Understanding the Epidemiology and Genetic Diversity of *Leptosphaeria maculans,* and Exploring Chemical Control Strategies to Manage Blackleg of Winter Canola (*Brassica napus)* in Northern Idaho

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Abstract

Blackleg disease of canola (*Brassica napus*), caused by the fungal pathogen *Leptosphaeria maculans,* is a major constraint of production worldwide. Blackleg can cause stem lesions and cankers, and in severe cases result in detrimental yield loss. In northern Idaho blackleg was first discovered in 2011 and is an emerging threat to canola seed production. Growers have access to multiple disease management practices for blackleg including crop rotations, stubble management, fungicide applications, and genetic resistance. An important element in overall management of blackleg is qualitative resistance where avirulence effector gene products in the pathogen are recognized by corresponding resistance gene products in the host. *Leptosphaeria maculans* isolates were collected from eastern Washington to elucidate the race structure. Greenhouse host plant differentials and PCR were used to characterize the following genes: *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm 9, AvrLm11, AvrLepR1, AvrLepR2,* and *AvrLepR3.* The highest frequency of effector genes present in the eastern Washington *L. maculans* pathogen population are *AvrLm5* (100%)*, AvrLm6* (100%)*, AvrLm7* (100%)*, AvrLm11* (92%)*, and AvrLepR1* (100%). Eighteen unique race structures were observed with the three most common as follows: *AvrLm3-5-6-7-11-LepR1-LepR2* (24%), *AvrLm5-6-7-11-LepR1-LepR2-LepR3* (16%), and *AvrLm3-5-6-7-11-LepR1-LepR2-LepR3* (15%). Determining *L. maculans* population demographics provides insight as to which canola cultivars with specific *R*-genes should be considered when growers and breeders are making management decisions. Other disease management practices should be used in conjunction with qualitative resistance to reduce the possibility of resistance breakdown events. Fungicides and disease forecasting have shown successful reduction of blackleg disease incidence when utilized in management programs. Ensuring successful disease prevention through foliar fungicide applications relies on identification of optimal spray timing for the region. Winter canola field trials were established on the Palouse (Moscow and Genesee, ID) and the Camas Prairie (Grangeville or Nezperce, ID) for 2 years to test the impact foliar fungicide application timing and fungicide seed treatment has on blackleg disease incidence, disease severity, and yield response in the region. Each trial was a split-plot experimental design where cultivar was the main plot and fungicide treatments were randomly assigned to the subplots across four replications. Treatments included: (1) two cultivars, Mercedes (resistant) and Amanda (susceptible); (2) fungicide seed treatment (Helix Vibrance)

or no seed treatment (clothianidin); and (3) four foliar fungicide application timings (fall only, spring only, fall and spring, and no application). Seed treatment was not effective in reducing blackleg disease incidence and severity. Disease incidence and severity was significantly reduced when foliar fungicides were applied. No application resulted in disease incidence of 16% and 27% and for Mercedes and Amanda, respectively. Disease was significantly reduced in both cultivars with a fall only application (6% to 11%) and a spring only application (2% to 9%). Applying in both the fall and spring resulted in the lowest disease incidence (1% to 4%) for each cultivar. No application of foliar fungicide resulted in a disease severity rating of 0.22 (0-5 scale with 0 = no disease). A fall only application (0.10), spring only application (0.09) and both fall and spring application (0.02) reduced stem severity, indicating the importance of foliar fungicides in preventing stem canker formation. Yield response to foliar application timing had a *p*-value of 0.059, indicating disease did not cause serious stem canker formation, but use of a foliar fungicide has the potential to improve yields. No spray plots yielded 4,748 kg/ha. A fall only and spring only application increased yield to 4,905 kg/ha and 4,835 kg/ha, respectively, and applying twice resulted in a yield of 5,004 kg/ha. Because blackleg is new to northern Idaho, there is limited knowledge as to when spores are released, and initial infection occurs. Therefore, Burkard volumetric spore traps were deployed adjacent to the winter canola field trials on the Palouse and Camas Prairie and used to determine when spores are moving. At both locations, ascospores were released between March and June under average weekly temperatures between 3 and 16ºC, relative humidity between 55 and 93%, and total weekly precipitation between 0 and 43 mm. Additional ascospore release may occur in the fall between September and October, but this was only observed on the Camas Prairie location in 2020. The results from this research contribute to our understanding of chemical control and the population structure of *L. maculans* in the region. This information is vital to developing a blackleg disease management plan specific to the production of winter canola in northern Idaho.

Acknowledgements

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Thank you to Rubisco Seeds for providing the seed necessary for the completion of my field trials. A huge thank you to Nathan and Christopher Riggers and to Ron and Mark Frei for providing a location for my field trials on the Camas Prairie. Equal appreciation goes out to Roy Patten and Brad Bull for providing a location for my field trials on the research farms.

Dedication

To my parents, you are the best cheerleaders a girl could ask for. Although you may not always understand my research, you consistently support my endeavors and I thank you for raising me to be the well-rounded individual I am today.

To my husband, who has kept me grounded during all the uncertainty life throws our way. Thank you for the unwavering support and encouragement to keep moving forward.

All my love.

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Chapter 1: Literature Review

1.1 Canola - Family and Origins

Canola (*Brassica napus*) is a member of the Brassicaceae (Cruciferae) family in genus *Brassica* and is characterized by its bright yellow flowers with four petals arranged in a cross that gives rise to the family name, meaning 'crux' in Latin (Brown et al., 2008). Canola is a cool season crop with winter and spring varieties, is self-pollinating, and follows epigeal emergence and an indeterminate growth habit (Koenig et al., 2011; Brown et al., 2008).

The relationship between members of the Brassica genus begins with three diploid species, *B. nigra, B. oleracea*, and *B. rapa*, forming the "Triangle of U" developed by Nagaharu U in 1935 (Figure 1.1). Through a natural hybridization and chromosome doubling, the allotetraploid species, *B. carinata*, *B. juncea*, and *B. napus* were developed (Rimmer et al., 2007). The major canola species are *B. napus, B. rapa,* and *B. juncea. Brassica napus* is the predominant species grown in the United States (Brown et al., 2008). *Brassica napus* was developed through an interspecific hybridization between *B. rapa* and *B. oleracea*, resulting in a species containing the A and C genome, making it an allotetraploid organism, or containing a complete diploid chromosome set from each parent (Long et al., 2011).

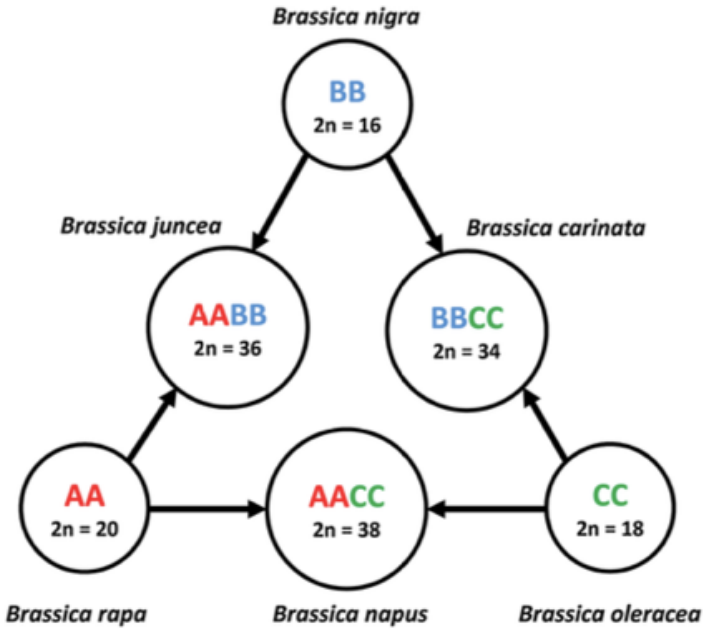

Figure 1.1: The triangle of U (Koh et al., 2017; Adapted from U, 1935).

Brassica napus originated in the Mediterranean over 3,000 years ago (Shahidi, 1990) and originally contained more than 40% euric acid along with glucosinolates in the oil, making it unhealthy and unpalatable (Lin et al., 2013). Through traditional plant breeding methods, Canadian scientists developed edible *B. napus,* or canola, in 1968 after the demand for industrial oil as machine lubrication declined once WWII ended (Brown et al., 2008). Canola contains less than 2% euric acid and less than 30 μmol/g of glucosinolates in the oil (Bonnardeaux, 2007). Canola is the world's only "made in Canada" crop as it is a contraction of 'Canada' and 'ola', meaning oil, and was granted this name in 1978 by the Rapeseed Association of Canada (Brown et al., 2008).

1.2 Uses of Canola

1.2.1 Edible Food Products

Roughly 45% of the canola seed consists of oil and is classified as an edible food oil due to the low euric acid content (less than 2%) (Brown et al., 2008). Although soybean oil is the most consumed vegetable oil (Lin et al., 2013), it does not possess the same health benefits that canola oil provides. Canola oil contains 7% saturated fat, the lowest of any vegetable cooking oil (Bushong et al., 2018). Due to low levels of saturated fat, there is less cholesterol in the blood stream which in turn reduces the risk of heart and other cardiovascular diseases, which are among the most common health issues in the United States (Johnson et al., 2007; Lin et al., 2013). Along with a low percentage of saturated fat, as compared to soybean oil with 15% (Brown et al., 2008), canola oil also possesses high levels of Omega-3 and is a good source of Vitamins E and K. Because of the nutritional value, health benefits, and being "generally recognized as safe" by the United States Food and Drug Administration in 1985, canola oil has slowly replaced other vegetable oils on the market, currently ranking third, thus providing an increase in demand to produce canola (Brown et al., 2008; Lin et al., 2013; Johnson et al., 2007).

1.2.2 Seed Meal Applications

Crushing canola seed for oil results in the by-product of seed meal. Along with low euric acid content in the oil, canola also possesses less than 30 μmol/g glucosinolates in the seed meal (Bonnardeaux, 2007). Glucosinolates are the compounds responsible for creating the 'spicy' taste when in contact with water. Less glucosinolates in canola make the seed meal more palatable to livestock and thus an ideal choice for feedstock managers along with the

high-protein content making canola meal an ideal supplement used to enhance livestock feed (Shahidi, 1990) and replacing soybean meal. Canola meal may have less crude protein than soybean meal, however, canola meal is slowly replacing the use of soybean meal in livestock feeds due to having higher levels of fiber and essential vitamins and minerals (Downey and Bell, 1990).

Even with multiple benefits to using canola meal in livestock feed there are drawbacks such as the presence of phenolic compounds. There are many types of phenolics that cause poor palatability (Bonnardeaux, 2007). For example, sinapine causes chickens to lay eggs that possess a fishy odor (Bonnardeaux, 2007). However, producers can adjust the amount of canola meal included in the livestock feed, making the phenolic levels low enough that problems do not arise. Even though phenolics may cause issues for livestock to enjoy their feed, many operations only add a ration of 10 to 15% canola meal and blend with another protein source to ensure animal nutrition is not completely affected (Evans et al., 2019; Bonnardeaux, 2007).

1.2.3 Canola as a Dual-Purpose Crop

Winter and Spring canola has recently been utilized as a dual-purpose crop, where forage is supplied to grazing livestock and seed is harvested at the end of the growing season (Lilley et al., 2015; Kirkegaard et al., 2008). Dual-purpose farming has been practiced for many years using wheat and other cereals, especially in Australia, and more recently in the Great Plains of the United States, where mixed farming operations are popular. Use as a dual-purpose crop is appealing due to canola forage providing more protein, less fiber, and more energy to livestock than traditional wheat and grain crops, in addition to low glucosinolate levels reducing the risk of glucosinolate-related animal health issues (Bushong et al., 2018; Dove and Kirkegaard, 2014). A disadvantage of using canola as a dual-purpose crop of grazing then harvesting for seed is the potential of yields being decreased by more than 50% in some cases (Bushong et al., 2018). However, if proper rotational grazing practices are conducted and the meristems of the plants are not damaged by removing grazing livestock from the field before stem elongation, there is still the ability to have a high yielding seed crop (Dove and Kirkegaard, 2014; Lilley et al., 2015). Due to the low fiber content, the canola needs to be planted in a mixture with a high fiber crop or have fiber source nearby such as supplemental hay due to the low fiber content in canola that can cause bloat in cattle (Bushong et al., 2018).

1.3 Canola Production Around the World

World production of canola in 2019 was roughly 68.20 million metric tons (mmt) (Canola Council of Canada, 2020b). Canada led production with nearly 19 mmt produced over a span of 8.5 million hectares in 2019 (Canola Council of Canada, 2020b). Of this, 3.5 million hectares serves as exports around the world, as Canada exports 90% of its seed (Canola Council of Canada, 2020b). The European Union follows with 16.83 mmt, China with 13.10 mmt, Australia with 2.33 mmt, then the United States with 1.55 mmt (USDA Foreign Agricultural Service).

Idaho accounts for 2.4% of canola grown in the United States, with 4 million kilograms produced in 2018 making Idaho number 5 in U.S. canola production, an increase since 2017 when ranked 7 (1.5 million kilograms). North Dakota is the U.S. leader in canola production with 1.3 billion kilograms produced and 680,000 hectares planted in 2018 (USDA NASS). Idaho saw an increase of hectares planted in 2018 with 17,000 as compared to 9,000 in 2017 (USDA NASS). A steady increase of the amount of canola produced has been observed in Idaho since 2012 where production was 28.8 million kg. The same increasing trend has been observed in North Dakota where production in 2012 was 895.2 million kg. This ever-increasing rate of production is due to the many uses and benefits of canola to human health and the agricultural industry.

1.4 Production of Canola in Northern Idaho

Idaho is considered a dry Mediterranean climate, consisting of mild winter and hot summer temperatures in some areas and extreme cold in winter with hot dry summers in other areas of the state (Kassam et al., 2012). Optimal temperature for best growth of canola is 12 to 30°C with maximum growth and development achieved at 20°C, temperatures that are easily attained in northern Idaho (Brown et al., 2008). Northern Idaho is part of the inland Pacific Northwest (IPNW) where most of the precipitation occurs from October to March, with the remaining 25% occurring from April to June (Kruger et al., 2017). The Palouse region of the IPNW is made up of 1.3 million hectares of rainfed farming where a 3-year rotation of spring small grain, grain legume, then winter wheat is typically used (Guy and Cox, 2002). Figure 1.2 shows the agroecological classes within the IPNW (Kruger et al., 2017), indicating the Palouse and Camas Prairie of northern Idaho falls into two different agroecological classes. The Palouse and parts of the Camas Prairie are in the annual class while the areas of the Camas

Prairie focused on winter canola production is in the annual crop-fallow transition class. Both regions follow either tillage, reduced tillage, or direct seed practices to grow the same crops under similar rotations.

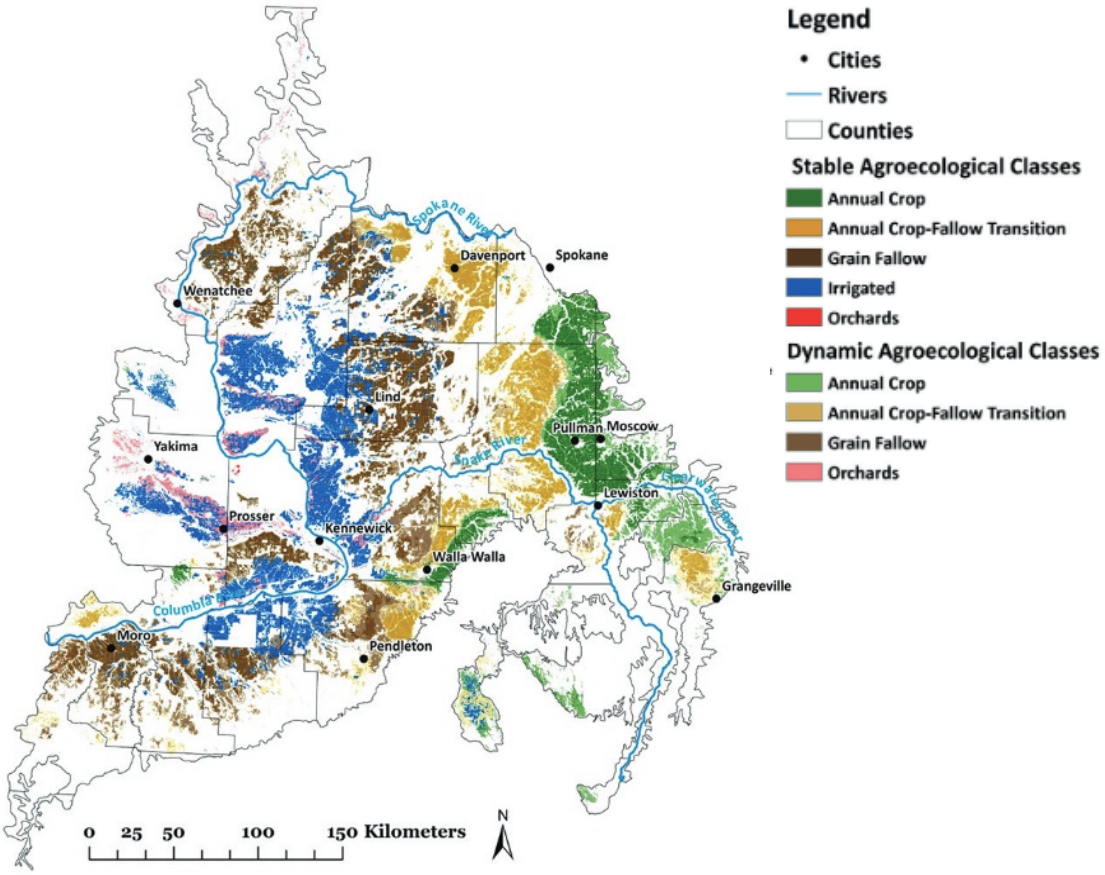

Figure 1.2: Agroecological classes of the inland Pacific Northwest (Kruger et al., 2017).

Under the annual agroecological class, 3- to 4-year rotations including winter wheat, spring wheat, spring barley, and pulses are traditionally practiced (Kruger et al., 2017). However, an increased interest in alternative crops has led to the introduction of canola into the rotation. Rotations under annual class on the Palouse often take the form of 3-years and include 1) winter wheat, spring wheat, spring canola, or 2) winter wheat, spring canola, pulse crop (Kruger et al., 2017).

Under the annual crop-fallow transition agroecological class, 2- to 3-year rotations including winter wheat, spring wheat, fallow is traditionally practiced (Kruger et al., 2017). Inclusion of canola into these rotations includes planting 1) winter wheat, fallow, winter

canola, or 2) winter wheat, spring wheat, spring canola (Kruger et al., 2017). Although both spring and winter canola can be included in the crop rotation, spring canola is better adapted in annual rotation following spring cereals, while winter canola follows fallow due to the need to seed in late summer in adequate moisture to allow for establishment of a large enough plant to survive the winter, and winter varieties are generally chosen over spring varieties due to higher yield potential (Brown et al., 2008).

Introduction of canola into cropping systems of northern Idaho adds multiple benefits to grower's operations including improved soil health and increased yield of cereal crops. Canola plants possess a large tap root that breaks up hard plow pans, reaching deep into the soil where other crops are unable to reach (Koenig et al., 2011). Having a large tap root and extensive root hair system provides canola the ability to maintain water soil content and bring nitrogen back into the soil while overall improving the soil structure (Martinez-Feria et al., 2016; Brown et al., 2008).

It has been shown that when canola is included in rotations, there is a 17 to 20% yield increase for small grains, and a decrease in pest and disease incidence of the small grains (Guy and Cox, 2002; Brown et al., 2008). It was shown in Australia, where climate is like that of northern Idaho, that incorporating canola in the cropping rotation leads to increased wheat yields due to the disease cycle being disturbed (Angus et al., 1991). Bushong et al. (2018) reported that local growers in the Great Plains region saw an increase in wheat yields up to 50% after introducing canola in the rotation.

1.5 Idaho Canola and Rapeseed Regulations

Canola produced in Idaho is for distribution as certified seed or sent to crushing facilities. All growers in Idaho must follow a set of regulations outlined by the Idaho State Department of Agriculture (ISDA). Regulations are in place to ensure edible and industrial rapeseed varieties do not cross pollinate and cause cross contamination along with preventing rapeseed from cross pollinating and contaminating other *Brassica* crops grown for seed in Idaho.

Under IDAPA 02.06.13 (2021), Idaho is split into 8 districts (Figure 1.3), with the 8th considered 'no district'. The only *Brassica* allowed in Districts 1, 5, 6, and 7 is canola. If growers want to plant rapeseed in these districts, they must obtain written approval from growers bordering their field. Rapeseed is traditionally only authorized to be grown in Districts

2 and 3, however spring and winter canola can be grown in both districts. Authorization to produce canola in District 2 and 3 required written consent from farmers bordering the field. Seed to be planted in Idaho must also be treated with EPA and State registered fungicides for the control of blackleg and all seed lots imported into the state must be tested (IDAPA, 2021).

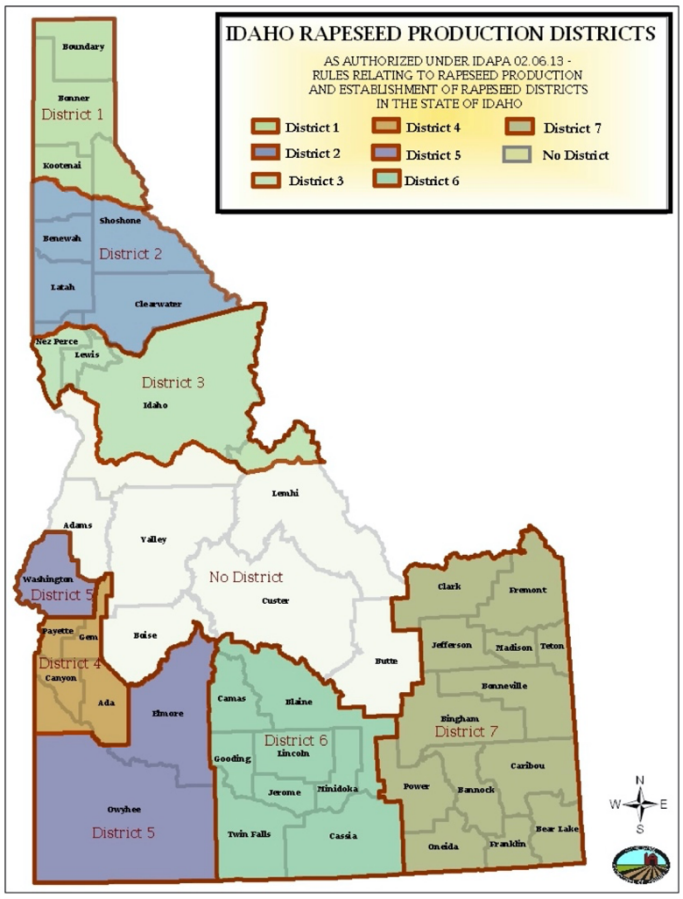

Figure 1.3: Idaho rapeseed production districts (IDAPA, 2021).

1.6 Major Diseases of Canola

1.6.1 Sclerotinia Stem Rot (White Mold)

Sclerotinia Stem Rot is caused by the soil borne fungal pathogen *Sclerotinia sclerotinium* and has a wide range of broadleaf hosts including peas, potatoes, and sunflowers. Initial infection occurs from infested seed or soil, where the pathogen can remain in the soil for several years as sclerotia which germinates and infects the roots and stem bases (Bailey et al., 2003). Ascospores are released from fruiting bodies in the soil that stick to the canola flower petals under wet and warm conditions and during petal drop, the highest rates of

germination and infection occur (Brown et al., 2008). The petals land on leaves and axils where the pathogen infects the plant and causes stem girdling, leading to premature ripening (Bushong et al., 2018). Methods of control include crop rotations of three to five years between hosts, planting certified seed that is free from sclerotia, and fungicide application before symptoms are present (Bailey et al., 2003; Brown et al., 2008; Bushong et al., 2018).

1.6.2 Alternaria Black Spot

Alternaria Black Spot is caused by the fungal pathogens *Alternaria brassicae* and *Alternaria raphani* that infect leaves, stems, and seed pods, and remains in infected stubble, seed, and even weed hosts (Bushong et al., 2018). *Alternaria* spp. cause spots on the stems and leaves that are grey to black in color with borders of purplish or black and if seed pods become infected, seed shrinkage occurs leading to yield losses of over 20% (Brown et al., 2008). Control is achieved through 3-year rotations and planting certified seed (Bushong et al., 2018).

1.6.3 Blackleg (Phoma Stem Canker)

Leptosphaeria maculans, an ascomycete fungus, is the causal agent of blackleg and can be found throughout the world. It is considered a polycyclic disease with modes of infection being sexual ascospores, asexual conidia, or infected seed lots. It is the most economically important disease of canola, and growing regions around the world follow a strict zero-tolerance policy to regulate the spread and management of the disease.

Blackleg was first described in the 1700s, where Tode identified the pathogen on dead cabbage plants in Germany (Henderson, 1918). Over 50 years later, blackleg was again described on *Brassica* species where it acted as a parasite on the host plant, by Desmaziere in 1849 France (Henderson, 1918). During the early stages of identification, the pathogen described was the anamorph *Phoma lingam* (Tode ex Fr.). Cunningham (1927) first noticed blackleg disease symptoms caused by *P. lingam* were similar, but differed in their level of severity, and he began grouping the fungal pathogen into two strains coining them weakly parasitic and highly parasitic based on the rapid or slow growth of the pathogen in culture and on the host.

Many studies and observations of blackleg pathogens have been conducted over the past 50 years to describe these weakly parasitic and highly parasitic strains. Production of yellow-brown pigment in vitro by avirulent and not by virulent strains were used as a grouping mechanism by McGee and Petrie (1978) and became a common method of reliably

characterizing the two strains. Humperson-Jones (1983) characterized virulent strains as those causing severe symptoms and stem cankers while avirulent strains produced mild symptoms. Petrie (1988) determined the growth of germ tubes differed for each strain and grouped them as weakly virulent and aggressive. Weakly virulent strains can produce longer germ tubes at the same rate of an aggressive strain producing a shorter tube under the same amount of time. The rapid rate of culture growth was used by Koch et al. (1989) to further group the two strains as aggressive and nonaggressive.

The phytotoxic compound sirodesmin was observed to be produced only by aggressive strains (Koch et al., 1989), resulting in strains grouped as Tox^{+} or Tox^{0} (Balesdent et al., 1992). Tox^{+}, or aggressive, isolates produce both sirodesmin PL and phomenoic acid while Tox^{0}, non-aggressive, isolates do not produce either compound. Pathogenicity of each group was defined, resulting in pathotype NA (non-aggressive) and pathotype A (aggressive) (Badawy et al., 1991). Pathotype A strains were then further divided into subgroups (A0-A4) with more detailed descriptions of disease symptoms. The presence of pigment and rate of which cultures grow are still a reliable means to identify the two strains apart under lab settings, as the two groups are usually indistinguishable based on spore size and morphology along with field symptoms being similar in appearance except for those regions in which severe disease symptoms do occur.

At the genetic level, Johnson and Lewis (1990) identified polymorphism between the two strains that suggested different species. Taylor et al. (1991) later identified distinct differences in chromosome band patterns and total number of chromosomes present in each strain, leading to further evidence of two species. Aggressive strains have 6 to 8 distinct chromosome bands and a genome size of 8.6×10^{6} base pairs, while non-aggressive strains have 12 to 14 distinct chromosome bands and a larger genome (1.6×10^{7} base pairs) than the aggressive strains. The teleomorph is considered part of the *Leptosphaeria* species complex, a term coined by Mendes-Pereira et al. (2003) that refers to the grouping of two species: *Leptosphaeria maculans* and *Leptosphaeria biglobosa.* It was not until 2001 that the distinction between the two species was described (Shoemaker and Brun, 2001) and are believed to have a common ancestor (Gudelj et al., 2004). It is difficult to microscopically differentiate between the two species, however when able to do so, ascospores of *L. biglobosa* contain a beak that is enlarged at the apex while *L. maculans* does not (Shoemaker and Brun, 2001).

1.7 Distribution and Economic Impact of Blackleg

Blackleg has a worldwide distribution, with *L. maculans* found in every canola growing region except China (Fitt et al., 2006; Van de Wouw et al., 2016). Blackleg has been present in Europe since the 1790s, Canada and Australia since the early 1970s and the United States since 1977 where blackleg is widespread among the Northern plains, Midwest and South (Agostini et al., 2013). It is speculated *L. maculans* spread from Canada south to North Dakota and other bordering states while *L. biglobosa* is less commonly found in the region (Bushong et al., 2018). Idaho was blackleg-free until 2011 when it was confirmed to be present on oilseed collected from Bonners Ferry, ID (Agostini et al., 2013). In 2015, blackleg was confirmed on winter canola from the Camas Prairie, ID, and regions throughout eastern Washington (Paulitz et al., 2017).

Worldwide canola production sees losses of more than $900 million per cropping season due to blackleg (Zhang and Fernando, 2017). Specifically in Canada, yield losses of up to 50% (Canola Council of Canada, 2017) have been reported in fields and current research from the University of Alberta predict that for every 20% increase in disease severity, there is an expected 17.2% loss in plant seed yield (Hwang et al., 2016). As blackleg continues to affect canola crops, the industry is going to continue to suffer losses. Therefore, it is important to understand the biology of this pathogen to develop optimal blackleg management strategies for each specific region that blackleg is present.

1.8 Disease Cycle, Symptoms, and Epidemiology of Blackleg

Leptosphaeria maculans and *L. biglobosa* produce multiple sources of inoculum including ascospores, conidia, and infected seeds. Conidia and ascospores can infect canola simultaneously and on virtually any portion of the plant, making the lifecycle of blackleg either monocyclic or polycyclic. When ascospores are the primary source of infection the disease is considered monocyclic unless conidia cause secondary infection, then blackleg is considered polycyclic (Li et al., 2007a). Canada, Australia, and Europe are faced with the polycyclic cycle of blackleg (Fitt et al., 2006; Zhang and Fernando, 2017).

Ascospores are the primary source of infection, released from mature pseudothecia under temperatures between 5 and 20°C, with 8 to 12°C ideal for optimal release, commonly occurring in conjunction with precipitation events (Toscano-Underwood et al., 2003; Rimmer et al., 2007). Guo and Fernando (2005) identified the potential of released ascospores to

disperse via wind 25 meters or further from the inoculum source where they then land on leaves of the canola plant.

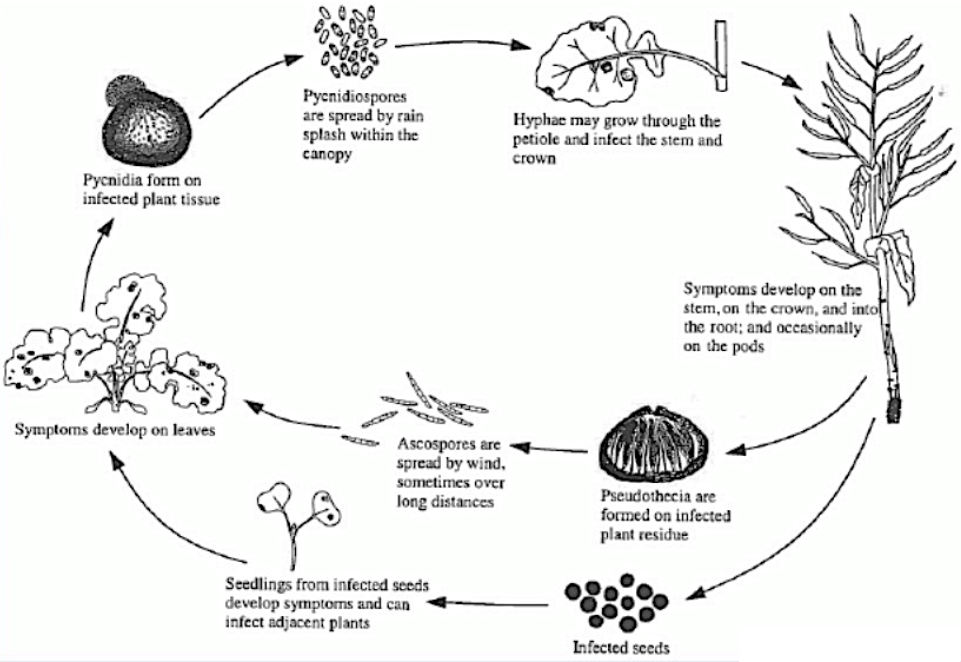

Figure 1.4: Blackleg disease cycle (Buchwaldt, 2007).

Ascospores germinate in humid and wet conditions to produce hyphae that cause infection via stomata and wounds, giving rise to leaf lesions (Rimmer et al., 2007; Bailey et al., 2003). It is from leaf and stem lesions that pycnidia form and create pycnidiospores, otherwise known as conidia, that are dispersed via water splash. As the infection colonizes the leaf tissues and becomes systemic, the hyphae travel through the leaf tissues to the stem where it travels through the stem via the xylem, producing stem lesions and eventually forming stem cankers at the base of the plant (Hammond et al., 1985; Hammond and Lewis, 1986) Stem cankers develop pseudothecia, the fruiting body responsible for producing ascospores. Ascospores overwinter on infected crop residue where they can survive two to five years (Huang et al., 2003). Within the pseudothecia on infected residue, the fungus undergoes sexual recombination, overwinters, and releases the next generation of ascospores under ideal environmental conditions to initiate infection once more.

Both species of *Leptosphaeria* have been found to co-exist on the same plant with *L. biglobosa* on the upper stem portion and *L. maculans* near the base (West et al., 2002).

Pathogen infection and blackleg onset occurs through the same process and disease cycle (Figure 1.4). However, *L. biglobosa* results in less aggressive disease symptoms identified as lesions and pith blackening while *L. maculans* is more aggressive, causing not only lesions and pith blackening, but also has the potential to develop stem cankers that may lead to extreme damage, causing yield loss and economic impact (Somda et al., 1998).

Leptosphaeria maculans and *L. biglobosa* can infect all above ground parts of the plant. Therefore, seed pod infection is an additional concern alongside stem cankers. Often caused by ascospores and/or conidia initiating secondary infection, rather than the pathogen traveling up the stem, infected seed pods can lead to infected seed, providing the third source of inoculum for the pathogen to spread (Van de Wouw et al., 2016). The greatest concern, especially for certified seed production, is infected seed introducing blackleg into regions otherwise deemed "blackleg-free".

1.9 Disease Management Practices

1.9.1 Certified Seed and Trade Restrictions

There is a constant risk of introducing blackleg into regions otherwise deemed blackleg free. It is speculated that *L. maculans* can be introduced via contaminated seed lots and plant debris and this potentially explains the worldwide distribution (Van de Wouw et al., 2016). To address this issue, certified seed is produced, and trade restrictions have been developed not only in the U.S. but in Canada, China and other canola growing regions of the world. For example, only *L. biglobosa* has been described in China and there is high concern that *L. maculans* will be introduced. A trade barrier between Canada and China was put into effect in 2009, and between Australia and China in 2012 to reduce the potential introduction of *L. maculans* in infected seed lots (Zhang and Fernando, 2017; Van de Wouw et al., 2016).

The Idaho state legislature enacted the Seed and Plant Certification Act of 1959 (ICIA, 2015) to ensure all seed available to the public would be of the highest genetic purity and quality, resulting in the certified seed certificate. Certification standards published by ICIA (2017) outlines production restrictions for certified oilseed to ensure seed meets the highest genetic purity and quality standard, along with ensuring canola seed is 99.99% blackleg free. Certified seed must be produced from a field that has been free from brassica crops for 3 years, a standard that aligns with common crop rotation practices in the area to reduce disease inoculum levels. Further certified seed standards include field isolation of 201 meters for

certified *B. napus* seed from a surrounding *B. napus* field. This ensures any infected surrounding fields will be unable to pass along ascospores or conidia to the field planted for certification along with ensuring no cross pollination can occur. All certified seed contains a certificate tag that allows the tracking of seed lots including location of production and destination. If the seed lot ends up containing blackleg, the origins are easily traced, and control methods can be in place to reduce the same origin producing and shipping further infected seed lots.

1.9.2 Genetic Resistance

An important element in overall management of blackleg in canola is through genetic resistance and is considered the most important aspect of breeding programs in Canada, Europe, and Australia (Long et al., 2011). There are two types of resistance, qualitative and quantitative. Quantitative resistance is expressed as adult plant resistance and is controlled by multiple genetic factors (Brun et al., 2010), making it non-race specific, and effectively reduces the severity of disease development in the stem (Delourme et al., 2006). Qualitative resistance refers to a single gene-for-gene interaction that is race specific. Qualitative resistance goes by many names including vertical and major resistance. It is based on a theory described by H. H. Flor (1946), through his work on the flax rust fungus, *Melampsora lini*. This disease management strategy has been available in commercial canola cultivars since the early 1990s after widespread blackleg outbreaks occurred in the 1980s (Zhang and Fernando, 2017).

The gene-for-gene interaction consists of avirulence effector genes that are complementary to resistance genes in the host, causing a hypersensitive response when both are present, thus rendering the pathogen unable to infect the plant. As of 2016, 16 avirulent genes have been identified in *L. maculans* including *AvrLm1-Lm11, AvrLepR1-LepR3, AvrLmS,* and *AvrLmJ1* (Liban et al., 2016). Eighteen resistance genes have been characterized in *B. napus*, *B. rapa,* and *B. juncea* cultivars, all located on the 'A' genome (Long et al., 2011) with 16 corresponding to those avirulent genes identified in the pathogen, including *Rlm1-11, RlmS* (Van de Wouw et al., 2009; Van de Wouw et al., 2016), *LepR1-LepR4* (Yu et al., 2005, 2008, 2013), and *BLMR1* and *BLMR2* (Long et al., 2011).

R-gene mediated resistance is advantageous due to the specificity and Mendelian inheritance, however it is not durable and often leads to 'boom-and-bust' cycles due to the pathogen's ability to undergo sexual recombination (McDonald, 2010; Zhang and Fernando,

2017). The boom refers to the growth of a variety containing a single *R*-gene over a large area for a long period of time. *Leptosphaeria maculans* has a high evolutionary potential and can overcome this resistance in a short amount of time, leading to an abundance of virulent isolates. The bust occurs when the cultivar is replaced with one offering better resistance, either due to the presence of a new *R*-gene or the introduction of multiple *R*-genes, known as pyramiding. The virulent pathogen population decreases which is referred to as a rotation of *R*-genes resulting in pathogen population manipulation and evolution (Brun et al., 2010; Marcroft et al., 2012; McDonald, 2010).

Along with the rotation of *R*-genes, a combination of qualitative and quantitative resistance in varieties provides improved durability of blackleg resistance (Zhang and Fernando, 2017). This has been supported through the effort of Brun et al. (2010) whose work shows by combining the two methods, *R*-gene resistance does not breakdown as rapidly, thus providing overall greater disease control and continued crop production. Although the combination of both quantitative and qualitative resistance is ideal, it is a difficult method to achieve through traditional breeding methods due to *R*-genes usually masking the effects of quantitative resistance (McDonald, 2010).

1.9.3 Chemical Control

Chemical control against blackleg disease consists of fungicide seed treatments at planting and in season foliar applications. Many active ingredients labeled for use against blackleg are available to growers, each categorized in a specific fungicide group by the Fungicide Resistance Action Committee (FRAC) where mode of action is identified and any level of resistance that may be present. Common groups include demethylation inhibitors (DMIs), phenyl amides (PA), carboxamides, quinone outside inhibitors (QoIs), and phenylpyrroles (PP). Pathogens can develop resistance to active ingredients, therefore many fungicides on the market will consist of multiple ingredients from different FRAC groups to provide optimal control.

Fungicides improve disease management in regions where resistant cultivars are not commercially available to all growers or do not possess full resistance towards the pathogen, which is common in Australia where high disease pressure is common (Khangura and Barbetti, 2002; Marcroft and Potter, 2008). However, planting directly into infested soil or adjacent to infected residues reduces the effectiveness of fungicide seed treatments (Bushong et al., 2018)

requiring foliar applications in conjunction with seed treatments. For example, Fraser et al. (2020) identified effective management of blackleg in susceptible cultivars when pyraclostrobin seed treatment was paired with foliar applications of pyraclostrobin & fluxapyroxad (Priaxor).

DMI fungicides are a common seed treatment and proven effective in managing seed-borne blackleg, however QoIs are the most used fungicide found in both seed treatments and foliar products (Fraser et al., 2020) for management of the blackleg pathogen. Common foliar fungicides on the market include Priaxor (pyraclostrobin & fluxapyroxad) and Quadris (azoxystrobin) (Bushong et al., 2018; Brown et al., 2008). Multiple efficacy tests have been conducted around the world to identify ideal fungicides to use as seed treatments and foliar applications for management of blackleg. Triazoles, DMI fungicides, are commonly used in Australia, however climatic conditions often play a large role in the efficacy of these fungicides (Marcroft and Potter, 2008; Khangura and Barbetti, 2002).

Once the pathogen colonizes the canola stem, fungicides are less effective at preventing further disease symptoms, so it is important to identify the optimal time for application (Zhang and Fernando, 2017). The most common method to determine the ideal application time is scouting fields for leaf lesions, although symptoms may not always be present when the plants are infected, and infection may have already reached the stem (West et al., 1999; West et al., 2001). Identifying the optimal time to apply fungicides depends on climate and the pathogen lifecycle. Identifying when spores are released and causing initial infection determines the time of year foliar applications should be made. Utilization of spore traps aids in identification of spore release and contributes to the development of disease forecasting publications. For example, optimum time of foliar application for winter canola in western Europe is in the fall, 6 months before symptoms appear on stems (West et al., 2001).

Once the optimal time to apply fungicides is determined, growers need to take into consideration the economic benefit to spray or not. Most resistant varieties will not benefit from having a fungicide application and often fungicides in Canada are only applied when significant production issues result from the disease (Zhang and Fernando, 2017).

1.9.4 Cultural Control

Due to *L. maculans* ability to survive on crop residues for an extended period and rate of residue breakdown being variable based on environmental conditions, cultural production

practices can contribute to the management and prevention of blackleg. The most common cultural practices being crop rotation, stubble management, and isolation. Although crop residues are valuable in conservation practices to reduce erosion and promote soil health and moisture (Cook, 2006), infected residues are problematic for blackleg disease management. Infected residues in no-till systems do not provide a significantly higher level of disease incidence as compared to conventional practices, rather the persistence of residue in the field provides a steady disease pressure (Bailey et al., 2000). Different environmental conditions contribute to the rate at which infected residue breaks down, and as the quantity of infected residue increases, so does the amount of inoculum (Kutcher et al., 2013). It was shown that residue breakdown is often slower under dry summers and cold winters, while rapid breakdown occurs under mild and wet conditions (Kutcher et al., 2013; West et al., 2001). Rather than leaving residues on the soil surface to slowly degrade, burying infected debris promotes faster breakdown of plant tissues and reduction of inoculum (Blenis et al., 1999). Huang et al. (2003) and Marcroft et al. (2003) showed infected residues buried for an extended period resulted in lower pseudothecia quantities while those stems recently left in the field or buried for a few months resulted in significantly higher levels.

In conjunction with burying infected residues, crop rotations of 3 to 4 years decrease the quantity of inoculum while breaking other pest and disease cycles and promoting soil health (West et al., 2001; Kutcher et al., 2013; Rimmer et al., 2007). Environmental conditions of the region need to be taken into consideration when determining an ideal crop rotation. For example, a 2-year rotation with cool and wet conditions will promote faster residue breakdown while warmer and dryer weather results in slower degradation (Kutcher et al., 2013). Crop choice within the rotation plays a large role in disease management and *Brassica* host crops should only be included in the rotation every 3 to 4 years (Kutcher et al., 2013). Blackleg damage seen in Canada and Australia are mainly attributed to short rotations and monoculture of canola. Crop rotations under both conventional and conservational practices have resulted in low disease incidence and severity when *Brassica* host crops are only included once every 3 to 4 years in the rotations with a diverse selection of non-host crops including wheat and flax (Guo et al., 2005; Guo et al., 2008).

1.10 Integrated Blackleg Management Strategy

Integrated disease management strategies are developed to provide growers with the tools necessary for optimal control against blackleg in canola. As outlined by Zhang and Fernando (2017) in Figure 1.5, integrated blackleg management includes utilizing as many control practices as possible that suit the grower's production practices. Resistant varieties should be planted in combination with diverse crop rotations and other cultural practices that will aid in inoculum reduction. To ensure an effective blackleg management strategy there needs to be continual monitoring of the pathogen population and how the environment and cropping system contribute to its epidemiology.

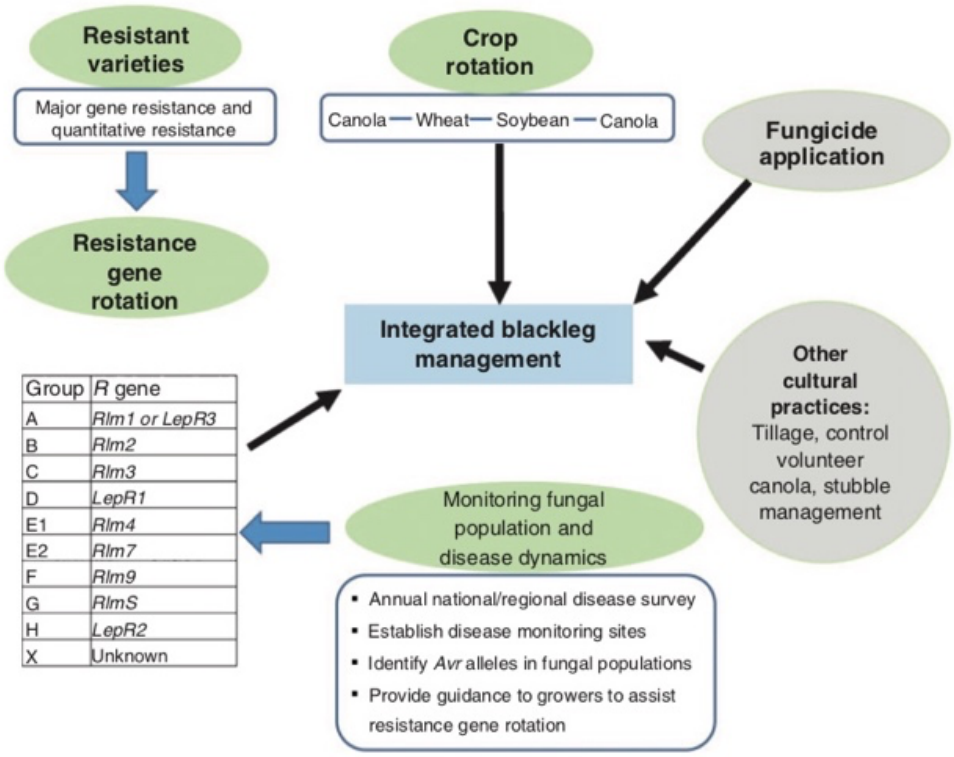

Figure 1.5: Integrated disease management strategy created by Zhang and Fernando (2017).

1.11 Research Objectives

Blackleg is new disease to northern Idaho; therefore, researchers and growers have limited knowledge regarding how the environment contributes to the epidemiology of *L. maculans* and spread of blackleg across the region. General management strategies are available for growers in the area to follow, however they are not adapted to suit the specific needs of northern Idaho canola production. Therefore, the main objective of this research is to contribute to the development of grower guidelines for best management practices of blackleg in canola through a means of understanding the epidemiology of *L. maculans* specific to northern Idaho. Specific project objectives include 1) characterization of *L. maculans* isolates collected from eastern Washington, 2) identification of spore production and dispersal in conjunction with weather patterns inducing initial infection, and 3) determining optimal timing to apply foliar fungicides to reduce disease incidence and identify impact of fungicides on agronomic characteristics.

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Chapter 2: Characterization of *Leptosphaeria maculans* Isolates Obtained from Eastern Washington

2.1 Introduction

Blackleg disease, also known as phoma stem canker, is caused by the fungal pathogen *Leptosphaeria maculans* and *L. biglobosa.* Blackleg follows a polycyclic disease cycle where wind-blow ascospores and rain-splashed pycnidiospores act as sources of inoculum, infecting canola via stomata and wounds, resulting in leaf and stem lesions. When infection is caused by *L. maculans,* stem cankers will form at the base of the plant, causing stem girdling and yield loss, thus making *L. maculans* induced blackleg the most economically important disease of canola worldwide (Somda et al., 1998; Fitt et al., 2006).

Blackleg has been present in Canada, Australia, and Europe since the 1970s (Bailey et al., 2003) where severe production impacts occur in spring and winter cultivars. In northern Idaho, blackleg was first identified in 2011 (Agostini et al., 2013) and confirmed in eastern Washington in 2015 (Paulitz et al., 2017) and does not currently cause severe production impacts. Rather, because *L. maculans* can infect all above ground parts of the plant, there is a concern of seed becoming infected and thus spreading the pathogen into other regions (Bailey et al., 2003) as well as impacting brassica seed production in the region.

An important element in overall management of blackleg in canola is through genetic resistance and this is considered the most important aspect of breeding programs in Canada, Europe, and Australia (Long et al., 2011). Qualitative resistance is a gene-for-gene interaction consisting of avirulence effector genes that are complementary to resistance genes in the host, causing a hypersensitive response when both are present, thus rendering the pathogen unable to infect the plant. Qualitative resistance has been available in commercial canola cultivars since the early 1990s after widespread blackleg outbreaks occurred in the 1980s (Zhang and Fernando, 2017).

To date, sixteen avirulent genes have been identified in *L. maculans* including *AvrLm1-Lm11, AvrLepR1-LepR3, AvrLmS,* and *AvrLmJ1* (Liban et al. 2016). The corresponding *R*-genes have been characterized in *B. napus*, *B. rapa,* and *B. juncea* cultivars (Long et al. 2011). *Rlm1* and *Rlm2* were the first *R*-genes available in commercial canola cultivars (Ansan-Melayah et al., 1998). After widespread and consistent use of *Rlm1* and *Rlm2*, surveys of the *L. maculans* populations in Canada, Europe, and North Dakota have

resulted in the absence of *AvrLm1* and *AvrLm2* (Rong et al., 2015; Balesdent et al., 2005; Nepal et al., 2014). Genetic resistance is an effective control method against blackleg, but breakdown of the *R*-gene resistance occurs due to the pathogens ability to undergo sexual recombination (McDonald, 2010; Zhang and Fernando, 2017). Similar breakdown events have occurred in Canada where *Rlm3* lost effectiveness in 5 years and *RlmS* was ineffective within 3 years (Zhang et al., 2016; Sprague et al., 2006).

Pathogen population surveys identify the race structure of isolates and provide insight as to which *R*-genes in canola will provide optimal control in the field, thus reducing the possibility of resistance breakdown events because the proper *R*-genes are utilized. Blackleg is a new disease to northern Idaho and eastern Washington; therefore, little is known regarding the *Leptosphaeria* species population in this region. In a previous study (Pickard, 2018), isolates of *L. maculans* from Idaho were characterized for the frequency of *AvrLm* genes. More recently, *L. maculans* isolates were collected from eastern Washington and the purpose of this study is to elucidate the race structure by identifying the presence of *AvrLm* genes in these isolates. The objectives of this research include 1) characterization of isolate race structure, 2) determination of the distribution of *AvrLm* genes across eastern Washington, 3) comparison of the eastern Washington *L. maculans* population demographics with *L. maculans* population demographics identified in northern Idaho and other countries, and 4) providing insight as to which *R*-genes should be considered when growers and breeders are making management decisions.

2.2 Materials and Methods

2.2.1 Isolate Culture Maintenance

Isolates were collected from canola fields in four eastern Washington counties (Adams, Garfield, Lincoln, and Spokane) with some isolates collected in Latah County, Idaho (Figure 2.1) by Dr. Timothy Paulitz (USDA-ARS Pullman, Wheat Health, Genetics and Quality Research Unit) and maintained on potato dextrose agar plates (PDA; 1 liter distilled water, 24 g potato dextrose broth, 18 g agar) at 4°C. Long term storage of cultures was completed by dipping sterile filter paper discs into a conidial suspension of the fungus in sterile distilled water (SDW) and allowed to dry for 4 hours. Discs were stored in 1.5 ml centrifuge tubes at -20°C.

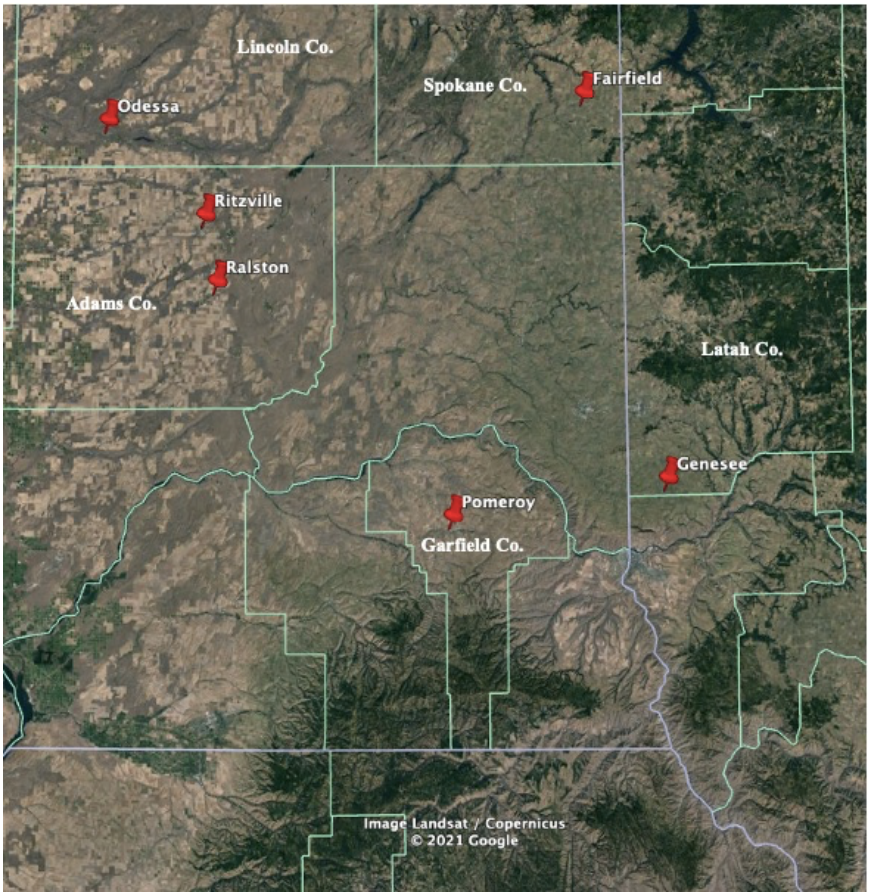

Figure 2.1: Map of isolate collection sites in eastern Washington.

2.2.2 DNA Isolation and Identification of Isolates

Agar plugs of each isolate were taken from the edge of actively growing mycelium and placed onto a sterile petri dish then flooded with ~10 ml potato dextrose broth (PDB; 1 liter distilled water, 24 g potato dextrose broth). Plates were stored in the dark for 7 days. Once mycelial mats were formed, genomic DNA was isolated using a FastDNA Kit (MP Biomedicals, Santa Ana, CA, USA). Mycelial mats were removed from the broth using sterile forceps, dried on sterile filter paper, and transferred to lysing tubes. Lysing tubes were placed in a Mini Beadbeater (Biospec Product, Inc., Bartlesville, OK, USA) and DNA was isolated following the protocol provided with the FastDNA kit. To ensure DNA was successfully isolated from each mycelial mat, DNA was loaded into a 1% agarose gel with ethidium bromide (10 mg/ml) and run at 100 V for 1 h. Results were observed under UV light and recorded. If no genomic DNA was present, the sample was reevaluated and if still no DNA

was observed, DNA was re-isolated from the sample. When DNA was observed by gel electrophoresis, the DNA samples were aliquoted and stored at 4°C until used for PCR reactions.

Internal transcribed spacer (ITS) sequences allow for identification of isolates at the genus and often at the species level (Bakkeren et al., 2000). The Washington isolate collection contains a combination of *L. biglobosa* and *L. maculans.* To distinguish the individual isolates, PCR amplification of the ITS region was conducted by adding the following to strip cap tubes: 6 μl of 5X buffer, 1.8 μl 2mM dNTPs (Thermo Fisher Scientific, Grand Island, NY, USA), 16.9 μl PCR water, 2 μl each of 10 pmol forward and reverse primer (Table 2.2), 0.3 ul GoTaq DNA Polymerase (Promega, Madison, WI, USA), and 1 μl DNA template (Bakkeren et al. 2000). DNA was amplified using an Eppendorf Ag Thermocycler (Eppendorf, Hamburg, Germany) with settings as follows: 1 cycle of 94°C for 3 min; 31 cycles of 92°C for 45 s, 60°C for 45 s and 72°C for 60 s; 1 cycle of 72°C for 10 min; and hold indefinitely at 15°C. PCR products were confirmed using electrophoresis as previously described and samples were compared to a 100 bp ladder (100 bp+ Gene Ruler, Thermo Fisher Scientific, Grand Island, NY, USA) with negative and positive controls. *Leptosphaeria maculans* and *L. biglobosa* ITS regions amplify at 580 bp and 555 bp, respectively (Fernando et al., *unpublished*).

Purification of PCR product was completed in a 1.5 ml centrifuge tube containing 1 μl ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems, Thermo Fisher Scientific, Grand Island, NY, USA) and 2.5 μl PCR product. Tubes were incubated at 37°C for 15 min followed by an additional 15 min at 80°C. Tubes were removed after incubation and allowed to cool to near room temperature and 14 μl PCR water was added. Clean PCR product was stored short term at 4°C until sequencing reactions could be performed.

ITS sequencing reactions were prepared in 0.2 ml 8-Tube PCR strip-cap-tubes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using 2.5 μl of 3.2 pmol ITS forward primer (Table 2.2) in all reactions. Depending on the intensity of the band on electrophoresis gels, 1 to 3 μl of PCR product was combined with PCR water to a final volume of 12.5 μl. Prepared samples were sent to Elim Biopharmaceuticals, Inc. (Hayward, CA, USA) for sequencing.

Using the software Geneious (Auckland, New Zealand), sequences were reviewed and edited. Using Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sequences were

analyzed and compared to authenticated standard isolates from previously published work to identify isolates as *L. maculans* or *L. biglobosa.*

2.2.3 Greenhouse Pathogenicity Assay

Agar plugs of each isolate were taken from the edge of actively growing mycelium and plated onto 20% V8 (800 ml DI water, 2 g calcium carbonate, 200 ml Low Sodium V8 Vegetable Juice, 18 g agar) and placed under UV plant and aquarium lights (GE Lighting, Inc., Cleveland, OH, USA) for 14 days at room temperature (22 to 24°C) (Liban et al., 2016). After 14 days, cultures were flooded with SDW, scraped with a sterile microscope slide to dislodge spores, and filtered through Miracloth (Calbiochem, La Jolla, CA, USA) using a funnel into a 40 ml centrifuge tube (Liban et al., 2016). Spore suspensions were centrifuged at 1,500 RPM for 10 min (Eppendorf centrifuge 5804R, Eppendorf North America, Enfield, CT, USA). Supernatant was poured off and inoculum was resuspended in 1 ml of SDW. The concentrated inoculum was transferred to 1.5 ml centrifuge tubes and stored at -20°C until day of inoculation.

Susceptible spring canola cultivar Westar was seeded into 72-cell seedling flats with cell dimension of 5.9 cm deep by 3.8 cm across filled with Pro Mix BX Mycorrhizae general purpose potting mix (Premier Tech Horticulture, Québec, Canada). Seedlings were spot watered daily and emerging true leaves were removed to prevent senescence (Liban et al., 2016). Growth conditions were 20 to 24°C with a 16-hour photoperiod. After 10 days, when seedlings were fully expanded and true leaves began to form, seedlings were inoculated with conidial spore suspensions.

On day of inoculation, concentrated conidial spore suspension was removed from storage and adjusted with SDW to a desired concentration range of 1.5 to $2.5x10^7$ spores/ml using a hemocytometer (Liban et al., 2016). Cotyledons of each seedling were wounded using modified forceps resulting in 4 wounds per plant and 24 wounds total per isolate. Of the 24 total wounds, 22 wounds for each isolate received a 10 μl drop of inoculum while the remaining two wounds received a 10 μl drop of SDW to serve as a negative control for each isolate. Drops on each wound were allowed to air dry before placing seeding flats into humidity tents for 48 hours (Van de Wouw et al., 2009). Once removed from high humidity, seedlings were fertilized with 20:20:20 (N:P:K) (Liban et al., 2016) and emerging true leaves removed. A confirmed *L. maculans* isolate, Phl010, provided by Dr. Dilantha Fernando

(University of Manitoba, Winnipeg, Canada), was used as a positive control for the pathogenicity assays. Seedlings were fertilized again 1 week after removal from high humidity. 14 days post inoculation, lesions were scored based on the IMASCORE rating system using infection classes 1 to 4 (Figure 2.2; Balesdent et al., 2001).

The IMASCORE rating system consists of six infection classes (IC) with 0 having no visible symptoms, corresponding to water control (Van de Wouw et al., 2009), and IC1 being a typical hypersensitive response. IC2 is a darker necrotic lesion and IC3 is a nonsporulating lesion that always contains a dark necrotic region around the wound and may show tissue collapse like IC4 to IC6 but does not always. IC4, IC5, and IC6 all have gray-green tissue collapse without a darkened margin. What sets the three groups apart is IC4 does not have sporulation, IC5 has few pycnidia and IC6 has many sporulating pycnidia. Resistant ratings are considered IC1 to IC3 whereas IC4 to IC6 are susceptible. Thus, isolates with a resistant response can be identified as *L. biglobosa* while those within IC4 or higher are considered *L. maculans*.

Figure 2.2: IMASCORE rating system; left to right rating 0-4 (Van de Wouw et al., 2009; Balesdent et al., 2001).

2.2.4 Characterization of Avirulence Genes by Host Plant Differentials

Isolates confirmed to be *L. maculans* through morphological characteristics, ITS sequencing, and pathogenic in the assay described above were screened in greenhouse host plant differentials to characterize avirulence effector genes of each isolate. Host differentials consisting of *B. napus* and *B. juncea* cultivars were used to characterize avirulence genes of each isolate confirmed to be *L. maculans.* Each differential line contained the following *R*-genes (Table 2.1): Westar (no *Rlm* genes, Delourme et al., 2004); Columbus (*Rlm1, 3*), Glacier (*Rlm2, 3*), Bristol (*Rlm2, 9*), 02.22.2.1 (*Rlm3*), Jet Neuf (*Rlm4*) (Balesdent et al., 2005);

Cutlass (*Rlm5, 6*, Liban et al., 2016); 01.23.2.1 (*Rlm7*, Dilmaghani et al., 2009); Goeland (*Rlm9*, Balesdent et al., 2006); Topas LepR1 (*LepR1),* Topas LepR2(*LepR2*), Topas LepR3 (*LepR3*) (Larkan et al., 2016).

One *L. maculans* isolate is screened on each cultivar for their disease response, allowing the differential determination of the presence of *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5-6, AvrLm7, AvrLm 9, AvrLepR1, AvrLepR2,* and *AvrLepR3* in each isolate. Protocol outlined above for the pathogenicity assay were followed for planting, conidial spore suspension preparation, and seedling inoculations to conduct the greenhouse differential screening. Inoculated seedlings were rated using the IMASCORE system 14 days post inoculation (Liban et al., 2016), as described above. A rating of 1 to 3 indicates resistance (the *Avr* gene is present) while a rating of 4 indicates susceptibility (the *Avr* gene is absent).

2.2.5 Characterization of Avirulence Genes by PCR Differentials

After screening *L. maculans* isolates in greenhouse host plant differentials, PCR was used to confirm the results of the host plant differentials along with identifying *Avr* genes that may be present but were not able to be identified in the host plant differential. This is due to not obtaining cultivars carrying the specific *R* gene, and some *Avr* genes are not expressed in the presence of others (Parlange et al., 2009; Plissonneau et al., 2016; Ghanbarnia et al., 2018).

Primers developed to identify *AvrLm1, AvrLm2, AvrLm3, AvrLm4-7, AvrLm5, AvrLm6,* and *AvrLm11* were utilized to complete PCR differentials (Table 2.2). To characterize each *Avr* gene in individual isolates, 1 μl of template DNA was added to the following reaction mix in strip cap tubes: 4 μl of 5X buffer, 1.2 μl 2mM dNTPs (Thermo Fisher Scientific, Grand Island, NY, USA), 11 μl PCR water, 1.3 μl each of 10 pmol forward and reverse primer (Table 2.3), 0.2 μl GoTaq DNA Polymerase (Promega, Madison, WI, USA) (Pickard, 2018). DNA was amplified using an Eppendorf Ag Thermocycler (Eppendorf, Hamburg, Germany) with settings as follows: 1 cycle of 94°C for 30 s; 31 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s; 1 cycle of 72°C for 5 min and hold indefinitely at 15°C. *AvrLm* PCR product results were confirmed using gel electrophoresis as previously described. Samples were compared to a 100 bp ladder (100 bp+ Gene Ruler, Thermo Fisher Scientific, Grand Island, NY, USA) with negative and positive controls.

2.2.6 Characterization of Mating Types

Characterization of mating type *MAT1.1* and *MAT1.2* of each isolate was completed using PCR. Reactions were prepared in strip cap tubes as follows: 4 μl of 5X buffer, 1.2 μl 2mM dNTPs (Thermo Fisher Scientific, Grand Island, NY, USA), 11 μl PCR water, 1.3 μl each of 10 pmol forward and reverse primer (Table 2.2), 0.2 μl GoTaq DNA Polymerase (Promega, Madison, WI, USA), 1 μl template DNA. Thermocycler settings were 1 cycle of 94°C for 30 s; 30 cycles of 55°C for 15 s, 72°C for 60 s, 72°C for 60 s; 1 cycle of 72°C for 5 min; and hold indefinitely at 15°C (Pickard, 2018). Mating type PCR product results were confirmed using gel electrophoresis as previously described and samples were compared to a 100 bp ladder (100 bp+ Gene Ruler, Thermo Fisher Scientific, Grand Island, NY, USA) with negative and positive controls. *MAT1.1* and *MAT1.2* amplify at 686 bp and 443 bp, respectively.

Table 2.1: List of cultivars used for greenhouse host differential.

Cultivars	Crop	*Rlm* genes	Reference
Westar	S. Canola	None	Delourme et al., 2004
Columbus	W. Canola	1, 3	Balesdent et al., 2005
Glacier	W. Canola	2, 3	Balesdent et al., 2005
Bristol	W. Canola	2, 9	Balesdent et al., 2005
02.22.2.1	W. Canola	3	Balesdent et al., 2005
Jet Neuf	W. Canola	4	Balesdent et al., 2005
Cutlass	S. Mustard	5, 6	Liban et al., 2016
01.23.2.1	W. Canola	7	Dilmaghani et al., 2009
Goeland	W. Canola	9	Balesdent et al., 2006
Topas LepR1	S. Canola	LepR1	Larkan et al., 2016
Topas LepR2	S. Canola	LepR2	Larkan et al., 2016
Topas LepR3	S. Canola	LepR3	Larkan et al., 2016

Table 2.2: List of primers for ITS, *Avr* genes, and *MAT* genes PCR.

Primer Name	Sequence (5' to 3')	Reference
UN-UP18S42 F	CGTAACAAGGTTTCCGTAGGTGAAC	Bakkeren et al., 2000
UN-LO28S576B R	GTTTCTTTTCCTCCGCTTATTAATATG	Bakkeren et al., 2000
*AvrLm1*F	CTATTTAGGCTAAGCGTATTCATAAG	Gout et al., 2006
*AvrLm1*R	GCGCTGTAGGCTTCATTGTAC	Gout et al., 2006
*AvrLm2*F	CGTCATCAATGCGTTCGG	Ghanbarnia et al., 2015
*AvrLm2*R	CTGGATCGTTTGCATGGA	Ghanbarnia et al., 2015
*AvrLm3*ext-SpelF	GAGAGAACTAGTCTGTTAAATGCCTGCTGT	Plissonneau et al., 2016
*AvrLm3*ext-XholR	GAGAGACTCGAGCGCGCTTATGTTAGAATC	Plissonneau et al., 2016
AvrLm4-7 ext – Lo (R)	GATGGATCAACCGCTAACAA	Parlange et al., 2009
AvrLm4-7 ext – Up (F)	TATCGCATACCAAACATTAGGC	Parlange et al., 2009

Table 2.2 continued: List of primers for ITS, *Avr* genes, and *MAT* genes PCR.

*AvrLm6*F	TCAATTTGTCTGTTCAAGTTATGGA	Fudal et al., 2007
*AvrLm6*R	CCAGTTTTGAACCGTAGTGGTAGCA	Fudal et al., 2007
*AvrLmJ1*F	ACAACCACTCTTCTTCACAGT	Van de Wouw et al., 2014
*AvrLmJ1*R	TGGTTTGGGTAAAGTTGTCCT	Van de Wouw et al., 2014
AvrLm11-L (uP119060_L) R	CAAGTTGGATCTTTCTCATTCG	Balesdent et al., 2013
AvrLm11-U2 (uP119060_U) F	TGCGTTTCTTGCTTCCTATATTT	Balesdent et al., 2013
MAT-1.1 (F)	CTCGATGCAATGTACTTGG	Cozijnsen and Howlett, 2003
MAT-1.2 (F)	AGCCGGCGGTGAAGTTGAAGCCG	Cozijnsen and Howlett, 2003
MAT-R	TGGCGAATTAAGGGATTGCTG	Cozijnsen and Howlett, 2003

2.3 Results

2.3.1 Characterization of Isolates

BLAST analysis of the ITS sequences obtained from the 100 eastern Washington fungal isolates resulted in one isolate identified as *L. biglobosa* and one as *Alternaria infectoria*. Pathogenicity assays for these two fungal isolates resulted in an IMASCORE rating of (1) confirming they are not *L. maculans.* Three isolates were contaminated and removed from the collection. This resulted in a working collection of 95 isolates identified as *L. maculans* through morphological characteristics, ITS sequencing, and pathogenicity testing. The 95 isolates were screened for avirulence effector genes and mating type genes using PCR and greenhouse host differentials. All isolate information and results are in Appendix A.

2.3.2 Characterization of Avirulence Genes by Host Plant Differentials

Brassica napus and *B. juncea* cultivars were used for a host plant differential screen, each carrying resistance genes specific to the following avirulence effector genes in *L. maculans* isolates: *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5-6, AvrLm7, AvrLm9, AvrLepR1, AvrLepR2, AvrLepR3.* The 95 isolates screened in this differential set resulted in the following frequencies: *AvrLm1* (2%), *AvrLm2* (0%), *AvrLm3* (0%), *AvrLm4* (26%), *AvrLm5-6* (100%), *AvrLm7* (100%), *AvrLm9* (2%), *AvrLepR1* (100%), *AvrLepR2* (94%), *AvrLepR3* (53%) (Figure 2.3).

2.3.3 Characterization of Avirulence Genes by PCR Differentials

All 95 isolates were screened using PCR to identify avirulence effector genes present in each fungal isolate. Isolates were screened using primers for seven genes known to confer avirulence: *AvrLm1, AvrLm2, AvrLm3, AvrLm4-7, AvrLm5, AvrLm6, AvrLm11* (Table 2.2). Presence of a PCR band at the correct size was indication that the avirulence gene was present. Frequency of each gene for the 95 isolates is as follows: *AvrLm1* (2%), *AvrLm2* (0%), *AvrLm3* (61%), *AvrLm4-7* (97%), *AvrLm5* (99%), *AvrLm6 (99%), AvrLm11 (91%)* (Figure 2.4). PCR with the primers for *AvrLm2* resulted in non-specific amplification with all *L. maculans* isolates. *AvrLm2* is an uncommon avirulence gene for isolates to carry and with all isolates in this study showing incorrect amplification size along with host plant differential screens being virulent, all isolates were deemed to not carry the avirulence effector gene (Balesdent et al., 2005; Rouxel et al., 2003; Nepal et al., 2014).

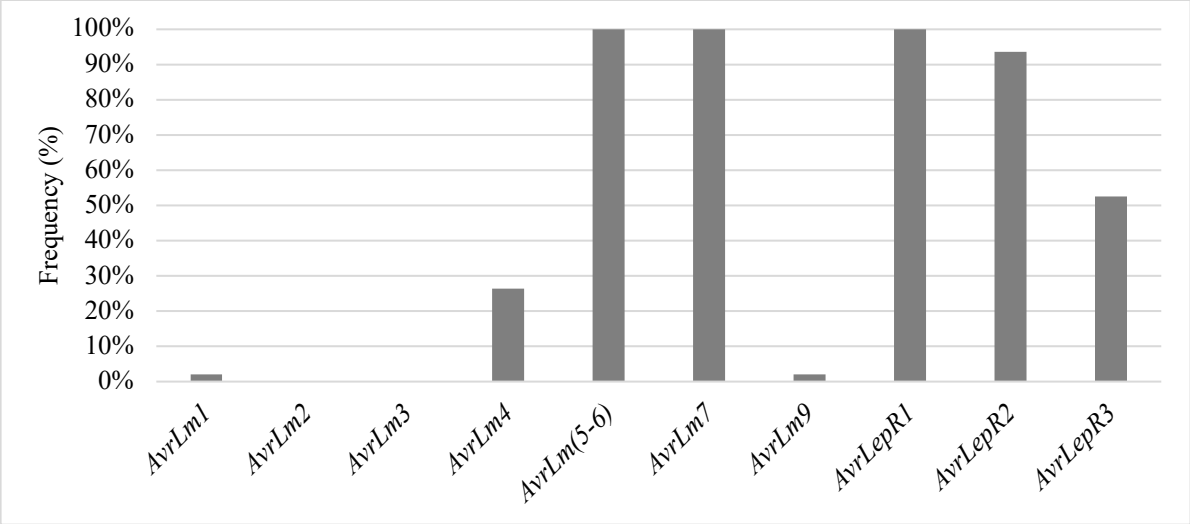

Figure 2.3: Frequency of *AvrLm* genes in Washington isolates identified through greenhouse host differentials.

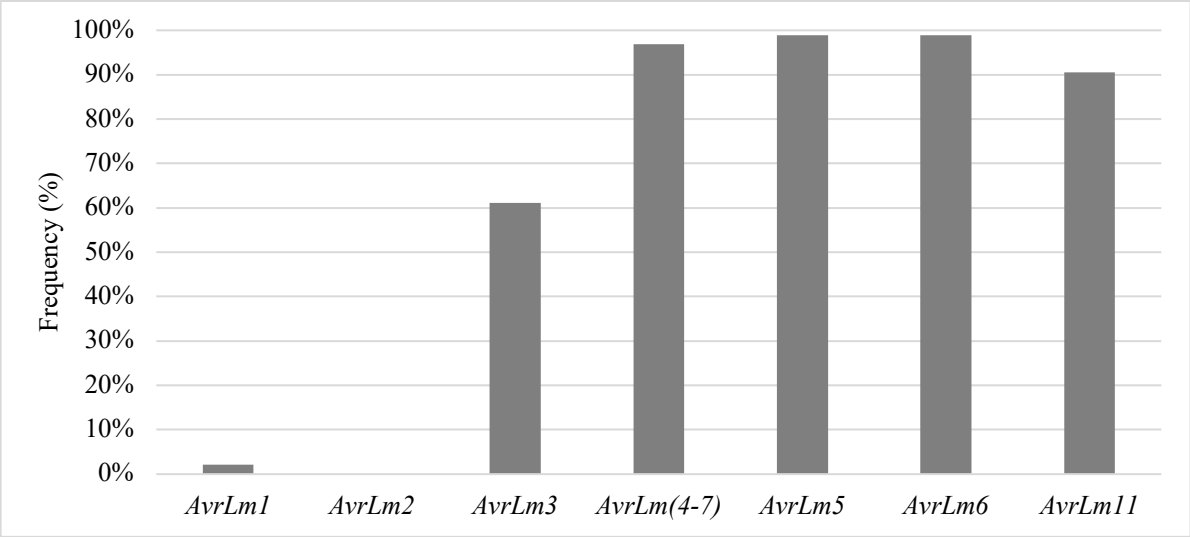

Figure 2.4: Frequency of *AvrLm* genes in Washington isolates determined by PCR.

2.3.4 Characterization of Race Structure in Eastern Washington

AvrLm gene expression can be masked by other *AvrLm* genes when present, requiring use of greenhouse host differentials and PCR differentials to determine presence of individual *AvrLm* genes. Combined PCR and greenhouse host differential *AvrLm* gene frequencies for the entire collection are as follows: *AvrLm1* (2%), *AvrLm2* (0%), *AvrLm3* (62%), *AvrLm4*

(27%), *AvrLm5* (100%), *AvrLm6* (100%), *AvrLm7* (100%), *AvrLm9* (2%), *AvrLm11* (92%), *AvrLepR1* (100%), *AvrLepR2* (94%), *AvrLepR3* (54%) (Figure 2.5).

Dividing the entire collection into counties that isolates were collected from resulted in 49 isolates from Adams County, 21 isolates from Garfield County, 14 isolates from Lincoln County, and 5 isolates from Spokane County. In addition to the 4 eastern Washington counties, 6 isolates from Latah County, Idaho are included in this collection. Overall, *AvrLm* gene frequencies for each county subpopulation are similar to each other, with a few exceptions (Figure 2.6). *AvrLm5, AvrLm6, AvrLm7,* and *AvrLepR1* are present within each county at a frequency of 100%. Garfield, Lincoln, and Spokane County isolates all possessed *AvrLm11* while lower frequencies were observed in the Latah County subpopulation (67%) and Adams County subpopulation (88%). *AvrLepR2* was also observed at high frequencies, with all subpopulations consisting of 83% to 100% of the isolates possessing the gene.

Lower frequencies of *AvrLm* genes were also observed. *AvrLepR3* was present in each county ranging from 47% to 67% of all isolates possessing the gene. *AvrLm2* was absent from all counties, as was *AvrLm9* except for 2% of the isolates from Adams County and 7% of the isolates from Lincoln County possessing *AvrLm9*. Latah county, located in northern Idaho, is the only subpopulation of isolates possessing *AvrLm1* (33%). The Latah County subpopulation possessed higher frequencies of *AvrLm3* (83%) and *AvrLm4* (67%) than the eastern Washington counties in which 50% to 63% of the isolates possessed *AvrLm3* and 20% to 33% of the isolates possessed *AvrLm4*.

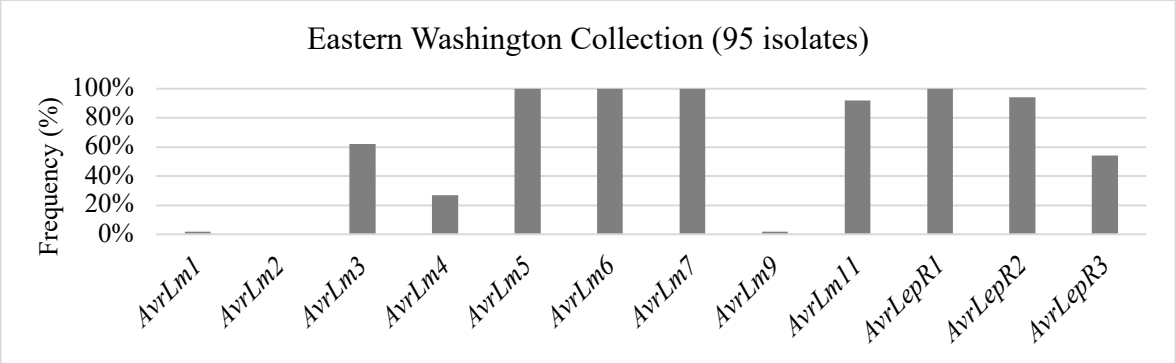

Figure 2.5: Eastern Washington collection gene frequency of *L. maculans AvrLm* genes using combined data from host differentials and *AvrLm*-specific primers.

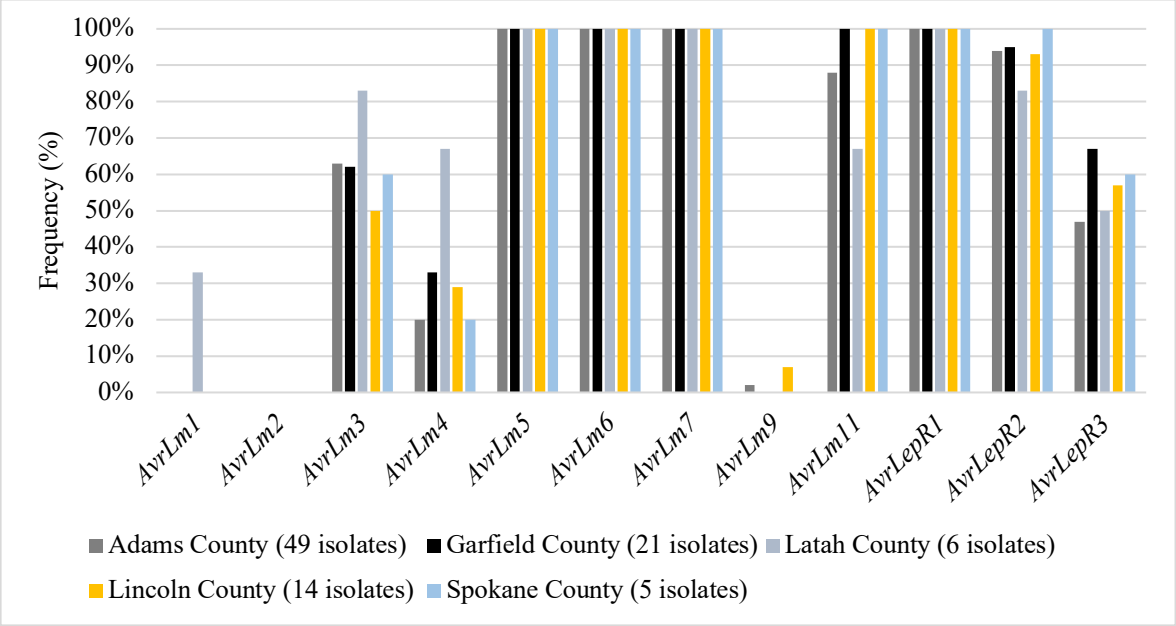

Figure 2.6: *AvrLm* gene frequencies of eastern Washington counties' *L. maculans* subpopulations using combined data from host differentials and *AvrLm*-specific primers.

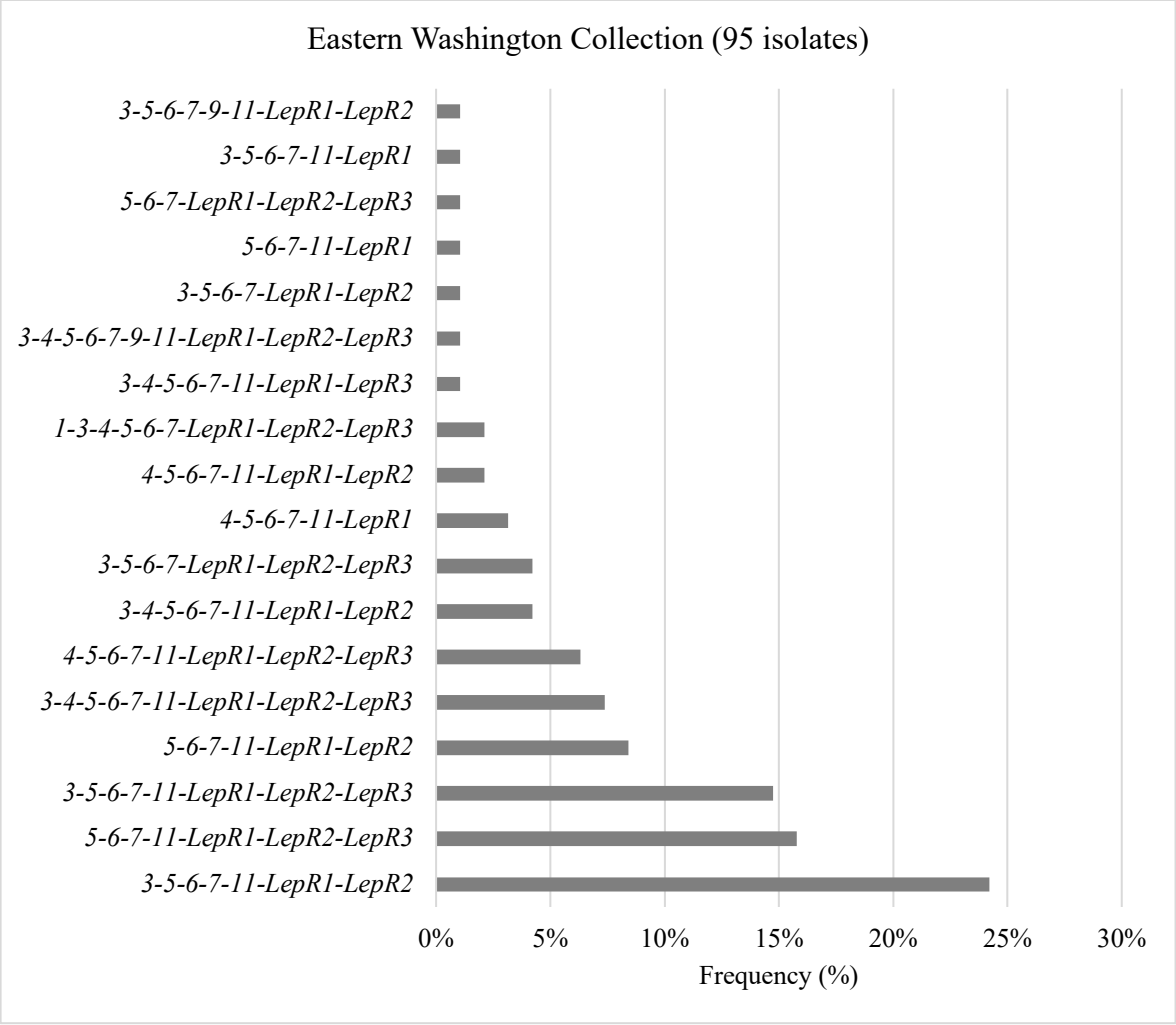

Figure 2.7: Characterized race structures and their frequencies within the eastern Washington *L. maculans* isolate collection.

2.3.5 Characterization of Mating Type

Each isolate was evaluated for the presence of both mating types *MAT1.1* and *MAT1.2* using PCR. Within the collection, 60 isolates (63%) possess *MAT1.1* while the remaining 35 isolates (37%) possess *MAT1.2.* Adams County had 49 isolates with 78% possessing *MAT1.1* and 22% possessing *MAT1.2.* Garfield County had 21 isolates with 29% possessing *MAT1.1* and 71% possessing *MAT1.2.* Lincoln County had 14 isolates with 71% possessing *MAT1.1* and 29% possessing *MAT1.2*. The six isolates from Latah County all possessed *MAT1.1* and all five isolates from Spokane County possessed *MAT1.2* (Figure 2.12).

Distribution of the two mating types within the entire eastern Washington collection was examined using chi-square analysis. When the distribution of 63% of the isolates possessing *MAT1.1* and 37% of isolates possessing *MAT1.2* was compared to a 1:1 distribution, chi-square analysis resulted in the eastern Washington isolate collection significantly deviating from a 1:1 distribution ($\chi^2_{1\ df}$ =6.76, p=0.009). The distribution of mating type within each county was also examined using chi-square analysis to identify variation from a 1:1 distribution. Each county resulted in the mating type distribution being significantly different from a 1:1 distribution with chi-square and p-values as follows: Adams County $\chi^2_{1\ df}$ =31.36, p=<0.00001; Garfield County $\chi^2_{1\ df}$ =17.64, p=0.00003; Latah County $\chi^2_{1\ df}$ =100.00, p=<0.00001; Lincoln County $\chi^2_{1\ df}$ =17.64, p=0.00003; and Spokane County $\chi^2_{1\ df}$ =100.00, p=<0.00001.

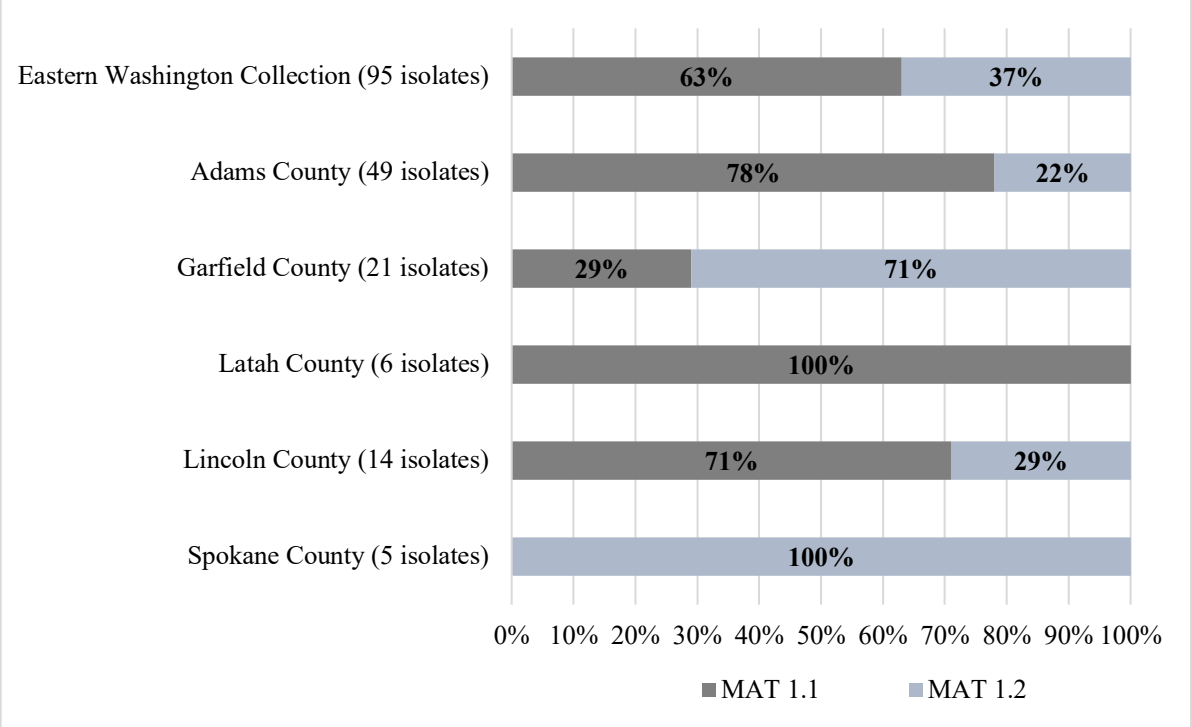

Figure 2.8: Frequency of mating type genes of *L. maculans* isolates in eastern Washington collection and county subpopulations.

2.4 Discussion

An important disease management tool for *L. maculans* in canola is through genetic resistance and is considered the most important aspect of breeding programs in Canada, Europe, and Australia (Long et al., 2011). The pathogen-host relationship between *L. maculans* and *Brassica napus* regarding qualitative resistance was not described until 1998. Previously, isolates were placed into pathogenicity groups (PG) (Mengistu et al., 1998). Using three cultivars, Westar, Quinta, and Glacier, they developed PG1, PG2, PG3, and PG4. PG1 included all nonaggressive isolates, PG2 were avirulent on Glacier and Quinta, PG3 were avirulent on Quinta, and PG4 isolates were completely virulent on Westar, Quinta, and Glacier. Ansan-Melayah et al. (1998) later identified the pathogen-host relationship was a gene-for-gene interaction and discovered Quinta carries the resistance gene *Rlm1* while Glacier carries *Rlm2* and the isolates within each PG carried the corresponding avirulence genes. Commercially available canola cultivars with qualitative resistance have been available since the early 1990s after widespread blackleg outbreaks occurred in the 1980s (Zhang and Fernando, 2017) and continues to be a strong component in blackleg management.

Determining population demographics of *L. maculans* for specific regions will provide insight as to which cultivars with specific *R*-genes should be considered when growers and breeders and making disease management decisions. Thus, *Leptosphaeria* species isolates were collected from eastern Washington to determine the regions pathogen population demographics. Through morphological characteristics, ITS sequencing and pathogenicity testing, isolates in the collection were confirmed to be *L. maculans* or *L. biglobosa.* Only one isolate collected from eastern Washington was confirmed to be *L. biglobosa* while the remainder were confirmed to be *L. maculans*, resulting in a collection of 95 isolates. *Leptosphaeria maculans* is the aggressive species, causing stem cankers and being more economically important than *L. biglobosa* (Fitt et al., 2006; West et al., 2001). *Leptosphaeria biglobosa* is only considered a minor problem because it does not cause severe symptoms, rather just minor lesions that do not lead to cankers (Somda et al., 1998). Because *L. maculans* is of greater importance, research concerning genetic resistance focuses on mechanisms between *L. maculans* rather than *L. biglobosa.* It has been suggested that *L. biglobosa* isolates do not carry avirulence effector genes, thus corresponding *R*-genes of canola cultivars are ineffective in preventing disease onset by this pathogen (Somda et al., 1998; Fitt et al., 2006).

The 95 *L. maculans* isolates were characterized through host plant differentials and PCR to determine race structure and mating type. All *AvrLm* genes were present in the collection at a frequency of 27% or higher, except for *AvrLm1* and *AvrLm9* at a frequency of 2% and *AvrLm2* absent from the collection (Figure 2.5). A similar distribution of *AvrLm* genes were present in each county. *AvrLm2* was absent from each county, *AvrLm1* was only present in Latah County (33%), *AvrLm9* was only present in isolates from Adams County (2%) and Lincoln County (7%), while the remaining *AvrLm* genes were present in each county at frequencies between 20 to 100%. The top three race structures in the collection were determined to be *AvrLm3-5-6-7-11-LepR1-LepR2* (24%), *AvrLm5-6-7-11-LepR1-LepR2-LepR3* (16%), *AvrLm3-5-6-7-11-LepR1-LepR2-LepR3* (15%) (Figure 2.6).

Both host plant differentials and PCR were needed to characterize the fungal isolates. The host set consists of only 11 *Rlm* genes while 18 *Rlm* genes have been characterized in *B. rapa, B. juncea,* and *B. napus* (Long et al., 2011). For example, *Rlm11* has been characterized in *B. rapa* (Balesdent et al., 2002; Balesdent et al., 2013; Long et al., 2011), but for this research, a cultivar containing *Rlm11* was unable to be obtained. Rather, to identify if isolates possess avirulence towards *Rlm11*, PCR primers created from the cloned *AvrLm11* gene were obtained (Balesdent et al., 2013).

Results of both differential sets were confirmed due to the presence of certain avirulence genes masking the expression of others. *Leptosphaeria maculans* isolates carrying *AvrLm7* will induce resistance responses in plants containing *Rlm7* or *Rlm4*, so it is referred to as *AvrLm4-7* (Parlange et al., 2009). Therefore, two cultivars, Jet Neuf (*Rlm4*) and 01.23.2.1 (*Rlm7*) were included in the greenhouse differential to identify if *AvrLm4* and/or *AvrLm7* was present in isolates of *L. maculans.* (Balesdent et al., 2005; Dilmaghani et al., 2009). *AvrLm3* expression is masked when *AvrLm4-7* is present in *L. maculans* (Plissonneau et al., 2016), therefore PCR screening for the presence of *AvrLm3* in the *L. maculans* isolate is required due to the host plant response being masked. When *Rlm3* and *AvrLm3* are present and interact with each other, along with the isolate containing *AvrLm4-7*, a susceptible response would occur. This occurred often in the host plant differentials, due to all isolates being avirulent towards 01.23.2.1 (*Rlm7*) and virulent towards the cultivar 02.22.2.1 (*Rlm3*) (Balesdent et al., 2005; Figure 2.3).

Ghanbarnia et al. (2018) discovered a similar masking interaction when *AvrLm4-7* and *AvrLm9* are present in the same isolate of *L. maculans*. If *AvrLm9* is present in *L. maculans* and the cultivar contains *Rlm9*, and *AvrLm4-7* is present, there will be a susceptible response on the host. When isolates in this study were screened in the greenhouse, the frequency of *AvrLm9* present in the *L. maculans* isolates was 2%. Since many of the *L. maculans* isolates in this study contain *AvrLm7*, there may be a discrepancy in the frequency of *AvrLm9* and isolates should be screened for the presence of *AvrLm9* using PCR. However, primer sets for *AvrLm9* are currently not available (Liu et al., 2020). Future research should include screening the *L. maculans* isolates in the collection for the *AvrLm9* gene via PCR once primer sets are available to confirm the host plant differential results.

Overall, the highest frequency of avirulent effector genes present in the eastern Washington *L. maculans* pathogen population are *AvrLm5* (100%)*, AvrLm6* (100%)*, AvrLm7* (100%)*, AvrLm11* (92%)*, and AvrLepR1* (100%). *L. maculans* isolates collected from northern Idaho were recently characterized (Pickard, 2018). When compared to isolates characterized in this research, it is observed both collections show similar trends. Within each region, *AvrLm5*, *AvrLm6, AvrLm7, AvrLm11, and AvrLepR1* are the most frequent avirulence genes present with 94 to 100% of the population possessing these genes. *AvrLm1*, *AvrLm2*, and *AvrLm9* are either absent or only present at a frequency less than 2%. *Leptosphaeria maculans* isolates collected and characterized from Alberta, Canada had a similar frequency of avirulence genes as observed in this study. Specifically, *AvrLm1* was absent and the highest frequency of avirulence genes were *AvrLm4-7* and *AvrLm6* (Rong et al., 2015). Additionally, isolates from North Dakota characterized by Nepal et al. (2014) resulted in more than 60% of the population placed into PG4 while the remainder were placed into PG1, PG2, and PG3, indicating that *AvrLm1* and *AvrLm2* are absent.

Previously noted are the similar frequencies of avirulent genes present between *L. maculans* isolates from eastern Washington/northern Idaho and those collected in Alberta, Canada (Rong et al., 2015). However, that is where the similarities between this region and Canadian isolates stop as isolates from this region are more closely related to isolates in Europe. European isolates collected from the UK, Germany, France, Portugal, and Poland do not carry *AvrLm2*, rarely carry *AvrLm3* and *AvrLm9*, and have a high frequency of *AvrLm6* and *AvrLm7* (Balesdent et al., 2005) present in the genomes of the *L. maculans* isolates, just

as over 50% of the population in this region is comprised. *Leptosphaeria maculans* isolates from Canada are thus genetically similar to those found in Australia, where nearly all avirulence genes are present in one combination or another (Balesdent et al., 2005).

Leptosphaeria maculans reproduces asexually and sexually, with sexual reproduction occurring via ascospores giving rise to race structure variation (Cozijnsen and Howlett, 2003). To sexually reproduce, two ascospores need opposite mating types (Cozijnsen and Howlett, 2003). Of the 95 isolates from eastern Washington, 63% possessed *MAT1.1* while the remaining 37% possess *MAT1.2.* Although both mating types are present, Chi-square analysis was used to examine if the distribution of the two mating types differed from a 1:1 ratio. Results of the chi-square analysis indicated the distribution of mating types significantly differed from a 1:1 ratio ($\chi^2_{1\ df}$ =6.76, p=0.009). However, if more isolates were included in the eastern Washington collection, the distribution of mating type may be closer to a 1:1 ratio, as observed in the northern Idaho pathogen population. Mating type alleles were more evenly divided amongst the northern Idaho *L. maculans* isolates with 55% possessing *MAT1.1* and 45% possessing *MAT1.2* (Pickard, 2018).

Both mating types were observed in eastern Washington isolate subpopulations collected from Adams, Garfield, and Lincoln County. Adams County (49 isolates) and Lincoln County (14 isolates) resulted in similar frequencies with 78% and 71% of isolates possessing *MAT1.1* and 22% and 29% of isolates possessing *MAT1.2*, respectively. Garfield County (21 isolates) saw an opposite distribution with 29% of isolates possessing *MAT1.1* and 71% of isolates possessing *MAT1.2*. The 6 isolates from Latah County all possess *MAT1.1* and all 5 isolates from Spokane county possess *MAT1.2*. Similar distributions of each mating type for isolates collected from eastern Washington and northern Idaho indicate the capability for sexual reproduction across the entire region. Ascospores can travel 25 meters or more from initial inoculum sources (Guo and Fernando, 2005), easily allowing the pathogen to travel extensively throughout the region. Along with mating type similarities, the eastern Washington and northern Idaho collections have similar frequencies of avirulence genes and are found on canola grown under the same climatic conditions and growing practices.

No matter how similar the frequency of avirulence genes is within pathogen populations around the world are, a noticeable decrease or absence in populations carrying *AvrLm1* and *AvrLm2* is easily detected. Surveys completed in western Canada show the

decline in *AvrLm1* frequency from 46% in 1997 to 13.7% in 2011 and *AvrLm2* from 96.6% in 1997 to 80.56% in 2011 (Kutcher et al., 2010; Liban et al., 2016). North Dakota isolates characterized by Nepal et al. (2014) showed 67% of the population can be classified in PG4 while the remainder fall into PG1, PG2, and PG3, suggesting that the race structure in North Dakota does not carry *AvrLm1* or *AvrLm2*.

Genetic resistance, although an effective method for overall management of blackleg, has a disadvantage. Single gene resistance leads to strong selection pressure on *L. maculans* populations (Zhang et al., 2016) leading to low and decreasing frequencies of avirulence genes as mentioned previously. Historical resistance breakdown events explain why genes eventually become absent within the pathogen population. Resistant cultivars became commercially available in the 1990s with *Rlm1* and *Rlm2* as the first resistance genes identified and incorporated into in *B. napus* cultivars (Mengistu et al., 1998; Ansan-Melayah et al., 1998). Continual use of these two resistance genes provided constant selection pressure on the pathogen, and through genetic recombination, *L. maculans* eventually overcame *Rlm1* and *Rlm2* in France and Canada (Rouxel et al., 2003; Zhang et al., 2016). Further work completed by Zhang et al. (2016) showed the direct relationship between extensive use of *Rlm3* in Canadian commercial cultivars and the nearly absent *AvrLm3* within the pathogen population. *Rlm3* was the first resistance gene introduced after identification of blackleg in Canada in the 1970 and continuous use to reduce disease outbreaks led to resistance breakdown (Zhang et al., 2016). *RlmS* was identified in *B. rapa* subs. *sylvestris* and this resistance gene was introgressed into *B. napus* cultivars and made commercially available first in Australia (Van de Wouw et al., 2009). Australia experienced similar resistance breakdown of *AvrLmS* over a span of less than 3 years due to consistent use of spring canola cultivars only carrying this resistance gene (Sprague et al., 2006).

While single gene resistance may be easily overcome, introducing multiple resistance genes into a single cultivar, otherwise known as pyramiding, provides more durable resistance (Zhang and Fernando, 2017). Rotation and pyramiding of resistance genes in commercially available cultivars are ideal, however, this can also be a challenge due to the requirement of needing to know the population structure and evolutional pressure (Van de Wouw et al., 2021; Zhang and Fernando, 2017). By conducting population surveys of the region, breeders and growers will have greater understanding of pathogen genetics and the ability to select for

cultivars conferring the greatest level of resistance. This will lead to fewer resistance breakdown events and ensure resistance genes are not overused which would lead to high selection pressure (Balesdent et al., 2005; Rouxel et al., 2003).

Enhancing overall genetic resistance against *L. maculans* can be achieved through a combination of qualitative and quantitative resistance (Zhang and Fernando, 2017; Brun et al., 2010). Through the effort of Brun et al. (2010) *Rlm6* was introgressed into two winter canola varieties, Darmor which carries quantitative resistance and Eurol which is susceptible. After a 5-year study, quantitative resistance in Darmor reduced pseudothecia formation while increasing the durability of *Rlm6*, thus reducing leaf lesion occurrence. In contrast, *Rlm6* was ineffective in preventing infection after 3 years. Overall, greater disease control and crop production can be provided through the combined genetic resistance. Although the combination of both quantitative and qualitative resistance is ideal, it is a difficult method to achieve through traditional breeding methods due to *R*-genes usually masking the effects of quantitative resistance (McDonald, 2010).

Canola acreage in Washington consists of 63% spring cultivars and 37% winter cultivars while Idaho acreage consists of 73% spring cultivars and 27% winter cultivars (Jim Davis, *personal communication*; PNW Canola Association, 2020). Of all winter canola grown in the region, roughly two-thirds of the cultivars are from Rubisco Seeds (Philpot, KY, USA) and are classified as having good to excellent blackleg resistance (Rubisco Seeds, 2021). The specific resistance genes in each cultivar are unavailable, except for the cultivar Kicker, which carries *RlmS*. Growers and breeders within the area do not know the specific resistance genes carried in winter canola cultivars, if any, making it difficult to determine how the pathogen population interacts with winter canola regarding genetic resistance.

RlmS was identified in *B. rapa* subs. *sylvestris* and this resistance gene was introgressed into *B. napus* cultivars and made commercially available first in Australia (Van de Wouw et al., 2009). The corresponding avirulence gene is present in *L. maculans* however, for this research a cultivar carrying *RlmS* was not obtained. Therefore, the pathogen population in this region may carry *AvrLmS* and the knowledge will not be available until further host plant differentials are conducted.

As for spring canola, about half of the acres planted consist of Croplan cultivars by WinField United, all with one or multiple of the following resistance genes: *Rlm1*, *Rlm3*,

LepR3, *RlmS* (Jim Davis, *personal communication*; WinField United, 2021). Based on the frequency of avirulence alleles identified in eastern Washington and northern Idaho isolates, growing spring cultivars carrying *Rlm1* and *Rlm3* will provide little to no resistance. *Leptosphaeria maculans* isolates in the region do not carry *AvrLm1* and if *AvrLm3* is present, the resistance response is null due to the entire population carrying *AvrLm7*. As for cultivars carrying *LepR3*, there will be moderate resistance to the region's population as the frequency in the eastern Washington collection is 54% and 40% in northern Idaho's *L. maculans* population.

Eighteen *Rlm* genes have been characterized and this project was only able to identify 12 corresponding *AvrLm* genes through host plant and PCR differentials (Long et al., 2011). Therefore, final race structure and pathogen population frequencies for eastern Washington and northern Idaho are incomplete. Future differentials need to be completed to solidify recommendations to growers regarding which cultivars to plant, especially for winter canola genetic resistance. In regard to this research, commercial cultivars carrying *Rlm5*, *Rlm6*, *Rlm7*, *Rlm11,* and *LepR1* will provide the greatest level of resistance against *L. maculans* in eastern Washington and northern Idaho until further differential screens can occur.

Sustainable production of quality crops is the top priority of growers and researchers. With crop production comes disease and methods of control are always evolving to provide optimal management strategies for specific regions. To ensure that genetic resistance remains the most effective method of blackleg disease control, pathogen population surveys should regularly be conducted along with identifying the common canola cultivars grown in a region and the resistance genes they carry. Obtaining this information will aid breeding programs and ensure growers have access to the best cultivars for blackleg resistance, thus resulting in reduction of blackleg disease across the region.

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Chapter 3: Determining Optimal Foliar Fungicide Application Timing for Management of Blackleg Disease of Winter Canola (*Brassica napus*) in Northern Idaho

3.1 Introduction

Blackleg disease, caused by the fungal pathogen *Leptosphaeria maculans,* is the most economically important disease of canola (*Brassica napus*) worldwide. Infection caused by wind-blown ascospores, and rain splashed conidia result in leaf and stem lesions. Severe infection will result in stem cankers at the base of the plant, causing stem girdling and reduction of seed pod fill, reducing yield (Bailey et al., 2003; Somda et al., 1998). All above ground parts of the plant can become infected, including seed pods and seed, leading to the potential for introduction of *L. maculans* into other regions and providing an additional source of inoculum via infected seed (Rimmer et al., 2007).

Methods of disease management include genetic resistance, cultural practices, planting clean seed, and use of fungicides as seed treatments and foliar applications. Genetic resistance is the most effective form of control against blackleg; however, resistance breakdown has occurred in Canada, Australia, and Europe (Rouxel et al., 2003; Zhang et al., 2016; Sprague et al., 2006) making use of other control methods a necessity. Fungicides labeled for foliar use and seed treatments against blackleg in canola have proven effective, especially when used in conjunction with low to moderately resistant cultivars (Khangura and Barbetti, 2002; Khangura and Barbetti, 2004; Marcroft and Potter, 2008; Fraser et al., 2020). Active ingredients that are effective against *L. maculans* include triazoles, methyl benzimidazole carbamates, and Qo inhibitors (Eckert et al., 2010; Fraser et al., 2020).

All canola seed is required to be treated with a fungicide labeled for use against seed-borne blackleg. Fluquinconazole and flutriafol are labeled for use in Australia and have shown effective reduction of disease and plant mortality (Khangura and Barbetti, 2004; Khangura and Barbetti, 2001; Marcroft and Potter, 2008). Foliar applications of flusilazole, tebuconazole, carbendazim, and benomyl were effective in preventing blackleg infection caused by conidia rather than ascospores, while flusilazole and tebuconazole applications resulted in the lowest disease incidence observed in winter canola (Eckert et al., 2010). Further work conducted on winter canola in Europe resulted in successful reduction of disease incidence when triazoles and methyl benzimidazole carbamates are applied as a mixture. Single fall applications of difenoconazole + carbendazim (West et al., 2002) and flusilazole + carbendazim (Huang et al.,

2011; Steed et al. 2007) resulted in low disease incidence. Other fungicides available for foliar use include propiconazole and pyraclostrobin + fluxapyroxad, both of which have proven effective in disease reduction in spring canola (Bailey et al., 2000; Fraser et al., 2020).

Weather conditions contribute to *L. maculans* development and spore dispersal, causing initial infection to be regionally dependent (West et al., 2001). Foliar fungicide applications act as a barrier for the plant, preventing germinating conidia and ascospores from infecting. Once the pathogen reaches the stem, fungicides are less effective in preventing further disease symptoms, thus it is important to identify the ideal time to make foliar fungicide applications (Zhang and Fernando, 2017). First identified in northern Idaho in 2011, blackleg is a new disease to the region (Agostini et al., 2013; Paulitz et al., 2017). Growers and researchers have limited knowledge concerning pathogen development and initial infection, thus the ideal time to spray fungicides on winter canola grown in the region is unknown.

Fungicides have successfully been used in control of blackleg on winter canola in Europe and spring canola in Canada and Australia. To successfully integrate fungicides into northern Idaho management strategies for blackleg disease control in winter canola, optimal spray timing needs to be identified along with determining further impacts caused by fungicide use on disease incidence and severity as well as crop yield and seed quality. Therefore, winter canola field trials in northern Idaho were established with the following objectives 1) determine the effect different timings of foliar fungicide applications have on disease incidence and severity in winter canola; 2) identify if fungicide seed treatments have an impact on disease incidence and severity; and 3) determine if fungicides impact winter canola seed yield and seed quality.

3.2 Materials and Methods

3.2.1 Locations

Winter canola field trials were planted at three locations in northern Idaho during the 2019-2020 and 2020-2021 growing seasons. In 2019-2020, the locations included: the University of Idaho Parker Plant Science Farm in Moscow, ID (46.7263, -116.9571), the University of Idaho Kambitsch Farm in Genesee, ID (46.5922, -116.9461), and the Camas Prairie near Grangeville, ID (45.933028, -116.9461). Each location followed a winter wheat – fallow – winter canola rotation. The 2020-2021 locations included: the University of Idaho Parker Plant Science Farm Arboretum in Moscow, ID (46.7160, -117.0215), the University of

Idaho Kambitsch Farm in Genesee, ID (46.5856, -116.9503), and the Camas Prairie near Nezperce, ID (46.2379, -116.2555). Each location followed a winter wheat – fallow – winter canola rotation.

3.2.2 Cultivars

The field trials consisted of two winter canola cultivars with different levels of resistance to blackleg. Mercedes conventional hybrid winter canola (non-GMO) was obtained from Rubisco Seeds (Rubisco Seeds Hybrid Canola, Philpot, KY, USA) and has "good" resistance to blackleg. The second cultivar, Amanda, is a winter canola cultivar from the University of Idaho (Moscow, ID, USA) that is susceptible to blackleg. The seeding rate of winter canola planted was comparable to that used by commercial growers being between 741,000 to 1,235,000 seeds per hectare (Jim Davis*, personal communication*). For this research, the field trials were planted at a higher seeding rate to ensure ideal emergence due to a germination rate of approximately 94% and to provide a thicker canopy cover that allows for a more conducive environment for the onset of blackleg disease. Mercedes was seeded at 4.5 g per plot, or 1,512,272 seeds per hectare, while Amanda was seeded at a rate of 5.0 g per plot, or 1,461,865 seeds per hectare.

3.2.3 Fungicide Treatments

To identify the impact of fungicide seed treatments on blackleg incidence and severity, half of each cultivar block was treated with Helix Vibrance (Syngenta, Greensboro, NC, USA). Helix Vibrance active ingredient (a.i.) and percentages are as follows: thiamethoxam (20.7%; Group 4 insecticide), difenoconazole (1.25%; Group 3 fungicide), mefenoxam (0.40%; Group 4 fungicide), fludioxonil (0.13%; Group 12 fungicide), and sedaxane (0.26%; Group 7 fungicide) for a total of 22.3% a.i. applied at the label rate of 1.5 liters per 100 kg of seed. Due to potential damage of seedlings caused by flea beetles, the remaining half block of each cultivar was treated with clothianidin (Group 4 insecticide) and served as the control. Concentrated clothianidin (96% a.i.) was applied to achieve an equivalent rate of clothianidin to that in Prosper Evergol (Bayer Crop Science, Calgary, AB, USA), a fungicide and insecticide seed treatment with clothianidin as one of the active ingredients. The label rate is 1.4 liters per 100 kg of seed, therefore the concentrated clothianidin was diluted at a 1:3 rate using DI water to achieve the proper label rate and active ingredient concentration of 22.3%.

Although two different insecticides were used, previous field trials comparing the effectiveness of flea beetle control in seedling canola showed equal control (Davis and Brown*, unpublished*).

Foliar fungicide applications consisted of a fall only, spring only, combination of fall and spring, or no application. Priaxor® Xemium® Brand Fungicide (BASF Corporation, NC, USA) containing active ingredients fluxapyroxad (14.33%; Group 7 fungicide) and pyraclostrobin (28.58%; Group 11 fungicide) was used for the foliar applications at the labeled rate of 600 ml/ha with the maximum amount applied per year of 1.2 liters/ha. A total solution of 265 liters/ha was applied using a CO_2 backpack sprayer for all foliar fungicide applications. Fall and spring foliar fungicide application for all three locations during the 2019-2020 and 2020-2021 growing seasons occurred in early October and early to mid-April, respectively, while plants were in the rosette growth stage, as the seedling to six-leaf stage is the most vulnerable growth stage of the plant (Bailey et al., 2003).

3.2.4 Planting and Experimental Design

The Moscow and Genesee field trials for both years were planted using a six-row, small-plot drill with a Hege seed distribution cone and John Deere double disk row openers with packer wheels on eighteen-centimeter spacings. The Camas Prairie locations (Grangeville and Nezperce) were planted using a small-plot, direct seed drill with a Hege seed distribution cone and five Flexi-Coil Stealth paired-row openers on twenty-five-centimeter row spacings with eight-centimeter-wide packer wheels. All plot dimensions were 1.5 m by 6.1 m at planting, with 1.5 m by 4.6 m retained after alleys between plots were sprayed.

The trials for the 2019-2020 growing season were seeded on 14 August 2019 and the trials of the 2020-2021 growing season were seeded on 25 August 2020. The 2019-2020 trial at Genesee was replanted on 11 September 2019 due to high temperatures scorching most seedlings from the first planting. Nearly half of the plots at the 2020-2021 trial in Genesee were lost due to herbicide residue in the soil. A Clearfield cultivar was planted as border plots on 29 September 2020 to protect the remaining plots.

The experimental design of the field trials is a split-plot within a randomized complete block. The main plot consisted of two winter canola cultivars, Mercedes and Amanda, blocked across four replications. The seed treatment (clothianidin or Helix Vibrance) and foliar fungicide application timing (none, fall, spring, both) were randomly assigned to the sub-plots within the main plot.

3.2.5 General Crop Management Practices

The University of Idaho Plant Science farms at both Moscow and Genesee were managed using standard tillage practices. Ground preparation consists of fall chisel plowing, application of fertilizer and cultivation in the spring, followed by summer rod weeding and herbicide application (Roundup; a.i. glyphosate; Monsanto, St. Louis, MO, USA; 2.4 liters/ha) to control weeds as needed and shallow cultivation immediately prior to seeding (Jim Davis*, personal communication*).

Fertilizer applications at the Genesee location were made 30 April 2019 for the 2019-2020 season and again on 10 April 2020 for the 2020-2021 growing season. Both applications were made at a rate of 113-34-0-23 kg/ha (N-P-K-S) using a ripper shooter with shanks on 31 cm spacing (The McGregor Co., Colfax, WA, USA). The fertilizer sources included anhydrous ammonia (NH_3, 82% N), ammonium polyphosphate (11-37-0), and ammonium thiosulfate (12-0-0-13) (Brad Bull*, personal communication*). Fertilizer was applied in 2019 and 2020 for the two seasons of Moscow trials at a rate of 109-35-0-23 kg/ha (N-P-K-S) using a 50:50 blend of ammonium phosphate sulphate (16-20-0-13) and urea (46-0-0-0) (Roy Patten*, personal communication*).

Cooperators at the Grangeville location use standard tillage practices. Ground preparation for the 2019-2020 season included fall moldboard plowing, spring and summer cultivation for weed control followed by herbicide application (Roundup; a.i. glyphosate; Monsanto, St. Louis, MO, USA; 2.1 liters/ha) prior to planting. Fertilizer was applied (17 July 2019) prior to seeding at a rate of 151-25-0-29 kg/ha (N-P-K-S) using anhydrous ammonia (NH_3, 82% N), ammonium polyphosphate (11-37-0), ammonium thiosulfate (12-0-0-13), and M-Struct (8-24-0; The McGregor Co., Colfax, WA, USA). Nitrogen stabilizer (N-Serve 24; Corteva agriscience, Indianapolis, IN, USA) was included in the fertilizer application at a rate of 1.8 liters/ha. The surrounding crop was seeded on 23 August 2019 using the canola cultivar Mercedes. An aerial application of herbicide and fungicide was made 18 May 2020 for control of cabbage seed pod weevil and blackleg consisting of the following mixture: Bifen 2 Ag Gold (a.i. bifenthrin; WinField, St. Paul, MN, USA) at a rate of 195 ml/ha; Max-In Boron (WinField, St. Paul, MN, USA) at a rate of 1.2 liters/ha; and Tilt (a.i. propiconazole; Syngenta, Greensboro, NC, USA) at a rate of 0.3 liters/ha (Mark Frei, *personal communication*).

The Nezperce cooperators follow minimum tillage practices. Ground preparation for the 2020-2021 growing season consisted of three applications of herbicide (Roundup; a.i. glyphosate; Monsanto, St. Louis, MO, USA) in the spring, followed by cultivation using a spike tooth harrow. Fertilizer at a rate of 34-39-0-17 kg/ha (N-P-K-S) was applied (25 June 2020) prior to seeding and an additional round of harrowing followed. The surrounding crop was seeded on 6 August 2020, using the canola cultivar Mercedes, and the field's borders were aerial sprayed with insecticide on 26 August 2020. An application of 124 kg/ha nitrogen fertilizer was applied on 4 November 2020 (Chris Riggers*, personal communication*).

Alleys between plots within the trials were made using 1.2 liters of Roundup per hectare (a.i. glyphosate; Monsanto, St. Louis, MO, USA) mixed with 2.4 liters of AgriStar® 2,4-D Amine 4 per hectare (a.i. 2,4-D amine; Albaugh LLC, Ankeny, IA, USA), resulting in a total solution of 152 liters/ha. Other pesticides were not applied to the Moscow and Genesee 2019-2020 trials; however, they were applied at the Genesee 2020-2021 trial. Warrior II (a.i. lambda-cyhalothrin; Syngenta, Greensboro, NC, USA) at a rate of 143 ml/ha was applied for control of flea beetles on 12 September 2020. Herbicide was sprayed on 6 October 2020 at the same location for control of grassy and thistle weeds. Herbicide used was Assure II (a.i. quizalofop-p-ethyl; DuPont, Wilmington, DE, USA) at a rate of 750 ml/ha, mixed with Stinger (a.i. clopyralid; Dow AgroSciences LLC, Indianapolis, IN, USA) at a rate of 300 ml/ha. The same herbicide application was made on 6 October 2020 at the Nezperce location (Jim Davis, *personal communication*).

3.2.6 Data Collection and Harvest

Data collection at all three locations included fall and spring plant stand counts, flowering date, mature plant height, blackleg disease incidence and severity, seed yield, estimated oil seed content, and estimated protein content of the seed.

Fall and spring plant stand counts were completed before the foliar fungicide applications by recording the number of seedlings present in a 1 m long section of the plot, repeated 3 times for an average stand count per plot. The number of plants per square meter was calculated to determine average plant stand. Fall stand counts determine the number of seedlings that germinated while spring stand counts determine the number of plants that survived the winter. Flowering notes consist of recording the number of days after January 1

that over 75% of the plants within each plot have fully flowered. Plant height was completed by recoding the average plant height in centimeters of each plot directly after flower petal drop.

Disease incidence and severity ratings were completed by following a modified protocol established by BASF (BASF Canada Inc., 2020) in which 15 stems were randomly selected and removed from each plot. Pycnidiospores generally germinate under moist conditions after 12 to 24 hours (Hall, 1992), allowing the identification of blackleg to be confirmed in a lab setting. Collected stems observed to have blackleg lesions were placed on filter paper moistened with sterile DI water in a petri dish. Stem pieces were then observed under a dissection microscope each day for the appearance of blackleg conidial sporulation. If present, the stem was deemed to be infected with blackleg. The number of stems confirmed to be infected with blackleg was used to calculate the percent disease incidence for each plot. The same 15 stems were cut at the soil line and rated for blackleg disease severity using the blackleg field rating scale (Figure 3.1) provided by the Canola Council of Canada (2017). Average severity rating was calculated for each plot. Additionally, stems observed to possess pith blackening at the cross section were used to determine the percentage of stems with tissue necrosis. Stem samples were collected the day before swathing at the Moscow and Genesee locations for both growing seasons, while the Camas Prairie locations each year had stem samples collected during harvest.

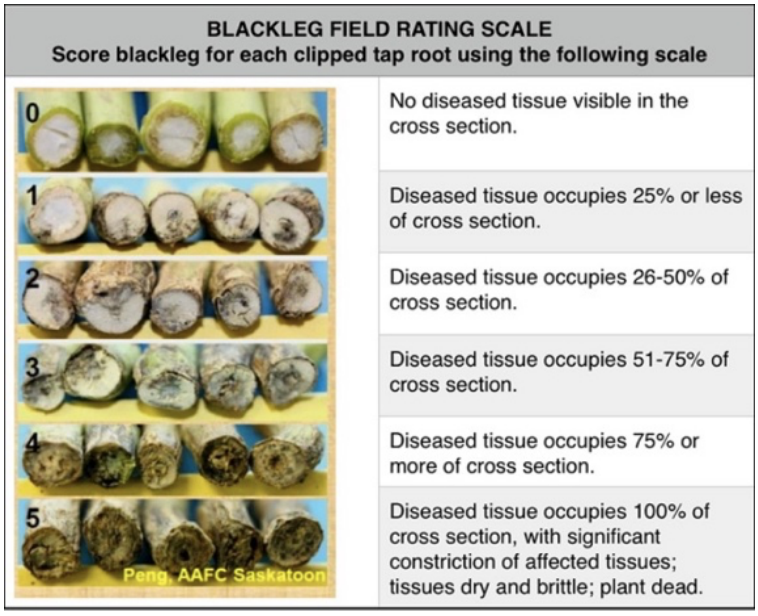

Figure 3.1: Blackleg field rating scale used for disease severity ratings (Canola Council of Canada, 2017).

For both growing seasons, Moscow and Genesee trials were swathed prior to harvest to allow for adequate seed pod dryness using a plot swather with 1.5 m header designed for canola (Swift Machine & Welding Ltd, Saskatchewan, Canada). A Zürn 110 plot combine harvester (Zürn Harvesting GmbH & Co. KG, Germany) was used to harvest plots at each location. For the 2019-2020 season, Moscow was swathed on 10 July 2020 and threshed on 16 July 2020. Genesee was swathed on 22 July 2020 and threshed on 29 July 2020. The Grangeville trial was direct cut harvested on 7 August 2020. For the 2020-2021 growing season, Moscow was swathed on 1 July 2021 and threshed on 9 July 2021. Genesee was swathed on 10 July 2021 and threshed on 19 July 2021. The Nezperce trial was direct cut harvested on 27 July 2021. Collected seed was placed in drying chambers for 1 week before processing.

Dried seed was weighed to determine the yield, in grams, of each plot and cleaned for subsample collection. Subsamples were placed into drying chambers for a week to ensure all seed was roughly at the same dry matter percentage before conducting seed quality tests. Subsamples were removed from drying chambers and analyzed to determine the estimated dry matter, oil, and protein content using a XDS near infrared spectrophotometer (FOSS, Sweden).

3.2.7 Data Analysis

Collected data was used for a combined analysis of treatment on response variables. Data was analyzed using the general linear model (GLM) procedure in SAS (SAS studio, online, 3.8 enterprise edition) to estimate any missing values in the analysis of variance. Within the analysis of variance, the combined effect of cultivars and disease treatments using orthogonal contrasts were as follows: 1) difference between cultivars; 2) difference between seed treatment and no seed treatment; 3) difference between no spray and spray; 4) difference between one spray and two sprays; 5) difference between fall spray and spring spray; 6) interaction between contrasts 2 and 3; 7) interaction between contrast 2 and 4; 8) interaction between contrast 2 and 5; 9) interaction between contrast 1 and 2; 10) interaction between contrast 1 and 3; 11) interaction between contrast 1 and 4; 12) interaction between contrast 1 and 5; 13) interaction between contrast 1 and 6; 14) interaction between contrast 1 and 7; and 15) interaction between contrast 1 and 8, each with one degree of freedom. Mean sum of squares are reported along with the following *p*-values: $0.1 > p > 0.05$; $0.05 > p > 0.01$; $0.01 > p > 0.001$; and $p < 0.001$.

Analysis of variance (ANOVA) was conducted over each individual location using GLM procedure in SAS. Significant differences between treatment means were identified using Fisher's least significant difference with an alpha value of 0.05. All statistical analysis and mean separation are in Appendix B.

3.3 Results

3.3.1 Plant Stand, Height, and Flowering

Fall stand counts were conducted to determine the number of seeds that germinated and emerged after planting in the fall to ensure adequate plant stand within plots. Cultivar significantly impacted fall plant stand and accounts for 44.6% of all variation (Table 3.1). Amanda fall plant stand was 74 plants/m^2 while Mercedes fall plant stand was 65 plants/m^2. Applying foliar fungicides compared to no application had a moderate impact on fall plant stand, with a *p*-value of 0.051. No application of fungicide resulted in a lower plant stand (67 plants/m^2) than applying foliar fungicides (71 plants/m^2).

An interaction between cultivar and seed treatment was observed with a *p*-value of 0.056 and is shown in Figure 3.2, indicating seed treatment affects cultivar differently. Amanda seed treated with Helix resulted in a higher fall plant stand (75 plants/m^2) than seed treated

with clothianidin (73 plants/m^2). The opposite trend occurred for Mercedes. Seed treated with Helix resulted in a lower fall plant stand (63 plants/m2) as compared to seed treated with clothianidin (67 plants/m^2).

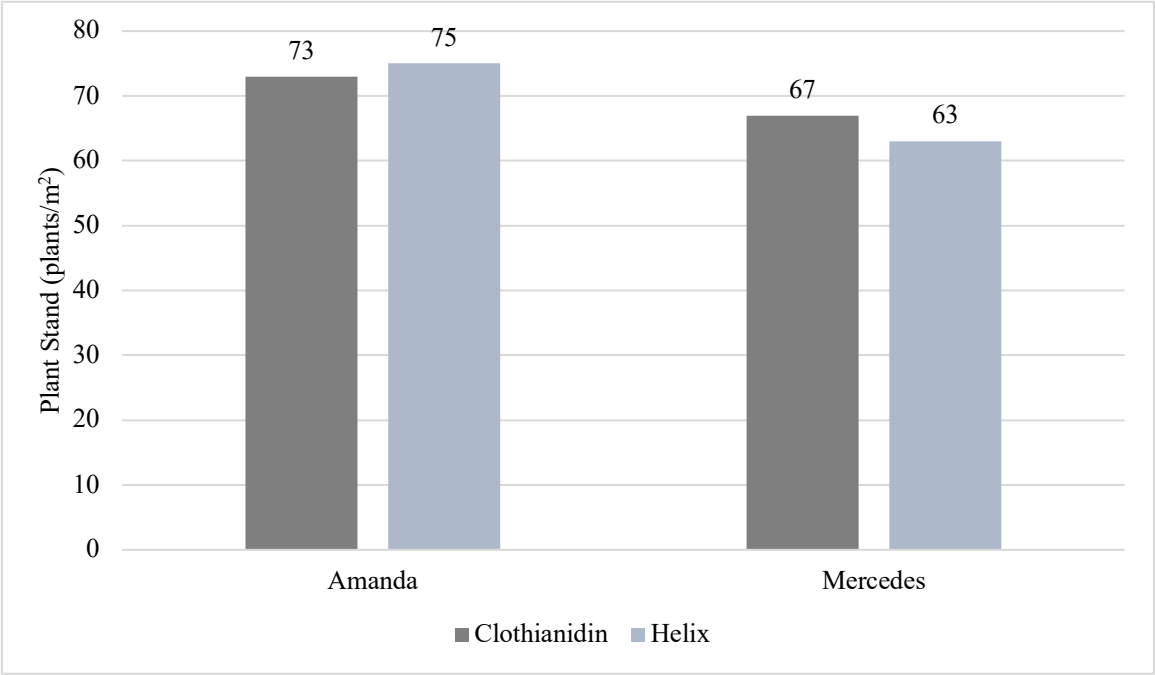

Figure 3.2: Amanda and Mercedes fall plant stand for seed treated with Helix Vibrance and clothianidin.

Stand counts were conducted again in the spring to determine the number of plants that survived the winter and identify plant stand within each plot. Spring plant stand significantly differed for each cultivar, accounting for 36.2% of all variation (Table 3.1). Mercedes spring plant stand of 48 plants/m^2 was less than Amanda plant stand (54 plants/m^2). Both cultivars experienced between 26 and 27% reduction in stand between fall and spring stand counts, indicating winter kill occurred, but not at a significant level.

Application of foliar fungicides compared to no application significantly impacted spring plant stand. Spraying fungicides resulted in plant stand of 52 plants/m^2, an increase compared to no fungicide application resulting in 48 plants/m^2. Although fungicide application increases spring plant stand, specific application time does not significantly differ between a

fall only application (53 plants/m^2), spring only application (50 plants/m^2), and fall and spring application (52 plants/m^2).

An interaction between seed treatment and application of foliar fungicides or no application was observed with a *p*-value of 0.077 (Figure 3.3). Spraying fungicide in combination with clothianidin resulted in a spring plant stand of 54 plants/m^2 while spraying in combination with Helix resulted in a lower plant stand of 50 plants/m^2. The opposite trend occurred when no foliar fungicide was applied in conjunction with use of clothianidin (47 plants/ m^2) or with Helix (49 plants/m^2).

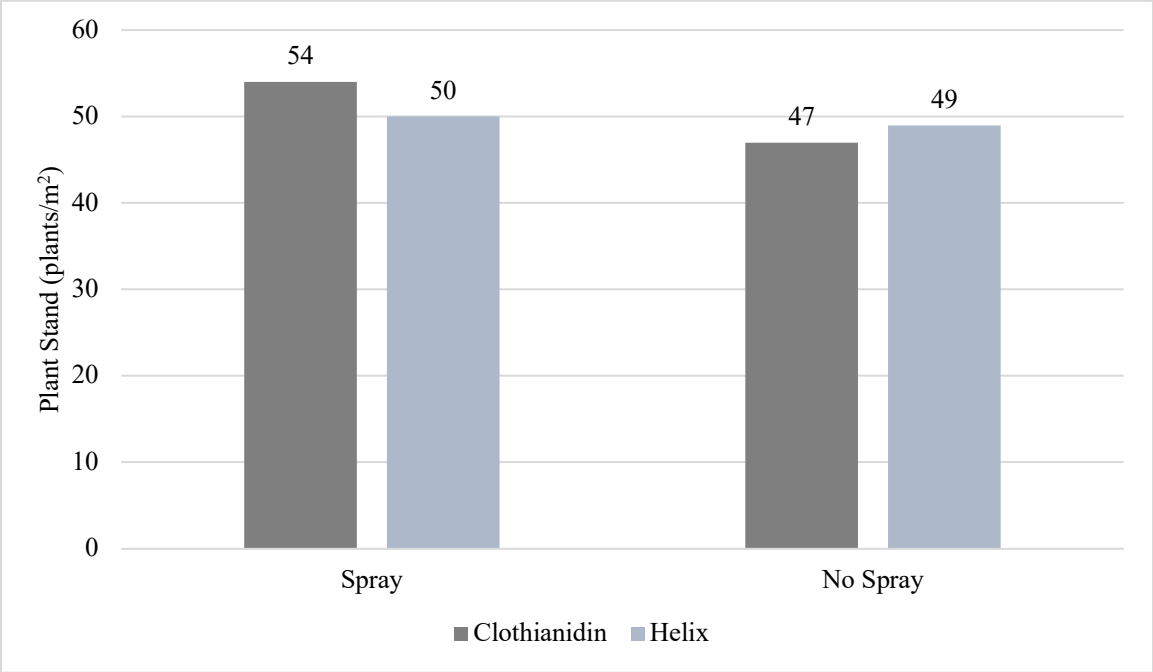

Figure 3.3: Spring plant stand after different seed treatments and foliar fungicide treatments.

Height was not significantly impacted by cultivar or blackleg disease treatments. Amanda and Mercedes both had a height of 158 cm. Cultivar only accounted for 10.4% of the total variation while applying or not applying foliar fungicides accounted for the largest portion of total variation (27.0%) with a *p*-value of 0.073 (Table 3.1). Further investigation of spray timing group means resulted in the same plant height (158 cm) for each treatment. Flowering notes consist of recording the number of days after January 1 that over 75% of the plants within each plot have fully flowered. The only impact on time of flowering was cultivar. Mercedes flowered earlier than Amanda, occurring 129 and 132 days after January 1, respectively.

Table 3.1: Percentage of the total sum of squares of all variation caused by cultivars and disease treatments from the analyses of variance of fall plant stand, spring plant stand, plant height, and flowering recorded on two canola cultivars grown in three environments over two years. In the analyses the effect of cultivar and disease treatment were partitioned using orthogonal contrasts.

		Fall Plant Stand		**Spring Plant Stand**		**Plant Height**		**Flowering**	
Source	**df**	**%SS**	***p***	**%SS**	***p***	**%SS**	***p***	***%SS***	***p***
(1) Between cultivars	1	44.6%	***	36.2%	***	10.4%		99.3%	****
(2) Seed treat v No seed treat	1	2.4%		5.0%		2.1%		0.0%	
(3) No Spray v sprayed	1	18.7%	*	27.1%	***	27.0%	*	0.0%	
(4) One spray v Two sprays	1	1.1%		0.3%		3.5%		0.0%	
(5) Fall spray v Spring spray	1	1.4%		2.7%		2.3%		0.2%	
(6) Interaction between (2) x (3)	1	2.0%		9.6%	*	0.7%		0.1%	
(7) Interaction between (2) x (4)	1	0.5%		1.3%		6.1%		0.1%	
(8) Interaction between (2) x (5)	1	1.8%		2.9%		3.2%		0.0%	
Interaction between (1) x (2)	1	17.9%	*	0.6%		0.0%		0.1%	
Interaction between (1) x (3)	1	0.0%		6.1%		4.6%		0.0%	
Interaction between (1) x (4)	1	3.1%		4.0%		9.6%		0.0%	
Interaction between (1) x (5)	1	1.8%		2.2%		15.0%		0.0%	
Interaction between (1) x (6)	1	4.1%		1.1%		1.4%		0.0%	
Interaction between (1) x (7)	1	0.2%		0.5%		13.9%		0.0%	
Interaction between (1) x (8)	1	0.4%		0.4%		0.1%		0.2%	
Total CV & Treatment SSq	**15**	**9,253**		**6,495**		**433.6**		**585.7**	

* $0.1 > p > 0.05$
** $0.05 > p > 0.01$
*** $0.01 > p > 0.001$
**** $p < 0.001$

3.3.2 Disease Incidence, Severity and Stem Necrosis

Disease incidence was calculated by taking stem samples and identifying presence of lesions to calculate the average percent of infected stems within the plot. Overall, seed treatment has no impact on disease incidence, while cultivar and spray timing have a significant impact on disease incidence.

Partitioning of cultivar and disease treatments using orthogonal contrasts resulted in foliar fungicide application accounting for the highest percentage of total variation (69.1%) and cultivar accounting for 16.7% of the total variation, both with *p*-values less than 0.001 (Table 3.2). Before determining significantly different group means between cultivars and spray timings, an interaction between spray timing and cultivar with a *p*-value less than 0.001 was investigated (Figure 3.4). The interaction indicates applying a foliar fungicide significantly reduces disease and affects each cultivar differently. The highest disease incidence for Amanda (27%) and Mercedes (16%) was observed when no fungicide was applied and significantly reduced to 8 and 3%, respectively, when fungicide was applied. Although fungicide application reduced disease incidence for each cultivar, the percent reduction of disease was 81% reduction for Mercedes and 70% reduction for Amanda.

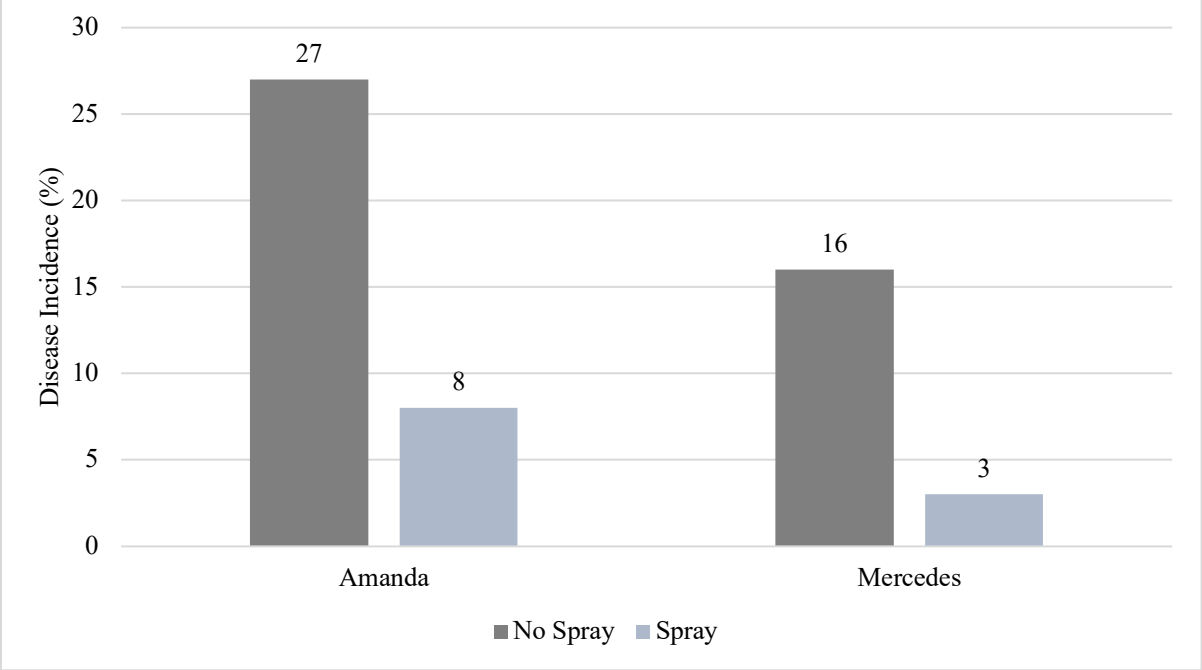

Figure 3.4: Disease incidence of Amanda and Mercedes after foliar fungicide application and no foliar fungicide application.

Different timing of foliar fungicide applications has a significant impact on disease incidence for each cultivar (Figure 3.5). The bars with lowercase letters indicate the treatment group included in the orthogonal contrast analyzed, with different lowercase letters being significantly different (Figure 3.5; Table 3.2). Fall only and spring only applications both reduced disease incidence compared to no application and are significantly different from each other. Amanda experienced 27% disease incidence with no foliar application which was reduced to 11% disease incidence when fungicides were only applied in the fall. The spring only application significantly reduced disease from 27% with no application to 9% disease incidence. The same trend occurred with reduction of disease in Mercedes. No foliar fungicide application resulted in 16% disease and a reduction to 6% disease with a fall only application and further reduction to 2% disease with a spring only application. While both a single application in the fall or in the spring significantly reduces disease, an application made in the fall and again in the spring significantly reduces disease to the lowest incidence. Disease incidence in Amanda and Mercedes after a fall and spring application was 4 and 1%, respectively.

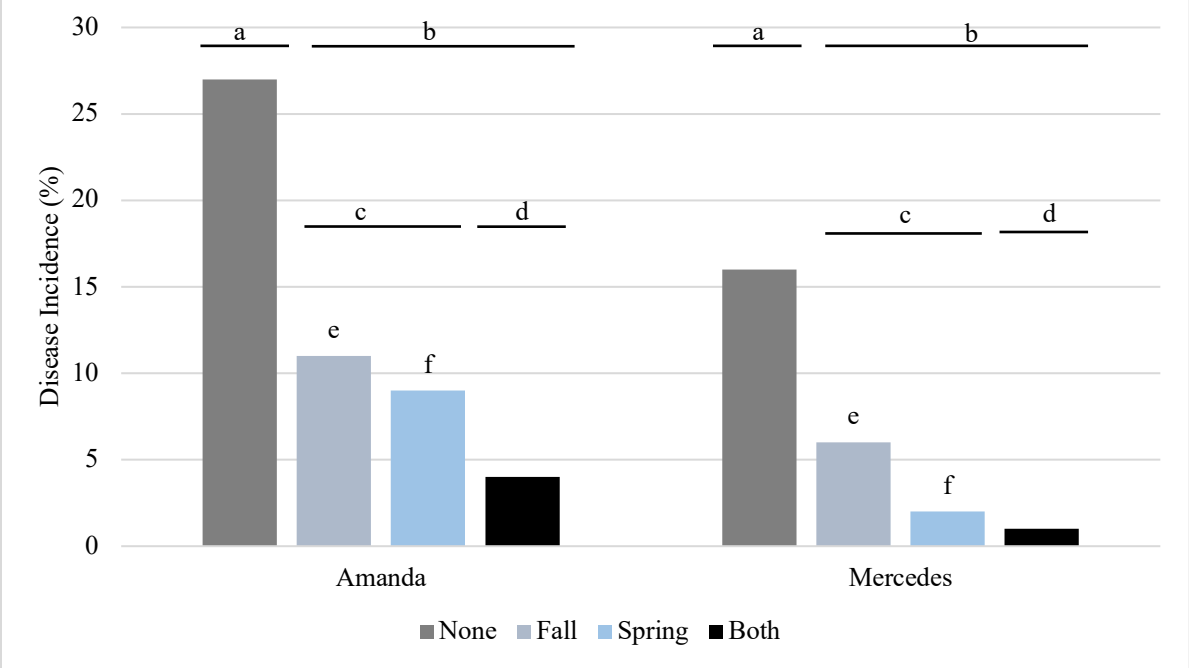

Figure 3.5: Disease incidence of Amanda and Mercedes after different foliar fungicide application times.

The randomly collected 15 stems were cut at the soil line and rated for blackleg disease severity on a scale of 0 to 5, with 5 being severe. Although moderate to high disease incidence was observed, plants experienced low disease severity. When collecting data, stem ratings were often 0 and 1, with few stems possessing ratings of 3 or 4 and no ratings of 5. Both cultivar and spray timing had a significant impact on disease severity. Mercedes experienced a disease severity rating of 0.13 while Amanda experienced less severe disease with a rating of 0.09. The strongest influence on disease severity was fungicide application compared to no application, accounting for 68% of the total sum of squares of all variation (Table 3.2; Figure 3.6). No application of fungicide resulted in a disease severity rating of 0.22. A fall only application (0.10) and spring only application (0.09) did not significantly differ in reduction of disease severity, but a single application in either the fall or spring was significantly higher than applying in the fall and spring where severity rating was significantly reduced to 0.02.

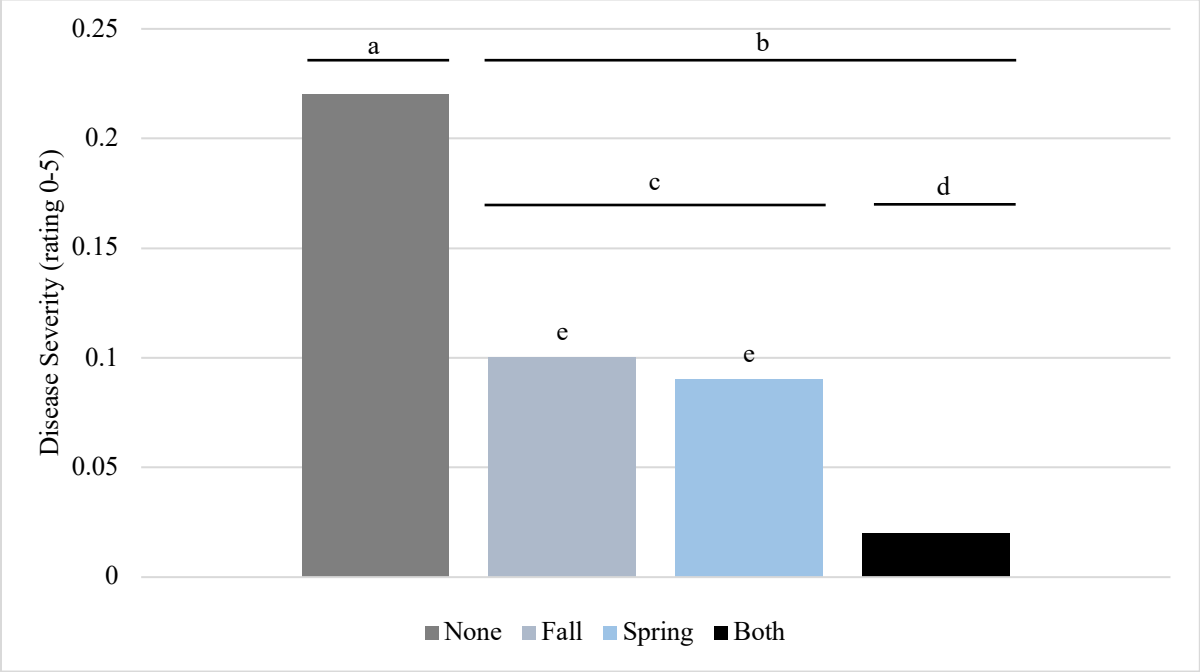

Figure 3.6: Impact of spray timing on blackleg disease severity recorded on two canola cultivars grown in three environments over two years.

Additionally, of the 15 stems collected and observed to possess pith blackening at the cross section, percentage of stems with tissue necrosis were calculated. A potential interaction (*p*-value of 0.092) between cultivar and number of foliar fungicide applications was investigated (Figure 3.7). The percent of stems with necrosis for Amanda and Mercedes was 8 and 12%, respectfully, when either a fall only or spring only application was made. Stem necrosis was significantly reduced to 3% for both cultivars when fungicide was applied in the fall and the spring. Although there is a reduction in necrosis of stems, Mercedes and Amanda responded differently to the fungicide application. Mercedes experienced 75% reduction while Amanda experienced 63% reduction.

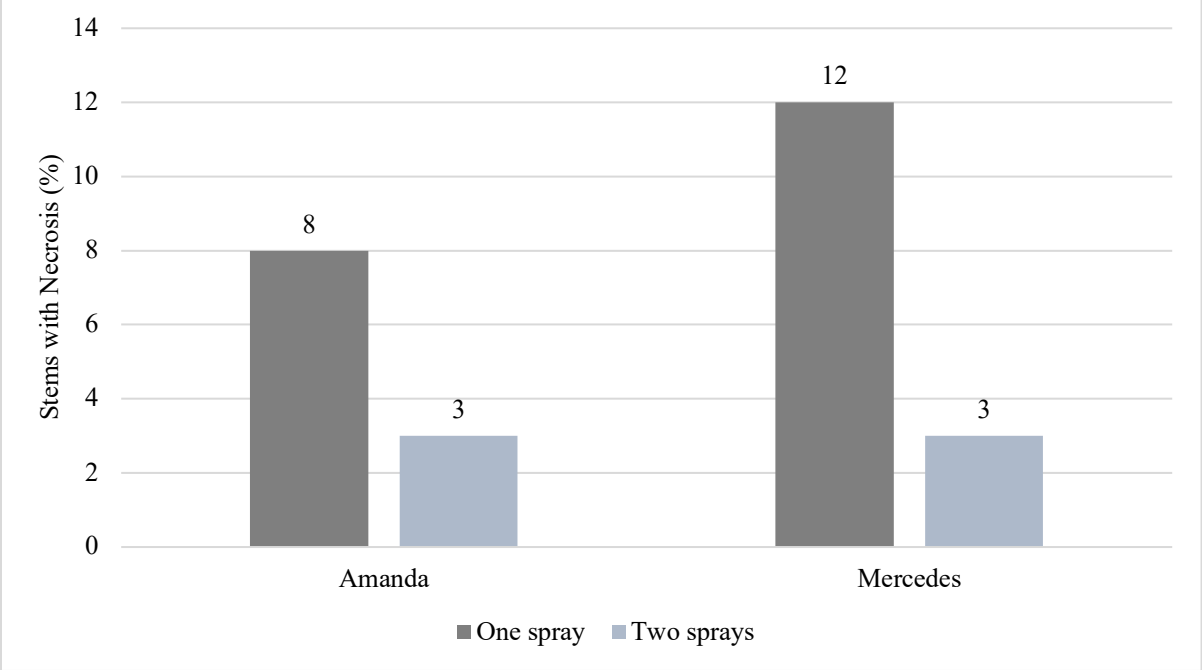

Figure 3.7: Number of Amanda and Mercedes stems with necrosis after one foliar fungicide application compared to the number of stems with necrosis after two foliar fungicide applications.

Application of foliar fungicides and spray timing reduced the percent of stems with necrosis (Figure 3.8). The highest percentage of stems with necrosis for Amanda (23%) and Mercedes (26%) was observed when fungicides were not applied. Application in the fall only (10 to 12%), spring only (5 to 13%), or in the fall and spring (3%) significantly reduced stem necrosis for both cultivars, with the "both" treatment being significantly lower than the fall only and spring only application.

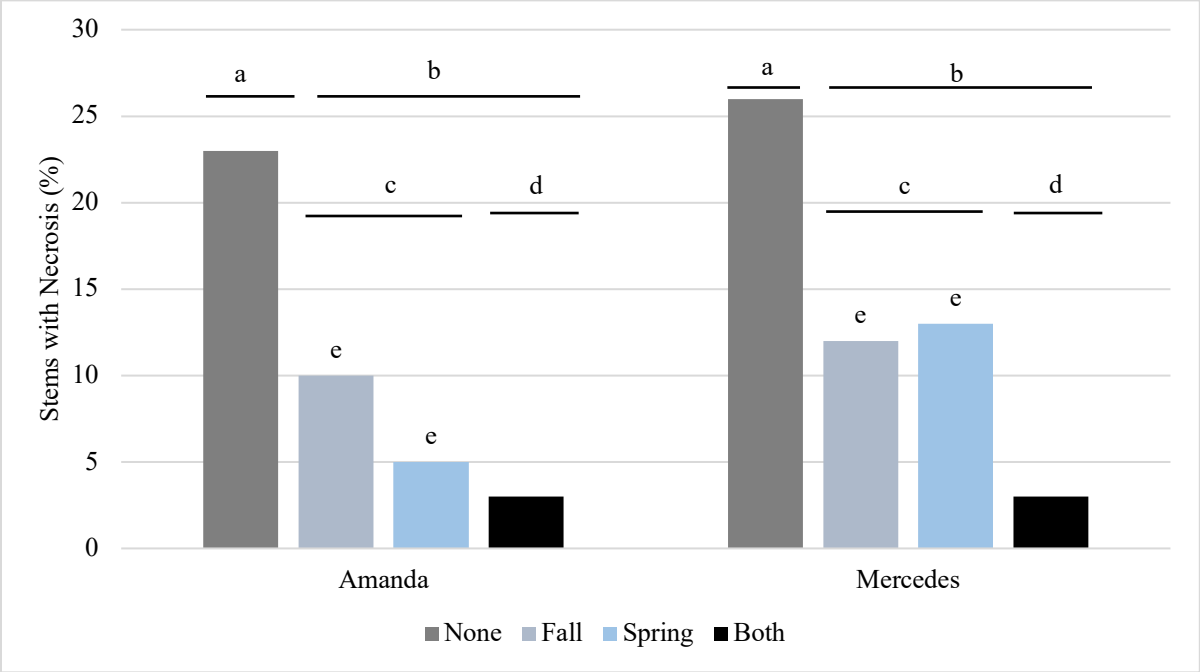

Figure 3.8: Number of Amanda and Mercedes stems with necrosis after different foliar fungicide application times.

Table 3.2: Percentage of the total sum of squares of all variation caused by cultivars and disease treatments from the analyses of variance of blackleg disease incidence, blackleg disease severity, and stem necrosis recorded on two canola cultivars grown in three environments over two years. In the analyses the effect of cultivar and disease treatment were partitioned using orthogonal contrasts.

		Disease Incidence		**Disease Severity**		**Stem Necrosis**	
Source	**df**	**%SS**	***p***	**%SS**	***p***	***%SS***	***p***
(1) Between cultivars	1	16.7%	****	8.8%	***	2.8%	
(2) Seed treat v No seed treat	1	0.1%		0.3%		0.7%	
(3) No Spray v sprayed	1	69.1%	****	68.1%	****	79.1%	****
(4) One spray v Two sprays	1	6.3%	****	15.5%	***	11.4%	****
(5) Fall spray v Spring spray	1	2.4%	***	0.5%		0.6%	
(6) Interaction between (2) x (3)	1	0.4%		0.1%		0.3%	
(7) Interaction between (2) x (4)	1	0.0%		0.1%		0.0%	
(8) Interaction between (2) x (5)	1	0.5%		0.5%		1.2%	
Interaction between (1) x (2)	1	0.0%		0.0%		0.1%	
Interaction between (1) x (3)	1	2.7%	***	1.3%		0.0%	
Interaction between (1) x (4)	1	0.8%		2.8%		1.7%	*
Interaction between (1) x (5)	1	0.0%		0.9%		1.4%	
Interaction between (1) x (6)	1	0.0%		0.1%		0.0%	
Interaction between (1) x (7)	1	0.0%		0.9%		0.4%	
Interaction between (1) x (8)	1	0.9%		0.1%		0.4%	
Total CV & Treatment SSq	**15**	**21,275**		**1.490**		**12976**	

* $0.1 > p > 0.05$
** $0.05 > p > 0.01$
*** $0.01 > p > 0.001$
**** $p < 0.001$

3.3.3 Seed Yield

Across both years and all locations, cultivar is the only treatment that had an impact on yield, accounting for 81% of the total sum of squares of all variation (Table 3.3). Mercedes yielded 5,128 kg/ha and was significantly higher than Amanda which yielded 4,617 kg/ha. Foliar fungicide application had a moderate impact on yield (*p*-value of 0.059) compared to no application of fungicide (Figure 3.8). A fall only application resulted in yield increasing to 4,905 kg/ha from the no spray treatment yield of 4,748 kg/ha. An increase in yield was also observed for the spring only treatment (4,835 kg/ha) compared to the no spray treatment. There is no significant difference in yield between the fall only and spring only treatment. An application in both the fall and spring resulted in the highest yield (5,004 kg/ha) compared to the single application times and no spray treatment. The increase in yield from the "both" treatment only accounts for 4.1% total variation with a *p*-value of 0.053, but indicates over multiple years and locations, this may become a significant trend and applying fungicide for control of blackleg will increase yields.

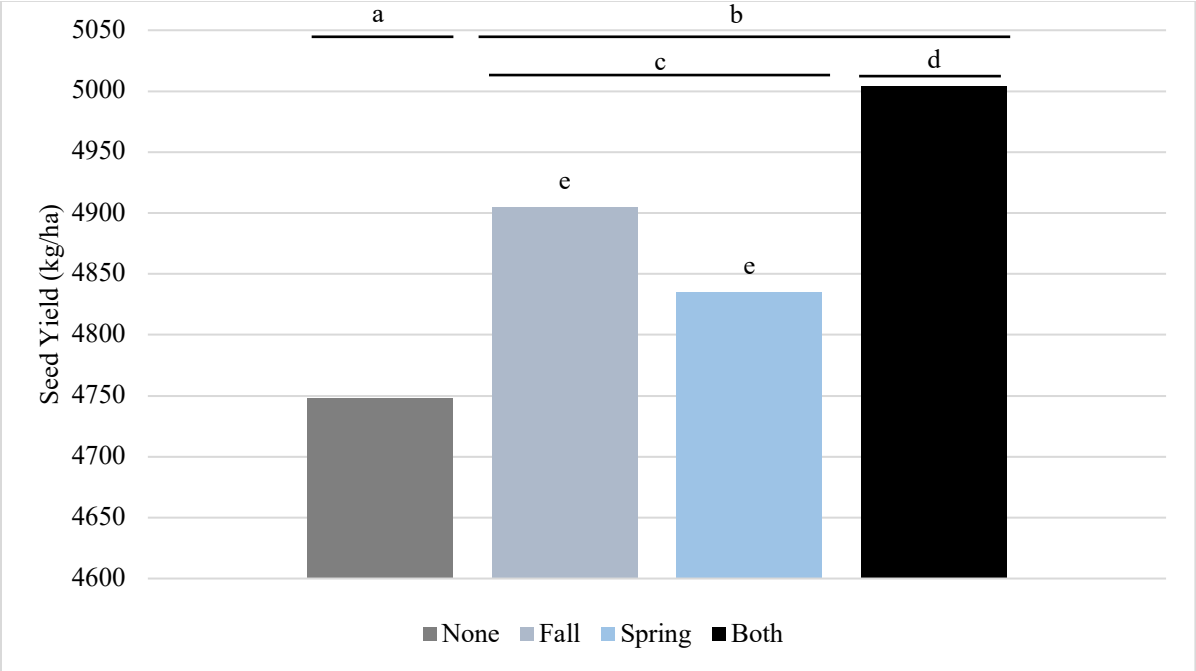

Figure 3.9: Impact of spray timing on yield recorded on two canola cultivars grown in three environments over two years.

3.3.4 Seed Quality

The largest influence on estimated oil seed content is cultivar, accounting for 95% of all variation (Table 3.3). Mercedes seed possess 42.5% estimated oil while Amanda seed possess a lower percentage of estimated oil at 40.1%. A potential interaction (*p*-value of 0.083) between number of foliar fungicide applications and seed treatment was investigated (Figure 3.9). When treated with Helix and sprayed either in the fall or spring, or both in the fall and spring, oil content was 42.5%. Seed treated with clothianidin and sprayed in either the fall or spring resulted in estimated oil seed content of 41.4%. Application in the fall and spring resulted in a slight reduction to 41.2% estimated seed oil content. Estimated seed protein content is only affected by cultivar. Amanda possessed 21.6% estimated seed protein content while Mercedes estimated seed protein content (20.8%) was significantly lower.

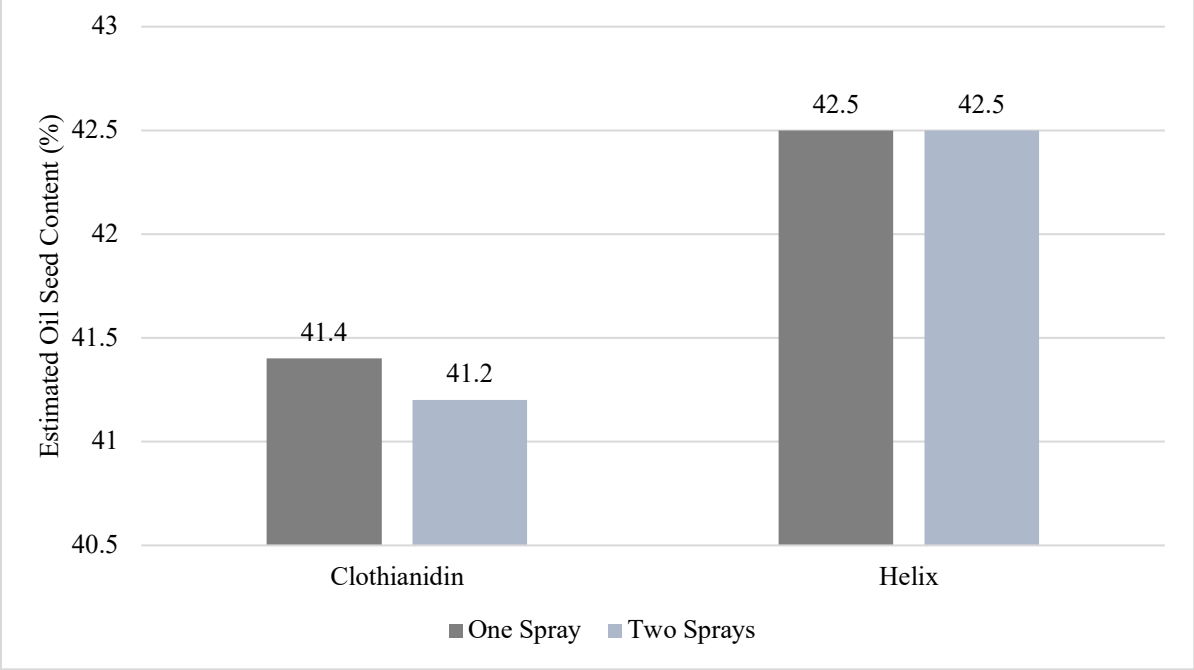

Figure 3.10: Impact of different seed treatments and number of foliar fungicide applications on estimated oil seed content.

Table 3.3: Percentage of the total sum of squares of all variation caused by cultivars and disease treatments from the analyses of variance of seed yield, seed oil content and seed protein recorded on two canola cultivars grown in three environments over two years. In the analyses the effect of cultivar and disease treatment were partitioned using orthogonal contrasts.

		Seed Yield		**Oil Content**		**Seed Protein**	
Source	**df**	**%SS**	***p***	**%SS**	***p***	**%SS**	***p***
(1) Between cultivars	1	81.3%	****	96.1%	****	86.7%	****
(2) Seed treat v No seed treat	1	0.9%		0.3%		1.0%	
(3) No Spray v sprayed	1	3.9%	*	0.0%		0.1%	
(4) One spray v Two sprays	1	4.1%	*	0.1%		1.5%	
(5) Fall spray v Spring spray	1	0.1%		0.1%		1.2%	
(6) Interaction between (2) x (3)	1	0.7%		0.1%		0.0%	
(7) Interaction between (2) x (4)	1	0.4%		1.0%	*	0.2%	
(8) Interaction between (2) x (5)	1	0.1%		0.3%		1.5%	
Interaction between (1) x (2)	1	1.9%		0.6%		1.0%	
Interaction between (1) x (3)	1	0.4%		0.1%		0.4%	
Interaction between (1) x (4)	1	2.0%		0.8%		1.7%	
Interaction between (1) x (5)	1	0.0%		0.0%		1.5%	
Interaction between (1) x (6)	1	0.0%		0.3%		1.7%	
Interaction between (1) x (7)	1	0.6%		0.1%		0.7%	
Interaction between (1) x (8)	1	3.6%	*	0.2%		0.7%	
Total CV & Treatment SSq	**15**	**24,108,773**		**445.2**		**51.82**	

* $0.1 > p > 0.05$
** $0.05 > p > 0.01$
*** $0.01 > p > 0.001$
**** $p < 0.001$

3.4 Discussion

Fungicides applied as seed treatments and foliar applications are an effective disease management tool to prevent onset of blackleg disease. Blackleg is new to northern Idaho and researchers and growers have limited knowledge as to when spores are released, and initial infection occurs. Therefore, field trials were established to identify the impact of fungicide seed treatments and foliar applications on blackleg disease incidence, severity, seed yield, and seed quality in winter canola cultivars grown in northern Idaho. Overall results will aid in determining the optimal time to apply foliar fungicides and successfully integrate fungicides into management practices of blackleg specific to northern Idaho.

Field trials included two cultivars, Mercedes (resistant) and Amanda (susceptible), each treated with clothianidin and Helix Vibrance as seed treatments. Foliar applications of Priaxor were made at a rate of 600 ml/ha under four different regimes: fall only, spring only, fall and spring, and no application. Results suggest blackleg inoculum is present and causing infection in the fall and spring. If only one fungicide application is made, the spring application is preferred, however, a fall and spring application results in further disease reduction. Spray timing had an impact on seed yield at the 10% significance level rather than the traditional 5% and no impact on seed quality. Fungicide seed treatment did not impact disease incidence, yield, or seed quality.

Cultivar had the greatest impact on plant stand, flowering time, and plant height along with seed yield and seed quality. Cultivar had a moderate impact on disease incidence with Mercedes showing lower disease incidence (1% to 16%) than Amanda (4% to 27%). Mercedes has “good” resistance towards blackleg, but specific *R-gene* information is unavailable (Rubisco Seeds, 2021), while Amanda was assessed as susceptible. Both cultivars experienced a significant reduction in disease when any foliar fungicide was applied. However, the disease reduction was greater for Mercedes (80%) than for Amanda (70%). Application of fungicides affects resistant and susceptible cultivars differently and although disease incidence is significantly different between cultivars, the percentage of disease reduction in Mercedes is only 10% higher than that of Amanda, suggesting that the resistance of Mercedes may not be strongly effective towards the pathogen population.

Disease incidence was significantly impacted by spray timing. Fraser et al. (2020) showed the effective use of Priaxor as a foliar fungicide when used on spring canola in Canada.

Based on the reduction of disease incidence from winter canola trials in northern Idaho, Priaxor also provides effective control against blackleg disease in winter canola. The highest percentage of disease was observed in the control plots for Amanda (27%) and Mercedes (16%). The fall only application for both cultivars resulted in a significant reduction of 11% incidence in Amanda and 6% in Mercedes, suggesting fall infection is caused by conidia and/or ascospores.

Although we are not necessarily seeing disease symptoms in the fall, there was a reduction in disease incidence from the fall only application. This suggests that the pathogen may be infecting in the fall but is asymptomatic due to leaves infected in the fall senescing after freezing in the winter before the pathogen reaches the stem. Initial infection in leaves is followed by symptomless colonization as the pathogen moves from the leaf vascular tissue through the petiole and into the stem (Rimmer et al., 2007). It is during this time, that if the leaves fall off in preparation for dormancy and the infection does not reach the stem, there is less likelihood of severe infection occurring in the stem. Fall infection may also be asymptomatic due to weather conditions. After initial infection occurs, temperatures above 20°C will result in symptoms rapidly, while less than 10°C will hinder appearance of symptoms although colonization may be occurring in the leaf tissue (Rimmer et al., 2007).

Disease incidence under the spring only foliar application was significantly lower for both cultivars (2% and 9%) than the fall only application (6% and 11%), indicating that inoculum is also present in the spring. Applying in the fall and again in the spring resulted in the lowest and significantly different level of disease incidence for Mercedes (1%) and Amanda (4%) compared to no application and a single application in either the fall or spring. Furthest reduction of disease under the “both” treatment indicates blackleg infection occurs in the fall and again in the spring, similar to Europe, where winter canola in infected twice a year (Fitt et al., 2006; Li et al., 2007; Zhang and Fernando, 2017; West et al., 2001). Because there is a significant reduction in disease incidence under the “both” treatment, there may be a benefit to applying fungicides twice a year in northern Idaho, but the economic feasibility of spraying once versus twice in the year needs to be considered and further data needs to be collected to solidify these suggestions.

Although there was a high percentage of disease observed in the control plots for both cultivars (16% to 27%), there was no significant impact on yield from seed treatment and spray

timing. Stem cankers are formed by the aggressive species, *L. maculans* (Somda et al., 1998). Once established, the cankers will girdle the stem base during pod filling, causing premature plant ripening and preventing complete seed pod fill (Bailey et al., 2003), thus reducing yields. Results indicate formation of stem cankers is rare and disease severity is low, but disease severity it affected by spray timing. Plants not treated with a foliar fungicide had disease severity rating of 0.22 and a foliar application in either the fall or spring significantly reduced severity to a rating to 0.10 and 0.09, respectively. Further significant reduction in disease severity was achieved under the "both" treatment, resulting in a severity rating of 0.02. If an additional spring infection is occurring, it is most likely attributed to ascospore release in May or June when temperatures reach 8 to 15°C (Toscano-Underwood et al., 2003; Rimmer et al., 2007), and there is not enough time remaining in the growing season to allow for canker formation and disease severity before harvesting the winter canola along with higher summer temperatures in northern Idaho.

Foliar fungicide application may improve winter canola yield in northern Idaho. Lowest yield was recorded under no application of fungicides (4,748 kg/ha) and increased under a fall only (4,905 kg/ha) and spring only (4,835 kg/ha) application. An even larger increase in yield was observed when both a fall and spring application was made, resulting in 5,004 kg/ha.

Seed treatment had no impact on any of the response variables. Disease incidence for clothianidin (10%) was only slightly higher than Helix Vibrance (9%). A similar occurrence was observed for the disease severity in which Helix Vibrance (0.10 severity rating) only slightly reduced disease severity compared to clothianidin (0.11 severity rating). Previous work on fungicides for seed borne blackleg are triazoles, but more specifically the active ingredients tested have been fluquinconazole and flutriafol (Khangura and Barbetti, 2004; Marcroft and Potter, 2008; Elliot and Marcroft, 2011) and proven successful in preventing infection. The active ingredients in Helix Vibrance consist of a triazole fungicide that should be effective against blackleg. Recent work by Upadhaya et al. (2019) showed that fungicides labeled for seed treatment in North Dakota, including Helix Vibrance, were only effective in reducing blackleg infection under greenhouse settings rather than field setting. Certified seed was used in the present study, reducing the possibility of infection caused by seedborne inoculum. Because Helix did not reduce disease incidence or severity, soilborne inoculum is most likely

not present and infection was caused by ascospore or conidia infection after the fungicide seed treatment protection ran its course. Although infested soils may not be of concern in the region, use of certified seed in conjunction with fungicide seed treatment will minimize the potential impact of seed borne inoculum causing initial infection.

Because *L. maculans* and *L. biglobosa* can cause blackleg on the same plant at the same time (West et al., 2002) determining the species demographic in northern Idaho would be ideal to optimize management programs, especially those relying on fungicides. For example, the UK and Poland control of blackleg relies on effective fungicide use which requires application in the fall before ascospores infect their winter canola and symptoms are observed (West et al., 1999; Kaczmarek et al., 2009). Previous work by Pickard (2018) identified the northern Idaho blackleg pathogen population mainly consists of *L. maculans* with very few isolates of *L. biglobosa.* However, further investigation into pathogen population demographics to ensure application of fungicides are economically feasible should be completed due to fungicides showing less effectiveness towards *L. biglobosa* (Eckert et al., 2010). Identification of species can be completed by collection of leaf lesion field samples in the fall and stem lesions in the spring (Brachaczek et al., 2016).

Along with species demographics, epidemiology studies are important and will provide detailed information on climatic conditions of specific regions and the impact weather has on spore release and timing of initial infection. Use of volumetric spore traps and molecular assays have proven successful in identifying main species, and timing of spore release corresponding to the weather (Guo and Fernando, 2005; Kaczmarek et al., 2009; Kaczmarek et al., 2012). Seeing success of epidemiology studies in other countries led to operation of spore traps adjacent to winter canola field trials in this current study. Results from the epidemiology study will be reported in the following chapter.

Based on this research, blackleg follows a polycyclic lifecycle in northern Idaho, and infection of winter canola occurs in the fall and spring. The climate of northern Idaho is conducive to initial infection and pathogen colonization, however, with cold winters followed by hot and dry summers, north Idaho climate does not appear to be conducive to extreme canker epidemics as seen in other countries. However, multiple years of data will be required to provide confirmation. Although there is no yield loss and currently no concern for severe blackleg epidemics of winter canola in northern Idaho, there is still concern of blackleg

infecting seeds, resulting in seed lot rejection and economic losses for growers and the seed industry. Thus, proper management plans should be followed to minimize blackleg incidence.

Fungicides should be effectively integrated into management plans as foliar applications made in the spring, or possibly in the fall and spring. Use of certified seed in conjunction with fungicide seed treatments and following ideal crop rotation practices should also be followed to minimize blackleg incidence. Completion of epidemiology studies will provide a solid understanding of the pathogen species demographics and lifecycle. After collecting a third year of data and combining results of this current study with those of the epidemiology study, spray timing guidelines can confidently be made to growers in the region.

3.5 References

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Chapter 4: Identifying the Timing of Spore Dispersal of the Fungal Pathogen *Leptosphaeria maculans* and *Leptosphaeria biglobosa* in Northern Idaho Through the use of Burkard Volumetric Spore Traps

4.1 Introduction

Leptosphaeria maculans and *L. biglobosa* are the two fungal species responsible for blackleg disease of canola (*Brassica napus*). Blackleg is the most economically important disease of canola worldwide. Multiple disease management strategies are practiced to prevent blackleg, including genetic resistance, fungicide use and crop rotation. Europe and Poland for example, extensively rely on effective fungicide use for management of blackleg which requires application before ascospores infect their winter canola (West et al., 1999; Kaczmarek et al., 2009). Therefore, optimizing blackleg management programs requires an understanding of the epidemiology of *Leptosphaeria* species due to climate conditions impacting the pathogen lifecycle and blackleg disease cycle from region to region.

In general, *L. maculans* and *L. biglobosa* follow the same disease cycle with sources of infection from wind-blown ascospores and rain splashed conidia (Rimmer et al., 2007). Ascospores are generally the primary source of inoculum, released from mature pseudothecia under temperatures between 5 and 20°C, with 8 to 12°C ideal for optimal release, commonly occurring in conjunction with precipitation events (Toscano-Underwood et al., 2003; Rimmer et al., 2007). However, climate conditions influence the development and distribution of spore release between regions. Specific epidemiology studies have been conducted in Canada, Australia, Europe, and Poland where *L. maculans* has been identified as the main species and follows a polycyclic disease cycle (Fitt et al., 2006; Li et al., 2007; Zhang and Fernando, 2017; West et al., 2001).

The Hirst trap was developed in 1952 as the first tool for aerobiological sampling consisting of a clock mechanism to move a glass slide for collection across the air intake (West and Kimber, 2015). Variations of the Hirst trap have been developed leading to the Burkard volumetric spore trap which has an internal rotating drum rather than a single glass slide for collection. Air sample analysis has been and continues to be completed by microscopy, however visual observation of spores can be inaccurate due to many species having a similar appearance (West and Kimber, 2015). Molecular techniques have become a popular choice in analyzing samples because of less time and more accurate results, especially when spores are

visually similar. Epidemiology studies of *L. maculans* using volumetric spore traps in conjunction with microscopy and molecular techniques have provided accurate timing of pathogen development and initial infection (Guo and Fernando, 2005; Kaczmarek et al., 2009; Kaczmarek et al., 2012).

Idaho is considered to experience a Mediterranean climate and is part of the inland Pacific Northwest where most of the precipitation occurs from October to March and the remaining 25% occurs from April to June (Kassam et al., 2012; Kruger et al., 2017). Winters are wet and cool with average monthly temperatures ranging from 14 to 30°C while summers are dry and warm with temperatures ranging from 20 to 35°C, or higher (Kassam et al., 2012). Weather conditions in northern Idaho are conducive for *Leptosphaeria* spp. spore development and dispersal. However, blackleg is new to northern Idaho, having been observed in 2011 (Agostini et al., 2013; Paulitz et al., 2017), and little is known about spore movement and timing. Objectives of the research are 1) identify the prominent method of *L. maculans* infection whether by ascospores or conidia; and 2) identify timing of spore release and pair with weather data to determine the conditions in the region conducive to spore release and disease infection.

4.2 Materials and Methods

4.2.1 Locations

Burkard seven-day recording volumetric spore traps (Figure 4.1; Burkard Manufacturing Co. Ltd., Hertfordshire, UK) were deployed adjacent to University of Idaho winter canola variety trials and blackleg winter canola trials at the University of Idaho Kambitsch Farm and Camas Prairie locations. For the 2019-2020 growing season, the trap at the Kambitsch Farm (46.5922, -116.9461) and Grangeville (45.9330, -116.2096) operated from 20 September 2019 until 17 December 2019, and again from 5 March 2020 until 2 July 2020. For the 2020-2021 growing season, the trap at the Kambitsch Farm (46.5856, -116.9503) and Nezperce (46.2379, -116.2555) operated from 1 September 2020 until 15 December 2020 and again from 16 March 2021 to 6 July 2021.

4.2.2 Operation of Spore Traps and Weather Stations

Burkard volumetric spore traps contain an internal drum for the use of catching air particles (Figure 4.2a). The traps operate through the use of a 12-volt deep cycle battery that powers a vacuum pump to cycle air through the trap through a 2 mm x 14 mm orifice and

deposit any particles on the tape, while the drum rotates on a manually wound clock mechanism. 'Melinex' tape is wound around the internal drum and then coated with a thin layer of Vaseline. Drums are exchanged on the same day every week and the internal clock is rewound to allow for another week of sampling.

Tape samples were processed using the protocol outlined by the Burkard company. A Perspex cutting block supplied by Burkard (Figure 4.2b) contains a guide for daily and hourly tape sectioning. Sterile forceps and scalpel were used to remove the tape from the drum and placed on the cutting block where it was divided into seven daily segments. These daily segments were divided into two corresponding pieces. One was set aside for microscope mounts and the other was placed into a sterile 2 ml screw top tube and placed in -20°C storage until needed for DNA isolation and qPCR.

An Onset HOBO USB micro station data logger (Onset Computer Corporation, Bourne, MA, USA) was deployed adjacent to the spore trap at each location. Rain, temperature, and relative humidity readings were recorded every 30 minutes and were analyzed to determine monthly and weekly averages for the growing season.

Figure 4.1: Burkard volumetric spore trap.

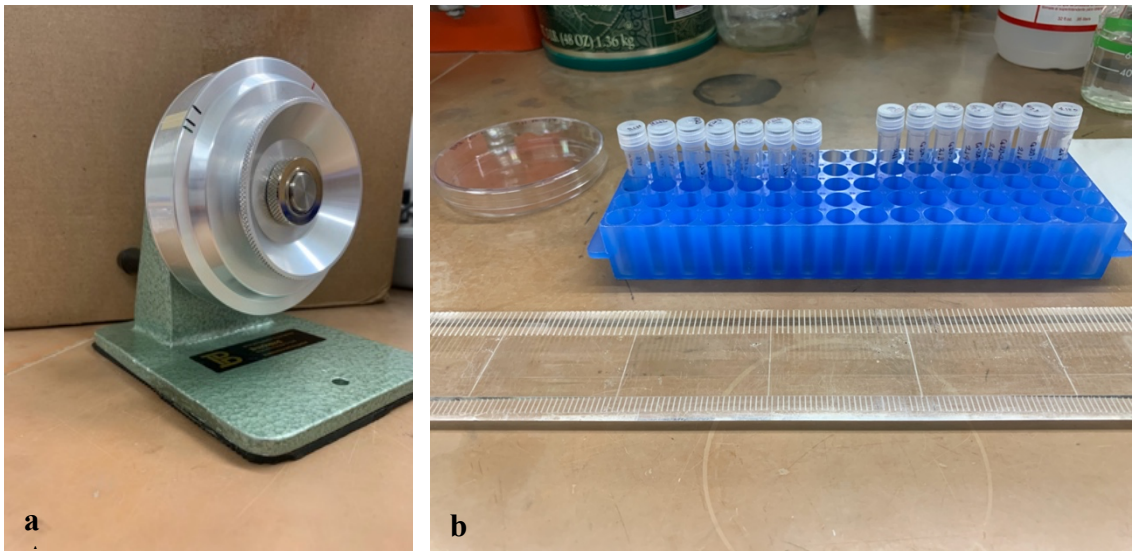

Figure 4.2: a) Internal drum of spore trap; b) Perspex cutting board.

4.2.3 Creation of Microscope Slides to Identify Presence of Ascospores

Microscope slides were made to identify the presence of ascospores from both *Leptosphaeria* species using an acid fuchsin mounting medium. *Leptosphaeria maculans* and *L. biglobosa* ascospores have similar morphology (Shoemaker and Brun, 2001), thus microscopic distinction between the two species is difficult. Acid fuchsin stain solution was prepared as follows: 750 ml sterile distilled water (SDW), 3.5 g Alfa Aesar acid fuchsin sodium salt (Thermo Fisher Scientific, Grand Island, NY) and 250 ml glacial acetic acid (Thermo Fisher Scientific) (Tylka 2020). 3 ml of the stain solution was mixed with the following to make the mounting medium: 250 ml glycerol (Thermo Fisher Scientific), 100 ml of 85% lactic acid (Millipore Sigma, Burlington, MA), and 50 ml of SDW (Sime et al., 2009).

A thin bead of polyvinyl alcohol mounting medium with DABCO (PVA; Sigma-Aldrich Co., St. Louis, MO) was placed on a glass microscope slide to hold the daily half segment in place. Slides were placed onto a hot plate set at 45ºC for 45 minutes to dry. A thin bead of acid fuchsin mounting medium was placed on a glass coverslip and set on top of the daily half segment and left on the hot plate to dry for an additional hour (Levetin, 2000). Slides were removed from the hot plate and allowed to cool and set overnight. Slides were analyzed under 1000x magnification using the single longitudinal traverse method for the presence of *Leptosphaeria* spp. ascospores that are 5-septate (Lacey and West, 2006; Figure 4.3). The number of spores observed for each daily segment was recorded and daily spore concentrations

were calculated using the following formula: Ascospore concentration (spores/m^3) = number of spores recorded * (width of tape/field diameter of objective lens) * (1/total volume of air sampled), where the width of tape is 7 mm, the field diameter of objective lens is 1.7 mm, and the total volume of air sampled is 14.4 cubic meters per day (Lacey and West, 2006; Kaczmarek et al., 2012; Guo and Fernando, 2005).

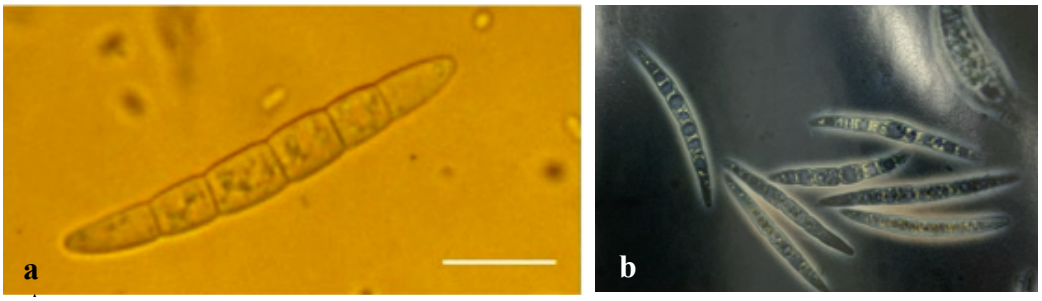

Figure 4.3: a) *Leptosphaeria maculans* ascospore, bar=10μm (Howlett et al., 2001); b) *L. maculans* ascospores (Rimmer et al., 2007).

4.3 Results

Monthly and weekly ascospore concentrations from microscope slides and corresponding weather data from 2019 to 2021 on the Palouse and Camas Prairie are in Appendix C. Overall, ascospore release was observed on the Palouse in the spring between April and June 2020 (Figure 4.4) and between March and May 2021 (Figure 4.5). Ascospore release on the Camas Prairie was observed between April and May 2020 (Figure 4.6) and between May and June 2021 (Figure 4.7). An instance of spore release was also observed on the Camas Prairie in September and October 2020 (Figure 4.7).

Spore release on the Palouse between April and June 2020 occurred mid to late April (0.57 ascospores/m^3) under a weekly total of 10 mm precipitation, an average temperature of 8^{o}C and average relative humidity of 64%. Spore release was later observed early to mid-May (0.57 ascospores/m^3) under less precipitation (6 mm) and higher temperature (12^{o}C) and relative humidity (66%) than spore release observed in April. Peak ascospore concentration occurred early to mid-June (1.14 ascospores/m^3) when precipitation was less than that of April and May (5 mm), temperature was the same as May (12^{o}C) and relative humidity was higher than April and May at 81%.

The following year on the Palouse, spore release occurred late March (0.29 ascospores/m^3) under similar precipitation amounts (9 mm) and relative humidity (83%), but colder temperatures (3°C) than observed for spore release between April and June 2020. Peak ascospore release occurred between 4 May and 25 May (1.14 ascospores/m^3) under temperatures ranging from 9 to 16°C, relative humidity from 55 to 68%, and precipitation of 1 mm, similar to May and June 2020 except May 2021 had no rainfall, and slightly higher temperatures and relative humidity.

Peak spore release on the Camas Prairie in 2019-2020 was between 16 April and 30 April (0.57 to 0.86 ascospores/m^3) when average weekly temperature was between 9 and 11°C, average weekly relative humidity was between 63 and 76% with total weekly precipitation between 12 and 13 mm. Final spore release in the spring of 2020 occurred the week of 14 May to 21 May, resulting in 0.86 ascospores/m^3 released under higher precipitation (43 mm) and relative humidity (93%) and a similar temperature (10°C) to weather associated with April spore release.

Increase in temperature on the Camas Prairie in 2020-2021 resulted in spore release occurring in September and October 2020, along with delayed ascospore release in the spring starting in May and ending in June. Ascospore release in September 2020 (0.29 ascospores/m^3) occurred at the beginning of the month. Unfortunately, the weather station was not operating and there is no weather data specific for the week associated with spore release. Spore release in October (0.57 ascospores/m^3) occurred at the beginning of the month, under 16 mm of precipitation, average weekly temperature of 10°C, and relative humidity of 77%.

Spore release on the Camas Prairie in spring 2021 occurred under similar weather conditions as to spring 2020 and fall 2020 on the Camas Prairie. Spores observed in May were release throughout the entire month, beginning on 4 May with a concentration of 0.29 ascospores/m^3. Higher concentrations were observed later in the month where 0.57 ascospores/m^3 and 0.86 ascospores/m^3 were observed the week of 18 May and 25 May, respectively. Weekly average temperature throughout May was between 9 and 12°C, relative humidity ranged from 71 to 82% and total weekly precipitation was between 5 and 13 mm. Ascospore release (0.29 ascospores/m^3) was observed a final time during the week of 8 June 2021 under 18 mm total weekly precipitation, average weekly temperature of 14°C, and relative humidity of 78%

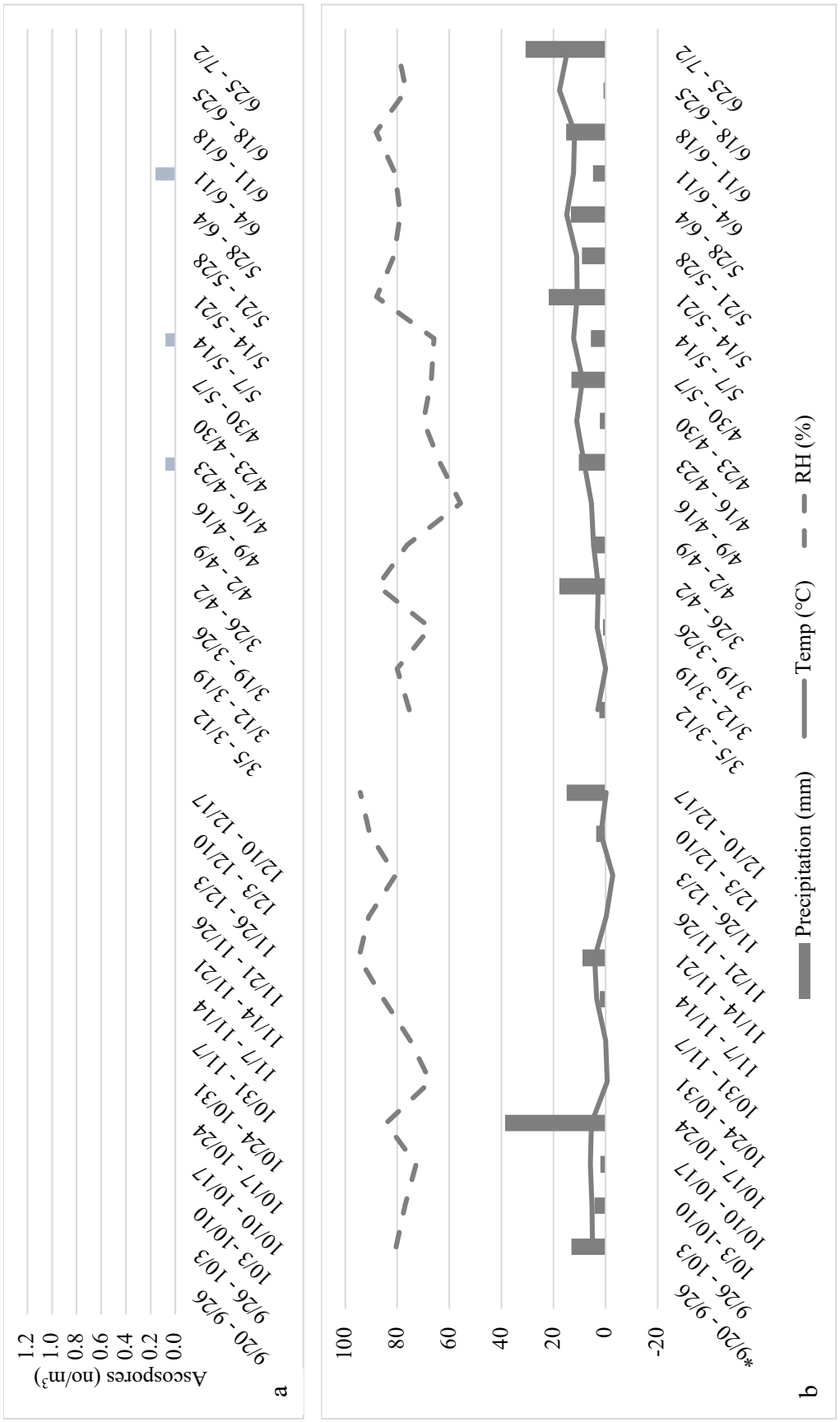

Figure 4.4: a) Weekly ascospore concentration and b) corresponding weather conditions on the Palouse (Genesee, ID) from September 2019 to July 2020. Asterisk '*' indicates weather station was not recording data.

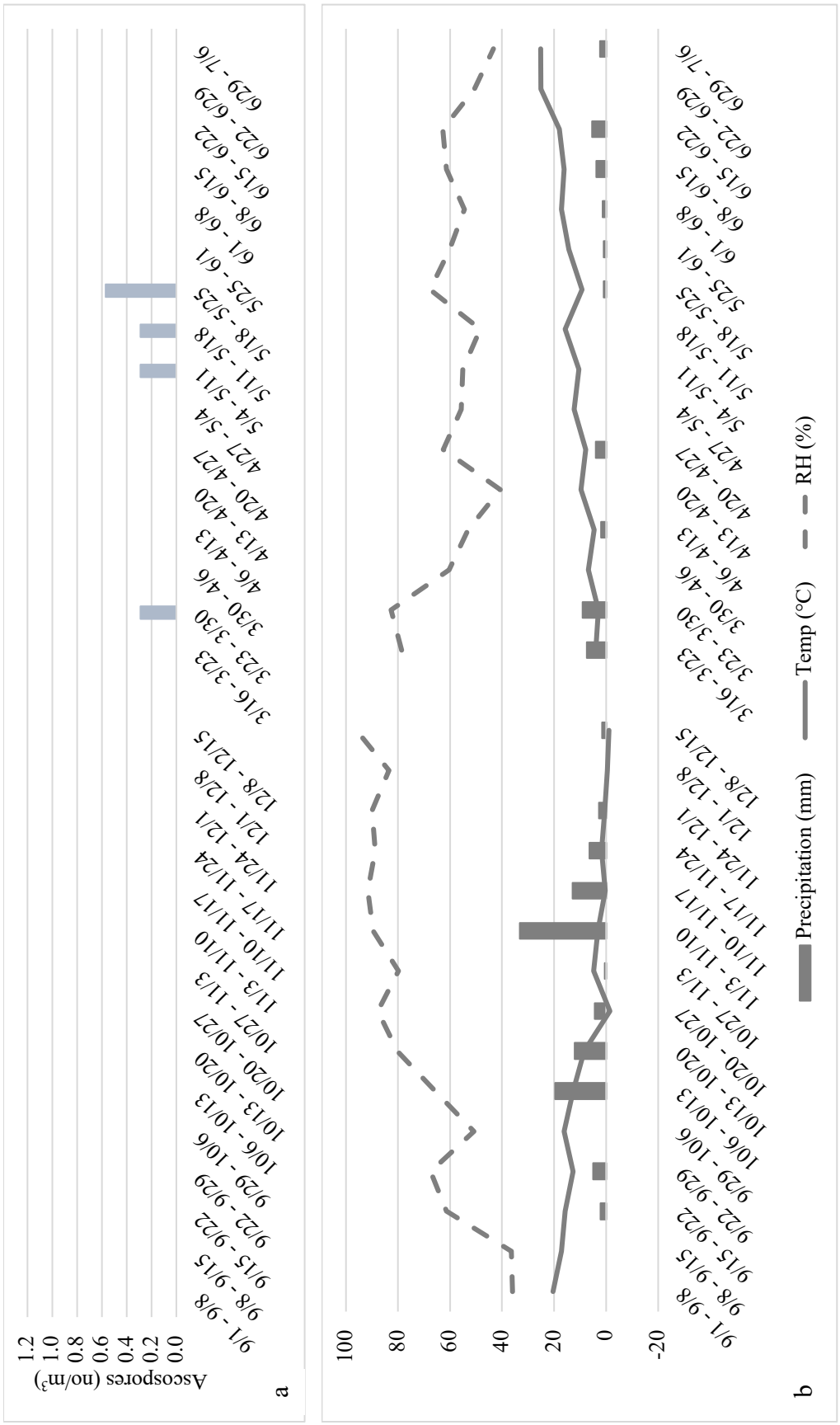

Figure 4.5: a) Weekly ascospore concentration and b) corresponding weather conditions on the Palouse (Genesee, ID) from September 2020 to July 2021.

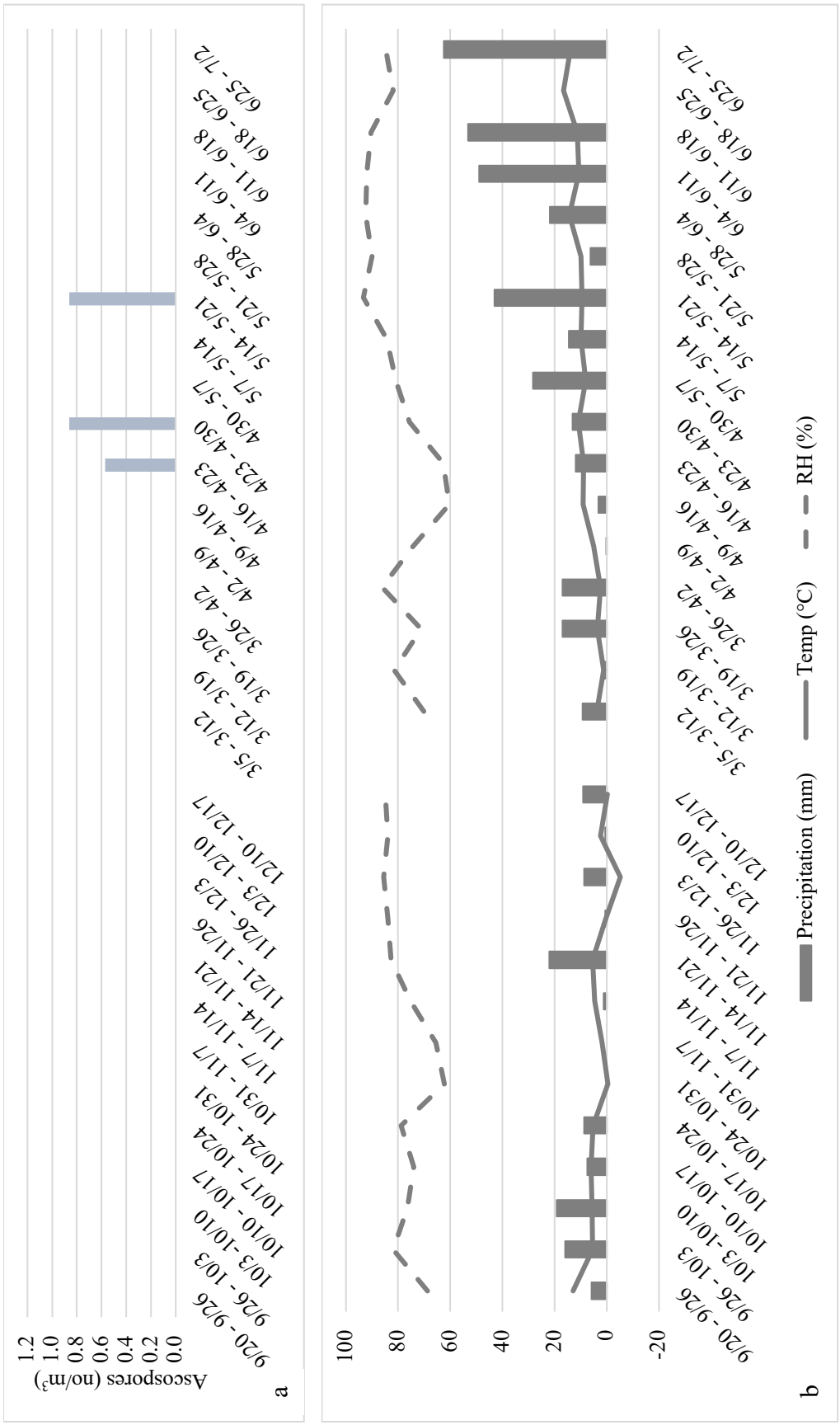

Figure 4.6: a) Weekly ascospore concentration and b) corresponding weather conditions on the Camas Prairie (Grangeville, ID) from September 2019 to July 2020.

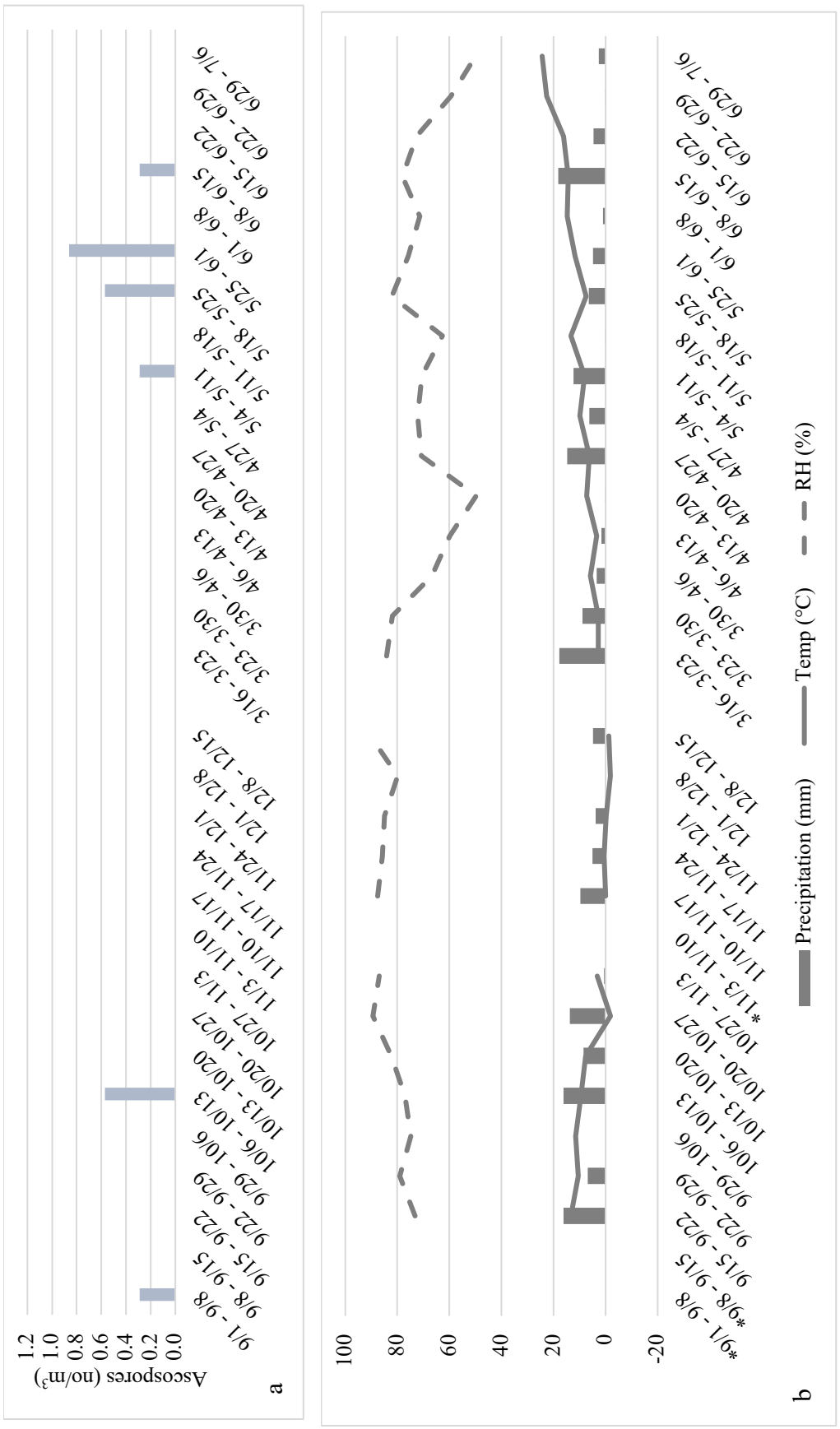

Figure 4.7: a) Weekly ascospore concentration and b) corresponding weather conditions on the Camas Prairie (Nezperce, ID) from September 2020 to July 2021. Asterisk '*' indicates weather station was not recording data.

4.4 Discussion

The epidemiology of *L. maculans* in northern Idaho is not well understood due to this pathogen being new to the region. Burkard volumetric spore traps were placed adjacent to winter canola field trials to determine time of ascospore release and weather conditions associated with release and time of the growing season that initial infection occurs. Results indicate ascospores are released in northern Idaho between March and June under average weekly temperatures of 3 to 16ºC, relative humidity of 55 to 93%, and total weekly precipitation between 0 and 43 mm. Additional ascospore release also occurred in the fall between September and October on the Camas Prairie in 2020, under similar weather conditions.

Weather conditions influence pathogen development and spore dispersal, with temperature and precipitation having the greatest impact. Spore release in northern Idaho occurs under similar weather conditions as Canada and Australia. Ascospore release in Canada occurs in June and July under temperatures of 13 to 18°C (Guo and Fernando, 2005; Zhang and Fernando, 2017). Spring canola in Canada is planted in late May, making the ascospore showers of minor concern due to the inability to cause severe stem cankers. Therefore, the crop damage and stem cankers observed in Canada are a result of conidia causing initial infection (McGee and Petrie, 1979). Differing amounts of rainfall across Australia influence spore release more than temperature. Khangura et al. (2007) identified spore release under high rainfall regions (>450 mm) occur between May and June while low rainfall areas (<325 mm) occurred later, in July and August with temperatures ranging from 13 to 18°C. Ascospores are the main source of inoculum in Australia, while conidia act as secondary infection, the opposite of what occurs in Canada (Li et al., 2007).

Northern Idaho *L. maculans* spore release and initial infection is more closely related to epidemiology of the pathogen in the UK than in Canada or Australia. Winter canola grown in the UK experiences initial infection from ascospores that are released in September and October between 5 and 24°C, leading to stem cankers observed in April and secondary infection caused by conidia (West et al., 1999; Gladders and Musa, 1980; Steed et al., 2007; Huang et al., 2005). Similar patterns of ascospore release occurs in Poland, however the Mediterranean region where weather is not as dry as the other areas of the country, experiences stem canker epidemics similar to the UK (Kaczmarek et al., 2012). Other areas of Poland will

experience spore release between September and October, but because of the dry and cold winters, leaves generally fall off before reaching the stem, thus cankers generally do not form (Huang et al., 2005). Spores were mainly released in the spring between March and June on the Palouse and Camas Prairie. Observation of ascospores in September and October in 2020 on the Camas Prairie, however, indicates that initial infection of winter canola may be caused by ascospores released in the fall, similar to what occurs in the UK and Poland.

This research has provided the beginnings of understanding the epidemiology of *L. maculans* in northern Idaho, but further work needs to be completed to gain a deeper understanding of spore release in the region. Spores were released under temperatures of 3 to 16ºC, relative humidity of 55% to 93%, and total weekly precipitation between 0 and 43 mm. However, under the same weather conditions during other weeks, no ascospores were observed. For example, spores were observed for the week of 16 April, 7 May, and 4 June on the Palouse in 2020. The remaining weeks in April and May experienced 3 to 22 mm of precipitation, temperatures between 9 and 15ºC, and relative humidity between 67 and 88%. Weather conditions conducive to spore release but no spores were observed.

Leptosphaeria maculans and *L. biglobosa* ascospores are difficult to distinguish from one another through microscopy (Shoemaker and Brun, 2001). Corresponding tape samples collected in this study will be used to quantify *L. maculans* DNA through TaqMan qPCR. Values will be compared to ascospore concentrations observed on microscope slides. This will allow for confirmation of *L. maculans* and provide ascospore concentration for other weeks in which spores were not observed, or indication of conidia causing infection during these weeks. Volumetric spore traps are made to collect air-borne particles, but if any conidia is cycled through the trap, it is difficult to identify them on the slides due to morphology (Kaczmarek et al., 2009), giving further support of qPCR to complete epidemiology studies of *L. maculans* in the region. Future work will consist of continued sampling to begin development of a disease forecasting schemes for growers to follow to ensure optimal disease prevention and control for blackleg disease of winter canola in northern Idaho.

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Chapter 5: Conclusion and Recommended Management Strategies for Blackleg Disease of Winter Canola in Northern Idaho

Several methods are in place for the management of blackleg in winter canola, including genetic resistance, chemical control, and cultural control and should be used in conjunction with each other as an integrated blackleg management program. To ensure an effective blackleg management strategy there needs to be continual monitoring of the pathogen population and how the environment and cropping system contributes to its epidemiology.

Leptosphaeria maculans isolates were collected from eastern Washington to elucidate the race structure and compare the population demographics with *L. maculans* population demographics identified in northern Idaho. The highest frequency of avirulent effector genes present in the eastern Washington *L. maculans* pathogen population are *AvrLm5* (100%)*, AvrLm6* (100%)*, AvrLm7* (100%)*, AvrLm11* (92%)*, and AvrLepR1* (100%). *Leptosphaeria maculans* isolates collected from northern Idaho were recently characterized and when compared to eastern Washington isolates, both collections have similar frequencies of avirulence genes. Within each region, *AvrLm5*, *AvrLm6, AvrLm7, AvrLm11, and AvrLepR1* are the most frequent avirulence genes present with 92 to 100% of the population possessing these genes. However, of the 18 *Rlm* genes characterized in *B. napus, B. juncea,* and *B. rapa,* this project was only able to identify 12 corresponding *AvrLm* genes. The pathogen population in this region may carry *AvrLmS, AvrLm8, AvrLm10, LepR4, BLMR1,* and *BLMR2,* but researchers do not have the knowledge until cultivars carrying these resistance genes are obtained and further host plant differentials are conducted. From this research, growers in northern Idaho and eastern Washington should select commercial cultivars carrying *Rlm5*, *Rlm6*, *Rlm7*, *Rlm11,* and *LepR1* to provide the greatest level of resistance against *L. maculans* until further differential screens can occur.

Through field trials and volumetric spore traps, timing of spore release and initial infection occurrence in winter canola was identified. Winter canola can be infected by *L. maculans* and *L. biglobosa* in both the fall and the spring. Data suggests that infection of leaves in September and October do not lead to pathogen colonization and stem canker formation, likely due to cold winters and hot dry summers. Infection between May and June, right before high temperatures are experienced in the region, gives rise to leaf and stem lesions. However, the high temperatures and low precipitation levels are not conducive for stem canker formation.

Although weather conditions may not be conducive for stem canker epidemics and yield loss of winter canola, integrated management practices should still be utilized to prevent the buildup of inoculum and potential infection of seeds. Use of Priaxor as a foliar fungicide applied to winter canola was effective in reducing blackleg in the fall and the spring. Therefore, growers in the region should be able to successfully reduce blackleg disease by applying fungicides in the spring. However, further benefit of spraying once in the fall and again in the spring may provide additional reduction in disease incidence, but additional data collection and economic evaluation is needed to provide definite guidelines to growers.

The ideal practices to follow for management of blackleg in our region include planting certified seed of resistant varieties and spraying a foliar fungicide to limit infection. These strategies should be used in conjunction with other cultural practices such as long crop rotations (3+ years) and residue management, to reduce the level of inoculum by promoting more rapid breakdown of infested residues. Blackleg is currently a minor problem in north Idaho canola production, but not practicing proper disease management methods could eventually lead to large disease outbreaks and extreme crop damage and yield loss, like epidemics previously seen in Canada and Australia.

This thesis has focused on understanding the biology and epidemiology of blackleg disease specific to northern Idaho along with focusing on blackleg management strategies in winter canola. While the majority of infected canola plants observed in northern Idaho have been winter seeded, spring canola is the major type grown in northern Idaho, so it would be beneficial to conduct similar fungicide studies in spring canola. Crop rotation and stubble management studies may also prove beneficial to investigate blackleg incidence in both winter and spring canola. Priaxor was used in this research, however many growers in northern Idaho use other, less expensive fungicides. Therefore, fungicide efficacy trials have been established to identify which fungicides available to growers will provide the greatest protection against blackleg in winter canola.

Appendix A: Eastern Washington *Leptosphaeria maculans* Isolate Collection

95 of *L. maculans* isolates collected from blackleg infected canola plants across the Palouse region of eastern Washington were genetically characterized through PCR and host plant differentials to identify *Avr* and mating type genes present in the population.

Table A.1: Collection site and genetic characterization of eastern Washington *Leptosphaeria maculans* isolates.

Isolate	County	Town	Greenhouse Race	PCR Race	Final Race	MAT
319015	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2-LepR3	1.2
319017	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2-LepR3	1.2
319018	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2-LepR3	1.2
319019	Adams	Ralston	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2	1.2
319021	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6	5-6-7-LepR1-LepR2-LepR3	1.2
319023	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6	3-5-6-7-LepR1-LepR2-LepR3	1.2
319025	Adams	Ralston	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319026	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319027	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319028	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319029	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319030	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319031	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319032	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319033	Adams	Ralston	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319034	Adams	Ralston	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319035	Adams	Ralston	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1

Table A.1 continued.

319036	Adams	Ralston	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319078	Adams	Ralston	4-(5-6)-7-LepR1	(4-7)-5-6-11	4-5-6-7-11-LepR1	1.1
319084	Adams	Ralston	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319085	Adams	Ralston	(5-6)-7-LepR1	3-(4-7)-5-6-11	3-5-6-7-11-LepR1	1.1
319087	Adams	Ralston	(5-6)-7-9-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-9-11-LepR1-LepR2	1.1
319089	Adams	Ralston	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319093	Adams	Ralston	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319095	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319097	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319099	Adams	Ritzville	4-(5-6)-7-LepR1	(4-7)-5-6-11	4-5-6-7-11-LepR1	1.1
319101	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2	1.1
319102	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2	1.1
319103	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319104	Adams	Ritzville	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319105	Adams	Ritzville	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319106	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2	1.1
319107	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319108	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319122	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319123	Adams	Ritzville	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319124	Adams	Ritzville	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319125	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319128	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319129	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319131	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1

Table A.1 continued.

319134	Adams	Ritzville	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319136	Lincoln	Odessa	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319138	Lincoln	Odessa	4-(5-6)-7-LepR1-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR3	1.1
319140	Lincoln	Odessa	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319141	Lincoln	Odessa	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319142	Lincoln	Odessa	4-(5-6)-7-9-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-9-11-LepR1-LepR2-LepR3	1.1
319143	Lincoln	Odessa	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.1
319144	Lincoln	Odessa	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319145	Lincoln	Odessa	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319149	Lincoln	Odessa	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319151	Adams	Ritzville	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319169	Latah	Genesee	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319170	Latah	Genesee	1-4-(5-6)-7-LepR1-LepR2-LepR3	1-3-(4-7)-5-6	1-3-4-5-6-7-LepR1-LepR2-LepR3	1.1
319171	Latah	Genesee	1-4-(5-6)-7-LepR1-LepR2-LepR3	1-3-(4-7)-5-6	1-3-4-5-6-7-LepR1-LepR2-LepR3	1.1
319172	Latah	Genesee	4-(5-6)-7-LepR1	(4-7)-5-6-11	4-5-6-7-11-LepR1	1.1
319173	Latah	Genesee	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319174	Latah	Genesee	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319175	Lincoln	Odessa	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319179	Lincoln	Odessa	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.1
319180	Lincoln	Odessa	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.2
319181	Lincoln	Odessa	4-(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2	1.2
319182	Lincoln	Odessa	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.2
319184	Garfield	Pomeroy	(5-6)-7-LepR1	(4-7)-5-6-11	5-6-7-11-LepR1	1.2
319185	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2	1.2
319186	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.1

Table A.1 continued.

319187	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319188	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319190	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.1
319191	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319192	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.2
319193	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.2
319195	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.2
319196	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.2
319197	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.2
319198	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.2
319199	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.2
319200	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2	1.2
319201	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.2
319202	Garfield	Pomeroy	4-(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2-LepR3	1.2
319204	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319205	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.1
319211	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.2
319214	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.2
319215	Adams	Ritzville	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319218	Spokane	Fairfield	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319220	Adams	Ritzville	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319223	Adams	Ritzville	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319225	Adams	Ritzville	4-(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	4-5-6-7-11-LepR1-LepR2-LepR3	1.1
319230	Spokane	Fairfield	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2
319233	Spokane	Fairfield	(5-6)-7-LepR1-LepR2-LepR3	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2-LepR3	1.2

Table A.1 continued.

319234	Spokane	Fairfield	(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-5-6-7-11-LepR1-LepR2	1.2
319237	Spokane	Fairfield	4-(5-6)-7-LepR1-LepR2	3-(4-7)-5-6-11	3-4-5-6-7-11-LepR1-LepR2	1.2
319247	Garfield	Pomeroy	(5-6)-7-LepR1-LepR2-LepR3	(4-7)-5-6-11	5-6-7-11-LepR1-LepR2-LepR3	1.2

Appendix B: Analysis of Variance for Winter Canola Field Trials

Collected data was used for a combined analysis of treatment on response variables. Data was analyzed using the general linear model (GLM) procedure in SAS (SAS studio, online, 3.8 enterprise edition) to estimate any missing values in the analysis of variance. Within the analysis of variance, the combined effect of cultivars and disease treatments using orthogonal contrasts into 1) difference between cultivars; 2) difference between seed treatment and no seed treatment; 3) difference between no spray and spray; 4) difference between one spray and two sprays; 5) difference between fall spray and spring spray; 6) interaction between contrasts 2 and 3; 7) interaction between contrast 2 and 4; 8) interaction between contrast 2 and 5; 9) interaction between contrast 1 and 2; 10) interaction between contrast 1 and 3; 11) interaction between contrast 1 and 4; 12) interaction between contrast 1 and 5; 13) interaction between contrast 1 and 6; 14) interaction between contrast 1 and 7; and 15) interaction between contrast 1 and 8, each with one degree of freedom.

Analysis of variance (ANOVA) was conducted over each individual location using GLM procedure in SAS. Significant differences between treatment means were identified using Fisher's least significant difference with an alpha value of 0.05.

Table B.1: Mean squares from the analysis of variance of fall plant stand, spring plant stand, plant height, blackleg disease severity and incidence, seed yield, seed oil content and seed protein recorded on two canola cultivars grown in three environments over two years.

Source	df	Fall Stand	Spring Stand	Height	Disease Severity	Disease Incidence	Yield	Oil	Protein
Year	1	1,136.3	4,167.1***	2,630.3***	3.060***	7,632.5***	26,006,235***	5,120.67***	1,418.05***
Error (1)	6	1,250.3*	674.9**	54.0	0.032	62.9	650,414*	3.58*	1.53
Site within year	4	13,113.3***	16,822.4***	12,810.6***	2.308***	5,272.2***	128,192,079***	301.68***	129.42***
Error (2)	11	523.2	454.6*	108.7**	0.016	132.9*	1,201,133***	3.69**	4.08***
CV & T	15	644.7	429.5**	30.5	0.104***	1,347.2***	1,639,194***	30.40***	3.55***
CV & T* (S w Y)	75	523.1	236.4	30.0	0.092***	340.4***	524,225***	1.95	1.03
Pooled Replicate Error	241	449.2	197.5	36.0	0.019	68.9	260,575	1.50	0.78

CV: cultivar
T: seed treatment and spray timing
Error (1) = Rep w Year
Error (2) = Rep * (Site w Year)

* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.2: Grangeville 2020 mean squares from analysis of variance for fall and spring stand, plant height, disease incidence and severity, yield, and estimated seed oil and protein content.

Source	df	Fall Stand	Spring Stand	Height	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	1,251.4	13.1	56.3	1,950.9***	0.46**	10,507,322.3***	235.24***	42.41***
Error (1)	3	421.3	96.9	19.8	230.4	0.26*	495,517.4	5.60**	0.61
ST	1	97.5	0.4	1.6	84.0	0.01	8,281.0	0.38	0.41
SP	3	768.3	206.3	63.5	9,352.5***	2.83***	303,698.5	0.90	0.31
ST*SP	3	288.2	19.6	31.8	159.9	0.03	489,096.3	0.98	0.24
C*ST	1	523.3	11.4	1.6	200.8	0.02	1,239,882.3*	8.63*	3.11*
C*SP	3	437.6	45.9	65.6	130.4	0.13	142,960.3	0.98	0.14
C*ST*SP	3	123.9	5.1	11.0	269.2	0.04	311,298.9	1.14	0.07
Error (2)	42	480.0	85.9	37.9	150.4	0.08	292,796.1	1.39	0.55

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.3: Genesee 2020 mean squares from analysis of variance for fall and spring stand, plant height, flowering, disease incidence and severity, yield, and estimated seed oil and protein content.

	df	Fall Stand	Spring Stand	Height	Flowering	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	5,513.1**	1,359.8**	1.6	109.0***	117.3*	0.01	1,602,439.5*	76.34***	0.10
Error (1)	3	996.7	336.8	38.0	2.4***	6.2	0.00	917,679.1*	5.30*	2.04*
ST	1	798.1	112.9	76.6	0.5	17.4	0.00	1,246,851.4*	6.96*	1.03
SP	3	233.3	197.4	19.8	0.1	467.3**	0.01**	288,503.8	0.21	0.11
ST*SP	3	917.3	336.9	38.0	0.3	8.1	0.00	369,810.9	2.17	0.29
C*ST	1	637.6	178.9	6.3	0.0	0.7	0.00	300,989.4	4.05	1.47
C*SP	3	1010.4	284.6	27.6	0.0	30.3	0.01*	699,746.9	0.89	0.29
C*ST*SP	3	751.1	418.2	49.0	0.4	17.4	0.00	259,972.8	4.50*	1.97*
Error (2)	42	533.3	172.0	38.7	0.3	83.8	0.01	283,845.5	1.47	0.58

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.4: Moscow 2020 mean squares from analysis of variance for fall and spring stand, plant height, disease incidence and severity, yield, and estimated seed oil and protein content.

	df	Fall Stand	Spring Stand	Height	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	833.8	3,249.0***	47.3	3,306.3***	0.00	4,154.1	123.49***	9.46***
Error (1)	3	208.5	127.3	113.9*	104.4	0.00	188,358.9	1.72	0.71
ST	1	192.5	25.0	47.3	0.7	0.00	24,414.1	0.13	0.01
SP	3	192.4	6.0	22.3	1,441.3***	0.01	416,798.4	0.06	0.07
ST*SP	3	91.9	138.2	18.1	47.00	0.01	42,920.1	0.28	0.08
C*ST	1	0.0	100.0	31.6	56.3	0.00	276,150.3	0.04	0.20
C*SP	3	585.7	162.5	1.4	341.4*	0.01	553,326.2	0.51	0.07
C*ST*SP	3	240.9	394.0*	6.6	21.1	0.00	333,730.2	1.62	0.61
Error (2)	42	285.3	141.1	37.9	94.4	0.01	195,803.1	0.71	0.25

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.5: Nezperce 2021 mean squares from analysis of variance for fall and spring stand, plant height, flowering, disease incidence and severity, yield, and estimated seed oil and protein content.

	df	Fall Stand	Spring Stand	Height	Flowering	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	530.4	78.7	58.5*	227.2***	1.2	0.00	12,774,876.7***	60.41***	44.27***
Error (1)	3	99.9	165.9	100.2***	0.3	45.5	0.00	1,516,605.8***	6.50	12.56***
ST	1	378.9	361.1	15.5	0.0	7.5	0.00	9,368.1	2.89	1.65
SP	3	49.1	12.9	34.8*	0.2	75.7*	0.00	236,451.7	2.37	2.12
ST*SP	3	78.1	40.4	4.1	0.2	6.9	0.00	57,399.7	1.15	0.98
C*ST	1	297.3	197.7	15.5	0.2	0.3	0.00	116,563.4	0.94	0.68
C*SP	3	293.2	114.1	30.8	0.1	0.6	0.00	84,445.1	3.78	0.91
C*ST*SP	3	2.8	55.2	27.8	0.1	56.7	0.00	461,569.8*	0.39	0.63
Error (2)	41	377.9	151.7	11.4	0.4	21.8	0.00	118,992.0	2.63	1.50

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.6: Genesee 2021 mean squares from analysis of variance for fall and spring stand, plant height, flowering, disease incidence and severity, yield, and estimated seed oil and protein content.

	df	Fall Stand	Spring Stand	Height	Flowering	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	82.2	4.7	8.6	58.4***	917.9***	0.00	2,778,123.5	16.95***	1.36
Error (1)	2	6.2	81.5	119.8	0.3	27.6	0.00	10,449.2	1.64*	1.39
ST	1	355.1	116.7	62.8	0.1	30.8	0.00	37,383.7	1.20	0.00
SP	3	1,275.2	822.0	81.9	0.1	692.7***	0.00	325,956.5	1.62*	0.63
ST*SP	3	554.5	133.0	49.8	0.1	69.3	0.00	85,681.8	1.94*	0.27
C*ST	1	3,685.9	307.8	84.0	0.2	15.1	0.00	1,121,347.3	0.64	1.18
C*SP	3	500.7	532.6	9.0	0.1	156.6*	0.00	310,477.6	0.48	0.20
C*ST*SP	3	389.3	175.8	26.0	0.1	345.2**	0.00	73,076.1	0.68	0.02
Error (2)	15	739.9	500.1	43.3	0.6	47.4	0.00	645,893.0	0.46	0.41

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.7: Moscow 2021 mean squares from analysis of variance for fall and spring stand, plant height, flowering, disease incidence and severity, yield, and estimated seed oil and protein content.

	df	Fall Stand	Spring Stand	Height	Flowering	Disease Incidence	Disease Severity	Yield	Oil	Protein
C	1	689.1	625.0	126.6	310.6***	0.00	0.00	12,643,287.0***	47.61***	0.85
Error (1)	2	1,700.7**	822.8*	55.7	1.7*	0.00	0.00	828,240.7***	0.95	0.59
ST	1	0.1	2.3	14.1	0.0	0.00	0.00	21,959.5	0.17	0.13
SP	2	212.0	143.9	9.4	1.1	0.00	0.00	304,182.6*	0.38	0.10
ST*SP	2	1,571.9**	1,194.7**	10.9	0.6	0.00	0.00	84,531.1	1.41	0.37
C*ST	1	12.3	105.1	100.0	0.0	0.00	0.00	9,867.4	0.15	0.38
C*SP	2	765.2	398.0	10.9	0.2	0.00	0.00	156,620.4	1.33	0.52
C*ST*SP	2	92.7	373.4	19.8	0.3	0.00	0.00	139,503.2	0.56	0.17
Error (2)	42	400.5	293.3	36.3	0.5	0.00	0.00	93,541.7	0.82	0.51

C: cultivar
Error (1): Cultivar*Replication
ST: seed treatment
SP: spray timing
Error (2): pooled replicate error
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

Table B.8: Impact of cultivar, seed treatment, and spray timing on fall plant density of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- plants m^{-2} ----------					
Mercedes	43[a]	59[a]	82[a]	76[a]	69[a]	65[a]
Amanda	52[a]	78[a]	89[a]	82[a]	69[a]	71[a]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- plants m^{-2} ----------					
Clothianidin	46[a]	72[a]	87[a]	77[a]	74[a]	68[a]
Helix	49[a]	65[a]	84[a]	82[b]	65[a]	68[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- plants m^{-2} ----------					
None	52[a]	66[a]	81[a]	79[a]	51[a]	64[a]
Fall	51[a]	74[a]	89[a]	77[a]	72[ab]	71[a]
Spring	37[b]	69[a]	84[a]	79[a]	82[b]	71[a]
Both	49[ab]	66[a]	87[a]	82[b]	69[ab]	66[a]

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.9: Impact of cultivar, seed treatment, and spray timing on spring plant density of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	plants m^{-2}					
Mercedes	28[a]	38[a]	63[a]	47[a]	52[a]	61[a]
Amanda	29[a]	47[a]	77[b]	49[a]	52[a]	67[a]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	plants m^{-2}					
Clothianidin	28[a]	44[a]	71[a]	50[a]	55[a]	64[a]
Helix	28[a]	41[a]	70[a]	46[a]	50[a]	64[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	plants m^{-2}					
None	27[a]	40[a]	71[a]	46[a]	36[a]	60[a]
Fall	33[b]	48[a]	70[a]	49[a]	54[ab]	67[a]
Spring	24[c]	40[a]	70[a]	48[a]	60[b]	65[a]
Both	30[d]	42[a]	70[a]	49[a]	56[ab]	65[a]

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.10: Impact of cultivar, seed treatment, and spray timing on plant height of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- cm ----------					
Mercedes	171[a]	172[a]	140[a]	148[a]	160[a]	161[a]
Amanda	173[a]	172[a]	138[a]	146[a]	161[a]	158[a]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- cm ----------					
Clothianidin	172[a]	171[a]	138[a]	147[a]	163[a]	159[a]
Helix	172[a]	173[a]	140[a]	148[a]	159[a]	160[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- cm ----------					
None	173[a]	171[a]	138[a]	147[a]	157[a]	158[a]
Fall	173[a]	171[a]	140[a]	145[a]	163[a]	158[a]
Spring	169[b]	173[a]	139[a]	148[a]	160[a]	159[a]
Both	172[ab]	172[a]	139[a]	149[a]	162[a]	160[a]

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.11: Impact of cultivar, seed treatment, and spray timing on flowering date of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- Julian Day ----------					
Mercedes	---	128[a]	---	134[a]	130[a]	123[a]
Amanda		131[b]		138[b]	133[b]	128[b]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- Julian Day ----------					
Clothianidin	----	129[a]	---	136[a]	131[a]	126[a]
Helix		129[a]		136[a]	132[a]	126[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- Julian Day ----------					
None	---	129[a]	---	136[a]	131[a]	125[a]
Fall		129[a]		136[a]	132[a]	126[a]
Spring		129[a]		136[a]	132[a]	125[a]
Both		129[a]		137[a]	132[a]	126[a]

Means followed by the same letter within each column are not significantly different from each other using LSD ($p < 0.05$).

Table B.12: Impact of cultivar, seed treatment, and spray timing on blackleg disease incidence in winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	% disease					
Mercedes	22[a]	3[a]	4[a]	3[a]	5[a]	0
Amanda	33[a]	6[b]	19[b]	3[a]	15[b]	0

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	% disease					
Clothianidin	29[a]	4[a]	11[a]	3[a]	10[a]	0
Helix	27[a]	5[a]	12[a]	4[a]	9[a]	0

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	% disease					
None	61.25[a]	12.50[a]	25.42[a]	6.67[a]	23.81[a]	0
Fall	28.33[b]	2.92[b]	7.50[b]	2.08[a]	10.00[b]	0
Spring	16.25[bc]	1.25[b]	7.92[b]	2.50[a]	5.83[b]	0
Both	5.42[c]	1.25[b]	4.58[b]	1.67[a]	1.33[b]	0

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.13: Impact of cultivar, seed treatment, and spray timing on blackleg disease severity of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020
	---------- severity scale 0 to 5, with 5 most severe----------		
Mercedes	0.6[a]	<0.1[a]	0.1[a]
Amanda	0.4[a]	<0.1[a]	<0.1[a]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020
	---------- severity scale 0 to 5, with 5 most severe----------		
Clothianidin	0.5[a]	<0.1[a]	0.1[a]
Helix	0.5[a]	<0.1[a]	<0.1[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020
	---------- severity scale 0 to 5, with 5 most severe----------		
None	1.02[a]	0.1[a]	0.1[a]
Fall	0.49[b]	<0.1[ab]	0.1[a]
Spring	0.41[b]	<0.1[b]	0.1[a]
Both	0.11[c]	<0.1[b]	<0.1[a]

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.14: Impact of cultivar, seed treatment, and spray timing on yield of winter canola in northern Idaho.

Cultivar	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- kg ha^{-1}----------					
Mercedes	7,714[a]	4,231[a]	3,924[a]	4,117[a]	5,869[a]	5,222[a]
Amanda	6,904[b]	4,548[a]	3,873[a]	3,210[a]	4,986[b]	4,333[b]

Seed Treatment	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- kg ha^{-1}----------					
Clothianidin	7,321[a]	4,529[a]	3,879[a]	3,690[a]	5,497[a]	4,759[a]
Helix	7,298[a]	4,250[a]	3,918[a]	3,652[a]	5,442[a]	4,796[a]

Spray Timing	Grangeville 2020	Genesee 2020	Moscow 2020	Nezperce 2021	Genesee 2021	Moscow 2021
	---------- kg ha^{-1}----------					
None	7,186[a]	4,323[a]	3,692[a]	3,584[a]	5,484[a]	4,593[a]
Fall	7,195[a]	4,536[a]	4,022[a]	3,735[a]	5,244[a]	4,859[a]
Spring	7,439[a]	4,238[a]	3,849[a]	3,556[a]	5,512[a]	4,753[a]
Both	7,417[a]	4,462[a]	4,032[a]	3,801[a]	5,636[a]	4,903[a]

Means followed by the same letter within each column are not significantly different from each other using Fisher's LSD ($p < 0.05$).

Table B.15: Impact of cultivar, seed treatment, and spray timing on estimated seed oil content of winter canola in northern Idaho.

	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Cultivar	2020	2020	2020	2021	2021	2021
	---------- estimated % oil ----------					
Mercedes	47.7[a]	43.8[a]	48.2[a]	35.3[a]	40.1[a]	38.7[a]
Amanda	43.8[b]	41.6[b]	45.4[b]	33.4[b]	38.4[b]	37.0[b]

Seed	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Treatment	2020	2020	2020	2021	2021	2021
	---------- estimated % oil ----------					
Clothianidin	45.7[a]	43.0[a]	46.8[a]	34.6[a]	39.2[a]	37.9[a]
Helix	45.8[a]	42.4[a]	46.9[a]	34.1[a]	39.3[a]	37.8[a]

Spray	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Timing	2020	2020	2020	2021	2021	2021
	---------- estimated % oil ----------					
None	45.7[a]	42.6[a]	46.7[a]	34.8[a]	38.8[a]	38.1[a]
Fall	45.7[a]	42.8[a]	46.8[a]	34.1[ab]	39.3[a]	37.9[a]
Spring	45.5[a]	42.6[a]	46.9[a]	34.6[a]	39.7[a]	37.7[a]
Both	46.1[a]	42.7[a]	46.9[a]	33.9[b]	39.2[a]	37.8[a]

Means followed by the same letter within each column are not significantly different from each other using LSD ($p < 0.05$).

Table B.16: Impact of cultivar, seed treatment, and spray timing on estimated seed protein content of winter canola in northern Idaho.

	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Cultivar	2020	2020	2020	2021	2021	2021
	---------- estimated % protein ----------					
Mercedes	18.5[a]	20.9[a]	17.3[a]	24.0[a]	23.0[a]	22.2[a]
Amanda	20.1[b]	20.8[a]	18.0[b]	25.7[b]	23.4[a]	22.5[a]

Seed	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Treatment	2020	2020	2020	2021	2021	2021
	---------- estimated % protein ----------					
Clothianidin	19.4[a]	20.7[a]	17.6[a]	24.6[a]	23.1[a]	22.4[a]
Helix	19.2[a]	21.0[a]	17.7[a]	25.0[a]	23.3[a]	22.3[a]

Spray	Grangeville	Genesee	Moscow	Nezperce	Genesee	Moscow
Timing	2020	2020	2020	2021	2021	2021
	---------- estimated % protein ----------					
None	19.2[a]	20.9[a]	17.6[a]	24.5[a]	23.4[a]	22.2[a]
Fall	19.4[a]	20.9[a]	17.6[a]	24.8[a]	23.4[ab]	22.3[a]
Spring	19.4[a]	20.8[a]	17.6[a]	24.5[a]	22.8[c]	22.4[a]
Both	19.2[a]	20.7[a]	17.7[a]	25.3[a]	23.2[b]	22.4[a]

Means followed by the same letter within each column are not significantly different from each other using LSD ($p < 0.05$).

Appendix C: *Leptosphaeria* Species Ascospore Concentrations in Northern Idaho

Number of ascospores per m^3 from microscope slides and corresponding weather data from 2019 to 2021 on the Palouse and Camas Prairie, partitioned into weekly and monthly data sets. Precipitation is a weekly or monthly total. Temperature and relative humidity are a weekly or monthly average. Time refers to when the drum was exchanged on the first day of the week.

Table C.1: Weekly ascospore concentrations and weather conditions for the Palouse (Genesee, ID) from September 2019 to July 2021.

Date	Time	Precipitation (mm)	Temp (°C)	RH (%)	Ascospore Concentration
9/20/19	10:00 AM	*	*	*	0
9/26/19	2:00 PM	13.21	5.12	80.46	0
10/3/19	1:15 PM	4.32	5.20	77.08	0
10/10/19	2:20 PM	2.03	5.94	72.82	0
10/17/19	1:32 PM	38.61	5.51	84.39	0
10/24/19	1:21 PM	0	-0.71	67.43	0
10/31/19	1:25 PM	0	0.01	74.99	0
11/7/19	1:20 PM	2.29	3.42	85.3	0
11/14/19	1:17 PM	8.89	4.30	94.81	0
11/21/19	1:00 PM	0	-0.24	90.97	0
11/26/19	10:49 AM	0	-2.76	80.79	0
12/3/19	1:05 PM	3.56	1.73	90.71	0
12/10/19	11:45 AM	14.99	-0.30	94.27	0
**					
3/5/20	10:00 AM	2.51	3.00	75.37	0
3/12/20	10:30 AM	0.25	-0.01	80.06	0
3/19/20	11:28 AM	1.02	3.24	67.25	0
3/26/20	11:26 AM	17.71	2.72	87.63	0
4/2/20	9:38 AM	3.79	4.69	76.28	0
4/9/20	11:11 AM	0	5.45	55.72	0
4/16/20	10:26 AM	10.39	8.34	63.78	0.57
4/23/20	10:11 AM	2.27	11.08	69.93	0
4/30/20	10:16 AM	13.2	8.97	66.93	0
5/7/20	9:35 AM	5.56	12.28	65.84	0.57
5/14/20	10:15 AM	21.88	10.83	87.95	0
5/21/20	9:50 AM	9	11.04	81.36	0
5/28/20	9:53 AM	13.37	15.05	78.82	0

6/4/20	9:51 AM	4.76	12.26	80.95	1.14
6/11/20	9:35 AM	15.16	11.88	88.27	0
6/18/20	8:58 AM	0.75	17.72	76.73	0
6/25/20	9:09 AM	30.7	14.56	79.90	0
**					
9/1/20	9:13 AM	0.00	20.44	35.99	0
9/8/20	8:45 AM	0.00	17.17	36.41	0
9/15/20	8:25 AM	2.27	15.72	61.42	0
9/22/20	8:53 AM	5.07	12.66	67.46	0
9/29/20	10:30 AM	0.00	16.14	50.92	0
10/6/20	8:45 AM	19.54	12.70	65.69	0
10/13/20	8:56 AM	12.17	8.48	80.19	0
10/20/20	8:45 AM	4.31	-1.44	87.83	0
10/27/20	8:30 AM	0.50	4.79	79.93	0
11/3/20	8:13 AM	33.23	3.27	89.61	0
11/10/20	8:36 AM	12.94	0.39	91.54	0
11/17/20	8:30 AM	6.33	1.69	88.86	0
11/24/20	8:45 AM	2.76	0.58	89.91	0
12/1/20	8:40 AM	0.00	-0.39	83.53	0
12/8/20	8:21 AM	1.52	-1.12	95.98	0
**					
3/16/21	10:00 AM	7.35	4.06	78.57	0
3/23/21	10:02 AM	9.11	3.03	82.68	0.29
3/30/21	9:42 AM	0.00	6.85	60.39	0
4/6/21	9:47 AM	2.02	4.56	53.14	0
4/13/21	10:17 AM	0.00	9.77	40.61	0
4/20/21	8:20 AM	4.03	7.93	62.60	0
4/27/21	9:09 AM	0.00	12.34	55.71	0
5/4/21	8:43 AM	0.00	10.57	55.13	0.29
5/11/21	8:48 AM	0.00	15.69	48.34	0.29
5/18/21	8:50 AM	1.00	9.27	67.70	0.57
5/25/21	8:45 AM	1.01	14.33	60.13	0
6/1/21	9:58 AM	1.27	17.25	54.40	0
6/8/21	8:58 AM	3.78	16.22	61.36	0
6/15/21	8:42 AM	5.32	18.00	62.79	0
6/22/21	8:47 AM	0.00	25.00	50.61	0
6/29/21	11:50 AM	2.29	25.17	43.25	0

*weather station not collecting data
**break for winter and summer months

Table C.2: Weekly ascospore concentrations and weather conditions for the Camas Prairie (Grangeville or Nezperce, ID) from September 2019 to July 2021.

Date	Time	Precipitation (mm)	Temp (°C)	RH (%)	Ascospore Concentration
9/20/19	5:00 PM	5.84	13.01	68.84	0
9/26/19	4:00 PM	16.00	5.42	81.88	0
10/3/19	3:30 PM	19.30	5.68	76.43	0
10/10/19	3:58 PM	7.37	6.02	74.00	0
10/17/19	3:13 PM	8.64	5.22	78.85	0
10/24/19	3:03 PM	0.00	-0.44	62.28	0
10/31/19	3:05 PM	0.00	1.79	65.61	0
11/7/19	3:00 PM	1.27	4.62	75.25	0
11/14/19	3:06 PM	22.10	5.41	82.76	0
11/21/19	2:54 PM	0.51	0.43	83.96	0
11/26/19	12:36 PM	8.64	-5.20	85.64	0
12/3/19	2:50 PM	0.51	2.39	83.88	0
12/10/19	1:17 PM	9.14	-0.18	84.95	0
**					
3/5/20	1:00 PM	9.38	3.85	70.21	0
3/12/20	12:58 PM	1.52	1.28	81.47	0
3/19/20	1:20 PM	17.00	3.60	71.20	0
3/26/20	5:19 PM	16.95	2.43	86.21	0
4/2/20	11:15 AM	0.25	5.13	74.17	0
4/9/20	11:11 AM	3.29	9.08	60.39	0
4/16/20	12:36 PM	11.92	8.79	62.71	0.57
4/23/20	11:56 AM	13.19	10.89	75.66	0.86
4/30/20	12:30 PM	28.40	8.10	80.90	0
5/7/20	11:56 AM	14.46	9.90	84.30	0
5/14/20	12:36 PM	43.10	9.52	93.33	0.86
5/21/20	12:00 PM	6.34	9.91	89.93	0
5/28/20	11:46 AM	21.80	14.35	92.35	0
6/4/20	11:16 AM	48.97	10.74	92.10	0
6/11/20	11:33 AM	53.22	11.39	90.32	0
6/18/20	10:55 AM	0.00	16.59	81.85	0
6/25/20	10:44 AM	62.47	13.94	84.65	0
**					
9/1/20	11:16 AM	*	*	*	0.29
9/8/20	10:15 AM	*	*	*	0

9/15/20	10:06 AM	16.21	13.24	73.12	0
9/22/20	10:46 AM	6.86	10.52	79.11	0
9/29/20	12:09 PM	0.00	11.43	74.79	0
10/6/20	10:22 AM	16.22	9.57	77.00	0.57
10/13/20	10:26 AM	8.57	7.76	82.34	0
10/20/20	10:18 AM	13.69	-1.88	89.36	0
10/27/20	9:53 AM	0.50	3.21	86.94	0
11/3/20	9:53 AM	*	*	*	0
11/10/20	10:15 AM	9.65	-0.08	87.66	0
11/17/20	11:20 AM	5.06	0.61	85.82	0
11/24/20	10:21 AM	3.80	-0.19	84.98	0
12/1/20	10:16 AM	0.00	-1.87	79.93	0
12/8/20	9:45 AM	4.82	-1.23	90.33	0
**					
3/16/21	11:35 AM	17.74	2.89	84.16	0
3/23/21	11:28 AM	8.85	2.78	81.91	0
3/30/21	11:20 AM	3.52	5.86	66.64	0
4/6/21	11:37 AM	1.51	3.48	59.78	0
4/13/21	11:53 AM	0.25	7.27	49.73	0
4/20/21	9:43 AM	14.70	6.27	70.79	0
4/27/21	10:36 AM	6.35	9.95	72.29	0
5/4/21	10:12 AM	12.42	8.08	70.66	0.29
5/11/21	1:39 AM	0.25	13.24	62.72	0
5/18/21	10:45 AM	6.53	7.51	82.05	0.57
5/25/21	10:15 AM	4.81	11.71	75.75	0.86
6/1/21	11:28 AM	1.01	14.82	71.33	0
6/8/21	10:32 AM	18.27	14.35	78.09	0.29
6/15/21	10:11 AM	4.57	16.15	72.56	0
6/22/21	10:13 AM	0.25	22.72	59.45	0
6/29/21	8:38 AM	2.54	24.38	49.95	0

*weather station not collecting data

**break for winter and summer months

Table C.3: Monthly ascospore concentrations and weather conditions for the Palouse (Genesee, ID) from September 2019 to July 2021.

Month	Rain (mm)	Temperature (°C)	Relative Humidity (%)	No. Ascospores/m^3
Sep ‘19	13.21	5.87	82.24	0
Oct ‘19	44.96	3.89	75.37	0
Nov ‘19	11.18	1.17	85.27	0
Dec ‘19	18.54	0.54	91.53	0
Jan ‘20	**			
Feb ‘20	**			
Mar ‘20	20.99	2.37	76.69	0
Apr ‘20	16.95	7.05	67.69	0.57
May ‘20	52.40	11.51	76.17	0.57
Jun ‘20	61.98	14.18	81.01	1.14
Jul ‘20	**			
Aug ‘20	**			
Sep ‘20	7.34	16.59	50.09	0
Oct ‘20	36.52	7.74	74.42	0
Nov ‘20	55.26	1.91	88.55	0
Dec ‘20	33.18	-0.27	90.49	0
Jan ‘21	**			
Feb ‘21	**			
Mar ‘21	16.71	3.07	78.96	0.29
Apr ‘21	6.05	8.30	53.44	0
May ‘21	2.01	12.21	57.88	1.14
Jun ‘21	10.37	19.70	56.39	0
Jul ‘21	2.29	23.39	45.24	0
Aug ‘21	**			

**break for winter and summer months

Table C.4: Monthly ascospore concentrations and weather conditions for the Camas Prairie (Grangeville or Nezperce, ID) from September 2019 to July 2021.

Month	Rain (mm)	Temperature (°C)	Relative Humidity (%)	Ascospore Conc.
Sep ‘19	21.84	9.86	75.37	0
Oct ‘19	35.31	4.14	73.12	0
Nov ‘19	32.51	1.60	78.20	0
Dec ‘19	9.65	0.81	84.22	0
Jan ‘20	**			
Feb ‘20	**			
Mar ‘20	45.33	2.97	76.60	0
Apr ‘20	42.60	7.83	71.24	1.43
May ‘20	99.15	10.18	87.45	0.86
Jun ‘20	167.18	13.17	88.05	0
Jul ‘20	**			
Aug ‘20	**			
Sep ‘20	62.05	11.96	75.88	0.29
Oct ‘20	38.98	6.06	81.84	0.57
Nov ‘20	18.51	0.17	86.15	0
Dec ‘20	26.85	-0.87	86.29	0
Jan ‘21	**			
Feb ‘21	**			
Mar ‘21	27.60	1.86	81.17	0
Apr ‘21	19.98	6.61	62.39	0
May ‘21	30.36	9.83	73.18	1.72
Jun ‘21	24.10	17.66	68.86	0.29
Jul ‘21	2.54	22.64	52.80	0
Aug ‘21	**			

**break for winter and summer months