 0.6, P = 0.004) (Figure 3.3, A). For *F. solani* f. sp. *pisi*, there was a positive relationship between disease severity and DNA quantity for conidia treatment (R^2= 0.85,

P = 0.001), but no significant relationship between DNA quantity from colonized wheat straw and disease severity (R^2= 0.8, P = 0.1) (Figure 3.3, B).

The DNA extraction efficiency from soil samples was examined by adding a known amount of *T. thynnus* NADH dehydrogenase gene to the soil alongside the inoculum. The average percent recovery was determined to be 26.7%, with a range between 7-48% for *F. avenaceum* and 24.5% for *F. solani* f. sp. *pisi*, with a range between 10-56%.

3.4 Discussion

An essential step toward developing a prediction model, based on estimation of pathogen inoculum using molecular assays, is correlating pathogen DNA quantity with disease severity (Ophel-Keller et al., 2008). In the current study, standard curves relating the root rot severity caused by *Fusarium* spp. to DNA levels in the soil were created using conidia or mycelia as inoculum sources. Previous studies on Fusarium root rot and wilts of chickpea (Bhatti et al., 1992), lentil (Erskine et al., 1996) and pea (Rush & Kraft, 1986) showed that disease severity was dependent on inoculum density, which is in agreement with the results of present study. Plants treated with a higher dose of inoculum had higher disease severity and there was a linear correlation between inoculum dose and disease severity. This relationship can be used to develop a soil assay whereby DNA quantity of *F. avenaceum* and *F. solani* f. sp. *pisi* is measured using qPCR and use to predict the risk and severity level of root rot within a field. Predictive tests of this kind of have been reported for a number of pathogens such as *F. graminearum* (Poole et al., 2015), *A. cochlioides* (Almquist et al., 2016), and *Plasmodiophora brassicae* (Wallenhammar et

al., 2012). However, a similar approach did not show a positive association between DNA quantity and disease severity under field conditions for *F. oxysporum* f. sp. *spinaciae* (Okubara et al., 2013) or Fusarium wilt of Chinese water chestnut caused by *F. commune* (Zhu et al., 2016).

Two inoculation methods, conidia suspension or colonized wheat straw, were tested in this study. For both *F. avenaceum* and *F. solani* f. sp. *pisi,* there was no significant difference between disease severity for either inoculation technique at equal inoculum concentrations, although the rate of increase in disease severity was higher for colonized wheat straw inoculation. For *F. solani* f. sp. *pisi* the pattern was reversed and disease severity was generally greater for the conidia suspension inoculum, as disease symptoms were initiated at a lower inoculum dose than for colonized wheat straw inoculum. For *F. avenaceum,* comparison of the two regression lines indicated inoculation method (conidia and colonized wheat straw) had a significant effect on disease severity and there was a significant difference between both slope and intercept. Similarly for *F. solani* f. sp. *pisi* inoculation method had an effect on disease severity, which was interpreted as a significant difference in intercepts. These results indicated that inoculum dose has a significant and positive effect on root rot severity and the effect is not similar for conidia and colonized what straw. The relationship was linear for both inoculum methods, and the regression slope was positive for both conidia suspension and colonized what straw.

Inoculation method influenced the results of the qPCR analysis and the amounts of DNA recovered from soil. For *F. avenaceum,* more DNA was obtained from the colonized wheat straw

methods compared with the conidia suspension methods. It might be due to higher DNA extraction efficiency in the colonized wheat straw compared with conidia suspension, which was quite low. Moreover, it is possible that the effect of soil inhibitors were stronger for conidia suspension which were presumably bound to soil particles, rather than mycelia on wheat straw, which are suspended in soil. There may also have been issues with lysing conidial cell walls compared to colonized wheat straw. For *F. solani* f. sp. *pisi* mycelium inoculation, however there was no association found between DNA quantity and disease severity and also between recovered DNA and initial inoculum concentration. Comparing the regression lines indicated that inoculum type had a significant effect.

The range of concentrations of CFU in the colonized wheat straw inoculum was smaller than the range of conidia suspension concentrations, which may have confounded the analysis of the relationship. This variation might also be related to differences in *Fusarium* species biology. *Fusarium avenaceum* has a broad host range, is pathogenic on cereal crops and able to use crop residue as a source of nutrition for growth (Fernandez et al., 2008), however, *F. solani* f. sp. *pisi* is not adapted as a saprophyte as well as *F. avenaceum*. Plant debris is considered a key source of inoculum for *F. avenaceum* survival (Dill-Macky et al., 2000; Kohl et al., 2007), whereas *F. solani* primarily survives as chlamydospores (long-term resting spores) (Leslie et al., 2008). Chlamydospores germinate to produce a germ tube that is responsible for plant infection (Dahlberg et al., 1982; Sneh et al., 1984; Couteaudier et al., 1990; Ohara et al., 2004). The mechanism of colonization and penetration may be different among species (Parry et al., 1985). Previous studies evaluated the aggressiveness of chlamydospores and conidia of *Fusarium* spp.

on disease severity and indicated an equivalent dose of chlamydospore inoculum caused a higher disease rate (Couteaudier et al., 1990; De Cal et al., 1997).

The association between disease severity and inoculum dose is also associated with the aggressiveness of the pathogen (Vandemark et al., 2005). Correlation between DNA quantity and disease severity of five isolates of *A. euteiches* indicated that for three isolates there was a significant positive correlation, while for two isolates this association was not significant (Vandemark et al., 2005), due to variation in pathogen aggressiveness. The standard curve developed in the current study was based on two isolates, which were chosen as they were moderately aggressive (Chapter 2). There may be wide variation in aggressiveness of *Fusarium* spp. isolates (Feng et al., 2010; Chittem et al., 2015), and results from Chapter 2 did indicate that there are differences in aggressiveness of isolates of these species recovered from diseased pea roots. Therefore, it will be challenging to provide standard curves for *Fusarium* spp. to correlate disease severity to DNA quantity under field condition where mixtures of aggressive isolates and species occur. Further studies may be required to create standard curves for isolates or mixtures of isolates with different levels of aggressiveness.

Li et al. (2015) found that DNA extractions from soil samples consistently varied and had efficiencies ranging from 23.8 to 39.2%. While the percent recovery for our trials encompassed a wider range, the average agrees with the findings of Li et al. (2015). Improving DNA extraction efficiency by testing various methods and selecting proper technique might improve the results. Despite the low extraction efficiencies, there was still a linear relationship between DNA

quantity and inoculum dose and disease severity. However, the recovered DNA quantity was likely underestimated in all of the soil samples due to the low percent recovery.

Due to the limitation of management strategies for pea root rot, this information on inoculum dose disease relationship will be a valuable means for early detection of the pathogen and avoidance of high risk fields. One of the essential components for a predictive model is the assessment of the relationship between disease severity and yield loss (Cooke, 2006; Hogg et al., 2007). A previous study on pea root rot indicated that pea fields infested by *A. euteiches* showed negative correlation between disease severity and yield loss (Persson et al., 1997). The information generated in the current study, combined with research regarding the relation between disease severity and yield loss might help to develop model to predict disease severity and estimate yield loss. However, evaluation of disease severity and yield loss under field conditions is likely to be challenging due to the impact of several other factors, such as interaction with other pathogens and pests, environmental variables, and soil physicochemical and biological features. Nonetheless, the standard curves developed in this study are the first step towards developing a predictive model.

3.5 Tables

Table 3-1. Pairwise comparisons of disease severity and recovered DNA quantity of equal inoculum dose from two inoculation methods (colonized wheat straw and conidia)

Inoculum concentration	Log inoculum dose	Mean disease severity		*P* value	Mean DNA quantity (ln ng g^{-1} soil)		*P* value
		Wheat straw inoculum	Conidia		Wheat straw inoculum	Conidia	
F. avenaceum							
10000	4	5.2	4.0	0.6	6.4	1.6	0.0001*
5000	3.69	5.0	3.5	0.4	5.2	1.5	0.001*
1000	3	4.0	2.9	0.7	4.0	0.7	0.002*
500	2.69	3.3	2.7	0.9	2.6	0	0.01*
F. solani* f. sp. *pisi							
100,000	5	3.7	4.4	0.1	7.0	5.1	0.9
10,000	4.69	2.9	4.2	0.7	6.6	4.4	0.9
5000	3.69	2.6	3.5	0.9	6.4	1.6	0.7

[a] Standard error (SE) *F. avenaceum* 0.6, *F. solani* f. sp. *pisi* 0.5 for disease severity

[b] Standard error (SE) *F. avenaceum* 0.3, *F. solani* f. sp. *pisi* 2.7 for recovered DNA quantity

* = statistical difference at ($P < 0.05$) according to the Tukey HSD test.

3.6 Figures

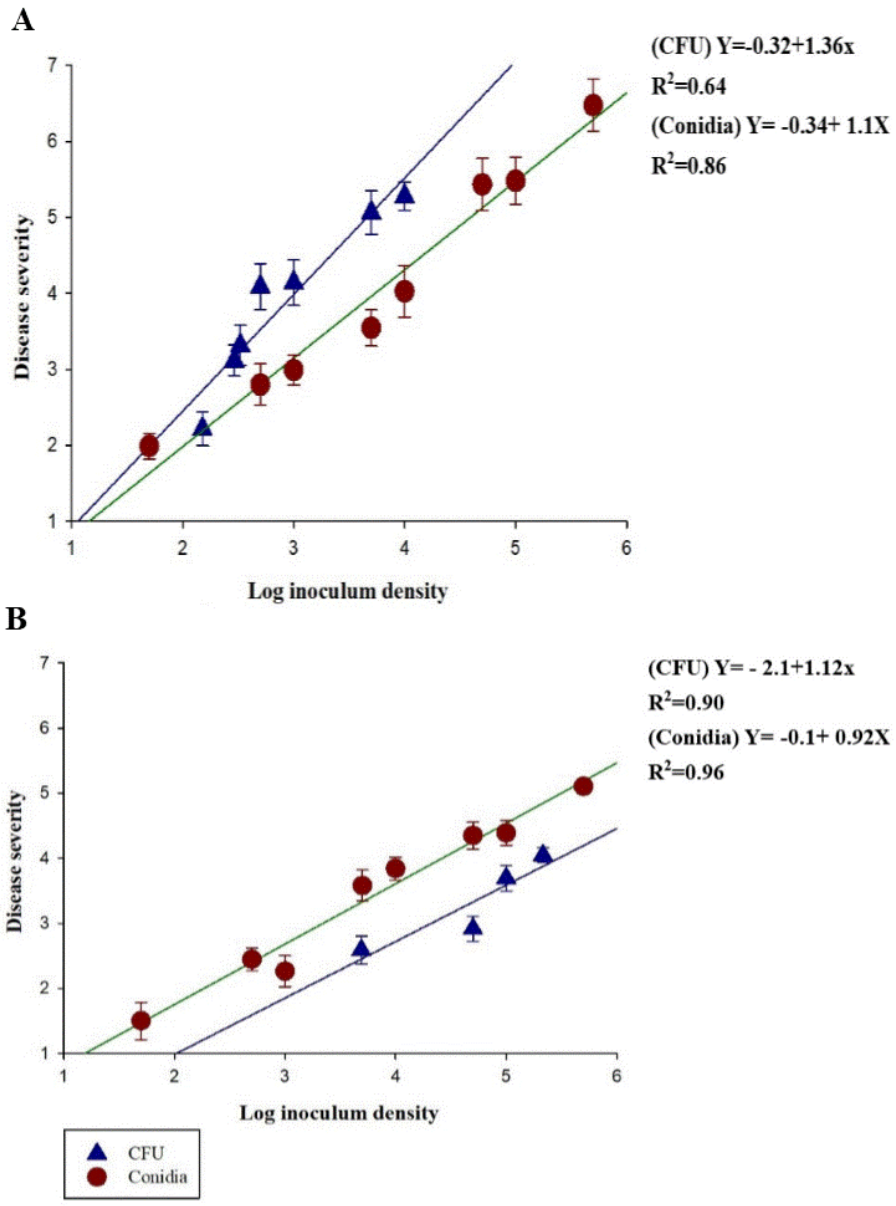

Figure 3-1. Association between disease severity and inoculum density of *F. avenaceum* (A) and *F. solani* f. sp. *pisi* (B) on pea cultivar CDC Meadow when inoculum was applied to soil as colonized wheat straw (colony forming units g^{-1} soil) or conidia suspension (conidia g^{-1} soil) at increasing concentration, under greenhouse condition.

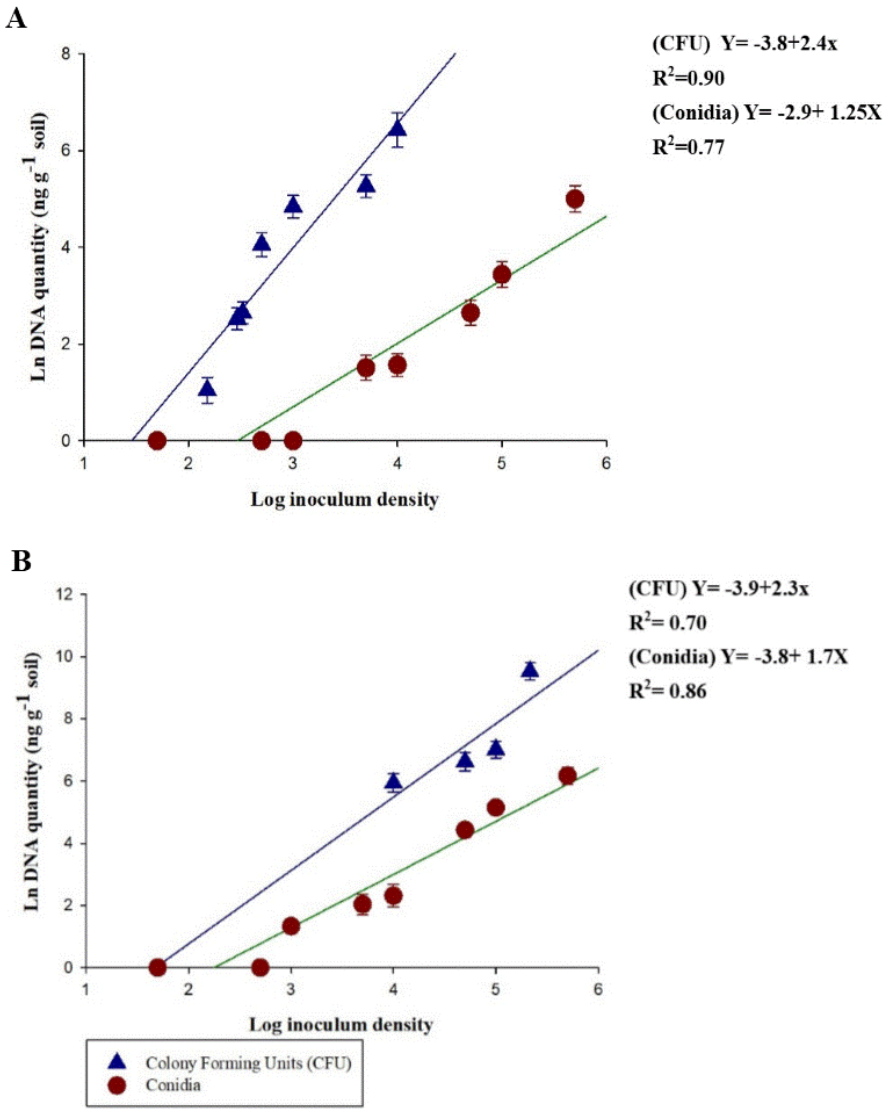

Figure 3-2. Association between DNA quantity and primary inoculum density of *F. avenaceum* (A) and *F. solani* f. sp. *pisi* (B) on pea cultivar CDC Meadow when inoculum was applied to soil as colonized wheat straw (colony forming units g^{-1} soil) or conidia suspension (conidia g^{-1} soil) at increasing concentration, under greenhouse condition.

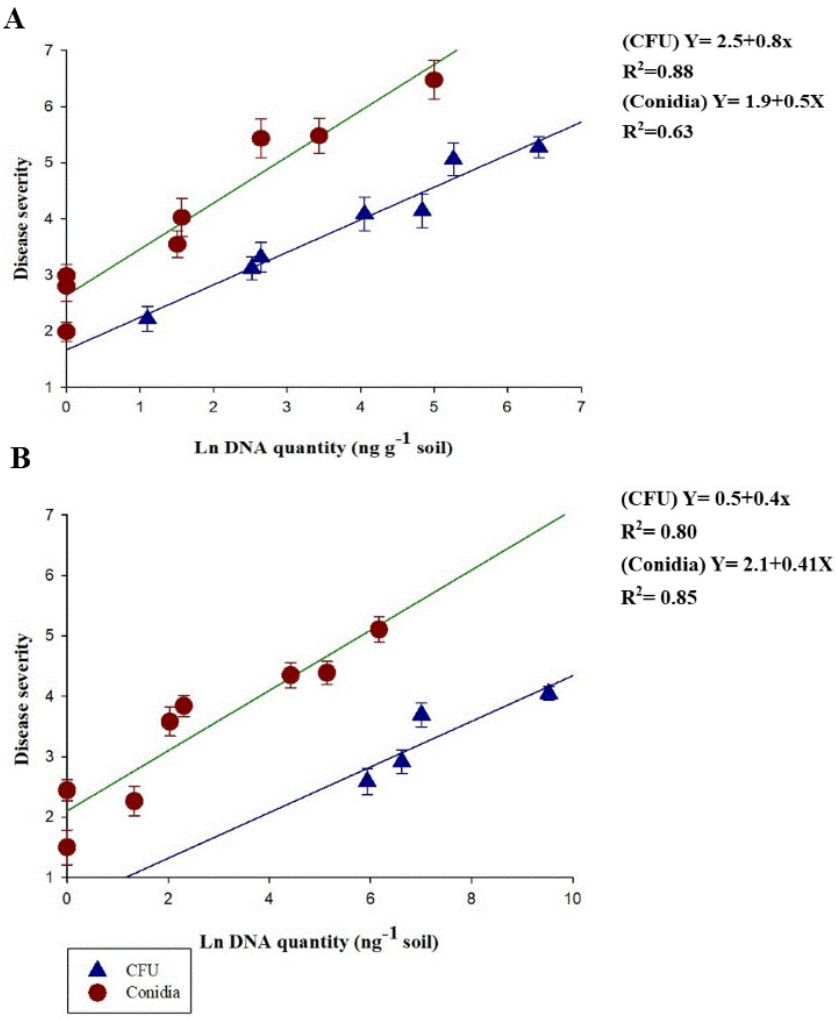

Figure 3-3. Association between disease severity and DNA quantity of *F. avenaceum* (A) and *F. solani* f. sp. *pisi* (B) on pea cultivar CDC Meadow when inoculum was applied to soil as colonized wheat straw (colony forming units g^{-1} soil) or conidia suspension (conidia g^{-1} soil) at increasing concentrations, under greenhouse condition.

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4 Determining the risk of pea root rot by greenhouse soil bioassay and qPCR analysis

4.1 Introduction

Root rot is a growing concern in the pea production areas of the Canadian Prairies. The main pathogens involved in pea root rot in Canada are *F. avenaceum*, *F. solani* f. sp. *pisi*, and *A. euteiches* (Feng et al., 2010 ; Chatterton et al., 2015; Esmaeili Taheri et al., 2016; Chatterton et al., 2018). *Pythium* spp. and *Rhizoctonia solani* are also involved in pea root rot, however they do not appear to be the predominant pathogens in Alberta (Hwang et al, 1989; Esmaeili Taheri et al., 2017). In addition to species and isolates, variation in root rot severity is related to climate, crop rotation sequence and agronomic practices (Slinkhard et al, 1994; Persson et al., 1997). Since there are no resistant cultivars or effective chemical controls for root rot diseases, avoidance of high-risk fields is the only practical management strategy. However, in order to utilize this strategy, an appropriate method is essential for determining root rot risk prior to planting pea. Estimation of inoculum potential is a common method of disease prediction for soil borne pathogens (Ophel-Keller et al., 2008). Precise assessment of inoculum levels in the soil and their relation to disease severity could help growers make decisions about planting pea in a specific field.

Identification and quantification of initial inoculum can be used for disease prediction, since pathogens associated with root rot are monocyclic and the only source of pathogen inoculum comes from resting or overwintering structures in the soil or crop residue (López-Escudero et al., 2007). There are multiple techniques for estimation of plant pathogen inoculum

in soil samples. Traditional methods like plating dilution series of soil on selective medium were a common method in past decades (Menzies, 1963; Elad et al., 1981). These methods do not provide accurate results for some pathogens like *A. euteiches* due to the low rate of germination of oospore on growth medium (Malvick et al., 1994). Another approach is soil indexing or a greenhouse bioassay where a susceptible cultivar is grown in soil samples collected from fields and monitored for disease severity. It is essential to identify both the potential and limitations of a greenhouse bioassay. This method is time consuming and labour intensive, while the main advantage of this method is that soil conditions as a key factor in disease severity are taken into account (Bruggenet al., 1996; Gatch, 2013). There are different types of bioassays such as the rolled towel (RT) which estimate inoculum density of *A. euteiches* by inoculating pea seeds with organic debris collected from infested fields (Krfat et al., 1990), and the most probable number (MPN) assay, that can used to estimate of pathogen inoculum in field soil samples (Malvick et al., 1994). Greenhouse bioassays are currently used in the United States for predicting spinach Fusarium wilt (Gatch et al., 2015) and root rot of sugar beet (Harveson et al., 2014), and were studied a number of decades ago as a means of predicting Aphanomyces root rot of pea and Fusarium root rot of pea (Oyarzun et al., 1994; Malvick et al., 1994; Reiling et al., 1998).

Disease prediction models based on estimation of DNA quantity are more rapid than greenhouse bioassays. Quantitative PCR (qPCR) analysis is a sensitive and precise method that can differentiate between closely related species (Yli-Mattila et al., 2008; Nicolaisen et al., 2009). This method has been used for quantification and detection of *Fusarium* species in cereal root, stem and crop residues (Hogg et al., 2007; Strausbaugh et al., 2005), *F. oxysporum* in soil

(Zhenggang et al., 2005; Lin et al., 2013) and *Fusarium* spp. in pea and soybean roots (Gao et al., 2004; Zitnick et al., 2018). Real time PCR has been used to quantify pathogen inoculum in soil or crop residue for predicting disease risk for a number of soil borne pathogens such as *Fusarium* spp. and *Rhizoctonia solani* associated with cereal root and crown rot (Poole et al., 2015), wheat take-all (Herdina et al., 2000) and Aphanomyces root rot of sugar beet (Almquist et al., 2016). However, the utility of using qPCR to determine incidence and levels of soil borne pathogens associated with root rot of pea, and how this relates to observed root rot severity in the field, has not been studied.

There is also limited information on the association between aboveground disease symptoms and root rot severity. Schwartz (2001) reported it is difficult to see foliar symptoms in dry bean plants infected by root rot pathogens even when the roots show a high level of disease severity. Wang et al. (2019) reported no significant correlation between Fusarium root rot severity in soybean plants and foliar symptoms, although pathogens were detected by qPCR in plants with and without foliar disease symptoms.

Although a diversity of *Fusarium* spp. were isolated from roots (Esmaeili Taheri et al., 2017), pathogenicity testing of this diverse group revealed that *F. avenaceum* and *F. solani* f. sp. *pisi* were the most aggressive species on pea (Chapter 2). These two species and *A. euteiches* were therefore chosen for further study with the hypothesis that they would be key drivers of pea root rot development. With the goal of developing the initial steps towards a pea root rot prediction system, the objectives of the present study were to: 1) evaluate a greenhouse soil

bioassay as a tool for predicting pea root rot; 2) develop a DNA-based assay for predicting pea root rot by comparing pathogen detection in roots versus soil samples and testing the use of qPCR to quantify pathogen DNA in soil and crop residues; and 3) compare disease severity, pathogen detection between foliar symptomatic and asymptomatic sites.

4.2 Material and methods

4.2.1 Sample collection and field survey

Samples were collected during 2013-2015 from 11 commercial pea fields located in different pea growing areas in Alberta. Overall 110 soil, crop residue, and root samples were collected from 10 sites, 5 sites in each field where root rot symptoms were obvious in the shoots (yellowing, stunting or dead) and 5 sites from asymptomatic sites (shoots appeared healthy) during the flowering stage in late June and early July. The sites were not chosen randomly in order to target an equal number of symptomatic and asymptomatic sites. At each site, five root samples and one bulk soil sample (1 L per site) to a depth of 20 cm, and a large Ziploc freezer bag of the previous crop residue were collected all at the same time. Root samples were returned to the laboratory, washed under running tap water for 10 min and rated on a 1-7 scale (where 1 is healthy, 7 is death) (Schneider & Kelly, 2000). Diseased roots with a rating of 4 - 6 were selected for DNA extraction and PCR analysis to determine pathogen composition in the roots as described by Chatterton et al. (2018). Soil samples were air dried for 7 days at room temperature, sieved through 2 mm sieves and then kept in cold storage at 4 ºC (Esmaeili Taheri et al., 2017) prior to use in the greenhouse bioassay and DNA extraction. Crop residues samples were

collected at the same time that soil samples were collected in 2013 and 2014. Crop residue samples were air dried for 7 days at room temperature (25 ºC), ground in a Wiley Mill and then a bead beater (TissueLyser II, Qiagen), prior to DNA extraction.

4.2.2 Greenhouse soil bioassay

A greenhouse bioassay experiment was conducted with soil samples collected from commercial pea fields in different regions of Alberta. Field soils were mixed with potting soil (mixture of sand, Cornell soil and peat moss 1:1:1 (vol/vol/vol)) (Boodley et al., 1972) at 100%, 50%, and 10% field. Autoclaved field and Cornell soil served as the controls, but were not included in the analysis. Seeds of pea cultivar CDC Meadow surface-sterilized for three minutes in 10% bleach with a drop of Tween 20 solution, and washed three times with sterile distilled water. Five surface-sterilized seeds were planted in each pot (0.5 L) containing 250 g of the field: potting soil mix. Plants were grown for four weeks in a greenhouse with a 16 h light day^{-1} and 18 °-21 °C, and watered manually twice per week. The roots were assessed visually for root rot severity using the 1-7 rating scale (Schneider & Kelly, 2000). The experimental design was a randomized complete block design (RCBD) with four replications for each treatment (field soil dilution level: 100%, 50% , 10%) and performed twice per soil samples collected each year. Soils were stored for approximately 4– 6 weeks between trial 1 and 2 each year.

4.2.3 Quantification of *F. avenaceum* and *F. solani* f. sp. *pisi* in agricultural soils and crop residues

Soil samples were used for quantification of *F. avenaceum*, *F. solani* f. sp. *pisi,* and *A. euteiches*. Total genomic DNA was extracted from 250 mg of soil using the PowerLyzer PowerSoil DNA Isolation kit (MoBio, Carlsbad, California, USA) according to the manufacturer's instructions with some modifications (Esmaeili Taheri et al., 2017). Genomic DNA was extracted from 40 mg crop residues using the Biosprint-96 DNA plant kit (Qiagen, Toronto, ON, CA) in a Biosprint instrument (Qiagen) according to the manufacturer's procedure. Extracted DNA was stored at -20 ºC prior to DNA analysis. DNA concentration was measured in a NanoDrop (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd., UK) and diluted to 10 ng μL^{-1} prior to DNA analysis. Specific primers and probes were designed based on the partial translation elongation factor alpha 1 (TEF-1 α) gene sequence from NCBI to quantify *F. avenaceum* (Nicolaisen et al., 2009) For detection of *F. solani* f. sp. *pisi* primers and probe were also based on the TEF-1 gene (Zitnick-Anderson et al., 2018). *Aphanomyces euteiches* primer and probes were based on the internal transcribed spacer (ITS) region of the rRNA for specific amplification of *A. euteiches* (Willsey et al., 2018).

To prepare standards, DNA was extracted from 7 - 10 day old cultures of *F. avenaceum* and *F. solani* f. sp. *pisi* growing on PDA using the Plant DNeasy kit (Qiagen) according to the manufacturer's directions. The DNA was quantified using NanoDrop (NanoVue Plus Spectrophotometer) and serially diluted to produce a six fold dilution standard curve ranging

from 10 ng μL^{-1} to 100 fg μL^{-1} *Fusarium* genomic DNA. For *A. euteiches,* the standard curve was based on gene copy number in six dilutions ranging from 2×10^9 to 2×10^4 gene copy μL^{-1}.

For *Fusarium* spp., reaction mixtures were made to a final volume of 25 µL containing: 2.5 µL template DNA, 12.5 µL, Environmental Master Mix 2.0 (Thermo Fisher Scientific), 2.5 µL of each primers (0.9 µM) and 2.5 µL of probes (0.25 µM) and 2.5 µL ultrapure H_2O. For *A. euteiches* reactions volume was 20 µL containing*:* 10 µL PrimeTime Gene Expression 2X Master Mix (IDT, Skokie, IL, USA), 0.2 µL of each primers (0. 5 µM) and 0.5 µL of probe (0.25 µM) , 2 µL of template DNA and 6.65 µL ultrapure H_2O, in 96-well fast plates real-time quantitative. Assays were run and analyzed on a QuantStudio 6 Flex Real-Time PCR System (Thermofisher) with the following cycling conditions: for *Fusarium* spp. was: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min, for *A. euteiches*, 95 °C 3 min, and 40 cycles of 95 °C 15 s, 60 °C, 1 min

4.2.4 Data Analysis

For analysis of the bioassay experiment, the effect of treatment (soil dilution), trial, and site (symptomatic and asymptomatic) on disease severity and their interaction were analyzed. The year and trial repeats did not meet homogeneity of variance assumption, using Levene's test for equal variance, therefore each trial was analysed separately. In addition, regression analysis was performed to determine if the greenhouse bioassay was an accurate predictor of disease levels in the field.

Root and soil samples collected from each site were tested by conventional PCR (Esmaeili Taheri et al., 2017), and qPCR analysis respectively, and the effects of year, and site (symptomatic and asymptomatic) was examined. Disease severity was assessed visually and disease incidence was calculated as the percent of sites that had root rot or tested positive for a specific root rot pathogen using conventional PCR (root samples) (Taheri et al 2017) and qPCR (soil samples). In order to compare frequency of pathogens detected in root and soil samples by PCR and qPCR in different years, an ANOVA analysis was conducted to compare the amount of each pathogen detected in different years and also to compare the amount of each pathogen detected in soil and crop residue in each year. Frequency values for incidence of pathogens was treated as continuous variables as described by McRoberts et al. (2003).

DNA quantity in soil and crop residue was determined by calculating the concentration from the cycle threshold (Ct) value using the regression equation calculated from the standard curve. All DNA quantities were converted to ln (x+1) for analysis and reporting. Only samples where DNA was present were used for calculating the mean DNA quantity.

Statistical analysis was performed with JMP 13.0 (SAS Inc.) using the Fit Model platform and least square means analysis and R version 3.4.1 (https://www.r-project.org). Means were separated using the Tukey-Kramer test. Significance for all statistical tests was defined as ($P < 0.05$).

4.3 Results

4.3.1 Greenhouse bioassays

Greenhouse bioassays were performed to determine their predictive value of root rot observed in the field and also to determine how much inoculum densities need to reduce risk of disease to zero risk. Generally, control plants grown in autoclaved soil were asymptomatic, all the plants in diluted soil, regardless of field soil percent, showed disease symptoms. Each trial was analyzed separately because variances were not homogeneous between repeated trials, and there was a significant effect of trial and year. For each year*trial ANOVA, the effect of treatment (field soil dilution level), but not site (foliar asymptomatic or symptomatic) or their interaction, on root rot severity was significant. Therefore, data from all sites were combined for analysis. Comparing disease severity under field and greenhouse condition showed that in 2014 the mean of field disease severity was 4.4, which was lower than mean of greenhouse bioassay disease severity at 100% in first trial (DS = 5.7). In addition, the greenhouse disease severity was reduced in the second trial (DS= 3.9). In 2015, the disease severity for 100% treatment (DS=3.6, DS=2.6) in both trials was lower than the mean of field disease severity, which were 4.1. In both years that the greenhouse bioassay was conducted, the disease severity was reduced in the second trial compared with the first trial. Regression analysis indicated there was significant association for trial 2 between greenhouse and field disease severity in 2014 (R^2= 0.42, P= 0.04) (Figure. 4.1 A), whereas in 2015, there was significant association for trial 1 (R^2= 0.55, P= 0.03)

(Figure.4.1 B). However for trial 1 in 2014 (R^2= 0.02 0, P= 0.7) and the second trial in 2015 (R^2= 0.3, P= 0.2), the associations were not significant (Figure. 4.1).

Disease symptoms were significantly higher in 100% field soil than in lower field soil ratios (50% and 10%) (Figures. 4.2 and 4.3). Plants showed the lowest level of disease severity at 10% inoculum levels (Figures. 4.2 and 4.3). Disease severity was significantly higher for the 100% treatment compared with 50% and 10% treatments except for the second trial in 2015 (Figure. 4.2 B), which did not show significant difference between 100% treatment and 50% treatment.

4.3.2 Field surveys and pathogen detection from root and soil samples

For all three tested pathogens, there were no significant differences between pathogens detection in samples collected from symptomatic and asymptomatic sites by PCR and qPCR (data not shown), therefore this factor was excluded from analysis. ANOVA analysis indicated that for *F. avenaceum* there was no significant effect of sample type (roots or soil) on frequency of pathogen detection, however there was an s effect of year (Table 4.1). The highest detection of *F. avenaceum* in roots, tested with PCR, was in 2014 (80%), followed by 2013 (58%) and 2015 (55%), which were not statistically different in any year (Table 4.2). Results of detection of *F. avenaceum* in soil samples by qPCR over the three years indicated that the pathogen was present in soil in 58% of samples in 2013, 78% in 2014 and 35% in 2015, which were not significantly different, except for 2014, which was significantly detected in higher frequency compared with 2015 (Table 4.2).

There were differences among years for frequency of detection of *F. solani* f. sp. *pisi,* moreover the interaction of type of samples (root/soil) and year was significant (Table 4.1). Results from root PCR showed that *F. solani* was detected in 33% of samples examined in 2013, 100% in 2014 and 85% in 2015, and frequency of detection in root samples was significantly higher in 2014 (P= 0.01) and 2015 (P= 0.04), compared with 2013. (Table 4.2). Soil sample analysis by qPCR indicated that *F. solani* f. sp. *pisi* was detected in 66% of tested samples in 2013, 56% in 2014, and 65% in 2015, which were not significantly different during all three years (Table 4.2).

Samples collected in 2013 were not tested for *A. euteiches*, because it was unknown at that time that it was present in Alberta. There was a difference between frequency of detection of *A. euteiches* by PCR in root and qPCR in soil (Table 4.1) and also a significant interaction between year and PCR (Table 4.1). Among samples tested for *A. euteiches,* 41% of roots (Table 4.2) were positive in 2014 and 91% in soil, (Table 4.2) while in 2015, 22% of roots (Table 4.2) and 97% of soil were positive (Table 4.2). Aphanomyces was detected with higher frequency in root samples in 2014 compared with 2015 (Table 4.2). Frequency of detection of *A. euteiches* in soil samples was higher than root samples in 2014 (P= 0.00001) and 2015 (P= 0.00001).

4.3.3 *Fusarium* spp. and *A. euteiches* quantification in soil and crop residues samples by qPCR analysis

The qPCR assay was also used to determine the DNA quantity of *F. avenaceum* and *F. solani* f. sp. *pisi* in the soil and crop residue samples collected from fields. A standard curve

relating DNA quantity to Ct value was generated and the linear relationship was used to calculate DNA quantity in field samples from measured Ct values (Figure. 4.4). The limit of detection was 100 fg for both *F. avenaceum* and *F. solani* f. sp. *pisi*. For *F. solani* f. sp. *pisi* qPCR efficiency was 97.8% and the coefficient of determination (R^2) was 0.99. A Ct value > 35 was considered negative (Figure. 4.4. A). For *F. avenaceum,* qPCR efficiency was 98.4%, coefficient of determination (R^2) was 0.99, and a Ct value > 36 was considered negative (Figure. 4.4. B). For *A. euteiches* qPCR efficiency was 98.8% and coefficient of determination (R^2) was 0.99, a Ct value > 40 was considered negative (Figure. 4.5).

Among samples positive for *F. avenaceum*, the average DNA quantity was 3.3 ng g^{-1} soil in 2013, 1.1 ng g^{-1} soil in 2014 and 2.5 ng g soil in 2015, amount of this pathogen the detected, in soil samples was higher in 2013 compared with 2014, 2014 was higher than 2015, while 2013 and 2015 was not different (Table 4.3).

The average amount of *F. avenaceum* quantified in crop residue was 9 ng g^{-1} in 2013, and 7.9 ng g^{-1} in 2014 with no difference between years (Table 4.4). Over the two years of survey and examining by qPCR, a significantly higher (P=0.0007) amount of *F. avenaceum* was detected in crop residues compared with soil samples (data not shown).

The quantity of *F. solani* f. sp. *pisi* DNA was of 2.9 ng g^{-1} soil in 2013, 1.9 ng g^{-1} soil in 2014, and 2.8 ng g^{-1} soil in 2015 (Table 4.3). There were no difference among the amount of pathogen detected in soil samples over the three years. (Table 4.3). *Fusarium solani* f. sp. *pisi* was not detected in 49 of the 62 crop residue samples. Mean detection in positive samples in

2013 was 5.8 ng g^{-1} crop residue and 4.2 ng g^{-1} crop residue in 2014 (Table 4.4). There was no difference between detected amount in 2013 and 2014 (P= 0.1) (Table 4.4), and no difference between the amount of pathogen detected in soil and crop residue samples (P= 0.2).

The average quantity of *A. euteiches* in 2014 (5.6 gene copies g^{-1} soil) was higher than in 2015 (7 gene copies g^{-1} soil) (Table 4.3). The highest detected amount was 11.5 gene copies g^{-1} soil in 2015, and the lowest was 2.8 gene copies g^{-1} soil in 2014. The amount of *A. euteiches* DNA quantified was higher (P= 0.04) in 2015 compared with 2014 (Table 4.3).

4.4 Discussion

Root rot is a major concern for pea production in Canada since it causes severe damage and yield reduction. Currently there is no practical approach to control root rot, and predicting the risk of root rot in the field prior to planting and field avoidance may be the only effective management strategy. In the present study, the use of a greenhouse bioassay and DNA analysis of soil and residue samples was evaluated for their potential to predict root rot. Greenhouse bioassays with pre-plant soils have been used previously to estimate of pathogen population to identify fields with high risk of bean root rot caused by *Fusarium solani* f. sp**.** *phaseoli***,** Aphanomyces root rot of sugar beet and pea (McFadden et al., 1989; Oyarzun, 1993; Malvick et al., 1994), and Fusarium wilt of spinach (Gatch et al., 2015). This method has shown great value as a disease predictor, when there is a high correlation between disease severity under field and greenhouse conditions. However, these results indicated that the greenhouse bioassay is not a robust predictor of root rot risk in Alberta, as there was only a weak correlation between field

and greenhouse severity. In the greenhouse bioassay, the effect of inoculum level on disease severity was also investigated. Inoculum levels were decreased by decreasing the proportion of field soil with natural inoculum using Cornell mix. The highest disease severity was observed in plants grown in 100% field soil, however, there were only small differences between doses of inoculum. Results showed that the disease-causing potential of field soil did not decline steadily when the proportion of field soil decreased, and in some cases field disease severity remained high despite a reduction to 10% field soil**.** The rate of inoculum decay under varying environmental conditions for *F. avenaceum* and *F. solani* has not been studied.

Using DNA analysis to estimate pathogen inoculum is currently used as a routine commercial method for screening plant pathogen inoculum in soil samples prior to planting to identify high-risk fields in Australia and Sweden for diverse plant diseases such as cereal, canola and pulse diseases (Ophel-Keller et al., 2008; Poole et al., 2015; Wallenhammar et al., 2016).

qPCR analysis was used to detect and quantify pathogen DNA in soil and crop residue, and the frequency of pathogen detection compared to corresponding root samples, which were tested by PCR analysis. qPCR analysis can provide a quantitative or qualitative assessment of pathogen biomass and has been used to quantify the amount of *Fusarium* spp. in soil and crop residue samples (Hogg et al., 2010). For example, Hogg et al. (2009) found that the populations of *F. culmorum*, *F. graminearum* and *F. pseudograminearum* were stable in spring wheat residues for up to 14 months after harvest. Conventional PCR has been used to detect pathogens

in plant samples although it is not an indicator of pathogen biomass (Henson et al., 1993; McCartney et al., 2003).

Results from this study indicated that both *F. avenaceum* and *F. solani* f. sp. *pisi* were widespread in Alberta. They were isolated from root and soil samples in the majority of fields over the three years of data collection. These results are also supported by previous studies conducted in Alberta, which reported these species as major pathogens (Hwang et al., 1994; Feng et al., 2010; Esmaeili Taheri et al., 2017). Comparing PCR results from roots and qPCR from soil samples indicated that for *F. avenaceum,* there was no significant difference between detection in root and soil samples, which shows that both PCR and qPCR are capable of pathogen detection, but biomass (quantification) levels estimated by qPCR were low. For *F. solani* f. sp. *pisi,* PCR detection in roots had a higher accuracy than qPCR detection of soils. In contrast, *A. euteiches* was detected in higher frequency in soil and qPCR analysis than in root samples. These differences between species are likely due to the pathogen's distribution patterns in soil samples, DNA extraction efficiency and recovery rates from soil samples, which has a wide range of variability for different soil types. These results suggested that the combination of both methods will be the most e accurate to monitor the presence of the pathogens in soil and root samples.

Although the three pathogens were frequently detected in soil samples using qPCR analysis, estimation of their biomass in soil was not correlated with field disease severity (data not shown). This result is in agreement with Okubara et al. (2013) who investigated Fusarium

wilt of spinach and found no correlation between DNA quantity of the pathogen and disease severity under field conditions. For Fusarium wilt of chestnut there was no correlation between the amount of pathogen DNA in the soil and disease severity. However, they found a positive correlation when they examined DNA quantity in stem tissues of the infected plants (Zhu et al., 2016). One of the reasons for this lack of correlation might be due to diverse soil types across Alberta and sampling from regions with a high amount of clay, which negatively affects the DNA extraction efficiency from the soil **(**Högfors-Rönnholm et al., 2018**).** DNA obtained from environmental samples like soils are challenging for molecular analysis since soil can contain PCR inhibitors like humic acid (Jizhong et al., 1996). Negative results obtained from qPCR analysis in the present study may therefore have been associated with low extraction efficiency and recovery of the pathogen DNA from soil (Frostegård et al., 1999; Krsek et al., 1999). Depending on soil type, the detection limit of qPCR could also change, as organic matter and clay content are key factors that affect the limit of detection (Almquist et al., 2016). Further research on improving DNA extraction efficiency and removing inhibitors by using new procedures is warranted (Lim et al., 2016). In addition, using an internal control prior to DNA extraction to normalise the results of extraction efficiency (Li et al., 2015) may improve the possibility of detecting correlation between disease severity and the amount of DNA.

There was an effect of year for *F. avenaceum* and *F. solani* f. sp. *pisi*, which is likely due to changes in environmental conditions, and there was a significant interaction between year and PCR for *F. solani* f. sp. *pisi*, and *A. euteiches*. Root rot development is associated with the interactions of several variables such as pathogen inoculum, soil structure, topographical factors

and environmental condition (Esmaeili Taheri et al., 2017). Therefore, the predominant pathogen might change each year depending on more favourable environmental conditions. Generally a higher quantity of *F. avenaceum* DNA was detected over the three years of data collection, but the amount of *F. solani* f. sp. *pisi*, DNA was higher in 2015. Taheri et al. (2017) reported a significant relationship between disease severity and frequency of isolation of *F. solani* f. sp. *pisi*, from pea roots in 2015, but this correlation was not observed in 2014. The increased levels of *F. solani* f. sp. *pisi*, in 2015 was likely due to the warmer and drier growing season, as these conditions are more favourable for *F. solani* f. sp. *pisi*, than *F. avenaceum* (Esmaeili Taheri et al., 2017). *Fusarium* spp. populations in soil has variation within and between season, and this variability is related to weather conditions (Bateman & Murray, 2001). For example, populations of *F. culmorum* fluctuated with changes in soil moisture and the fungal population was enhanced after rainfall, but the association between soil moisture and *F. solani* f. sp. *pisi*, population in soil was not stable (Bateman & Murray, 2001).

One source of inoculum of *Fusarium* spp. is crop residues and DNA analysis identified a high amount of *F. avenaceum* in crop residues. *Fusarium avenaceum* was the predominant species isolated from oat and wheat residues in a study in Norwegian cereal cropping systems (Hofgaard et al., 2016). Unlike most other *Fusarium* spp., *F. avenaceum* does not produce chlamydospores or other resting structures and therefore survives primarily on crop debris (Leslie et al., 2008). The current study also confirmed a high quantity of *F. avenaceum* on previous crop residue, which was mostly cereal residue. However, despite high levels of *F. avenaceum* in some crop residue samples, there was no strong correlation between the amount of

F. avenaceum DNA in crop residues and disease severity. *Fusarium avenaceum* is commonly isolated from cereal crowns, roots (Backhouse et al., 2004), heads (Kang et al., 2005), and can be a component of the Fusarium head blight complex (Bottalico et al., 2002; Nicholson et al., 2003). Therefore, cereal plant debris appears to be an important source of *F. avenaceum* inoculum and survival. However, *F. avenaceum* isolated from pea roots had little to no pathogenicity on cereal seedlings (Chapter 2). More research is required to determine the ecology of *F. avenaceum* on crop residue and determinants of its saprophytic versus pathogenic capabilities on diverse hosts. In contrast to *F. avenaceum*, *F. solani* f. sp. *pisi*, was rarely detected on cereal crop residues. Relatively few studies have examined the survival of *F. solani* f. sp. *pisi*, in crop residue but this result is in agreement with a previous study that this pathogen survives in soil but not crop residue (Nash, 1961).

Sites were chosen for sampling based on visual observation of shoot health (green, vigorous plants compared to yellow, stunted plants). However, there was no statistical difference in root rot severity between the field and greenhouse ratings or pathogen detection between symptomatic and asymptomatic sites. This indicates that above-ground disease symptoms may not be a good indicator of below-ground disease symptoms. In many cases, root rot was severe even when there were no visible disease symptoms on the shoots. The fact that the inoculum potential, as measured by the greenhouse bioassay, was similar between symptomatic and asymptomatic sites would indicate that there are site-specific factors that influence shoot symptom expression in the field. Disease development under field conditions is influenced by the interaction of several components that cannot be replicated easily under controlled

experimental conditions in the greenhouse. These variables may enhance or decrease the disease severity in the greenhouse compared with field conditions. Fluctuations in disease suppression over short distances in the field were attributed to changes in biological and physical properties of the soil (Rekah et al., 2001). The effect of soil compaction, temperature, and moisture on pea root rot caused by *Fusarium* spp. was investigated under controlled conditions, and results from this study indicated that by increasing the soil compaction and temperature disease severity increased (Tu, 1994). However, in the present study these factors were not included in the experiments.

In our study, the soil was homogenized and ground before use and the difference in disease severity between field and greenhouse might be related to the important role of soil structure in disease development. Disease levels were always lower in the second trial compared to the first. Changes in pathogen composition and soil structure during soil storage for four weeks at 4^{o} C may account for this variation. Soils were also air-dried prior to testing to ensure that all soils started with the same moisture levels. However, the procedure of drying could affect soil microbial activity through enhancing C and N mineralization (Mikha et al., 2005). Furthermore some bacterial species may die during the drying process due to sensitivity to osmotic stress (Van Gestel et al., 1993; Martí et al., 2012). Therefore, differences in disease severity might be related to soil processing.

qPCR analysis was a precise method for pathogen detection in soil, although it did not provide a strong association between the detected amount of DNA with disease severity,

however, it was a good predictor of pathogen incidence. It is probable that by improving current DNA extraction methods or analysis, the correlation with pathogen DNA quantity may be improved. Moreover, advanced molecular technique like droplet digital PCR, which is more sensitive and not affected by PCR inhibitor (Dingle et al., 2013) , might improve quantification. When molecular methods and traditional techniques have been applied together, these provide better results (Capote et al., 2012). Findings from this study suggested that inoculum density was an important factor in disease severity but that use of qPCR does not accurately predict root rot risk. It may be essential to consider other factors like environmental variables and soil properties to improve the prediction outcome.

4.5 Tables

Table 4-1. ANOVA results for frequency detection of *F. avenaceum*, *F. solani* f. sp *pisi* and *A. euteiches* in root and soil samples by PCR and qPCR (2013-2015)

Effect test	**F Value**	**Pr > F**
F. avenaceum		
Sample (root/soil)[a]	0.83	0.36
Year	10.46	0.0001*
Site	0.002	0.95
Sample (root/soil)*Year	0.88	0.41
F. solani* f. sp. *pisi		
Sample (root/soil)	1.872	0.19
Year	4.3	0.014*
Site	1.19	0.27
Sample (root/soil)*Year	7.40	0.0008*
A. euteiches		
Sample (root/soil)	121.65	0.001*
Year	1.20	0.27
Site	0.19	0.66
Sample (root/soil)*Year	4.7	0.03*

[a] Frequency of pathogen in root, as determined by PCR, compared to frequency of pathogen in soil, as determined by qPCR

Table 4-2. Frequency of detection of *F. avenaceum*, *F. solani* f. sp. *pisi* and *A. euteiches* by PCR and qPCR in root and soil samples (2013-2015)

Fungi/ Year	Soil (qPCR)	Roots (PCR)
F. avenaceum		
2013	58% (0.1) [a] A[b] a[c]	58% (0.1)[a] A a
2014	78% (0.06) A a	80% (0.08) A a
2015	35% (0.07) B a	55% (0.07) A a
F. solani f. sp. *pisi*		
2013	66% (0.1) A a	33% (0.1) B a
2014	56% (0.05) A a	100% (0.07) A b
2015	65% (0.06) A a	85%(0.06) A b
A. euteiches		
2014	91% (0.05) A a	41% (0.06) A b
2015	97% (0.06) A a	22% (0.06) B b

[a] Standard error of the mean (SEM)

[b]Means with the same upper case letter are not significantly different among years ($P < 0.05$) according to the Tukey-Kramer test

[c]Means with the same lower case letter are not significantly different between sample type (root/soil) ($P < 0.05$) according to the Tukey-Kramer test

Table 4-3. Estimated DNA quantity of *F. avenaceum, F. solani* f. sp. *pisi* and *A. euteiches* in 102 tested soil samples (2013-2015)

Fungi/ Year	DNA quantity (ln ng g^{-1})	SE[a]	Group letter*
F. avenaceum			
2013	3.3	0.03	a
2014	1.1	0.001	b
2015	2.5	0.01	ab
F. solani f. sp. *pisi*			
2013	2.9	0.10	a
2014	1.9	0.12	a
2015	2.8	0.41	a
A. euteiches			
2014	5.6**	0.10	a
2015	7.0	0.12	b

[a] The standard error of mean (SEM) for soil samples examined by qPCR
* Means with the same letter are not significantly different ($P < 0.05$) according to the Tukey-Kramer test
** DNA quantity of *A. euteiches* reported as gene copy number g^{-1}soil

Table 4-4. Estimated DNA quantity of *F. avenaceum* and *F. solani* f. sp. *pisi* in crop residue in 62 tested samples (2013-2014)

Fungi/Year	DNA quantity (ln ng g^{-1})	SE[a]	Group letter
F. avenaceum			
2013	9.0	0.1	a
2014	7.9	0.1	a
F. solani f. sp. *pisi*			
2013	5.8	0.05	a
2014	4.2	0.05	a

[a] The standard error of mean (SEM) for crop residue samples examined by qPCR

* Means with the same letter are not significantly different ($P < 0.05$) according to Tukey-Kramer test

4.6 Figures

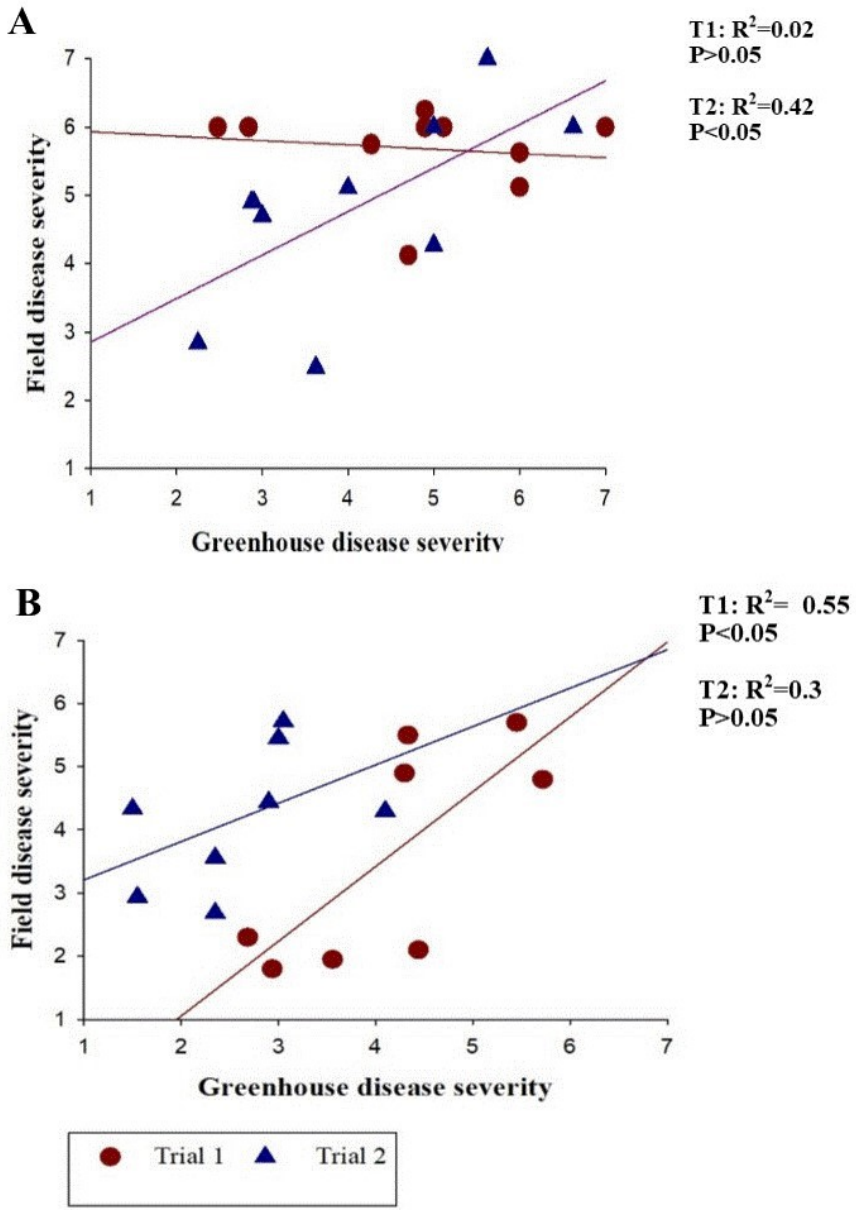

Figure 4-1. Regression analysis between pea root rot disease severity observed in a greenhouse bioassay with field soil compared to that observed in the corresponding field during disease surveys in 2014 (A), and 2015 (B). The greenhouse bioassay was performed twice, but trials could not be combined for analysis.

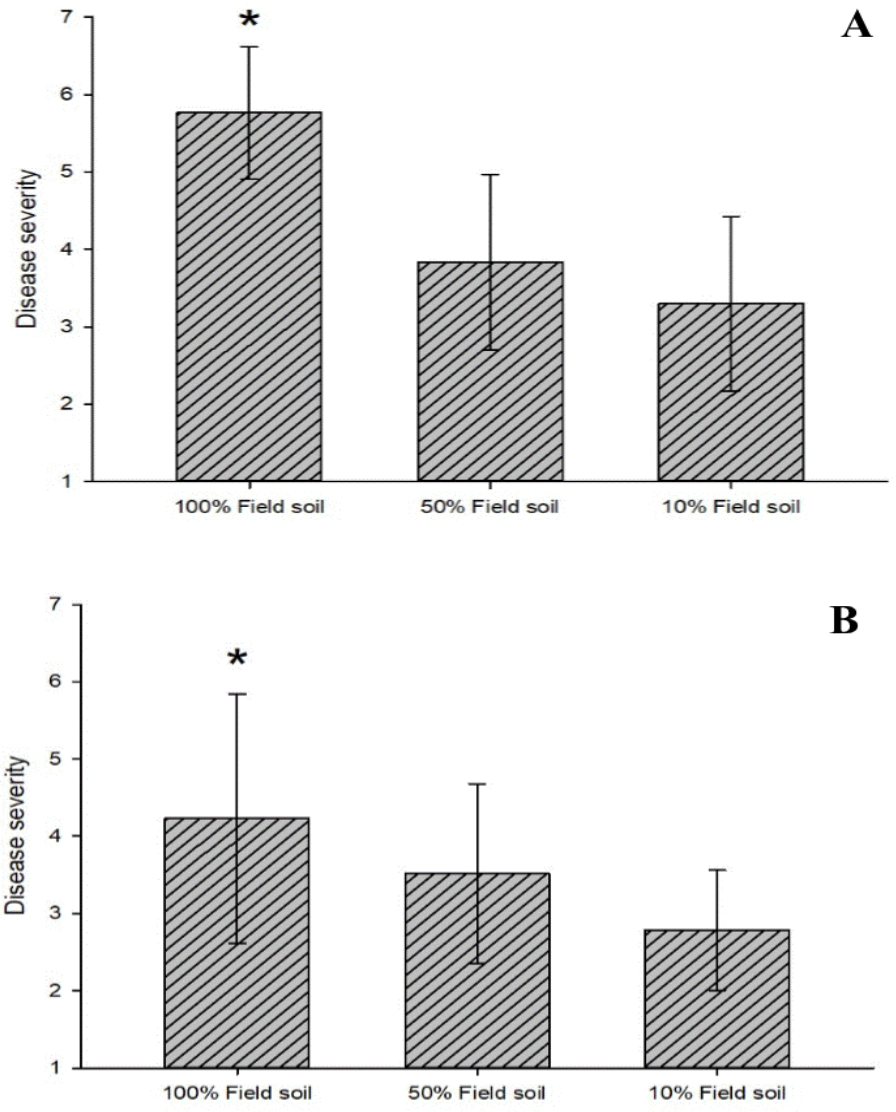

Figure 4-2. Disease severity changes by diluting field soil with autoclaved soil in: 100%, 50% and 10%: 2014 trial 1 (A); trial 2 (B) * = statistical difference between disease severity in different treatment at ($P < 0.05$) according to the Tukey-Kramer test.

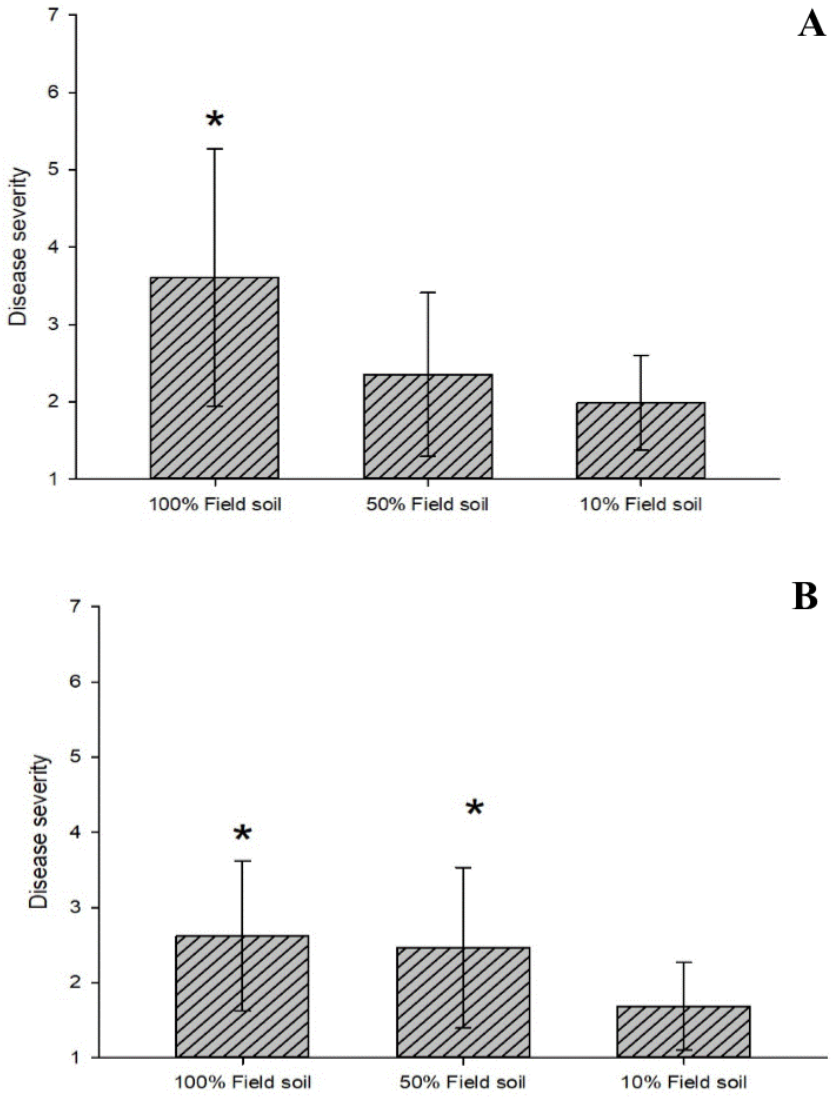

Figure 4-3. Disease severity changes by diluting field soil with autoclaved soil in: 100%, 50% and 10%: 2015 trial 1 (A), trial 2 (B) * = statistical difference between disease severity in different treatment at ($P < 0.05$) according to the Tukey-Kramer test.

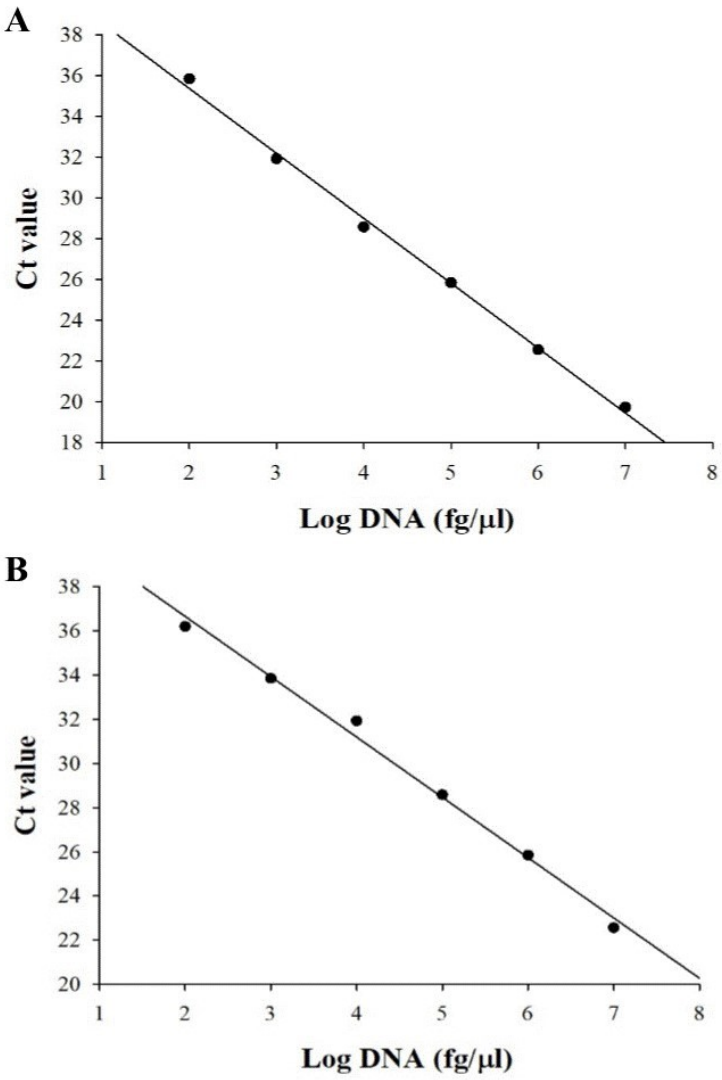

Figure 4-4. Standard curve of DNA concentration (log_{10}) of *F. solani* f. sp. *pisi* (A) and *F. avenaceum* (B) versus quantification cycle (Ct). Genomic DNA, extracted from 10 day old cultures of each pathogen, was used in six, 10 fold serial dilutions to generate standard curve on the QuantStudio6.

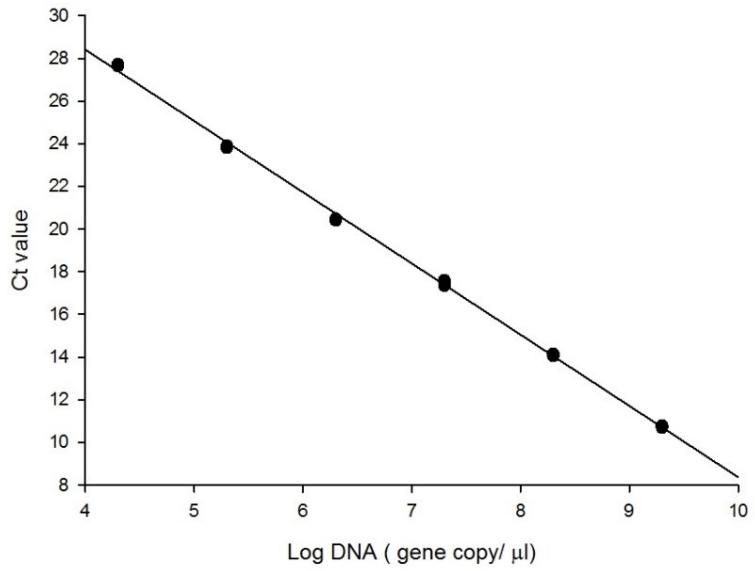

Figure 4-5. *Aphanomyces euteiches* standard curve generated for log10 and quantification cycle (Ct) with six, 10 fold serial dilution of Ae1.2_ITS1 gene copy.

4.7 References

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5 Predicting pea root disease using soil DNA analysis and environmental factors

5.1 Introduction

Root rot is an increasing concern for pea producers. *Fusarium avenaceum*, *F. solani* f. sp. *pisi* and *A. euteiches* are the three main pathogens in the Canadian Prairies (Chatterton et al., 2014; Chatterton et al., 2015; Chatterton et al., 2018). Root rot symptoms increase under biotic and abiotic stresses (Liu et al., 2016; Pandey et al., 2017). Inoculum concentration (Rush & Kraft, 1986), soil chemical properties (Heyman et al., 2007), soil compaction, temperature and moisture (Tu, 1994) are important factors that influenced pea root rot development. In addition, the composition of pathogenic species of *Fusarium* can also be influenced by soil properties (Bulluck et al, 2002), tillage system (Steinkellner et al., 2004) and weather conditions (Bateman et al., 2001; Doohan et al., 2003). Soil, weeds and crop residues are important sources of inoculum of *Fusarium* spp. (Leslie et al., 2006). Therefore, they might be more problematic in minimal and no tillage cropping systems (Postic et al., 2012), which is common in the Canadian Prairies (Awada et al., 2014).

There are numerous risk prediction models for airborne plant diseases based on single or multiple variables such as primary inoculum levels, temperature, rainfall and relative humidity (DeWolf et al., 2003; Schoeny et al., 2007; Soubeyrand et al., 2008; Pan et al., 2011). There are fewer models developed for soil borne plant pathogens, likely related to difficulties in data collection from soil for several variables including inoculum quantity, rainfall, soil physical,

biological and chemical features that can influence disease severity (Roget, 2001). However, despite the complications for modeling soil borne pathogens, during recent decades. The number of disease prediction models have increased and some are commercially accessible (Ophel-Keller et al., 2008; Wallenhammar et al., 2012). Due to the important role of environmental factors like rainfall and temperature on disease development, disease prediction models which relied mainly on DNA quantity might not always provide precise results. However, combination of DNA quantity and environmental parameters improved the model accuracy and provide greater estimation of disease severity and incidence for a cereal root rot disease (Poole et al., 2015). Predictive models use DNA testing of pre-sowing soil samples and correlate pathogen quantity and environmental parameters with disease severity to identify fields with a high risk of disease and yield loss to avoid planting in those fields (Roget, 2001; Ophel-Keller et al., 2008; Bithell et al., 2012; Wallenhammar et al., 2012; Poole et al., 2015).

Root rot diseases are generally caused by monocyclic pathogens and thus the initial inoculum might be predictor of disease development. Therefore, assessment of the amount of inoculum can provide valuable information about disease severity and incidence (Ophel-Keller et al., 2008; Poole et al., 2015; Wei et al., 2015; Shishido et al., 2016). Specific qPCR assays have been developed for the detection and quantification of *A. euteiches* (Vandemark et al., 2005; Gangneux et al., 2014) and *Fusarium* spp. associated with pea root rot (Zitnick-Anderson et al., 2018). By quantification of pathogen DNA in soil samples, the risk of disease severity could be predicted before planting which would allow growers to avoid high risk fields, decreasing the risk of economic loss (Ophel-Keller et al., 2008; Bithell et al., 2012).

Soil physical conditions play a principal role in plant disease development due to their impact on root development and nutrients accessibility by plants and microorganisms (Ghorbani et al., 2008). The study of the impact of abiotic elements such as soil texture and pH, and biotic factors like the soil microbial community on disease development provide valuable information for disease management (Ghorbani et al., 2008).

There is limited information about the influence of environmental parameters on pea root rot severity and incidence. Tu (1994) showed that high soil mositure and temperature increased root rot severity caused by *Fusarium* species. In addition, high soil moisture also favours *A. euteiches* infection (Papavizas, 1974). Due to the increasing prevalence of root rot disease in the Canadian Prairies, further research is required to determine the impact of these parameters on disease severity and incidence. It is important to develop methods for routine soil testing so that only low risk fields be chosen for planting pea.

The objective of the current study was to: (I) examine the potential of a regression model to predict pea root rot severity based on DNA quantity of *F. avenaceum*, *F. solani* f. sp. *pisi* and *A. euteiches*, rainfall and temperature, (II) and determine the association between root rot severity and soil physical and chemical properties.

5.2 Material and methods

5.2.1 Field surveys and sample collection

During, 2016-2017 over 260 samples were collected from 19 fields across Alberta, 15-20 soil and crop residue samples collected from each field in a W pattern in late April prior to planting (1 L per site) to a depth of 20 cm. A large Ziploc freezer bag of the previous crop residue was collected at the same time and from the same site. In 2016, two regions were sampled: Lethbridge and Lacombe, in 2017, 3 regions were sampled: Calgary, Lacombe and Drumheller. In late June, five root samples from each site were collected and returned to the laboratory, washed under running tap water for 10 min and rated on a 1-7 scale (Schneider & Kelly, 2000). Soil samples were sieved through 20 mm sieves, and samples kept at -20 °C prior to DNA extraction. Crop residues were air dried for 7 days at room temperature (25 °C), ground in a Wiley Mill and were then homogenized with a TissueLyser II (Qiagen, Toronto, ON, Canada). After processing, 70 g of each soil sample was homogenized, air dried and used for physical analysis.

Samples were characterized for texture types by hydrometer method (Bouyoucos et al., 1962), EC and pH were measured by saturated paste extract method (McKeague et al., 1978). Chemical properties including: potassium (K) was extracted with $NH_4CH_3CO_2$ and measured by AAS (Atomic Absorption Spectroscopy) (Hamm et al., 1970). Calcium (Ca) and magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) , contents were determined by ammonium acetate extract and measured by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectroscopy) (McKeague et al., 1978; Jones Jr, 1999). Nitrate-nitrogen (No_3N) and sulfate-sulfur (SO_4S) (Hamm et al., 1970). Overall in 2016 and 2017 for each individual soil property 190 pairs of observations was conducted. The average precipitation and maximum

temperature were collected from the Environment Canada weather station data closest to each sampled field (monthly and total rainfall, mean temperature April-July) (Table 5.1).

5.2.2 Analysis of soil and crop residue sample by qPCR

Soil samples were used for quantification of *F. avenaceum*, *F. solani* f. sp. *pisi*, and *A. euteiches*. Total genomic DNA was extracted from 250 mg of soil using the PowerLyzer Power Soil DNA Isolation kit (MoBio, Carlsbad, California, USA) according to the manufacturer's instructions. Genomic DNA was extracted from 40 mg crop residues using the Biosprint-96 DNA plant kit (Qiagen, Toronto, ON, CA) in a Biosprint instrument (Qiagen) according to the manufacturer's procedure. Extracted DNA was stored at -20 °C prior to DNA analysis. DNA concentration was measured by NanoDrop (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd., UK) and diluted to 10 ng μL^{-1} prior to DNA analysis. To quantify *F. avenaceum* specific primers and probes were designed based on the partial translation elongation factor alpha 1 (TEF-1 α) gene sequence from NCBI to quantify. For detection of *F. solani* f. sp. *pisi* the primers and probe was also based on the TEF-1 gene (Zitnick-Anderson et al., 2018). *Aphanomyces euteiches* primer and probes were based on the ITS region (Willsey et al., 2018). To prepare standards for *Fusarium* spp., DNA was extracted from 7 - 10 day old cultures of *F. avenaceum* and *F. solani* f. sp. *pisi* growing on PDA using the Plant DNeasy kit (Qiagen) according to manufacturer's directions. The DNA was quantified using a NanoDrop (NanoVue Plus Spectrophotometer) and serially diluted to produce a six-fold dilution standard curve ranging from 10 ng μL^{-1} to 100 fg μL^{-1} *Fusarium* genomic DNA. For *A. euteiches*, the standard

curve was based on gene copy number in six dilutions ranging from 2×10^9 to 2×10^4 gene copies μL^{-1}. For *Fusarium* spp., qPCR reactions were made to a final volume of 25 µL containing: 2.5 µL template DNA, 12.5- µL, Environmental Master Mix 2.0 (Thermo Fisher Scientific), 2.5 µL of each primer (0.9 µM) , 2.5 µL of probes (0.25 µM) and 2.5 µL ultrapure H_2O. For *A. euteiches* reaction volume was 20 µL containing: 10 µL PrimeTime Gene Expression 2X Master Mix (IDT, Skokie, IL, USA), 0.2 µL of each primers (0.5 µM) and 0.5 µL of probe (0.25 µM), 2 µL of template DNA and 6.65 µL ultrapure H_2O, in 96-well fast plates real-time quantitative. Assays were run and analyzed on a Quant Studio 6 Flex Real-Time PCR System (Thermofisher) under the following cycling conditions: *Fusarium* spp., 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min; for *A. euteiches*, 95 °C 3 min, and 40 cycles of 95 °C 15 s, 60 °C, for 1 min.

5.2.3 Data analysis

A regression tree was used to carry out analysis between estimated DNA quantity of the pathogens, rainfall, temperature and disease severity, from the combined 2016 and 2017 data sets. DNA quantity in the soil was determined by calculating the concentration from the cycle threshold (Ct) value using the regression equation calculated from the standard curve. The DNA quantity was converted to ln (x+1) for analysis and reporting. Stepwise forward selection was used to build a regression model. To assess the relationship between root rot severity and soil properties, pearson correlation coefficients were used. Statistical analysis was performed with R version 3.4.1 and R studio (https://www.r-project.org). The DNA quantity recovered was

converted to conidia per gram of soil based on the standard curve create as in Chapter 3. For the regression model, DNA quantities from soil and crop residues were added together.

5.3 Results

5.3.1 Soil and crop residue qPCR analysis

Aphanomyces euteiches was the predominant pathogen quantified from soil and was detected from 68% of the soil samples, followed by *F. avenaceum* in 65% and *F. solani* f. sp. *pisi* in 63% of the soil samples. The range of DNA quantity for *F. avenaceum* was between 0.3 - 8.5 ng g^{-1} soil and for *F. solani* f. sp. *pisi* DNA quantity was 0.5 - 5.1 ng g^{-1} soil (Table 5.2). *Aphanomyces euteiches* quantity ranged between 7.3 - 14.8 gene copy number g^{-1} soil (Table 5.3). *Fusarium avenaceum* ranged between 1058 - 105,944 conidia g^{-1} soil and *F. solani* f. sp. *pisi* ranged between 253 - 31,435 conidia g^{-1} soil (Table 5.2).

5.3.2 Soil physical and chemical analysis

Pearson correlation coefficients were not significant for the relationship between pH, texture characteristics and disease severity (Table 5.4). Analysis of the soil physical properties indicated the pH ranged from 5.9 to 7.5 in 2016 and between 6.1 to 7.4 in 2017. Soil type textures were classified in three groups including: sandy-loam, clay and clay-loam. Two fields were classified as sandy-loam, 5 fields as clay and 13 fields as clay-loam. Disease severity in clay-loam was DS= 3.5, sandy-loam DS= 2.6 and clay DS= 2, but there was no significant difference between classes. Pearson correlation analysis for the soil properties found that Zn (r = 0.3, P=

0.001) and NO_3N (r = 0.3, P= 0.01) had significant positive correlations with root rot severity. There was a negative association with Ca (r = -0.2, P = 0.01) and Mg (r=-0.1, P = 0.03), while there were no significant correlations with other examined soil properties and disease severity (Table 5.5)

5.3.3 Regression tree model

All data was combined and stepwise forward multiple regression analysis carried out with DNA quantities, rainfall, temperature and root rot severity. Root rot severity was associated with the total amount of rainfall from April to July ($P < 0.001$). The next significant parameter on pea root rot development was mean temperature from seeding to flowering which was correlated with disease severity ($P < 0.0008$). Among quantities of pathogens, quantity of *F. solani* f. sp. *pisi* ($P< 0.0001$) and quantity of *A. euteiches* ($P< 0.005$) showed a significant effect on the regression model. *Fusarium avenaceum* DNA quantity explained 3.6 -5.1%, and the amount of *F. solani* DNA explained 2.1-5.8% of disease severity. *Aphanomyces euteiches* DNA quantity in soil explained 1.4- 6% of disease severity (Figure.5.1).

The model Akaike information criterion (AIC) value was 176, and the overall the model was significant at $P< 0.05$ ($R^2= 0.3$). Comparing actual disease severity observed under field condition and predicted root rot severity with a regression model indicated a significant relationship ($R^2=0.3$, $P <0.00001$) (Figure. 5.2).

5.4 Discussion

Predictive models have great potential to be used in the effective management of plant diseases (DeWolf et al., 2007; Giosuè et al., 2010; Poole et al., 2015; Webb et al., 2017). In the current study, the possibility of developing a prediction tool for pea root rot based on pathogen DNA quantities in pre-sowing soil and crop residue samples, precipitation and temperature was investigated. According to procedures described in various studies, predicting plant disease requires a multivariate approach (Bourke, 1970; DeWolf et al., 2007; Ophel-Keller et al., 2008). Models based on the quantity of pathogen initial inoculum in soil have been applied commercially for several soil borne diseases like cereal root rot (Ophel-Keller et al., 2008; Poole et al., 2015) and clubroot of canola, these service which offers quick and reliable prediction of disease severity and corresponding yield loss, are accessible by government offices and facilities (Wallenhammar et al., 2012; Poole et al., 2015; Wallenhammar et al., 2016).

One of issues in constructing a multiple linear regression model is multicollinearity, which defines as correlation between predictor variables, it would reduce model accuracy (Heiburger & Holland, 2004). In order to examine the relationship between variables for multicollinearity variance inflation factor (VIF) for each independent variable should be calculated , VIF> 5 indicated that there is correlation between predictor variables (Heiburger & Holland, 2004).

One method to overcome this issue is using backward elimination for variables selection in regression model, it select the best parameters and exclude variables which are not valuable in model for prediction. Akaike information criterion (AIC) uses for compare models and selecting

the best model, among different fitted models the one that has lower AIC indicates a better model (Heiburger &Holland, 2004; Faraway, 2016). Two essential criteria for a good model: it has smallest number of variables required to fit model and has high adjusted R^2 value. Thus the best model choice will balance between lower model complexity (AIC) and the goodness-of-fit (R^2 value) (Faraway, 2016)

The quantity of three pathogens: *F. avenaceum, F. solani* f. sp. *pisi,* and *A. euteiches* causing pea root rot was estimated in pre-sowing field soil and crop residue samples by qPCR. Under greenhouse conditions, *F. avenaceum* and *F. solani* f. sp. *pisi* DNA levels were linearly correlated with disease severity (Chapter 3). However, in the current study, there was only a weak association between DNA quantity of pathogens and disease severity under field condition. This result suggests that pathogen DNA levels may not be a valuable predictor of pea root rot severity, although presumably pathogen inoculum levels should be related to disease levels. For example, Hollaway et al. (2013) found that *Fusarium* spp. DNA quantity provided an accurate prediction of yield loss caused by crown rot in cereal crops. The weak correlation found in this study might be attributed to poor DNA extraction efficiency from soil samples (Okubara et al., 2013; Li et al., 2015) and also the presence of PCR inhibitors in DNA extracts from soil (Braid et al., 2003; Schrader et al., 2012). The key point to consider is that due to great variation in soil characteristics, the extraction efficiency of DNA from soils with different characteristics may also vary. Therefore, accurate results from soil DNA analysis may require specialized and improved procedures for each soil type with specific physical and chemical features (Zielińska et al., 2017). Another potential reason for the poor correlation between disease severity and DNA

quantity is soil type. A stronger correlation between DNA quantity and take-all disease severity was observed among fields with similar soil types (Roget, 2000). Field pea is planted across a broad range of soil types in Alberta, and the same inoculum dose-disease response might not occur in all soil types. *Fusarium* isolates recovered from pea roots in Alberta showed a wide range of aggressiveness (Chapter 2), but the genetic markers for aggressiveness are not known. This could be another possible reason for the lack of correlation between field disease severity and DNA quantity. The TEF-1 primer set used in this study targets all *F. avenaceum* or *F. solani* f. sp. *pisi* isolates, and therefore non-pathogenic isolates would also be quantified.

Based on the regression tree, *F. avenaceum* DNA quantity in soil explained 3.6 -5.1%, *F. solani* f. sp. *pisi* DNA 2.1-5.8% and *A. euteiches* DNA 1.4- 6% of disease severity. A previous study indicated the impact of DNA quantity on disease development varied greatly among years. For instance, DNA quantity of *Gaeumannomyces graminis*, *F. pseudograminearum*, and *Pratylenchus* spp. predicted cereal root rot between 2% and 16% in two different years (Poole et al., 2015). Year to year variation is likely related to the impact of other variables on pathogen populations and disease development (Poole et al., 2015). In the case of a plant disease complex associated with more than one pathogen, the role of DNA quantity of each pathogen on disease severity is different (Poole et al., 2013; Poole et al., 2015).

One potential source of inoculum for *Fusarium* spp. is crop stubble and straw (Hogg et al., 2010; Hofgaard et al., 2016). To determine whether the inoculum level of *Fusarium* spp. in crop debris is a valuable disease predictor, correlation was conducted between DNA quantities in crop residue and disease severity. Assessments of pathogen DNA in crop residue indicated high

quantity of *F. avenaceum* (data not shown). It was found in almost all samples tested which is reasonable since previous studies indicated cereal crop stubble is the main source of *F. avenaceum* inoculum (Hofgaard et al., 2016), however, there was not a strong association between DNA quantity and disease severity, adding the DNA quantity in the crop residue and soil into one combined value for analysis improved the significance of the model. This would indicate cultivation of wheat as a host of *F. avenaceum* and also crop residue left in the field are likely the reasons for increased disease severity . *Fusarium solani* f. sp. *pisi* was not detected in the majority of crop residue samples, which is likely due to its survival strategy of producing chlamydospores that remain in the soil.

There is little information on the influence of environmental factors on pea root rot development (Tu, 1994). One of the key considerations for evaluating the impact of environmental factors on pea root rot development is that there are various pathogens, with different epidemiology associated with root rot. Depending on the pathogen, the key environmental parameters that affect disease development might be different. For wheat take-all and root rot diseases, rainfall and temperature are major variables for disease prediction (Yang et al., 1997; Hollaway et al., 2013; Poole et al., 2015;). Rainfall and temperature have effects on the dominant *Fusarium* spp. each year (Bateman et al., 2001; Poole et al., 2013), as the optimal conditions for each *Fusarium* species are different (Doohan et al., 2003; West et al., 2012). Higher precipitation increased, the *F. culmorum* population, while it had the reverse impact on the *F. pseudograminearum* population (Poole et al., 2013). *Fusarium solani* was the more dominant species in a field season with lower precipitation, while *F. avenaceum* was favored by

more moisture (Taheri et al., 2016). Similarly, *A. euteiches* caused more damage under high soil moisture (Papavizas et al, 1974). Results from the present study showed an effect of rainfall on disease development, but suggested that a higher amount of rainfall resulted in a lower level of disease. However, Sippell (Sippell et al., 1982) reported that Fusarium root rot on bean caused high disease severity when summer rainfall was high. Similarly, soils with a high clay content and high moisture increased the occurrence of of *A. euteiches* (Pfender & Hagedorn, 1983). In 2016, two regions were sampled with different amounts of rainfall, but disease severity was higher in Lethbridge which had less rainfall than Lacombe. In 2017, Calgary and Lacombe areas had similar amount of rainfall, but there was difference between disease severity, which might be explained by the influence of variables rather than rainfall.

Several studies showed the important role of temperature on population dynamics of *Fusarium* spp. and determined the optimum conditions for each species (Saremi et al., 1999; Doohan et al., 2003). Optimum temperature for *F. avenaceum* growth was 20-25 ^{o}C (Brennan et al., 2003), while for *F. solani* it was 30.5 ^{o}C (Scruggs et al., 2016). Root rot development on chickpea caused by *F. solani* f. sp *pisi* increased at higher temperatures (Bhatti et al., 1992). In the current study there was negative effect of temperature on disease development; high temperatures caused less disease. Surprisingly, results of the regression tree suggested that environmental variables have greater potential to predict the risk of disease than pathogen levels in the soil.

The possible association between root rot severity under field conditions and soil physical features (pH, EC, texture) was also investigated. Soil physical features can impact root

development. For example, the combination of improper drainage and high clay content reduced root growth compared with a well drained and sandy loam soil (Bengough et al., 2005). Root rot of cauliflower and wheat caused by *R. solani* was lower in clay soil and higher in sandy soil due to the possible mechanism of faster propagation of the pathogen in sandy soil (Chauhan et al., 2000; Sivasithamparam et al., 2000). Sanogo et al. (2001) examined the impact of soil texture on sudden death syndrome of soybean and reported higher disease severity in soil with high sand content, this was explained by lower organic matter in these soils, which was correlated with a lower activity of antagonistic microorganisms. There are several studies demonstrating that soil clay increases pea root rot severity while high calcium content contributes to pea root rot suppressiveness (Persson et al., 2000; Heyman et al., 2007). Field pea in Alberta is grown across a broad range of soil types (McKay et al., 2003), but our results demonstrated no significant correlations between disease severity and soil type. Similar results were found in another study across the Canadian prairies, where there were no differences between root rot incidence, severity and soil zone (Chatterton et al., 2018).

Soil texture and the predominant soil particles could play a critical role in recovery of DNA extraction from soil samples since soil microorganism adhere to soil particles (Daniel, 2005). DNA extraction is more challenging in soil with predominantly clay particles (Cai et al., 2006), and previous studies showed low extraction efficiency in soil with high clay content (Roose-Amsaleg et al., 2001; Lakay et al., 2007). Most of the soil samples in our study were classified as having a high clay content, which is likely one of the possible causes of low DNA recovery from the samples. The risk of root rot caused by *A. cochlioides* on sugar beet is

predictable by soil DNA test, although soil clay content affected the limit of detection (Almquist et al., 2016).

The association between soil properties and Fusarium wilt of spinach demonstrated significant positive correlation of 15 soil properties such as Ca, Fe and NH_4N with disease severity (Gatch et al., 2015). It has been reported that calcium content is associated with soil suppressiveness against some soil-borne pathogens (Serrano et al., 2012). Our results indicated a negative correlation between root rot severity and Ca, which is consistent with previous research that demonstrated a negative impact of Ca on pea root rot disease caused by *A. euteiches* (Heyman et al., 2007). Another element that was negatively correlated with root rot was NO_3-N. A high level of NO_3N fertilizers was related to reducing Fusarium wilt and was likely associated with an increased soil pH that decreased the accessibility of Mn and Fe (Dordas, 2008). Nitrogen form influenced Fusarium root rot development on tomato. A high level of ammonium nitrate generally increased disease severity or had no effect, while a high level of nitrate-N (NO_3-N**)** decreased disease severity (Duffy et al., 1973).

Another factor which may have influenced the results is sampling depth. The effect of depth on populations of *F. solani* on soybean revealed that the greatest population of pathogens were in the 0-15 cm depth, which might be related to a higher activity of the soil microbial community in this depth (Rupe et al., 1999). For *A. euteiches* the highest pathogen density was detected at 10-40 cm (Moussart et al., 2009).

There was a positive correlation between Zn and disease severity in our study, and to the best of our knowledge there are no reports on the impact of Zn on pea root rot. There was an inverse correlation between root rot severity and Mg content in soil. Previous study investigate influence of Mg level on pea root rot caused by *A. euteiches* and results indicated, it had no significant effect on root rot enhancement or reduction (Persson et al., 2000). Investigating and distinguishing numerous factors associated with the impact of soil physical and chemical features on disease development is complex and the potential role of soil features on pea root rot development requires more research.

It is important to investigate the inoculum levels of pathogens related to disease development and yield losses. However, our findings suggest that rainfall and temperature markedly influenced root rot severity, and disease severity was more strongly related to rainfall and temperature than to DNA quantity of the associated pathogens. The impact of environmental parameters on pea root rot is unclear and very few studies have investigated these parameters. The relationship between DNA quantity and disease severity were consistent during two years of study, but additional research is required to overcome the challenges associated with DNA extraction from soil and optimization of methods for various soil types in Alberta. In addition, more sensitive DNA analysis techniques like digital droplet PCR (dd PCR), which is not affected by PCR inhibitors (Dingle et al., 2013; Racki et al., 2014), may improve DNA quantification results. To verify a disease prediction model, several years of data collection across a broad range of soil types and environments are required, however the influence of some factors like soil type, crop sequence and soil microbial community will be difficult to incorporate (Almquist

et al., 2016). The quantity of pathogenic DNA is a predictor of disease severity and yield reduction, but is insufficient to support grower decisions. Further research is required to build a more robust model.

5.5 Tables

Table 5-1. April through July precipitation, mean temperature and location of sampled fields in 2016-2017

Field number	Location	Disease severity	Rainfall *					Temperature (April-July)[a]
			April	May	June	July	Total	
2016								
1	Lethbridge	1.5	13.8	67.5	12.8	32.4	126.5	13.3
2	Lethbridge	5.9	13.8	67.5	12.8	32.4	126.5	13.3
3	Lethbridge	1.4	13.8	67.5	12.8	32.4	126.5	13.3
4	Lethbridge	4.4	13.8	67.5	12.8	32.4	126.5	13.3
5	Lethbridge	6.5	13.8	67.5	12.8	32.4	126.5	13.3
6	Lacombe	2.1	15.1	79.1	27.7	119.9	240	12.3
7	Lacombe	2	15.1	79.1	26.7	119.9	240	12.3
8	Lacombe	2.45	15.1	79.1	26.7	119.9	240	12.3
2017								
9	Calgary	2.2	56.4	17.3	41.2	67.7	182.6	12.4
10	Calgary	2.5	56.4	17.3	41.2	67.7	182.6	12.4
11	Calgary	1.5	56.4	17.3	41.2	67.7	182.6	12.4
12	Calgary	1.6	56.4	17.3	41.2	67.7	182.6	12.4
13	Calgary	1.5	56.4	17.3	41.2	67.7	182.6	12.4
14	Lacombe	3.6	24.7	45.2	69.7	39.7	179.3	11.8
15	Lacombe	3.3	24.7	45.2	69.7	39.7	179.3	11.8
16	Lacombe	2.1	24.7	45.2	69.7	39.7	179.3	11.8
17	Drumheller	2	16.3	35.8	61.1	21.3	134.5	13.9
18	Drumheller	2.5	16.3	35.8	61.1	21.3	134.5	13.9
19	Drumheller	1.4	16.3	35.8	61.1	21.3	134.5	13.9

[a] Environmental data obtained from environment Canada weather station located closest to sampled field.

* Units for rainfall is (millimeters), and air temperature is degrees Celsius.

Table 5-2. Mean DNA quantity of *F. avenaceum* and *F. solani* f. sp. *pisi* recovered from 260 combined soil and crop residue samples (2016-2017)

Field number/ Year	*F. avenaceum*		*F. solani* f. sp. *pisi*	
	Ln DNA (ng g^{-1} soil)	Conidia g^{-1} soil	Ln DNA (ng g^{-1} soil)	Conidia g^{-1} soil
2016				
1	6.6	8558	4.1	44366
2	6.1	6687	3.4	17190
3	5.5	4849	2.6	5817
4	4.4	3817	3.6	22539
5	2.4	913	3.7	25808
6	4.0	2855	2.5	5080
7	7.8	21127	1.3	1000
8	6.9	18687	1.5	1311
2017				
9	3.5	1224	3.4	17190
10	5.1	3432	3.7	25808
11	5.5	9864	4.1	44366
12	4.6	2405	3.2	13111
13	5.7	7560	2.3	3874
14	4.8	2924	1.7	1719
15	6.4	8455	2.1	2955
16	6.1	6147	5.6	338385
17	7.0	10902	5.4	258086
18	7.3	14317	4.8	114504
19	1.9	237	4.3	58170

[a] Recovered DNA quantity converted to conidia per gram of soil base on standard curve created in Chapter 3.

Table 5-3. Estimated DNA quantity of *A. euteiches* recovered from 260 soil tested soil samples (2016-2017)

Field number	*Aphanomyces euteiches DNA* Ln (gene copy* number g soil^{-1})
2016	
1	9.1
2	9.3
3	8.8
4	9.0
5	9.2
6	10.8
7	9.7
8	9.0
2017	
9	9.4
10	8.9
11	10.4
12	8.5
13	9.4
14	9.6
15	11.2
16	9.2
17	9.5
18	9.5
19	9.6

*based on partial ITS gene target of which are there are ~100 copies per cell (Gangneux et al., 2014)

Table 5-4. Pearson correlation analysis of association between soil parameters and pea root rot severity under filed condition

	Sand	Silt	Clay	EC[a]	pH
Correlation coefficient	0.07	0.1	-0.1	-0.2	-0.2
P value	0.8	0.6	0.5	0.3	0.4

* = Probability of a significant correlation (P<0.05) between soil parameters and pea root rot severity

[a] EC electrical conductivity

Table 5-5. Pearson correlation analysis of association between soil properties and pea root rot severity under field condition, 190 samples tested.

	So_4S	Cu	Fe	Mn	Zn	K	NO_3N	P	Ca	Mg
Correlation coefficient	0.1	-0.008	-0.01	0.07	0.3	-0.002	0.3	-0.1	-0.2	-0.1
P value	0.1	0.9	0.9	0.3	0.001*	0.9	0.01*	0.2	0.01*	0.03*

* = Probability of a significant correlation ($P<0.05$) between soil properties and pea root rot severity

5.6 Figures

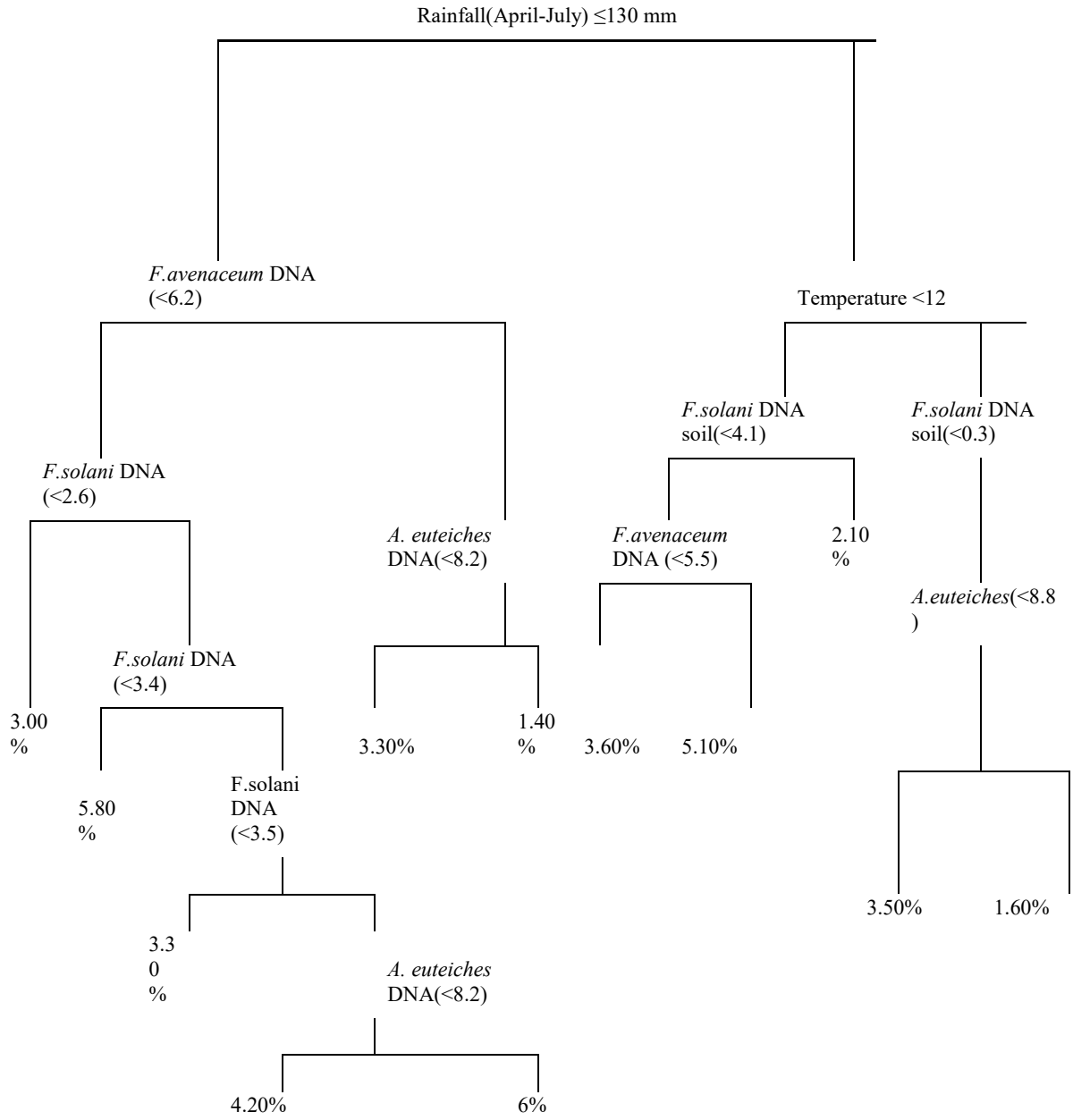

Figure 5-1. Regression tree of predicted root rot disease severity established based on DNA quantity of *Fusarium avenaceum*, *F. solani* f. sp. *pisi* in soil and crop residue, monthly average of precipitation and maximum temperature (April-July). Units for rainfall is (millimeters), *Fusarium* spp. DNA (Ln DNA + 1 ng g soil $^{-1}$), *Aphanomyces euteiches* DNA

(Ln gene copy number g soil^{-1}) and air temperature in degrees Celsius. Predicted pea root rot severity by the regression model is represented by the proportional numbers at the bottom.

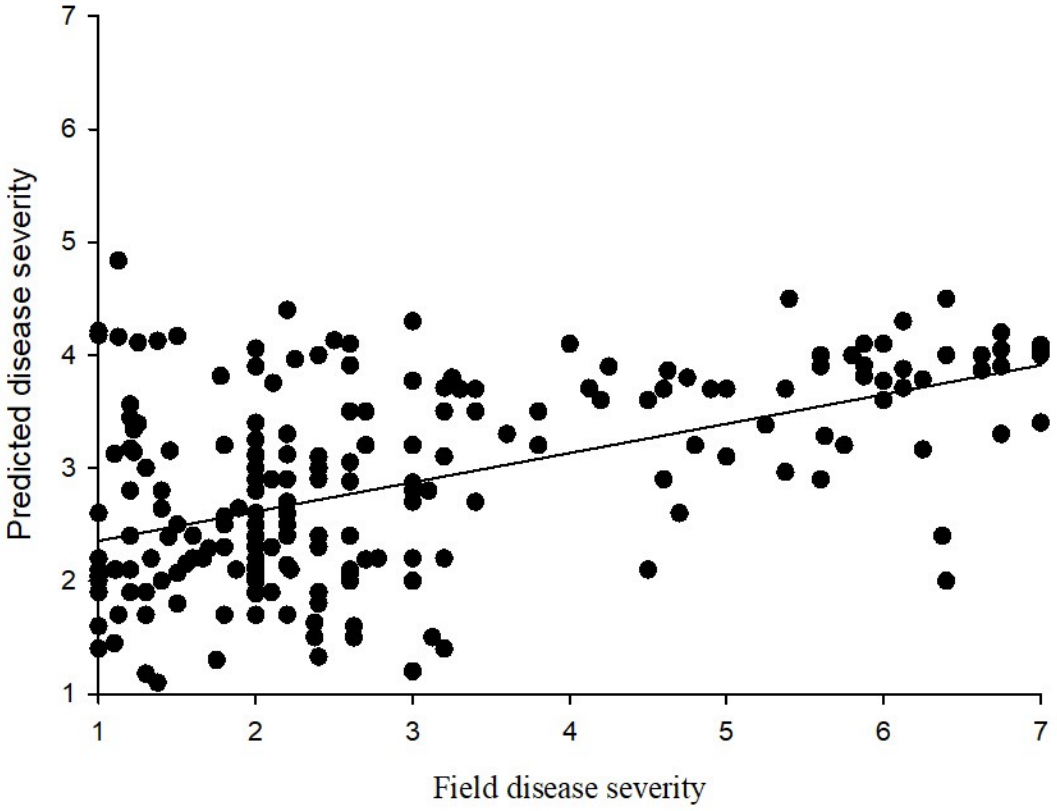

Figure 5-2. Regression analysis (R^2=0.3, P<0.00001) of predicted pea root rot severity according to regression model plotted against actual measured field disease severity observed during two years survey in Alberta.

5.7 References

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6 General discussion

6.1 Summary of significant results

Root rot is a wide spread disease in pea production areas of the Canadian prairies. It is a disease complex caused by several soil-borne pathogens. *Fusarium avenaceum*, *F. solani* and *A. euteiches* are the most problematic pathogens in Alberta (Feng et al., 2010; Chatterton et al., 2015; Esmaeili Taheri et al., 2017). Depending on the associated pathogens, type of survival structure, dormancy and environmental conditions, root rot pathogens can remain in the field for several years. Disease management is challenging since there is no effective management strategy to control the disease (Kraft et al., 2001; Gossen et al., 2016;). Understanding all different aspects of root rot including: the associated pathogens, their aggressiveness and host range, pathogen interactions and influence of environmental parameters, will help to assess management strategies. The primary goal of the current study was to investigate the possibility of developing a prediction model to estimate disease severity prior to planting and allow producers to avoid high risk fields. The objectives of the current research were to: (1) evaluate the aggressiveness and host range of *Fusarium* species associated with pea root rot in Alberta, (2) determine the relationship between *F. avenaceum* and *F. solani* f. sp. *pisi* inoculum level and disease severity using qPCR, (3) using a greenhouse soil bioassayto evaluate changes in disease severity by changing the soil inoculum levels and evaluate this method to predict root rot risk, (4) develop a prediction model for pea root rot based on pathogen DNA quantity, rainfall and temperature. The host range, pathogenicity and aggressiveness of *Fusarium* spp. isolated during field surveys was examined (Chapter 2). The most aggressive species were *F. solani* and *F.*

avenaceum. Moreover, there were differences between isolates of *F. solani* f. sp. *pisi* *F. avenaceum* and *F. oxysporum,* which demonstrated wide virulence variability in these species. Greenhouse host range tests were performed to identify other possible hosts of *F. avenaceum* and *F. solani*. Alternative hosts were mostly other pulse crops; planting these crops in rotation with pea is expected to increase pathogen inoculum.

A previous study identified pathogenicity genes linked to the host specificity of *F. solani* (Rep & Kistler, 2010). However, our results demonstrated a lack of host specificity of

F. solani f. sp. *pisi* which is in agreement with recent study that showed it has several symptomatic and asymptomatic hosts (Šišić et al., 2018). Examined isolates of *F. avenaceum* and *F. solani* f. sp. *pisi* species did not cause disease symptoms on the cereal crops tested while previous reports showed a high level of cross-pathogenicity between *Fusarium* spp. isolated from soybean, corn and wheat (Parikh et al., 2018). While there is limited information about cross-pathogenicity of associated species pathogenic on pea, further research is essential to investigate cross-pathogenicity.

Pathogenicity and host range experiments were conducted under greenhouse conditions. Under field conditions results might be different, due to the impact of other variables like soil conditions, environmental parameters, and the interaction of several pathogens. Pathogenic *Fusarium* spp. on soybean generally showed lower root rot severity under field conditions compared with greenhouse conditions (Arias et al., 2013). Another step toward understanding the aggressiveness of *Fusarium* spp. would be testing the isolates under field conditions, however, it will be difficult to do this since *Fusarium* spp. are widespread and finding fields in

which this fungus are not already present would be difficult. Additionally, in the current study, only single isolates of each species were examined, while interactions of *Fusarium* spp. with other associated pathogens like *A. euteiches* could increase the disease severity (Willsey et al., 2018). Due to the complexity of interactions between plants and microbes and their role in plant health, understanding of these interactions would be critical for selecting cultivars resistant to multiple pathogens (Wille et al., 2018).

A greenhouse bioassay was conducted with soil samples to evaluate the reduction of disease severity due to reducing natural inoculum in field soil (Chapter 4). Results showed disease severity was reduced when inoculum dose was reduced by half, which indicated the role of inoculum on disease progress. This method had some benefits like considering soil microbial community (Windels, 1996; Harveson et al., 2014). Greenhouse bioassays have provided various results for different pathogens. For instance, bioassays are a reliable method to predict root rot caused by *A. cochlioides* and *R. solani* in sugar beet, but they did not provide accurate estimation of *Rhizomonia* and *Fusarium* wilt damage (Harveson et al., 2014). In the current study, the majority of pea plants planted in field soil showed root rot symptoms and considerably greater disease severity was observed in the 100% and 50% treatments while lowest symptoms were observed on plants that grew in the 10% treatments. The results of greenhouse bioassay varied between trials and, for some trials, correlations between disease severity and treatment levels were weak.

DNA analysis was conducted to detect pathogens in soil by qPCR and in root samples by PCR, . Results confirmed the presence of pathogens in both symptomatic and asymptomatic sites

(Chapter 4). qPCR analysis indicated that generally higher quantity of *F. avenaceum* was detected in crop residue than in soil, while the reverse pattern was observed for *F. solani.* This finding suggests wheat crop residue is an important source of inoculum for *F. avenaceum.* Therefore, the common wheat-pea rotation in the Canadian prairies might be one of the possible factors promoting root rot, and management of straw might benefit to reduce pathogen inoculum. Further investigation is required regarding the population dynamics of *Fusarium* species associated with pea root rot in crop residue, and monitoring the survival and aggressiveness of isolates recovered.

6.2 Limitation and challenges of study

Application of qPCR for quantification and detection of *F. avenaceum* is challenging because it does not differentiate between pathogenic and non-pathogenic isolates. This is likely to influence the correlation between DNA quantity recovered and disease severity. A similar issue has been reported for other *Fusarium* species like *F. oxysporum* (Okubara et al., 2013). Another reason for the poor correlation between estimated DNA quantity and disease severity is likely attributed to virulence variability of isolates of *F. avenaceum* (Feng et al., 2010; Chittem et al., 2015), *F. solani* (Chapter2) and *A. euteiches* (Wicker et al., 2001). It is possible to use molecular markers to categorise *Fusarium* spp. isolates based on aggressiveness (Miedaner et al., 2001; Mishra et al., 2003; Cumagun et al., 2004). Feng et al. (2010) reported an association between ITS and CPN60 sequences and virulence variability among *F. avenaceum* isolates

pathogenic on pea, and it might be beneficial to classify virulence of the isolates recovered based on molecular features.

Greenhouse trials, in conjunction with qPCR analysis of soils, were conducted to determine the association between pathogen inoculum dose, disease severity and soil DNA quantity (Chapter 3). Results showed that the relationship between disease severity and inoculum dose of each pathogen was dependent on inoculum type. For *F. solani* mycelium treatment, despite high inoculum concentration, disease severity was not high and did not show a significant linear correlation, while for *F. avenaceum* mycelium treatment inoculum dose was correlated with disease severity. To our knowledge, no information is available about the aggressiveness or infectivity variation between mycelia and conidia. This variability might be related to optimum temperature and moisture for germ tube or mycelial growth. Quantification of DNA did not always result in a linear relationship with initial inoculum dose. Moreover, there were limits to the sensitivity of detection, and greenhouse experiments indicated that inoculum concentration >1000 conidia g^{-1} soil for *F. avenaceum* and >500 conidia g^{-1} soil for *F. solani* f. sp. *pisi* were not detectable. The qPCR assay was more sensitive for detection of pure genomic DNA, while for DNA extracted from soil, sample processing and PCR inhibitors are likely to affect the results (Van der Heyden et al., 2018). To the best of our knowledge, these are the first standard curves to relate *F. avenaceum* and *F. solani* inoculum density with DNA quantification and disease severity. To develop a predictive model based on initial inoculum in soil, an essential step is understanding the relationship between DNA quantity and disease severity. Further study

is needed to develop standard curves generated with isolates with different levels of aggressiveness.

Identifying the best disease predictors was the main goal of this project. Pathogen DNA levels in soil and crop residue, environmental parameters and soil properties or combination of these features were investigated for this purpose (Chapter 5). Collectively our results indicated that there was a weak but significant association between *Fusarium* spp. and *A. euteiches* DNA quantity in pre-plant soil and crop residue and disease severity. These results were similar to results reported by Okubara et al (2013) where a positive correlation between disease severity and detected amount of *F. oxysporum* was not observed. However, there are several successful examples of this approach for predicting various soil-borne plant diseases (Wallenhammar et al., 2012; Okubara et al., 2014; Poole et al., 2015; Wallenhammar et al., 2016). In our model, environmental parameters were considered a strong driver for disease prediction, both temperature and rainfall playing important roles in disease development. The influence of soil physical, chemical and biological characteristics on plant disease development is complicated and it does not always show a consistent pattern in different regions (Höper et al., 1996; Datnoff et al., 2007; Gatch et al., 2015). Assessment of soil physical properties indicated no correlation with disease severity, and among the examined chemical properties, a negative correlation was observed for Ca and Mg and a positive correlation of NO_3N and Zn with root rot severity.

One of the factors which may have influenced the correlation analysis is DNA quantity and quality (Feinstein et al, 2009; Demeke et al., 2010). The quality of extracted DNA can be influenced by soil physical and chemical features (Feinstein et al., 2009). Depending on the soil

type, optimized DNA extraction methods may vary (Lakay et al., 2007), and extraction is more challenging when the predominant soil particle is clay (Cai et al., 2006). Most of the field sampling areas in our study had a high clay content. Recently, an indirect extraction technique was described as an optimal method for soil with high clay content (Högfors et al., 2018). Part of the low correlation between DNA quantity and disease severity under field condition is likely attributed to low extraction efficiency. Therefore, we conclude that it is likely that changing the DNA extraction method to improve the recovered DNA quantity and quality might improve the results.

Due to the association of several pathogens with root rot, and the various differences in requirements for growth and infection, like moisture, temperature and nutrient necessities, (Holub et al., 1991; Doohan et al., 2003; Gossen et al., 2016; Wu et al., 2018), developing a prediction model for the pea root rot complex is challenging. Association between root rot severity and environmental parameters needs further investigation, and in more fields and larger area of sampling with more detailed information about climatic variables. Current study focused mostly on pathogen quantity, while there are other aspects that require further study. One of the factors which is likely to have great impact on disease development under field conditions is the soil microbial community (Weller et al., 2002; Garbeva et al., 2004), which was not investigated in the current study. Furthermore, new PCR technology like droplet digital PCR may enhance the accuracy of results, because it is less subject to the influence of PCR inhibitors (Dingle et al., 2013: Račkiet al., 2014).

6.3 Future directions

One of the most effective management strategies recommended for many soil-borne plant diseases is the use of resistant cultivars (Martin et al, 2003; Katan, 2017). However, it has been very difficult to breed for resistance to root rot pathogens, and despite 20 –30 years of research, there are still no resistant cultivars available commercially. Developing cultivars resistant to multiple pathogens might be the most effective management strategy for pea root rot diseases together with cultural practices (Kraft & Kelly, 2001; Gossen et al., 2016). Due to the complexity of interactions between plants and microbes, and their key role in plant health, an understanding of their interplay will be critical for developing cultivars resistant to multiple pathogens (Wille et al., 2018).

Our knowledge could be improved by applying new technologies like next generation sequencing, it is possible to investigate synergistic interactions between multiple pathogens, as well as role other microorganisms which have interactions with pathogens by this method, better understanding about these associations effectively improve disease management strategies (Knief., 2014; Nejat et sl., 2017),

Another alternative strategy for disease management could be the application of organic amendments and cover crops that have suppressive impacts on pea root rot pathogens (Bonanomi et al., 2010; Hossain et al., 2012; Hossain et al., 2015). However, due to inconsistent effects reported for this approach, it needs more research.

Combination of breeding and using advanced tools to understand the interactions between host plants and pathogenic and non-pathogenic soil organisms soil may lead to development of management strategies for soil-borne plant pathogens (Wille et al., 2018). In spite of the importance of the association between disease severity and yield loss, there is limited research in this area. Estimation of yield loss due to root rot was achieved through relating the yield of healthy plants and inoculated plants, and calculated based on seed dry weight (Basu et al., 1976; Basu, 1978). More research is required to investigate if root rot severity is a reasonable indicator of yield loss or not.

Due to the lack of an effective management strategy, having a prediction model which can help growers to know the disease potential in fields prior to planting would be beneficial. Therefore, a key consideration is evaluating the factors which have the greatest impact on disease and exclude the features with slight impact. The current study identified the major pea root rot pathogens in Alberta, and our results indicated that one of the reasons for root rot promotion is the wide host range of associated pathogens and cultivation of susceptible host in rotation with pea, which increases pathogen inoculum in the soil. PCR and qPCR analysis showed the presence of pathogens in soil and roots collected from symptomatic and asymptomatic sites. Crop residue was identified as a major source of inoculum of *Fusarium* spp. In a greenhouse experiment, root rot severity was positively correlated with primary inoculum density and also recovered DNA quantity from soil; disease severity increased gradually with inoculum density. The minimum of conidia detected by qPCR was 500-1000 spores g^{-1} of soil. Therefore, an inoculum density less than this amount is not detectable. Moreover depending on

soil clay content, it is likely that extraction efficiency is reduced and the limit of detection might increase. It is expected that improved DNA extraction methods might result in a stronger correlation.

The results from the current study indicated the need of specific DNA extraction methods for each soil type. Soil texture may be an important factor in extraction efficiency. qPCR provided valuable results for detection of pathogens in soil samples, though not in quantification, and it might be useful for accrue detection of some pathogens like Aphanomyces which their detection by PCR in plant tissue was significantly lower than detection in soil samples. Environmental parameters play a critical role in root rot development but, optimal conditions for each root rot pathogens are varied. Therefore, the dominant pathogen might change each year depending on weather conditions. Results from the current study indicated spring rainfall and temperature were the principal parameters which had the greatest impact on disease severity.

Due to the involvement of several pathogens in the root rot disease complex, which have different epidemiology and biology, as well as complex interactions that affect disease development (Willsey., 2018) disease prediction will be challenging. Moreover, some of the factors like the influence of the soil microbial communities and crop sequences may not be easily quantified for inclusion into a model. Finally, there is a need to evaluate the usefulness of prediction models in many fields, environments and over a long period of time. Those are considerations for future research to address the pea root rot issue in Alberta.

6.4 References

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