Yield losses and pyraclostrobin sensitivity in blackleg (*Leptosphaeria maculans*) of canola

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Plant Science

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Abstract

Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not., is an important disease of canola (*Brassica napus* L.) worldwide. In Canada, blackleg is managed mainly by the cultivation of resistant or moderately resistant canola hybrids and the application of fungicides. Field experiments were conducted in central Alberta in 2017 and 2018 to determine the relationship between blackleg severity and yield in two moderately resistant hybrids '73-15RR' and '1950RR'. Seed yield per plant was found to decrease as a consequence of *L. maculans* infection, with regression analysis showing that the relationship between yield and disease severity was best explained by quadratic equations. Sensitivity to the fungicide pyraclostrobin, a strobilurin that is commonly applied as a foliar and seed treatment for blackleg and other diseases, was compared in *L. maculans* collections made in Alberta in 2011 and 2016. The half-maximal effective concentration (EC_{50}) of pyraclostrobin was determined using agar and microtiter plate assays, and two discriminatory doses of the fungicide were selected to identify highly insensitive isolates in the collections. The mean EC_{50} was approximately 4× greater for the isolates collected in 2016 versus those collected in 2011. While almost all isolates were still sensitive to pyraclostrobin, this increase in the EC_{50} suggests that proper fungicide stewardship is warranted for the sustainable long-term management of *L. maculans*.

Preface

This thesis is an original work by me, Yixiao Wang. I conducted all of the experiments and wrote the first draft of all chapters. Dr. Stephen Strelkov, my supervisor, reviewed and provided editorial revisions and suggestions for improvement of each chapter. Dr. Alireza Akhavan, a Research Associate in our group, also helped to review Chapter 2 and provided suggestions on this study. A colleague, Dr. Robert Conner (Agriculture and Agri-Food Canada), also proofread most of the thesis. I incorporated or addressed all of the revisions as needed. Finally, Dr. Strelkov and my co-supervisor, Dr. Hwang, approved the thesis for submission.

For the work outlined in Chapter 2, Dr. Akhavan provided suggestions with respect to experimental design, statistical advice, and technical guidance. Most of the isolates of *Leptosphaeria maculans* included in Chapter 2 were isolated by Drs. Yue Liang and Enid Perez-Lara (U of Alberta). Mr. Zhiyu Yu (U of Alberta) provided assistance with data collection. Mr. George Turnbull (Alberta Agriculture and Forestry) provided assistance with the application of pesticides and herbicides for weed and pest control, and helped to harvest the field plots in Chapter 3. Many undergraduate and graduate students from the University of Alberta Plant Pathology Lab assisted with field plot preparation and weeding.

Financial support for this research was provided through grants to Drs. Strelkov and Hwang from the Canola Agronomic Research Program (Alberta Canola, SaskCanola, the Manitoba Canola Growers Association), as well as the Canadian Agricultural Partnership (Agriculture and Agri-Food Canada and the Canola Council of Canada). I also gratefully acknowledge in-kind support from Alberta Agriculture and Forestry and the U of Alberta.

Acknowledgements

I would like to acknowledge my supervisor Dr. Stephen Strelkov and co-supervisor Dr. Sheau-Fang Hwang for their guidance, support, and mentoring throughout the creation of this thesis. I would like to thank Dr. Alireza Akhavan and Mr. George Turnbull for their assistance with technical expertise, and for many dedicated hours. My heartiest gratitude goes to Dr. Sheau-Fang Hwang for inspiring me to learn more about plant pathology and providing space, materials, seed, and equipment for the research projects. I would like to thank Dr. Rong-Cai Yang for reading this thesis and serving as the arms-length examiner during my defense.

I would like to thank Dr. Robert Conner for critically reviewing this manuscript. I really appreciate the help from Dr. Qixing Zhou and Mr. Heting Fu for their technical guidance and assistance. I thank my fellow graduate student Mr. Longfei Wu for assisting me in the field. I gratefully appreciate the help I received from summer students in the lab and in the field.

In addition, I would like to thank my dearest friend Mr. Zhiyu Yu for supporting and helping me through this entire process, who never ever let me give up!

Lastly, I would like to thank my mom and dad, Yanling and Yanfeng for their constant encouragement and support over the years. I could not have succeeded without what you contribute to my life.

I would like to acknowledge the Canola Agronomic Research Program (Alberta Canola, SaskCanola, Manitoba Canola Growers Association), along with the Canadian Agricultural Partnership (Agriculture and Agri-Food Canada and the Canola Council of Canada), for financial support. I also thank Alberta Agriculture and Forestry and the U of Alberta for in-kind support of my work.

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1.1 Canola

1.1.1 Introduction to canola

Canola or rapeseed (*Brassica napus* L.) is a cultivated crop of the Brassicaceae family. Other species in this family include rutabaga (*B. napobrassica* (L.) Mill.), cabbage (*B. oleracea* L.), turnip (*B. rapa* L.), and mustard (*Sinapis alba* L.) (Casséus 2009). While canola/rapeseed is grown worldwide, most production occurs in the European Union, Canada, China and India (The Statistics Portal 2019). The evolution and relationship between the six major Brassica species are depicted in what has become known as the "Triangle of U" (Figure 1.1), named after the Korean-Japanese botanist Woo Jang-choon. Originally, rapeseed was cultivated and used as a source of lamp oil, and later as cooking oil in Asia and Europe (Casséus 2009). With the development of steam power, rapeseed oil became an essential lubricant for steam engines on naval and merchant ships. During the Second World War in Europe and Asia, supplies of rape oil were cut off which led to increased rapeseed production in Canada (Casséus 2009). In the 1970s, canola, an oilseed crop, was developed by plant breeders on the Prairie Provinces. By minimizing undesirable compounds such as erucic acid and glucosinolate, food-grade quality oil was extracted from canola (Casséus 2009).

Importance of canola

Although canola/oilseed crops are cultivated in many regions, growth cycles and seed yields differ because of specific soil and environmental conditions as well as the varieties grown (Oplinger et al. 1989). For instance, farmers in Europe plant high-yielding winter rapeseed crops, which have a much longer growing season compared with canola grown in Canada that has only

3-4 months from planting to maturity. Canola is a good source of cooking oil due to its low glucosinolate and erucic acid content; the meal is used as animal feed because of its high protein content; and the oil is also used as biofuel feedstock because of its exceptional cold weather performance (CCC 2017).

According to a recent report by the United States Department of Agriculture (USDA 2018), canola accounts for approximately 12% (~70.22 million metric tons) of the total oilseed production worldwide. Canada and the European Union are the top two producers, with 21.1 and 19.6 million metric tons, respectively, produced in 2018. Other major producers include China, India, and Japan (USDA 2018). Indeed, canola is the most profitable commodity for Canadian farmers (CCC 2017; Statistics Canada 2018). By province, Saskatchewan, Alberta, and Manitoba account for 99% of Canada's canola production, with small acreages grown in British Columbia, Ontario, Quebec and the Maritimes (AAFC 2018). A recent study indicated that canola contributes $26.7 billion to the Canadian economy each year (CCC 2017). Over the past decade, the economic impact of the canola value chain has increased by over 250% (CCC 2016).

Main diseases of canola in western Canada

The high economic returns associated with canola production create financial incentives for growers to cultivate this crop over wide acreages and often in short rotations. Consequently, various diseases are of concern in canola production. While blackleg is the focus of this dissertation, three other canola diseases also warrant mention, namely clubroot, Alternaria black spot and Sclerotinia stem rot.

Clubroot

Clubroot, caused by *Plasmodiophora brassicae* Woronin, is a serious soil-borne disease of canola and other crucifers that causes the development of characteristic galled roots on infected plants. These root galls reduce the capacity of plants to take up water and nutrients from the soil, resulting in wilting, stunting, and premature and uneven ripening of the crop (Hwang et al. 2012). Infected plants produce a lower seed yield and quality (oil content) (Hwang et al. 2012). On the Canadian Prairies, the first cases of clubroot on canola were found in 12 fields located in central Alberta in 2003 (Tewari et al. 2005). The disease has continued to spread over the past 15 years. As of 2018, there were 3,044 confirmed clubroot field infestations in Alberta, distributed across much of the province (Strelkov et al. 2019). In Saskatchewan, where clubroot had not been documented until recently, 43 fields with symptomatic canola plants were found in 2018. Similarly, the presence of *P. brassicae* DNA has been confirmed in 294 individual fields in Manitoba, and visual symptoms of clubroot have been observed in 33 fields in that province (Manitoba Agriculture 2018). Disease management strategies for clubroot include long rotations out of canola and other susceptible crops, planting of clubroot-resistant cultivars, sanitization practices to restrict the movement of pathogen inoculum, and the application of soil amendments and lime to create unfavorable conditions for the pathogen (Strelkov et al. 2011).

Alternaria black spot

Alternaria black spot is caused by the fungi *Alternaria brassicae* (Berk.) Sacc., *A. alternata* L. and *A. raphani* J.W. Groves & Skolko, and is a common disease on the Canadian prairies (Conn et al. 1990). *Alternaria* spp. can infect canola plants at all growth stages, with disease severity affected by moisture and temperature conditions (Conn et al. 1990). Symptoms of infection include brown, black or greyish-white leaf spots with a dark border, as well as lesions on the pods and stems (Nowicki et al. 2012). *A. brassicae* can cause severe economic

losses because of reduced seed germination and seedling vigour, in addition to pre- and post-emergence damping-off (Nowicki et al. 2012). Nevertheless, while all canola varieties are susceptible to Alternaria black spot, *B. napus* is more tolerant than *B. rapa* (CCC 2017; Nowicki et al. 2012). Since nearly all canola grown in Canada is now of the *B. napus* type, black spot is not as prominent on the prairies as it was several decades ago, when many canola varieties were *B. rapa* (CCC 2017). Foliar fungicides and seed treatments are effective for the management of black spot (Mamgain et al. 2013), while a combination of crop rotation and burying of infected crop residues also can reduce the incidence of the disease (Bailey et al. 2003).

Sclerotinia stem rot

The causal agent of Sclerotinia stem rot is *Sclerotinia sclerotiorum* (Lib) deBary., which causes significant economic losses in many dicot crops including canola in Canada and worldwide (Turkington et al. 2011). With increases in canola production, Sclerotinia stem rot has become more serious, resulting in yield losses of up to 100% (Fernando et al. 2007). The first visual symptoms of this disease appear at the end of flowering, after the plant has been infected for 2-3 weeks (Purdy 1979). Water-soaked lesions or areas of very light brown discolouration develop on the leaves, main stems and branches and eventually expand, giving the plant a bleached appearance (GRDC 2014; Purdy 1979). Effective stem rot control has been achieved with fungicide application, which can increase seed yields and reduce dockage due to sclerotia contamination of the seed (Bradley et al. 2006; Turkington et al. 2011). In addition, studies have examined the potential for foliar application of biocontrol agents to limit petal infection by ascospores (Fernando et al. 2007).

Blackleg

Blackleg (also known as phoma stem canker) of canola, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. and De Not., is of major economic importance in many regions (West et al. 2001) and the focus of this thesis. The blackleg fungus infects canola from the seedling stage onwards, resulting in girdled stems, restricted water and nutrient uptake, and yield losses (Howlett et al. 2001). Blackleg has posed a threat to canola production in western Canada since the mid-1970s (Kutcher et al. 2011). It is a stubble-borne disease, surviving as pseudothecia on infected crop residues; in the spring, the pseudothecia release wind-borne ascospores, which serve as the primary inoculum to initiate the disease cycle (Bokor 1972). Infection eventually results in the production of asexual fruiting bodies (pycnidia) that in turn release rain-splashed pycnidiospores, which are the secondary inoculum that propagates the disease (Howlett 2004). When pycnidiospores, even at relatively low concentrations, are co-inoculated with ascospores, they can induce severe disease symptoms (Li 2006). For the past two decades, blackleg disease has been effectively controlled by practicing integrated disease strategies including fungicide application, crop rotations, and planting resistant cultivars.

1.2 *Leptosphaeria maculans*

1.2.1 Taxonomy and classification

Leptosphaeria maculans (anamorph *Phoma lingam* Tode ex Fr.) belongs to the Pleosporales, which is the largest order in the fungal class Dothideomycetes, phylum Ascomycota. For many years, *L. maculans* was classified under the Loculoascomycetes, before a revision of the taxonomic subdivisions (Rouxel & Balesdent 2005). The characteristics of the Pleosporales include perithecioid ascomata, the presence of cellular pseudoparaphyses,

bitunicate asci, and ascospores of various shapes, pigmentations and septations (Zhang 2012). The majority of species in this order are saprobes of dead organic material, but some species are parasites of living plants, epiphytes or endophytes (Zhang et al. 2009).

The order Pleosporales consists of 28 families including the Leptosphaeriaceae (Lumbsch & Huhndorf 2010), with species that are saprobes or parasites (Zhang 2012). Distinguishing characteristics of this family include relatively thick peridia, bitunicate cylindrical asci and hyaline to brown, transversely septate ascospores in their sexual morph (Hyde et al. 2013). Small- to medium-sized solitary ascomata may be scattered or in groups and they are immersed, subglobose containing interascal filamentous pseudoparaphyses (Ariyawansa et al. 2015). Bitunicate, broad asci bearing eight fusiform, transversely 3–5-septate, hyaline to yellow-brown ascospores are found inside of the ascomata (Ariyawansa et al. 2015). The genus *Leptosphaeria* was included in this family in a recent classification; *L. maculans* is the most studied species and economically important plant pathogen in this family.

Leptosphaeria maculans is a ubiquitous pathogen of the Brassicaceae, attacking cultivated Brassicas such as canola, broccoli, cabbage, kale and rapeseed, as well as numerous wild species (Rouxel & Balesdent 2005). Tode (1791) was the first to link a fungal species, named *Sphaeria lingam* Tode, with dried stems of red cabbage (*Brassica oleracea*). Desmazières (1849) recognized this fungus as a pathogen of cabbage, renaming it *Phoma lingam* (Tode) Desm. Although the teleomorph or sexual stage of *P. lingam* was proposed as *Leptosphaeria maculans* (Desm.) Ces. & De Not., this was not scientifically demonstrated until the mid 1900s (Rouxel & Balesdent 2005). From the end of the 1900s to the early 2000s, severe epidemics of stem canker were reported in Australia, North America and Europe (West et al. 2001). *Leptosphaeria maculans* has been shown to be highly variable and is divided into two groups.

Isolates that are highly virulent or aggressive were classified as group A (Tox^{+}). Group B (Tox^{0}) consists of weakly virulent or nonaggressive isolates. These groups differ in culture, genetics, metabolite production, as well as the leaf and stem symptoms they cause, although they infect the same hosts and the spores look similar (West et al. 2001). Two species, *L. maculans*, which belongs to group A, and *Leptosphaeria biglobosa* Shoemaker and H. Brun, which belongs to group B, co-exist in most *B. napus* growing regions of the world (Fernando et al. 2016). *Leptosphaeria maculans* is more aggressive than *L. biglobosa* and survives in infected plant residues after harvest. Ascospores and pycnidiospores released from infected residues in the next growing season act as the primary inoculum to continue the life cycle of *L. maculans* and *L. biglobosa*. Under cool temperatures, the fruiting bodies of *L. maculans* mature faster than those of *L. biglobosa* (Kaczmarek & Jędryczka 2012). Nonetheless, *in planta* and *in vitro* experiments showed that the mycelium of *L. biglobosa* grows faster than that of *L. maculans* (Kaczmarek & Jędryczka 2012).

1.2.2 Distribution of blackleg

Blackleg of crucifers has been recorded for over 220 years (Henderson 1918), and it is ubiquitous in most canola/rapeseed producing regions of the world (Gugel & Petrie 1992). In 1950, severe epidemics occurred near Paris, France, and because of increasing rapeseed production, the disease had become endemic in most regions of France by 1968 (Gugel & Petrie 1992). Yield losses of up to 80% were reported (Gugel & Petrie 1992). In Germany and England, blackleg occurred in almost in all of the rapeseed growing regions by 1977 and 1978 (Cook & Evans 1978; Krüger 1979). Three to four years after the introduction of rapeseed to Australia, outbreaks of blackleg occurred and devastated many crops in the early 1970s (McGee 1973). In Canada, *L. biglobosa* was identified on adult rapeseed plants in 1961; however, the weakly

virulent strain did not cause significant damage (Vanterpool 1961; Thomas 1984). In 1975, however, *L. maculans* was detected in central Saskatchewan and became widespread during the 1980s (Gugel & Petrie 1992). As Canadian canola production has expanded, blackleg also has spread throughout Alberta, Manitoba, and Ontario. In addition, blackleg has been reported in the U.S., China, and Brazil (Fernando et al. 2003; Zhang & Fernando 2018). In most countries, blackleg appears to be caused by *L. maculans*; in China, however, only *L. biglobosa* has been reported on rapeseed and cruciferous vegetable crops in recent years (Cai et al. 2014).

1.2.3 Epidemiology, symptoms, and life cycle

Blackleg is a polycyclic disease, since *L. maculans* produces both primary and secondary inocula (Figure 1.2). The primary inoculum involves the release of air-borne ascospores produced in pseudothecia on infected crop residues and seed from the previous growing season (Hall 1992). As a facultative saprophyte, *L. maculans* can survive on canola residues for 1 to 5 years; some cruciferous crops also can act as alternative hosts for overwintering by the pathogen (Hall 1992). Pseudothecia and ascospores are the sexual structures produced on stem residues (Rouxel & Balesdent 2005). Large numbers of ascospores are produced under rainy or very humid weather conditions. Infections coincide with the emergence of young, susceptible plants. The spores are spread by the wind and can travel for kilometers (Rouxel & Balesdent 2005). Because winters are long and cold in western Canada, ascospores are released from May to August for spring canola infection (West et al. 2001). Huang et al. (2003) reported that when the temperature is between 5-20°C, ascospores can remain viable for up to 30 days in a dry environment.

After release, the ascospores colonize the cotyledons or leaves of seedlings, germinate and produce hyphae under humid or wet conditions at 4-28°C (West et al. 2001). The pathogen infects the leaves mainly via stomatal pores and wounds, with the fungal hyphae growing into intercellular spaces between the mesophyll cells (Chen et al. 1996; Hammond et al. 1985). After the pathogen penetrates the leaves, it grows within the plant via xylem vessels and causes development of light green or beige-coloured circular lesions. The lesions grow to 1-2 cm in diameter and become pale brown with tiny dark fruiting bodies (pycnidia) that produce asexual pycnidiospores (conidia) (West et al. 2001). This is the anamorph or asexual stage of the fungus, known as *P. lingam* (Kaczmarek and Jedryczka 2011). Under optimal temperature and wetness conditions, the single-celled pycnidiospores ooze out of the pycnidia as pink or purple masses. The pycnidiospores serve as the secondary inoculum and are dispersed by rain-splash over a diameter of several meters to adjacent plants (Rouxel & Balesdent 2005). Although secondary infections caused by pycnidiospores are rare in Canada, these spores can infect wounded leaves, petioles and stems (West et al. 2001). After the fungus has infected the leaf, it continues to grow biotrophically through the lamella and petiole to reach the stem. After this phase, the fungus grows in the intercellular spaces towards the crown at the base of the stem and the upper root, but the plant remains symptomless (Rouxel & Balesdent 2005). Towards the end of the growing season, the fungus becomes necrotrophic and basal stem canker lesions develop. Those symptoms are associated with plant lodging and yield loss (West et al. 1999). If a plant is infected with blackleg, dry blackened necrotic tissues can be observed in cross-sections of the crown (Hayward et al. 2012). The first six leaves appear to be the most susceptible to infection and result in the most severe stem cankers, causing extensive damage and yield losses to the crop

(McGee & Petrie 1979). The mycelium of *L. maculans* overwinters on infected plant residues after harvest and produces new pseudothecia and pycnidia on the crop debris.

1.2.4 Blackleg management strategies

The successful long-term management of blackleg requires a combination of cultural, biological, genetic and chemical strategies that depend on knowledge of genetics, disease epidemiology and the environment in different regions.

Cultural control

Crop rotation

Since *L. maculans* is a facultative saprophyte, it is possible for the pathogen to survive on infected plant residues for a number of years. Petrie (1995b) reported that *L. maculans* could survive and continue to produce viable ascospores on canola residues for up to 7 years in Canada. Hence, a fundamental blackleg management strategy is crop rotation. By adjusting the growth, survival and reproduction of the causal organism, crop rotation reduces pathogen inoculum concentration to a low level to avoid a build-up of disease. However, due to the higher economic returns associated with canola vs. most other crops that can be grown in western Canada, growers tend to grow canola continuously or in short rotations in much of this region (Kutcher et al. 2013). Short rotations do not allow sufficient time for canola residues to decompose completely, enabling *L. maculans* to survive and develop on infected crop debris (Kutcher et al. 2013). Despite planting resistant cultivars, blackleg incidence and severity increase when canola is grown more frequently than 1 in 4 years (Kutcher et al. 2013).

The emergence of new pathogen races and the air-borne, long-distance dispersal of the ascospores have negative effects on rotation as a blackleg management strategy. A reduction in the movement of ascospores and pycnidiospores between fields may be achieved by isolating infected canola crops. In western Canada, it is recommended that canola crops be planted at least 50 to 100 m from each other to decrease blackleg risk (Guo & Fernando 2005). However, in Australia the recommendation for an effective buffer distance between the source of infection and other canola fields is 500 m (Marcroft et al. 2004). Either way, this likely will result in a reduction in disease, rather that its elimination, since *L. maculans* ascospores can travel kilometers in the wind.

In addition to canola stubble residue, volunteer canola and cruciferous weed species such as wild mustard (*Sinapis arvensis* L.) can serve as alternative hosts for *L. maculans* in non-canola years. Volunteer plants and weeds can act as an inoculum bridge without effective weed control practices, reducing the effectiveness of crop rotation (Kutcher et al. 2011). Therefore, good weed management is an important blackleg control measure when combined with crop rotation and the appropriate buffer distances.

Scouting

It is critical to scout for and accurately identify blackleg, as other diseases may cause similar symptoms. Knowledge of blackleg incidence and severity can assist growers or agronomists in evaluating the need for and the effectiveness of current control strategies, as well the need for alternative approaches to disease management (Kutcher et al. 2011).

Blackleg diagnosis can be a challenge to some extent. The symptoms resulting from infection by *L. biglobosa* vs. *L. maculans* can have a different impact on canola. *Leptosphaeria*

biglobosa produces superficial cankers and tissue discoloration, which have relatively little impact on yield (Cai et al. 2018). In contrast, *L. maculans* has the ability to cause severe, yield-limiting blackleg symptoms, including deep-seated cankers that can girdle the stem (Kutcher et al. 2011). Additionally, blackleg can be confused with other diseases such as Verticillium stripe, which also presents as discoloured stems and premature ripening (Hwang et al. 2017).

There are three main scouting periods for blackleg: prior to planting, the vegetative stage (3 - 6 leaf stage), and prior to or at swathing (Kutcher et al. 2011). Before seeding, scouting a field for the presence of canola residue and pseudothecia is essential to assess the risk of blackleg infection for the new growing season (Kutcher et al. 2011). Fields under continuous monoculture or short canola rotations are likely to contain large amounts of partially decomposed canola residue, which would create favorable conditions for pathogen growth (Kutcher et al. 2011). At the vegetative stage, fields can be scouted in a 'w-pattern', with at least 50 plants examined for the presence of lesions on the cotyledons and early true leaves (Kutcher et al. 2011). If these lesions occur on > 10% of the plants, they indicate the presence of the pathogen and the potential for a disease epidemic (Kutcher et al. 2011). To identify and quantify blackleg severity, scouting just prior to swathing or at swathing is best (Kutcher et al. 2011). At that time, the basal cankers are easy to observe and lesions with black pycnidia may be present on the upper canopy including pods. Scouting fields in a w-pattern and pulling at least 50 plants are recommended. Researchers or agronomists clip off the plant at the soil line and examine the cross-section of the stem for blackened tissue. A 0-5 scale is used to evaluate the severity of infection and disease incidence, where 0 = no disease in a cross-section of the stem base; 1 = decay on < 25% of the cross-section; 2 = decay on 25%-50% of the cross-section; 3 = decay on 51%-75% of the cross-section; 4 = decay on > 75% of the cross-section; and 5 = death of the

plant (Hwang et al. 2016). This information can be used to help identify best management practices in the next growing season.

Forecasting models

The maturity of *L. maculans* pseudothecia is highly related to rainfall and temperature. The timing of ascospore release is triggered by rainfall events. Several studies have been conducted in Europe and Canada to predict pseudothecial maturity based on weather conditions (Petrie 1995b; Pérès et al. 1999; West et al. 1999). Salam et al. (2003) developed a forecasting system for pseudothecial maturity and the timing of ascospore showers in Western Australia, based on weather conditions in relation to blackleg disease. A combination of two important weather factors was considered in this model, daily mean temperature and daily total rainfall. The "Blackleg Sporacle" forecast model demonstrated that when rainfall exceeded 4 mm weekly and average temperatures were between 16 - 25°C, weather conditions were optimal for maturation of the pseudothecia (Salam et al. 2003). Since the Blackleg Sporacle forecast model had not been evaluated outside of Australia, it was re-calibrated (Salam et al. 2007) as two new weather-based forecasting models: the SporacleEzy and the Improved Blackleg Sporacle models, which were tested in Canada, Australia, Poland, France and the United Kingdom. The Improved Blackleg Sporacle model takes into account the impact of low temperatures on pseudothecial maturation, which matches the weather conditions in many regions, especially at high latitudes. The SporacleEzy model is a simplified version of the Improved Blackleg Sporacle model, since it considers two fewer parameters (temperature - threshold - duration and rain - threshold - duration). Results showed that the Improved Blackleg Sporacle model accurately predicted the onset of seasonal ascospore release in all five countries, and provided more accurate predictions than the SporacleEzy model (Salam et al. 2007).

Forecasting models can help growers to adjust sowing dates to avoid the release of large quantities of ascospores. Ideally, forecasting models should be combined with yield loss models that help to determine the risk of losses (and the need for disease control actions) associated with certain levels of blackleg disease. The impact of blackleg on yields has been examined in a limited number of studies. Most recently, Hwang et al. (2016) evaluated the relationship between blackleg severity and yield losses under field conditions in Alberta. This study resulted in the development of a yield loss model which indicated that for each unit increase in blackleg disease severity, there was a corresponding decline of 17.2% in yield (Hwang et al. 2016). The precision of the model may be limited, however, because it was developed with an old, open-pollinated canola cultivar ('Westar') that is completely susceptible to blackleg. Hence, the blackleg yield model of Hwang et al. (2016) should be refined using hybrid canola cultivars that carry at least some resistance to *L. maculans*, since these are much more representative of the canola varieties currently being grown in Canada.

Biological control

Numerous studies have been carried out in recent decades to identify biological control agents effective against blackleg. For example, a bacterial isolate *Paenibacillus polymyxa* Prazmowski (strain ATCC 202127) that produces antifungal peptides was reported as a potential biological control agent to suppress the growth of *L. maculans* and other fungi (Kharbanda et al. 2003). The strain was reported to be effective when applied with canola seeds at planting. In addition, *Paenibacillus polymyxa* strain PKB1, *Pseudomonas chlororaphis* (PA23), and *Bacillus amyloliquifaciens* (BS6) also inhibited the growth of *L. maculans* (Kharbanda et al. 1999; Fernando et al. 2007). Compared with pesticides and fungicides, biological control is environmentally friendly, leaving no residues in the soil. Unfortunately, however, most

biological control agents have been evaluated only under controlled environmental conditions. Practical applications of biocontrol for the management of blackleg are few and remain to be confirmed; however, if perfected and commercialized, biological products might offer a safe and effective control method for growers.

1.3 Genetic Resistance

Currently, one of the most effective blackleg disease management strategies is the cultivation of resistant canola cultivars. There are two types of resistance: qualitative (syn. major gene or race-specific) resistance and quantitative (syn. minor gene or adult plant) resistance. Qualitative resistance usually is controlled by single dominant genes, which are expressed during initial infection at the seedling stage. The resistance gene products recognize or interact with small proteins (elicitors) produced by matching avirulence genes in the pathogen in a highly specific, gene-for-gene manner, triggering a defense response (Wagner & Simons 1994). This type of resistance may last for several years if the corresponding avirulence gene is dominant in the pathogen population (Balesdent et al. 2002). Although qualitative resistance provides complete resistance to the pathogen, it often can be broken down easily because of the strong adaptative ability of *L. maculans* (Huang et al. 2009). In contrast, quantitative resistance is controlled by many minor genes and confers partial resistance. Resistance is triggered when the pathogen attacks the stem tissue, protecting the adult plant (West et al. 2001). Quantitative resistance is more durable than qualitative resistance, and is important for plant breeding (Delourme et al. 2006). A blackleg resistant rapeseed cultivar 'Major' that carried the resistance gene *Rlm4* was introduced in Europe in 1971 (Delourme et al. 2006). In 1978, a French winter cultivar 'Jet Neuf' also became available to growers, which carried both the R gene *Rlm4* and

race-nonspecific resistance. This cultivar was used for pathotyping in Canada and was an important source for breeding material (Rimmer 2006).

In Canada, most canola cultivars are resistant or moderately resistant to blackleg (Rimmer 2006; CCC 2017). Field resistance ratings are based on the severity of blackleg relative to a susceptible check, the canola 'Westar'. There are four resistance ratings: Resistant "R" (disease severity of 0-29.9% of 'Westar'), Moderately Resistant "MR" (30-49.9% of 'Westar'), Moderately Susceptible "MS" (50-69.9% of 'Westar'), and Susceptible "S" (70-100% of 'Westar'). These ratings, however, do not provide any information regarding the basis of the resistance found in a cultivar. In an effort to indicate the resistance source (and potentially enable growers to rotate sources of resistance), a new blackleg labelling system was adopted beginning in 2018. Letters (A, B, C, D, E_1, E_2, F, G, H, X) have been added to the resistance ratings to denote the Resistance Group (RG) classification of specific cultivars. Different RGs indicate the presence of different blackleg resistance genes. Resistance Group A indicates the presence of the *Rlm1* or *LepR3* gene in a cultivar, RG B indicates the *Rlm2* gene, RG C the *Rlm3* gene, RG D the *LepR1* gene, RG E1 the *Rlm4* gene, RG E2 the *Rlm7* gene, RG F the *Rlm9* gene, RG G the *RlmS* gene, RG H the *LepR2* gene, and RG X indicates the presence of unknown resistance genes (CCC 2017). For example, if a variety is labelled 'R (AX)', it is rated as Resistant (blackleg severity <30% that if 'Westar'), and carries the *Rlm1* or *LepR3* resistance gene as well as other unidentified resistance gene(s).

The deployment of blackleg resistant canola cultivars has been the most powerful approach for the management of this disease. However, strong selection pressure can lead to shifts in the virulence of *L. maculans* populations, resulting in the breakdown of resistance. The highly resistant canola 'Surpass 400' was first released in Australia in 2000 as a tool for blackleg

management (Li & Cowling 2003). In 2003, however, severe damage from blackleg infection occurred on 'Surpass 400' in two individual canola fields (Li et al. 2005). Similarly, a French study found that the large-scale cropping of cultivars carrying the *Rlm1* resistance gene decreased the proportion of *L. maculans* isolates harbouring the matching avirulence gene, *AvrLm1* (Rouxel et al. 2003). If growers plant the same variety in a short rotation in the same region, the risk of severe blackleg infection will be high. A possible approach to increasing the longevity of resistance is to combine, in the same cultivars, major gene resistance with quantitative resistance, thereby providing a more full-spectrum resistance to blackleg (Brun et al. 2010; Delourme et al. 2014; Marcroft et al. 2012). It is also recommended that farmers rotate the RG designation of the cultivars they plant in their fields, so that *L. maculans* populations will be exposed to different resistance genes every year, helping to reduce the risk of selection for new virulent pathogen race(s) (CCC 2017).

1.4 Chemical Control

Although the deployment of resistant canola is one of the most commonly practiced blackleg management strategies, short rotations still result in increasing disease levels. Consequently, growers have started to apply more fungicides as an additional control for this disease (Kutcher et al. 2011).

Fungicide treatments for *L. maculans* control include seed treatments and foliar sprays. The first seed treatment for blackleg in Canada was adopted in 1978 (Guo & Fernando 2005). The benefits of applying seed treatments include limiting the survival of seed-borne inoculum to prevent the spread of blackleg into non-infested regions (Gugel and Petrie 1992). However, it cannot protect seedlings infection from ascospores nor adult plants from later infection (Sprague

& Burgess 2001). A study examined the performance of the fungicide fluquinconazole as a seed treatment on canola (Marcroft and Potter 2008). The results showed that blackleg severity and plant mortality were reduced greatly at high inoculum concentrations. Fluquinconazole increased yield in cultivars with moderate to low levels of resistance, but had no significant effect on the yield of cultivars with high resistance (Marcroft and Potter 2008).

In the early 1990s, triazole fungicides became available in western Canada for use against blackleg. Susceptible canola cultivars showed reduced symptoms and increased yield in response to foliar fungicide application. However, blackleg-resistant canola varieties did not show any response to foliar sprays (Kutcher et al. 2011). Foliar infection by *L. maculans* can occur throughout the growing season, since ascospores are released constantly (Gugel and Petrie 1992). Therefore, the timing of foliar fungicide application can be a challenge and a single application may not be sufficient. Optimal fungicides application may benefit from an accurate forecasting model, based on the seasonal and diurnal patterns of spore dispersal, which is coordinated with crop stage (Kutcher et al. 2011).

In western Canada, the first strobilurin fungicide (azoxystrobin, Quadris®) became available in the early 2000's and a second (pyraclostrobin, Headline®) in 2010 (Kutcher et al. 2011). There are now several foliar products with different modes of action registered for blackleg control on canola in Canada, including triazoles (propiconazole) and strobilurins (azoxystrobin and pyraclostrobin) (Alberta Agriculture and Forestry, 2018). Strobilurin fungicides are one of the most important classes of agricultural fungicides.

1.4.1 Strobilurin fungicides

Strobilurins are synthetic analogs of a group of natural fungicidal derivatives of β-methoxyacrylic acid, which includes one derived from *Strobilurus tenacellus* Pers. (Bartlett et al. 2002). These natural fungicides help *S. tenacellus* outcompete microbes that exist in rotting wood, but they are sensitive to sunlight. Therefore, industry chemists transformed these natural fungicides into stable synthetic fungicides, which were less subject to photochemical degradation (Vincelli 2002). The first strobilurins became available in 1996. There are six active ingredients currently available in Canada within this chemical family, including azoxystrobin, fenamidone, pyraclostrobin, trifloxystrobin, picoxystrobin, and fluoxastrobin (Alberta Agriculture and Forestry 2018). Global sales of strobilurin fungicides for crop use reached $3.8 billion USD in 2014, and they are now the largest fungicide category by market value worldwide (Reportlinker 2016).

Strobilurin fungicides are also known as quinone outside inhibitors (QoI fungicides), since they are able to inhibit mitochondrial respiration through binding of the quinone outside (Qo) oxidizing site of cytochrome b, which is a part of the cytochrome bc1 enzyme complex (complex III). By binding to the Qo site, strobilurins block electron transfer and disrupt the energy cycle to prevent the formation of ATP (adenosine triphosphate) (Bartlett et al. 2002). This creates an energy deficiency for the fungus, eventually causing death (Bartlett et al. 2002). In addition, reactive oxygen species (ROS) are produced because of the inhibition of the electron transport chain; ROS are highly damaging to the mitochondria and other cellular components (Inoue et al. 2012).

Strobilurin fungicides are active against four major groups of plant pathogens: oomycetes, deuteromycetes, ascomycetes and basidiomycetes (Bartlett et al. 2002). Because strobilurins are site-specific and pose little risk to human health and/or the environment, they

have become the most widely-applied fungicides worldwide (Vincelli 2002). These fungicides serve as protectants because they deprive energy from the fungal spore during the germination process (Vincelli 2002). Strobilurin fungicides are locally systemic, meaning they are able to move through the lamina; they are absorbed by the cuticle and can be found on both leaf surfaces (Vincelli 2002). Although these fungicides are effective for disease control, they are highly specific, so they are at risk for the buildup of fungicide-resistant pathogen strains (Vincelli 2002).

1.4.2 Strobilurin fungicide sensitivity monitoring

The tendency for a fungus to build up resistance to a fungicide is heritable, typically arising from a very low rate of genetic mutation. These insensitive individuals initially may be less affected by the labelled fungicide application rate, but under selection pressure from fungicide use over time, the isolates may evolve into a dominant insensitive sub-population (Ma & Michailides 2005). There are two types of fungal mutations associated with fungicide resistance: single-gene resistance and polygenic resistance (Laskin 2009). Generally, single-gene resistance occurs as a result of a single mutation in one gene, while polygenic resistance requires multiple mutant genes (Laskin 2009). Single-gene resistance results in a qualitative change in fungicide sensitivity and is associated mostly with single-site fungicides. In contrast, polygenic resistance leads to quantitative changes as the pathogen gradually shifts toward insensitivity. There is no sudden loss of fungicide efficiency and with higher rates and/or more frequent applications, the fungus still can be controlled (Vincelli 2002).

The extensive use and single-site activity of strobilurin fungicides suggest that there is a high risk of developing insensitive pathogen subpopulations. Worldwide, there is an increasing

number of pathogens of field crops, fruits, and vegetables that are resistant to strobilurins, since a single mutation in the pathogen can overcome the effects of these fungicides (Vincelli 2002). The first fungal isolates reported to be resistant to strobilurin fungicides were discovered on wheat (*Triticum aestivum* L.) in 1998, when strobilurin resistant isolates of powdery mildew (*Blumeria graminis tritici* (DC.) Speer Marchal) were detected in northern Germany (Bartlett et al. 2002). The mechanism of resistance involved a point mutation in the cytochrome b gene in which an amino acid glycine was substituted with alanine at position 143 (G143A) in the cytochrome b protein (Bartlett et al. 2002). Later, this target site mutation was discovered in other pathogens including: *Alternaria* spp., *B. graminis* f. sp. *hordei*, *Didymella bryoniae* Fuckel, *Mycosphaerella fijiensis* Morelet, *Pyricularia grisea* (Cooke) Sacc., *Podosphaera fusca* (Fr.) U. Braun & Shishkoff, *Pseudoperonospora cubensis* (Berk. & M. A. Curtis) Rostovzev, *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, *Podosphaera fuliginea* (Schltdl.) U. Braun & S. Takam., and *Venturia inaequalis* (Cooke) G. Winter (Kim & Hwang 2007). This type of mutation can result in high fungicide tolerance, but it has no effect on cytochrome b activity, indicating no negative effects on the fitness of individuals (Gisi et al. 2002). Another mutation can result in an amino acid change from phenylalanine to leucine at position 129 (F129L) of the *cyt b* gene (Bartlett et al. 2002). This target site mutation was identified in *Pythium aphanidermatum* Edson and *Pyricularia grisea* (Cooke) Sacc. (Kim & Hwang 2007; Ma & Michailides 2005). In addition to these mutations, new alternative mechanisms may contribute to strobilurin resistance (Ma & Michailides 2005). Miguez et al. (2004) reported that the *in vitro* sensitivity of *M. graminicola* to strobilurin fungicides was reduced by activating an alternative oxidase (AOX).

1.4.3 Pyraclostrobin

Pyraclostrobin is a broad-spectrum strobilurin fungicide developed by BASF in 2000 (Bartlett et al. 2002). It was registered on diverse crops for the control of several diseases, including blackleg of canola. Pyraclostrobin was first registered as a foliar fungicide in Canada in 2003, as Headline EC and Cabrio EG Fungicide, for use on various crops (Government of Canada 2011). Headline EC was registered on canola in 2010 (BASF 2019). In 2015, BASF registered the fungicide Priaxor for control of blackleg of canola in western Canada. Priaxor combines two products with different modes of action: pyraclostrobin and fluxapyroxad. This product not only controls blackleg and increases yield, but also reduces the occurrence of fungicide resistance (BASF 2014).

Pyraclostrobin acts as a protective fungicide, suppressing spore germination and preventing infection. It also has some curative properties, since it can achieve a relatively efficient level of control after infection has already begun, and has translaminar activity, moving through the leaf from the sprayed side to the non-sprayed side (Bartlett et al. 2002; Ivic 2010). Since pyraclostrobin is a locally systemic fungicide, it is able to move a short distance to bind in the leaf cuticle. Therefore, the rainfastness period of the pyraclostrobin product Headline EC is only 1 hour (BASF 2014).

Due its effectiveness, pyraclostrobin has been applied widely on many crops including fruits, cereals, pulses and canola. This widespread use of pyraclostrobin has resulted in numerous reports of fungicide insensitivity, including in Canada and the northern U.S. For example, Bowness et al. (2016) studied the pyraclostrobin sensitivity of *Didymella pinodes* (Berk. & A. Bloxam) Petr. isolates from Saskatchewan, Alberta, Washington, and North Dakota. Using radial growth assays, they found that of 324 isolates studied, 19 were insensitive, 304 were sensitive, and one showed intermediate insensitivity to this fungicide (Bowness et al. 2016). Wise et al.

(2009) collected *Ascochyta rabiei* (Pass.) Labr. isolates from the Northern Great Plains and the Pacific Northwest of the U.S. and tested them using an *in vitro* spore germination assay. Their results showed that 65% of isolates collected from North Dakota in 2005, 2006, and 2007 and from Montana in 2007 were resistant to pyraclostrobin. Akhavan et al. (2017) studied pyraclostrobin sensitivity in *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) from Alberta, Saskatchewan and Manitoba. Using microtiter assays, they determined there was a shift in the sensitivity of some *Ptt* and *Ptm* isolates to pyraclostrobin, but no qualitative or extreme insensitivity was found (Akhavan et al. 2017). While pyraclostrobin sensitivity has been evaluated in many important plant pathogens, studies with *L. maculans* are much rarer. Fraser et al. (2017) examined the sensitivity to pyraclostrobin of *L. maculans* isolates collected from Alberta in 2011; their analysis, based on both conventional growth plate and microtiter plate assays, indicated that all of the isolates were sensitive to this fungicide.

1.5 Objectives

1.5.1 Sensitivity study

With the intensive cropping of canola in western Canada (West et al. 2001), the risk of blackleg disease has increased. Although fungicides are an effective tool for disease control, their repeated use can result in the development of insensitivity in fungal populations. Due to their single-site activity, strobilurins are at particular risk; it is, therefore, important to continue monitoring populations of the blackleg pathogen for fungicide sensitivity. The first objective of this thesis will be to evaluate the pyraclostrobin sensitivity of a collection of *L. maculans* isolates recovered from Alberta in 2016, and to compare this sensitivity with that of isolates collected in

Alberta in 2011 (Fraser et al. 2017). I hypothesize that the sensitivity of *L. maculans* to pyraclostrobin in these two collections will have shifted.

1.5.2 Yield loss model study

Through better understanding of the relationship between blackleg severity and yield losses, the agronomic efficacy and economic benefits of disease management measures can be more accurately evaluated. As a result, growers will be able to make more informed crop management decisions. Therefore, the second objective of this thesis is to establish a relationship between blackleg disease severity and the yield of canola hybrids, by refining the recent yield loss model of Hwang et al. (2016). I hypothesize that increasing blackleg severity will be associated with significant decreases in canola yield, even in moderately resistant canola.

1.6 Figures

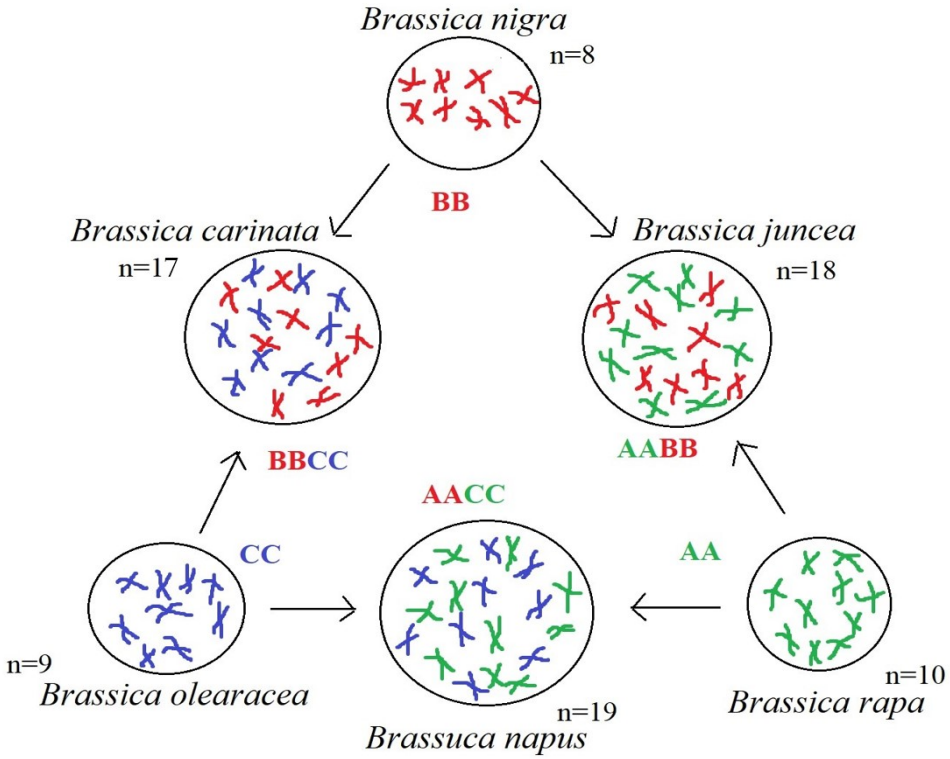


Figure 1.1 The "Triangle of U" explaining the genetic relationship between different *Brassica* species (A, B, C = genome; n = chromosome number) (reproduced from Cruz & Dierig, 2014).

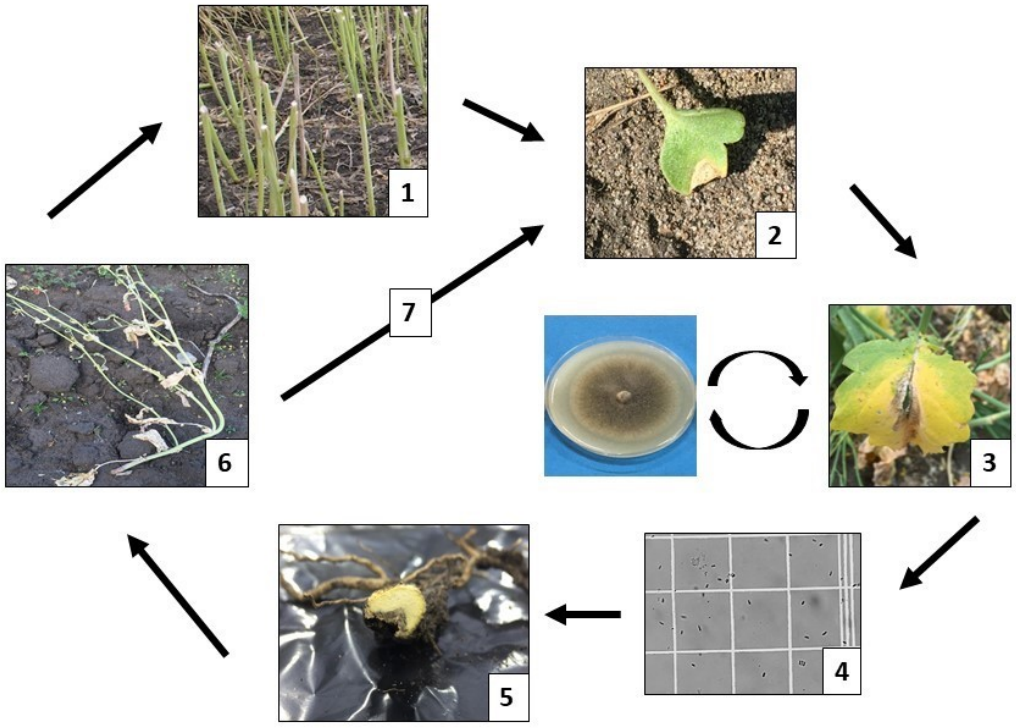


Figure 1.2 Life cycle of *Leptosphaeria maculans* on *Brassica napus* (based on Howlett et al., 2001). The fungus survives as a saprophyte and overwinters as pseudothecia on stubble (1). Ascospores or pycnidiospores land on cotyledons or young leaves (2). Greyish lesions develop on leaves, where black pycnidia can be observed. Pycnidiospores are released from the pycnidia (as seen on the Petri dish) and initiate secondary cycles of infection (3). Pycnidiospores spread through rain splash (4). The fungus grows inside the vascular tissues towards the stem base (5). Throughout growing season leisons form on leaves, stems and seed pods (6). Infected pods and seeds may give rise to infected seedlings (7).

Chapter 2: Decreased Sensitivity of *Leptosphaeria maculans* to Pyraclostrobin in Alberta, Canada

2.1 Introduction

Blackleg (phoma stem canker) caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not. is the most economically important disease of canola (syn. rapeseed, *Brassica napus* L.) worldwide (Fitt et al. 2006; Howlett 2004; West et al. 2001). The disease can cause significant yield losses and is considered a major constraint to canola production (Gugel & Petrie 1992; Hwang et al. 2016). Blackleg emerged as a major production challenge for western Canadian canola growers in the 1970s and 1980s, with the first blackleg resistant cultivars becoming available in the early 1990s (Kutcher et al. 2011). Blackleg resistant cultivars enabled farmers to manage the disease quite successfully, particularly when these varieties were combined with a 4-year crop rotation (Kutcher et al. 2013). In recent years, however, high economic returns, market opportunities and cultivar improvements have led to the more intense production of canola and shorter crop rotations in western Canada (Kutcher et al. 2013). From 2010 to 2015, blackleg incidence in this region increased (Zhang & Fernando 2017), resulting in renewed interest in the management of this disease (Kutcher et al. 2011).

On the Prairies of western Canada (Alberta, Saskatchewan and Manitoba), fungicides represent an important tool in blackleg management. There are four registered active ingredients, including propiconazole, fluxapyroxad, azoxystrobin and pyraclostrobin (Canola Council of Canada 2017). Azoxystrobin and pyraclostrobin are broad-spectrum strobilurin fungicides. The strobilurins are single-site mode-of-action chemicals, also known as quinine outside inhibitors (QoI) (Bartlett 2002). These fungicides bind to the Qo site of the cytochrome bc1 enzyme

complex (complex III) of the mitochondria, inhibiting mitochondrial respiration (Gisi et al. 2002). This prevents the formation of adenosine triphosphate (ATP) and results in an energy deficiency in the fungus (Gisi et al. 2002). Strobilurin fungicides are particularly effective at inhibiting spore germination, which is an energy-demanding stage of fungal development (Bartlett 2002). Inhibition of the respiratory electron transport chain also results in the build-up of reactive oxygen species, which are very damaging to cells and hence contribute to fungal control (Inoue et al. 2012).

Pyraclostrobin has been used widely on various crops in Canada since 2002 (Government of Canada: Health Canada Pest Management Regulatory Agency 2011), and is the active ingredient in the commonly applied fungicide Headline EC (pyraclostrobin, 250 g L^{-1}; BASF 2018). This product was registered on canola in 2010 and was first used in 2011. Because of the site-specific activity of pyraclostrobin, G143A, G137R and F129L mutations at the cytochrome b gene can result in the appearance of fungicide-resistant or tolerant fungal strains (Vincelli 2002). In the case of *L. maculans*, no pyraclostrobin resistance or specific mutation has been reported to date. The repeated application of pyraclostrobin could, however, select for a fungicide-resistant subpopulation of *L. maculans*, as has been reported for other fungal pathogens (Forcelini et al., 2016; Vincelli 2002).

Fraser et al. (2017) examined the pyraclostrobin sensitivity of a collection of *L. maculans* isolates made from Alberta in 2011, the year following the registration of this fungicide on canola. All of the isolates tested in this earlier study were sensitive to pyraclostrobin, although the potential for future shifts in sensitivity was noted (Fraser et al. 2017). Periodic monitoring is important for proper fungicide stewardship and ensuring continued product efficacy. In this context and in the present study, the fungicide sensitivity of an *L. maculans* collection from

Alberta made in 2016, after five years of pyraclostrobin application on canola, was investigated in growth plate and microtiter plate assays.

2.2 Materials and Methods

2.2.1 Collection and preparation of single-spore isolates

A collection of 351 *L. maculans* single-spore isolates was obtained from infected canola stubble from 18 fields surveyed throughout Alberta in 2016. Stubble was collected from a 1-m^2 area at each of five points along the arms of 'W' sampling pattern in each field. Single-spore isolations were made following Rong et al. (2015) and stored as pycnidiospores in 1.5 mL microcentrifuge tubes at -80°C in sterilized 20% glycerol. The identity of these isolates as *L. maculans* was confirmed by means of a PCR assay with β-tubulin-based primers (Rong et al. 2015). Twelve single-spore isolates obtained from infected canola stubble in Alberta in 2011 (Fraser et al. 2017) were also included in this study and regarded as baseline isolates.

2.2.2 Conventional growth plate assay

Determining the mean EC_{50}

A subset of 38 *L. maculans* isolates collected in 2016 was randomly selected from the entire collection and used to determine the effective concentration of pyraclostrobin required to inhibit mycelial growth by 50% (EC_{50}). The EC_{50} of the 12 isolates collected in 2011, previously analyzed by Fraser et al. (2017), also was determined. To revive the isolates, stored pycnidiospores were removed from storage in the microcentrifuge tubes and placed on Petri dishes (10-cm diameter) filled with V8 growth medium (composition per L: 850 mL distilled water, 150 mL V8® Original Vegetable Juice, 1.5 g $CaCO_3$, 15.0 g agar). After 10 days, 6-mm plugs of the developing colonies were transferred to fresh V8 medium amended with different

concentrations of pyraclostrobin. Briefly, a fungicide stock solution was prepared by adding 1 mL of Headline 250 EC (pyraclostobin, 250 g L^{-1}; BASF Canada Inc., Mississauga, ON) to 49 mL sterilized distilled water, and used to obtain final concentrations of active ingredient in the amended plates of 0, 0.0125, 0.025, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75 and 0.875 mg L^{-1} by serial dilution. The appropriate amounts of fungicide were added shortly after autoclaving when the medium had cooled to 50 to 60°C. Although Fraser et al. (2017) did not report any significant difference between the EC_{50} values of isolates tested in the presence or absence of salicylhydroxamic acid (SHAM), 0.1 g L^{-1} of this chemical was included in the present assays as a precaution against alternative respiration (Wood & Hollomon 2003). The Petri dishes were placed under fluorescent lighting at 20 ºC ± 2 ºC for a period of 10 days, at which time two orthogonal measurements of colony diameter (growth) were made using a digital caliper and averaged. The experiment was arranged in a completely randomized design with five replicates (Petri dishes) per treatment, and was repeated independently. The percentage of radial growth inhibition relative to the un-amended control was determined using the equation:

$$[(average\ growth\ on\ unamended - \text{growth on the amended medium}) / (average\ growth\ on\ unamended\ medium)] \times 100$$

Testing the pyraclostrobin sensitivity of *L. maculans* isolates

To evaluate the sensitivity of *L. maculans*, two discriminatory doses of pyraclostrobin were used to screen a collection of 251 single-spore isolates from 2016 and 12 single-spore isolates from 2011. The dosages were 4× and 50× the EC_{50} value of the 12 isolates from 2011. Therefore, V8 growth medium containing pyraclostrobin at 0.28 or 3.5 mg L^{-1} and SHAM at 0.1g L^{-1} was used to determine the sensitivity of the examined fungal isolates. Inoculation of the cultures and preparation of the medium in Petri dishes were as described above. The Petri dishes

were incubated under fluorescent lighting for a 24 h period at 20 ºC ± 2 ºC for 10 days. Two orthogonal measurements of the mycelial growth of each isolate were made and averaged using a digital caliper, with inhibition of radial growth expressed as a percentage of the un-amended controls as described above. The treatments included four replicates (Petri dishes) for each isolate plus four control replicates that did not contain any fungicide. Treatments were arranged in a completely randomized design, and the experiment was independently repeated.

2.2.3 Microtiter plate assay

Determining the mean EC_{50}

The same set of isolates used in the conventional growth plate assay was also used in the microtiter plate assay. Fifty isolates including 12 isolates collected in 2011 and 38 isolates collected in 2016 were used to determine the mean EC_{50}. In order to increase spore density, the spores were collected and immersed in YBA medium (composition per liter of sterilized water: 20 g yeast extract, 20 g Bacto peptone, 40 g sodium acetate). Streptomycin (100 mg L^{-1}) was added to the medium to avoid bacterial contamination. YBA medium is relatively low in nutrients. Therefore, the fungus cannot gain sufficient energy to undergo alternative respiration while it still provides sufficient nutrients for fungal growth (Spiegel & Stammler 2006). Hence, SHAM was not included in the YBA medium. The fungal spore concentration was adjusted to 2 × 10^4 spores per mL. Fifty microliters of the spore suspension, containing about 1000 spores, was transferred into each well of a 96-well microtiter plate, which contained 50 µL of the fungicide solution of different concentrations prepared in YBA medium. Formulated Headline 250 EC fungicide was diluted to achieve the end concentrations, with the EC_{50} determined based on 12 concentrations of pyraclostrobin: 0 (control), 0.0005, 0.001, 0.0015, 0.002, 0.0025, 0.003,

0.005, 0.01, 0.015, 0.05, 0.15 mg L^{-1}. The combined solution was mixed thoroughly in the microplate. Experiments were conducted using a completely randomized design with four replicate wells assigned for each fungicide concentration–isolate combination. The plates were incubated for 7 d at 18 °C in darkness. The endpoint optical density (OD) of pycnidiospore germination was measured with a spectrophotometer (BioTek, Winooski, VT) set at a wavelength of 405 nm for the initial and the final reading. For each isolate and concentration, final values for absorbance were obtained by subtracting the values of the initial reading from those of the final reading, and then growth inhibition was calculated using the equation: $1 - [(absorption\ for\ each\ treatment/absorption\ of\ control)]$, and presented as a percentage.

Testing the pyraclostrobin sensitivity of *L. maculans* isolates

Two discriminatory doses of pyraclostrobin were used to screen 251 single-spore isolates from 2016 and 12 single-spore isolates from 2011. To be consistent with the conventional growth plate assay, the discriminatory doses were 4× and 50× the EC_{50} value obtained for the 2011 isolates. Therefore, medium containing concentrations of 0 (control), 0.006, and 0.075 mg L^{-1} pyraclostrobin were evaluated. Briefly, after incubating for 7 d at 18 °C in darkness, fungal density was measured at a wavelength of 405 nm. The growth inhibition was calculated using the formula described above. The experiment was arranged in a completely randomized design with treatments replicated four times for each isolate, and the entire experiment was repeated.

2.2.4 Statistical analysis

Data were analyzed with R: A Language and Environment for Statistical Computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013). The normality of the EC_{50} values for 50 *L. maculans* isolates (12 isolates from 2011 and 38 isolates from 2016)

were evaluated with the Shapiro-Wilk test using the statistical package shapiro.test. The homogeneity of variance was tested using the Levene test (LeveneTest package). To assess the EC_{50} concentration, a log10-transformed fungicide concentration was employed to linearize fungal growth inhibition data and a regression line was calculated. The EC_{50} values for each isolate were calculated accordingly and all values were averaged to obtain the mean EC_{50}. The mean EC_{50} values of the isolates collected in 2016 and 2011 were compared with a two-sided t-test. The mean EC_{50} values of the isolates from 2011 calculated in the current study were also compared with the corresponding values calculated by Fraser et al. (2017) using a two-sided paired t-test, in order to ensure the reproducibility of the data. The mean growth inhibition values of all of the 263 isolates used in the discriminatory dose experiment in the growth plate and microtiter plate assays were displayed in a histogram.

2.3 Results

2.3.1 Determination of EC_{50} values of baseline isolates

Conventional growth plate assay

In the conventional growth plate assay, the EC_{50} values of the 12 isolates from 2011 ranged from 0.02 mg L^{-1} to 0.11 mg L^{-1}, with a mean of 0.07 mg L^{-1} (Figure 2.1; Table 1). A two-sided paired t-test revealed no significant difference between EC_{50} values calculated in the current study and those calculated by Fraser et al. (2017) for the same set of isolates ($P = 0.31$) (Table 1). The EC_{50} values of the 38 isolates from 2016 ranged from 0.03 mg L^{-1} to 0.64 mg L^{-1}, with a mean of 0.28 mg L^{-1}(Figure 2.1). Five of these isolates had predicted EC_{50} values greater than the maximum concentration of fungicide employed; therefore, to avoid out-of-range

estimates, their estimated EC_{50} values were excluded from the calculation of the mean EC_{50} for the 2016 isolates (and only the other 33 isolates were used).

Microtiter plate assay

In the microtiter plate assay study, the EC_{50} values of the *L. maculans* isolates from 2011 ranged from 0.0001 mg L^{-1} to 0.0066 mg L^{-1}, with a mean of 0.0015 mg L^{-1} (Figure 2.2) and a left-skewed distribution. The EC_{50} values of the isolates from 2016 ranged from 0.0002 mg L^{-1} to 0.0489 mg L^{-1}, with a mean of 0.0049 mg L^{-1} (Figure 2.2).

2.3.2 Pyraclostrobin sensitivity of *L. maculans* isolates

Conventional growth plate assay

Two hundred and fifty one *L. maculans* isolates randomly selected from a total of 351 isolates collected from Alberta in 2016, along with another 12 isolates collected in 2011, were assessed for pyraclostrobin sensitivity. When tested with the lower (0.28 mg L^{-1}) of the two discriminatory doses, growth inhibition of the 263 isolates ranged from 15.7% to 82%, relative to the control treatment (no pyraclostrobin). Ninety four (36%) of the isolates had a growth inhibition <50%, 91 (35%) had a growth inhibition of 50% to <60%, 63 (24%) isolates had a growth inhibition of 60% to <70%, 14 (5%) had a growth inhibition of 70% to <80%, and one isolate had a growth inhibition of 80% to <90%. No isolate had a growth inhibition >90% (Figure 2.3a). The 12 isolates from 2011 all had a growth inhibition >50%. When tested with the second (higher) discriminatory dose (3.5 mg L^{-1}) of pyraclostrobin, one isolate had a growth inhibition of <50%, seven (3%) had a growth inhibition of 50% to <60%, 19 (7%) had a growth inhibition of 60% to <70%, 80 (30%) had a growth inhibition of 70% to <80%, 98 (37%) had a growth inhibition of 80% to <90%, 55 (21%) had a growth inhibition of 90% to <100%, and

three (1%) isolates had a growth inhibition of 100% (Figure 2.3b). The growth inhibition of the 263 isolates ranged from 40.9% to 100%, relative to controls that had not been treated with pyraclostrobin. The 12 isolates from 2011 were all in the highly sensitive category (inhibition >90%).

Microtiter plate assay

In the presence of 0.006 mg L^{-1} of pyraclostrobin (4× the mean EC_{50} of the *L. maculans* isolates collected in 2011), growth inhibition of the 263 isolates ranged from 20% to 87.8%, relative to the no pyraclostrobin controls. Eighty-one (31%) isolates had a growth inhibition of <50%, 94 (36%) had a growth inhibition of 50% to <60%, 61(23%) had a growth inhibition of 60% to <70%, 18 (7%) had a growth inhibition of 70% to <80%, and nine (3%) isolates had a growth inhibition of 80% to <90%. No isolates had a growth inhibition >90% (Figure 2.4a). At the higher discriminatory dose of 0.075 mg L^{-1} of pyraclostrobin (50× the EC_{50} of the isolates collected in 2011), seven (3%) isolates had a growth inhibition of 50% to <60%, 19 (7%) had a growth inhibition of 60% to <70%, 72 (27%) had a growth inhibition of 70% to <80%, 103 (39%) had a growth inhibition of 80% to <90%, and 62 (24%) isolates had a growth inhibition of 90% to <100% (Figure 2.4b). Growth inhibition ranged from 50.3% to 99.5% across the 263 isolates, relative to the control treatment with no pyraclostrobin.

A two-sided paired t-test was used to compare the results obtained with the conventional growth plate and microtiter plate assays. There was no significant difference ($P = 0.05$) in the results from both assays and the data were left-skewed at the 4× EC_{50} discriminatory dose. At the 50× EC_{50} discriminatory dose, the results from both assays exhibited a right-skewed distribution and were not statistically different at $P = 0.06$.

2.4 Discussion

Numerous studies have evaluated the sensitivity of different plant pathogens to pyraclostrobin, given the high risk for the development of resistance to this fungicide. Pyraclostrobin resistant isolates of *Mycosphaerella pinodes* were reported from infected field pea crops in two provinces of Canada and two states of the USA (Bowness et al. 2016). Akhavan et al. (2017) also identified an isolate of *Pyrenophora teres* f. *maculata (Ptm)* (net blotch of barley) from Saskatchewan with decreased sensitivity to pyraclostrobin. In contrast, isolates of *Phytophthora cactorum* (Lebert and Cohn) from infected strawberry in the USA (Rebollar-Alviter et al. 2007) and isolates of *Pyrenophora tritici-repentis* (Died.) Drechsler from infected wheat in Canada (MacLean et al. 2017) showed no decrease in sensitivity to this compound. In Canada, pyraclostrobin was first registered on cereals, pulses, and vegetables in 2001, then on canola in 2010 (Government of Canada: Health Canada Pest Management Regulatory Agency 2011). Limited information is available, however, on the pyraclostrobin sensitivity of *L. maculans* populations in Alberta. Fraser et al. (2017) evaluated the sensitivity in a collection of 117 *L. maculans* isolates from Alberta in 2011, but did not find any evidence of insensitivity to pyraclostrobin at that time.

Isolates with no history of exposure to a fungicide are typically used as a baseline in fungicide sensitivity studies. The isolates characterized by Fraser et al. (2017) were collected in the first year that pyraclostrobin was registered for blackleg control on canola, and hence there is some possibility that these isolates may have been exposed to the fungicide. Nonetheless, since no *L. maculans* isolates collected prior to the registration of pyraclostrobin in Alberta are available, the same baseline isolates used by Fraser et al. (2017) were included in the current study, along with 38 isolates randomly selected from a collection of *L. maculans* isolates made

from Alberta in 2016. A two-sided paired t-test indicated that the EC_{50} values obtained by Fraser et al. (2017) were not statistically different from those calculated in this study for the same set of isolates ($P = 0.31$). The mean EC_{50} for the 2016 isolates was four-fold greater than for the 2011 isolates. Moreover, testing with a discriminatory dose of pyraclostrobin 4× greater than the mean EC_{50} of the 2011 isolates found that approximately a third (80) of the isolates were inhibited <50%; at a discriminatory dose 50× greater than the mean EC_{50}, one isolate was inhibited <50%. These results suggest that the pyraclostrobin sensitivity of *L. maculans* isolates in Alberta decreased from 2011 to 2016. Repetitive use of a fungicide with the same mode of action could lead to the development of insensitivity or resistance in fungal populations (Brent & Hollomon 2007). Given that the isolates collected in 2016 may have been exposed to pyraclostrobin several more times than those collected in 2011, it is likely that the selection pressure imposed by the frequent application of this product resulted in an increase in the proportion of the *L. maculans* population exhibiting insensitivity. Differences in fungicide sensitivity also may be influenced by the inherent diversity in isolates of different fungal populations across a region (Kutcher et al. 2007), and (or) different fungicide application regimes (Kutcher et al. 2011).

Five of the *L. maculans* isolates used to determine the mean EC_{50} of the 2016 fungal collection via the growth plate assay had predicted EC_{50} values greater than the maximum concentration of pyraclostrobin employed and, therefore, were excluded from the calculation of the final mean EC_{50}. This may have led to a slight underestimation of the mean EC_{50} for the 2016 isolates, further supporting the conclusion that this value had increased for *L. maculans* between 2011 and 2016. The EC_{50} values obtained with the microtiter plate assay were lower than those calculated based on the conventional growth plate assay. Quinine outside inhibitors have a direct effect on spore germination (Olaya et al. 1998), so it is expected that the EC_{50}

values would be much lower in a microtiter plate assay compared with a growth plate assay (Bartlett et al. 2002). Since pyraclostrobin mainly supresses conidial germination, the microtiter plate assay is likely the preferred and most sensitive method for determining sensitivity of *L. maculans* isolates to this fungicide (Patel et al. 2012; Wise et al. 2008). Nonetheless, growth inhibition of the isolates at the two discriminatory doses evaluated, in both the conventional growth plate and microtiter plate assays, was not significantly different.

Three amino acid substitutions, G143A, G137R and F129L, in cytochrome b may contribute to resistance or tolerance to QoI fungicides (Grasso et al. 2006; Sierotzki & Frey 2007). Based on the current results, only one isolate (16CW239002) appeared to be insensitive or even resistant to pyraclostrobin. Further studies are necessary to determine whether any of the known mutation(s) are associated with this observed insensitivity. Indeed, since the mutations associated with pyraclostrobin insensitivity in *L. maculans* have not been characterized to date, the molecular basis underlying the observed insensitivity should be explored.

To the best of our knowledge, this is the first report of *L. maculans* isolates exhibiting decreased sensitivity to pyraclostrobin. Nonetheless, while insensitivity to pyraclostrobin has not been reported previously in this pathogen, Van de Wouw et al. (2017) found that the sensitivity of Australasian isolates of *L. maculans* to fluquinconazole has also decreased. This suggests the potential for multiple resistance issues in this important fungus and highlights the need for judicious use of fungicides in the management of blackleg of canola. Strategies such as the rotation of fungicides with different modes of action, along with the adoption of cultural and other control methods as part of an integrated disease management plan, will be important for the sustainable and long-term mitigation of blackleg.

2.5 Tables

Table 2.1 The effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in a conventional growth plate assay for 12 isolates of *Leptosphaeria maculans* originally collected in 2011 (Fraser et al. 2017).

Isolate	EC_{50} value determined in the current study[a]	EC_{50} values determined by Fraser et al. (2017)
11C78103	0.02	0.07
11C78301	0.05	0.05
11C78384	0.03	0.02
11C78401	0.09	0.04
11P125062	0.07	0.13
11P125232	0.10	0.08
11P125384	0.08	0.04
11S54201	0.05	0.04
11S54041	0.09	0.03
11W189011	0.11	0.17
11L999014	0.05	0.17
11L999241	0.06	0.24
Average	0.07	0.09

[a]A paired t-test indicated that there was no significant difference between the mean EC_{50} values determined by Fraser et al. (2017) and in the current study ($P = 0.31$).

2.6 Figures

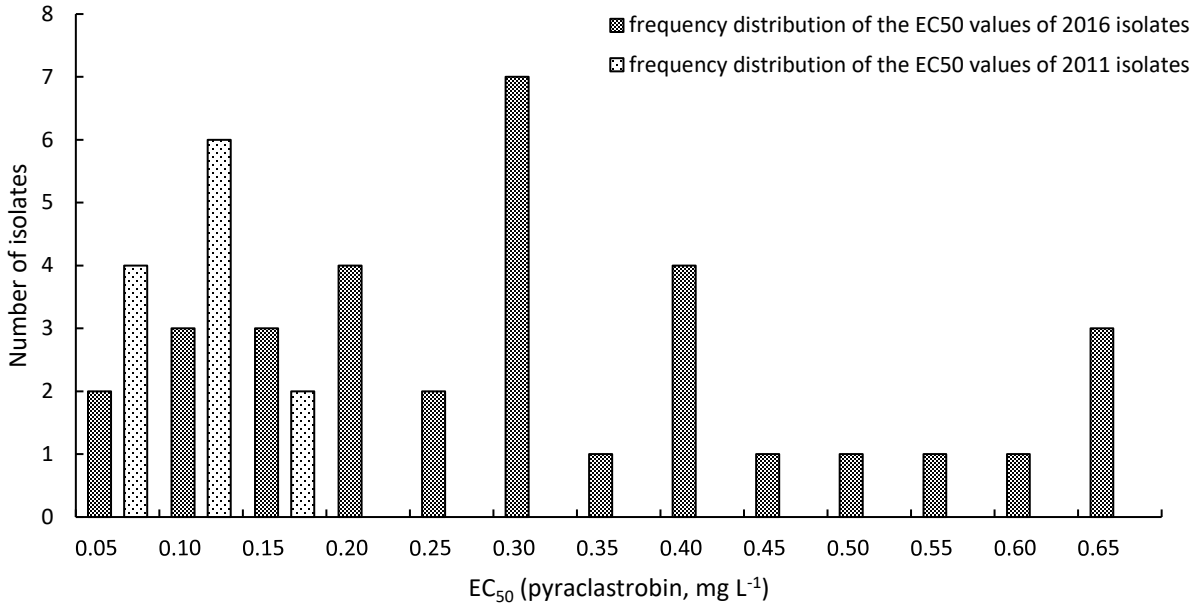


Figure 2.1 Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 33 isolates of *Leptosphaeria maculans* collected in 2016 and 12 isolates of *L. maculans* collected in 2011 from Alberta, Canada. Values were determined based on a conventional growth assay. Individual isolates are grouped in class intervals of 0.05 mg L^{-1}.

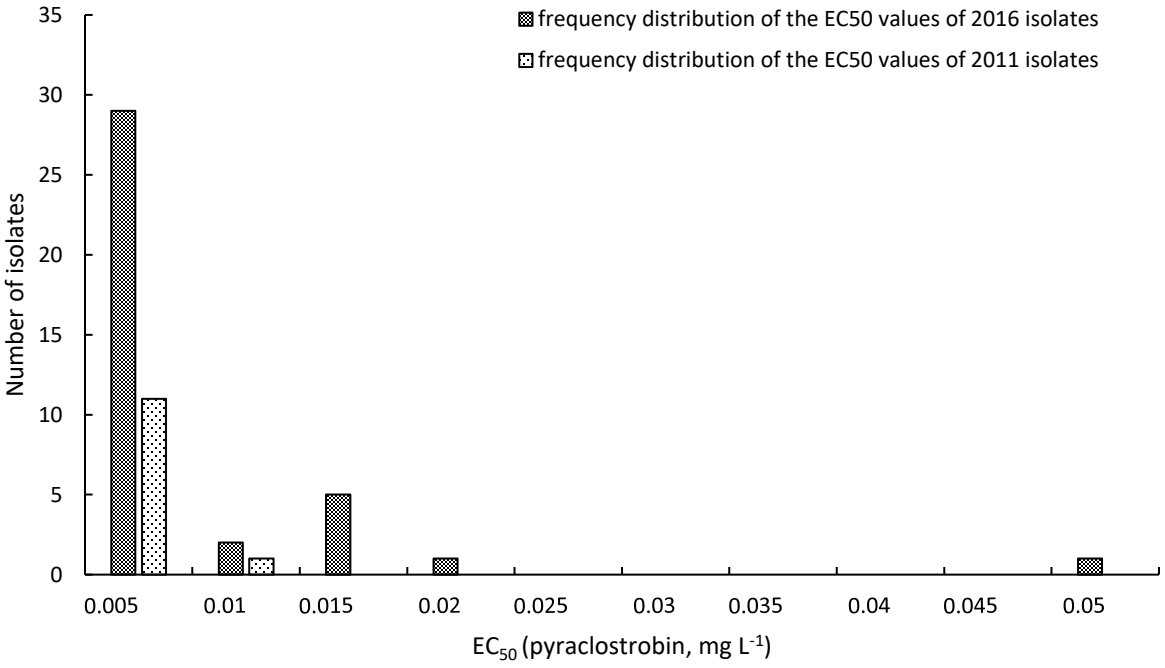
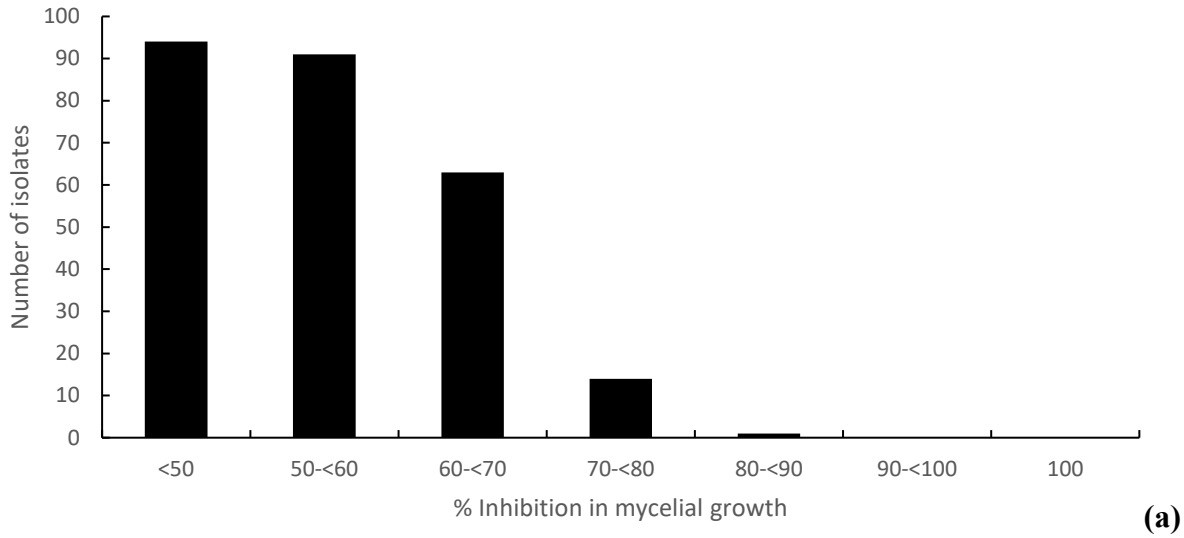


Figure 2.2 Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 38 isolates of *Leptosphaeria maculans* collected in 2016 and 12 isolates of *L. maculans* collected in 2011 from Alberta, Canada. Values were determined based on a microtiter plate assay. Individual isolates are grouped in class intervals of 0.0005 mg L^{-1}.



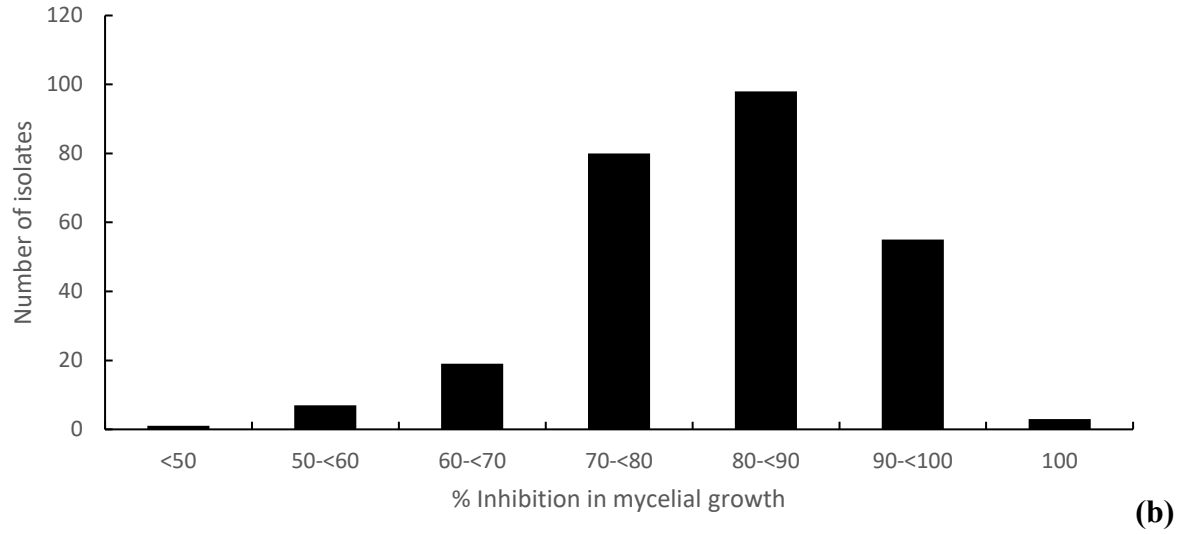
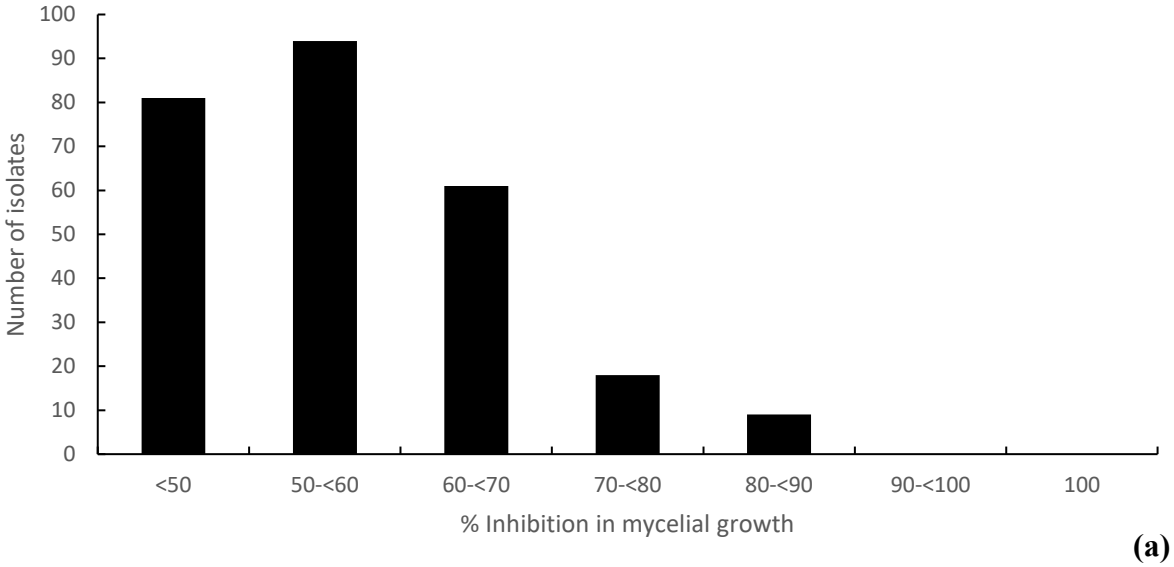


Figure 2.3 Frequency distribution of the inhibition of mycelial growth in 263 *Leptosphaeria maculans* isolates tested with a discriminatory dose of pyraclostrobin (0.28 mg L^{-1}) representing 4× the EC_{50} for baseline isolates collected in 2011 **(a)**, and a discriminatory dose of pyraclostrobin (3.5 mg L^{-1}) representing 50× the EC_{50} for baseline isolates collected in 2011**(b)**, based on conventional growth plate assays. The inhibition of mycelial growth is expressed as a percentage relative to a control treatment with no pyraclostrobin included.



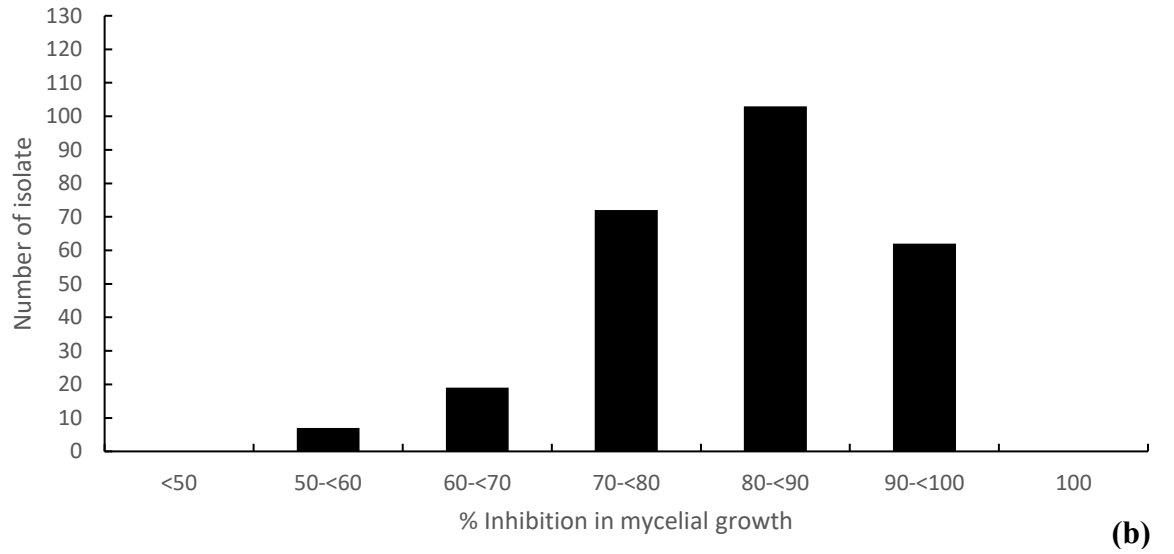


Figure 2.4 Frequency distribution of the inhibition of mycelial growth in 263 *Leptosphaeria maculans* isolates tested with a discriminatory dose of pyraclostrobin (0.006 mg L^{-1}) representing 4× the EC_{50} for baseline isolates collected in 2011 **(a)**, and a discriminatory dose of pyraclostrobin (0.075 mg L^{-1}) representing 50× the EC_{50} for baseline isolates collected in 2011 **(b)**, based on microtiter plate assays. The inhibition of mycelial growth is expressed as a percentage relative to a control treatment with no pyraclostrobin included.

Chapter 3: Yield Losses in Canola in Response to Blackleg Disease

3.1 Introduction

Blackleg (phoma stem canker), caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. and de Not. (anamorph *Phoma lingam* (Tode) Desm.), is an important disease of canola (oilseed rape; *Brassica napus* L.) (West et al. 2001). The disease is a major constraint to the production of this crop across many regions, including Europe, Canada and Australia (Barbetti 1975; Gugel & Petrie 1992; West et al. 2001). Infection can result in the development of lesions on cotyledons, leaves, stems and pods (Sivasithamparam et al. 2005). Blackleg also causes the formation of basal stem cankers, which restrict water and nutrient uptake and reduce yield (Howlett et al. 2001). *Leptosphaeria maculans* is a stubble-borne pathogen that survives on infected canola residues for 1 to 5 years (Hall 1992). The fungus produces wind-borne ascospores that can travel 5-8 kilometers from the source, as well as asexual pycnidiospores that spread to adjacent plants via rain-splash over a diameter of several meters (Bokor et al. 1975; Howlett 2004; Rouxel & Balesdent 2005). In western Canada, the average incidence of blackleg was 11% and symptoms of basal infection occurred on about 70% of canola crops surveyed in 2017 (Harding et al. 2018; McLaren et al. 2018; Ziesman et al. 2018).

Blackleg can be managed in a variety of ways, including by implementing longer rotations out of canola. Due to the higher economic returns associated with canola, however, many growers in western Canada tend to grow this crop continuously or in short rotations (Kutcher et al. 2013). Fungicidal seed treatments and foliar applications are also widely used to manage blackleg (West et al. 2001), but fungicide application did not have a major impact on yield in blackleg-resistant canola varieties (Kutcher et al. 2013). Furthermore, the need for

multiple applications of a fungicide can reduce the practicality of chemical control (Kutcher et al. 2011). The planting of blackleg resistant cultivars is the most effective means to manage this disease (West et al. 2001). However, in recent years, there have been several reports (Chen & Fernando 2006; Van de Wouw et al. 2014; Zhang et al. 2016) of shifts in the virulence of *L. maculans* populations, resulting in the loss or erosion of cultivar resistance.

By improving understanding of the relationship between blackleg severity and yield losses, the agronomic efficacy and economic benefits of control measures can be evaluated more accurately. An earlier study by Rempel et al. (1991) suggested that when disease severity was high, yield losses occurred in all seven genotypes they examined. They reported that canola yield and blackleg severity had a negative linear relationship (Rempel et al. 1991). More recently, Hwang et al. (2016) found that there were strong negative relationships between blackleg severity and seed yield and pod number in the blackleg-susceptible and open-pollinated canola 'Westar'. However, despite the importance of blackleg and its negative impacts on yield, the relationship between disease severity and yield has not been examined in hybrid canola, which represents the vast majority of the canola acreage in western Canada. As such, the objective of this study was to establish the relationship between blackleg severity and the yield of canola hybrids under conditions in western Canada.

3.2 Materials and Methods

3.2.1 Inoculum preparation

All experiments were conducted with wheat grain inoculum produced following Hwang et al. (2014). Briefly, cultures of *L. maculans* were grown in Petri dishes on V8 medium (composition per L: 850 mL distilled water, 150 mL V8 Original Vegetable Juice, 1.5 g $CaCO_3$,

15.0 g agar). The cultures were incubated for 21 days at room temperature (RT) under fluorescent lighting to encourage pycnidiospore production. Colonies of *L. maculans* were cut into small pieces and mixed with water-soaked wheat grains (900 mL wheat grains to one *L. maculans* culture) that had been sterilized in an autoclavable bag. The inoculated grain was incubated at RT for 21 days and then put in a dryer for 2 days. After drying, the grain was ground into 2 mm-diameter granules using a grain mill.

3.2.2 Field experiments

Field experiments were conducted over two years (2017 and 2018) at two sites located at the Crop Diversification Centre North (CDCN), Alberta Agriculture and Forestry, Edmonton, AB (53°39′ N, 113°22′ W). Two canola hybrids, '1950RR' (Canterra Seeds, Winnipeg, MB, Canada) and '73-15RR' (Monsanto, Winnipeg, MB), rated as moderately resistant to blackleg, were included in the experiments. At one site (site 1), the experiment was arranged in a randomized complete block (RCB) design with four replicates. Each plot consisted of four rows, 6 m in length and 1.5 m in width with 0.25-m spacing between the rows. Adjacent plots were separated by a 1 m buffer space, with 2 m between replications. Each row was seeded with 0.7 g of seed using a push seeder. The grain inoculum was applied at seeding by placing it in the push seeder along with the seeds. No grain inoculum was included in the control treatment. The seeding dates were 20 June 2017 and 24 May 2018. At the second site (site 2), the experiment was organized in an RCB design with six replications. Each plot was 1.5 m × 1.5 m, with a 1 m x 1 m treatment area in the center, 1 m spacing between plots, and a 2 m buffer between replications. The plots were hand-seeded at a rate of 25 seeds per row together with the inoculum. The plots were seeded on 7 June 2017 and 22 May 2018.

3.2.3 Disease and yield assessments

To assess the impact of blackleg on canola yield at site 1, all plants within a 1 m^2 area in the center of each plot were carefully dug up with a shovel and bagged. The remainder of each of the plots was harvested with a small-plot combine using a straight cut header, and the seed was weighed to determine overall yield. At site 2, all of the plants were dug up. Both sites were harvested at maturity on 18 September 2017 and 24 September 2018. Any soil was brushed off the roots, the plants were clipped at the soil line and the stem cross-sections were examined visually for blackened tissue. The plants were rated for blackleg severity on a 0–5 scale, where: 0 = no disease in a cross-section of the stem base; 1 = decay on <25% of the cross-section; 2 = decay on 25%–50% of the cross-section; 3 = decay on 51%–75% of the cross-section; 4 = decay on >75% of the cross section; and 5 = death of the plant (Guo & Fernando 2005). Average blackleg disease severity per 1 m^2 plot area was determined. For each plant, the height, number of pods and seed yield were recorded individually. The pods from each plant were threshed manually, and the seeds were cleaned and weighed individually.

3.2.4 Statistical analysis

Statistical analysis was carried out with R: A Language and Environment for Statistical Computing (R Core Team, R Foundation for Statistical Computing, Vienna Austria, 2013). Cultivar was considered as fixed effect, and replication and site-year and their interaction as random effects. Analysis of variance was performed, and a paired t-test was conducted to compare the mean disease severity for the two sites over two years for each cultivar. Regression analysis was used to establish the relationship between blackleg severity and both pod number and seed yield. Adjusted R^2 values and the F-test were used to examine whether the regression

was a good fit to the data. Residual data were tested for normality with the Shapiro-Wilk test in R shapiro.test stats. Regression equations were used to evaluate the loss of pod number and seed yield with increases in disease severity. In addition, the yield of plants with no blackleg symptoms was used as a point estimate, with different yield data points at each disease severity transformed into yield percentages relative to canola yield with no disease. Regression analysis was performed to estimate yield loss percentage per unit increase in disease severity.

3.3 Results

At site 1, the mean blackleg disease severity on the canola '73-15RR' was 1.5 in 2017 and 1.8 in 2018; at site 2, these values were 2.2 in 2017 and 1.5 in 2018 (Figure 3.1). A paired t-test indicated that the mean disease severity of '73-15RR' was not significantly different between the two sites over the two years (P=0.78). In the case of the canola '1950RR', mean blackleg severity was 2.2 in 2017 and 2.0 in 2018 at site 1, and 2.2 and 1.5 at site 2 (Figure 3.1). As was the case with '73-15RR', a paired t-test also indicated that the mean disease severity on '1950RR' was not significantly different between the two sites over the two years of the experiment (P=0.53). When averaged over the two site-years (2017–2018), the mean disease severity was 1.8 on '73-15RR' and 2.0 on '1950RR', which also did not differ significantly (P=0.32) (Figure 3.1).

Seed yield and pod number per plant decreased with increasing blackleg severity. In the case of the hybrid '73-15RR', the average seed yield ranged from 0.07 to 13.01 g per plant and the average pod number ranged from 14 to 164 pods per plant. Regression analysis showed that relationships between disease severity and pod number and seed yield were best explained by quadratic equations in all site-years. When averaged across site years for '73-15RR', the

regression model was $y = -6.977x^2 + 7.1194x + 158.91$ ($R^2 = 0.96$) for pod number vs. disease severity (Figure 3.2a), and $y = -0.559x^2 + 0.4369x + 11.995$ ($R^2 = 0.96$) for seed yield vs. disease severity (Figure 3.2b). Average seed yield and pod number for the canola hybrid '1950RR' were higher than for '73-15RR', ranging from 0.56 to 15.17 g seed per plant and from 18 to 210 pods per plant. When averaged across all site years for '1950RR', the regression models were $y = -9.9525x^2 + 16.106x + 186.78$ ($R^2 = 0.91$) for pod number vs. disease severity (Figure 3.3a), and $y = -0.5265x^2 - 0.1836x + 13.947$ ($R^2 = 0.93$) for seed yield vs. disease severity (Figure 3.3b).

The regression models for percent yield losses vs. disease severity were $y = 4.8217x2 - 3.7691x - 3.4744$ ($R^2 = 0.96$) for the hybrid '73-15RR', and $y = 4.0815x2 + 1.4236x - 8.1147$ ($R^2 = 0.93$) for '1950RR' (Figure 3.4). Plants with a blackleg severity of 0 had a slightly lower seed yield loss than plants with a severity of 1 for both canola hybrids; when disease severity was rated as 1, seed yield increased by 1.42g and 2.27g for '73-15RR' and '1950RR', respectively. However, as severities increased further to 2 to 5, yields began to decrease. In '73-15RR', the percentage yield loss increased by 15.5-99.4% in plants with disease severities of 2-5, relative to plants with disease severities of 0-1. In the case of '1950RR', the percentage yield loss increased by 12.6.7-95.7% in plants with disease severities of 2-5 compared with plants with disease severities of 0-1.

3.4 Discussion

In this study, the average blackleg severity rating was approximately 2 on the canola hybrids '73-15RR' and '1950RR', consistent with their classification as moderately resistant to this disease. The deployment of resistant canola cultivars has been the primary blackleg

management strategy used by Canadian growers and was highly effective in the past. With shifts in the virulence of *L. maculans* populations in western Canada (Chen & Fernando 2006; Kutcher et al. 2007), however, blackleg incidence has been increasing in recent years (Harding et al. 2018; McLaren et al. 2018; Ziesman et al. 2018). Consequently, resistant or moderately resistant canola cultivars are more likely to develop severe blackleg symptoms and suffer yield losses. Zhang et al. (2016) reported that the *Rlm3* resistance gene was predominant in 206 varieties/breeding lines tested from western Canada. Unfortunately, the corresponding avirulence gene, *AvrLm3,* occurred at low frequencies in the *L. maculans* population, suggesting that *Rlm3* resistance has lost its effectiveness (Zhang et al. 2016).

There have been several previous studies (Hwang et al. 2016; Rempel et al. 1991; Zhou et al. 1999) on blackleg severity-yield relationships. In an earlier report from Canada, Rempel et al. (1991) evaluated blackleg severity and yield loss in rapeseed under field conditions, and found a negative linear relationship between these parameters. They also noted that different genotypes had intrinsically large differences in their regression coefficients, which played a significant role in disease and yield loss assessment (Rempel et al. 1991). More recently, Hwang et al. (2016) examined the relationship between pod number and blackleg severity, as well as between seed yield and blackleg severity, in western Canada using a blackleg-susceptible and open-pollinated cultivar ('Westar'). Their results indicated that both of these relationships could be expressed by negative linear equations, and that seed yield per plant was reduced by 17.2% for each unit increase in disease severity. In contrast, a four-year study described the relationship between yield loss and blackleg severity with a logarithmic equation (Hall et al. 1993). Hall et al. (1993) surveyed both winter and spring oilseed rape in Ontario and recorded blackleg severity and 1000-seed weight to establish the model. Their results showed that the

reduction in 1000-seed weight in winter oilseed rape was higher than in spring oilseed rape (Hall et al. 1993). Zhou et al. (1999) studied the relationship between both blackleg incidence and severity and yield of winter oilseed rape under field conditions in the UK, using a critical point model, and found that early stem cankers affected yield the most because they caused severe damage later (Zhou et al. 1999). In another study from the UK, Sansford et al. (1996) established a yield loss model based on blackleg incidence on the stems prior to harvest. They reported a strong and significant relationship between yield losses and disease incidence as well as disease severity (Sansford et al. 1996).

The results of the current study indicated that blackleg severity-yield loss relationships were explained by quadratic equations, in which slight *L. maculans* infection (disease severity of 1) was associated with a small increase in yield relative to plants with no disease at all. When disease severity increased to ≥ 2, however, yields began to decrease dramatically. While plants of both '73-15RR' and '1950RR' with a blackleg severity rating of 1 generally produced more pods and suffered less yield loss than plants rated 0, the non-symptomatic plants tended to have poorer growth relative to the plants rated 1. Similar results were observed by Hwang et al. (2016), who suggested that late emerging (and generally less vigorous) plants may have escaped infection and hence were rated as zero. It also is possible that the slight decrease in yield associated with plants rated 0 vs. 1 may reflect the energy expended by the former in stopping infection; an array of structural, chemical, and protein-based defenses are triggered in response to attempted infection by a fungus (Freeman & Beattie 2008). Regardless, when blackleg severity increased to ≥ 2, yield losses increased significantly. While the R-squared values in the current analysis suggested that quadratic equations explained the blackleg severity-yield loss relationships quite well, overfitting of the regression models could be an issue (Burnham &

Anderson 2004). It may be useful, therefore, to use the Akaike information criterion (AIC) and/or Bayesian information criterion (BIC), which introduce penalty terms for the number of parameters in a model, for selection of the best model for the data (Burnham & Anderson 2004).

Unsurprisingly, yield losses in the moderately resistant hybrids '73-15RR' and '1950RR' appeared to be generally lower than what was reported earlier for the open-pollinated cultivar 'Westar' (Hwang et al. 2016). The latter does not carry any known blackleg resistance and is regarded as universally susceptible, while the hybrids in this study developed average disease ratings of ca. 2, as noted earlier. It is also possible that infection may have less of an effect on hybrids relative to open-pollinated varieties; a study from Australia showed significant differences in the yield of hybrids and open-pollinated canola, with hybrids having the higher yields (Zhang et al. 2016). Nonetheless, it is clear that infection of canola by *L. maculans* can result in significant yield losses, even in moderately resistant varieties, particularly at higher disease severities. As such, the adoption of integrated strategies, potentially with the rotation of resistance gene groups (Peng 2017) may be necessary for the successful long-term management of blackleg of canola. Additionally, all studies used decay percentage of the intersection area to evaluate disease severity on a 0-5 scale which is the categorical predictor in regression analysis. Further studies using the continuous predictor which is based on the actual decay percentage of the intersection area may be achieved through the use of imaging technology.

3.5 Figures

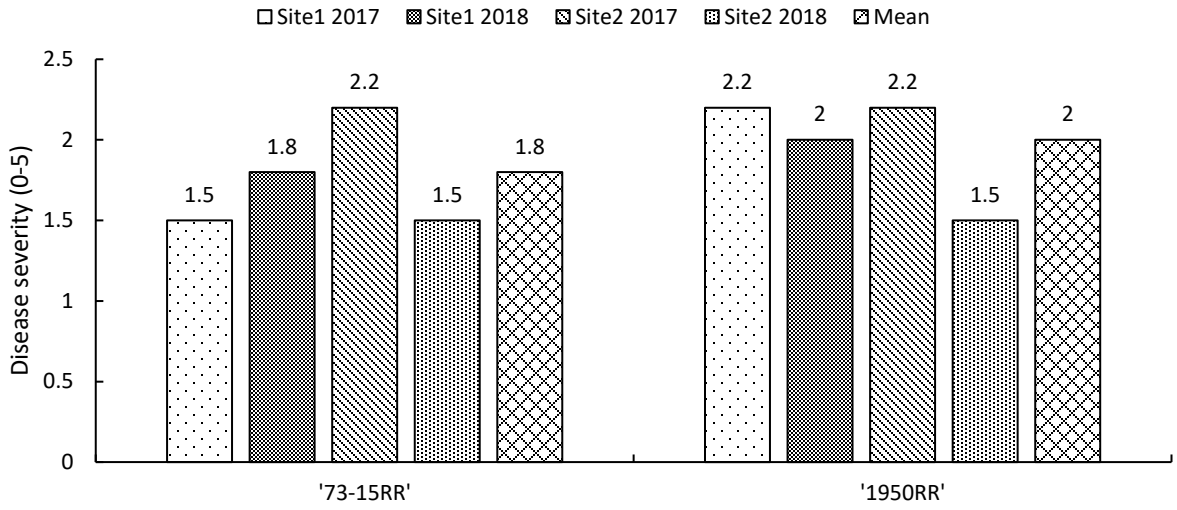


Figure 3.1 Mean disease severities of the canola hybrids '73-15RR' and '1950RR' under field conditions. Data were collected over four site years at two locations near Edmonton, Alberta (Crop Diversification Centre North, 2017 and 2018). Blackleg severity were assessed on a 0-5 scales, where: 0 = no disease and 5 = death of the plant. Paired t-tests indicated that the mean disease severity of '73-15RR' and '1950RR' were not significantly different between the two sites over the two years (P=0.78 and P=0.53 respectively)

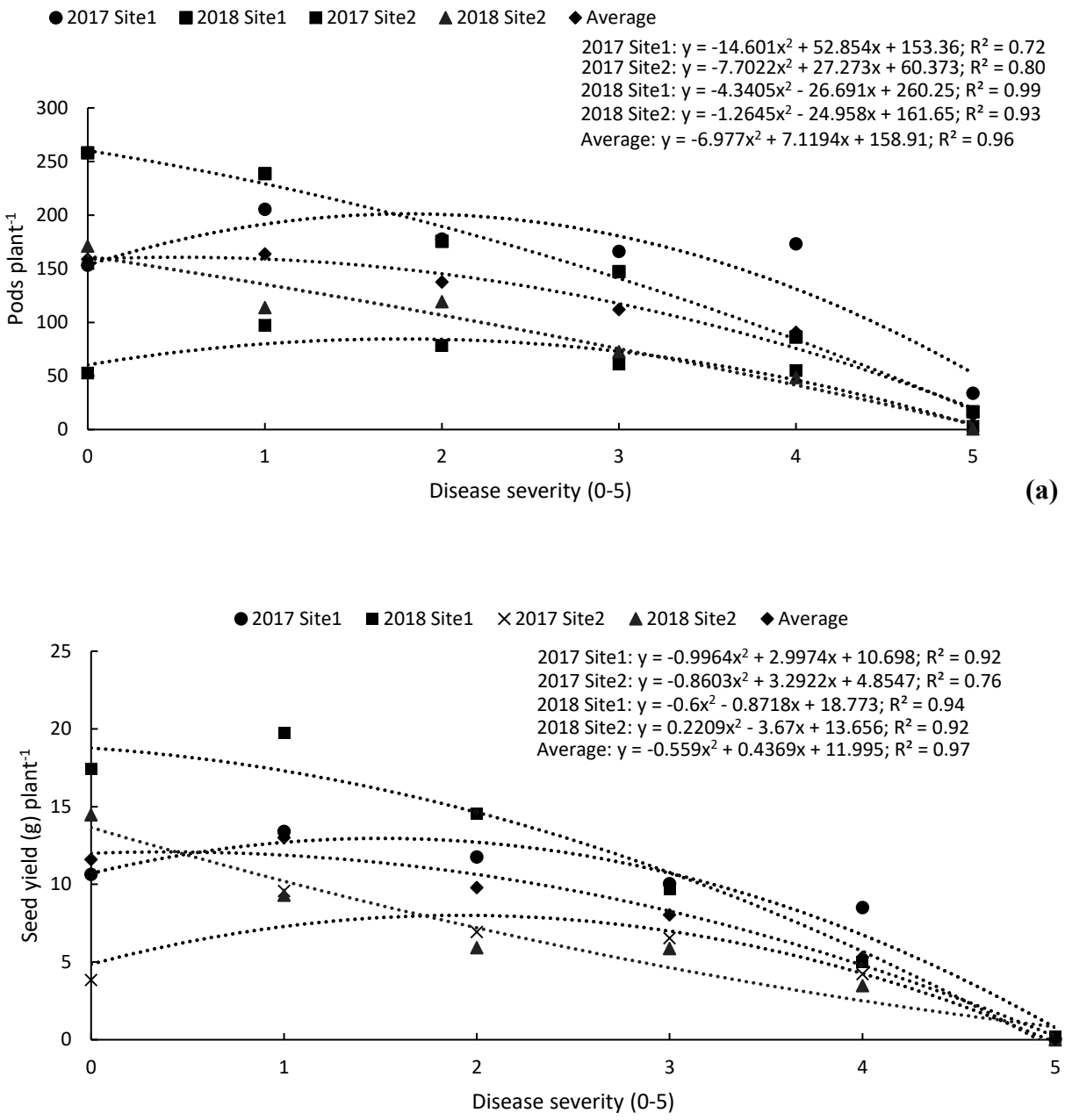
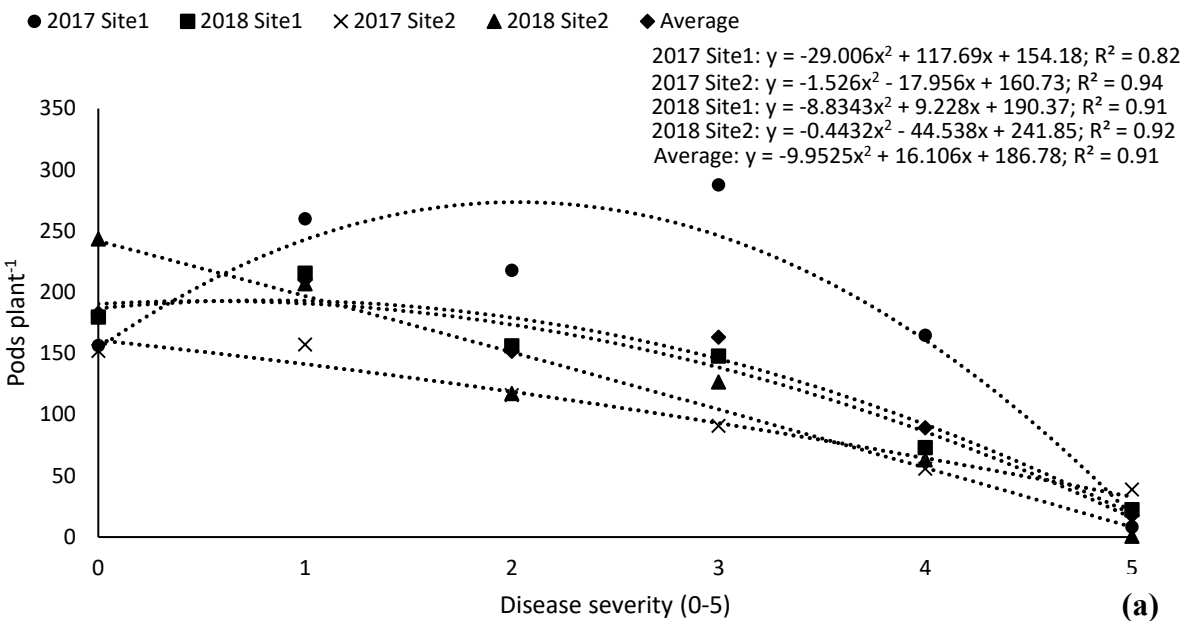


Figure 3.2 Relationship between blackleg severity and pods per plant **(a)** and seed yield per plant (g) **(b)** in the canola hybrid '73-15RR' under field conditions. Data were collected ov four site years at two sites in Edmonton, Alberta, 2017–2018. Each point represents the mean of four replications × four site-years. Blackleg severity was assessed on a 0-5 scale, where: 0 = no disease and 5 = death of the plant.



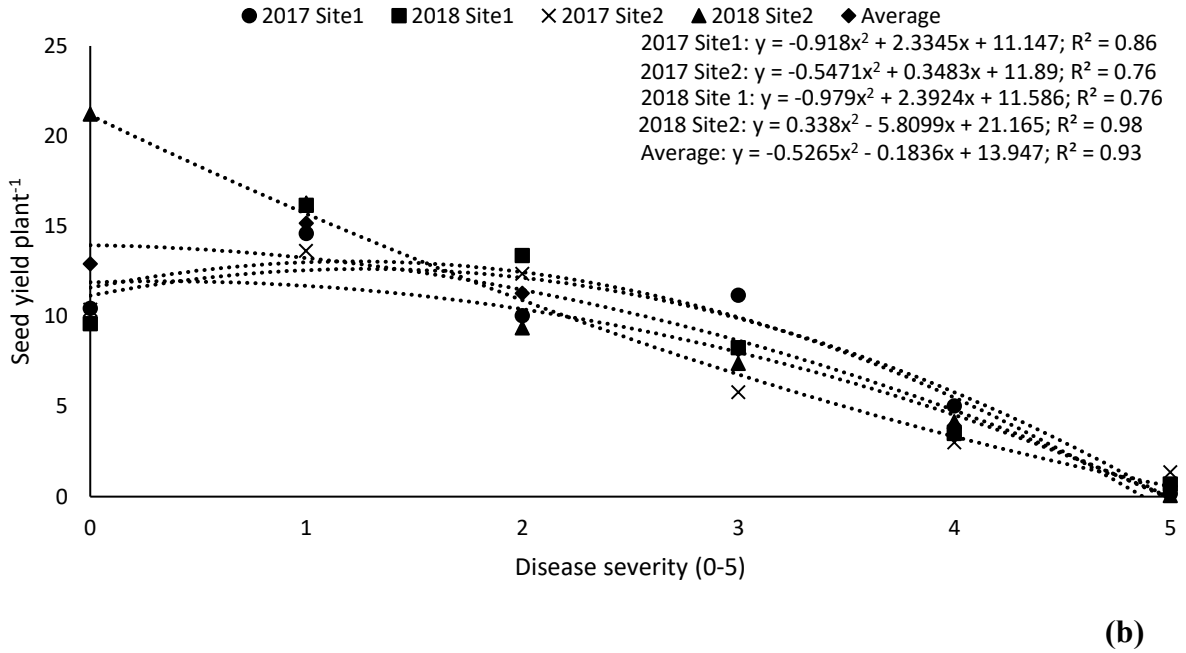


Figure 3.3 Relationship between blackleg severity and pods per plant **(a)** and seed yield per plant (g) **(b)** in the canola hybrid '1950RR' under field conditions. Data were collected over four site years at two sites in Edmonton, 2017–2018. Each point represents the mean of six replications × four site-years. Blackleg severity was assessed on a 0-5 scale, where: 0 = no disease and 5 = death of the plant.

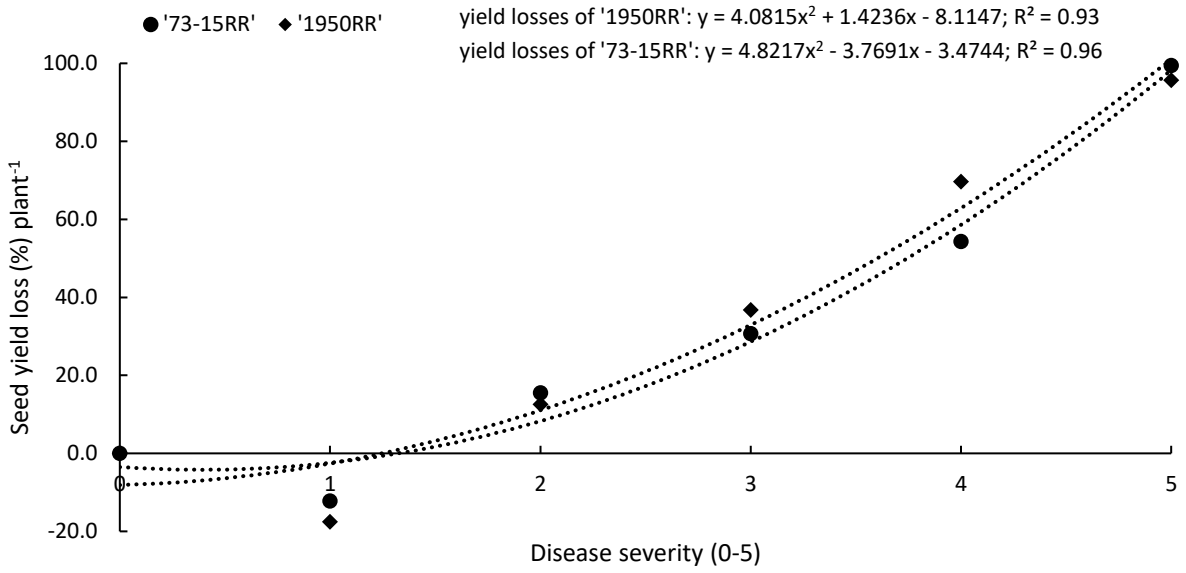


Figure 3.4 Relationship between blackleg severity and yield loss in the canola hybrids '73-15RR' and '1950RR' under field conditions at Edmonton in 2017–2018. The yield loss data were estimated using the y-intercept in the equation averaged over four site-years. The data points were transformed into the percentage of the yield (% maximum yield).

Chapter 4: Conclusion

4.1 General Conclusion

There are many possible strategies for the management of blackleg of canola. However, a single strategy cannot constrain the disease sustainably and/or completely, since each control measure has its own limitations. An integrated approach to blackleg management is, therefore, crucial for effective disease control (Kutcher et al. 2011). Cultural practices such as crop rotation, along with the deployment of resistant hosts and the application of fungicides as needed, can all contribute to successful blackleg management.

Growers use both foliar and seed fungicides to manage blackleg. The active ingredients in these fungicides include propiconazole, azoxystrobin and pyraclostrobin (Alberta Agriculture and Forestry 2018). With a limited choice of products, intensive use of the same mode of action can result in a build up of insensitive isolates, eventually resulting in fungicide resistance in the pathogen population (Brent & Hollomon 2007). Pyraclostrobin is a strobilurin with a single site-specific mode of action, so it is at high risk of establishing insensitivity (Fungicide Resistance Action Committee 2014). Fraser et al. (2017) evaluated pyraclostrobin sensitivity in *L. maculans* isolates collected from Alberta in 2011, and found that the fungal population from this province was sensitive to pyraclostrobin at that time (Fraser et al. 2017). However, pyraclostrobin sensitivity needs to be periodically re-examined. Therefore, in this thesis, the sensitivity to pyraclostrobin in *L. maculans* isolates collected from Alberta in 2016 was examined and compared with the results of Fraser et al. (2017).

In addition to fungicides, the deployment of resistant canola cultivars is the most common method of blackleg control (Peng et al. 2012). Although foliar fungicides have positive

effects on reducing blackleg severity and increasing yield in blackleg susceptible canola varieties, they do not have a large impact on the yield of blackleg resistant canola varieties (Kutcher & Brandt 2008; Kutcher et al. 2013). In the past decade, shifts in the virulence of *L. maculans* populations have been reported, which resulted in the breakdown of genetic resistance (Chen & Fernando 2006; Li et al. 2004; Rouxel et al. 2003). By understanding the relationship between blackleg disease severity and the yield of hybrid canola, the agronomic efficacy and economic benefits of management measures can be assessed more accurately. Therefore, the relationships between blackleg disease severity and yield of two canola hybrids were established under field conditions in Alberta.

4.2 Sensitivity Study

In Chapter 2, 251 single-spore isolates of *L. maculans* were collected from infected canola stubble from 18 counties in Alberta in 2016. Two methods were used to study fungicide sensitivity, including a conventional radial growth plate assay and a microtiter plate assay. Thirty-eight isolates selected randomly from the collection were used to determine the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}). In addition, the EC_{50} of 12 *L. maculans* isolates collected in 2011 (Fraser et al. 2017) was also established, enabling comparisons with the isolates from 2016 and the identification of any shifts in the pyraclostrobin sensitivity of fungal populations. While were no isolates were available that had been collected prior to the registration of pyraclostrobin on canola, the isolates from 2011 had at most been exposed to this fungicide for one year (Fraser et al. 2017). Therefore, the isolates from 2011 served to establish a baseline sensitivity to pyraclostrobin. The EC_{50} of the isolates collected in 2016 was four times greater than that of the 12 isolates collected in 2011, suggesting a reduction in the sensitivity of *L. maculans* over this five-year period.

Two discriminatory doses were used to test the pyraclostrobin sensitivity of the collection of 251 isolates from 2016 and the 12 isolates from 2011. One discriminatory dose was 4× the EC_{50} value of the 2011 isolates and the other was 50× the EC_{50} value of the 2011 isolates. If any isolates were inhibited by <50%, those isolates were regarded as insensitive to pyraclostrobin (Fraser et al. 2017). When tested with the discriminatory dose that was 4× the EC_{50} value of the 2011 isolates, approximately 90 of the isolates from 2016 showed <50% inhibition. This indicated that the sensitivity to pyraclostrobin in *L. maculans* had shifted. When tested with the discriminatory dose that was 50× the EC_{50} value of the 2011 isolates, other isolates were inhibited more than 50% and only one isolate from 2016 was inhibited around 50%. This isolate may be highly insensitive or even resistant to pyraclostrobin, since the dosage applied was so high. The results obtained using the growth plate and microtiter plate assays showed no significant differences.

Pyraclostrobin is an effective tool for the management of blackleg; however, insensitivity to pyraclostrobin in other fungal species developed after just two years of exposure (Wise et al. 2009). The shifts in the pyraclostrobin sensitivity of *L. maculans* observed between the earlier study by Fraser et al. (2017) and the current thesis suggest the need for judicious use of this fungicide. Indeed, the careful stewardship of this product is essential for its sustainable use as a blackleg management tool. It also may be important to consider use of other fungicides, with different modes of action, for control of this disease. Further monitoring of the pyraclostrobin sensitivity of *L. maculans* populations in Alberta is recommended, as further shifts may be expected in the future.

4.3 Yield Loss Study

Two moderately resistant canola hybrids were used in Chapter 3 to determine the relationship between blackleg severity and yield under field conditions in central Alberta. The relationships between disease severity and pod number and seed yield were best explained by quadratic equations. Similarly, the relationship between percent yield loss and disease severity was also explained by quadratic equations. In contrast, an earlier study from Alberta, based on a conventional, completely susceptible canola cultivar 'Wester', showed a linear relationship between blackleg and yield loss (Hwang et al. 2016). The results from this thesis may be more applicable to the present situation in western Canada, however, since nearly all canola acreage is planted to hybrid cultivars that are resistant or moderately resistant to blackleg. Moreover, the results from the current study suggest that minor blackleg infection does not necessarily have a negative effect on canola yield. Further research on more canola hybrids may be beneficial, since different blackleg-resistant varieties may carry different resistance genes, which could influence their response to infection by *L. maculans*.

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