THE CHARACTERIZATION OF FUNGICIDE RESISTANCE, POPULATION STRUCTURE, AND AGGRESSIVENESS OF FUNGAL SPECIES ASSOCIATED WITH ASCOCHYTA BLIGHT OF PULSE CROPS IN MONTANA

by

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DEDICATION

I dedicate this thesis to my wife Olajumoke for her unflinching support for the advancement of my career for the last nine years and always. To my daughter Ifedayo, and son Ayokunle for understanding that daddy needs to go to school and work and sometimes may not spend adequate time with them. And to all who cherish hard work and the determination to contribute positively to humanity.

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ABSTRACT

Ascochyta blight (AB) of pulse crops causes yield loss in Montana, where 1.24 million acres were planted to pulses in 2018. Pyraclostrobin and azoxystrobin, quinone outside inhibitor (QoI) fungicides, have been the choice of farmers for the management of AB in pulses. QoI-fungicide-resistant *Didymella rabiei* isolates were found in one chickpea seed lot each received from Daniels, McCone and Valley Counties, MT, from seed produced in 2015 and 2016. Multiple alignment analysis of amino acid sequences showed a mutation that replaced the codon for amino acid 143 from GGT to GCT, introducing an amino acid change from glycine to alanine (G143A), which is reported to be associated with QoI resistance. Under greenhouse conditions, disease severity was significantly higher on pyraclostrobin-treated chickpea plants inoculated with QoI-resistant isolates of *D. rabiei* (QoI-R) than sensitive isolates (QoI-S) (p-value = 0.001). *D. rabiei*-specific PCR primer pair and probes were developed to discriminate QoI-R and QoI-S isolates. In North America, AB of dry pea is caused by a complex of fungal pathogens (*Didymella pisi*, *Peyronellaea pinodes*, and *Peyronellaea pinodella*). *D. pisi* is the predominant causal pathogen of AB of dry pea in Montana resulting in yield losses. Thirty-three microsatellite markers (SSR) were developed and used to analyze the genetic diversity and population structure of 205 *D. pisi* isolates from four geographical regions of Montana. Unweighted Neighbor-joining, principal coordinate, and population structure analyses grouped these 205 isolates into two major sub-groups. The clusters did not match the geographic origin of the isolates. Analysis of molecular variance showed 85% of the total variation within populations and only 15% among populations. There was moderate genetic variation in the total populations (PhiPT = 0.153). Recently, a shift in pathogen composition has been observed in Montana from *D. pisi* to *P. pinodes* and *P. pinodella*. Also, a *Phoma sp.* was found associated with AB contaminated dry pea seeds and included in this study. Mycelial growth and sporulation were evaluated at different temperatures. Also, the pathogenicity of *Phoma sp*. and the difference in aggressiveness among the fungal pathogens was evaluated. At all temperatures, *Phoma sp.* had the highest growth rate (p-value = < 0.001) and produced more spores than the other species (p-value = < 0.001). *P. pinodes* caused greater disease severity than the other species when inoculated on pea plants (cv. Carousel, p-value ≤ 0.001). The *Phoma sp.* was not pathogenic. Peameal agar was used to visually discriminate between fungal species. Diagnosis of AB of dry pea is challenging because of the complex of pathogens involved. Also, they have slow growth rate and different morphotypes. Currently, there are no PCR-based assays developed for *D. pisi* or any of the fungal pathogens associated with the AB complex of dry pea. *D. pisi* specific SYBR green SSR-qPCR and conventional SSR-PCR assays were developed for rapid detection and quantification of *D. pisi* both *in-planta* and *in-vitro.*

CHAPTER ONE

INTRODUCTION

Ascochyta blight of (AB) of pulse crops is a host-specific disease caused by fungal species including *Didymella rabiei* on chickpea, a species complex consisting of *Didymella pisi* (Barilli *et al*., 2016), *Peyronellaea pinodes*, and *Peyronellaea pinodella* on field pea (Aveskam *et al*., 2010). Davidson *et al* (2009) reported and characterized a fourth causal agent, *Phoma koolunga,* which has become widespread in South Australia. Recently, *Phoma herbarum* and *Phoma glomerata* have also been shown to be associated with the ascochyta blight complex on field peas in Australia (Li, *et al.*, 2011; Tran *et al.*, 2014; and Liu, *et al.*, 2016). In North America, *P. koolunga*, *Phoma herbarum*, and *Phoma glomerata* have not been reported. On lentil, Ascochyta blight is caused by *Didymella lentis* (Barilli *et al*., 2016). Ascochyta blight can infect pulse crops at all developmental stages and cause over 40–50% yield reduction under conditions suitable for disease development (Mondal *et al*., 2005 and Wise *et al*., 2011). In faba bean, 90% losses have been reported (Pande *et al*., 2005 and Barilli *et al*., 2016). Symptoms of AB can develop on foliage, stems, and also cause seed rot or shrunken, discolored seeds. AB is seed- and residue-borne. In the field, disease onset is normally post-flowering (growth stage R1) through plant maturity (growth stage R8) (Ye *et al*., 2000 and Gossen *et al*., 2011). In addition, *D. rabiei*, *D. pisi*, and *P. pinodes* can subsist in the sexual and/or asexual forms (pseudothecia, pycnidia, and perithecia respectively), producing ascospores and conidia that provide a source of inoculum for disease epidemics (Tivoli and Banniza, 2007; Chilvers *et al*., 2009 and Wise *et al*., 2011).

The initial distribution of disease symptoms may reflect the source of primary inoculum. Under field conditions, scattered patches may suggest seedborne inoculum, while disease symptoms on field edges suggests inoculum moving in from neighboring fields. A uniform pattern, or one associated with patches of residue from previous crops, suggests residue-borne inoculum. Because moisture is essential for infection and blight development, infection sites may be localized in lowlands or densely planted patches of plants (Mondal *et al*., 2005, Tivoli and Banniza, 2007, Wise *et al*., 2011, and Sivachandra-Kumar and Banniza 2017).

Management of this disease would benefit from the availability of resistant germplasm. The lack of a single or multigenic source of resistance to AB in chickpea and dry pea have confounded management (Darby and Lewis, 1986; Kraft, *et al*., 1998; and Sharma and Ghosh, 2016). In addition, what tolerance has been deployed has not been rapidly adopted by farmers due to market demands. Foliar fungicides are effective but enhance selection for fungicide resistance (Davidson, *et al*., 2007 and Owati *et al*., 2017). Thus, disease control often relies on an integrated approach including the use of certified disease-free seeds, crop rotations of at least 3 years, tillage to bury plant debris, and fungicide seed treatment to reduce seed transmission (Gossen and Derksen, 2003; Kinane, *et al*., 2005; and Wise *et al*., 2011). The success of these practices requires an understanding of the epidemiology of the pathogens, the recombination potential, diversity and adaptability to the environment, and pathogen composition and aggressiveness.

Chickpea and dry pea production in Montana

Chickpea is an annual cool-season legume that requires an optimal daytime temperature range of 70 to 84 °F, and nighttime temperatures of 64 to 70 °F. Chickpea is a self-pollinated crop that grows to 15-30 inches in height with small, feathery to large leaves (McVay, *et al.,* 2013). Mature pods usually contain 1-2 seeds. Chickpea has an indeterminate growth pattern which allows plants to develop new leaves and pods on upper branches after flowering has begun (McVay, *et al.,* 2017). This can often lead to difficulty with homogenous maturation of the crop, and desiccation is a common practice near harvest. Swathing is also used, although may be deleterious to seed quality and is less common.

In Montana, chickpea is planted with a 6 to 12-inch row spacing in the early spring (February – April) to improve yield potential. Yield is reduced by flower abortion when temperatures are >90 ^{o}F, and early planting allows plants to flower prior to the onset of high daily temperatures, normally occurring in July-August. There has been a rapid increase in the production of chickpea in Montana. In the last five years, acreage planted to chickpea increased from 31,500 to 346,000 acres (United States Department of Agriculture National Agricultural Statistics Service USDA-NASS, 2018). In 2018, Montana accounted for 42% of the total national production of chickpea (USDA-NASS, 2018).

Dry pea is self-pollinated, resulting in pods about three inches long containing four to nine seeds. Dry pea is an annual or winter annual grain legume species with tendrils it uses to climb on supporting structures including neighboring plants. Current widely planted varieties of dry pea in Montana are semi-dwarf, semi-leafless with white flowers, clear

seed coats, and a large seed size (7.5 – 9 mm). Other genetic characteristics include increased stem stiffness and improved resistance to lodging during maturity. This enhances the upright growth of the plants and facilitates ease of harvesting with minimal losses. These characteristics have not been shown to impact the severity of ascochyta blight (Bretag and Brouwer, 1995).

In Montana, dry pea is seeded in the spring or winter. Winter pea has historically had poor survival and is not a common practice, although research is ongoing. Spring dry pea are planted in late March or early May in a tilled or no-tilled cropping system in a 6 to 12 inches row spacing. Flowering occurs after the 10- to the 14-leaf stage, approximately about 50 to 60 days after seeding, and plants attain maturity in less than 90 days (Miller, *et al*., 2002). Dry pea yield is limited by the number of flowering days, high temperature at flowering (≥ 80 ^{o}F), and available soil water. Temperatures above 80 ^{o}F during flowering can cause the flower to blast. Also, pea has a shallow root system (≤ 2 ft) and is sensitive to drought. When available soil water is 2 ft to 4 ft, dry pea yield is limited (Miller *et al*., 2002).

Montana leads in the production of dry pea in the US, where 415,000 acres were planted in 2018 (USDA-NASS, 2018). This accounts for 47% of the total production in the US. Being a high-water-use efficiency crop, it is of great importance to the semi-arid region like Montana. In addition, dry pea provide a nitrogen credit to subsequent crops because of their capacity to fix atmospheric nitrogen, saving synthetic nitrogen application needs for future crops. Montana growers include pulse crops in rotation with many crops including cereals such as wheat and barley, corn, potatoes, sugar beets, etc.

<u>Pathogens causing Ascochyta blight in chickpea and dry pea</u>

Didymella rabiei **.** *Didymella rabiei* is a heterothallic fungus that selectively attacks chickpea plants. The sexual and asexual stages of *D. rabiei* produce distinct spore types. Sexual spores referred to as ascospores are produced in dark fruiting bodies (pseudothecia) on overwintered chickpea residues in contact with moist soil. Asexual spores called conidia are produced abundantly in similarly dark, raised fruiting bodies (pycnidia) embedded in diseased tissues. Ascospores are ellipsoid to biconic hyaline, two-celled with a constriction at the septum. The length of the ascospores is approximately 9.5–16 by 4.5-7µm (Pande, *et al.,* 2005). Conidia are oval to oblong, slightly bent at one or both ends and measure 6–12 by 4-6µm (Punithalingham and Holiday, 1972). The draft genome of *D. rabiei* is 34.65 Mb in size and is predicted to code for ~10,600 proteins (Verma, *et al*., 2016).

The fungus grows on a variety of artificial nutrient media, but prefers chickpea meal dextrose agar (Pande, *et al.,* 2005). Cultures grow optimally at 20 ^{o}C +/- 1 ^{o}C under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark) producing a pale cream-colored mycelium in which black to brown pycnidia are immersed (Pande, *et al.,* 2005). Variability in morphology and color often occurs and isolates often produce unicellular conidia (CAB International 2000).

Didymella pisi. *D. pisi* is heterothallic and does not produce chlamydospores (Kraft *et al*., 1998; and Chilvers, *et al.,* 2009). It produces globose brown pycnidia on dry pea leaves and debris (Chilvers, *et al.,* 2009). Conidiophores are hyaline, short and measure approximately 6 –14 by 3-8μm. Conidia are hyaline, straight, and septate with rounded ends and measure approximately 10–16 by 3-4.5 μm (Punithalingham and Gibson, 1972). Colonies on oatmeal agar produce abundant pycnidia with carrot red colored spore exudate (Ahmed, *et al.,* 2015). *D. pisi* secretes ascochitine, a metabolite toxic to *Pisum* species that has been associated with pathogenicity. The toxin is not produced by *P. pinodes* or *P. pinodella* (Foremska, *et al*., 1990 and Marcinkowska *et al.,* 1991).

Peyronellaea pinodes. *P. pinodes* is homothallic and produces chlamydospores in culture either singly or catenated (in chains) (Punithalingham and Holiday, 1972). Generally, the fungus grows on a variety of artificial media producing a light to dark gray mycelia. At 20-30 mm mycelial radial growth under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark) at 20 ^{o}C +/- 1 ^{o}C, pseudothecia and pycnidia become arranged in a concentric ring (Onfroy, *et al.,* 1999). On dry pea debris, it produces pycnidia with conidia and pseudothecia containing ascospores. Conidia are hyaline, slightly constricted at the septum, and 8–16 by 3-4.5 μm in size (Punithalingham and Holiday, 1972). *P. pinodes* produces ascospores which develop from pseudothecia on infested plants stubble and senescent plant material (Punithalingham and Holiday, 1972; Tivoli *et al*., 1996; Onfroy, *et al.,* 1999; and Tivoli and Banniza, 2007). Ascospores are not produced by *D. pisi or P. pinodella*, only conidia are produced. The *in-vitro* production of pseudothecia or pycnidia on media is dependent on nutrient availability. While

pseudothecia will be produced on minimal media, the production of pycnidia requires highly nutritive media (Hare and Walker, 1944; and Tivoli, *et al.*, 1996). In addition, the production of pseudothecia and pycnidia is temperature dependent, the optimum temperature of pseudothecia is 16 ^{o}C. High temperatures decrease the number or stop the development of pseudothecia. Pycnidial production is reduced at low temperature and increases with light (Hare and Walker, 1944).

Peyronellaea pinodella. *P. pinodella* is heterothallic and produces chlamydospores (Bowen, *et al.*, 1997). Cultures on media are dark with an irregular pattern of pycnidia and turn black at maturity due to the abundant production of chlamydospores. Chlamydospores are dark brown, often spherical and smooth and exist singly or catenated (Punithalingham and Gibson, 1976). Under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark) at 20 ^{o}C +/- 1 ^{o}C, colonies form alternating zones of mycelium and pycnidium resulting into concentric rings around the initial mycelia plug (Punithalingham and Gibson, 1976). The pycnidia are sub-globose and larger than those of *P. pinodes* with an approximate size of 200-300 µm. *P. pinodella* conidia are hyaline and often non-septate, but smaller than those of *P. pinodes* at 4.5–9 by 2-3 µm (Punithalingham and Gibson, 1976).

Disease cycle

D. rabiei**.** In the field, in addition to seed, air-borne ascospores are another major source of primary inoculum that can initiate blight infection (Fig. 1). Air-borne ascospores are produced abundantly on infested crop residues that overwinter on the soil surface (Wiese, *et al*., 2001). These ascospores are released in the spring and early summer under favorable temperature moisture conditions. The air-borne ascospores infect chickpea leaves and produce small water-soaked necrotic spots which rapidly enlarge and coalesce (Galloway and Macleod, 2003 and Pande, *et al.,* 2005). Once infections are established, numerous asexual spores (conidia) are produced on symptomatic plant tissues. These conidia then cause the secondary spread of disease within the field (Fig. 1) (Wiese, *et al.,* 2001; and Pande, *et al.,* 2005). Conidia can be produced under minimal moisture conditions. Spores are spread by rain splash and wind to infect tissues on the same or adjacent plants. The development of concentric rings on lesions is a characteristic symptom of *D. rabiei*. Lesions that develop on leaves and pods appear circular with brown margins and a gray center which contains the pycnidia, while lesions on stems are more elongated. Inoculum may also be dispersed with infested living plant parts, crop residues, and contaminated machinery. Infested crop residues and seed are primarily responsible for the long-term perpetuation of the fungus.

D. pisi, P. pinodes, and P. pinodella. Primary inoculum (conidia or ascospores) of all species that cause AB in dry pea is spread either by rain splash or wind onto emerging crops. Only *P. pinodes* produces ascospores, the other two pathogens produce conidia (Punithalingham and Holiday, 1972; Tivoli *et al*., 1996; and Onfroy, *et al.,* 1999). Regardless of the type of inoculum, the initial infection causes black spots on leaves which coalesce under moist conditions resulting in leaf necrosis (Roger, *et al.,* 1996 and Tivoli, *et al.,* 2007). Pycnidia develop within the lesions and conidia (pycnidiospores) are spread to neighboring plants via rain splash (Schoeny, *et al.,* 2008) (Fig 2). The dispersal of conidia over short distances is responsible for secondary inoculum (Tivoli, *et al.,* 2007), thus increasing disease severity and early tissue senescence. Tissue senescence initiates the production of pseudothecia. After rainfall, pseudothecia release airborne ascospores which are dispersed over long distances (Roger, *et al.,* 1996; Tivoli, *et al.,* 2007; and Schoeny, *et al*., 2008). Once AB is established, abscission of infected flowers and pods often occur. The pathogens subsist on crop residue, seed, and in the soil.

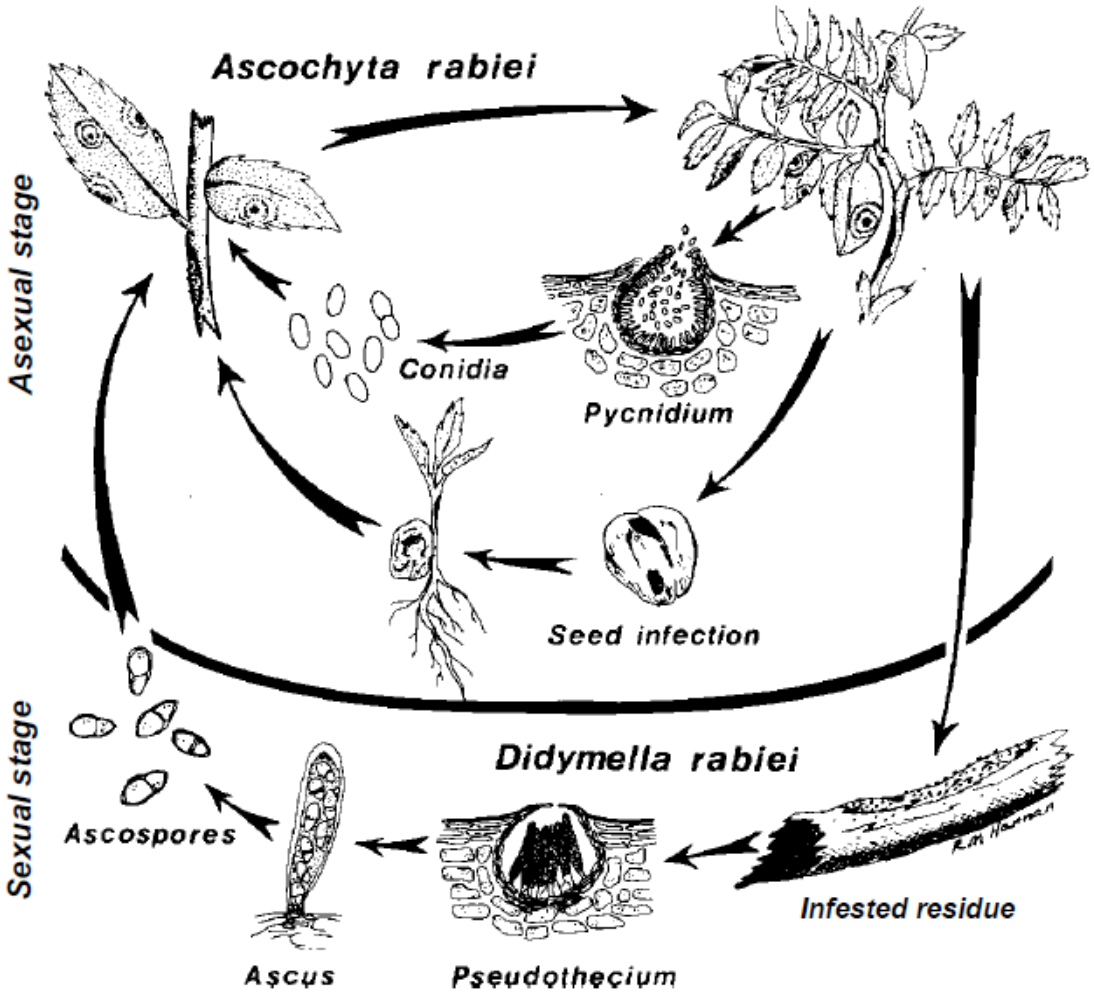

Figure 1. Disease cycle of ascochyta blight of chickpea caused by *Didymella rabiei* (Wiese, *et al*., 2001)

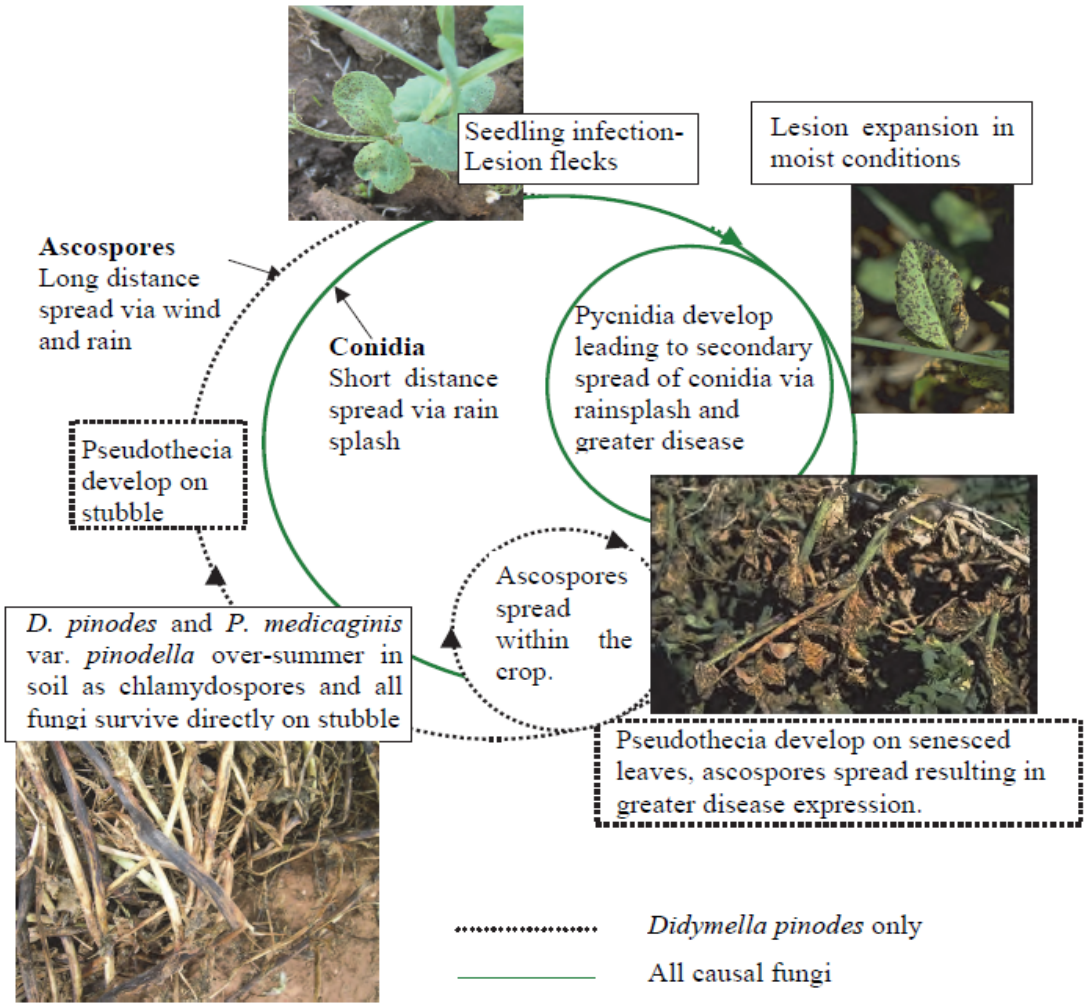

Figure 2. Disease cycle of ascochyta blight of dry pea caused by *Didymella pisi*, *Peyronellaea pinodes*, and *Peyronellaea pinodella* (Davidson, 2012)

Disease Control

Cultural control. Agronomic practices that reduce the main sources of inoculum are the most effective disease management strategies. Cultural practices such as planting disease-free seeds, crop rotation with non-host crops, and burial of plant stubbles in the soil are all effective measures to reduce the amount of inoculum (Kraft, *et al*., 1998; Bailey, *et al*., 2001; and Pande, *et al.,* 2005). Marzani *et al.,* (2013) demonstrated that intercropping, which is the simultaneous cultivation of multiple crops on the same field, is effective in the control of ascochyta blight of chickpea. Intercropping of chickpea with faba bean and oilseed rape reduced AB incidence in chickpea. Similarly, a 40% reduction in AB incidence was reported when dry pea was intercropped with barley (Kinane, *et al*., 2005).

Under low disease pressure, agronomic practices such as late sowing date and seeding rate can reduce the incidence and severity of AB (Pande, *et al.,* 2005; Schoeny, *et al*., 2008; and Davidson, 2012). Furthermore, late maturing dry pea varieties have been reported to exhibit lower disease severity than early maturing varieties. This may be associated with the later development of necrotic tissues and consequently, the later development of pseudothecia (Tivoli, *et al*., 1996 and Davidson, 2012). Ascochyta blight is more severe on crops planted on nutrient-depleted or in waterlogged soils (Davidson, 2012). Addition of potassium fertilizers in soils with high nitrogen content was reported to enhance chickpea yields and reduce AB (Kader, *et al.*,1990 and Pande, *et al.,* 2005).

Chemical control**.** Several foliar and seed treatment fungicides have proved to be effective in the control of AB in dry pea and chickpea. Depending on the price of the seed treatment, growers may choose to not apply fungicide where yield or crop value is expected to be low (Pande, *et al*., 2005 and Davidson, 2012). In addition, continuous applications of both seed and foliar fungicides encourage selection for fungicide resistance in the pathogen population (Wise *et al*., 2008, Lonergan *et al*., 2015; Bowness *et al*., 2016; and Owati, *et al.,* 2017).

Fungicides are widely used to achieve an acceptable level of disease control (Davidson and Kimber, 2007 and Lonergan *et al*., 2015). Broad spectrum protectant fungicides (chlorothalonil) are typically applied pre-flowering and can delay the onset of AB; however, once symptoms appear it is imperative for the grower to apply fungicides that provide a high level of control and move beyond the site of application in plant tissues due to canopy closure (Gan *et al*., 2006; Davidson and Kimber, 2007; Wise *et al*., 2009; and Lonergan *et al*., 2015). Quinone outside inhibitor (QoI) fungicides are highly effective, locally systemic, and commonly used for the control of AB. In addition, the US Environmental Protection Agency (EPA) approved the use of pyraclostrobin (Headline®) to benefit plant health on federally issued labels (Agweb, 2009). This plant health benefit of the QoI-fungicide was reported by Dimmock and Gooding (2002) to prolong grain filling in wheat crops, which may be associated with increased yield. Furthermore, QoI-fungicide has been reported to lower transpiration rates and also reduce the rate of senescence in wheat plants (Petit *et al*., 2012 and Mahoney *et al*., 2014). No studies of this type have been done in dry pea or chickpea.

Integrated disease management. Management of AB requires an integrated approach including the use of certified disease-free seeds, crop rotations of at least 3 years, tillage to bury plant debris, fungicide seed treatment to reduce seed transmission, the use of resistant cultivars and foliar fungicides for prevention or treatment of disease symptoms (Gossen and Derksen, 2003; Kinane, *et al*., 2005; and Wise *et al*., 2011). The use of resistant varieties and cultural practices can reduce AB. However, resistant varieties are not widely available in the Northern Great Plains. Breeding for resistance to AB is challenging in chickpea and dry pea. This is because this trait is reported to be rare in the genetic resources available for chickpea (Sharma and Ghosh, 2016). In addition, negative genetic correlation has been reported between resistance to AB and other desirable traits. This was illustrated by Lichtenzveig *et al*. (2002), who pointed out the negative genetic correlation that existed when combining good resistance to AB and early flowering in chickpea. Current studies in the Middle East, North America, and Australia are targeted at developing AB-resistant chickpea genotypes, especially resistance to pathotype IV of *D. rabiei*, which is considered highly virulent (Bayaa *et al*., 2004; Imtiaz *et al*., 2011; and Sharma and Ghosh, 2016).

Similarly, in dry pea, use of resistant varieties is the most efficient approach for AB management. However, no single gene resistance to AB of dry pea has been found (Kraft, *et al*., 1998). In addition, AB in dry pea is caused by a complex of fungal pathogen, thus, this poses a challenge in securing gene resistance to AB of dry pea due to the genetic variability that exists in the pathogens (Darby and Lewis, 1986).

Summary and research objectives

Ascochyta blight is one of the major threats to chickpea and dry pea production in Montana. Currently, there are no AB resistant varieties of chickpea or dry pea in the Great Plains of North America (Darby and Lewis, 1986; Bayaa *et al*., 2004; Imtiaz *et al*., 2011; Gossen, *et al*., 2011; and Sharma and Ghosh, 2016). In chickpea, the disease is caused by *D. rabiei* while a complex of fungal pathogens is responsible for the disease in dry pea (Davidson, *et al*., 2009; Li, *et al.*, 2011; Tran *et al.*, 2014; Liu, *et al.*, 2016; and Barilli, *et al*., 2016). In Montana, growers often rely on cultural practices and fungicide treatments to achieve acceptable levels of disease control. However, insensitivity to QoI fungicides has been reported in North Dakota and Canada (Gossen, *et al.*, 2004; Wise, *et al*., 2008; and Delgado, *et al.*, 2013). These are regions in geographically close proximity to Montana which facilitates the exchange of germplasm. This heightens the need to monitor the development of QoI-fungicide resistance in Montana chickpea fields. As earlier stated that AB of dry pea is caused by a complex of fungal pathogens, *Didymella pisi* tends to be more prevalent in Montana compared to other fungal species that cause Ascochyta blight in dry pea (Owati *et al*., 2017). While *P. pinodes* and *P. pinodella* are the prominent species in other dry pea producing regions of North America such as North Dakota and Canada (Alberta, Saskatchewan, Manitoba) (Wallen, *et al*., 1967; Gossen, *et al*., 2011; and Sivachandra-Kumar and Banniza, 2017). Literature has shown that understanding the genetic diversity and structure of plant pathogen can be used as a base to develop or improve disease management strategies (Peixoto-Junior *et al*., 2014). In this light, 33 SSR loci in *D. pisi* genome were targeted to determine the genetic diversity of the pathogen population in Montana.

Regional differences in the composition of pathogens causing AB in dry pea occur worldwide. In Australia, *P. pinodes*, *P. pinodella*, and *P. koolunga* are the primary pathogens associated with AB of dry pea, and *D. pisi* is rarely isolated (Davidson, *et al.*, 2009). *D. pisi* tends to be the prevalent pathogen of AB in Europe and Asia and a recent epidemic of AB in Spain was associated with *D. pisi* (Chilvers, *et al.*, 2007 and Kaiser, *et al.*, 2008). In North America, Montana is host to *D. pisi*, while *P. pinodes* and *P. pinodella* are prevalent in North Dakota and pea production areas of Saskatchewan and Alberta. The frequency of germplasm exchange between these areas creates a potential for a shift in pathogen composition of the AB complex in dry pea. This information is important for variety recommendations and breeding efforts. The concern is that the aggressive *P. pinodes* and *P. pinodella* in North Dakota in the US and in Saskatchewan in Canada (Kraft, *et al.*, 1998; Gossen, *et al*., 2011; Ahmed *et al.*, 2015; and Sivachandra-Kumar and Banniza 2017) may gradually dominate in Montana, where they have been occurring at insignificant levels to date. There is an urgent need to characterize these pathogens in the context of Montana's dry pea fields and to develop a media-based diagnostic tool that will be used to monitor the changes in the pathogen population.

Sequential to the development of the media-based diagnostic tool for monitoring AB complex pathogens of dry pea, molecular-based assays that are rapid, specific, and sensitive were developed to monitor the dynamics of the pathogen population. Currently, there is no rapid and sensitive diagnostic assay developed for *in-vitro* and *in-planta* detection of any of the pathogen in the ascochyta blight complex of dry pea or any of the pathogens associated with ascochyta blight of pulse crops. Therefore, the development of

such an assay will facilitate rapid diagnosis of AB of dry pea and can be used in epidemiology studies which can serve as a basis to improve our understanding and management of this disease.

The objectives of the research presented in this thesis were to (1) detect and characterize QoI-resistant *Didymella rabiei* causing ascochyta blight of chickpea in Montana; (2) design microsatellite markers and apply them for the genetic diversity study and population structure of *Didymella pisi* associated with ascochyta blight of dry pea in Montana; (3) characterize and detect fungal species associated with ascochyta blight of dry pea in Montana; and (4) develop and apply real-time and convention PCR assays for rapid and sensitive detection of *Didymella pisi* associated with ascochyta blight of dry pea.

Linking statement

The research in this thesis is presented in six chapters, including four research chapters, two (chapters 2 and 3) of which has been published in peer-reviewed journal.

Chapter 1 consists of an introduction to the thesis and a review of literature of ascochyta blight of chickpea and dry pea. A summary of research objectives is presented at the end of this first chapter.

Chapter 2 reports the detection of QoI fungicide-resistant isolates of *D. rabiei* the causal pathogen of ascochyta blight of chickpea. The study went further to evaluate the isolates for insensitivity to QoI fungicide under greenhouse conditions, characterize the mechanism of resistance, and develop a qPCR for specific, sensitive, and rapid detection and quantification of QoI resistant isolates.

Chapter 3 shows the development and application of microsatellite markers developed for *D. pisi* genome to study the genetic diversity and population structure of *D. pisi* which is the most prevalent of the fungal pathogens associated with ascochyta blight of dry pea in Montana

Chapter 4 presents research in the characterization of fungal species associated with ascochyta blight of dry pea in Montana. The study evaluated the effects of temperature on the growth and development of these fungal species. In addition, the study demonstrated the use of a selective media that can effectively and consistently discriminate among the fungal species associated with AB of dry pea in North America.

In Chapter 5, non-polymorphic microsatellite markers developed in chapter 2 were selected and evaluated for specific detection of *D. pisi in-vitro* and *in-planta*. Validated markers were used in a qPCR and conventional PCR assays for specific, sensitive, and rapid detection and quantification of *D. pisi*.

Chapter 6 presents a general discussion of all the finding presented in this thesis. It follows a logical flow of discussion from QoI resistance in *D. rabiei* (Chapter 2) to rapid and specific detection of *D. pisi* (Chapter 5).

CHAPTER TWO

THE DETECTION AND CHARACTERIZATION OF QOI-RESISTANT *DIDYMELLA RABIEI* CAUSING ASCOCHYTA BLIGHT OF CHICKPEA IN MONTANA.

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CHAPTER TWO

THE DETECTION AND CHARACTERIZATION OF QOI-RESISTANT *DIDYMELLA RABIEI* CAUSING ASCOCHYTA BLIGHT OF CHICKPEA IN MONTANA

Abstract

Ascochyta blight (AB) of pulse crops (chickpea, field pea, and lentils) causes yield loss in Montana, where 1.2 million acres was planted to pulses in 2016. Pyraclostrobin and azoxystrobin, quinone outside inhibitor (QoI) fungicides, have been the choice of farmers for the management of AB in pulses. However, a G143A mutation in the cytochrome b gene has been reported to confer resistance to QoI fungicides. A total of 990 isolates of AB-causing fungi were isolated and screened for QoI resistance. Out of these, 10% were isolated from chickpea, 81% were isolated from field peas, and 9% isolated from lentil. These were from a survey of grower's fields and seed lots (chickpea = 17, field pea = 131, and lentil = 21) from 23 counties in Montana sent to the Regional Pulse Crop Diagnostic Laboratory, Bozeman, MT, United States for testing. Fungicide-resistant *Didymella rabiei* isolates were found in one chickpea seed lot each sent from Daniels, McCone and Valley Counties, MT, from seed produced in 2015 and 2016. Multiple alignment analysis of amino acid sequences showed a missense mutation that replaced the codon for amino acid 143 from GGT to GCT, introducing an amino acid change from glycine to alanine (G143A), which is reported to be associated with QoI resistance. Under greenhouse conditions, disease severity was significantly higher on pyraclostrobin-treated chickpea plants

inoculated with QoI-resistant isolates of *D. rabiei* than sensitive isolates (p-value = 0.001). This indicates that where resistant isolates are located, fungicide failures may be observed in the field. *D. rabiei*-specific polymerase chain reaction primer sets, and hydrolysis probes were developed to efficiently discriminate QoI- sensitive and - resistant isolates.

Introduction

The production of cool season pulse crops including chickpea (*Cicer arietinum* L.), field pea (*Pisium sativum* L.), and lentil (*Lens culinaris* Medik) in the Northern Great Plains of the United States is rapidly increasing. Montana is the leading producer of field peas and lentil in the United States, where 1.2 million acres were planted to pulses in 2016 (United States Department of Agriculture and National Agriculture Statistics Service, 2016). However, an increase in pulse production is accompanied by potentially yield-limiting diseases. Chief among these diseases is Ascochyta blight (AB). This is a host-specific disease caused by fungal species including *Didymella rabiei* (Kovachevski) v. Arx (anamorph *Ascochyta rabiei* (Pass) Labr) on chickpea, a species complex consisting of *Didymella pisi* (Barilli *et al*., 2016), *Peyronellaea pinodes*, and *Peyronellaea pinodella* on field pea (Aveskam *et al*., 2010), and *Didymella lentis* Kaiser, Wang and Rogers (anamorph *A. lentis* Vassiljevsky) on lentil (Barilli *et al*., 2016). AB can infect crops at all developmental stages and cause over 40–50% yield reduction under conditions suitable for disease development (Mondal *et al*., 2005; Wise *et al*., 2011). In faba bean, 90% losses have been reported (Pande *et al*., 2005 and Barilli *et al*., 2016). Symptoms of AB can develop on foliar and stem parts of the plant and also cause seed rot. AB is seed- and residue-borne. In the field, disease onset is normally post-flowering (growth stage R1)

through plant maturity (growth stage R8). “Infected seeds from diseased pods may be small, shrunken or discolored” (Ye *et al*., 2000 and Gossen *et al*., 2011). In addition to seed as a source of inoculum, *D. rabiei*, *D. pisi*, and *P. pinodes* also can subsist in the sexual and/or asexual forms (perithecia, pycnidia, and pseudothecia, respectively), producing ascospores and conidia that can provide a source of inoculum for disease epidemics (Tivoli and Banniza, 2007; Chilvers *et al*., 2009 and Wise *et al*., 2011).

Management of AB requires an integrated approach including the use of certified disease-free seeds, deep seeding depth, crop rotations of at least 3 years, tillage to bury plant debris, fungicide seed treatment to reduce seed transmission, the use of resistant cultivars and foliar fungicides for prevention or treatment of disease symptoms (Gossen and Derksen, 2003 and Wise *et al*., 2011). The use of resistant varieties and cultural practices can reduce AB, however, resistant varieties are not widely available in the Northern Great Plains. Breeding for resistance to AB is challenging in chickpea. This is because this trait is reported to be rare in the genetic resources available for chickpea (Sharma and Ghosh, 2016). In addition, negative genetic correlation has been reported between resistance to AB and other desirable traits. This was illustrated by Lichtenzveig *et al*. (2002), who pointed out the negative genetic correlation that existed when combining good resistance to AB and early flowering in chickpea. Current studies in the Middle East, North America and Australia are targeted at developing AB-resistant chickpea genotypes, especially resistance to pathotype IV *D. rabiei*, which is considered highly virulent (Bayaa *et al*., 2004; Imtiaz *et al*., 2011; and Sharma and Ghosh, 2016). Integration of molecular tools and conventional breeding approaches are being used to accelerate introgression of

AB-resistance genes in chickpea genotypes (Sharma and Ghosh, 2016). Fungicides are still widely used to achieve an acceptable level of disease control (Davidson and Kimber, 2007 and Lonergan *et al*., 2015). Broad spectrum protectant fungicides (chlorothalonil) are typically applied pre-flowering and can delay the onset of AB; however, once symptoms appear it is imperative for the grower to apply fungicides that provide a high level of control and move beyond the site of application in plant tissues due to canopy closure (Gan *et al*., 2006; Davidson and Kimber, 2007; Wise *et al*., 2009; and Lonergan *et al*., 2015). This concern is heightened in chickpea which is more susceptible to AB when compared with field peas and lentils. Thus, fungicides are frequently applied to chickpea fields and sparingly in field peas and lentils. Three registered fungicide classes that provide a premium level of control for the management of AB include succinate dehydrogenase inhibitors (SDHI; FRAC code 7), demethylation inhibitors (DMI; FRAC code 3), and quinone outside inhibitors (QoI; Fungicide Resistance Action Committee [FRAC] code 11) (Burrows, 2013 and Lonergan *et al*., 2015). These classes of fungicides are considered to have high to medium risk of resistance development (Fungicide Resistance Action Committee, 2015, 2016). This concern is elevated by the site-specific mode of action (MOA) of the fungicides, the polycyclic nature of the disease, airborne spores of the AB pathogens, and of the option of sexual reproduction for most species, allowing rapid mutation and allow inheritance by offspring. The polycyclic nature of the disease predisposes growers to repeat fungicide applications as disease severity can increase rapidly when the weather is favorable, particularly with AB of chickpeas (Banniza *et al*., 2011; Lonergan *et al*., 2015; and Fungicide Resistance Action Committee, 2016).

Currently, of the three classes of fungicide, QoI fungicides are the choice of most pulse growers for pre- and post-infection management of AB in the United States and Canada (Wise *et al*., 2008; Delgado *et al*., 2013; Lonergan *et al*., 2015; and Bowness *et al*., 2016). Prior to 2007, it was the only available fungicide MOA on pulse crops and resistance developed rapidly in North Dakota and Canada (Gossen and Anderson, 2004; Wise *et al*., 2011; and Bowness *et al*., 2016). In 2012, SDHIs were registered for use and these have largely been released as blends with other fungicide MOAs due to the high risk of resistance development. In addition, grower's preference of QoI- fungicides for disease control in pulse fields got a boost when the US Environmental Protection Agency (EPA) approved the use of pyraclostrobin (Headline ®) to benefit plant health on federally issued labels (Agweb, 2009). This plant health benefit of the QoI-fungicide was reported by Dimmock and Gooding (2002) to prolong grain filling in wheat crops. Furthermore, QoI-fungicide was reported to lower transpiration rates and also reduce the rate of senescence in wheat plants (Petit *et al*., 2012 and Mahoney *et al*., 2014).

This QoI class of fungicide inhibits mitochondrial respiration in the cytochrome bc1 complex (also known as respiratory chain complex III). The cytochrome bc1 complex facilitates electron transfer from ubiquinol to cytochrome c and links this transfer to proton translocation across the bc1 complex membrane via a mechanism called the proton-motive Q cycle (Brandt and Trumpower, 1994), resulting in ATP/energy production. The fungicide binds to the center of the quinone (Qo) site of the cytochrome bc1 complex (complex III) on the positive side of the inner mitochondrial membrane. This causes

depletion of adenosine triphosphate (ATP) that ultimately halts spore germination due to energy inadequacy (Grasso *et al*., 2006a; Wise *et al*., 2009; and Delgado *et al*., 2013).

Resistance to QoI fungicides has been reported in wheat pathogens such as *Microdochium nivale Blumeria graminis* f. sp. *tritici*, *Microdochium majus, Ourcosphaerella graminicola*, (Sierotzki *et al*., 2000; Amand *et al*., 2003; Walker *et al*., 2009; and Patel *et al*., 2012), and several other fungal pathogens including *Cercospora sojinia, Colletotrichum graminicola, Alternaria alternata, Botrytis cinerea, Pyricularia grisea, Podosphaera fusca, Pythium aphanidermatum, Pyrenophora teres*, and *Pseudoperonospora cubensis* (Ishii *et al*., 2001; Gisi *et al*., 2002; Avila-Adame *et al*., 2003; Kim *et al*., 2003; Ma *et al*., 2003; Sierotzki *et al*., 2007; Banno *et al*., 2009; Samuel *et al*., 2011; and Zeng *et al*., 2015). In addition, QoI resistance has been reported in *D. rabiei* in North Dakota and Canada (Gossen and Anderson, 2004; Wise *et al*., 2009; and Delgado *et al*., 2013). "The mechanism of resistance of *D. rabiei* has been attributed to single amino acid replacement in the cytochrome b protein of the *cytochrome bc1* complex" (Delgado *et al*., 2013). Currently, three amino acid substitutions are found in the cytochrome b protein of fungal plant pathogens that confer different degrees of resistance to QoI fungicides (Grasso *et al*., 2006a and Delgado *et al*., 2013). Low levels of resistance are bestowed by a substitution from phenylalanine to leucine at position 129 (F129L) and a substitution from glycine to arginine at position 137 (G137R) while a high level of resistance is conferred by the amino acid change from glycine to alanine at position 143 (G143A). Fungicide insensitivity have been categorized to two types: quantitative and qualitative. With quantitative insensitivity, the pathogen becomes less sensitive to the

fungicide, although higher rates of the fungicide are still effective. Qualitative insensitivity predisposes the pathogen to become completely insensitive to the active ingredient and disease control is no longer achieved at recommended field application rates. Insensitivity to the QoI-fungicides has previously been reported to be qualitative (Delgado *et al*., 2013 and Bowness *et al*., 2016). QoI-resistant *D. rabiei* isolates have been identified in North Dakota and Montana (Wise *et al*., 2008, 2009), where the mechanism of resistance was identified as the G143A mutation in the former (Delgado *et al*., 2013). However, in Montana, there has not been a statewide survey to monitor for resistance to QoI fungicide in pulse crops. There is an urgent need to develop a robust screening and monitoring strategy for QoI resistance to help prevent the spread of QoI-resistant AB pathogens in the rapidly increasing pulse acreage in Montana. Thus, the objectives of this study were to (1) determine the presence of resistance to QoI fungicides in AB pathogens from chickpea, field pea, and lentil in Montana; (2) determine the mechanism of resistance associated with QoI-resistant isolates; and (3) develop a robust multiplex real-time PCR diagnostic tool for screening and monitoring of QoI resistance.

Materials and Methods

A Collection of D. rabiei, D. pisi, and D. lentis Isolates**.** Isolates of *D. rabiei*, *D. pisi*, and *D. lentis* were obtained from four general sources. Most isolates were obtained from chickpea, field pea and lentil seed lots submitted by growers in 23 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT for testing during the 2014, 2015, and 2016 growing seasons (Table 1). A second set of isolates were obtained from chickpea and field pea production fields in Montana where QoI fungicides

had been applied. Fields containing chickpea and field pea plants with AB symptoms were sampled on a “W” pattern, with samples taken at a set of intervals of approximately 15 m. The third set of isolates were collected from chickpea and field pea plants with AB symptoms sampled by growers and submitted to the Schutter Diagnostic Laboratory, Bozeman, MT. Finally, some isolates were also obtained courtesy of Julie Pasche at North Dakota State University, Fargo, ND, United States.

For a standard AB seed test, seed (chickpea n= 600, field pea = 400, and lentil = 400) were sterilized in a 1% free chlorine solution for 10 min (International Rules for Seed Testing [IRST], 2017). The solution was drained, and seeds were air-dried in the biological cabinet for 30 minutes. Dried seeds (n = 10 per plate) were plated on potato dextrose agar (PDA) (Alpha Biosciences Inc., Baltimore, MD, United States). Mycelial growth was noticed from the plated seeds after 11 to 14 days incubation at 20 ^{o}C +/- 1 ^{o}C in the presence of a routine cycle of cool white fluorescent light (12 h light followed by 12 h dark). The presence of AB pathogens was confirmed by viewing the conidia at 40x magnification.

From plants, isolates were also obtained from a single lesion on symptomatic leaves and stems by cutting the tissue into 3- to 4-cm sections. Stem or leaf sections were immersed in 1% Sodium hypochlorite for 30 s and rinsed for 30 s in sterile distilled water. Sterilized stem or leaf sections were air-dried in a biological cabinet, placed on PDA and incubated under the conditions described above. Confirmation of the pathogen was conducted as previously described. Conidium of individual isolates from infected seed lots (n = 5 to 10) and from symptomatic leaves or stems were incubated on PDA under the conditions previously described. To isolate single spores, three pycnidia from a 10-day old

culture were dropped into 2 ml screw cap tube (MP Biomedicals) containing five ceramic beads (MP Biomedicals), 300 ml of sterile water and 0.05% (v/v) tween-20. The mixture was homogenized using a Beadbug homogenizer (Benchmark BeadBug Homogenizer, Benchmark Scientific, NJ, United States) for 60 s at 4000 rpm. The supernatant was removed into a clean 1.5 ml eppendorf tube and diluted 100-fold in sterile water. From the diluted suspension, 100 ml was inoculated on fresh PDA plates and incubated at 20 ^{o}C +/- 1 ^{o}C under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark). Single spores germinated after 3–5 days. Isolates were stored long-term as conidia on sterile filter paper and as mycelia in 15% sterilized glycerol at –80 ^{o}C (Skoglund *et al*., 2011).

Table 1 | Isolates of *Didymella rabiei*, *D. pisi*, and *D. lentis* were obtained from chickpea, field pea, and lentil seed lots sent by growers to the Regional Pulse Crop Diagnostics Laboratory (RPCDL) in Bozeman, MT, United States for planting during 2014, 2015, and 2016 growing season.

Collection location by county	**Number of seed lots sampled**	**Total number of isolates**	**Isolates with quinone outside inhibitor (QoI) resistance[a]**
Cascade	6	39	0
Chouteau	2	3	0
Daniels	28	235	5
Dawson	7	38	0
Gallatin	5	16	0
Glacier	2	5	0
Hill	7	45	0
Liberty	2	6	0
McCone	24	92	4
Musselshell	1	1	0
Philips	4	9	0
Pondera	5	6	0
Richland	3	16	0
Roosevelt	23	133	0
Sheridan	15	81	0
Teton	2	4	0

Valley	24	213	2
Yellowstone	2	9	0
Toole	1	2	0
Broadwater	1	2	0
Flathead	1	5	0
Blaine	2	15	0
Garfield	2	15	0
Total	169	990	11

[a]An isolate was considered resistant if it has the G143A mutation in its cytochrome b gene.

Screening of Didymella spp. Isolates for QoI Fungicide Resistance Using a Discriminatory Dose. A total of 990 AB causing isolates were screened for QoI resistance. Of these, 10% were from chickpea, 81% from field peas, and 9% from lentil from seed lots (chickpea = 17, field pea = 131, and lentil = 21) submitted to the RPCDL from 23 counties in Montana. Screening of the isolates was conducted using an *in vitro* agar plate assay according to published methods (Wise *et al*., 2008) with some modifications. Stock solutions of technical grade formulations of pyraclostrobin (99% active; BASF Corporation, Research Triangle Park, NC, United States) were prepared at a concentration of 5 mg/ml and diluted in acetone. Salicylhydroxamic acid (SHAM; Sigma–Aldrich) was dissolved in methanol and added to all fungicide-amended media at a concentration 100 mg/ml. SHAM minimizes the effect of the alternative oxidative pathway that some fungi use to evade QoI fungicide toxicity in *in vitro* fungicide sensitivity assays (Olaya and Köller, 1999; Bartlett *et al*., 2002; Wise *et al*., 2008, 2009; and Lonergan *et al*., 2015). *D. rabiei* and other AB pathogens can utilize this alternative pathway in the presence of QoI fungicides. SHAM has been reported to have no side effects on conidial germination (Wise *et al*., 2008). The 0 mg/ml treatment served as a control and was amended with 100 mg/ml SHAM, 1 ml of acetone, and 1 ml of methanol per liter.

In addition to agar plate assay, isolates from all the three hosts were screened using a mismatch amplification mutation assay PCR (MAMA-PCR) (Delgado *et al*., 2013). This PCR-based assay was used to detect mutant isolates of *D. rabiei* bearing the A143 allele of the *cytochrome b* gene. Isolates that had a mycelial growth on fungicide amended media

that was 70% of the control plate (without pyraclostrobin fungicide) and that also amplified with the MAMA-PCR were selected for total RNA extraction. Only 11 isolates of *Didymella rabiei* met the two criteria. No isolates of *D. pisi* or *D. lentis* met either of the criteria.

Total RNA Extraction**.** Selected isolates were cultured on PDA at 22 ^{o}C for 7 days at 12 h light. Total RNA was isolated from the fungal isolates using the RNeasy Plant Mini kit (QIAGEN) with alterations in the starting process. Fresh fungal mycelium of each isolate (100 mg) from a 7-day old culture was scraped into a 2 mL screw cap tube (MP Biomedicals) containing 450 mL RLC buffer. The mycelium was disrupted using the BeadBug Benchtop homogenizer (Benchmark Scientific, NJ, United States) set at 3500 rpm for 60 s, and centrifuged at 13, 000 *g* for 1 min. About 400 mL lysate was then transferred to a QIAshredder spin column placed in a 1.5 mL collection tube. From this stage onward, the protocol followed the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000c at 260 nm (Thermo Scientific, United States) and adjusted to a final concentration of 100 ng/mL.

Synthesis of Complementary DNA, RT-PCR, and Sequencing**.** The first-strand complementary DNA (cDNA) was synthesized using a RevertAid-Reverse Transcriptase kit (Thermo scientific). The cDNA was used in a PCR assay to amplify the coding sequence for amino acid codons 127–276 of the *cytb* gene from *D. rabiei*. This region has been reported to have the G143A mutation and other mutations that confer resistance to QoI fungicides (Fraaije *et al*., 2002 and Delgado *et al*., 2013).

Standard PCR was conducted in a T100 Biorad thermocycler (Bio-Rad Inc.) with Phusion High-Fidelity PCR master mix, 10 *p*mol each of primer (Delgado *et al*., 2013) and 50 ng of cDNA template in a final volume of 50 mL. The reaction conditions were: 94 ^{o}C for 5 min, followed by 35 cycles at 94 ^{o}C for 30 s, 55 ^{o}C for 1 min and 72 ^{o}C for 1 min. PCR was terminated with an extension at 72 ^{o}C for 5 min. PCR products were analyzed on ethidium bromide-stained 1.5% (w/v) agarose gels run in the 1x tris-acetate-EDTA buffer and exposed to UV light to visualize DNA fragments. Isolates with an expected product of 675 bp were purified directly from PCR products using alcohol precipitation. The purified PCR products were sequenced with primer pair used for the amplification (Table 2), in both directions (MCLAB DNA sequencing services).

Effect of G143A Mutation on *D. rabiei* Fungicide Sensitivity on Disease Control**.** Greenhouse trials were conducted to determine the level of *in vivo* disease control attainable with QoI fungicides against isolates classified as susceptible or resistant to QoI fungicides based on sequencing results. Five QoI-sensitive *D. rabiei* isolates (AR-405, AR-407, AR-419, AR-439, and AR-430) (Wise *et al*., 2008) and five QoI-resistant isolates (AR-R001 to AR-R005) were included in the trial (Table 3). The five QoI-resistant isolates were isolated from a chickpea seed lot submitted to the RPCDL for seed testing, and five QoI-sensitive isolates were from a baseline population (Lonergan *et al*., 2015). The QoI sensitivity of these five isolates were determined using pyraclostrobin amended PDA, MAMA-PCR, and mutation analysis of their *cytb* gene.

The greenhouse experiments were performed following Pasche *et al*. (2004, 2005) and Wise *et al*. (2009). Briefly, chickpea seeds (cv. Troy) were obtained from Washington

State Crop Improvement Association (WSCIA). Troy is a moderately resistant chickpea cultivar. The seeds were tested free of seed borne AB and were sown at one plant per pot in 80 ml plastic cones filled with a mixture of peat and Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA, United States) at ratio 1:1, and grown at 22 ^{o}C. Fourteen days after planting, chickpea plants were treated with commercial formulations of pyraclostrobin (Headline, 2.09 EC; BASF Corporation) at concentrations of 0, 0.1, 1.0, 10, and 100 mg a.i./ml of water. Fungicides were applied to runoff using a CO_2-powered Generation III Research Track sprayer (DeVries Manufacturing, United States). About 24 h after fungicide application, chickpea plants were inoculated with a conidial suspension obtained from 14 old culture of QoI-resistant and sensitive *D. rabiei* isolates. Within an hour made conidial suspensions were adjusted to a concentration of 3 x 10^5 conidia/ml and applied to chickpea plants. Inoculum from each isolate was applied to plants using hand-held spray bottles. Chickpea plants were placed in a mist chamber and held at >90% relative humidity for 36 h at a 14 h photoperiod under artificial lighting. After 11 days, disease severity was assessed visually based on the percent leaf area infected of the whole plant (Reddy and Singh, 1984). The experiment was laid out as a randomized completely block design (RCB). Nine replicates (one plant per replicate) were included in each experiment, and the disease severity was calculated for each observational unit. Percent disease control was calculated by: "[1 – (% diseased tissue/% disease on 0 mg/ml control)] 100" (Wise *et al*., 2009). Homogeneity of the variances from the two greenhouse experiments was determined by the Levene's test (Levene, 1960). Data were converted to percent disease control to enhance direct comparisons between QoI-sensitive and resistant

isolates at each fungicide concentration and analyzed using the generalized linear mixed-effects model in lm4/ nlme statistical package (R Core Team, 2013).

Development of a Multiplex Hydrolysis Probe Assay for the Detection of QoI-Resistant (G143A) and QoI-Sensitive *D. rabiei* Isolates**.** For simultaneous detection and differentiation of the *D. rabiei* G143A mutants from the sensitive isolates, a primer pair (A243 and A244) and two hydrolysis probes (A245res and A246ses) were designed for a multiplex real-time PCR assay to amplify a 92 bp fragment of the *cytb* gene. The 5^0 ends of the probes A245res and A246ses were labeled with 6-carboxy fluorescein (FAM) and cyanine 5 (Cy5), while their 3^0 ends were labeled with Iowa black-FQ and Iowa black-RQ quencher, respectively. The primers were designed to flank the region of the G143A mutation, while the two fluorogenic dyes enable the multiplex differentiation between resistant and sensitive alleles. To enhance the efficiency of the probes both probes were designed to have T_m values at least 7 C higher (69 ^oC) than that of the primers (62 ^oC), and their GC content was higher than 45%. The multiplex TaqMan assay was optimized in a final volume of 20 mL containing 10 mL of EconoTaq Plus 2x master mix according to manufacturer's recommendations (Lucigen Corporation, Middleton, WI, United States), 25 pM of each primer (A-243, A-244), 10 pM of each probe (A-245res, A-246sens) and 3 mL of DNA extract. Cycling parameters were 4 min at 94 ^oC, followed by 35 cycles of 15 s at 94 ^oC and 30 s at 64 ^oC and a final extension at 72 ^oC for 5 min completed the PCR. Primers and hydrolysis probes were synthesized by Integrated DNA Technology (IA, United States). The assay was developed, evaluated and analyzed on the Biorad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

In addition, evaluation of the assay efficiency was determined by plotting cycle thresholds for a six times tenfold dilution starting with 1000 ng of DNA obtained from resistant and sensitive isolates against DNA concentration to yield the standard curves. The result from experiments in the multiplex assay was compared to the uniplex assay using tenfold dilutions from 1000 ng to 1 pg DNA extracts from only isolates that contained the G143A mutation.

Table 2 | Primers pairs used for amplification of the cytochrome b gene fragment of *Didymella rabiei* and for detecting the G143A mutation.

Primers	**Primer sequence (5^0 –3^0)**	**Annealing temperature**	**Reference**	**Primer pair purpose**
A99	TATTATGAGAGATGTAAATAATGG	46 ºC	Delgado *et al*., 2013	Sequencing of cytochrome b gene
A100	CCTAATAATTTATTAGGTATAGATCTTA	46 ºC	Delgado *et al*., 2013	Sequencing of cytochrome b gene
A243	GCTTTCCTGGGTTACGTTCT	64 ºC	This study	Multiplex TaqMan PCR
A244	CCAACTCATGGTATAGCACTCAT	64 ºC	This study	Multiplex TaqMan PCR

A245res	FAM-TGGGCAAATGTCACTATGAGCTGCTACAG-BHQ1	64 ºC	This study	QoI-resistant (A143 allele)	probe
A246sens	Cy5-TGGGCAAATGTCACTATGAGGTGCTACAG-BBQ	64 ºC	This study	QoI-sensitive (G143 allele)	probe

Table 3 | List of *D. rabiei* isolates used for the in vivo assay.

Isolate ID	**QoI status**	**Location by state**
AR-R001	Resistant	Montana
AR-R002	Resistant	Montana
AR-R003	Resistant	Montana
AR-R004	Resistant	North Dakota
AR-R005	Resistant	North Dakota
AR 405	Sensitive	Idaho
AR 407	Sensitive	Idaho
AR 439	Sensitive	Washington State
AR 411	Sensitive	Idaho
AR 430	Sensitive	Idaho

Results

From the screening, only 11 isolates of *D. rabiei* amplified with the MAMA-PCR and also had a mycelial growth on fungicide amended media that was 70% of the control plate (without pyraclostrobin fungicide). Multiple alignment analysis of amino acid sequences of the *cytb* gene of the detected QoI-resistant *D. rabiei* isolates showed a mutation that replaced the codon for amino acid 143 from GGT to GCT, resulting in an amino acid change from glycine to alanine (G143A) (Figure 1). Other known mutations such as (F129L) and (G137R) were not found in the protein sequences of our QoI-resistant isolates. However, none of the isolates of *D. pisi* and *D. lentis* amplified using MAMA-PCR.

Effect of the G143A Mutation on *D. rabiei* Fungicide Sensitivity**.** Independent analysis of greenhouse disease control experiments showed that variances were homogeneous, and the two experiments were combined for further analysis (*p*-value = 0.05). Disease severity was significantly higher on chickpea plants inoculated with G143A mutant isolates at all concentrations of pyraclostrobin. Percent disease control from the non-treated was calculated to directly compare the two isolate groups. Disease control of G143A mutant isolates was significantly reduced in the pyraclostrobin treatments when compared to wild type isolates at all fungicide concentrations (*p*-value <0.001) (Figure 2). About 75% disease control was observed at 10 and 100 mg/ml in wild-type isolates while <25% disease control was observed in G143A mutant isolates.

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AR-R001  VNNGWLIRYLHSNTASAFFFIVYLHIGRGMYYGSYRAPRTLVWTIGTVIFILMMATAFLG 60
AR-R002  VNNGWLIRYLHSNTASAFFFIVYLHIGRGMYYGSYRAPRTLVWTIGTVIFILMMATAFLG 60
AR-R003  VNNGWLIRYLHSNTASAFFFIVYLHIGRGMYYGSYRAPRTLVWTIGTVIFILMMATAFLG 60
AR-405   VNNGWLIRYLHSNTASAFFFIVYLHIGRGMYYGSYRAPRTLVWTIGTVIFILMMATAFLG 60
AR-407   VNNGWLIRYLHSNTASAFFFIVYLHIGRGMYYGSYRAPRTLVWTIGTVIFILMMATAFLG 60

                 G143A
                   *
AR-R001  YVLPYGQMSLWAATVITNLMSAIPWVGQDIVEFIWGGFSVNNATLNRFFSLHFVLPFVLA 120
AR-R002  YVLPYGQMSLWAATVITNLMSAIPWVGQDIVEFIWGGFSVNNATLNRFFSLHFVLPFVLA 120
AR-R003  YVLPYGQMSLWAATVITNLMSAIPWVGQDIVEFIWGGFSVNNATLNRFFSLHFVLPFVLA 120
AR-405   YVLPYGQMSLWGATVITNLMSAIPWVGQDIVEFIWGGFSVNNATLNRFFSLHFVLPFVLA 120
AR-407   YVLPYGQMSLWGATVITNLMSAIPWVGQDIVEFIWGGFSVNNATLNRFFSLHFVLPFVLA 120

AR-R001  ALALMHLIVLHDTAGSGNPLGVSGNYERISFAPYFIFKDLITVFAFIFVLSLFVFFMPNV 180
AR-R002  ALALMHLIVLHDTAGSGNPLGVSGNYERISFAPYFIFKDLITVFAFIFVLSLFVFFMPNV 180
AR-R003  ALALMHLIVLHDTAGSGNPLGVSGNYERISFAPYFIFKDLITVFAFIFVLSLFVFFMPNV 180
AR-405   ALALMHLIVLHDTAGSGNPLGVSGNYERISFAPYFIFKDLITVFAFIFVLSLFVFFMPNV 180
AR-407   ALALMHLIVLHDTAGSGNPLGVSGNYERISFAPYFIFKDLITVFAFIFVLSLFVFFMPNV 180
```

Figure 1 **|** The partial protein sequence of the cytochrome b gene of five isolates of *Didymella rabiei* with different Qol sensitivities. Star indicates the G143A amino acid substitution responsible for decreased sensitivity to Qol fungicides. Dark gray highlighted areas represent 100% identities.

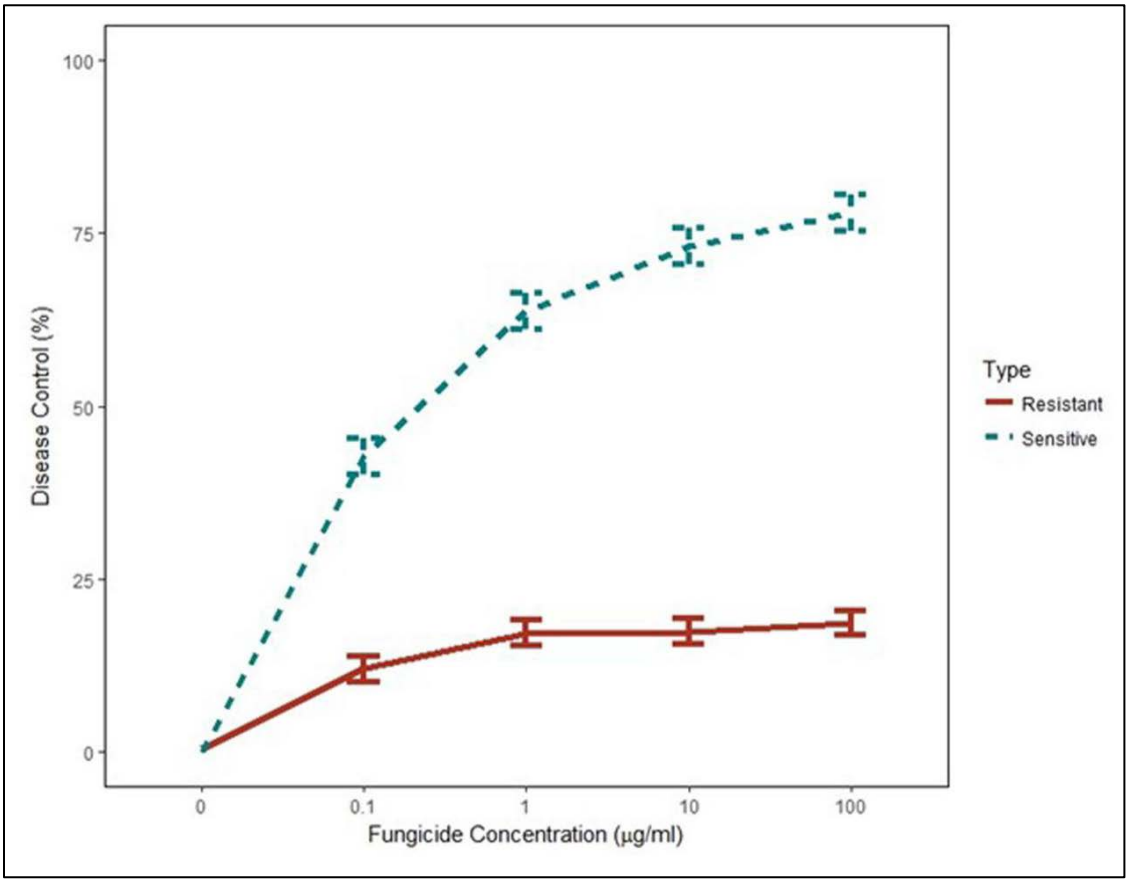

Figure 2 | Mean in vivo percent disease control for five Qol-sensitive and five Qol-resistant *Didymella rabiei* isolates to pyraclostrobin fungicide concentration (mg/ml). Values include standard errors of disease control measurements obtained from one plant across nine replications in two experiments.

Detection of QoI-Resistant (G143A) and QoI-Sensitive *D. rabiei* Isolates Using a Multiplex Hydrolysis Probe Assay. By deploying the single nucleotide polymorphism (SNP) hydrolysis probe assay, it was possible to detect the G143A mutation and discriminate between QoI- resistant and QoI-sensitive isolates. The SNP could be distinguished using specific probes in which a nucleotide proximal to the 3^0 is complementary to one allele but forms as a mismatch with the second allele. The annealing temperature was validated in a temperature gradient assay, the optimum annealing temperature was 64 ^oC for both multiplex and uniplex (data not shown). The optimum concentration of primers and probes that gave the highest reporter fluorescence and the lowest threshold cycle was 20 and 10 mM, respectively, in both tests. To confirm the assay can simultaneously detect the two alleles, DNA from wild-type (QoI-sensitive) and G143A mutant (QoI-resistant) isolates were mixed in the same proportion. Satisfactory discrimination was achieved between the two alleles (Figure 3). This emphasized the accuracy of this assay, by its capacity to detect either of the alleles both in a multiplex or uniplex assays, respectively. Standard curves were constructed based on the tenfold dilution series of the wild-type and mutant isolates (Figure 4). There was linearity in the amplification across the DNA dilutions and correlation coefficients for the standard curve of the DNAs from wild types and G143A mutant isolates were 0.998 and 0.991, respectively (Table 4). The y-axis on the amplification plot measures the relative fluorescence units (RFU), a measure of the amplified DNA, while cycling threshold value (cq) on the x-axis, is inversely proportional to the initial concentration of nucleic acid template in each sample, which correlates to the number of copies in each sample

Table 4 | Slope, efficiencies, correlation coefficients (R^2), and y-intercepts from amplification of serial dilutions of DNA from *Didymella rabiei* isolates sensitive and resistant to quinone outside inhibitor fungicides using TaqMan single-nucleotide polymorphism assay to detect G143A mutation.

Allele	**Slope**	**Efficiency (%)**	R^2	***y*-intercepts**
Wild-type-sensitive	3.39	97.2	0.998	37.705
Mutant-resistant	2.59	142.7	0.991	36.531

Table 5 | Distribution of Ascochyta isolates collected per crop in 2014–2016 from Montana.

Crop	**Number of Counties sampled**	**Total Number of seed lots**	**Total number of isolates**	**Isolates with QoI resistance**
Chickpea	9	17	88	11
Field pea	17	131	810	0
Lentil	7	21	92	0
Total		169	990	11

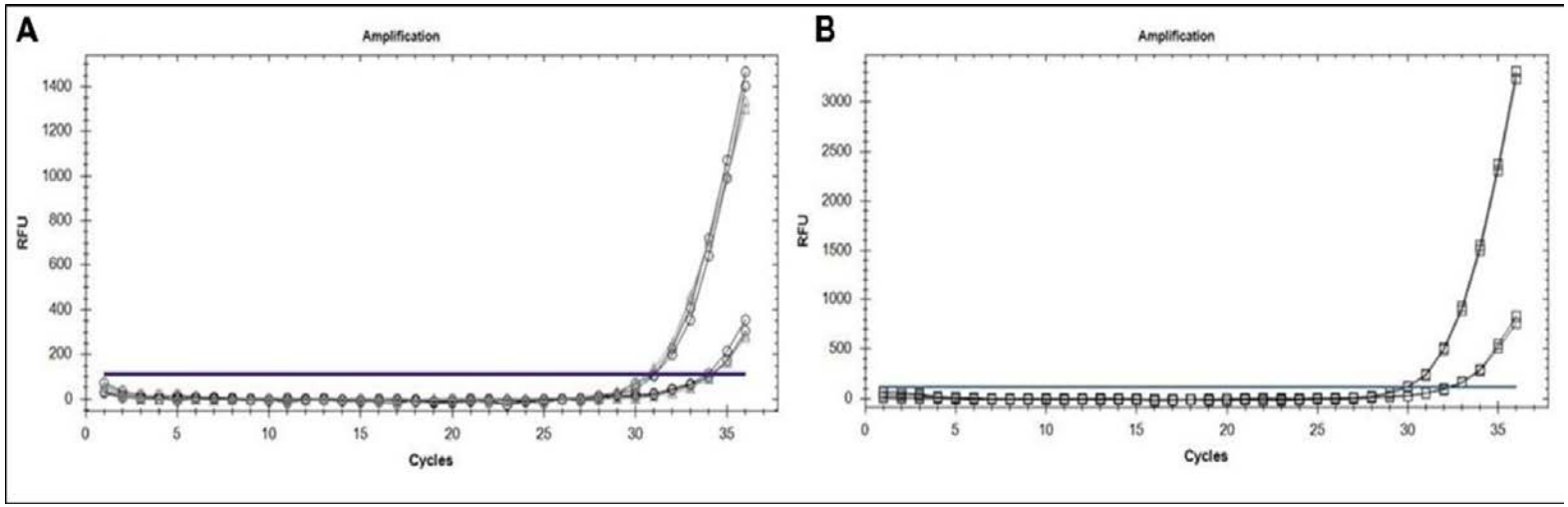

Figure 3 | **(A)** Amplification curves showing the detection efficiency of sensitive (o) and resistant (Δ) alleles using the mixtures of DNA from Qol-resistant and Qol-sensitive isolates as a template. **(B)** Amplification curves showing the detection efficiency of resistant (□) alleles in uniplex TaqMan real-time PCR using DNA from Qol-resistant isolates as a template

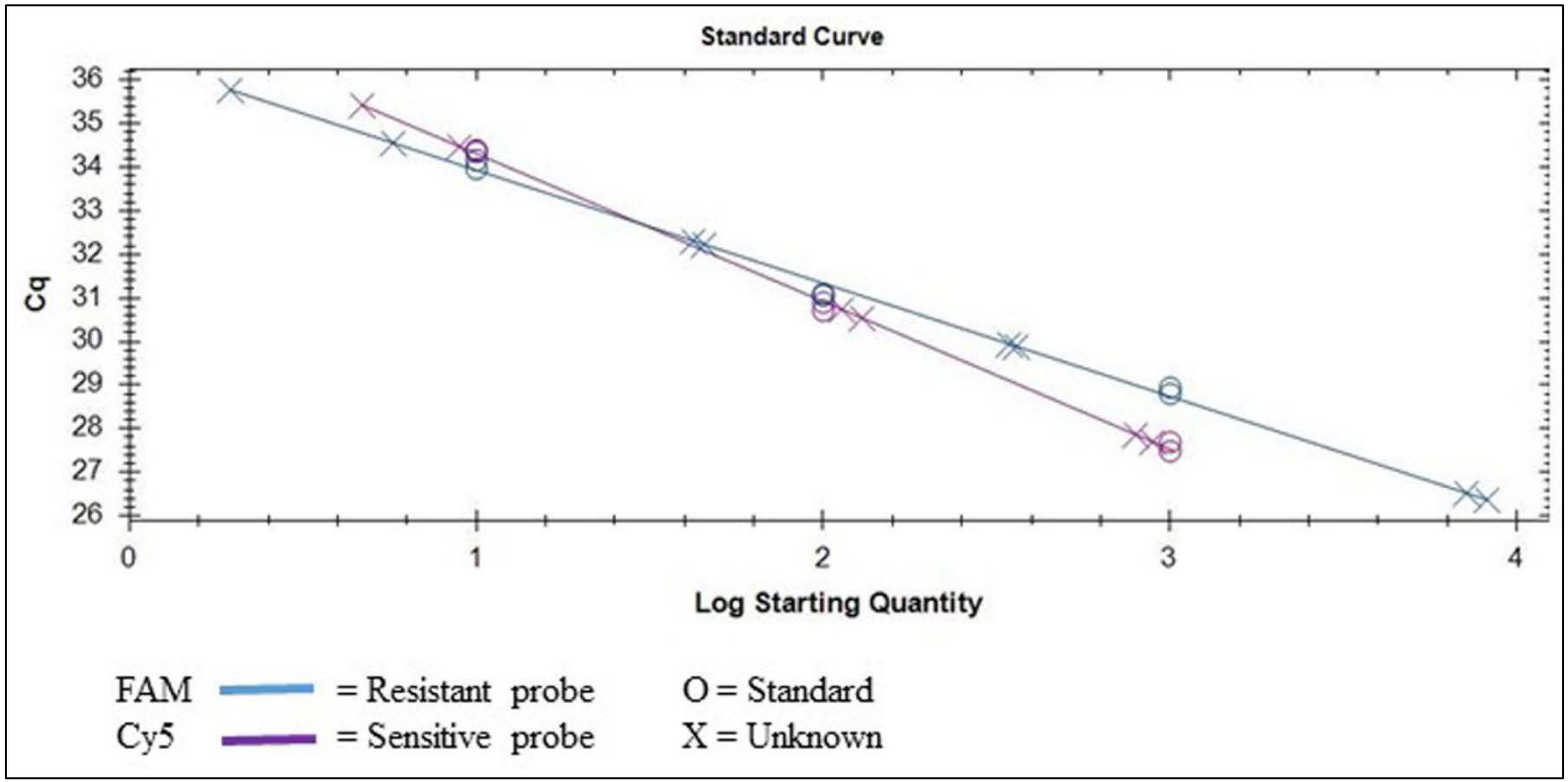

Figure 4 | Standard curve obtained by using the multiplex SNP TaqMan assay to detect the G143A mutation in *D. rabiei* isolates collected from Montana.

Discussion

According to our findings, the G143A mutation is responsible for QoI fungicide resistance in *D. rabiei* isolates from Montana chickpea fields. The gene structure of the *cytochrome b* gene of *D. rabiei* appears to be favorable for the development of a SNP associated with QoI resistance at codon 143 (Delgado *et al*., 2013). In contrast, the G143A mutation has not been reported in fungal species that have an intron downstream of codon 143 (Grasso *et al*., 2006b; Sierotzki *et al*., 2007; Banno *et al*., 2009; Samuel *et al*., 2011; Delgado *et al*., 2013; and Zeng *et al*., 2015). Thus, there is no reported case of QoI resistance in this type of *cytochrome b* gene structure. For example, fungal species such as *A. solani* do not show a G143A mutation due to the lethal effect of proximal exonic flanking sequences on the 5^0-splice (Lambowitz and Belfort, 1993; Pasche *et al*., 2005; Grasso *et al*., 2006b; Sierotzki *et al*., 2007; Banno *et al*., 2009; and Delgado *et al*., 2013). However, G143A mutation has been reported to be responsible for QoI fungicide resistance in *C. sojina*, the causal organism of frog eye leaf spot of soybean (Zeng *et al*., 2015) and in *B. cinerea*, the causal organism of gray mold (Samuel *et al*., 2011). In these two organisms, the mutation does not occur in the 5^0-splicing site. QoI fungicides do not control fungi bearing the G143A mutation, while those containing the F129L and G137R are controlled to some degree, but at a lower level than wildtype isolates. Similar results were obtained in a study in North Dakota (Wise *et al*., 2009 and Delgado *et al*., 2013), where the mechanism of resistance of QoI-resistant *D. rabiei* isolates was reported to be a G143A mutation. Noticeably, all the three counties where QoI-resistant *D. rabiei* isolates were

found are near North Dakota indicating the QoI-resistant isolates detected could have either spread from North Dakota via seed or were selected for in Montana chickpea fields.

Quinone outside inhibitor fungicide resistance was observed in isolates of *D. rabiei* collected in Montana during the 2012 and 2015 growing seasons (M. Burrows, Personal communication). Prior to this, QoI-resistant isolates were reported in 2009 from chickpea fields in North Dakota (Wise *et al*., 2009) and have been largely maintained in the population (Delgado *et al*., 2013). From both seed testing results and the current in-field survey of Montana pulse fields, the frequency of resistant isolates is thus far low (B. Agindotan, personal communication, March 2016). This is different from the report of high frequency of QoI-resistant isolates in North Dakota (Wise *et al*., 2009; and Delgado *et al*., 2013). This may be due to the comparatively low relative humidity and reduced disease pressure in Montana vs. North Dakota. The relative humidity level in North Dakota is 12.1% higher than Montana in chickpea growing areas (ClimaTemps, 2015). Though this study was targeted at AB of chickpea, field pea, and lentil, only 11 isolates of *D. rabiei* from three seed lots from a larger study of 88 isolates from 17 seed lots were confirmed to be resistant to pyraclostrobin. All isolates contained the G143A mutation which confers resistance to all QoI fungicides (Table 5). The frequency of QoI resistance might be on the rise in *D. rabiei* because, from observations during the 2016 crop year, USDA-NASS statistics and grower testimonies, there is increasing chickpea acreage and multiple applications of fungicides to ward off potential fungal attacks. Many of these applications were QoI products solely and in combination with either chlorothalonil or SDHI fungicides such as fluxapyroxad. The proportion of QoI fungicide applied solely is higher than that

applied in combination with other fungicides. However, continuation of the practice of applying multiple applications of fungicides including high-risk products such as QoI and SDHI fungicides will select for resistance development. These active ingredients are available as seed treatments and foliar products. Although education is underway, fungicide decisions are often driven by the price and efficacy of the product more than the MOA.

In contrast with chickpea, field pea and lentil fields are rarely treated with fungicides. This is due to the low foliar disease occurrence in Montana to date. Since seed testing was started in Montana in 2000, the percent of seed lots with at least one seed of 500 infected by AB has increased from 0% (2000–2002) to as high as 25% through 2009. Since the 2010 crop year, the level has increased to 60%. It was 80% in 2017. To date, we have very rarely observed QoI resistance in AB pathogens recovered from a field pea seed lots and never observed QoI resistance in AB recovered from lentil seed lots. This correlates with the low frequency of fungicide application in these crops.

Differences in disease control were observed when QoI-resistant and QoI-sensitive *D. rabiei* isolates were inoculated on pyraclostrobin-treated chickpea plants. Applications of pyraclostrobin at a concentration of 100 mg/ml provided less than 25% control of disease on chickpea plants infected with QoI-resistant isolates. This amount of control is unacceptable in field production. Disease severity of AB was higher on fungicide-treated chickpea plants inoculated with QoI-resistant *D. rabiei* isolates that on plants inoculated with QoI-sensitive isolates study. Several studies have not observed a fitness cost associated with G143A substitution in cytb in fungal pathogens (Chin *et al*., 2001; Avila-

Adame *et al*., 2003; Karaoglanidis *et al*., 2011; and Veloukas *et al*., 2014). However, once established, resistance is likely to be preserved in the population due to the selective advantage if QoI fungicides continue to be applied. This lack of disease control in QoI-resistant isolates was confirmed in a report from North Dakota (Wise *et al*., 2009). In that study, <50% disease control was achieved with the applications of 100 mg a.i/ml pyraclostrobin to chickpea plants inoculated with QoI-resistant isolates. Considering this, monitoring of QoI resistance in AB pathogens infecting pulse crops is important to prevent the establishment of resistant populations. Due to the widespread occurrence of AB pathogens in seed lots in Montana, favorable environmental conditions will likely lead to a widespread epidemic of the pathogen. Frequent fungicide applications in those circumstances will lead to the establishment of resistant isolates and then additional management strategies will need to be used more effectively to manage fungicide resistant AB pathogens. The risk of fungicide resistance development can be lowered by limiting the number of applications of single-site fungicides with the same MOA, rotation among fungicides with different biochemical modes of action or by using blends of fungicides with different modes of action. This approach was very effective in the control of fungicide-resistant strains of sclerotinia dollar spot and pythium blight in turfgrass (Couch, 2002, 2003).

The development of QoI resistant isolates in the epicenter of pulse production in Montana could cause significant problems for the industry, which in 2016 occupied 1.2 million acres and was valued at $322 million dollars (United States Department of Agriculture and National Agriculture Statistics Service, 2016). To contain the spread of

QoI- resistance, monitoring is key. PCR-based tools like the one developed in this study are needed to monitor the shift in fungicide sensitivity of field population of fungal pathogens. Furthermore, integrated pest management practices including crop rotation, tillage to bury infested residue, and host resistance (where available) can help reduce the risk of fungicide resistance (Zeng *et al*., 2015). There should be caution in the application of foliar fungicides for reasons other than disease control including 'plant health benefit.' Other practices to prevent fungicide resistance development include restricting the number of QoI fungicide applications to two to four per season as specified on the label, using QoIs preventatively rather than after disease has developed, not allowing sequential applications of QoI products, as specified on the label, rotating fungicide modes of action when multiple applications are necessary, using pre-mixtures or tank mixtures different modes of action, and always applying the recommended labeled rate (Kitchen *et al*., 2016). The minimum recommended rates of each fungicide in the tank mix should be used (Damicone and Smith, 2009). Failure to practice these guidelines will apply additional selection pressure on *D. rabiei* and other fungal pathogens which will consequentially result in fungicide resistance development.

Various polymerase chain reaction- based methods such as PCR-RFLP and CAPS methods have been developed for the detection of G143A mutation. However, limited quantitative methods such as allele-specific real-time PCR have been developed for a few pathogens (Samuel *et al*., 2011; Zeng *et al*., 2015). The real-time PCR assay enhances high throughput and accuracy during QoI-sensitivity screening of isolates when compared to the conventional *in vitro* test using fungicide amended PDA plates. This test takes about 7 to 10 days to get a result, in addition to the logistics required to screen multiple isolates. The

hydrolysis probes were designed to have T_m values at least 7 C higher (69 ^oC) than that of the primers (62 ^oC), and their GC content was higher than 45%, improving the specificity of the assay in discriminating sensitive from resistant alleles. Furthermore, modification of the primers and probes avails this assay the potential to monitor G143A mutation in *D. pisi*, *D. lentis*, and *A. alternata*, thus it can serve as a tool to monitor QoI resistance in other fungal pathogens and also for routine screening of fungal isolates in diagnostic laboratories. In addition, this technique is suitable for future research targeted at determining modification in the frequency of G143 and A143 alleles and also to determine the fitness of QoI-resistant and sensitive isolates of fungal pathogens.

Conclusion

This study was successful in detecting the presence of QoI-resistant *D. rabiei* isolates, characterizing the mechanism of resistance and developing a diagnostic tool for QoI resistance in *D. rabiei* that will allow high throughput and accurate screening of G143A mutants. Other researchers have been successful in developing a molecular technique to detect the G143A mutation (Delgado *et al*., 2013). This is a qualitative assay that cannot be used to monitor the frequency of G143 and A143 alleles both in individual isolates and in mixed field populations. The assay reported in this study streamlines the detection process. This process could be used for large-scale surveys, as well as rapid identification of insensitivity to QoI fungicides. Furthermore, this technique can be used in studies to determine changes in the frequency of G143 and A143 alleles in *D. rabiei*.

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CHAPTER THREE

FIRST MICROSATELLITE MARKERS DEVELOPED AND APPLIED FOR THE GENETIC DIVERSITY STUDY AND POPULATION STRUCTURE OF *DIDYMELLA PISI* ASSOCIATED WITH ASCOCHYTA BLIGHT OF DRY PEA IN MONTANA.

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FIRST MICROSATELLITE MARKERS DEVELOPED AND APPLIED FOR THE GENETIC DIVERSITY STUDY AND POPULATION STRUCTURE OF *DIDYMELLA PISI* ASSOCIATED WITH ASCOCHYTA BLIGHT OF DRY PEA IN MONTANA.

Abstract

Didymella pisi is the predominant causal pathogen of ascochyta blight of dry pea causing yield losses in Montana, where 415,000 acres were planted to dry pea in 2018. Thirty-three microsatellite markers were developed for dry pea pathogenic fungus, *Didymella pisi*, these markers were used to analyze genetic diversity and population structure of 205 isolates from four different geographical regions of Montana. These loci produced a total of 216 alleles with an average of 1.63 alleles per microsatellite marker. The polymorphic information content values ranged from 0.020 to 0.990 with an average of 0.323. The average observed heterozygosity across all loci varied from 0.000 to 0.018. The gene diversity among the loci ranged from 0.003 to 0.461. Unweighted Neighbor-joining and population structure analysis grouped these 205 isolates into two major sub-groups. The clusters did not match the geographic origin of the isolates. Analysis of molecular variance showed 85% of the total variation within populations and only 15% among populations. There was moderate genetic variation in the total populations (PhiPT = 0.153). Information obtained from this study could be useful as a base to design strategies for improved management such as breeding for resistance to ascochyta blight of dry pea in Montana

Key words: *Didymella pisi*, Microsatellite, Polymorphism, Genetic diversity, Population structure

1.0. Introduction

The production of dry pea (*Pisium sativum* L) in the Northern Great Plains of the United States is rapidly increasing. Montana leads in the production of dry pea in the USA, where 415,000 acres were planted in 2018, accounting for 47% of the total production in the US. Also, in the last five years, the area planted with pulse crops in Montana increased from 686,000 acres in 2014 to 1.2 million acres in 2018, with dry pea accounting for 50.5% of the increase (United States Department of Agriculture National Agricultural Statistics Service USDA-NASS, 2018). Being a high-water-use efficiency crop, it is of great importance to the semi-arid region like Montana (McVay *et al*., 2013 and Miller *et al.*, 2005). In addition, pulse crops provide nitrogen credit to subsequent crops because of their capacity to fix atmospheric nitrogen (McVay *et al*., 2013 and Miller *et al.*, 2005). Thus, Montana growers include dry pea in rotation with cereals such as wheat and barley. This increase in the production of dry pea in Montana is associated with increasing export demands due to the high quality of dry pea seeds from the United States and increasing domestic interest in pulse crops as a ‘clear label’ source of protein in food and drink manufacturing (USDA-Economic Research Services 2017, Wendy *et al.*, 2012). Furthermore, the Food and Agriculture Organization of the United Nations (FAO) declared 2016 as the International Year of Pulses (FAO, 2016). This initiative promoted public awareness of the nutritional benefits of pulse crops and encouraged increased consumption

of pulses. Finally, fractionation of dry pea into components desired by consumers, especially its protein content, will drive the future demand for dry pea production.

As the acres planted to dry pea increase, so does the disease risk. Ascochyta blight (AB) has been reported to cause 15% yield loss under favorable conditions (Skoglund *et al.,* 2011). All parts of the dry pea plant and every growth stage can be infected by the fungus. Symptoms of the disease can develop on leaves, stems, and pods and can also cause pre-emergence seed rot. AB is a seed- and residue-borne. The lesions on pods are purplish-brown and infected seeds may be small and discolored (Gossen *et al.,* 2011). Ascochyta blight is often referred to as a disease complex because it is caused by a few closely related seed-borne fungal pathogens which can exist together on a host or independently of each other (Davidson *et al.,* 2009 and Tivoli and Banniza, 2007). Prior to 2009, three fungal pathogens were associated with this disease: *Didymella pisi* (Barilli *et al*., 2016), *Peyronellaea pinodes* and *Peyronellaea pinodella* (Aveskam *et al.,* 2010). Davidson *et al.,* (2009) characterized a fourth causal agent, *Phoma koolunga*. This pathogen and other species such as *Phoma herbarum* (Li *et al.,* 2011), and *Phoma glomerata* (Tran *et al.,* 2014) were both shown to be pathogenic on dry pea and have been associated with ascochyta blight only in Australia.

Seed infection is one of the major survival mechanisms of fungi species causing ascochyta blight of dry pea and an important mode of transmission into previously uninfected areas. The impact of seed-borne inoculum is determined by climatic factors such as precipitation and temperature. Thus, areas with low precipitation often produce disease-free seeds in the field (Sivachandra-Kumar and Banniza, 2017). In addition, some

species can also produce significant wind-borne inoculum (Sivachandra-Kumar and Banniza, 2017 and Tivoli and Banniza, 2007). In addition to seed as a source of inoculum, *D. pisi*, and *P. pinodes* also can overwinter on crop residue as pycnidia and pseudothecia, which produce conidia and ascospores, respectively (Chilvers *et al*., 2009 and Tivoli and Banniza, 2007). Generally, primary inoculum (asexual conidia or sexual ascospores) of all the pathogens are dispersed by rain and wind onto emerging dry pea crops and cause outbreaks of disease (Schoeny *et al.,* 2008). However, ascospores are more efficient than conidia at disseminating the fungus because the ascospores are airborne (Chilvers *et al*., 2009; Tivoli and Banniza, 2007; and Wise *et al.,* 2011).

Management of AB on dry pea requires an integrated approach, this often includes cultural practices, chemical treatment, and use of resistant cultivars (Kinane and Lyngkjaer, 2002 and Wise *et al.,* 2011). The use of resistant varieties is the best efficient approach for AB management. However, no single gene resistance to AB of dry pea has been found (Kraft *et al*., 1998). In addition, this difficulty in securing gene resistance to AB of dry pea may be due to the genetic variability that exists in the pathogen (Darby and Lewis, 1986).

Didymella pisi tends to be more prevalent in Montana compared to other species of fungus that cause Ascochyta blight in dry pea. This is based on seed tests of 130 dry pea seed lots submitted by growers from 23 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT during the 2015 and 2016 growing seasons. About 98% of the ascochyta isolates detected and characterized were *D. pisi* (Owati *et al.,* 2017). However, *P. pinodes* and *P. pinodella* are the prominent species in other dry pea producing regions of North America such as North Dakota and Saskatchewan (Sivachandra-Kumar and Banniza, 2017 and Wallen *et al*., 1967).

D. pisi is heterothallic, the formation of sexual spores enables genetic diversity due to sexual recombination, which is associated with increased aggressiveness, development of fungicide resistance, and loss of resistant cultivars (Chilvers *et al*., 2009). *D. pisi* secretes ascochitine, a metabolite toxic to *Pisum* species and associated with pathogenicity (Foremska *et al.,* 1990 and Marcinkowska *et al*., 1991). An epidemic in Spain was attributed primarily to *D. pisi* (Chilvers *et al*., 2009 and Kaiser *et al*., 2008). Thus, it is important to understand the genetic diversity and population structure of the fungus and define the spatial distribution of the pathogen. This information is vital in the development and implementation of effective disease management strategies. For instance, in Brazil, the development of effective disease management strategies was achieved for the control of sugarcane brown rust after understanding the population genetic variability of *Puccinia melanocephala* its causal pathogen (Peixoto-Junior *et al*., 2014).

DNA markers have been widely adopted for analyzing the population dynamics of plant pathogens due to their high levels of precision and accuracy (Milgroom and Peever, 2003; Moges *et al.,* 2016; and Peixoto-Junior *et al*., 2014). They are rapid, highly specific, and have a low detection limit (Milgroom and Peever, 2003 and Moges *et al.,* 2016). Microsatellites also referred to as simple sequence repeats (SSRs), are among the most variable types of DNA sequences in eukaryotic genomes and are widely used for the study of plant pathogens (Ellegren, 2004). Simple sequence repeats (SSRs) are tandemly repeated tracts of DNA with about 1– 6 base pair (bp) long units (Tóth *et al*., 2000). They are ubiquitous and relatively abundant in prokaryotes and eukaryotes (Moges *et al*., 2016; Peixoto-Junior *et al*., 2014; and Tóth *et al*., 2000).

Although SSRs are ubiquitous and evenly distributed in eukaryote genomes, fungal genomes are known to contain fewer SSR sequences than other eukaryotes (Dutech *et al*., 2007; Huntley and Golding, 2000; Katti *et al*., 2001; Lenz *et al*., 2014; and Moges *et al*., 2016). However, in the presence of polymorphic loci, SSRs are useful for population genetic studies. In this light, SSRs have been used for the study of the genetic diversity of various plant pathogenic fungi including *Didymella rabiei* (Baite *et al.,* 2017 and Bayraktar *et al.,* 2007) *Sclerotinia subarctica* and *S. sclerotiorum* (Winton *et al*., 2007), *Puccinia melanocephala* (Peixoto-Junior *et al*., 2014), *Colletotrichum spp* (Marulanda *et al*., 2014), and *Colletotrichum gloeosporioides* (Moges *et al*., 2016). Pathogen diversity studies offer information about the organism's future evolutionary potential, which is useful not only in the screening for resistance genes but also in defining fungicide use (Ciampi *et al.,* 2011; Marulanda *et al*., 2014; and McDonald and Linde, 2002). In addition, temporal and spatial information on genetic diversity and population structure of plant pathogen are highly important to understand the evolutionary adaptability and the pathogen's potential to overcome the potential resistance of the host plant (Ciampi *et al.,* 2011 and McDonald and Linde 2002).

Despite the importance of *D. pisi* on dry pea as the predominant pathogen associated with AB of dry pea in Montana, its population genetic diversity has not been determined. Thus, the objective of this study was to develop microsatellite markers and analyze the genetic diversity and population structure of *D. pisi* populations from the major dry pea production regions of Montana.

2.0. Materials and Methods

2.1. Fungal Isolate Collection. Two hundred and five isolates of *D. pisi* were previously obtained from two sources (Owati *et al.*, 2017) (Table 1). A total of 171 isolates were collected in 2016 and 34 isolates were collected in 2017 (Table 1). About 90% of the isolates were obtained from AB contaminated seed lots submitted by growers in 15 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT, for seed testing intended for planting in 2016 and 2017 (Table 1) (S1 Table). The second set of isolates were obtained in 2016 and 2017 during a survey of dry pea production fields in Montana (Table 1) (S1 Table).

2.2. Fungi isolation and DNA extraction. A total of 205 *D. pisi* isolates were recovered on PDA from AB contaminated dry pea seed and infected foliage collected from major dry pea production areas of Montana from 2016 to 2017. The isolates were assigned to four populations based on their geographic origin (Table 1).

The total genomic DNA was extracted from a 10-day-old culture grown on PDA using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instruction. The quality and concentration of extracted DNA were estimated using the NanoDrop 2000c Spectrophotometer at 260 nm (Thermo Fisher Scientific, Walthum, MA) and visualized in 1% agarose gel stained with SYBR Safe DNA gel stain under ultraviolet-light (Bio-Rad Universal Hood II Gel Doc systems, Hercules, CA). DNA was stored at -20°C until further use.

Table 1. Geographic origins of *Didymella pisi* population on *Pisium sativum*, the number of isolates from seed and plant sources and year of collection (Owati *et al*., 2017).

Region	**No. of isolates**	**No. of isolates from seed**	**No. of isolates from plants**	**Counties represented**	**Year**
Central	29	26	3	Cascade, Teton, and Judith Basin	2016
	7	7	0	Cascade, Teton, and Judith Basin	2017
North Central	32	30	2	Philips, Glacier, Hill, and Chouteau	2016
	9	9	0	Philips and Chouteau	2017
North East	77	67	10	Valley, Daniels, Sheridian, Garfield, Roosevelt,	2016

				Richland, and Dawson	
	18	16	2	Daniels, McCone, Garfield, and Roosevelt	2017
South West	31	23	8	Gallatin	2016
	2	2	0	Gallatin	2017
Total	205	180	25		

2.3. Genome sequencing and assembly. Whole genome DNA libraries were constructed using next-generation Illumina MiSeq sequencing technology (MCLAB) for one representative *D. pisi* isolate. The libraries were sequenced and 250 bp x 2 paired-end reads were obtained. The quality and nucleotide distribution of the sequences were determined using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Galaxy (https://usegalaxy.org/).

The removal of the indexed adapters, trimming of poor-quality sequence from the end of each sequence, and genome assembly was done using Galaxy (https://usegalaxy.org/) and CLC Genomic Workbench 10 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). SSR markers were developed from the assembled sequences.

2.4. SSR identification and primer design. A genome-wide SSR marker database was developed for *D. pisi*. *De novo* genome assembly was performed to obtain long contigs followed by a microsatellite search and primer design. SSR motifs were identified using simple sequence repeat locator (SSRLocator) (Da Maia *et al*., 2008) and an online simple sequence repeat identification tool (SSRIT) (Moges *et al.*, 2016). The results obtained using these two search tools were compared and validated using tandem repeats finder (TRF) version 4.09 (https://tandem.bu.edu/trf/trf.basic.submit.html) and primers were designed for selected SSR loci using BatchPrimer version 3 (https://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). The primers were validated using OligoAnalyzer version 3.1 (http://www.idtdna.com/calc/analyzer). The

parameters for primer design included product sizes of 180 to 500 bp; primer size of 18 to 22 bp with optimal length 20 bp; primer melting temperature (Tm) of 50 °C to 60 °C with an optimum at 55 °C; the GC% of 45% to 65% with an optimum of 50% and primers were at least 5 bp away from the SSR locus.

2.5. PCR amplification and genotyping. Standard gradient PCR was performed to determine the optimal annealing temperature for the PCR amplification for each SSR locus. The PCR assay was optimized in a final volume of 20 µl containing 10 µl of EconoTaq Plus 2x master mix according to manufacturer's recommendations (Lucigen Corporation, Middleton, WI, United States), 10 pM of each forward and reverse primer, and 2.5 µl DNA (50 ng). Cycling parameters were 4 min at 94 ˚C, followed by 30 cycles of 30 s at 94 ˚C, 30 s at 56.6 ˚C, 30 s at 72 ˚C, and a final extension at 72 ˚C for 5 min. To reveal polymorphisms and for allele identification, the PCR products were analyzed on SYBR Safe-stained 2.5% (w/v) agarose gels run in the 1x sodium-borate buffer (Brody and Kern, 2004) and exposed to UV light to visualize DNA fragments. The amplicon sizes were estimated using a 100-bp DNA ladder (Thermo Fisher Scientific, Walthum, MA). The SSR markers were scored for the presence or absence of the corresponding bands among the test isolates (S1 Fig. 1) (Moges *et al.*, 2016).

2.6. SSR polymorphism and genetic diversity. SSR markers were used to analyze the genetic diversity of 205 *D. pisi* isolates from Montana. Basic statistics, including the major allele frequency, the number of alleles per locus, heterozygosity, polymorphic information content (PIC), and gene diversity were estimated using GenAlEx version 6.503 (Peakall and Smouse, 2012). For individual SSR marker, the amount of polymorphism estimated by gene diversity was calculated for all 205 isolates (Nei, 1987). The PIC for each locus, which estimates the discriminatory power of the markers, was computed by PIC $=1 - \sum Pi2$, where Pi^2 referred to the sum of the ith allelic frequency of each microsatellite locus for the genotypes (Anderson *et al*., 1993 and Botstein *et al.*, 1980).

In addition, the number of effective alleles per locus, number of different alleles per locus, number of private alleles, observed heterozygosity, expected heterozygosity, unbiased heterozygosity, banding pattern across the population, and Shannon's Information Index were estimated for each population using GenAlEx version 6.503 (Peakall and Smouse, 2012).

2.7. Population structure and gene flow. To assess the statistical distribution of gene diversity and estimate the variance components of the populations, analysis of molecular variance (AMOVA) based on co-dominant SSR loci was estimated using GenAlEx version 6.503 (Peakall and Smouse, 2012). In addition, population differentiation, PhiPT of the total populations and pairwise PhiPT among all pairs of populations were determined, and significance was tested based on 1000 bootstraps. Furthermore, the Principal Coordinate Analysis (PCoA) was computed using GenAlEx version 6.503 (Peakall and Smouse, 2012) to show the pattern of genetic variation of the populations of *D. pisi* isolates. An

unweighted neighbor-joining dendrogram for the 205 isolates belonging to the four populations of *D. pisi* was constructed based on the Euclidean distances using Paleontological Statistics (PAST) version 3 (Hammer *et al*, 2001). The tree was bootstrapped with 1000 replicates.

Using the SSR loci data, the detection of admixture and pattern of population structure were inferred using a Bayesian model-based clustering algorithm designed in STRUCTURE version 2.3.4 (Falush *et al.*, 2003, 2007; Hubisz *et al*., 2009; Moges *et al*., 2016; and Pritchard *et al.*, 2000). For this, two independent analyses were computed with and without prior information about the populations (Moges *et al*., 2016). The first run assigned the collection site as the putative population origin for individual isolates while the second run was without location assignment and letting the STRUCTURE software assign individuals to a population (Moges *et al*., 2016). To determine the most appropriate number of populations (K), a burn-in period of 25,000 was used in each run, and data were collected over 100,000 Markov chain Monte Carlo (MCMC) replications from K =1 to K = 10 (Moges *et al*., 2016). This procedure group individuals into populations and estimates the proportion of membership in each population for individuals (Moges *et al*., 2016 and Pritchard *et al.*, 2000). The K value was determined by the log probability of data (Ln P(D)) based on the rate of change in LnP(D) between successive K (Earl and Von Holdt, 2012 and Moges *et al*., 2016). The optimum K value was predicted following the simulation method of (Evanno *et al*., 2005) using the web-based software STRUCTURE HARVESTER version 0.6.92 (Earl and Von Holdt, 2012).

3.0 Results

3.1. De-novo assembly of sequence and Marker development. *De novo* assembly of the sequence data produced 4,283 contigs covering 33.8 Mb with an average N50 of 25.2 Kb and an average contig length of 9.2 Kb. Each of the thirty-three SSR primer pairs successfully produced a clear single amplicon at annealing temperatures of 54 ºC or 55 ºC (Table 2). Multiplex PCR amplifications were performed using three to five SSR primer pairs in each PCR based on annealing temperatures.

Table 2. Characteristics of 33 polymorphic microsatellite markers developed in this study for population genetic diversity analysis of *Didymella pisi* isolates.

Locus	**Forward primer Sequence (5'-3')**	**Reverse primer Sequence (5'-3')**	**Tm (ºC)***	**Allele size (bp)**	**Repeat motifs**
AJO-1	CAGCACACTCAACATAGCAG	CTTAACCACTCACCTGACAAC	55	253	(AG)11
AJO-2	AAAAGAGAGAGGAACCAAGC	AAGGGAGGGTGAAGGTAGT	55	304	(GA)11
AJO-3	TCGTACGATTACTCTCCTCAC	GAGAAATAGCAGTAGGGTGCT	54	214	(CT)8

AJO-4	ACAGGGTGACCTTTGCTT	GACCATTGAAGGACAGAGG	55	368	(CA)12
AJO-5	AGCAGGCATTACGTTTAACT	GGTAAGATGCGAGTACGAAT	54	249	(AGC)6
AJO-7	ATAACAACCAACCTCTGACG	GGAGCAATAGGTGATCTTCTC	55	486	(ACC)6
AJO-8	CGAGCTCTACAACTACCTCCT	GCCTCCTCTTTGAGTATCTTC	55	402	(CGA)6
AJO-9	GGTGGACTGAGTTCTGTGTAG	TAGCGTGCTCTTGAGGATTA	55	199	(TTC)7
AJO-10	CCTCTGTCCTTCAATGGTC	GTTAGCAGGGTAGCGAAAG	55	332	(GTC)8
AJO-11	CTACGGAGTGCTCTACAAAGA	AGCTTGCTCACAGCATAAAG	55	354	(CTG)7
AJO-12	CTAGAATCGTGCTTGTTGC	GAGTCTCCCTGTCTTTGTCC	55	406	(TCG)7

AJO-13	AAGAGCTCAACCTAACACCTC	CTCTCTCTCTTCACACCCAAT	55	248	(GGT)8
AJO-14	CCAGTGTAACTCCTGTAATGC	GTCTCCATACAGACGACAGG	55	290	(CAG)10
AJO-15	GCACGACGAAACAGTAGTTC	AGGAGGTTGAAAGTTCGAG	55	255	(TGAG)6
AJO-16	GTACCCATTCATCGTACAGG	AGCCCAAAAGAGCAGAGT	55	209	(AATC)7
AJO-17	GAGTTGAGTTGAGTTGAGTCG	GAAAGACAGGGTAGGTAGGTG	55	385	(GGAT)5
AJO-18	GTACAGTGCAACACAGCAGTA	ACTCGAGACCACATAGCATT	55	405	(AAGG)6

AJO-19	CATGAGTCGGACTTGTTTTC	GTACGTCCATATCTGCGATT	55	401	(AATC)7
AJO-20	GTTGTCAGGTGAGTGGTTAAG	GTAGGTAGGCGTGTATCTGTG	54	230	(AGTG)7
AJO-22	AAGATGAGACTCGGAGAAGAG	CTTATATCCTGAACGCCTTG	55	235	(TGAGA)5
AJO-23	GATAGCCTGGTCATCATTTC	ATCCACAGTGATAGCGAATC	54	316	(ACAGT)5
AJO-25	CTTAATCTCAACCCACATCG	TGCTGTAAGCACACAGACAC	55	298	(CATCT)5
AJO-26	GGTTGGATTGTGGTGTAGTAG	GAGGGTGCTAGGTGACTTACT	54	199	(ATCGC)6

AJO-27	CGTAGTATGTGCAGGAAGAAG	GAGGGTGCTAGGTGACTTACT	55	390	(ATCGC)6
AJO-28	CACCCTACTGCTATTTCTCCT	TCTGATCTCCTTCTTGCAGT	55	211	(TACTC)5
AJO-29	ACAGTGTGTCTGTCTCGTCTC	GAAGTCATGAACAGGAGGAA	55	269	(TTCTT)4
AJO-30	TAGTGGGAGTGTGCTAGTGTC	GTGGATTAGGTTGAGGGTAGT	55	172	(GGTGA)4
AJO-31	CGAGACTCACACACACAATC	GTAGATACCCAACCCGACTAC	55	354	(AAGTCA)4
AJO-32	ACATGACCTCGACTGCTG	GCTGGACGTCGAGTTGTC	56	206	(CCACGC)8

AJO-33	CCATACGTTGACGTAGACAG	ACATTCCATACCCTCTGTACC	54	378	(TGGTAC)5
AJO-34	CCCCTAACACTTTCTCTCTCT	ACTTTTCAACCTACCGTCCT	54	398	(TAACTC)4
AJO-35	GTCCAGAAGGGACAAAGAC	ACTACGGGGTAGTTGCTTACT	54	396	(CCTGCA)6
AJO-36	AGGTGAGTGGAGGTGATAAGT	GTTTGTCGACACAGACAGAG	54	237	(TGTGCT)5

*Tm = Melting Temperature

3.2. SSRs polymorphism and gene diversity. The polymorphism and diversity of the different SSR loci are presented in Table 3. The 33 polymorphic SSR markers detected a total of 216 alleles with an average of 1.63 alleles per marker. The PIC values for the markers ranged from 0.02 (AJO-7) to 0.99 (AJO-29), with an average of 0.323 per marker. Seven SSR loci were highly polymorphic (PIC ≥ 0.5), six were moderately polymorphic (0.5 < PIC > 0.25), and twenty were slightly polymorphic (PIC < 0.25).

The frequency of the major alleles in each locus ranged from 0.003 to 0.995, with an average of 0.831. The number of effective alleles was in the range of 1.003 to 1.874, with an average of 1.420. Gene diversity, defined as the probability that an individual from a population will be heterozygous at a given locus (Moges *et al*., 2016), ranged from 0.003 (AJO-29) to 0.461 (AJO-30), with an average of 0.246. Furthermore, a low level of heterozygosity (0.000 to 0.180) was observed in the *D. pisi* isolates used in this study. In addition, one SSR locus had no heterozygosity while one displayed less than 0.01 heterozygosity. The expected heterozygosity ranged from 0.000 to 0.362 (Table 3). Based on the Hardy-Weinberg equilibrium (HWE) exact test for all populations, 32 loci (96.9%) exhibited significant deviation from HWE corrected for multiple comparisons (P <0.001), by having less expected levels of heterozygosity. However, one locus (AJO-29) did not show significant departure from HWE. These results are not surprising, especially when most of the *D. pisi* isolates used in the study were assumed to be from the asexual population.

Table 3. Diversity indices of the 33 microsatellite loci used in the study

Locus	**MAF**	**Na**	**GD**	**He**	**Ho**	**uHe**	**PIC**	**Ne**	**SI**
AJO-1	0.273	2.00	0.232	0.034	0.017	0.236	0.956	1.360	0.378
AJO-2	0.248	2.00	0.226	0.015	0.007	0.229	0.954	1.308	0.380
AJO-3	0.956	2.00	0.314	0.085	0.043	0.317	0.114	1.472	0.491
AJO-4	0.934	1.75	0.296	0.168	0.084	0.299	0.177	1.502	0.436
AJO-5	0.965	1.75	0.250	0.134	0.067	0.252	0.086	1.378	0.386
AJO-7	0.992	1.25	0.073	0.127	0.064	0.073	0.029	1.103	0.117
AJO-8	0.906	1.75	0.293	0.238	0.119	0.296	0.271	1.508	0.432
AJO-9	0.984	1.25	0.094	0.177	0.088	0.095	0.058	1.151	0.141
AJO-10	0.880	1.75	0.320	0.233	0.117	0.323	0.328	1.569	0.463
AJO-11	0.675	1.75	0.322	0.250	0.125	0.325	0.663	1.587	0.463
AJO-12	0.987	1.25	0.088	0.162	0.081	0.089	0.048	1.137	0.135
AJO-13	0.791	1.75	0.369	0.239	0.119	0.373	0.450	1.726	0.514
AJO-14	0.912	2.00	0.390	0.118	0.059	0.394	0.203	1.667	0.575

AJO-15	0.955	1.75	0.275	0.149	0.074	0.278	0.104	1.436	0.415
AJO-16	0.956	1.50	0.188	0.237	0.119	0.189	0.141	1.321	0.279
AJO-17	0.912	1.75	0.313	0.203	0.102	0.316	0.238	1.554	0.455
AJO-18	0.961	1.50	0.202	0.241	0.120	0.204	0.095	1.338	0.296
AJO-19	0.731	2.00	0.440	0.163	0.081	0.445	0.553	1.797	0.631
AJO-20	0.921	1.25	0.123	0.362	0.181	0.124	0.271	1.242	0.171
AJO-22	0.831	1.75	0.314	0.254	0.127	0.318	0.457	1.542	0.457
AJO-23	0.938	1.50	0.194	0.268	0.134	0.196	0.203	1.344	0.285
AJO-25	0.804	1.75	0.339	0.242	0.121	0.342	0.486	1.621	0.483
AJO-26	0.970	1.50	0.172	0.205	0.103	0.174	0.095	1.275	0.263
AJO-27	0.941	1.75	0.276	0.191	0.096	0.278	0.168	1.460	0.413
AJO-28	0.968	1.50	0.175	0.211	0.106	0.177	0.104	1.285	0.266
AJO-29	0.003	0.50	0.003	0.000	0.000	0.003	0.990	1.003	0.008
AJO-30	0.632	2.00	0.461	0.065	0.032	0.467	0.674	1.874	0.653
AJO-31	0.995	1.25	0.062	0.105	0.052	0.062	0.019	1.082	0.104

AJO-32	0.493	2.00	0.401	0.038	0.019	0.406	0.794	1.685	0.589
AJO-33	0.992	1.25	0.073	0.127	0.064	0.073	0.029	1.103	0.117
AJO-34	0.897	2.00	0.365	0.172	0.086	0.369	0.280	1.614	0.547
AJO-35	0.765	1.75	0.329	0.250	0.125	0.332	0.566	1.601	0.471
AJO-36	0.985	1.50	0.143	0.157	0.079	0.144	0.039	1.199	0.230
Mean	0.832	1.64	0.246	0.170	0.085	0.248	0.323	1.420	0.365

MAF = Major allele frequency; **Na =** Number of different allele; **GD** = Genetic diversity; **He** = Expected heterozygosity; **Ho** = Observed heterozygosity; **uHe** = Unbiased heterozygosity; **PIC** = Polymorphic information content; **Ne** = Number of effective allele; and **SI** = Shannon's information index

3.3. Population genetic diversity. A summary of the genetic diversity estimates for the four populations of *D. pisi* is presented in Table 4. The number of different alleles (Na), and effective alleles (Ne) averaged across all loci ranged from 1.212 to 2.00 and 1.161 to 1.662, respectively for the four populations (Southwest, Northcentral, Central, and Northeast). The Northeast population had the highest while the Southwest population had the lowest Na, and Ne values. Average observed heterozygosity (Ho) was in the range of 0.033 to 0.385, with a mean of 0.161 across all loci. Genetic diversity was the lowest in Southwest population (He = 0.094) and the highest in Northeast population (He = 0.379), and its value averaged over all populations and loci was 0.246 (SE = 0.017). The percentage of polymorphic loci (PL) ranged from 24.24% in the Southwest to 100% in the Northeast, with an average of 65.91%. Based on the Shannon's Index (I), a higher diversity was observed in the Northeast (I = 0.557) population and was least in the Southwest (I = 0.139) population.

Table 4. Summary of the population diversity indices averaged over 33 microsatellite loci for isolates of *D. pisi* from four geographic regions in Montana.

Population	**Na**	**Ne**	**PL**	**Ho**	**He**	**I**
Southwest	1.212	1.161	24.24	0.385	0.094	0.139
Central	1.697	1.452	72.73	0.033	0.268	0.401
Northcentral	1.636	1.403	66.67	0.150	0.242	0.363
Northeast	2.00	1.662	100	0.076	0.379	0.557
Overall	1.636	1.420	65.91	0.161	0.246	0.365

Na = Number of different allele; **Ne** = Number of effective allele; **PL** = Percentage of polymorphic loci; **Ho** = Observed heterozygosity; **He** = Expected heterozygosity; and **SI** = Shannon's information index

3.4. Population genetic structure and gene flow. Analysis of molecular variance (AMOVA) showed that the differences among isolates within the population accounted for 85% and the differences among the different populations accounted for 15% of the total genetic variation detected (Table 5). There was significant genetic variation among the populations based on the randomization test (PhiPT = 0.153 at $P < 0.001$). The pairwise PhiPT values of the genetic distance among all populations were significant ($P < 0.01$, Table 6). The PhiPT value between northcentral and central populations was the smallest (0.000) while the PhiPT value between northeast and southwest populations was the largest (0.254). A similar pattern of differentiation among subgroups was observed using Nei's genetic distance. The Nei's genetic distance value between northcentral and central populations was the smallest (0.015) distance while the genetic distance value between northeast and southwest populations was the largest (0.237, $P < 0.001$, Table 7).

Table 5. Analysis of molecular variance among and within populations of *D. pisi* populations from Montana based on 33 SSR loci.

Source	**Degree of freedom**	**Sum of squares**	**Mean squares**	**Estimated Variance**	**Variation (%)**	**P-value**
Among populations	3	90.744	30.248	0.575	15	< 0.001
Within population	201	641.608	3.192	3.192	85	< 0.001
Total	204	732.351		3.767	100	< 0.001

Table 6. Pairwise genetic distance based on PhiPT matrix, a measure of divergence among the *D. pisi* populations from Montana.

Population	**SW**	**NE**	**NC**
NE	0.254		
NC	0.085	0.131	
C	0.130	0.103	0

PhiPT matrix is related to variance in allele frequency among the population

Table 7. Pairwise genetic distance based Nei's Genetic Distance among the *D. pisi* populations from Montana.

Population	**SW**	**NE**	**NC**
NE	0.237		
NC	0.050	0.097	
C	0.065	0.079	0.015

Nei's Genetic Distance measures genetic differences over time due to mutation and genetic drift

The unweighted Neighbor-joining dendrogram grouped the 205 isolates of the four populations into two major clusters (Fig 1A and 1B). From the 205 isolates, 106 and 99 isolates were grouped together in Cluster I and II respectively. The overall topology of the dendrogram indicated the presence of two clades in *D. pisi* associated with dry pea in Montana. Several sub-clades were observed for the populations indicating genetic variability within and among isolates in each population.

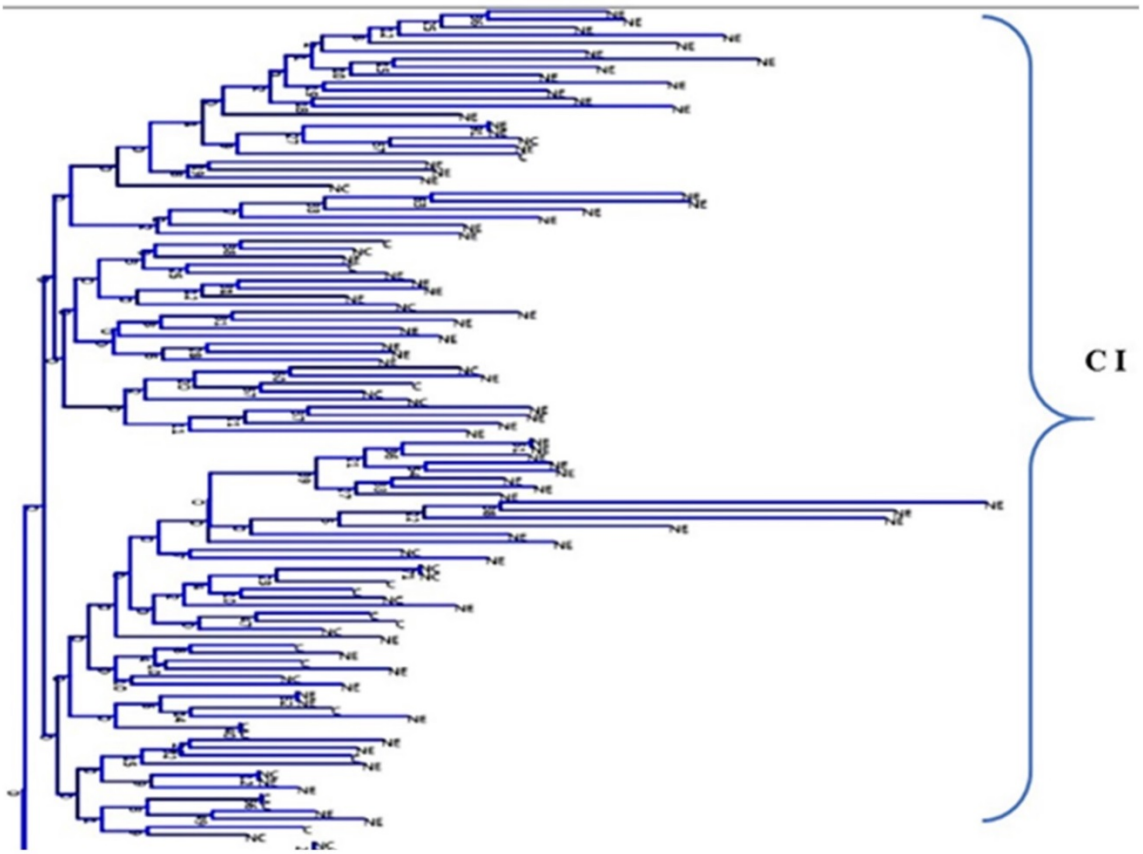

Figure 1A**.** Unweighted Neighbor-joining tree using the simple matching similarity coefficient based on 33 microsatellite markers for the 205 isolates of *Didymella pisi* isolated from dry pea in Montana. The tree shows the clustering pattern of isolates (C I = cluster 1) from the four *D. pisi* populations

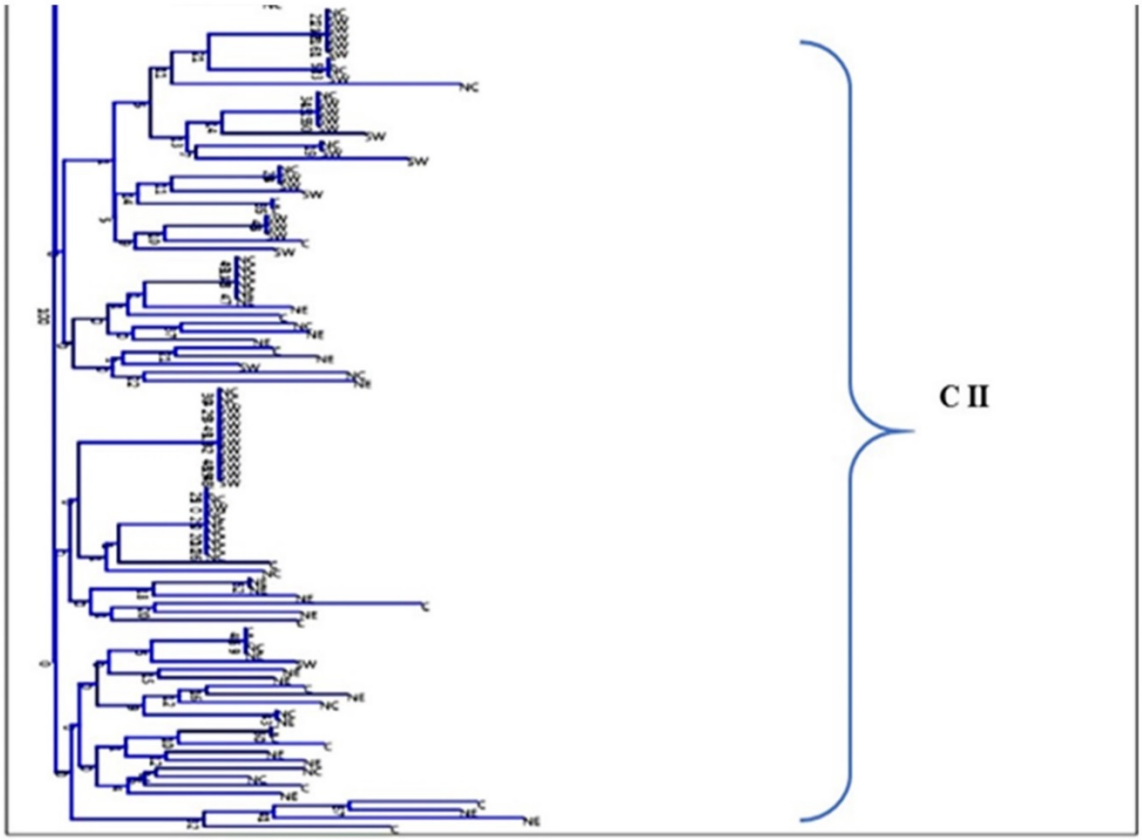

Figure 1B**.** Unweighted Neighbor-joining tree using the simple matching similarity coefficient based on 33 microsatellite markers for the 205 isolates of *Didymella pisi* isolated from dry pea in Montana. The tree shows the clustering pattern of isolates (C II = cluster 2) from the four *D. pisi* populations.

A similar pattern of clustering was observed in the Principal Coordinate Analysis (PCoA) based on the 33 microsatellite loci (Fig 2). Based on the Evanno *et al.*, (2005) method on STRUCTURE outputs, it predicted K = 2 to be the most likely number of clusters (Fig 3A and 3B).

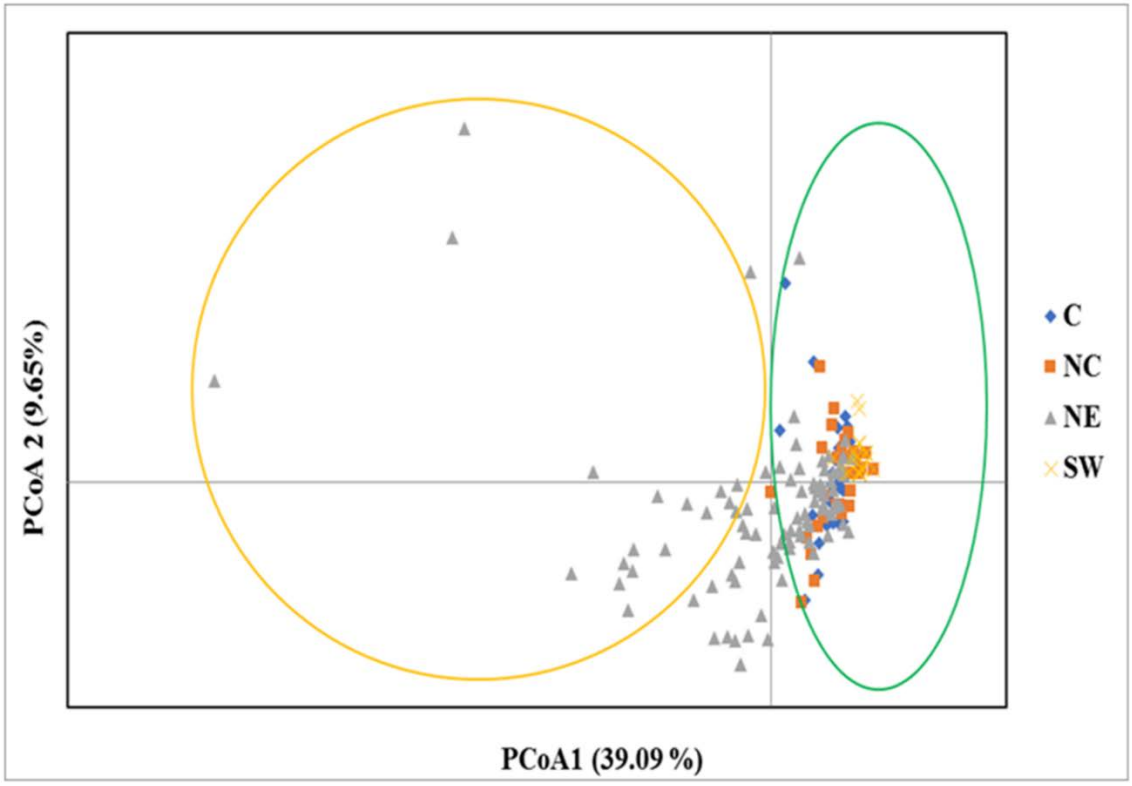

Figure 2. Principal coordinates analysis (PCoA) bi-plot showing the clustering of the 205 *D. pisi* isolates based on 33 microsatellite loci. The four populations are color coded as follows: C, Central region (blue); NC, Northcentral region (orange); NE, Northeast region (gray); SW, Southwest region (gold). Percentages of variation explained by the first 3 axes (1, 2, and 3) are 39.09, 9.65, and 8.08 %, respectively.

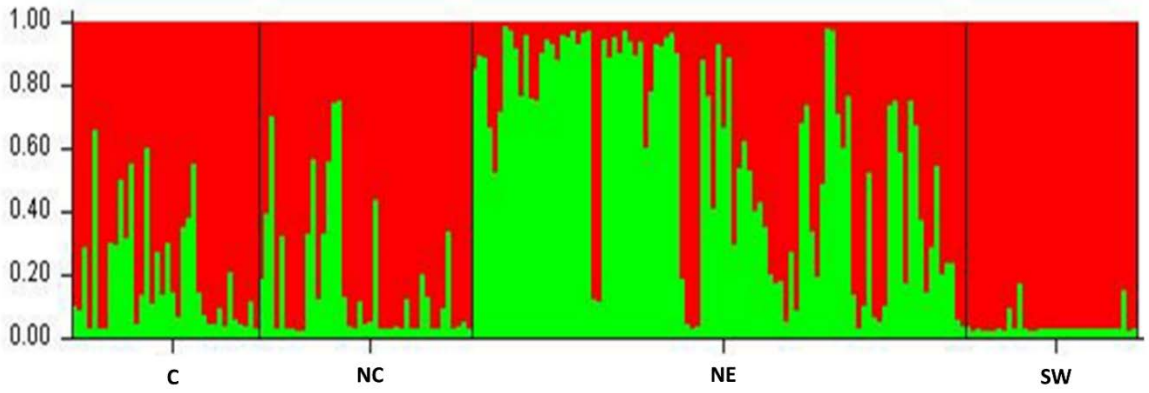

Figure 3A. Bayesian model-based estimation of population structure (K = 2) for the 205 *D. pisi* isolates in four pre-determined populations (x-axis): Central Montana (C), Northcentral Montana (NC), Northeast Montana (NE), and Southwest Montana (SW). Each group is separated by a black vertical line. Numbers in the y-axis show the coefficient of assignment.

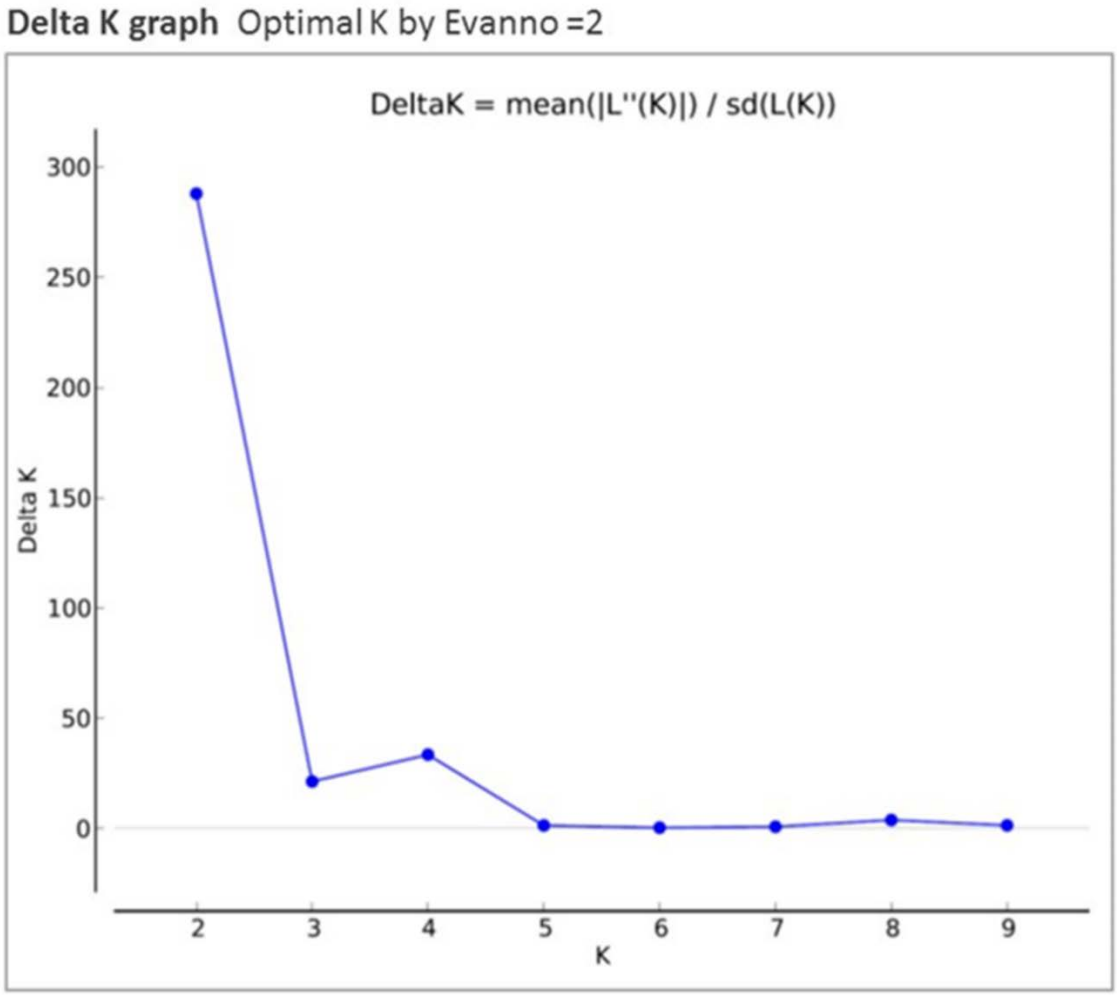

Figure 3B. The relationship between K and Delta K based on the Evanno method (Evanno, *et al.*, 2005). K = appropriate number of subpopulations

4.0 Discussion

Ascochyta blight of dry pea is caused by a complex of fungal pathogens. *D. pisi* is the predominant causal agent in Montana and has also been reported to cause an epidemic in Europe (Kaiser *et al*., 2008). However, SSR markers have only been developed for *Didymella rabiei*, the causal pathogen of ascochyta blight of chickpea (Baite *et al.,* 2017). The present study is the first report of *D. pisi* SSR markers development and their use for a genetic diversity and population structure study. This study has increased the number of population genetic studies on pathogenic fungi. Currently, population genetic studies of fungal pathogens are low when compared with other organisms (Dutech *et al.,* 2007 and Zane *et al*., 2002). Information on pathogen genetic diversity and population structure on the landscape scale are important to understand the potential of pathogen populations to spread, increase aggressiveness, develop fungicide resistance, and overcome host resistance (Ciampi *et al.,* 2011 and Marulanda *et al.,* 2014).

In this study, 33 polymorphic markers were developed and used to evaluate the genetic diversity of *D. pisi* isolates from dry pea. The polymorphism detected by the markers ranged from slightly informative to highly informative. The PIC value of a marker, which refers to the discriminatory power of a locus while accounting for the number and relative frequency of the alleles.

The AMOVA results supported the presence of genetic diversity in the *D. pisi* population in Montana. The highest percentage of variation (85%) was within populations of *D. pisi* isolates. However, the gene diversity observed among the Montana *D. pisi* populations was low. This might be associated with the introduction of a few genotypes of

the fungus into Montana as well as low level of seeds coming from outside Montana. Also, the incidence of *D. pisi*, unlike *P. pinodes* is very low in North Dakota and Canada, which are neighboring regions to Montana with high production of dry pea (Gossen *et al*., 2011 and Sivachandra-Kumar and Banniza, 2017). High genetic diversity was detected in the northeast region of Montana while genetic diversity was lowest in the southwest. Additionally, Shannon's index value was highest in the northeast and lowest in southwest Montana. This is an information statistic index, which assumes all types are represented in a sample and that they are randomly sampled (Heip *et al.*, 1998; Moges *et al.*, 2016; and Morris *et al*., 2014). The genetic variability between these two regions is expected because the northeast region is the epicenter of dry pea production in Montana and might be due to the early introduction of the fungus in this region. This allows time for genetic mutation, genetic drift, and recombination which results in greater genetic diversity (McDonald, 1997 and Moges *et al.*, 2016). This can be associated with the high incidence of ascochyta blight recorded on seed lots sent to the RPCDL for seed test from NE region than from other regions of Montana. These differences may also be due to environmental conditions, geography, and differences in alternative host species diversity that may be associated with generating variability within populations (McDonald 1997 and Moges *et al.*, 2016).

Several factors such as single nucleotide mutation and recombination, gene gain and gene loss, horizontal gene transfer, loss of heterozygosity, genome rearrangement, and conditionally dispensable chromosomes have been associated with genetic variation in fungi (McDonald, 1997 and Taylor *et al.*, 2017).

Didymella pisi is a heterothallic fungus. It is known that a heterothallic fungal pathogen that exhibits sexual recombination poses a greater threat when they inbreed due

to the emergence of new genotypes (McDonald, 1997 and Taylor *et al.*, 2017). However, genetic diversity may not be affected by the mating system because fungi that reproduce exclusively through asexual reproduction may have a similar number of alleles at individual loci as those that reproduce sexually (McDonald, 1997 and Moges *et al.*, 2016). Thus, gene diversity is affected by the age of a population, population size, and selection processes (McDonald, 1997). For instance, populations that have evolved over a long time at one location are expected to have more alleles than newly introduced populations, because there has been more time for mutation to introduce new variants and for genetic drift to increase the frequencies of new alleles to detectable levels (McDonald, 1997).

Information on the population structure of *D. pisi* populations from different locations improves the understanding of the biology of the pathogen, evolutionary, and potentially adaptive genotypic diversity in the species (Marulanda *et al.*, 2014). The *D. pisi* isolates from the four geographic regions of Montana are closely related as reflected by the high genetic identity among populations. Furthermore, based on the population genetic analyses of the Montana *D. pisi* isolates, the population was categorized into two sub-populations. STRUCTURE analysis, PCoA, and the unweighted Neighbor-joining algorithm supported and indicated admixture among the two populations. Evidence of admixture among isolates from the different regions does not support geographical separation of isolates into distinctly isolated sub-populations. The PhiPT value (0.153) between the *D. pisi* populations evaluated in this study indicated moderate differentiation among the groups that might be attributed to gene flow among regions. The moderate degree of diversity in populations of *D. pisi* shown in this study may be attributed to the dispersal of the clonal inoculum over long distances that may allow for pathogen spread in

dry pea-growing areas in Montana. This dispersal may be associated with the exchange of dry pea germplasm between growers, seed elevators, and researchers.

The microsatellite markers developed in this study were used to understand the genetic diversity and population structure of *D. pisi* isolates from dry pea growing regions of Montana. Despite regional variations, the observed genetic diversity in all four populations was lower than expected, suggesting inter-regional exchanges of planting materials and dispersal of inoculum among the regions. This study generated information that can be used to further understand the pathogen biology and its evolutionary potential and provide the basis for other studies on disease development, host-pathogen interactions, and development of disease management strategies which includes development and use of resistant dry pea varieties. In addition, information generated from this study can be used to design novel specific primers for characterization of *D. pisi*

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Authors Contribution

AO and BA conceived and designed the experiments. AO performed the experiment, analyzed the data, and wrote the manuscript. MB and BA reviewed the manuscript.

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CHAPTER FOUR

CHARACTERIZATION AND DETECTION OF FUNGAL SPECIES ASSOCIATED WITH ASCOCHYTA BLIGHT OF DRY PEA IN MONTANA

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CHAPTER FOUR

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Abstract

Montana is the leading producer of peas in the US, where 415,000 acres were planted in 2018, accounting for 47% of the total production in the US. The predominant foliar fungal disease of dry pea is Ascochyta blight (AB). This disease is caused by a complex of fungal pathogens including *Didymella pisi*, *Peyronellaea pinodes*, and *Peyronellaea pinodella*. *D. pisi* is the predominant pathogen in Montana. Recently, a shift in pathogen composition has been observed in northeastern Montana from *D. pisi* to *P. pinodes* and *P. pinodella*. In addition, a *Phoma sp.* was found associated with dry pea seeds and included in this study. To characterize these fungi, we evaluated the effects of temperature (15, 20, 25, and 30 ^{o}C) on mycelial growth rate and sporulation. At all temperatures, *Phoma sp.* had the highest growth rate (p-value = < 0.001) and produced more spores than the other species (p-value = < 0.001). Pathogenicity assays on dry pea indicated that *P. pinodes* caused greater disease severity than the other species when inoculated on pea plants (cv. Carousel, p-value ≤ 0.001). The *Phoma sp.* was not pathogenic. Peameal agar was used to visually discriminate between fungal species and could be a useful diagnostic tool. Results from this study will be used to understand the changes in the composition of fungal species causing AB in dry pea.

Introduction

Ascochyta blight (AB) of dry pea (*Pisium sativum*) can be a yield-limiting disease. Under favorable conditions, AB can cause 15- 60 % yield loss in dry pea (Wallen, 1965; and Xue, *et al.,*1996; 1997; and Ahmed, *et al.,* 2015). AB pathogens are seed-borne and can overwinter on infected plant debris and soil (Ali, *et al*., 1982; Bretag *et al.,* 2006; and Davidson, *et al*., 2011). All parts of the dry pea plant are susceptible to disease (Bretag, *et al*., 2001; Davidson *et al.,* 2009; 2011; and Gossen *et al.,* 2011).

Ascochyta blight of dry pea is a disease complex caused by a few closely related fungal pathogens which can exist together on a host or independently of each other (Trivoli *et al*., 2006 and Davidson, *et al*., 2009). Before 2009, three fungal pathogens were reportedly associated with this disease: *Didymella pisi* (Barilli *et al*., 2016), *Peyronellaea pinodes*, and *Peyronellaea pinodella* (Aveskamp *et al.,* 2010). Davidson *et al* (2009) reported and characterized a fourth causal agent, *Phoma koolunga,* which has become widespread in South Australia. Recently, *Phoma herbarum* and *Phoma glomerata* have also been shown to be associated with the ascochyta blight complex on field peas in Australia (Li, *et al.,* 2011; Tran *et al.,* 2014; and Liu, *et al.,* 2016). In the Great Plains of North America, the association *of P. koolunga, Phoma herbarum,* and *Phoma glomerata* with the AB complex have not been reported.

The pathogens causing AB in North America are similar, but there are differences in spore characteristics, appearance on media, survival structures, and toxin production. *D. pisi* is heterothallic and does not produce chlamydospores (Kraft *et al*., 1998; and Chilvers, *et al.,* 2009). Globose brown pycnidia are present on dry pea leaves and debris (Chilvers,

et al., 2009). Conidiophores are hyaline and short while the conidia are hyaline, straight, and septate. Colonies on oatmeal agar produce abundant pycnidia with carrot red colored spore exudate (Ahmed, *et al.,* 2015). About 80% seed infection with *D. pisi* was recorded in Canada in the 1950s (Skolko, *et al*., 1954). *P. pinodes* is homothallic and produces chlamydospores in culture either singly or catenated (Punithalingham and Holiday, 1972). On dry pea debris, it produces pycnidia with conidia and pseudothecia containing ascospores. Conidia are hyaline and septate. On potato dextrose agar, colonies of *P. pinodella* are dark with an irregular pattern of pycnidia. The pycnidia are sub-globose and larger than those of *P. pinodes*. *P. pinodella* conidia are hyaline, but smaller than those of *P. pinodes* and non-septate (Punithalingham and Gibson, 1976). *D. pisi* secretes ascochitine, a metabolite toxic to *Pisum* species that has been associated with pathogenicity. The toxin is not produced by *P. pinodes* or *P. pinodella* (Foremska, *et al*., 1990 and Marcinkowska *et al.,* 1991).

Differences in spore production described above led to some differences in the disease cycle among the different species causing AB. Asexual conidia or ascospores are the primary source of inoculum of all the different species associated with AB of dry pea. Ascospores of *P. pinodes* develop from pseudothecia on infected stubble, while *D. pisi* and *P. pinodella* produce conidia from pycnidia (Tivoli and Banniza, 2007). Regardless of the type of inoculum, the initial infection causes black spots on leaves which coalesce under moist conditions resulting in leaf necrosis (Roger, *et al.,* 1996 and Tivoli, *et al.,* 2007). Pycnidia develop within the lesions and conidia (pycnidiospores) are spread to neighboring plants via rain splash (Schoeny, *et al.,* 2008). The dispersal of conidia over short distances is responsible for secondary inoculum (Tivoli, *et al.,* 2007), thus increasing disease severity

and early tissue senescence. Tissue senescence initiates the production of pseudothecia. After rainfall, pseudothecia release airborne ascospores which are dispersed over long distances (Roger, *et al.*, 1996; Tivoli, *et al.*, 2007; and Schoeny, *et al*., 2008)

Transmission of the fungi causing AB from infected to uninfected areas is primarily achieved through the dissemination of infected seeds (Tivoli and Banniza, 2007 and Sivachandra-Kumar and Banniza 2017). Infected seeds serve as a source of inoculum and the impact of seed as a source of inoculum is influenced by the amount of rainfall and soil-borne inoculum. Once established, crop residue is the primary source of inoculum. Rain splash transfers inoculum over short distances to adjacent susceptible plants (Sivachandra-Kumar and Banniza 2017). *D. pisi* and *P. pinodes* overwinter as pycnidia and pseudothecia which produce conidia and ascospores, respectively, in the spring. Ascospores of *P. pinodes* are airborne and can be dispersed over long distances (>200 m) (Salam *et al*., 2011). This serves as the most important source of inoculum in the epidemic of ascochyta blight of dry pea (Tivoli and Banniza, 2007; Chilvers *et al.*, 2009; and Wise *et al.*, 2011). In addition to seed and infested residue, soil can serve as an important source of inoculum to the pea crop. *P. pinodes* and *P. pinodella* can survive in the soil for a few to several years, while *D. pisi* can only survive for few months (Wallen *et al.*, 1967). Reduction in grain yields has been correlated with the inoculum density of soil-borne ascochyta blight fungi (Bretag and Ward, 2001). The pathogens can survive as chlamydospores or mycelia. For instance, *P. pinodes* has been isolated from soil not sown to peas for 20 years while *P. pinodella* has been isolated from soil after five years of not planting peas (Wallen *et al.*, 1967; 1968; and McDonald and Peck 2009).

Regional differences in the composition of pathogens causing AB in dry pea occur worldwide. In Australia, *P. pinodes*, *P. pinodella*, and *P. koolunga* are the primary pathogens associated with AB of dry pea, and *D. pisi* is rarely isolated (Davidson, *et al.*, 2009). *D. pisi* tends to be the prevalent pathogen of AB in Europe and Asia and a recent epidemic of AB in Spain was associated with *D. pisi* (Chilvers, *et al.*, 2007 and Kaiser, *et al.*, 2008). In North America, *D. pisi* is prevalent in most of Montana (Owati *et al.*, 2017), while in North Dakota and Canada (Alberta, Saskatchewan, Manitoba), the prevalent pathogens are *P. pinodes* and *P. pinodella* (Gossen, *et al*., 2011; Ahmed, *et al.*, 2015; Sivachandra-Kumar and Banniza 2017).

Montana, North Dakota, and pea production areas of Saskatchewan and Alberta are geographically proximal to each other. There is a frequent exchange of seed materials between these areas. There is concern that there will be a shift in pathogen composition of the AB complex in dry pea. This information is important for variety recommendations and breeding efforts. The concern is that the aggressive *P. pinodes* and *P.pinodella* in North Dakota in the US and in Saskatchewan in Canada (Kraft, *et al.*, 1998; Gossen, *et al*., 2011; Ahmed *et al.*, 2015; and Sivachandra-Kumar and Banniza 2017) may gradually dominate in Montana, where they have been occurring at insignificant levels to date. In Canada, a shift in species from *D. pisi* to *P. pinodella* and *P. pinodes* occurred after the introduction of a *D. pisi*-resistant dry pea variety named Century in 1961 (Gfeller and Wallen, 1961 and Sivachandra-Kumar and Banniza 2017).

In 2017, a field survey and seed tests sent to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) for testing indicated that 5% of the fungal species isolated from AB infected plants and contaminated seedlots was *P. pinodes* and *P. pinodella* while the

predominant pathogen isolated was *D. pisi.* Out of 500 dry pea seed tests sent to RPCDL for planting in 2017 to 2018, 325 dry pea seedlots were contaminated with AB, predominantly *D. pisi*. *P. pinodes* was identified in samples from northeastern Montana, which has a longer history of pulse production and is geographically near dry pea production areas in neighboring states and provinces. There is an urgent need to characterize these pathogens in the context of Montana's dry pea fields and to develop a tool that will be used to monitor the changes in the population dynamics of the fungal pathogens associated with AB of dry pea in Montana. Thus, the objectives of this study were to (1) determine the optimum temperature required for the mycelia growth rate and sporulation of these fungal species; (2) determine differences in the aggressiveness and pathogenicity among these fungal species; and (3) develop a diagnostic tool for screening and monitoring these fungal species.

Materials and Methods

Fungal Isolation**.** Isolates of *D. pisi, P. pinodes, P. pinodella,* and an uncharacterized *Phoma sp.* associated with dry pea seed lots in Montana were obtained from three sources. *D. pisi, P. pinodes,* and *Phoma sp.* were isolated from AB-contaminated seed lots submitted by growers to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT for seed testing during the 2018 cropping season. Isolation from seed followed methods described by Owati (2017). A set of *P. pinodes* isolates were obtained courtesy of Dr. Julie Pasche at North Dakota State University, Fargo, ND, United States. Isolates of *P. pinodes* and *P. pinodella* were obtained courtesy of Dr. Frank Dugan at USDA-ARS, Pullman, WA, United States.

Effects of temperature on the mycelial growth and sporulation of D. pisi, P. pinodes, P. pinodella, and Phoma sp. Three isolates each of *P. pinodes*, *D. pisi*, and *Phoma sp.* and two isolates of *P. pinodella* were used for this study (Table 1). To evaluate the effect of temperature on the mycelial growth rate of the fungal pathogens, 5 mm mycelial plugs from 10-day old cultures of each isolate were placed on 20 ml of freshly prepared potato dextrose agar (PDA) (Alpha Biosciences Inc., Baltimore, MD, United States). The plates were incubated at 15, 20, 25, and 30 ^{o}C under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark). Radial mycelial growth was measured at 15 days post-inoculation using a digital caliper. This measurement facilitated direct comparisons among *D. pisi, P. pinodes, P. pinodella,* and *Phoma sp.* The growth rate was calculated using the formula:

Radial mycelial growth rate = mycelial diameter after 15 DPI – mycelial diameter at 0 DPI (mm)/ number of days cultured (days)

The experiments were organized as a randomized complete block design (RCBD) with four replicates of each isolate per treatment. The whole experiment was conducted twice. The sporulation experiment was set up as described above using 1/3 PDA. The experiments were laid out as a RCBD with four replicates of each isolate per treatment. The whole experiment was conducted twice. To prepare the conidial suspension, 10 ml of sterile, distilled water was added to *D. pisi, P. pinodes, P.pinodella,* and *Phoma sp.* 14-day old cultures and conidia were dislodged using a sterile glass rod. The conidial count from each plate was estimated using a hemocytometer viewed with a phase contrast Leica TCS

SP5 imaging system (Leica Microsystems Inc, Illinois). To enhance comparison among the fungal species, the spore count for each isolate for each species was log transformed.

Table 1**:** List of fungi isolates used in the *in-vivo* and *in-vitro* studies

Isolate ID	**Species**	**Location by state**	**Source**
F7 (2)	*P. pinodes*	Montana	Dry pea leaves
AWP7B1I0	*P. pinodes*	Idaho	Chilvers, *et al*., 2009
MP-I Scil	*P. pinodes*	Washington	Chilvers, *et al*., 2009
PMP-3	*P. pinodella*	Washington	Chilvers, *et al.*, 2009
AWP 4B1IO	*P. pinodella*	Idaho	Chilvers, *et al*., 2009
D -305	*D. pisi*	Montana	Dry pea seed
D -234	*D. pisi*	Montana	Dry pea seed
D - 249	*D. pisi*	Montana	Dry pea seed
PH-1	*Phoma sp*	Montana	Dry pea seed
PH-2	*Phoma sp*	Montana	Dry pea seed
PH-3	*Phoma sp*	Montana	Dry pea seed

Evaluation of the pathogenicity and aggressiveness of the fungal pathogens associated with ascochyta blight of dry pea**.** Greenhouse trials were conducted to determine the relative level of symptom severity caused by each fungal species. Dry pea seeds (cv. Carousel) which tested free of ascochyta blight were sown at one plant per pot in 80-ml plastic cones filled with a mixture of peat and Sunshine Mix 1 at ratio 1:1. At 12 days after planting, field pea plants were inoculated with three isolates each of *P. pinodes*, *D. pisi*, and *Phoma sp.* and two isolates of *P. pinodella* (Table 1). Conidial suspensions were prepared as described above from 14-day old cultures and adjusted to a concentration of 3×10^5 conidia/ml.

Conidial suspensions were applied to plants to run-off using hand-held spray bottles within an hour after preparation. Plants were placed in a mist chamber and held at > 90% relative humidity for 48 h at a 14-h photoperiod under artificial lighting before being placed on greenhouse benches. In order to facilitate comparison between disease symptoms caused by different fungal species on dry pea, the disease severity was estimated as the percentage of symptomatic leaves of the total number of leaves per plant inoculated with the spore suspension 10 days post inoculation (Seem, 1984; and Chen, *et al*., 2004). This method was used because using a disease severity scale designed for one of the pathogens would introduce bias for estimating disease severity for the other species. The experiments were arranged as an RCBD with five replicates (one plant per replicate) and conducted twice.

The development of a diagnostic tool for discriminating among fungal species associated with ascochyta blight of dry pea**.** Three isolates each of *P. pinodes*, *D. pisi*, and *Phoma sp.* and two isolates of *P. pinodella* were used for this study. A 5 mm mycelia plug of each isolate was inoculated on three media: PDA, oatmeal agar (OMA) (2.5% oat powder, 1.5% agar, w/w) and pea meal agar (PMA) (2.5% pea powder, 1.5% agar, w/v). The plates were incubated at 20 ^{o}C under a diurnal regime of cool white fluorescent light (12 h light followed by 12 h dark). To discriminate among the fungal species, the mycelial morphology was observed. The consistency of the mycelial morphology on the media was assessed by inoculating isolates from different geographical locations on the media (Table 1).

To determine the effects of the different media on the sporulation of the fungal species, conidial suspensions were prepared according to the methods previously described from three plates each of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp*. The conidia were counted at 3, 6, 9, and 12 days post-inoculation using a hemocytometer and viewed with a phase contrast Leica TCS SP5 imaging system (Leica Microsystems Inc, Illinois). The experiments were laid out as an RCBD with 12 replicates (one plate per replicate). The whole experiment was conducted twice.

To enhance comparison among the fungal species, the spore count for each isolate for each species was log transformed. Homogeneity of the variances from the two experiments was determined by the Levene's test (Levene, 1960). Data were analyzed using the generalized linear mixed-effects model in the lm4/ nlme statistical package (Bates, *et al.*, 2015 and Pinheiro, *et al*., 2018). A Tukey honestly significant difference for

multiple means comparison was computed using the multcomp package (Hothorn, *et al*., 2008).

To validate the efficiency of PMA as a diagnostic tool for discrimination among fungal species associated with AB of dry pea, 44 isolates collected from AB infected dry pea seeds lots in Montana and 10 isolates collected from symptomatic leaves of AB infected pea plants in North Dakota were screened. The internal transcribed spacer gene (ITS) of the isolates from Montana were also sequenced for taxonomic confirmation. The taxonomic identity of the isolates from North Dakota was confirmed using the morphology of characterized isolates as a reference (Chilvers, *et al.*, 2009). The morphology of typed isolates of *P. pinodes, P. pinodella* from Chilvers, *et al.*, (2009) and *D. pisi* and *Phoma sp.* from RPCDL on PMA were used as a reference for taxonomic identification of the isolates from North Dakota when inoculated on PMA. All the isolates were cultured on PMA at 22 ^{o}C as described above.

Statistical Analyses**.** Each experiment was conducted twice. Homogeneity of variances from the two experiments was determined by the Levene's test (Levene, 1960). In addition, data were analyzed using the linear mixed-effects model in the lm4/ nlme statistical package in R (Bates, *et al.*, 2015 and Pinheiro, *et al*., 2018). A Tukey honestly significant difference for multiple means comparison was computed using the multcomp package (Hothorn, *et al*., 2008)

Results

Effects of temperature on the mycelial growth rate and sporulation of D. pisi, P. pinodes, P. pinodella, and Phoma sp. This experiment evaluated the effects of temperature on the growth rate and sporulation of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp*. Independent analysis of the *in-vitro* experiments showed that variances were homogeneous, and the two experiments were combined for further analysis (P-value = 0.46). There was no differences in growth rate within isolates of same species (P-value = 0.16). Mean growth rate (mm/day) initially increased for all the species from 15 ^{o}C to 25 ^{o}C and then decreased at 30 ^{o}C (Figure 1). The mycelial growth rate was higher in *Phoma sp.* than in the other species (P-value= 0.0001, Table 2). At 15 ^{o}C, the growth rate of *D. pisi* and *P. pinodes* was 1.7 and 0.9 mm/day, while *P. pinodella* and *Phoma sp.* were 1.9 mm/day and 4.6 mm/day, respectively (Fig 1).

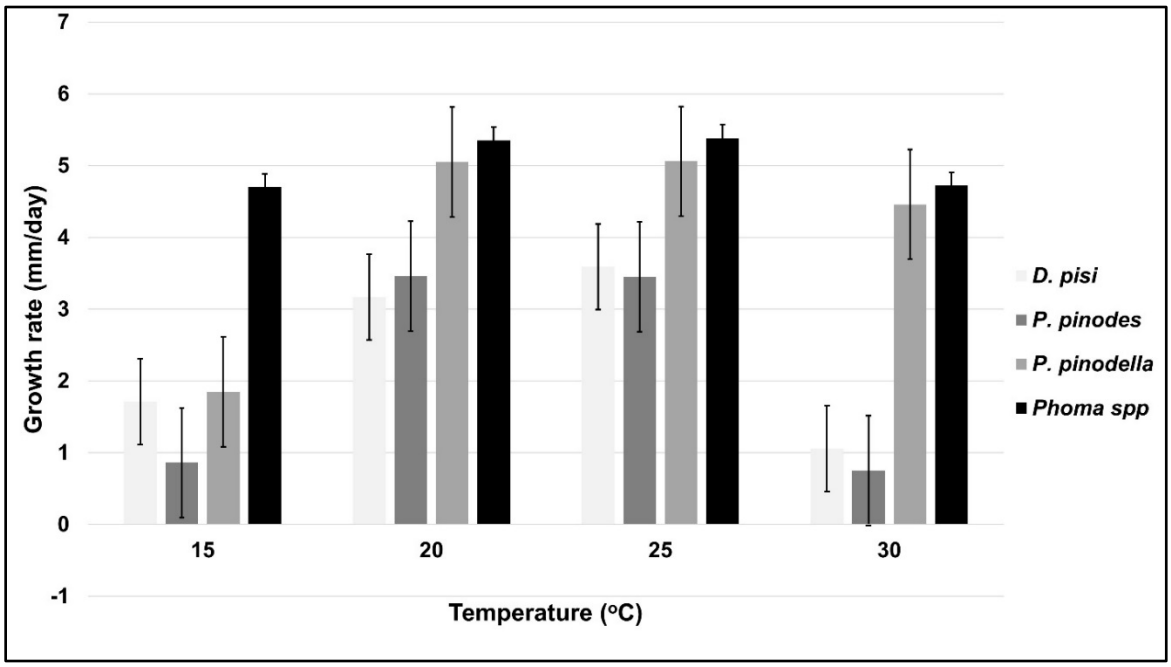

Figure 1**.** The mean growth rate of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* at four temperatures. Brackets represent standard errors of the mean growth rate.

Table 2 Tukey HSD pairwise means comparison of the mycelia growth rate at four temperatures of fungal species associated with ascochyta blight of dry pea

Fungal Species	**Mean (mm/day)**	**Critical difference (*w*)***
Didymella pisi	2.383	a
Peyronellaea pinodes	2.130	a
Peyronellaea pinodella	4.105	b
Phoma sp.	5.037	c

*= Means with different letters are significantly different across the four temperatures (P-value = < 0.0001)

The variances of the two sporulation experiments were homogenous and were combined for further analysis (P-value = 0.20). There were no significant differences within isolates of the same species (P-value = 0.167). The number of spores for all fungal species increased from 15 ºC to 25 ºC, followed by a decrease at 30 ºC (P-value = 0.0001) (Fig. 2). The number of spores was higher in *Phoma sp*. than in other species (Table 3)

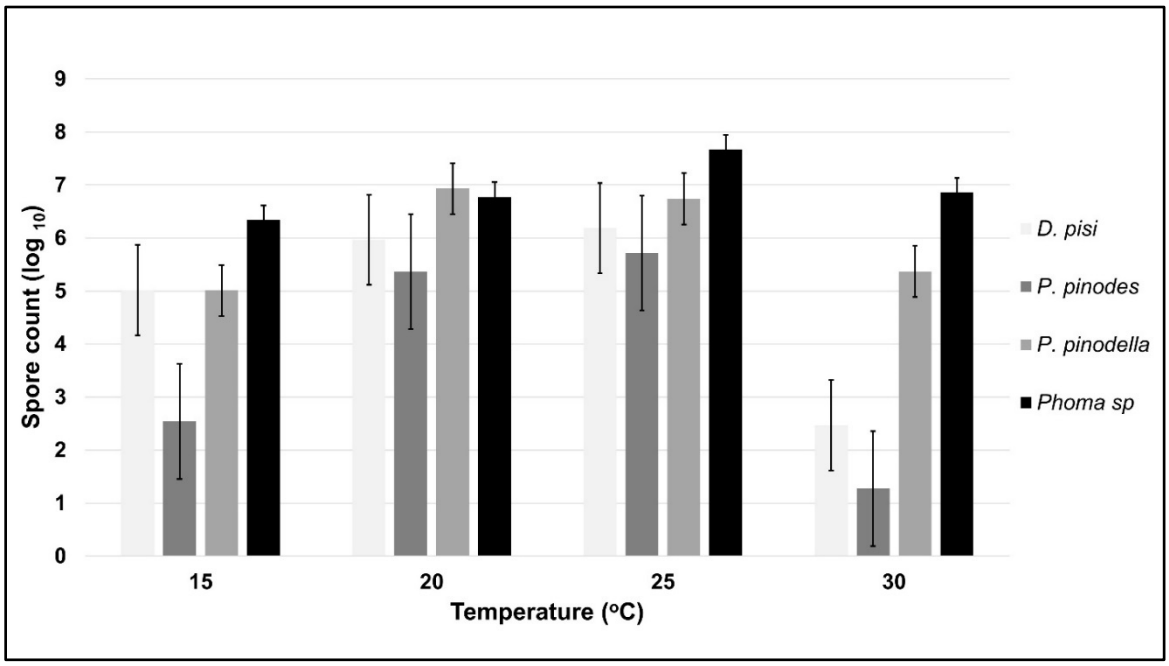

Figure 2**.** The mean spore count of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* at four temperatures. Brackets represent standard errors of the mean growth rate.

Table 3 Tukey HSD pairwise means comparison of the log spore count at four temperature of fungal species associated with ascochyta blight of dry pea

Fungal Species	**Mean**	**Critical difference (*w*)***
Didymella pisi	5.012	b
Peyronellaea pinodes	3.725	a
Peyronellaea pinodella	6.013	b
Phoma sp.	7.012	c

*= Means with different letters are significantly different across the four temperatures (P-value = < 0.0001)

Evaluation of the pathogenicity and aggressiveness of the fungal pathogens associated with ascochyta blight of dry pea. Independent analysis of greenhouse pathogenicity and aggressiveness experiments showed that variances were homogeneous, and the two experiments were combined for further analysis (P-value = 0.53). Disease severity was significantly higher on pea plants inoculated with *P. pinodes* isolates than with other fungal isolates (P-value = 0.0001). The *Phoma sp.* was not pathogenic on dry pea (P-value = 0.0001) (Fig. 3a and 3b). About 68 % disease severity was observed on plants inoculated with *P. pinodes*, 30 % disease severity was observed on dry pea plants inoculated with *P. pinodella*, and while < 25 % disease severity was observed in dry pea plants inoculated with *D. pisi* (Fig. 3a and 3b). In addition, pea plants inoculated with *P. pinodes* and *P. pinodella* developed lesions within 5 days, this is in contrast with pea plants inoculated with *D. pisi* where lesions were detected at 8 days post inoculation (data not shown).

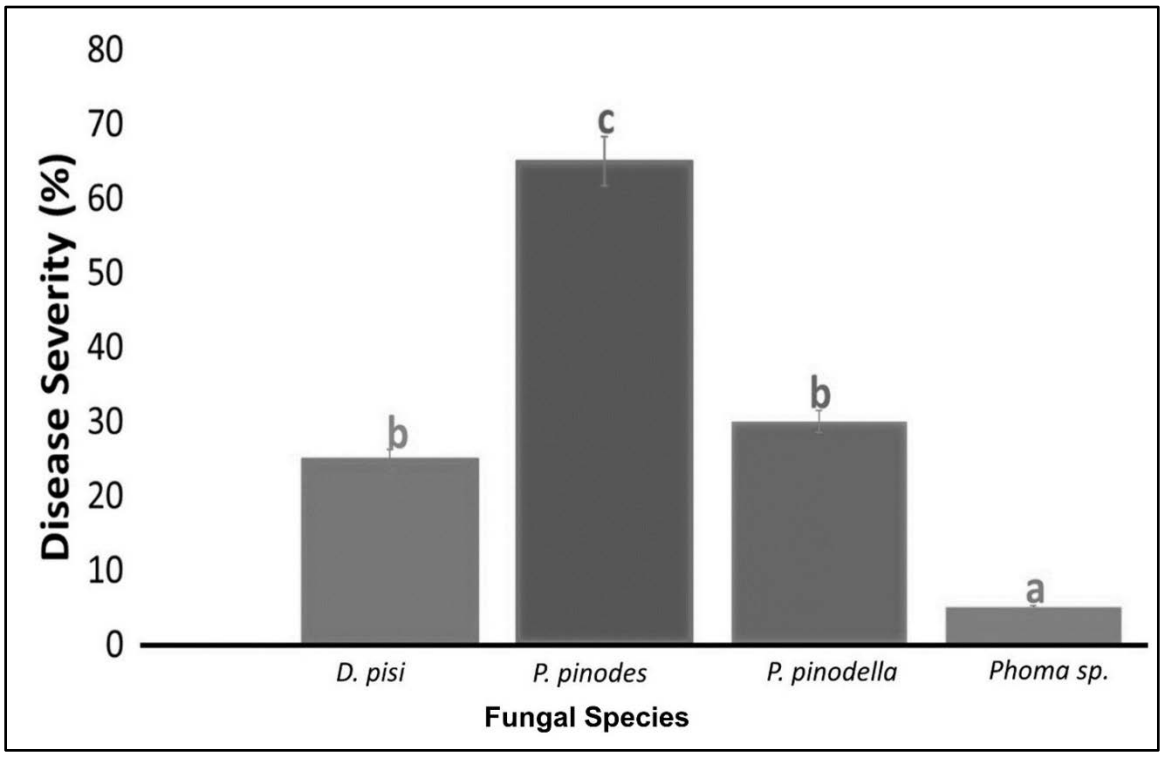

Figure 3a. The mean disease severity of dry pea cv. Carousel inoculated with *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp*. Brackets represent standard errors of disease severity estimates.

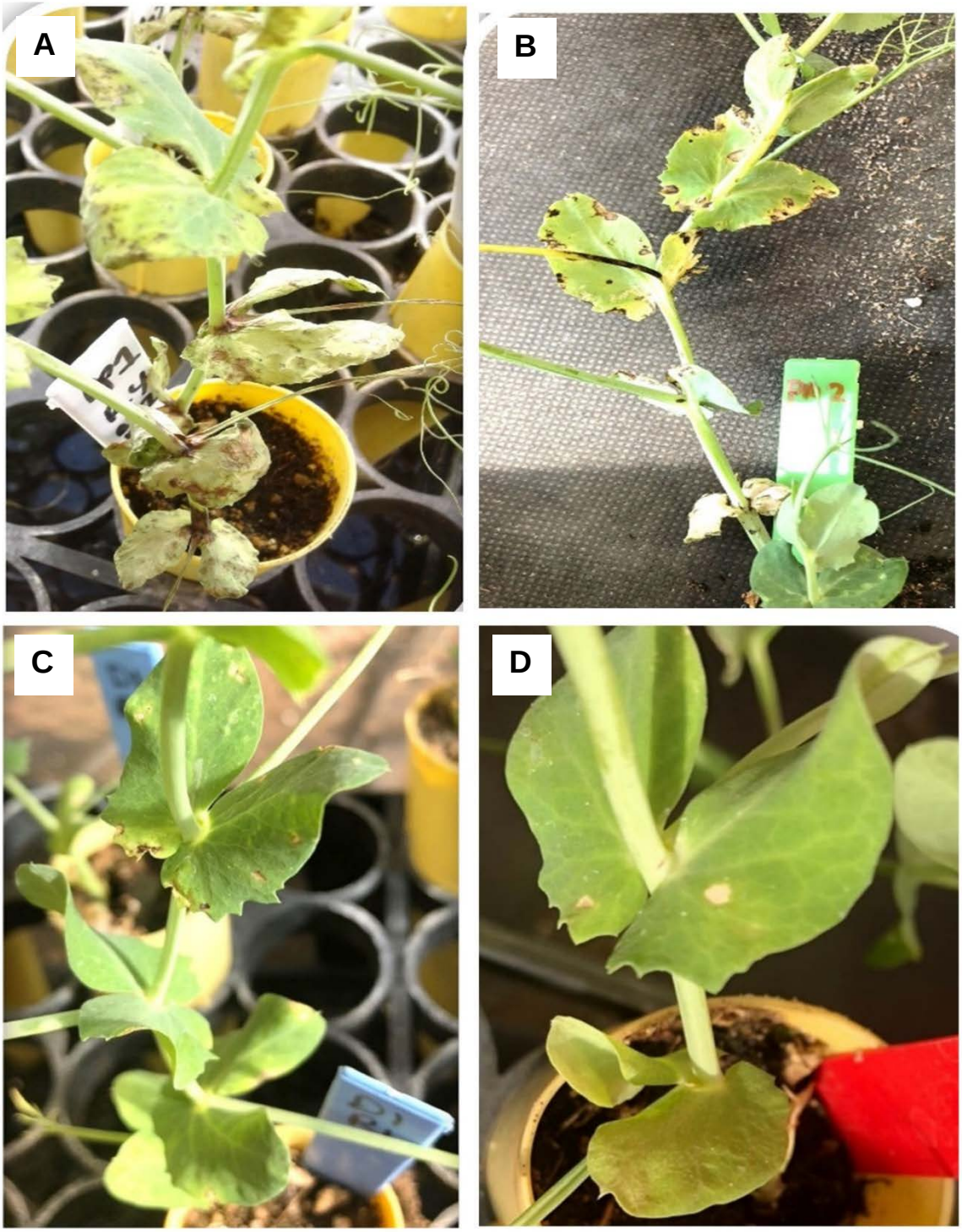

Figure 3b. Ascochyta blight symptoms severity on the pea plants at 10 days post inoculation with A= *P. pinodes*, B = *P. pinodella*, C = *D. pisi*, and D = *Phoma sp.*

The development of a diagnostic tool for discriminating among fungal species associated with ascochyta blight of dry pea**.** Isolates used for the previous experiment (Table 1) were used for this study. Inoculation of mycelial plugs of *D. pisi, P. pinodes, P. pinodella*, and *Phoma sp.* on PMA, PDA, and OMA resulted in distinctly different and consistent morphology. The characteristic carrot red morphology of *D. pisi* was observed on both PMA and OMA. On OMA, *P. pinodes* and *P. pinodella* produced sparsely distributed gray mycelia and *Phoma sp.* produced dark purplish-gray mycelia with a white halo. OMA could not be used to discriminate between *P. pinodes* and *P. pinodella*. On PDA, *D. pisi, P. pinodes*, and *P. pinodella* produced dense white fluffy mycelia while *Phoma sp.* produced brown mycelia with a white halo. These morphological observations indicated that PDA cannot be used to discriminate among the fungal species. However, on PMA, *P. pinodes* produced dense gray mycelia with a dense white halo, *P. pinodella* produced dense white mycelia with water droplets, and *Phoma sp.* produced brown mycelia with concentric rings (Fig 4). The characteristic white halo observed in *P. pinodes* on PMA allowed a clear discrimination between *P. pinodes* and *P. pinodella*. The morphological consistency of the fungal species was observed only on PMA (Fig. 4 and 5).

Conidial production was tested on the three media to determine if there were any differences between the species. There were no significant differences within isolates of the same species (data not shown). There was a linear increase in conidial production from 3 – 12 DPI for all the fungal species when inoculated on PDA, OMA, and PMA. Conidial production in *Phoma sp*. was significantly higher across the incubation period when compared with other fungal species (P-value ≤ 0.0001) (Fig. 6). Also, conidial production

was significantly higher across the entire incubation period for all the fungal species when cultured on peameal agar (PMA) than on the other media (P-value ≤ 0.0001) (Fig. 6).

Peameal agar was used to characterize seed- and plant-derived isolates of fungi associated with ascochyta blight symptoms from Montana and North Dakota. Out of the 44 isolates collected from dry pea seeds from Montana, four isolates were *P. pinodes.* Morphology matched ITS sequencing results (accession numbers of reference isolate not shown). Ten isolates collected from symptomatic pea plants from North Dakota, were *P. pinodes.* (Table 4).

Table 4: Distribution of Ascochyta blight fungal isolates characterized using pea meal agar

Source of isolates	**Fungal ID**		**# of Isolates**	**State of origin**
	Based on PMA	**Based on typed isolates**		
AB symptomatic dry pea seed lots	*D. pisi*	*D. pisi*	39	Montana
AB symptomatic pea leaves	*P. pinodes*	*P. pinodes*	4	Montana
AB symptomatic pea leaves	*P. pinodes*	*P. pinodes*	10	North Dakota

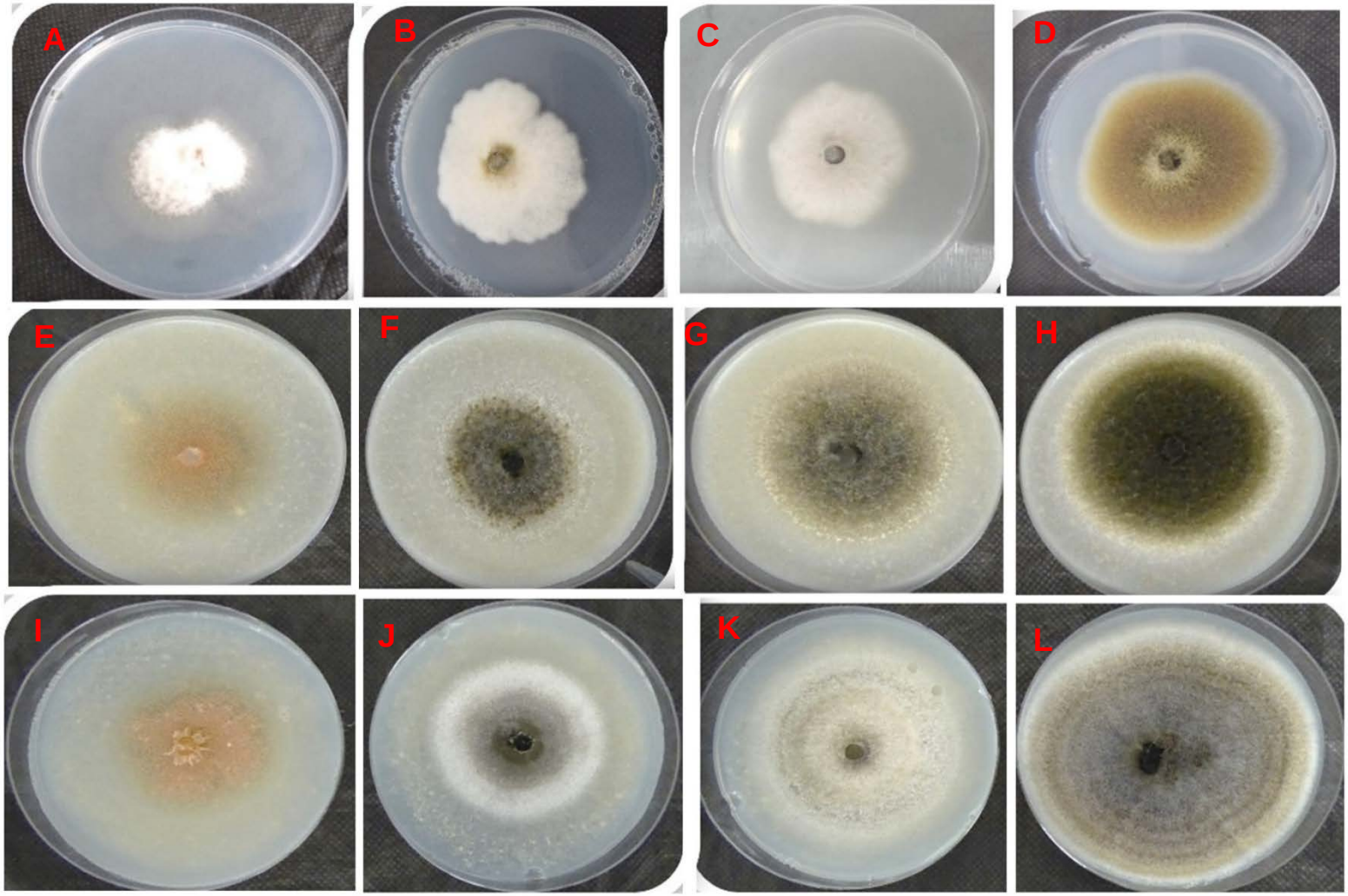

Figure 4. Morphological discrimination of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* when inoculated on potato dextrose agar, oatmeal agar, and peameal agar. (A-D) *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* inoculated on

PDA, (E-H) *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* inoculated on OMA, and (I-L) *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* inoculated on PMA.

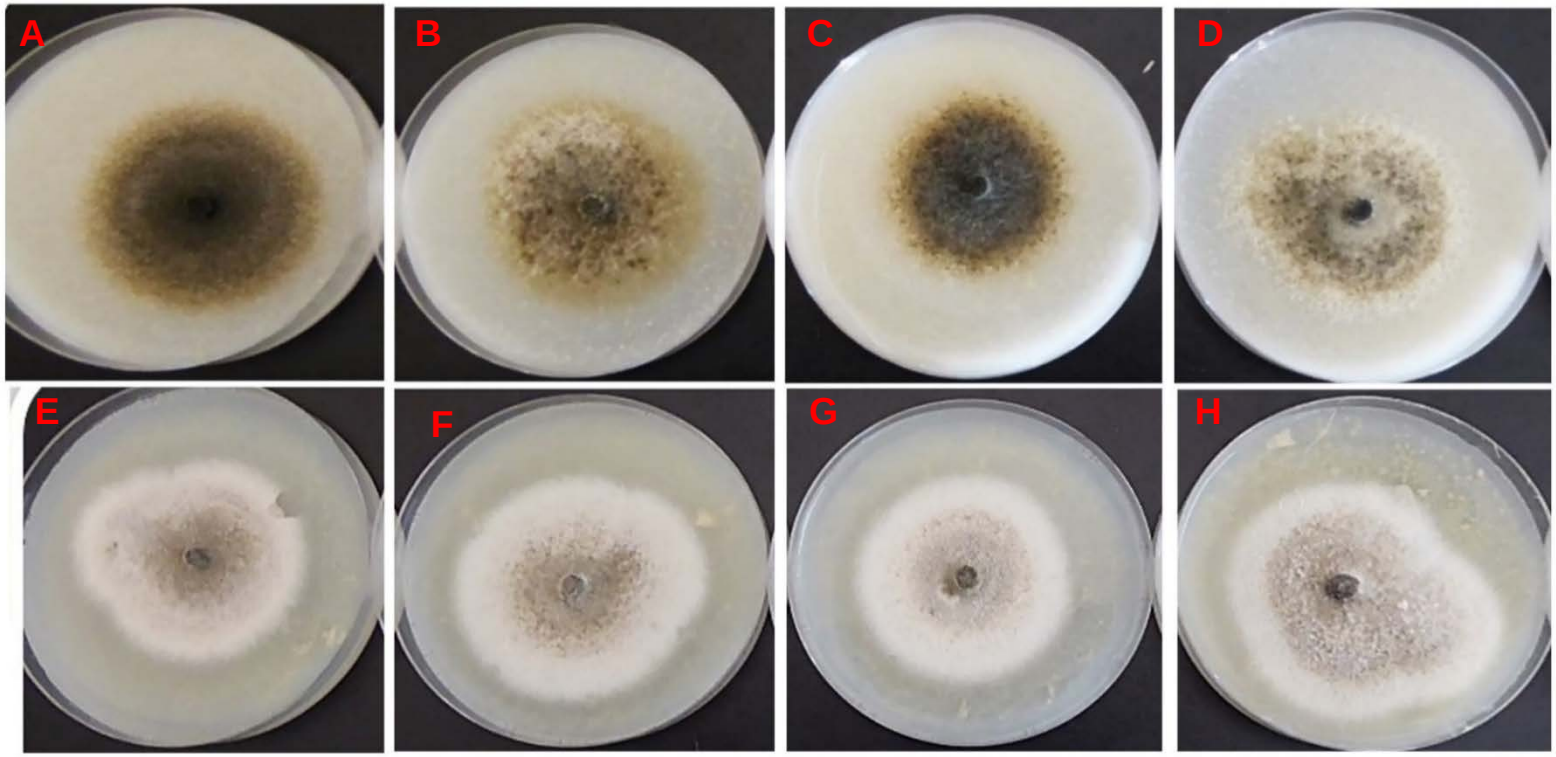

Figure 5. Morphological inconsistency of four geographically different isolates of *P. pinodes* when inoculated on oatmeal agar (A-D) and consistency of the isolates when inoculated on peameal agar (E-H).

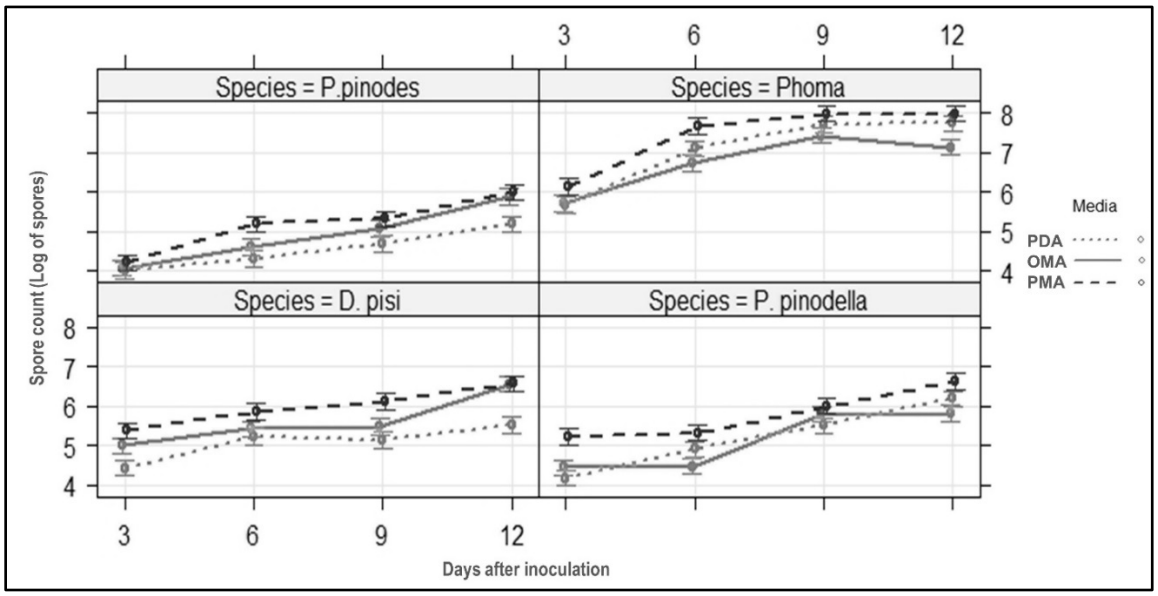

Figure 6. The mean log spore count of *D. pisi*, *P. pinodes*, *P. pinodella*, and *Phoma sp.* inoculated on potato dextrose agar, oatmeal agar, and peameal agar. Brackets represent standard errors of the mean.

Discussion

The mycelial growth rate and sporulation of the four fungal species associated with AB of dry pea were evaluated at different temperatures. Highest growth and development of all fungal species occurred at 20 ^{o}C and 25 ^{o}C. Summarily, their growth and development were impacted by the low temperature of 15 ^{o}C and a high temperature of 30 ^{o}C.

Similarly, we found that conidia were produced over a temperature range from 15 to 30 ^{o}C. Peak sporulation occurred at 25 ^{o}C for *Phoma sp.* and *D. pisi*, while *P. pinodes* and *P. pinodella* had peak sporulation at 20 ^{o}C. These fungal species are adaptable to varying temperatures. Thus, they can cause AB of dry pea in generally hot climates observed in Australia (Davidson, *et al.,* 2009; Li, *et al.,* 2011; Tran *et al.,* 2014; and Liu, *et al.,* 2016) and in cold climates of northern North America (Chilvers, *et al*., 2009; Gossen, *et al.,* 2011; Ahmed *et al.,* 2015; and Sivachandra-Kumar and Banniza 2017). In addition, the developmental responses of these pathogens to varying temperatures showed their adaptive potential to a climatic condition. This evolutionary adaptation may also be expressed in their ability to overcome the potential resistance of the host plant (Darby, *et al.,* 1986). *D. pisi* and *P. pinodella* both have low and high sporulation, respectively. Both pathogens are heterothallic (Bowen, *et al.,* 1997 and Chilvers, *et al.,* 2009). The formation of sexual spores in these pathogens enables genetic diversity due to sexual recombination, which is associated with increased aggressiveness, development of fungicide resistance, and loss of resistant cultivars (Chilvers, *et al*., 2009 and Owati, *et al*., 2017). Furthermore, *P. pinodes* produce ascospores from pseudothecia during rainfall (Roger, *et al.,* 1996 and Tivoli, *et al.,* 2007; and Schoeny, *et al*., 2008). These ascospores are windborne and travel

long distances to infect healthy pea plants, the consequences of this are increased epidemics of AB caused by *P. pinodes* and its adaptability to different geographical zones (Davidson, *et al*., 2009 and Gossen, *et al*., 2011).

The effects of temperature on the growth and sporulation of phytopathogenic fungi are well documented. Our results concurred with the findings of Terefe *et al*. (2015) where they reported that mycelia radial growth, sporulation, conidial size, and sclerotial formation of *Botrytis fabae* increased with increase in temperature and peaked at 22°C but progressively declined at increased temperature. A similar pattern of mycelia growth due to the effects of temperature was observed *Botrytis cinerea* (Fernández *et al*., 2014). Also, Peetz, *et al* (2009) reported that temperatures ≥ 30 ^{o}C significantly reduced the sporulation and infectivity of conidia from *Podosphaera macularis.* Furthermore, Copes, *et al* (2004), found that a low temperature of 6 ^{o}C and a high temperature of 30 ^{o}C reduced the sporulation of *Botryosphaeria dothidea*, *B. obtusa*, and *B. rhodian* compared to the ideal temperature of 24 ^{o}C.

The temperature range of 20 ^{o}C to 25 ^{o}C coincides with the average temperature (18 ^{o}C to 27 ^{o}C) during active growing stages (flowering to maturity) of dry pea in Montana (Western Region Climate Center WRCC). This implies that our environment is conducive for all the fungal species associated with AB of dry pea. Furthermore, the adaptability of these pathogens and the potential to disperse their propagules heighten the concerns to monitor the frequency of the invasive species (*P. pinodes* and *P. pinodella*) to Montana. *D. pisi,* which is the predominant species in Montana, had the lowest growth rate and lowest sporulation in culture among the four species evaluated. Symptoms on plants indicate it is not a highly aggressive species. This suggested that the fungus is a slow-growing pathogen

and produces a low amount of inoculum, thus, the pathogen will colonize dry pea plants at lower rate giving room for the plant to marshal its defense system to halt the pathogen's invasion or recover from the infection. This may explain why there are low incidences of significant yield losses associated with this pathogen in Montana dry pea fields. This is in contrast to the report of an epidemic of AB of dry pea in Spain (Chilvers, *et al.,* 2007 and Kaiser, *et al.,* 2008). *P. pinodes* and *P. pinodella* showed higher growth rate in culture and thus suggested to be capable of colonizing infected dry pea plants at a higher rate. The *in-vivo* responses of these pathogens may not be predictable by *in-vitro* studies.

P. pinodes caused increase symptom severity on variety Carousel compared to the other fungal pathogens in the AB complex. Although, only one dry pea variety was used in this study, further studies are needed to evaluate the response of multiple dry pea genotypes to ascochyta blight causing pathogens. The results of our study may explain the frequent cases of significant yield losses associated with this disease in North Dakota and Canada (Gossen, *et al.,* 2011). Also, the pathogenic variation that exist in *P. pinodes* population in Canada increases the challenge in selecting AB tolerant dry pea genotypes (Xue *et al*., 2001 and Ahmed *et al*., 2015). Out of the 335 field pea lines screened for partial resistance to *P. pinodes* in Canada, only seven lines expressed promising resistance to *P. pinodes* (Xue *et al*., 2001).

The uncharacterized *Phoma sp.* found associated with dry pea seed were not pathogenic on dry pea plants. There were no lesions on the pea leaves or stems. This is in contrast with the recently identified *Phoma koolunga*, *Phoma herbarum*, and *Phoma glomerata* which have been reported to be associated with AB of dry pea in Australia

(Davidson, *et al.*, 2009; Li, *et al.*, 2011; Tran *et al.*, 2014; and Liu, *et al.*, 2016). These pathogens have not been found in North America.

Peameal agar was an effective tool to consistently discriminate among the fungal species associated with AB of dry pea using visual characteristics. Previous studies have used OMA to discriminate between *D. pisi* and other fungal pathogens in the AB complex (Jones, 1927; and Ahmed, *et al*., 2015), OMA cannot consistently discriminate among all three fungal species associated with AB of dry pea in North America. To our knowledge, this is the first study that showed that PMA can be used to morphologically discriminate among the three fungal pathogens. This tool is of importance in North America where there is geographical variation in the predominant species associated with AB of dry pea. Since the pathogens differ in aggressiveness on our varieties, this indicates that management recommendations to farmers may change in the future as *P. pinodes* becomes more predominant in the landscape. This tool has been used to characterize fungal isolates from two states. All 10 isolates collected from dry pea plants symptomatic for AB from North Dakota were *P. pinodes*. This tool will be used to monitor changes in population dynamics of fungal species associated with AB of dry pea in North America with special emphasis on Montana where a shift in pathogen population from *D. pisi* to *P. pinodes* is currently being observed. This information is needed to advise growers on the need to modify their AB disease management strategies. For instance, most growers in Montana sparingly apply foliar fungicide treatment for the control of AB of dry pea. This recommendation may need to be changed where *P. pinodes* becomes the predominant pathogen, unless variety resistance is identified in currently available germplasm.

Sporulation of the species varied when inoculated on PDA, OMA, and PMA. PMA induced rapid sporulation in these pathogens when compared with other media (Fig 6). Across the three media used in this study, all the fungal species used in this study produced approximately 10^6 spores within 6 days when inoculated on PMA while it took 9 to 12 days to produce lower spore counts when inoculated on PDA and OMA. PMA can be used in the rapid preparation of inoculum needed for greenhouse or field trials, thus reducing the average days of incubation, amount of petri plates, and labor required for preparation of inoculum.

This study has characterized the fungal pathogens associated with AB of dry pea in Montana. It demonstrated the adaptability of the fungal pathogens to varying temperatures. Furthermore, the study supported previous results that *P. pinodes* is the most aggressive of all the fungal species associated with AB of dry pea in North America. In addition, the study found that the uncharacterized *Phoma sp.* associated with AB contaminated dry pea seeds in Montana is not pathogenic on dry pea plants.

Peameal agar is a selective media that can discriminate among the fungal species associated with AB of dry pea in North America. It also showed that PMA is a substrate that can be used for rapid sporulation of the fungal species. Thus, this medium serves as a diagnostic and epidemiological tool that can be used to identify and monitor changes in the population dynamics of fungal species associated with AB of dry pea.

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CHAPTER FIVE

THE DEVELOPMENT AND APPLICATION OF REAL-TIME AND CONVENTION SSR-PCR ASSAYS FOR RAPID AND SENSITIVE DETECTION OF *DIDYMELLA PISI* ASSOCIATED WITH ASCOCHYTA BLIGHT OF DRY PEA.

Contributions of Author and Co-Authors

Manuscript in Chapter 5

Author: Ayodeji Owati

Contributions: Conceived, designed, and conducted the experiments. Analyzed the data and wrote the manuscript.

Co-Author: Bright Agindotan

Contributions: Conceived the experiments and reviewed the manuscript

Co-Author: Mary Burrows

Contributions: Reviewed the manuscript

Manuscript Information

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CHAPTER FIVE

THE DEVELOPMENT AND APPLICATION OF REAL-TIME AND CONVENTION SSR-PCR ASSAYS FOR RAPID AND SENSITIVE DETECTION OF *DIDYMELLA PISI* ASSOCIATED WITH ASCOCHYTA BLIGHT OF DRY PEA.

Abstract

Didymella pisi is the primary causal pathogen associated with ascochyta blight (AB) of dry pea in Montana. Diagnosis of AB is challenging because there are a number of different species that cause AB worldwide and that can co-occur in the same geographic area. Additionally, agar plate identification of *D. pisi* is challenging due to its slow growth rate and different morphotypes. Currently, there is no polymerase chain reaction (PCR)-based assays developed for specific detection of *D. pisi* or any of the fungal pathogens associated with the AB complex of dry pea. In this study, we evaluated simple sequence repeats (SSRs) primer pairs that have consistently amplified a single band in 205 isolates of *D. pisi* from 13 counties in Montana for their specificity and sensitivity in real-time and conventional SSR-PCR in the presence of other fungal and plant DNA. The specificity of the assay was determined by testing genomic DNA of 10 dry pea varieties, fungal species in the ascochyta blight complex which share high sequence similarities of fingerprinting genes with *D. pisi*, and fungal species commonly associated with dry pea. To avoid false negative PCR results, plant and fungal DNA generic primer pairs were included as controls in a one-tube conventional multiplex SSR-PCR, to amplify at least any fungal or host plant DNA in the absence of *D. pisi* SSR target. SYBR green real-time SSR-PCR detection was

carried out using same primer pairs as in conventional PCR but in a uniplex format with controls and *D. pisi* in separate reactions. *D. pisi* was specifically amplified, while other fungi and host DNA were not. Also, sensitivity experiments showed that the detection limit of the assays was 0.01 ng of pure genomic DNA of *D. pisi* for both assays. In addition, the detection limit for SSR-qPCR was 100 conidia. Therefore, these qPCR and PCR assays are rapid, specific, and sensitive diagnostic tools for the detection, quantification, and monitoring of *D. pisi.*

Introduction

In 2017, a total of 1 million acres of dry peas (*Pisum sativum*) were harvested in the United States, representing a farm-gate value of $170 million (United States Department of Agriculture National Agricultural Statistics Service (USDA NASS, 2017). In Montana during 2017 cropping season, a total of 470, 000 acres of dry pea were harvested representing a farm gate value of $42 million. Also, Montana accounted for 47% of the total US dry pea production in 2018 (USDA NASS 2018). Changes in pathogen populations and the risk of moving pathogens to new areas pose a great risk to the production of high quality and disease-free dry pea seeds required in the export markets.

Six fungal species have been associated with ascochyta blight of dry pea (Aveskamp *et al.*, 2010; Barilli *et al*., 2016; Davidson *et al.*, 2009; Li *et al.*, 2011; Tran *et al.*, 2014; and Trivoli *et al*., 2007). All these pathogens can exist together on a host or independently of each other (Davidson *et al.*, 2009 and Trivoli *et al*., 2007). However, *Didymella pisi*, *Peyronellaea pinodes*, and *Peyronellaea pinodella* are the only three fungal pathogens

associated with ascochyta blight (AB) of dry peas in the Great Plains of North America. In Australia, *Phoma koolunga*, *P. glomerata*, and *P. herbarum* are the additional pathogens associated with the disease. (Aveskamp *et al.*, 2010; Davidson *et al.*, 2009; Li *et al.*, 2011; and Tran *et al.*, 2014). These pathogens are associated with shrinking and discoloration of dry pea seeds, and lesions on foliar parts of pea plants (Bretag *et al.*, 2001; 2006; Gossen, *et al.*, 2011; and Skoglund *et al*., 2011). In Montana, the predominant fungal species associated with AB of dry pea is *D. pisi* (Owati *et al.*, 2017). *Didymella pisi* is seed-and residue-borne and the dry pea plants are susceptible to the pathogen at every growth stage (Davidson *et al.*, 2009; 2011; and Gossen *et al.*, 2011). Under favorable conditions, the pathogen can cause 15- 30% yield loss in dry peas (Ahmed *et al.*, 2015; Wallen, 1965; and Xue *et al.*, 1996; 1997).

Current identification methods for *D. pisi* are laborious and time-consuming. Identification using an artificial medium like potato dextrose agar requires between 9 – 12 days for the fungus to grow and sporulate (International Rules for Seed Testing [IRST]; and Owati *et al*., 2017). Furthermore, molecular identification of this pathogen is challenging, because of the high sequence similarities of fingerprinting genes such as *Internal transcribed spacer genes* and intergenic sequences that exist among AB-associated pathogens. To date, the use of multiple gene sequences has been used to identify this pathogen (Liu *et al*., 2016). Confounding diagnostics further is the fact that the other two pathogens (*Peyronellaea pinodes* and *P. pinodella*) associated with AB of dry pea in the Great Plains of North America have similar symptom expression on pea plants as *D. pisi.* These challenges complicate diagnosis, reinforcing the need for an accurate and

sensitive assay to diagnose this pathogen and track changes in pathogen populations over time.

For both initial and rapid evaluation of AB diseased plant samples, the detection of *D. pisi* for diagnostic purposes in the field and laboratory have focused on the use of symptoms, the morphology of asexual structures (pycnidia), and sequencing of the fungus internal transcribed spacer (ITS) region and other conserved genes. The use of artificial media is laborious and time-consuming due to the slow growing nature of *D. pisi* and the need for single spore isolation to obtain pure cultures (Ahmed *et al.,* 2015 and Davidson *et al*., 2009). Multi-loci gene sequencing used for the diagnosis of *D. pisi* is not rapid and requires pure cultures which might take 10-14 days for mycelial growth. An additional 3-5 days are needed for DNA isolation, polymerization chain reaction (PCR), and sequencing of amplicons (Liu *et al*., 2016). Thus, a rapid, sensitive, and specific pathogen detection method will facilitate pathogen identification and assist with more timely and effective disease management strategies such as tracking changes in pathogen populations and determining the need for targeted fungicide applications (Wang *et al.,* 2006).

The use of molecular biology techniques including PCR has been used widely for detection and identification of phytopathogenic fungal species. Both conventional and real-time PCR assays have been developed for both *in-vitro* and *in-planta* detection and quantification of a number of foliar, root, and soil phytopathogenic fungi (Bonants *et al*., 1997; Bhat *et al*., 2010; Cao *et al.,* 2007; Chilvers *et al*., 2007; Henson *et al.,* 1993; Hughes *et al.,* 1998; Hussain, 2005; Keller *et al*., 1995; Kuzdraliński *et al*., 2017; Li *et al.,* 2013; Lin *et al.,* 2009; Moricca *et al.,* 1998; Moukhamedov *et al.,* 1994; Nieplod *et al.,* 1995; Phan *et al*., 2002; Wang *et al.,* 2006; 2007; and Wang *et al*., 2015). Zitnick-Anderson *et*

al. (2018) developed a multiplexed qPCR for the identification and quantification of seven *Fusarium sp*. associated with root rot of dry pea. Also, Rojas *et al* (2017) developed a qPCR and recombinant polymerase amplification assays (RPA) for genus- and species-specific detection and quantification of *Phytophthora sojae* and *P. sansomeana* root rot pathogens of soybean from plant tissue and soil samples.

Currently, there is no rapid, sensitive, and specific detection assay developed for *in-vitro* and *in-planta* detection of any of the pathogens in the ascochyta blight complex of dry pea. The objectives of this study were to (i) develop a specific, conventional SSR-PCR assay for the detection of *D. pisi* (ii) develop an SSR-qPCR assay for specific detection and quantification of *D. pisi*, and (iii) validate the qPCR and PCR assays both in the presence of host materials and other fungal species.

Materials and Methods

Fungal isolates and DNA extraction. Fungal genera associated with AB of dry peas and other genera commonly associated with dry peas were used in this study including *Didymella, Peyronellaea, Phoma, Alternaria, Stemphylium, Nigospora, Fusarium, Botrytis, Collectotrichum, Sclerotina, and Cladosporium* were collected from symptomatic dry pea leaves and seeds from Montana, Idaho, and Washington. DNA of *Phoma koolunga* was provided courtesy of Dr. Jenny Davidson, South Australia Research and Development Institute, Urrbrae, South Australia. Single spore cultures of each isolate were maintained on potato dextrose agar (PDA) at 22 ^{o}C with a 12 h diurnal photoperiod. Total genomic DNA was extracted from 10-day-old cultures grown on PDA using the DNeasy Plant Mini Kit (QIAGEN, Germantown, MD) according to the manufacturer's instruction with

modification of the starting process. The quality and concentration of extracted DNA were estimated using the NanoDrop 2000c Spectrophotometer at 260 nm (Thermo Fisher Scientific, Walthum, MA). The DNA samples were stored at -20 °C until further use.

Development of conventional Simple Sequence Repeats- PCR (SSR-PCR) for specific and sensitive detection of *D. pisi*

Screening of SSR primers for *D. pisi* specificity. Nine SSR primer pairs (A311, A313, A315, A318, A321, A324, A325, A326, and A342) (Owati *et al.*, 2019) which amplified a single band in 205 *D. pisi* isolates from Montana were selected for this study. These primers were screened for their specificity to *D. pisi* using host plant DNA and other fungal DNA templates in uniplex PCR reaction. Total genomic DNA was extracted from the foliage of 10 widely planted dry pea varieties in Montana courtesy of Dr. Kevin McPhee of Montana State University, Bozeman, using a cetyltrimethylammonium bromide (CTAB) procedure (Abarshi *et al.*, 2010) (Tables 2).

The PCR was optimized in a total volume of 25 µl containing 12.5 µl of Dream Taq Green PCR 2X Master mix (Thermo Fisher Scientific, Walthum, MA), 10 pM of each forward and reverse primer, and 2.5 µl DNA (50 ng). Amplification parameters were 4 min at 94 ˚C, followed by 30 cycles of 30 s at 94 ˚C and 30 s at 56.6 ˚C and a final extension at 72 ˚C for 5 min. The PCR products were analyzed on SYBR Safe-stained 2.5% (w/v) agarose gels run in the 1x sodium-borate buffer (Brody and Kern, 2004) and exposed to blue light to visualize DNA fragments. The amplicon sizes were estimated using a 100-bp DNA ladder (Thermo Fisher Scientific, Walthum, MA) (Owati *et al*., 2019).

Conventional multiplex SSR-PCR for *D. pisi* detection. Only SSR primers pairs that did not amplify host DNA were used for further experiments (Table 1). Each of the four *D. pisi*-specific SSR primer pairs (A311-F/R, A313-F/R, A315-F/R, and A318-F/R) together with fungal internal transcribed spacer (ITS) and *Rubisco L*-gene (RBCL) primer pairs (Table1) were tested for compatibility in conventional multiplex detection of *D. pisi.* The ITS and RBCL primers were used to amplify fungal and host plant DNA internal control DNA targets, respectively. DNA was extracted from two fungal-infected dry pea leaf samples, pure cultures of four fungal species in the AB complex, and 9 other fungal species of dry pea (Table 3) and used at 50 ng for the PCR to test also for the specificity of the multiplex PCR. The PCR reaction mix and cycling conditions were as described above except 10 pM of each of the ITS and RBCL primers were added to amplify the internal controls (Nassuth *et al.,* 2000 and White *et al.,* 1990).

Conventional multiplex SSR- PCR assay sensitivity tests. The sensitivity of the SSR-PCR assay was assessed by diluting DNA extracted from *D. pisi* culture. One hundred ng/µl genomic DNA of *D. pisi* was serially diluted 10-fold from 100 ng to 10 fg/ ul. The diluted DNA was used as template in a multiplex PCR as previously described with ITS primers to amplify fungal internal control target. Furthermore, to determine the sensitivity of this assay in the presence of host DNA, DNA from *D. pisi* was serially diluted 10-fold from 100 ng to 10 fg in 50 ng of DNA from dry pea (cv. Aragorn). Diluted DNA was used as template in a multiplex PCR assay with *D. pisi* SSR, ITS, and RBCL primers to specifically amplify *D. pisi*, fungal ITS and plant RBCL targets, respectively.

Table 1. Primer pairs used for specific amplification of *D. pisi*, internal transcribed spacer, and *Rubisco L*-gene

Primers	**Primer sequence (5'-3')**	**Repeat motifs**	**Size (bp)**	**Reference**	**Purpose**
A311-F	AGCAGGCATTACGTTTAACT	(AGC)6	249	Owati *et al.*, 2019	*D. pisi* amplification
A311-R	GGTAAGATGCGAGTACGAAT	(AGC)6	249	Owati *et al.*, 2019	*D. pisi* amplification
A313-F	ATAACAACCAACCTCTGACG	(ACC)6	486	Owati *et al.*, 2019	*D. pisi* amplification
A313-R	GGAGCAATAGGTGATCTTCTC	(ACC)6	486	Owati *et al.*, 2019	*D. pisi* amplification
A315-F	GGTGGACTGAGTTCTGTGTAG	(TTC)7	199	Owati *et al.*, 2019	*D. pisi* amplification

A315-R	TAGCGTGCTCTTGAGGATTA	(TTC)7	199	Owati *et al.*, 2019	*D. pisi* amplification
A318-F	CTAGAATCGTGCTTGTTGC	(TCG)7	406	Owati *et al.*, 2019	*D. pisi* amplification
A318-R	GAGTCTCCCTGTCTTTGTCC	(TCG)7	406	Owati *et al.*, 2019	*D. pisi* amplification
ITS 1	TCCGTAGGTGAACCTGCGG	[1]NA	550	White, *et al.*, 1990	Internal control for fungi DNA
ITS 4	TCCTCCGCTTATTGATATGC	NA	550	White, *et al.*, 1990	Internal control for fungi DNA

RBCL-H680	TGGACTTGATTTTACCAAAGATGATG	NA	642	Nassuth, *et al.*, 2000	Internal control for plant DNA
RBCL-C1321	TGTCCTAAAGTTCCTCCACC	NA	642	Nassuth, *et al.*, 2000	Internal control for plant DNA

[1]NA = Not Applicable

Table 2**.** List of dry pea varieties used in the specificity test of the *D. pisi* markers

Name of dry pea variety	**PCR amplification using *D. pisi* specific markers**	**PCR amplification using *Rubisco L*-gene markers**
Hyline	-	+
Treasure	-	+
CDC Leroy	-	+
Banner	-	+
Aragorn	-	+
Ginny	-	+
Montech 4193	-	+
MSGA-Jet Set	-	+
Early Star	-	+
Nette 2010	-	+

Table 3**.** Specificity test panel for conventional multiplex polymerase chain reaction assay

Fungi Species and Isolate ID	**PCR amplification[a]**	**ITS Amplification**	**Origin**
Alternaria alternata -15	-	+	Montana
Alternaria alternata - 44	-	+	Montana
Alternaria alternata - 49	-	+	Montana
Botrytis cinerea -2	-	+	Montana
Botrytis cinerea -3	-	+	Montana
Botrytis cinerea -4	-	+	Montana
Cladosporium sp - 84	-	+	Montana
Colletotrichum lentis- 63	-	+	Montana
Colletotrichum lentis -74	-	+	Montana
Colletotrichum lentis -80	-	+	Montana
Didymella pisi - 01-12	+	+	Montana
Didymella pisi - 01-13	+	+	Montana
Didymella pisi - 01-14	+	+	Montana
Didymella pisi - 01-15	+	+	Montana
Fusarium avenaceum - 6	-	+	Montana
Fusarium avenaceum - 13	-	+	Montana
Fusarium avenaceum - 33	-	+	Montana
Nigrospora oryzae - 8	-	+	Montana
Nigrospora oryzae - 20	-	+	Montana

Nigrospora oryzae - 25	-	+	Montana
Peyronellaea pinodella - 001	-	+	Washington
Peyronellaea pinodella - 002	-	+	Idaho
Peyronellaea pinodella - 003	-	+	North Dakota
Peyronellaea pinodes - AP1	-	+	Idaho
Peyronellaea pinodes - AP2	-	+	Washington
Peyronellaea pinodes - AP3	-	+	Montana
Phoma koolunga - T040	-	+	Australia
Phoma koolunga - FT07013	-	+	Australia
Phoma koolunga - FT15012	-	+	Australia
Phoma sp - PH1	-	+	Montana
Phoma sp - PH2	-	+	Montana
Phoma sp - PHM	-	+	Montana
Sclerotina sclerotium - 36	-	+	Montana
Sclerotina sclerotium - 38	-	+	Montana
Stemphylium vesicarium - 12	-	+	Montana
Stemphylium vesicarium - 14	-	+	Montana
Stemphylium vesicarium - 41	-	+	Montana
No Template	-	+	

[a]*D. pisi* specific primers were used for the PCR amplification

SYBR green real-time SSR-PCR amplification parameters

SYBR green qPCR amplifications were performed on the Biorad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States). The annealing temperature of the assay was optimized in a gradient qPCR. The SYBR green real-time was optimized in a total volume of 20 µl containing 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, United States), 10 pM of each forward and reverse primer, and 2.5 µl DNA (50 ng). Amplification parameters were 4 min at 94 ˚C, followed by 30 cycles of 30 s at 94 ˚C and 30 s at 59 ˚C and a final extension at 72 ˚C for 5 min.

Specificity and sensitivity tests of the SYBR green quantitative real-time SSR-PCR assay

Mycelial DNA. To determine the specificity of the real-time SSR-PCR assay, 50 ng of mycelial genomic DNA each of the target and non-target fungal species listed in Table 3 was used individually as a template. Primer pairs A311-F/R and A315-F/R specific to *D. pisi* were evaluated in the validation stage.

To determine the efficiency and sensitivity of the assays, a 10-fold *D. pisi* mycelial genomic DNA serial dilution from 100 ng to 10 fg was amplified using primer pair A311-F/R. Real-time quantitative (q) PCR efficiency was calculated with the formula: efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$. The slope was calculated from the linear regression between DNA $\log_{10}$ (template DNA concentrations) and quantification cycle (Cq) values using the Bio-Rad CFX Manager software package (Bio-Rad Laboratories, Hercules, CA, United States).

A similar experiment was conducted to determine the detection limit of the real-time qPCR assay in the presence of host plant leaf DNA using primer pair A311-F/R. Mycelial genomic DNA from *D. pisi* was serially diluted 10-fold from 100 ng to 1 fg in 50 ng genomic DNA from pea leaf (cv, Aragorn).

Conidial DNA. In order to quantify the number of spores that could be detected in an assay, a conidial suspension was obtained from three 15-day old cultures of *D. pisi*. This experiment was conducted twice. To prepare the suspension, the petri plate was flooded with 10 ml of sterile distilled water and conidia were dislodged using a sterile glass rod. The conidial count was estimated using a hemocytometer viewed with a phase contrast Leica TCS SP5 imaging system (Leica Microsystems Inc, Illinois). The spore concentration was adjusted to 10^5 conidia/ml and serially diluted 10-fold to 10 conidia/ml. DNA was extracted from each spore concentration using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instruction with modification of the starting process and tested with the specific qPCR assays. The efficiency and slope of the assay were computed as previously described.

Validation of the SYBR green real-time PCR assay. To validate these assays, genomic mycelial DNA of 30 isolates of *D. pisi* (Table 5) isolated from AB symptomatic dry pea seeds and plants from Montana were spiked with genomic DNA of dry pea at 20 ng *D. pisi* + 50 ng host plant DNA per 20 µl PCR reaction. Two replicates of each isolate were used in this experiment. The samples were tested using the PCR assays according to the methods previously described.

Data Analyses. Real-time results were collected and analyzed, and qPCR standard curve plots and DNA concentration correlations were plotted, using the Bio-Rad CFX Manager software package (Bio-Rad Laboratories, Hercules, CA, United States).

Results

Specificity and sensitivity of conventional PCR assay. Out of the nine SSR primer pairs screened with host pea DNA (data not shown), only four primer pairs: A311-F/R, A313-F/R, A315-F/R, and A318-F/R (Table 1) did not amplify host DNA of 10 dry pea varieties (Table 2) (Figure 1) and non-target fungal pathogens associated with dry pea, but specifically amplified and detected *D. pisi* isolates (Table 3). In addition, internal control templates were amplified in the multiplex PCR detection of *D. pisi* with *Rubisco L*-gene primers (642 bp product) and *Internal Transcribed Spacer* (550 bp product) primers when the host plant and non-target fungal were present, indicating the primers were compatible with SSR primers in the multiplex PCR reactions (Figures 2, 3 and 4). The detection limit of *D. pisi* in the conventional multiplex PCR both in the presence and absence of host DNA was 0.01 ng DNA of *D. pisi* (Figure 4).

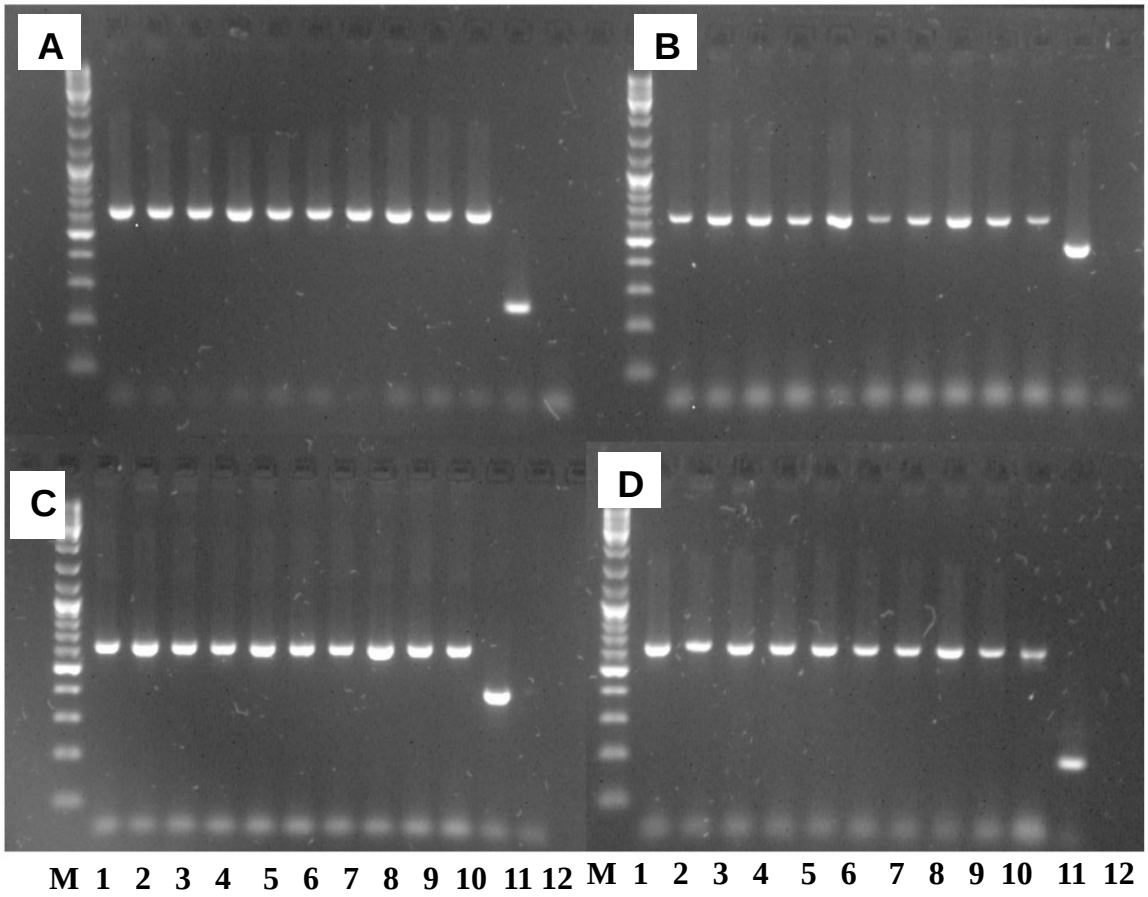

Figure 1. *Didymella pisi* SSR-primers specifically amplified *D. pisi* but not pea plant DNA in duplex PCR. (A) Duplex PCR with A311 SSR primer and RBCL primer pairs, (B) Duplex PCR with A313 SSR primer and RBCL primer pairs, (C) Duplex PCR with A318 SSR primer and RBCL primer pairs, and (D) Duplex PCR with A315 SSR primer and RBCL primer pairs. A311, A313, A318, and A315 are SSR primer pairs that specifically amplified 249, 489, 406, and 199 bp products of *D. pisi* respectively. RBCL = *Rubisco L*-gene primers amplified 642 bp product from host plant DNA, serving an internal PCR control. Lane M, 100 bp DNA ladder (100 – 1500 bp); lanes 1-10, dry pea varieties (cv. Hyline, Treasure, CDC Leroy, Banner, Aragorn, Ginny, Montech 4193, MSGA-Jet Set, Early Star, and Nette 2010); lane 11, *D. pisi* DNA positive control, and lane 12, no template DNA.

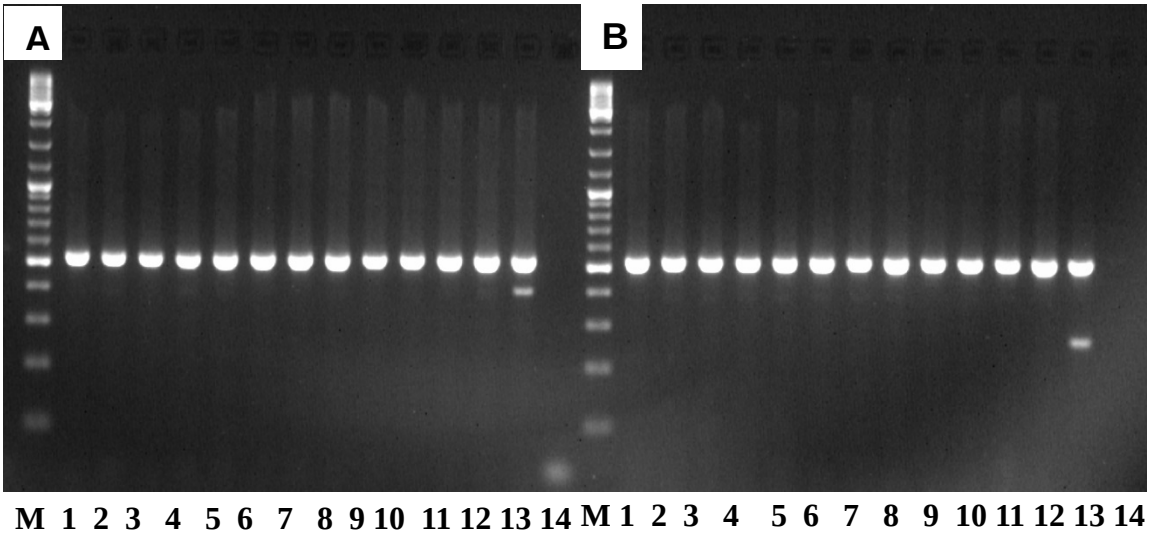

Figure 2. *Didymella pisi* SSR-primers specifically amplified *D. pisi* but not any other fungi in duplex PCR (A) Duplex PCR with A313 SSR and ITS-1 & ITS-4 primer pairs; (B) Duplex PCR with A311 SSR and ITS-1 & ITS-4 primer pairs. A311 and A313 are SSR primer pairs that specifically amplified 249 bp and 489 bp products of *D. pinodes*, respectively. ITS-1 & ITS-4 = *Internal transcribed spacer* generic primers amplified 550 bp product of all the fungal DNA, serving an internal fungal PCR control. Lane M, 100 bp DNA ladder (100 – 1500 bp); lanes 1-4, *P. pinodes* isolates; lanes 5-8, *P. pinodella* isolates; lanes 9-12, *Phoma* sp. isolates; lane 13, *D. pisi* DNA positive control, and lane 14, no template DNA.

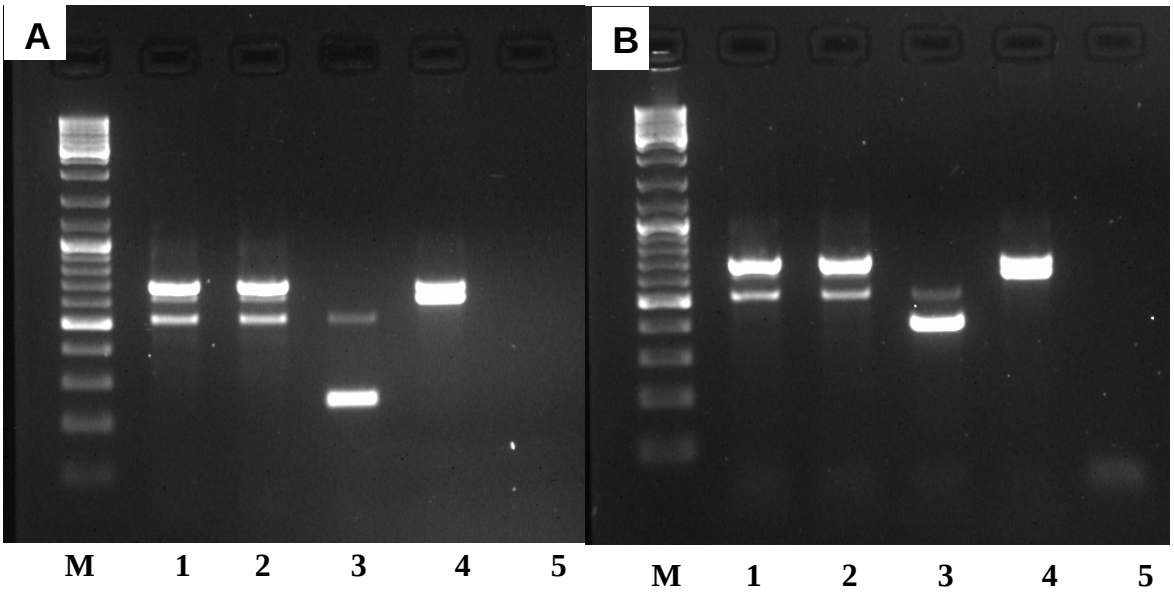

Figure 3. Multiplex PCR detection of *D. pisi*, fungal ITS and *host plant Rubisco* L-*gene*. (A) Multiplex PCR with A311 SSR, ITS-1/ ITS-4, and RBCL primer pairs; (B) Multiplex PCR with A313 SSR, ITS-1/ITS-4, and RBCL primer pairs; primer pairs A311 and A313 are SSR primer pairs that specifically amplified 249 bp and 489 bp products of *D. pisi*, respectively. ITS-1 & ITS-4 = Internal transcribed spacer generic primers that amplified 550 bp ITS product of any of the fungal DNA, serving an internal fungal PCR control. Lane M, 100bp DNA ladder (100 – 1500 bp); lanes 1-2, dry pea leaf samples infected with *Alternaria alternata.*; lane 3, *D. pisi* DNA positive control; lane 4, healthy dry pea leaf sample; and lane 5, no template.

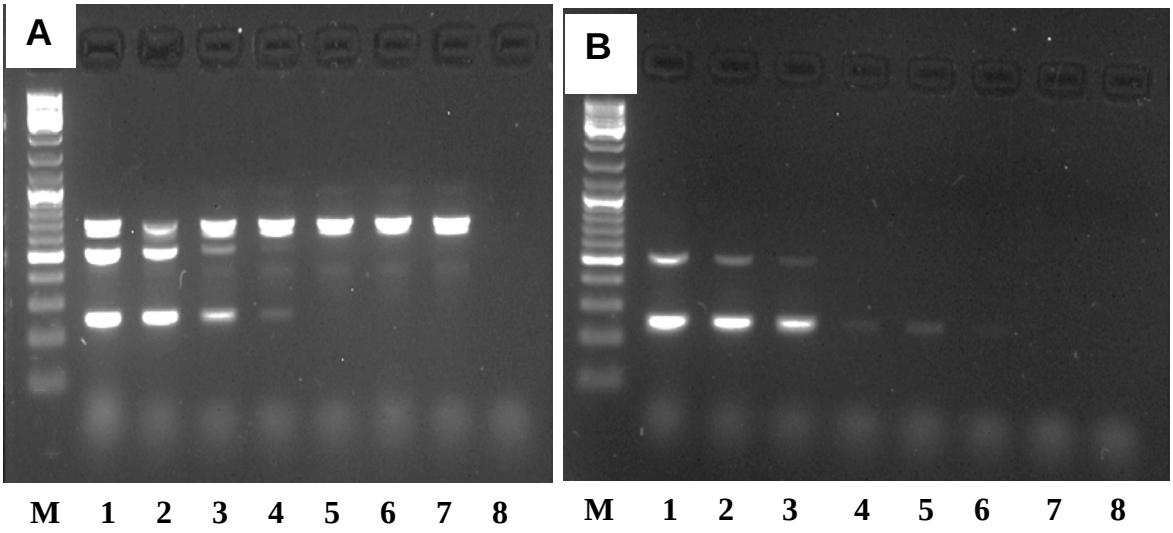

Figure 4. Sensitivity of multiplex PCR detection of *Didymella pisi* in the presence and absence of host plant DNA**. (**A) Multiplex PCR with A311, ITS-1/ ITS-4, and RBCL primer pairs; (B) Duplex PCR with A311 and ITS-1 & ITS-4 primer pairs. A311 and A313 are SSR primer pairs specifically amplified 249 bp and 489 bp products of *D. pisi*, respectively. ITS-1 & ITS-4 = Internal transcribed spacer generic primers that amplified 550 bp ITS product of any of the fungal DNA, serving an internal fungal PCR control. (A) Lane M, 100 bp DNA ladder (100 – 1500 bp); Lanes 1-7, 10-fold serial dilution of *D. pisi* DNA from 100 ng to 0.00001 ng in 50 ng DNA of pea (cv. Aragorn); and lane 8, no template and (B) Lane M, 100 bp DNA ladder (100 – 1500 bp); Lanes 1-7, 10-fold serial dilution of *D. pisi* DNA from 100 ng to 0.00001 ng.

Specificity and Sensitivity of the SYBR green Real-time SSR-PCR assay. The SYBR green PCR assay was specific and sensitive for the detection of *D. pisi* both in the presence of the DNA of dry pea host and other fungal species. The qPCR detected all the *D. pisi* isolates at 50 ng of DNA with quantification cycle values (Cq) range of 19.53 to 20.73. Non-target fungi species were not detected at 30 cycles (Table 3). The correlation coefficients for the standard curve of the DNAs from *D. pisi* pure cultures and conidial suspension were 0.999 and 0.980, respectively (Table 4). The quantification limit for the qPCR was 0.01 ng of mycelia DNA and 100 *D. pisi* conidia.

Validation of the SSR- qPCR assay. To validate the SSR-qPCR assay, DNA of 30 samples of *D. pisi* isolated from AB contaminated seed lots mixed with 50 ng of DNA of dry pea plants were evaluated. There was consistency in the sensitivity and specificity of the assay. The mean Cq values of the samples tested ranged from 18.15 to 19.52 and a standard deviation that ranged from 0.01 to 0.31 (Table 5).

Table 4. The efficiency of Real-time PCR using *D. pisi* specific primer on different sources of DNA

Sources of DNA	**Efficiency (%)**	**Slope**	**Intercept**	**R^2**	**Linear range of Cq values (min – max)**
D. pisi pure mycelial culture	96.5	- 3.408	26.280	0.999	19.2 – 28.5
D. pisi conidial suspension	115.6	-2.996	32.433	0.980	17.5 – 29.7

Table 5. SYBR green real-time PCR validation of *Didymella pisi* specific assay using genomic DNA of different fungal species associated with dry pea and genomic DNA of *D. pisi* isolates from Montana spiked with dry pea DNA samples.

Fungal species	**Cq values**	**[a]Standard deviation**	**Fungal species**	**Cq values**	**[a]Standard deviation**
Alternaria alternata	ND[b]	nil	*D. pisi*-01	18.31	0.310
Alternaria alternata	ND	nil	*D. pisi*-02	19.33	0.028
Alternaria alternata	ND	nil	*D. pisi*-03	19.33	0.064
Botrytis cinerea	ND	nil	*D. pisi*-04	19.48	0.097
Botrytis cinerea	ND	nil	*D. pisi*-05	19.31	0.019
Botrytis cinerea	ND	nil	*D. pisi*-06	19.23	0.032
Cladosporium sp	ND	nil	*D. pisi*-07	19.44	0.023
Colletotrichum lentis	ND	nil	*D. pisi*-08	19.40	0.018
Colletotrichum lentis	ND	nil	*D. pisi*-09	19.37	0.020
Colletotrichum lentis	ND	nil	*D. pisi*-10	19.52	0.030
Didymella pisi	20.73	0.031	*D. pisi*-11	19.37	0.021
Didymella pisi	20.37	0.021	*D. pisi*-12	19.19	0.014
Didymella pisi	19.73	0.019	*D. pisi*-13	19.28	0.250
Didymella pisi	19.50	0.022	*D. pisi*-14	19.19	0.123
Fusarium avenaceum	ND	nil	*D. pisi*-15	18.89	0.057

Fusarium avenaceum	ND	nil	*D. pisi*-16	18.93	0.160
Fusarium avenaceum	ND	nil	*D. pisi*-17	18.15	0.143
Nigrospora oryzae	ND	nil	*D. pisi*-18	19.34	0.013
Nigrospora oryzae	ND	nil	*D. pisi*-19	19.36	0.018
Nigrospora oryzae	ND	nil	*D. pisi*-20	19.46	0.041
Peyronellaea pinodella	ND	nil	*D. pisi*-21	19.35	0.095
Peyronellaea pinodella	ND	nil	*D. pisi*-22	19.47	0.044
Peyronellaea pinodella	ND	nil	*D. pisi*-23	19.18	0.127
Peyronellaea pinodes	ND	nil	*D. pisi*-24	19.39	0.031
Peyronellaea pinodes	ND	nil	*D. pisi*-25	19.36	0.174
Peyronellaea pinodes	ND	nil	*D. pisi*-26	19.45	0.092
Phoma koolunga	ND	nil	*D. pisi*-27	19.28	0.101
Phoma koolunga	ND	nil	*D. pisi*-28	19.04	0.154
Phoma koolunga	ND	nil	*D. pisi*-29	19.28	0.186
Phoma sp	ND	nil	*D. pisi*-30	19.16	0.027
Phoma sp	ND	nil	nil	nil	nil
Phoma sp	ND	nil	nil	nil	nil
Sclerotina sclerotium	ND	nil	nil	nil	nil
Sclerotina sclerotium	ND	nil	nil	nil	nil
Stemphylium vesicarium	ND	nil	nil	nil	nil

No template control	ND	No template control	ND

[a]Based on two replicates; ND[b] = Fluorescent signal not detected.

Discussion

Ascochyta blight (AB) is a threat to dry pea production in the Great Plains of North America, Europe, and Australia (Ahmed *et al.,* 2015; Chilvers *et al.,* 2007; Davidson *et al*., 2009; Kaiser *et al.,* 2008; Li *et al.,* 2011; and Tran *et al.,* 2014). In Montana, *D. pisi* is the most prevalent fungal pathogen in the AB complex (Owati *et al*., 2017 and 2019). Currently, rapid, specific, and sensitive molecular diagnostic assays are lacking. This hinders diagnosis and epidemiological studies, as several closely related species cause similar symptoms on dry pea plants. Greater understanding of the molecular differences in the species (Ahmed *et al.,* 2015) will allow us to design specific primers to rapidly identify members of AB complex and monitor changes in species composition on the landscape and recommend control measures.

In this study, a rapid, specific, and sensitive qPCR assay was developed to specifically detect and quantify *D. pisi* using SSR primer pairs. In addition, conventional PCR assays were developed for use in laboratories without qPCR capabilities. Both methods specifically detected only *D. pisi*; not closely related fungal pathogens associated with AB complex of dry pea, other fungal pathogens commonly found affecting dry pea, and host dry pea host DNA. The assays were sensitive enough to detect 0.01 ng of genomic *D. pisi* DNA of pure culture. In addition, the qPCR assay was sensitive enough to detect 100 conidia of *D. pisi*. This is four orders of magnitude less than the 10^6 conidia/mL routinely used to cause infection in controlled experiments (Ahmed *et al*., 2015). This shows the robustness of our assay and suggests that it can be used to detect *D. pisi* from infected, non-symptomatic plants.

Various PCR and qPCR assays have been developed for the diagnosis of crop pathogens (Abdullah *et al.*, 2018; Gramaje *et al*., 2013; Rojas *et al*., 2017; Wang *et al*., 2015; and Zitnick-Anderson *et al.*, 2018). Most of the qPCR and PCR based detection assays for plant pathogens were developed based on the intergenic sequences and internal transcribed spacer sequences of the rRNA (Bhat *et al*., 2010; Rojas *et al*., 2017; and Wang *et al*., 2015). This approach does not work well to differentiate closely related pathogens that share high sequence homology like those associated with the ascochyta blight complex of dry pea (Phan *et al*., 2002). The approach presented here, of designing the primers to flank loci of simple sequence repeats from contigs generated from Illumina next-generation sequencing reads, was instrumental to its high specificity and sensitivity. SSR loci are ubiquitous and offer a unique target in the fungal genome (Canfora *et al*., 2016 and Owati *et al.*, 2019). This characteristic makes SSR markers unique for the development of specific primers for pathogen detection. The specificity and sensitivity of our results are similar to the results of Confora *et al.*, (2016), who firstly reported the use of SSR primer-based qPCR for specific detection of fungal pathogens. The study developed an SSR-qPCR assay for the specific detection and quantification of *Beauveria bassiana* and *B. brongniartii* in culture and soil.

To our knowledge, this study represents the first study where this novel approach was used to develop specific markers for the detection and quantification of a member of Ascochyta blight complex in dry pea or ascochyta blight causal pathogen in another pulse crop.

The need for consistent and accurate PCR-based diagnostic assays for the detection of plant pathogens cannot be over-emphasized. The consequences of inaccurate diagnosis

can range from the misinformed usage of pesticides to hindrances in international trade. Some of the previously developed PCR and qPCR assays for the detection of plant pathogens lacked the inclusion of internal controls (Bonants *et al.,* 1997; Bhat *et al.,* 2010; Cao *et al.,* 2007; Chilvers *et al.,* 2007; and Wang *et al.,* 2006). Internal controls are essential in the assay to control for background amplification and prevent false negative reports. Hence, our PCR based assay was designed to multiplex the detection of *D. pisi* with those of ITS and *Rubisco L*-gene, which are internal control targets of fungi and plants, respectively. This approach is consistent with other PCR and qPCR assays, where internal controls are included in the assays for the detection of Soybean *Fusarium virguliforme* the causal agent of soybean sudden death syndrome and *Phytophthora sojae* and *P. sansomeana* root rot pathogens of soybean (Rojas *et al*., 2017 and Wang *et al*., 2015).

The development of the assays presented here is timely. Montana shares local and international borders with North Dakota and Canada. The pathogen composition of ascochyta blight complex of dry pea differs geographically. In North Dakota and Canada (Alberta, Saskatchewan, Manitoba), the pathogen complex consists of *Peyronellaea pinodes*, *Peyronellaea pinodella*, and rarely *D. pisi* (Ahmed *et al.,* 2015; Chilvers *et al*., 2009; Gossen *et al.,* 2011; and Sivachandra-Kumar and Banniza 2017). The proximity of these regions and exchange of germplasm is anticipated to influence changes in population dynamics of the pathogen complex (Sivachandra-Kumar and Banniza 2017). The qPCR and PCR assays developed in this study allow for rapid, accurate identification and quantification of *D. pisi*. These tools will be used for monitoring changes in pathogen composition and for epidemiological studies. Also, the *D. pisi* specific primers could potentially be multiplexed with specific primers for other members of the complex. In

addition, these assays may be used to screen for ascochyta blight resistance in dry pea where visual estimation or culture-based methods are difficult or not practical.

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Authors Contribution

AO and BA conceived the experiments. AO designed and performed the experiments, analyzed the data, and wrote the manuscript. MB and BA reviewed the manuscript.

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CHAPTER SIX

LINKING DISCUSSION

This study has provided new information on the distribution of QoI- fungicide resistant *D. rabiei* isolates in Montana and investigated the population diversity and structure of pathogens causing ascochyta blight. Conventional and molecular based tools for detection and monitoring changes in population dynamics of AB were developed.

QoI fungicide resistant isolates of *D. rabiei* were found in three counties in Montana. According to our findings, the G143A mutation is responsible for QoI fungicide resistance in *D. rabiei* isolates from Montana chickpea fields. The gene structure of the *cytochrome b* gene of *D. rabiei* appears to be favorable for the development of a SNP associated with QoI resistance at codon 143 (Delgado *et al*., 2013). In contrast, the G143A mutation has not been reported in fungal species that have an intron downstream of codon 143 (Grasso *et al*., 2006b; Sierotzki *et al*., 2007; Banno *et al*., 2009; Samuel *et al*., 2011; Delgado *et al*., 2013; and Zeng *et al*., 2015). Similar results were obtained in a study in North Dakota (Wise *et al*., 2009 and Delgado *et al*., 2013), where the mechanism of resistance of QoI-resistant *D. rabiei* isolates was reported to be a G143A mutation. Noticeably, all the three counties where QoI-resistant *D. rabiei* isolates were found are near North Dakota indicating the QoI-resistant isolates detected could have either spread from North Dakota via seed or were selected for in Montana chickpea fields.

The frequency of QoI resistance might be on the rise in *D. rabiei* because, from observations during the 2016 -2018 crop years, USDA-NASS statistics and grower testimonies, there is increasing chickpea acreage and multiple applications of fungicides to

ward off potential fungal attacks. Many of these applications were QoI products solely and in combination with either chlorothalonil or SDHI fungicides such as fluxapyroxad. The proportion of QoI fungicide applied solely is higher than that applied in combination with other fungicides. However, continuation of the practice of applying multiple applications of fungicides including high-risk products such as QoI and SDHI fungicides will select for resistance development. These active ingredients are available as seed treatments and foliar products. Although education is ongoing, fungicide decisions are often driven by the price and efficacy of the product more than the mode of action. In contrast with chickpea, field pea and lentil fields are rarely treated with fungicides. This is due to the low foliar disease occurrence in Montana to date. Since seed testing was started in Montana in 2000, the percent of chickpea seed lots with at least one seed of 500 infected by AB has increased from 0% (2000–2002) to as high as 25% through 2009. Since the 2010 crop year, the level has increased to 60%. It was 80% in 2017. To date, we have very rarely observed QoI resistance in AB pathogens recovered from a field pea seed lots and never observed QoI resistance in AB recovered from lentil seed lots. This correlates with the low frequency of fungicide application in these crops.

Differences in disease control were observed when QoI-resistant and QoI-sensitive *D. rabiei* isolates were inoculated on pyraclostrobin-treated chickpea plants. Applications of pyraclostrobin at a concentration of 100 mg/ml provided less than 25% control of disease on chickpea plants infected with QoI-resistant isolates. This amount of control is unacceptable in field production. Disease severity of AB was higher on fungicide-treated chickpea plants inoculated with QoI-resistant *D. rabiei* isolates that on plants inoculated

with QoI-sensitive isolates study. Several studies have not observed a fitness cost associated with G143A substitution in *cytb* gene in fungal pathogens (Chin *et al*., 2001; Avila-Adame *et al*., 2003; Karaoglanidis *et al*., 2011; and Veloukas *et al*., 2014). However, once established, resistance is likely to be preserved in the population due to the selective advantage if QoI fungicides continue to be applied. This lack of disease control in QoI-resistant isolates was confirmed in a report from North Dakota (Wise *et al*., 2009). In that study, <50% disease control was achieved with the applications of 100 mg a.i/ml pyraclostrobin to chickpea plants inoculated with QoI-resistant isolates.

Considering this, monitoring of QoI resistance in AB pathogens infecting pulse crops is important to prevent the establishment of resistant populations. A real-time PCR assay developed in this study enhances high throughput and accuracy during QoI-sensitivity screening of isolates when compared to the conventional *in vitro* test using fungicide amended PDA plates. This test takes about 7 to 10 days to get a result, in addition to the logistics required to screen multiple isolates. The hydrolysis probes were designed to have T_m values at least 7 C higher (69 ^{o}C) than that of the primers (62 ^{o}C), and their GC content was higher than 45%, improving the specificity of the assay in discriminating sensitive from resistant alleles. This technique can be used in studies to determine changes in the frequency of G143 and A143 alleles in *D. rabiei*. Also, this process could be used for large-scale surveys, as well as rapid identification of insensitivity to QoI fungicides. Furthermore, modification of the primers and probes avails this assay the potential to monitor G143A mutation in *D. pisi*, *D. lentis*, and *A. alternata*, thus it can serve as a tool to monitor QoI resistance in other fungal pathogens and also for routine screening of fungal isolates in diagnostic laboratories.

Ascochyta blight of dry pea is caused by a complex of fungal pathogens. *D. pisi* is the predominant causal agent in Montana and has also been reported to cause an epidemic in Europe (Kaiser, *et al*., 2008). However, simple sequence repeat markers (SSR) also known as microsatellite markers have only been developed for *Didymella rabiei*, the causal pathogen of ascochyta blight of chickpea (Baite, *et al.,* 2017). The present study is the first report of *D. pisi* SSR markers development and their use for a genetic diversity and population structure study. This study has increased the number of population genetic studies on pathogenic fungi. Currently, population genetic studies of fungal pathogens are low when compared with other organisms (Zane, *et al*., 2002; Dutech, *et al.,* 2007). Information on pathogen genetic diversity and population structure on the landscape scale are important to understand the potential of pathogen populations to spread, increase aggressiveness, develop fungicide resistance, and overcome host resistance (Ciampi, *et al.,* 2011; and Marulanda, *et al.,* 2014).

The results of analysis of molecular variance (AMOVA) supported the presence of genetic diversity in the *D. pisi* population in Montana. The highest percentage of variation (85%) was within populations of *D. pisi* isolates. However, the gene diversity observed among the Montana *D. pisi* populations was low. This might be associated with the introduction of a few genotypes of the fungus into Montana as well as low level of seeds coming from outside Montana. Also, the incidence of *D. pisi*, unlike *P. pinodes* is very low in North Dakota and Canada, which are neighboring regions to Montana with high production of dry pea (Gossen, *et al*., 2011; Sivachandra-Kumar and Banniza, 2017). High genetic diversity was detected in the northeast region of Montana while genetic diversity was lowest in the southwest (SW). The genetic variability between these two regions is

expected because the northeast (NE) region is the epicenter of dry pea production in Montana and might be due to the early introduction of the fungus in this region. This allows time for genetic mutation, genetic drift, and recombination which results in greater genetic diversity (McDonald, 1997; and Moges, *et al.*, 2016). This can be associated with the high incidence of ascochyta blight recorded on seed lots sent to the RPCDL for seed test from NE region than from other regions of Montana. These differences may also be due to environmental conditions, geography, and differences in alternative host species diversity that may be associated with generating variability within populations (McDonald, 1997; and Moges, *et al.*, 2016).

Information on the population structure of *D. pisi* populations from different locations improves the understanding of the biology of the pathogen, evolutionary, and potentially adaptive genotypic diversity in the species (Marulanda, *et al.*, 2014). The *D. pisi* isolates from the four geographic regions of Montana are closely related as reflected by the high genetic identity among populations. Furthermore, based on the population genetic analyses of the Montana *D. pisi* isolates, the population was categorized into two sub-populations. STRUCTURE analysis, PCoA, and the unweighted Neighbor-joining algorithm supported and indicated admixture among the two populations. Evidence of admixture among isolates from the different regions does not support geographical separation of isolates into distinctly isolated sub-populations.

The microsatellite markers developed in this study were used to understand the genetic diversity and population structure of *D. pisi* isolates from dry pea growing regions of Montana. Despite regional variations, the observed genetic diversity in all four populations was lower than expected, suggesting inter-regional exchanges of planting

materials and dispersal of inoculum among the regions. This study generated information that can be used to further understand the pathogen biology and its evolutionary potential and provide the basis for other studies on disease development, host-pathogen interactions, and development of disease management strategies which includes development and use of resistant dry pea varieties. In addition, information generated from this study can be used to design novel specific primers for characterization of *D. pisi*

Due to the recent observations in shift in pathogen composition from *D. pisi* to *P. pinodes* in Montana dry pea fields, this study investigated the biology of the fungal species associated with ascochyta blight of dry pea in North America. The study evaluated the effects of temperature on the growth and development and also determine the pathogenicity and aggressiveness of these pathogens. The mycelial growth rate and sporulation of the four fungal species associated with AB of dry pea were evaluated at different temperatures. Highest growth and development of all fungal species occurred at 20 ^{o}C and 25 ^{o}C. Summarily, their growth and development were impacted by the low temperature of 15 ^{o}C and a high temperature of 30 ^{o}C. These fungal species are adaptable to varying temperatures. Thus, they can cause AB of dry pea in generally hot climates observed in Australia (Davidson, *et al.,* 2009; Li, *et al.,* 2011; Tran *et al.,* 2014; and Liu, *et al.,* 2016) and in cold climates of northern North America (Chilvers, *et al*., 2009; Gossen, *et al.,* 2011; Ahmed *et al.,* 2015; and Sivachandra-Kumar and Banniza 2017). In addition, the developmental responses of these pathogens to varying temperatures showed their adaptive potential to a climatic condition. This evolutionary adaptation may also be expressed in their ability to overcome the potential resistance of the host plant (Darby, *et al.,* 1986). *D. pisi* and *P. pinodella* both have low and high sporulation respectively. Both pathogens are

heterothallic (Bowen, *et al.,* 1997 and Chilvers, *et al.,* 2009). The formation of sexual spores in these pathogens enable genetic diversity due to sexual recombination, which is associated with increased aggressiveness, development of fungicide resistance, and loss of resistant cultivars (Chilvers, *et al*., 2009 and Owati, *et al*., 2017).

The temperature range of 20 ^{o}C to 25 ^{o}C coincides with the average temperature (18 ^{o}C to 27 ^{o}C) during active growing stages (flowering to maturity) of dry pea in Montana (Western Region Climate Center WRCC). This implies that our environment is conducive for all the fungal species associated with AB of dry pea. Furthermore, the adaptability of these pathogens and the potential to disperse their propagules heighten the concerns to monitor the frequency of the invasive species (*P. pinodes* and *P. pinodella*) to Montana. In Canada, a shift in species from *D. pisi* to *P. pinodella* and *P. pinodes* occurred after the introduction of a *D. pisi*-resistant dry pea variety named Century in 1961 (Gfeller and Wallen, 1961 and Sivachandra-Kumar and Banniza 2017).

P. pinodes caused increase symptom severity than the other fungal pathogens in the AB complex studied here. This is consistent with previous findings that *P. pinodes* is the most aggressive in the complex and *D. pisi* is the weakest (Kraft, *et al.,* 1998 and Chilvers, *et al*., 2009). Moreover, the uncharacterized *Phoma sp.* used in this study was not pathogenic on dry pea, there were no lesions on the pea leaves or stems. This is in contrast with the recently identified *Phoma koolunga*, *Phoma herbarum,* and *Phoma glomerata* which have been reported to be associated with AB of dry pea in Australia (Davidson, *et al.,* 2009; Li, *et al.,* 2011; Tran *et al.,* 2014; and Liu, *et al.,* 2016) but they are yet be associated with AB of dry pea in North America.

Considering this shift in population from *D. pisi* to *P. pinodes*, a selective media was developed and used to discriminate among these pathogens. Peameal agar was an effective tool to and consistently discriminate among the fungal species associated with AB of dry pea using visual characteristics. Previous studies have used OMA to discriminate between *D. pisi* and other fungal pathogens in the AB complex (Jones, 1927; and Ahmed, *et al*., 2015), OMA cannot consistently discriminate among all the three fungal species associated with AB of dry pea in North America. To our knowledge, this is the first study that showed that PMA can be used to morphologically discriminate among the three fungal pathogens. This tool is of importance in North America where there is geographical variation in the predominant species associated with AB of dry pea. This tool has been used to characterize fungal isolates from two states. This tool will be used to monitor changes in population dynamics of fungal species associated with AB of dry pea in North America with special emphasis on Montana where a shift in pathogen population from *D. pisi* to *P. pinodes* is currently being observed. This information is needed to advise growers on the need to modify their AB disease management strategies. For instance, most growers in Montana sparingly apply foliar fungicide treatment for the control of AB of dry pea. This recommendation may need to be changed where *P. pinodes* becomes the predominant pathogen.

Furthermore, sporulation of the species varied when inoculated on PDA, OMA, and PMA. Across the three media used in this study, all the fungal species used in this study produced approximately 10^6 spores within 6 days when inoculated on PMA while it took 9 to 12 to produced lower spores count when inoculated on PDA and OMA. PMA induced rapid sporulation in these pathogens when compared with other media (Chapter 4). PMA

can be used in the rapid preparation of inoculum needed for greenhouse or field trials, thus reducing the average days of incubation, amount of petri plates, and labor required for preparation of inoculum. Peameal agar is a selective media that can discriminate among the fungal species associated with AB of dry pea in North America and also is a substrate that can be used for rapid sporulation of the fungal species. Thus, this medium serves as a diagnostic and epidemiological tool that can be used to identify and monitor changes in the population dynamics of fungal species associated with AB of dry pea.

In Montana, *D. pisi* is the most prevalent fungal pathogen in the AB complex. Currently, rapid, specific, and sensitive molecular diagnostic assays are lacking. This hinders diagnosis and epidemiological studies, as several closely related species cause similar symptoms on plants. Greater understanding of the differences in the species (Ahmed, *et al.*, 2015) will allow us to address changes in species composition on the landscape and recommend control measures.

In this study, a rapid, specific, and sensitive qPCR assay was developed to detect and quantify *D. pisi* from plant tissue and pure cultures. In addition, conventional PCR assays were developed for use in laboratories without a qPCR facility. These assays were specific to *D. pisi* when tested with closely related fungal pathogens including those associated with AB complex of dry pea and other fungal pathogens commonly found affecting dry pea. The need for consistent, accurate PCR-based diagnostic assays for the detection of plant pathogens cannot be over-emphasized. The consequences of inaccurate diagnosis can range from the misinformed usage of pesticides to hindrances to international trade.

The development of the assays presented here is timely. Montana shares local and international borders with North Dakota and Canada. In these territories, the pathogen composition of ascochyta blight complex of dry pea differs to the composition obtained in Montana. In North Dakota and Canada (Alberta, Saskatchewan, Manitoba), the pathogen complex consists of *Peyronellaea pinodes* and *Peyronellaea pinodella* and rarely *D. pisi* (Chilvers, *et al*., 2009; Gossen, *et al.*, 2011; Ahmed *et al.*, 2015; and Sivachandra-Kumar and Banniza 2017). The proximity of these regions and exchange of germplasm is suspected to influence changes in the population dynamics of pathogen composition (Sivachandra-Kumar and Banniza 2017). Thus, emphasizing the need for this assay to be used as a monitoring tool. The qPCR and PCR assays developed in this study allows for rapid, accurate identification, and quantification of *D. pisi*, these potentials will be used for monitoring changes in pathogen composition and for epidemiological studies. In addition, the *D. pisi* specific primers can be multiplexed with specific primers of other members of the complex for qPCR detection of all the fungal pathogens causing AB in North America. This will serve as a detection tool to predict disease consequences. Also, these assays may be used to screen for ascochyta blight resistance in dry pea where visual estimation or culture-based methods are difficult or not practical.

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