Sensitivity of *Leptosphaeria maculans* isolates to pyraclostrobin and assessment of fungicide efficacy in the management of blackleg 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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University of Alberta

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ABSTRACT

Blackleg (*Leptosphaeria maculans*) disease is endemic to canola (*Brassica napus*) worldwide. With shortened canola rotations and increasing levels of blackleg in Alberta, Canada, growers may look to fungicides as an attractive disease management tool. However, the improper or intensive use of fungicides can result in insensitivity to these products in pathogen populations. Pyraclostrobin is a strobilurin fungicide that was first registered on canola for blackleg control in 2010. The objective of this thesis project was to determine the pyraclostrobin sensitivity of *L. maculans* populations in Alberta, and examine the efficacy of seed and foliar fungicides in managing blackleg. A group of 113 single-spore *L. maculans* isolates from Alberta were evaluated for pyraclostrobin sensitivity in a growth plate assay, and no insensitive isolates were found. These results were confirmed when a subset of 41 isolates were further evaluated for sensitivity in a microtiter assay. Various seed and foliar fungicide treatments, some of which contained pyraclostrobin, were tested on blackleg-susceptible and moderately resistant canola cultivars under greenhouse and field conditions. A combination of the experimental seed treatment BAS 720 F and the foliar treatment Priaxor, both of which contain fluxapyroxad and pyraclostrobin, often decreased blackleg levels and improved seed yield, especially in the susceptible cultivar under high disease pressure. Pyraclostrobin may represent an effective and sustainable blackleg management tool for canola growers in Alberta, as long as fungicide stewardship is practiced and included as a component of an integrated pest management plan.

PREFACE

This thesis is an original work by Michelle Fraser. Ms. Fraser wrote the first draft of all chapters, which were examined by Dr. Stephen Strelkov who then provided editorial revisions and suggestions on each chapter. Dr. Sheau-Fang Hwang and Mr. Wayne Barton also provided suggestions with respect to Chapter 2. Revisions were incorporated by Ms. Fraser. Ms. Fraser conducted all of the experiments described in this document. However, many individuals assisted with the research described in Chapters 2 and 3.

In Chapter 2, Dr. Hafiz Ahmed provided suggestions with respect to experimental design, statistical advice, and technical guidance, while Mr. Alireza Akhavan provided suggestions for the project design. Mr. Akhavan and Dr. Rong-Cai Yang also provided statistical advice. Many of the isolates of *Leptosphaeria maculans* included in Chapter 2 were originally collected by Dr. Yue Liang. Tara McLeod provided assistance in filling and inoculating Petri dishes. Technical staff from the Crop Diversification Centre North (CDCN) and grad students from the University of Alberta Plant Pathology Lab assisted in data collection. Mr. Gerd Stammler and Ms. Simone Miessner conducted the microtiter test, but the statistical analysis was conducted by Ms. Fraser.

Mr. Gerald Martens treated the canola seeds with seed fungicides and supplied the foliar fungicides used in Chapter 3. Mr. George Turnbull provided assistance with the seeding of field plots, the application of fungicide treatments and herbicides for weed maintenance, and harvesting the field plots. Mr. Yalong Yang and Mr. Heting Fu helped to maintain the plants in the greenhouse, which included watering, fertilizing, and controlling pests. Many undergraduate and grad students from the University of Alberta Plant Pathology Lab assisted in data collection for the greenhouse and field trials, as well as weeding and plot maintenance for field trials. Lubica Paparcikova (BASF) provided field space in Camrose for the field experiment and

assisted with harvest. Nicole Caillou (BioVision) conducted the seed quality tests. Financial support for this research was provided to Drs. Strelkov and Hwang by Agriculture and Agri-Food Canada and SaskCanola through the Growing Forward 2 Program.

ACKNOWLEDGMENTS

I would like to acknowledge my supervisor Dr. Stephen Strelkov, co-supervisor Dr. Sheau-Fang Hwang, and committee member Wayne Barton for their guidance, support, and mentoring throughout the creation of this thesis. I would like to thank Hafiz Ahmed and George Turnbull for their assistance with project design, technical expertise, and for many dedicated hours. I especially acknowledge Dr. Sheau-Fang Hwang for providing space, materials, seed, and equipment for the research projects. I would like to thank Dr. Jocelyn Ozga for reading this thesis and serving as the arms-length examiner during my defense.

I would like to thank Qixing Zhou, Yalong Yang, and Heting Fu their technical guidance and assistance, Alireza Akhavan for his time and guidance on statistical analysis, and Yue Liang and Tara McLeod for their assistance with the sensitivity project. I gratefully appreciate the help I received from fellow grad and summer students in the lab and the field. I would like to thank Gerald Martens for providing material for the experiments, Lubica Paparcikova for accommodating my field trial in Camrose and assistance with harvest, Gerd Stammler and Simone Miessner for conducting the microtiter test, and Nicole Caillou for conducting the seed quality tests. I would also like to thank Gayle Rankin and Kelly Dunfield for mentoring me during my teaching assistantship. I would also like to acknowledge Agriculture and Agri-Food Canada and SaskCanola for financial support provided to my supervisors through the Growing Forward 2 Program.

Lastly, I would like to thank my family, friends, and spouse Cohen for all of their support when I needed it the most. I could not have completed this journey without the support from my fellow grad students, whom which will always remain true friends to me.

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1.1 CANOLA

1.1.1 Introduction to canola

Canola (*Brassica napus* L. and *Brassica rapa* L.) is an oilseed crop bred from rapeseed, which belongs to the Brassicaceae family (often referred to as the mustard family). Other plants in this family include mustard (*Sinapis arvensis*), cauliflower (*B. oleracea)*, cabbage (*B. oleracea)*, turnip (*B. rapa)*, and broccoli (*B. oleracea)* (Casséus 2009). Canola and rapeseed are grown in many parts of the world, with the highest production in China, Canada, India, France, Germany and Australia (FAOSTAT 2014). Rapeseed originated in Asia and has been cultivated for centuries in Asia, India, and Europe for lamp and cooking oil (Busch et al. 1994). It was later used as a lubricant for steam engines (Casséus 2009).

During the Second World War, the transportation of marine lubricants to Canada was halted; therefore, the production of rapeseed began in the prairies as an alternative. Rapeseed oil was an appropriate candidate for use in steam engines, since its high erucic acid content allowed the oil to stick easily to metal. The end of the war and the switch to diesel engines led to a significant reduction in the demand of oilseed lubricants and, in turn, rapeseed production eventually stopped (Busch et al. 1994). Also during this time and up until the 1960's, Canada was dependent on importing oilseeds for edible uses (Casséus 2009).

Canada was not a major producer of plant based oils since most of the edible oil crops could not survive Canadian winters; a solution to this was to look into the use of rapeseed as an edible oil (Busch et al. 1994). Although rapeseed oil had been consumed by other cultures for

centuries, it was claimed to have undesirable compounds with potential toxic effects at high levels; these compounds included erucic acid (a long chain fatty acid) and glucosinolates (sulphur compounds that give vegetables in the mustard family their characteristic sharp taste) (Casséus 2009). In the 1970's, through cross breeding experiments, plant breeders from Agriculture and Agri-Food Canada and the University of Manitoba developed canola, an oilseed crop bred from rapeseed with minimal levels of erucic acid and glucosinolates (Rempel et al. 2014). Canola is an abbreviation of "Canadian oil" (Casséus 2009) and has an internationally regulated standard since its chemical and nutritional content is different from rapeseed . To be called canola, the oil component must contain less than 2% erucic acid, while the oil free solid component must contain less than 30 μmol of glucosinolates per gram of meal(Rempel et al. 2014). By the 1980's, canola had replaced almost all rapeseed production in Canada (Casséus 2009).

Plant anatomy and development

Canola and rapeseed are cool-season crops, and are seeded and harvested using the same machinery as cereal crops (Canola Council of Canada 2016a). Polish canola (*B. rapa*) has a short growing season and produces brown and yellow colored seed, while Argentine canola (*B. napus*) has a longer growing season and produces black seed (Canola Council of Canada 2016b). Canola is seeded very shallow in a moist seeding bed and emerges within 4 to 15 days. The leaves first form a rosette and the plant eventually grows a long stalk with small yellow flowers. Plant height ranges from 0.7 to 1.7 m. Canola has a raceme inflorescence and flowers indeterminately. Siliques (pods) develop from fertilized flowers, growing to about 6 to 9 cm in length and producing 15 to 25 small round seeds each. Once the pods fill and 40% of the pods

are yellow or brown, the crop is swathed, dried, and harvested. Canola can also be direct cut when all the pods are mature and moisture is 8% or less (Edwards and Hertel 2011).

Utilization

Of the canola that is produced in Canada, 90% of it is exported to other countries in the form of seed, oil, and meal (Canola Council of Canada 2016c). The seed oil content ranges from 35% to 45%, while seed meal consists of 36% to 39% protein (Edwards and Hertel 2011). After the canola is harvested, 50% of the seed is trucked to one of the 13 crushing and refining plants across Canada (Rempel et al. 2014). Once the oil is extracted and refined, it can be used in many edible products such as salad dressings, margarine, and for cooking and frying (Rempel et al. 2014). As a result of its high smoke point, canola oil makes a good candidate for use in deep frying. Canola oil has a low proportion of saturated fats compared with other vegetable oils and, if substituted for saturated fats, it may reduce the risk of coronary heart disease. Canola oil also is used in many non-edible products including bio-diesel, cosmetics, detergents, inks, lacquers, lubricants, pharmaceuticals, plastics and much more. The meal that remains after crushing is very high in protein, and vitamins B and E (Casséus 2009). Canola meal is a good source of feed for use in aquaculture and in animal feed industries (Rempel et al. 2014).

Production and trade

Worldwide

When comparing worldwide crop production data, canola is included in the category of rapeseed (Casséus 2009). The total worldwide production of rapeseed in 2013 was about 72.5 million tonnes. Canada and China are the top two rapeseed producers, and produced 17.9 and

14.4 million tonnes in 2013, respectively. Other major producers include India, France, Germany, and Australia (FAOSTAT 2014).

Canada

The prairie provinces of Saskatchewan, Alberta, and Manitoba produce 99% of Canada's canola, while British Columbia, Ontario, Quebec and New Brunswick produce the other 1% (Statistics Canada 2014a). In 2013, Saskatchewan, Alberta, and Manitoba produced 8.9, 6.0 and 2.9 million metric tonnes of canola, respectively. The average canola yield across Canada was 2,200 kg/hectare (Statistics Canada 2014a) with an average value of $621 per tonne (Statistics Canada 2016). In 2013, canola had the largest cash farm receipts at $7,333,345, compared with wheat at $5,350,846 (Statistics Canada 2014b), and although more wheat was produced than canola, this makes canola Canada's most valuable crop (Casséus 2009). From 1961 to 2012, Canada has been the largest exporter of rapeseed/canola in the world (FAOSTAT 2014). In 2012, the top canola seed export destinations were China, Japan, and Mexico, while the United States was the top exporter destination of canola meal (Rempel et al. 2014).

Diseases of canola

The high economic returns associated with the growing of canola have led to intensive production of the crop, which has in turn resulted in the emergence of various disease issues. While the pathogens of canola are almost always present in a crop, disease severity will vary depending on factors such as location, environmental conditions, farming practices, and cultivar. Pathogens of canola can maintain themselves in the soil, on canola seed and residues, and through other susceptible host plants including related weeds (Canola Council of Canada 2014). The major soil borne fungal diseases of canola that infect roots and young seedlings include

seedling blight and damping off (*Rhizoctonia solani* Kühn, *Pythium* spp., and *Fusarium* spp.), foot rot or basal stem rot (*R. solani* and *Fusarium* spp.), and brown girdling root rot (*R. solani* and associated fungi). Soil borne diseases that affect the above ground portions of canola plants include downy mildew (*Peronospora parasitica* Fr.) and sclerotinia stem rot (*Sclerotinia sclerotiorum* Lib.). Another serious soil-borne disease of canola is clubroot, which is caused by the protist *Plasmodiophora brassicae* Woronin. Aster yellows disease, caused by a bacteria-like phytoplasma, is transmitted by leafhoppers (*Macrosteles quadrilineatus* Forbes*)* and affects the inflorescence of canola plants. Stubble- or residue-borne diseases of canola include white rust or staghead (*Albugo candida* Pers.), alternaria black spot (*Alternaria brassicae* Berk. and *Alternaria raphani* Groves and Skolko), white leaf spot and gray stem (*Pseudocerosporella capsellae* Ellis and Everh.), and blackleg (*Leptosphaeria maculans* Desm.) (Martens et al. 1988). Rouxel and Balesdent (2005) describe *L. maculans* as being "exceptional in its ability to develop contrasting life modes" and having a "high level of plasticity in terms of nutrient requirements and acquisition"; these qualities are what makes *L. maculans* a serious risk to the production of canola and rapeseed worldwide.

1.2 *LEPTOSPHAERIA MACULANS*

1.2.1 Taxonomy and classification

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.) is an ascomycete fungus that belongs to the Pleosporales, which is the largest order in the class Dothideomycetes. For some time, *L. maculans* was classified in the Loculoascomycetes, but was reclassified as part of the Pleosporales after taxonomic revisions. Fungi belonging to the order

Pleosporales have uniloculate ascomata and the presence of pseudoparaphyses. The asci of Pleosporales can be described as being bitunicate, cylindrical, clavate or cylindro-clavate, and sometimes obclavate or spherical, and containing hyaline or colored ascospores (Rouxel and Balesdent 2005). Fungi in this order are ubiquitous saprophytes, meaning they can live on dead organic material, but some also are parasites of living plants (Kaczmarek and Jedryczka 2011). The order Pleosporales also includes the pathogens *Phaeosphaeria nodorum* E.Müller (glume blotch of cereals), and the genera *Cochliobolus* (common root rot of wheat and barley), *Alternaria* (black spot of canola), and *Pyrenophora* (seedling blight and leaf blotch of oats, leaf stripe of barley, and tan spot of wheat) (Rouxel and Balesdent 2005).

The order Pleosporales is comprised of 28 families, including the Leptosphaeriaceae. Characteristics that distinguish *Leptosphaeria* from the other genera in this family are cylindrical asci with short pedicels, and lightly pigmented, fusoid, multiseptate ascospores. Fungi in the *Leptosphaeria* genus produce small to medium sized solitary ascomata that may be found scattered or in groups. They may be found partly immersed or on the host tissue surface, and contain asci with 8 cylindrical ascospores. The ascomata of *Leptosphaeria* aresubglobose, broad or narrow, conical, papillate, and ostiolate. The genus *Leptosphaeria* contains 26 species, including *L. maculans* (Zhang et al. 2012). *Leptosphaeria maculans* is a pathogen of the Brassicaceae family, affecting host species mainly of the genus *Brassica* (Rouxel and Balesdent 2005), which includes cabbage, cauliflower, broccoli, mustard, rapeseed, and canola.

Tode (1791) was the first to associate the cause of dried stems of red cabbage with a fungus, which he named *Sphaeria lingam*. Recognizing the fungus as a pathogen of cabbage in 1849, Desmazières renamed it *P. lingam*. The teleomorph or sexual stage of *P. lingam* was later recognized as *L. maculans*. Until recently, *L. maculans* was considered to be one species divided

into two groups of isolates: highly virulent or aggressive (group A, Tox^{+}) isolates and weakly virulent or non-aggressive (group B, Tox^{0}) isolates. After many studies showing that isolates of group A could not cross with isolates of group B, as well as the identification of physiological and morphological characteristics distinct to each group, it was suggested that group A and group B are in fact different species: *L. maculans* (aggressive) and *L. biglobosa* (non-aggressive). The two species exist as a complex on rapeseed and canola, although *L. maculans* is more damaging to the host (Fitt et al. 2006). Both species have similar life cycles, but *L. biglobosa* differs in terms of pseudothecial morphology, germination, growth, diffusion of pigmentation, biochemical traits, molecular patterns, and pathogenicity (Fitt et al. 2006). The fruiting bodies of *L. maculans* mature faster (Kaczmarek and Jedryczka 2011) and the ascospores of *L. biglobosa* do not survive as long as those of *L. maculans* (Fitt et al. 2006). On agar medium, *L. biglobosa* produces an abundance of mycelium, grows rapidly, and produces few pycnidia, while *L. maculans* is associated with slow growth, less mycelium, and extensive sporulation (Kaczmarek and Jedryczka 2011).

1.2.2 Epidemiology, symptoms, and life cycle

Leptosphaeria maculans, causing blackleg disease of canola (synonym: Phoma stem canker), is a polycyclic pathogen that produces primary and secondary inoculum. The primary inoculum consists of ascospores released from pseudothecia on infected canola residue left over from the previous growing season (Hall 1992). *Leptosphaeria maculans* survives as a saprophyte on canola residue as long as the debris persists (Rouxel and Balesdent 2005), which may be anywhere from 1 to 5 years (Hall 1992). Pseudothecia and ascospores are sexual structures produced from the sexual reproduction between isolates of opposite mating types found on the same piece of residue (Rouxel and Balesdent 2005). The release of ascospores

from pseudothecia is initiated mainly by the onset of rainfall, or heavy dew and high humidity (West et al. 1999). The ascospores are spread by wind and usually are dispersed within 500 meters of the source of inoculum (West et al. 1999), although they can travel distances of up to 5 kilometers (Kaczmarek and Jedryczka 2011). In western Canada, ascospores are released from May to August (West et al. 2001), with the number of spores peaking in June and July (Hall 1992). There are reports that ascospores can survive for 30 days at temperatures between 5-20°C under dry conditions (Kaczmarek and Jedryczka 2011) and up to 6 weeks after release (West et al. 1999).

After release, the ascospores colonize the cotyledons and leaves of young canola plants (Kaczmarek and Jedryczka 2011). Under moist conditions and temperatures between 4 to 28°C, the ascospores germinate and produce hyphae (West et al. 2001), which then infect the plant via stomata or wounds (Kaczmarek and Jedryczka 2011). Once inside the leaf, the hyphae grow through intercellular spaces and between mesophyll cells (Kaczmarek and Jedryczka 2011). At this point, *L. maculans* is necrotrophic (Rouxel and Balesdent 2005) and causes light green or beige colored circular lesions (Kaczmarek and Jedryczka 2011) about 1 to 2 cm in diameter (West et al. 2001). The early development of lesions on cotyledons and leaves may correlate with the incidence of stem cankers that develop later on. There are reports, however, of stem cankers developing in the absence of other external symptoms (Hall 1992). Eventually small black fruiting bodies, called pycnidia, can be seen in the lesions and result from asexual reproduction. This is the anamorphic stage of *L. maculans* where the fungus is called *P. lingam* (Kaczmarek and Jedryczka 2011). Under warm, moist conditions, pycnidiospores ooze out from the pycnidia in pink or purple masses. Pycnidiospores serve as secondary inoculum and are spread by rain splash over a diameter of several meters to neighboring plants (Hall 1992). Heavy

rainfall and continuous periods of wetness also are important factors in the spread of pycnidiospores (Barbetti 1976). Although it is rare, the fungus also can infect the siliques and consequently the seed inside. Infected seed may lead to the development of infected seedlings and spread of disease to uninfested fields (West et al. 2001).

After the fungus has infected the leaf, it enters a biotrophic stage of its development (Hayward et al. 2012). In this stage, there is symptomless colonization of the petiole and stem tissue (Hall 1992). The fungus grows between the xylem parenchyma and cortex or through xylem vessels, and makes its way into the stem cortex (Hayward et al. 2012). Blockage of the xylem vessels restricts water transport and causes premature ripening of the plant, ultimately contributing to yield loss (Kaczmarek and Jedryczka 2011). Near the end of the growing season, the fungus reaches the crown and roots of the plant where it becomes necrotrophic once again (Hayward et al. 2012) and produces crown cankers and stem lesions (West et al. 2001). Dry blackened necrotic tissue can be seen by examining cross-sections of the crown (Hayward et al. 2012). In severe infections, stem cankers can girdle and even sever the stem, leading to reduced water transport, premature ripening, and lodging (West et al. 2001). Canola is most susceptible to petiole and stem infection at the cotyledon and 1 to 2 leaf stage (Hall 1992), and therefore the incidence of stem infection is higher in younger plants (Hall 1992). Severe yield loss also is associated with stem infection that occurs before the 6 leaf stage (Gugel and Petrie 1992). After harvest, *L. maculans* colonizes canola stubble and produces new pycnidia. Pycnidiospores can also infect canola stubble, which consequently increases the levels of inoculum and production of pseudothecia (West et al. 2001). In contrast, the stem lesions caused by *L. biglobosa* are confined mainly to the upper stem (Fitt et al. 2006) and are pale brown in color with dark margins (Dilmaghani et al. 2012).

1.2.3 Importance of blackleg on canola

Worldwide

Blackleg is endemic to most regions where rapeseed or canola is grown (Gugel and Petrie 1992) and causes severe yield losses in Europe, Australia, and Canada (Chen and Fernando 2006). The worldwide distribution of this disease may be attributed to the transmission of different *Brassica* seed and crops carrying *L. maculans* and *L. biglobosa* (Fitt et al. 2006).

Only three years after the emergence of the rapeseed industry in Australia in 1968, there were major blackleg epidemics in the southern and western areas of the country. The disease was so devastating that by 1974, the area sown to rapeseed had decreased by 96% (Gugel and Petrie 1992).

Leptosphaeria maculans has caused serious damage to rapeseed crops in Europe. Blackleg is the most important disease of rapeseed in Germany (Gugel and Petrie 1992) and the United Kingdom (Toscano-Underwood et al. 2001). The disease was first seen on susceptible rapeseed cultivars in the United Kingdom in 1977 and 1978, with reported yield losses of 50% (Toscano-Underwood et al. 2001). Severe epidemics also were eported in France in 1950 and again in the late 1960's (Gugel and Petrie 1992).

There is evidence that where the less damaging *L. biglobosa* is dominant, it will soon be replaced by *L. maculans*, which is currently expanding its range geographically (Rouxel and Balesdent 2005). This poses a risk to rapeseed production in China. To date, only *L. biglobosa* has been isolated in China, where large areas of susceptible rapeseed and other *Brassica* crops are grown. If *L. maculans* is introduced, there is a risk that China will face a severe blackleg epidemic (Fitt et al. 2006).

Western Canada and Alberta

Blackleg was first found on rapeseed/canola in western Canada after 1961, at which time it was caused by the weakly virulent strain (*L. biglobosa*). It was not until 1975 that the highly virulent strain (*L. maculans)* was first discovered on canola stubble in central Saskatchewan. After the introduction of the high yielding canola cultivar 'Westar' in 1984, blackleg spread to most regions of Saskatchewan. The disease was first discovered in Alberta in 1986, and by 1989 it had spread in the central and southern areas of the province. Around the same time that blackleg had spread to Alberta it was also found in Manitoba, and by 1988 it was found in 62% of canola crops surveyed in that province. Over this period of epidemics in western Canada, the average incidence and yield losses associated with blackleg ranged from 5 to 15% and 15 to 30%, respectively. The highest incidence and yield loss were reported in Saskatchewan in 1985, at 90% and 56%, respectively (Gugel and Petrie 1992). The introduction of blackleg resistant canola cultivars in the early 1990s and the adoption of longer rotations led to a reduction in the severity of yield and quality losses; however, these practices have not eliminated the pathogen or the disease. Since the late 1990's, changes in the virulence of *L. maculans* have been reflected in the emergence of races with virulence phenotypes, which have caused severe levels of disease in canola cultivars that were previously blackleg resistant (Kutcher et al. 2011).

The prevalence and incidence of blackleg basal stem cankers ranged from 3% to 55% and from 2% to 25% in 2010 and 2013, respectively, in Saskatchewan canola crops (Dokken-Bouchard et al. 2011; Miller et al. 2014). In Manitoba, the prevalence ranged from 58% in 2010 to 75% in 2013, while the incidence remained relatively stable at about 13% (McLaren et al. 2014; McLaren et al. 2011). In Alberta, the prevalence (percent of fields with infected plants per region) and incidence (percent of infected plants per field) of blackleg also has been increasing,

and in recent years were the highest amongst the three prairie provinces; the prevalence ranged from 53.4% in 2010 to 97% in 2012, while the incidence ranged from 8.7% in 2010 to 23% in 2012 (BASF 2013; Lange et al. 2011). Increases in the incidence and severity of blackleg are likely the result of short crop rotations and intensive canola production which may have led to a breakdown in genetic resistance of canola cultivars (Lang 2012). Indeed, although the cropping of resistant cultivars may help to limit yield losses, blackleg continues to be an important disease issue in the Alberta.

1.2.4 Management of blackleg in canola

Crop rotation

The management of blackleg in canola should consist of an integrated approach, as there are many cultural and chemical disease management tools available. Two of the most important cultural control methods include crop rotation and the cropping of blackleg resistant cultivars (Kharbanda and Tewari 1996). Continuous canola monocultures or short rotations do not allow canola residues to decompose completely, allowing a build-up of *L. maculans* inoculum on infected crop debris (Kutcher et al. 2013). Regardless of whether a cultivar was resistant or susceptible, Kutcher et al. (2013) observed an increase in blackleg incidence and severity when canola was grown more frequently than 1 in 4 years. In addition to canola stubble residue, *L. maculans* also can survive on volunteer canola and related weed species; therefore, good weed control is an important component of successful blackleg management (Kutcher et al. 2011).

Genetic resistance

To aid in the management of blackleg, there is a strong focus on the breeding of canola cultivars with resistance to *L. maculans*. Canola exhibits both qualitative and quantitative

resistance to *L. maculans*. Qualitative resistance is expressed at the seedling stage and is race specific. This type of resistance follows the gene-for-gene model, meaning that resistance results when a resistance gene product in canola recognizes the corresponding elicitor molecule encoded by a *L. maculans* avirulence gene. Very little is known about cultivar resistance to *L. biglobosa*, although there is indication that resistance genes effective against *L. maculans* are not effective against *L. biglobosa* (Fitt et al. 2006). Qualitative resistance offers complete resistance to the pathogen, but it can be easily overcome as a result of selection towards virulent *L. maculans* races (Huang et al. 2009). Indeed, the continuous cropping of canola genotypes with the same basis of resistance can lead to the breakdown of that resistance (Marcroft et al., 2012). Currently, 12 major resistance genes have been identified that correspond to 12 *L. maculans* avirulence genes (Marcroft et al. 2012). In contrast to qualitative resistance, quantitative resistance is expressed at the adult plant stage and is race non-specific. Quantitative resistance is controlled by many minor genes and confers partial resistance. It is, however, more durable than qualitative resistance (Huang et al. 2009).

All cultivars of *B. napus* registered in Canada either have moderate or high blackleg resistance, although their specific genes for resistance are usually unknown (Kutcher et al. 2011). Marcroft et al. (2012) demonstrated that rotation of canola cultivars with different known resistance genes will reduce selection pressure for virulence in fungal populations, and consequently prolong the effectiveness of resistance. Results presented by Marcroft et al. (2012) also suggest that if the same cultivar is grown in the same region over several years, then it is best to maximize the distance to the previous year's residues. For example, there is a high risk that severe blackleg infection will develop if the same canola cultivar is grown consecutively for 3 years within 2 km of the same cultivar sown (Australian Government: Gains Research and

Development Corporation 2011), or if the same canola cultivar is grown within 100 m of 1-year-old stubble of the same cultivar (Department of Agriculture Government of Western Australia n.d.). The heterothalic nature of *L. maculans* promotes genetic diversity and may lead to more virulent genotypes within the pathogen population. Finding opposite mating types on resistant cultivars may be more difficult for the pathogen, however, since population sizes are smaller than on susceptible cultivars (Aubertot et al. 2006).

Residue management

Tilling and burning crop residue are additional cultural methods recommended for blackleg management in canola (Kharbanda and Tewari 1996). While the burning infected canola residue is practiced in Australia, it is not recommended or practical in western Canada due to the risk of grass fires (Gugel and Petrie 1992). Depending on the soil conditions and moisture levels, burying plant residue may increase its rate of decomposition (Gugel and Petrie 1992). Guo et al. (2005) concluded that tillage reduces blackleg incidence and severity when a simple rotation is used, while there is a reduced effect of tillage when using a diverse rotation. By contrast, Kutcher and Malhi (2010) found little relationship between blackleg severity and residue burning and tillage. Zero tillage practices resulted in higher canola yields, although this was attributed to retained soil moisture rather than a disease management effect (Kutcher and Malhi 2010).

Biological control

The bacterium *Paenibacillus polymyxa* Prazmowski (strain ATCC 202127) produces antifungal peptides that are active against *L. maculans* and other fungi. The strain can be applied to growing medium, plant seeds, and the plants themselves (Kharbanda et al. 2003). It was found that the bacterial strain decreased the growth of *L. maculans* in culture and on canola

leaves, stems, and stubble (West et al. 2001). The activity of the peptides of *P. polymyxa* against *L. maculans* makes it suitable as a possible biological control agent for blackleg disease in canola (Beatty and Jensen 2002). Hammoudi et al. (2012) also found a 50% reduction in blackleg symptoms on seedling leaves when canola seeds were treated with the bacteria *Pseudomonas chlororaphis* Guignard and Sauvageau, *Pseudomonas fluorescens Migula,* and *Gliocladium catenulatum* Gilman and Abbott, with the most effective control on root collars and stem bases provided by treatment with *Serratia plymuthica* Lehmann and Neumann.

Flooding

Peluola et al. (2013) found that flooding canola basal stems infected with *L. maculans* reduced pathogen recovery slightly after 2 weeks, while the pathogen could not be recovered after 6 weeks. Flooding may eradicate the inoculum of *L. maculans* in stubble, although this method is only practical in western Canada where spring flooding is common or in countries like China where rice paddy crops are grown (Peluola et al. 2013).

Sowing date

Blackleg severity can be reduced by adjusting the time of sowing in order to avoid the release of large quantities of ascospores (Kharbanda and Tewari 1996). In field experiments conducted in France, Aubertot et al. (2004) found that seeding winter rapeseed earlier in the season resulted in reduced crown canker severity. By seeding earlier, the period of susceptibility to infection was shifted away from the time of major ascospore release. While ascospore release various between regions and years, factors that affect crop development and emergence also must be considered when trying to reduce levels of disease (Aubertot et al. 2004). In field experiments in Australia, Khangura and Barbetti (2004) also found a reduction in blackleg

disease and an increase in yield when sowing canola crops before the maturation of pseudothecia. Canola sown later in the season required fungicide protection, and yields were lower as a result of a shortened growing season (Khangura and Barbetti 2004). In Canada, ascospores are released throughout the season. Therefore, the sowing date has little effect on the levels of blackleg (Kharbanda and Tewari 1996).

Forecasting models

The timing of ascospore release depends on the maturation of pseudothecia, which is related to ambient temperature and wetness; this is why the timing of ascospore release varies greatly between countries and even between locations within a country. There are very few reports on the development of prediction models for ascospore release, and they are usually restricted to the region where they were developed (Salam et al. 2007). The "Blackleg Sporacle" forecast model was developed in Australia to predict the onset of ascospore release based on temperature and rainfall. The model predicts pseudothecia maturation will occur with a 10-day average temperature of $< 22°C$ and ≥ 4 mm of weekly rainfall (Salam et al. 2003). Salam et al. (2007) tested the performance of the "Improved Blackleg Sporacle" model and the "SporacleEzy" model in Canada, Australia, Poland, France and the United Kingdom. The Improved Blackleg Sporacle model incorporates the impact of low temperatures on pseudothecial maturation, while the SporacleEzy is a simplified version of the Blackleg Sporacle model which excludes the duration threshold of rain and temperature. The Improved Blackleg Sporacle model performed well in all 5 countries in which it was evaluated, while the SporacleEzy model performed better than the Improved Blackleg Sporacle model in Canada, Australia, Poland and the UK (Salam et al. 2007).

Through the use of linear models, Ghanbarnia et al. (2012) found that total rainfall per week (*R*) was significantly correlated with mean disease incidence and severity when canola plants were infected with pycnidiospores of *L. maculans* at the cotyledon, three-leaf, and six-leaf stages. However, average maximum temperature per week was significantly correlated only with disease at the cotyledon and 6-leaf stages. To evaluate the combined effects of the two parameters on disease development, Ghanbarnia et al. (2012) also developed a non-linear model. The model was accurate in demonstrating the relation between the timing of infection and growth stage, and the main effect of rainfall in disease development. The success of the models developed by Ghanbarnia et al. (2012) suggests that they could be used to develop more comprehensive models to evaluate blackleg epidemics (Ghanbarnia et al. 2012). These types of models would be beneficial in the decision making process regarding sowing date and the use of fungicide treatments.

1.3 FUNGICIDAL CONTROL

Despite the availability and widespread cropping of blackleg resistant canola cultivars, growers in western Canada are reporting significant production issues related to the disease. While the deployment of resistant cultivars is one of the most important tools for managing blackleg, the adoption of short wheat-canola rotations has resulted in increased disease levels, and growers are beginning to apply more fungicides as a control measure (Kutcher et al. 2011).

In western Canada, fungicides are commonly used on canola as seed treatments and foliar treatments. Not only do seed treatments eradicate seedborne inoculum and prevent the spread of blackleg into un-infested areas, they also protect emerging seedlings from airborne ascospores with their systemic activity (Gugel and Petrie 1992). Marcroft and Potter (2008) evaluated the efficacy of the fungicide fluquinconazole as a seed treatment on canola. Results from the study

showed a reduction in blackleg symptoms, including reduced severity of internal infection and plant mortality, with the best results observed under high inoculum loads. Cultivars with low to moderate levels of disease resistance showed an increase in yield when treated with fluquinconazole, while the fungicide gave no yield advantage to cultivars with high blackleg resistance (Marcroft and Potter 2008).

When applied to susceptible canola cultivars, foliar fungicides have been shown to reduce blackleg symptoms and increase yields (Kutcher et al. 2011). However, mixed results have been reported with respect to the efficacy of foliar fungicides to control blackleg (Gugel and Petrie 1992). The timing of application of foliar fungicides may be difficult to determine since ascospores are released continuously throughout the growing season (Gugel and Petrie 1992), and usually more than one fungicide application is required for effective protection (Kutcher et al. 2011). Foliar fungicides should be applied early in the season, when plants are at the most susceptible stage, and at peak ascospore release, which may require the use of forecasting models (Gugel and Petrie 1992).

In an experiment conducted by Wherrett et al. (2003), infected canola residues were dipped in a number of different chemicals. Twenty of the treatments, including the fungicides fluquinconazole and flutriafol, delayed the development of pseudothecia and, in turn, the treatments reduced the amount of ascospores released. Results from this study suggest the potential for treatments to control *L. maculans* on old infected canola residues in order to prevent seedling infection (Wherrett et al. 2003).

While investigating the application timing of foliar fungicides, Steed et al. (2007) found that fluzilazole and carbendazin gave the best control of blackleg and yield response when there were

early epidemics of phoma leaf spot (leaf spots caused by *L. maculans*). The results also suggest that the application of foliar fungicides is unnecessary when epidemics of phoma leaf spot occur later in the season (Steed et al. 2007).

Khangura and Barbetti (2004) evaluated different combinations of seed (fluquinconazole), foliar (flusilazole), and in-furrow (flutriafol) fungicide treatments for the control of blackleg in canola. All fungicide treatments reduced disease severity and increased yields. Results from the study suggest a potential for the combination of seed and foliar fungicide treatments to control blackleg, especially when sowing susceptible cultivars under higher disease pressure (Khangura and Barbetti 2004)

There are currently five seed treatment (Table 1.1) and six foliar products (Table 1.2) registered for the control of blackleg on canola in Alberta, some of which contain strobilurin fungicides (Alberta Agriculture and Rural Development 2014). Strobilurins are one of the most important and recently developed classes of microbial fungicides (Kim and Hwang 2007).

1.3.1 Strobilurin fungicides

The active ingredients in strobilurin fungicides are synthetic analogs of naturally occurring β-methoxyacrylates isolated from basidiomycete fungi such as *Strobilurus tenacellus* Pers. Natural strobilurins are susceptible to photochemical degradation and, with chemical structural modification, scientists were able to transform them into stable synthetic fungicides useful for disease control (Balba 2007). The first synthetic strobilurin fungicides were developed in the 1990's and early 2000's by modifying the highly volatile and photochemically unstable strobilurin A molecule (Figure 1.2). They included azoxystrobin, kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin, and pyraclostrobin (Bartlett et al. 2001).

The molecular structure of all strobilurin fungicides include a pharmacophore that is bridged to a side chain by an aromatic ring (Huang et al. 2007). In strobilurin fungicides, the methoxyacrylate moiety is the pharmacophore (Figure 1.2), and therefore, the component which binds to the target enzyme (Gisi et al. 2002). There are nine classes of strobilurin fungicides (Fungicide Resistance Action Committee 2014), all of which bind similarly to a target site (Gisi et al. 2002). These different classes are analogs of natural strobilurins, and are defined by the modification of the methoxyacrylate moiety. Different chemicals within these classes are distinguished by their distant side chain (Balba 2007).

Strobilurin fungicides have single-site activity and inhibit mitochondrial respiration (Gisi et al. 2002). Fungicides that have single-site activity are active against one point in a metabolic pathway, or on a critical enzyme or protein. This activity is very different than in multi-site fungicides, which are active against several metabolic points within a fungus (Mueller et al. 2013). Strobilurin fungicides are also referred to as quinone outside inhibitors (Q_oIs), since they bind to the quinone outside (Q_o) oxidizing site. The Q_o site is located on the cytochrome bc1 enzyme complex (complex III) in the mitochondria. By binding to the Q_o site, electron transfer is blocked and the formation of ATP (adenosine triphosphate) is prevented. The fungus then dies as a result of an energy deficiency (Gisi et al. 2002). Binding of the Q_o site also leads to the formation of reactive oxygen species (ROS), which can damage the mitochondria and may contribute to death of the fungus (Inoue et al. 2012).

Strobilurin fungicides are active against most major fungal and fungal-like plant pathogens including the oomycetes, deuteromycetes, ascomycetes and basidiomycetes (Kim and Hwang 2007). Their site-specific toxicity, rapid activity and rapid environmental degradation make strobilurin fungicides some of the best-selling and most widely applied fungicides worldwide.

This class of fungicides are used mainly as protectants, since they are highly active at preventing fungal spore germination. Strobilurins are locally systemic and will spread out easily on a leaf surface. They are absorbed by the leaf cuticle and have translaminar activity. Nonetheless, only limited amounts of the fungicide moves systemically in the xylem (Balba 2007). Strobilurins are effective in controlling plant pathogens; however, their unique site-specific activity puts them at risk for the selection of fungicide insensitive fungal isolates.

1.3.2 Insensitivity issues associated with strobilurin fungicides

Within a pathogen population there may be individuals with naturally occurring insensitivity to certain fungicides that arise from a very low rate of genetic mutation (Ma and Michailides 2005). These insensitive individuals initially may be present at low frequencies, but with the selection pressure resulting from the intensive use of fungicides, they may increase over time to form an insensitive sub-population (Gisi et al. 2002). Certain mutations can affect the ecological fitness of the insensitive pathogens; if the fitness is negatively affected by the mutation, insensitive individuals cannot compete in the absence of the fungicide with wild type individuals. In this case, eliminating the use of the fungicide will decrease the frequency of insensitive individuals. Some mutations, however, do not affect the fitness of the isolates and they may survive with or without selection pressure from the fungicide (Ma and Michailides 2005).

There are two types of mutations in plant pathogenic fungi that confer fungicide insensitivity: major- or single-gene resistance and polygenic resistance. In major-gene resistance, the fungus becomes insensitive as a result of a single mutation in one gene. Single-gene resistance leads to a qualitative change in fungicide sensitivity, meaning that the mutant strain is completely unaffected by the fungicide application. Single-gene resistance is usually observed with most systemic, single-site fungicides (Georgopoulos and Skylakakis 1986),

including the strobilurins (Ma and Michailides 2005). Polygenic resistance involves the interaction of many mutant genes. The result of polygenic resistance is quantitative; exposure to the fungicide causes a gradual shift towards insensitivity. Unlike major-gene resistance, there is not a sudden loss of effectiveness of the fungicide, but rather disease control decreases gradually as isolates become less sensitive to the fungicide (Georgopoulos and Skylakakis 1986). Polygenic resistance has been reported to demethylation inhibitors (DMI) (Brent and Hollomon 2007) and succinate dehydrogenase inhibitors (Fungicide Resistance Action Committee 2016b). There is evidence that at least four different mechanisms can confer insensitivity to the DMI fungicides (Brent and Hollomon 2007), while over 27 mechanisms can confer insensitivity to the SDHI fungicides (Sierotzki and Scalliet 2013). The resistance mechanisms against some fungicide classes are unclear or have not been determined. In the case of the phenylamides, for example, the resistance mechanism may be based on 1 or 2 major genes, and possibly several minor genes (Fungicide Resistance Action Committee 2016a).

Fungicides with single-site activity are at a higher risk of developing insensitivity compared with ones with multi-site activity (Agrios 2005), since it only takes a single mutation in the pathogen to overcome the effects of the fungicide activity (Mueller et al. 2013). For this reason the strobilurins, which specifically target the Qo site of the mitochondria, have a moderate to high risk of causing the development of insensitivity in fungal populations (Gullino et al. 2000). Strobilurins were first introduced commercially in 1996, and soon after, insensitive isolates of *Blumeria graminis* DC. f. sp. *tritici* Marchal were detected on wheat in 1998 (Bartlett et al. 2001). The mechanism of insensitivity to strobilurin fungicides was identified as a point mutation in the mitochondrial cytochrome b (*cyt b*) gene (Gisi et al. 2002). The mutation leads to an amino acid substitution of glycine with alanine at position 143 (G143A) in the cytochrome

b protein (Kim and Hwang 2007), which in turn prevents the binding of the fungicide inhibitor to the enzyme complex (Gisi et al. 2002). There is also no effect of the mutation on the activity of the enzyme in the metabolic pathway, indicating that the fitness of insensitive individuals may not be affected (Gisi et al. 2002). Since all of the strobilurin classes have very similar binding activity to the Qo enzyme, fungal isolates that develop insensitivity to one strobilurin will exhibit cross-resistance to all QoIs (Gisi et al. 2002). Cross-resistance is defined as a situation when a pathogen becomes insensitive to one fungicide molecule in a fungicide class, and thereby becomes insensitive to all other molecules in that same class (Brent and Hollomon 2007). Unlike cross-resistance, multiple resistance occurs when a pathogen is insensitive to two or more independent classes of fungicides, as exposure to each fungicide selects independent mutations (Brent and Hollomon 2007).

The G143A mutation is correlated with strobilurin insensitivity in a variety of fungi and their hosts, and has been identified in isolates of *Alternaria spp., B. graminis* f. sp. *hordei, Didymella bryoniae* Fuckel*, Mycosphaerella fijiensis* Morelet*, Pyricularia grisea* Hebert*, Podosphaera fusca* Fr.*, Pseudoperonospora cubensis* Berkeley & Curtis*, Plasmopara viticola* Berk. and Curtis*, Sphaerotheca fuliginea* Schlecht.*, Venturia inaequalis* Cooke (Kim and Hwang 2007). A second mutation responsible for strobilurin insensitivity is the substitution of phenylalanine with leucine at position 129 (F129L) of *cyt b*. This mutation was identified in *Pythium aphanidermatum* Edson (Kim and Hwang 2007) and also was found in *P. grisea* (Ma and Michailides 2005).

Some fungi have been shown to develop *in vitro* insensitivity to QoIs through a cyanide-insensitive respiratory pathway involving an enzyme termed alternative oxidase (AOX). When the electron flow in the respiratory electron transport chain is interrupted, AOX will accept

protons from ubiquinol, reduce oxygen to water, and synthesize ATP. The fungus is able to regain metabolic activity. However, the efficiency of ATP formation is low in this process. It is believed that this pathway serves as protective role against oxidative stress associated with the formation of ROS (Inoue et al. 2012). It is important to note that this alternative respiration pathway has been observed only *in vitro* and is believed to be inhibited by plant flavones under natural conditions (Wise et al. 2008). As such, it is unlikely to be important in conferring insensitivity to otherwise strobilurin-sensitive fungi on host plants, but may represent a confounding factor in experiments conducted *in vitro* (Wise et al. 2008).

Fungicide resistance management should be included as part of an integrated disease management plan. A few examples of fungicide resistance management strategies include following the manufacturer's recommended dose, restricting the number of treatment applications per season, and applying the product only when necessary (Brent and Hollomon 2007). Fungicide products should not be used exclusively; they should be applied in a mixture with a companion or partner fungicide with a different mode of action. By mixing fungicides, the selection pressure from the at-risk fungicide will be reduced. While the pathogen may be insensitive to one fungicide, it will be controlled by the other fungicide in the mixture (Brent and Hollomon 2007). Chemical manufacturers often sell products in formulated or pre-packed mixtures to ensure some level of resistance management. Growers may notice a gradual or complete loss of the effectiveness of some fungicides when fungicide resistance becomes an issue. In order to determine if management strategies are effective, or to detect a potential resistance problem, fungicide insensitivity can be monitored by testing samples of target pathogen populations from the field through bioassays or genetic assays (Brent and Hollomon 2007).

There are various ways to detect strobilurin insensitivity in fungi. Molecular methods for detecting strobilurin insensitivity include polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR, and allele-specific real-time PCR analyses. Often, companies and public research laboratories use the G143A marker with allele-specific real-time PCR to detect and monitor strobilurin insensitivity, since it is a rapid, highly sensitive, and cost effective method. The speed of this method makes it ideal for helping growers to detect fungicide insensitive fungal genotypes in their fields in order to facilitate resistance management decisions (Ma and Michailides 2005).

Fungicide insensitivity also can be studied using conventional phenotypic bioassays. This involves collecting fungal isolates and testing their degree of sensitivity to certain fungicides. Usually the baseline sensitivity of a fungal population is determined by testing the fungicide sensitivity of fungal isolates that have never been exposed to a particular fungicide. This baseline indicates the natural range of sensitivity within that fungal population. A baseline is necessary to determine shifts in sensitivity after the fungus is exposed to the fungicide (Brent and Hollomon 2007). Bioassays are traditionally carried out by plating fungal isolates on agar medium amended with the fungicide in question, and measuring the inhibition of mycelial growth or conidial germination (Vega et al. 2012). To determine the baseline of a fungal population, unexposed isolates are plated on medium amended with various concentrations of the fungicide. The concentration at which fungal growth is effectively inhibited by 50% (EC_{50}) is the baseline concentration for the unexposed isolates. The isolates to be screened for insensitivity are grown on medium amended with this baseline concentration of the fungicide and are classified as sensitive or insensitive based on whether or not their growth is inhibited (Chang et al. 2007).

Conventional plating methods are an effective way to detect and monitor fungicide insensitivity, however, this process can be time consuming, laborious, and requires space for a large number of Petri dishes (Vega et al. 2012). On the other hand, microtiter plate assays are quicker and require fewer resources; they involve growing the pathogen in multi-well plates containing liquid medium and the fungicide in question. Growth is then measured with a spectrophotometer (Brent and Hollomon 2007). PCR methods can also be used for monitoring, and although these methods are rapid, a disadvantage to them is that they do not detect fungicide insensitivity that may have arisen via other mechanisms. For example, PCR analysis with primers designed to detect the G143A mutation will not detect insensitivity caused by the F129L mutation or any other silent mutations. Phenotypic assays such as the traditional plating method and microtiter assay can detect fungicide insensitive genotypes (Vega et al. 2012). Since alternative respiration via AOX has been observed *in vitro*, however, this may lead inaccurate interpretations of insensitivity in plating and microtiter sensitivity assays. By including the compound salicylhydroxamic acid (SHAM) in *in vitro* tests of sensitivity to QoI fungicides, the alternative respiration pathway is blocked and true fungicide insensitivity can be assessed accurately (Wise et al. 2008).

1.3.3 Pyraclostrobin

Pyraclostrobin is a broad spectrum strobilurin fungicide that is registered for use on various crops including blackleg of canola. Pyraclostrobin was developed by BASF in 2000 (Bartlett et al. 2002) and was first registered in Canada in 2003 as a broad-spectrum foliar fungicide, Headline EC, for use in various crops including pulses, wheat, barley, rye, potato, and sugar beets (Government of Canada: Health Canada Pest Management Regulatory Agency 2003, 2011). In 2010, Headline EC was also registered for canola (BASF 2014c). Pyraclostrobin was

registered as a seed treatment for wheat, barley, and corn in 2011 (Government of Canada: Health Canada Pest Management Regulatory Agency 2011), and for canola in 2012 (Government of Canada: Health Canada Pest Management Regulatory Agency 2012). In June of 2014, BASF announced the registration of Priaxor for the control of blackleg of canola in Western Canada for 2015. Priaxor is a product with two effective modes of action containing pyraclostrobin and fluxapyroxad. The combination of the two fungicides is intended to aid in management of fungicide insensitivity in the control of blackleg (BASF 2014a).

Pyraclostrobin has protectant (prevents infection from occurring), curative (stops pathogen in the early stages of infection from developing further), and translaminar activity (local systemic ability to move through the leaf to the non-sprayed side) (Bartlett et al. 2002; Mueller et al. 2013). Unlike contact fungicides, which remain on the surface of the plant tissue, locally systemic fungicides such as pyraclostrobin are able to stop the pathogen after it enters into the plant tissue (Mueller et al. 2013). Considering it disrupts ATP synthesis in the mitochondria, pyraclostrobin is very effective at preventing spore germination which is an energy demanding stage in the life cycle of *L. maculans* (Bartlett et al. 2002). After application, pyraclostrobin is tightly bound in the leaf cuticle. While pyraclostrobin is not truly systemic, it provides disease protection on both sides of the leaf as a result of its translaminar activity. As an result of this ability to enter the leaf, Headline has a rainfast period of only 1 hour, which contributes to the effectiveness of the product (BASF 2014b).

There are many studies exploring the sensitivity of various plant pathogens to pyraclostrobin. However, studies examining the sensitivity of *L. maculans* to pyraclostrobin are limited. Rebollar-Alviter et al. (2007) collected isolates of *Phytophthora cactorum* Lebert and Cohn from infected strawberries in seven states of the U.S.A. By using mycelium growth and

zoospore germination assays, they determined that the isolates did not show insensitivity to QoI fungicides. Nonetheless, based on relative growth of mycelia and activity against zoospore germination, the isolates appeared to be inhibited more by pyraclostrobin than azoxystrobin (Rebollar-Alviter et al. 2007). Isolates of *Pyrenophora tritici-repentis* Died. collected from North Dakota, U.S.A., showed no insensitivity to pyraclostrobin, indicating the fungicide is still effective in managing tan spot of wheat in that region (Patel et al. 2012). In California, Avenot et al. (2008) tested the sensitivity of *Alternaria alternata* Fr. isolates from pistachio (*Pistacia vera*) to pyraclostrobin, boscalid (succinate dehydrogenase inhibitor), and Pristine (pyraclostrobin and boscalid). Out of 59 isolates that had been exposed to Pristine, 56 were insensitive to pyraclostrobin, and most were sensitive to boscalid. Seven of these isolates were insensitive to both boscalid and Pristine. Results from this study suggest that the insensitivity to Pristine results mainly from multiple-resistance to the two fungicides, since pyraclostrobin and boscalid do not share the same site of action (Avenot et al. 2008).

There are a limited number of studies involving pyraclostrobin sensitivity in Canada. Gossen and Anderson (2004) collected 88 isolates of *Didymella rabiei* Kovatsch from chickpea in Saskatchewan and examined their sensitivity to azoxystrobin and pyraclostrobin. Mycelial growth of most of the isolates was inhibited by pyraclostrobin at 1ppm. There was only one isolate that was highly insensitive to azoxystrobin. This one isolate also showed some insensitivity to pyraclostrobin, indicating the possibility of cross-resistance (Gossen and Anderson 2004). Chang et al. (2007) evaluated the sensitivity of 66 *Ascochyta rabiei* Pass. (anamorph *D. Rabiei*) isolates, collected from chickpea in Alberta, to pyraclostrobin and two multi-site activity fungicides, chlorothalonil and mancozeb. Based on colony growth, 49 of the 66 isolates were insensitive to one or more of the fungicides, with some insensitive to two or

three. Of these isolates, 24% were insensitive to pyraclostrobin. Thirty-seven isolates were evaluated using a conidial germination assay, with four found to be insensitive to chlorothalonil, 12 insensitive to pyraclostrobin, and 10 insensitive to both of the fungicides (Chang et al. 2007).

1.4 OBJECTIVES

1.4.1 Sensitivity study

The intensive production of canola in western Canada is resulting in increased levels of blackleg in this crop. While the application of fungicides can be an effective tool for disease management, their overuse can lead to the development of insensitive pathogen populations. As a result of their high selectivity, continued application of strobilurin fungicides may result in the development of insensitivity in *L. maculans*. Although pyraclostrobin has been registered on canola for only 4 years, this fungicide has been registered in Canada for over a decade for application on other crops. Insensitivity to strobilurin fungicides can develop quickly, even within a matter of a couple of years. Therefore, it is important to monitor populations of the blackleg pathogen for fungicide insensitivity. An objective of this M.Sc. project was to survey a selection of *L. maculans* isolates from Alberta for their sensitivity to pyraclostrobin. The sensitivity of the isolates was evaluated phenotypically through colony growth and microtiter assays. Although no baseline isolates were available, an EC_{50} value was calculated using isolates from the population being evaluated. The aim of this study was to determine the level of pyraclostrobin sensitivity or insensitivity in *L. maculans* populations from Alberta. Since pyraclostrobin was registered for the management of canola only shortly prior to the collection of the fungal isolates, it was hypothesized that no insensitive isolates would be found.

1.4.2 Fungicide efficacy experiments

A second objective of this thesis was to investigate the potential utility of fungicides as a component of a sustainable approach for the management of blackleg of canola. Specifically, the efficacy of various seed and foliar fungicides (some of which contain pyraclostrobin) was evaluated in field and greenhouse experiments. It was hypothesized that canola treated with a combination of seed and foliar fungicides containing pyraclostrobin would provide the best control of blackleg and improve yields, especially when applied on a more susceptible cultivar.

Understanding the characteristics of the current population of *L. maculans* and developing management strategies for the control of blackleg disease of canola will assist in maximizing yields of this crop in a sustainable manner.

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1.6 TABLES

Table 1.1 Seed treatment products to control blackleg on canola

Product name [z]	Company	Active Ingredient(s)
Gaucho CSFL/ Gaucho 480	Bayer Crop Science	Carbathiin: 47.6g/L Thiram: 95.3 g/L
Helix Vibrance	Syngenta	Difenconazole: 1.25% Metalaxyl- M: 0.39% Fludioxonil: 0.13% Sedaxane: 500 g/L
Prosper Evergol	Bayer Crop-Science	Penflufen: 10.7 g/L Trifloxystrobin: 7.15 g/L Metalaxyl: 7.15 g/L
Rancona RS	Chemtura	Ipconazole: 9.38 g/L

[z] Based on Crop Protection 2014, Alberta Agriculture and Rural Development

Table 1.2 Foliar fungicide products to control blackleg on canola

Product name [z]	Company	Active Ingredient(s)
Tilt 250 EC/	Syngenta	Propiconazole: 250 g/L
Bumper 418 E	MANA Canada	Propiconazole: 418 g/L
Pivot 418 EC	IPCO	Propiconazole: 418 g/L
Propel	Loveland Canada Products Inc.	Propiconazole: 250 g/L
Headline EC	BASF Canada	Pyraclostrobin: 250 g/L
Quadris	Syngenta	Azoxystrobin: 250 g/L

[z] Based on Crop Protection 2014, Alberta Agriculture and Rural Development

1.7 FIGURES

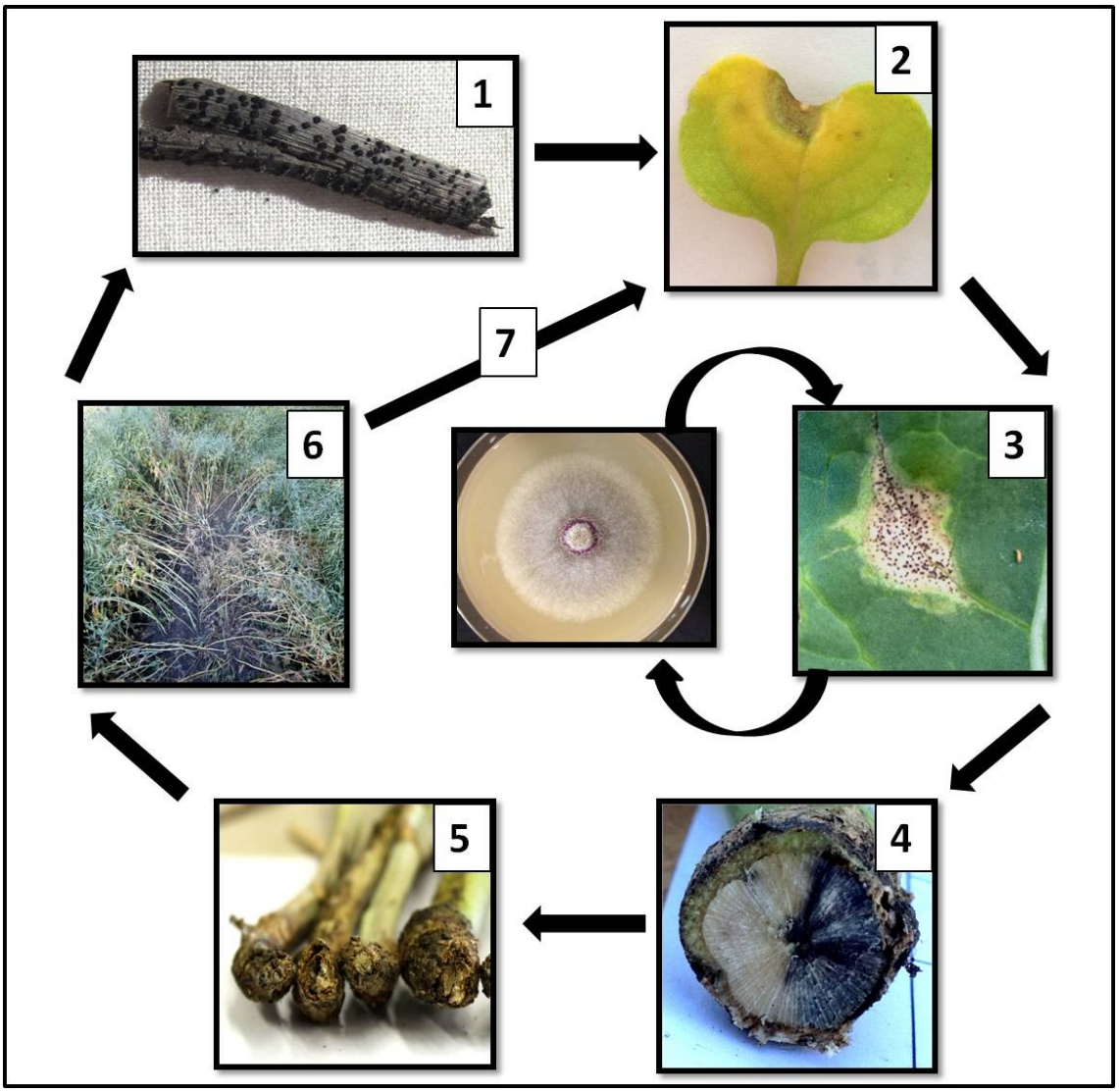

Figure 1.1 Disease cycle of *Leptosphaeria maculans* (based on Ash 2000). The pathogen overwinters as pseudothecia on canola stubble residue (1). Airborne ascospores are released from the pseudothecia and infect canola seedlings and basal rosette leaves (2). Greyish lesions develop on leaves, where black pycnidia can be seen. Pycnidiospores are released from the pycnidia in a pink colored mucilaginous liquid (as seen on the Petri dish) and initiate secondary cycles of infection (3). The fungus grows inside the vascular tissues towards the stem base (4). Stem cankers develop, leading to girdling and lodging (5 & 6). Infected pods and seeds may give rise to infected seedlings (7).

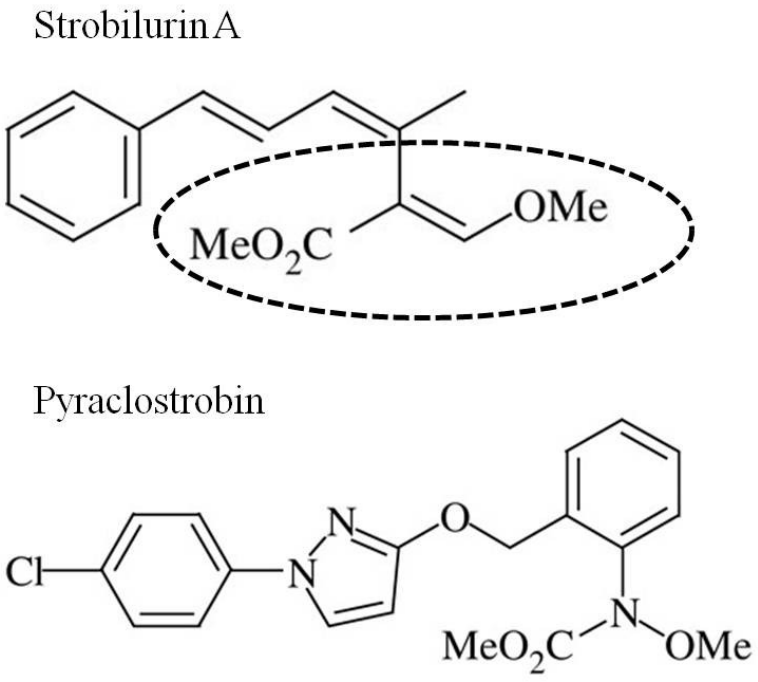

Figure 1.2 Chemical structure of strobilurin A and pyraclostrobin (Smith et al. 2008). The circle on strobilurin A denotes the methoxyacrylate moiety pharmacore.

2 CHAPTER 2: SENSITIVITY OF *LEPTOSPHAERIA MACULANS* TO PYRACLOSTROBIN IN ALBERTA, CANADA

2.1 INTRODUCTION

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.) is an ascomycete fungus belonging to the order Pleosporales and causes blackleg disease (synonym: Phoma stem canker) in canola rapeseed (*Brassica napus* L.) (Rouxel and Balesdent 2005). Blackleg is one of the most serious diseases of canola in western Canada and caused significant yield losses in the 1970s and 1980s, when canola production expanded (Gugel and Petrie 1992). In recent years, the intensive production of canola and lack of diverse crop rotations has led to an increase in blackleg disease in Alberta (Kutcher et al. 2011).

There are very few fungicidal active ingredients available for the management of blackleg in canola in Alberta (Alberta Government 2015), one of which is the broad spectrum fungicide pyraclostrobin. Pyraclostrobin has been used on various crops since 2003 as the foliar treatment Headline EC (pyraclostrobin, 250 g L^{-1}; BASF Canada Inc., Mississauga, ON). Headline EC was registered on canola in 2010 and first used in 2011, while in 2012 the active ingredient pyraclostrobin was registered as a seed treatment in combination with boscalid (Government of Canada: Health Canada Pest Management Regulatory Agency 2011, 2012). Pyraclostrobin is a strobilurin fungicide, also known as a quinone outside inhibitor (QoI) (Balba 2007). Strobilurin fungicides have single-site activity and inhibit mitochondrial respiration in fungi. Specifically, they act on the Qo site located on the cytochrome bc1 enzyme complex (complex III) of the mitochondria (Gisi et al. 2002). By interrupting the activity in the electron transport chain, QoI fungicides deprive the fungus of energy by preventing the formation of ATP (Gisi et al. 2002)

and cause a build-up of electrons in the mitochondria (Inoue et al. 2012). The overabundance of electrons results in the formation of reactive oxygen species (ROS) that damage the mitochondria and contribute to the death of the fungus (Inoue et al. 2012). Strobilurin fungicides, such as pyraclostrobin, have protectant and translaminar activity and are very effective at inhibiting spore germination, which is an energy demanding stage in the fungal lifecycle (Bartlett et al. 2002).

Strobilurin fungicides are one of the most widely applied fungicide classes worldwide (Balba 2007). However, because of their site-specific activity, they have a high risk of selecting for insensitive individuals within a fungal population (Gullino et al. 2000). There are two types of fungicide resistance: polygenic, which results in a gradual shift towards insensitivity due to an interaction of many mutant genes, and major gene resistance, which leads to complete insensitivity as a result of a mutation in one gene (Georgopoulos and Skylakakis 1986). With intensive use and repeated sprays of fungicides from the same class, insensitive individuals will be selected and the proportion of insensitive individuals will increase. Therefore, fungicide applications will become less effective or eventually ineffective at controlling the disease (Georgopoulos and Skylakakis 1986). Strobilurin insensitivity is associated with a point mutation in the cytochrome b gene, which leads to an amino acid substitution of glycine with alanine at position 143 (G143A) in the cytochrome b protein (Kim and Hwang 2007). This mutation has been identified in isolates of various fungal species (Fungicide Resistance Action Committee 2013; Kim and Hwang 2007). Although less common than the G143A mutation, a second mutation associated with strobilurin insensitivity is the substitution of phenylalanine with leucine at position 129 (F129L) of the cytochrome b protein, which has been identified in *Pyrenophora teres* Drechs., *Alternaria solani* Kuhn (Fungicide Resistance Action Committee

2013), *Pythium aphanidermatum* Edson (Kim and Hwang 2007), and *P. grisea* (Ma and Michailides 2005).

To ensure fungicide resistance management strategies are effective and gain insight into possible insensitivity issues, it is important to monitor the sensitivity of pathogens to frequently used fungicides (Brent and Hollomon 2007). Molecular methods, such as allele-specific real-time PCR, are commonly used to detect specific mutations responsible for fungicide insensitivity (Ma and Michailides 2005). While allele-specific real-time PCR is rapid and highly sensitive, its specificity means it does not detect other mechanisms of insensitivity. In contrast, phenotypic bioassays can detect the presence of any fungicide insensitive genotypes (Vega et al. 2012). Two examples of phenotypic bioassays for fungicide insensitivity include the conventional mycelial growth plate assay and the microtiter plate assay. When using these methods, the concentration at which fungal growth is effectively inhibited by 50% (EC_{50}) must be determined for a set of isolates, which serves as a sensitivity baseline relative to the pathogen population being assessed. With the growth plate assay, this involves plating the isolates on growth medium amended with different concentrations of the fungicide and measuring inhibition of colony growth. Isolates that have never been exposed to the fungicide are typically used to determine the EC_{50} in order to detect changes in sensitivity within the population being assessed. It is sometimes difficult, however, to obtain such isolates if they were not collected prior to the introduction of the fungicide (Russell 2004). The EC_{50} dose may be used in the future to monitor and detect any shifts in sensitivity of the pathogen population (Russell 2004). The microtiter method involves growing the pathogen in multi-well plates that contain liquid medium amended with various concentrations of the fungicide. A growth indicator dye is added to the wells and

spore germination and subsequent growth is measured by monitoring color change with a spectrophotometer (Brent and Hollomon 2007).

Although pyraclostrobin has been registered only recently for the control of blackleg on canola, there are concerns regarding the frequency of insensitive isolates initially present in the pathogen population when the fungicide became commercially available. While most canola cultivars grown in Alberta have moderate to high resistance to blackleg, there is always a risk that genetic resistance will be overcome, especially if the same cultivar is planted repeatedly in short rotations (Marcroft et al. 2012). If genetic disease resistance becomes ineffective, growers may become more dependent on fungicides for blackleg management. Therefore, it is important to provide a current baseline assessment of the sensitivity of *L. maculans* populations to determine the relative merits of and any risks associated with fungicidal products containing this active ingredient. Understanding the current baseline sensitivity, especially for high risk products such as pyraclostrobin, ensures these fungicides remain a sustainable tool for blackleg management in canola, alongside extended crop rotations and strategic deployment of resistance genes.

2.1.1 Objectives

The objectives of this study were to: 1) determine the EC_{50} value of a set of *L. maculans* isolates to pyraclostrobin fungicide by conducting a radial growth plate assay; and 2) assess the pyraclostrobin sensitivity of a collection of *L. maculans* isolates from canola in Alberta via mycelial growth plate and microtiter plate assays. It was hypothesized that no insensitive isolates would be found, since pyraclostrobin was registered only recently for use on canola to manage blackleg.

2.2 MATERIALS AND METHODS

2.2.1 Collection and Preparation of Single-spore *L. maculans* Isolates

A collection of single-spore *L. maculans* isolates described by Rong et al. (2015) was used in this study. These isolates were obtained in 2011 from infected canola stubble residues from 6 fields located in the Alberta counties of Camrose, Lacombe, Ponoka, Strathcona and Wetaskiwin (Table 2.1). Within each field, plant residues were collected in a 1 m^2 area at each of 5 points along a 'W' sampling pattern. Each isolate had been identified as either *L. maculans* or *L. biglobosa* by means of a PCR assay with β-tubulin based primers (Rong et al. 2015). The isolates were collected after the introduction of QoI fungicides and the registration of pyraclostrobin on canola. However, the history of pyraclostrobin use in these fields at the time of sample collection was unknown. Since the isolates could have been have been exposed to pyraclostrobin, they may not represent true baseline isolates. The isolates were stored as frozen concentrated stocks of pycnidiospores in 1.5 mL microcentrifuge tubes in sterilized distilled water (SDW) at -20°C until use.

2.2.2 Determining the Mean EC50

To assess the relative sensitivity of the isolates that were collected, it was necessary to first determine the effective concentration of pyraclostrobin required to inhibit the growth of *L. maculans* by 50% (EC_{50}). Determination of the EC_{50} was performed according to Ahmed et al. (2014) with some modifications. A total of 13 isolates were randomly selected from different locations to serve as 'baseline' sensitivity isolates. To revive the isolates, the pycnidiospores were thawed on ice and 1 µL of the spore suspension stock of each isolate was added to 500 µL of SDW. The spore solution was mixed gently and pipetted onto 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) and spread over the surface of the medium with a sterilized metal plate. After 5 days, 7-mm-plugs of the developing colonies were transferred to fresh V8 medium amended with the pyraclostrobin fungicide. Headline 250 EC (pyraclostrobin, 250 g L^{-1}; BASF Canada Inc., Mississauga, ON) was used as the source of pyraclostrobin. To prepare the fungicide amended plates, a stock solution was first prepared by adding 20 µL of the formulated product to 3980 µL of SDW. Appropriate amounts of fungicide were added to cooled autoclaved V8 medium just prior to pouring the plates. The concentrations of active ingredient in the amended plates were: 0.025 mg L^{-1}, 0.125 mg L^{-1}, 0.250 mg L^{-1}, 0.375 mg L^{-1}, 0.625 mg L^{-1}, and 0.875 mg L^{-1}. The experiments were arranged in a completely randomized design with 10 replicates (Petri dishes) per treatment. Four replicate control dishes that did not contain fungicide were included for each isolate.

The plates were placed on a table under fluorescent lighting for a 24 h period at 20°C ± 2°C. A period of 10 days was sufficient to detect significant growth differences between the fungicide amended and control plates. Using digital calipers, the radial growth of each isolate was determined by measuring the diameter of the colony at its widest point, and by taking a second measurement perpendicular to the first. The two measurements were averaged and then converted to a percentage of radial growth of the un-amended control using the equation: [(*average growth on un-amended – growth on amended*) / (*average growth on un-amended*)] × 100.

2.2.3 Effect of SHAM and Pyraclostrobin on Mycelial Growth

Some fungal pathogens have been observed to exhibit *in vitro* insensitivity to QoIs through an alternative respiratory pathway that relies on the alternative oxidase (AOX) enzyme. It is believed that this pathway is responsible for protecting the pathogen against oxidative stress

(Inoue et al. 2012). The compound salicylhydroxamic acid (SHAM) blocks alternative respiration; therefore, it is important to include SHAM in sensitivity studies to avoid inaccurate interpretations of insensitivity (Wise et al. 2008). To determine if *L. maculans* exhibits alternative respiration when exposed to pyraclostrobin *in vitro*, the same 13 baseline isolates that were used to determine the EC_{50} were tested on V8 growth medium containing salicylhydroxamic acid (SHAM, 99%; Sigma-Aldrich, St. Louis, MO) in addition to pyraclostrobin. The single-spore isolates were cultured and plated onto medium amended with pyraclostrobin at 0.025 mg L^{-1}, 0.125 mg L^{-1}, 0.250 mg L^{-1}, 0.375 mg L^{-1}, 0.625 mg L^{-1}, or 0.875 mg L^{-1}, as described above, as well as with SHAM at a concentration of 100 µg mL^{-1} (Wise et al. 2008). To prepare a solution of SHAM, salicylhydroxamic acid was dissolved in methanol. There were a total of 10 replicates (Petri dishes) per treatment for each isolate. There also were 10 replicate control dishes amended with the same concentration of SHAM, but which did not contain any fungicide. After 10 days, the diameters were measured and growth inhibition calculated as indicated previously.

2.2.4 Testing the Sensitivity of *L. maculans* Isolates

2.2.4.1 Conventional Growth Plate Assay

To detect highly insensitive isolates, a discriminatory dose 69-fold greater than the EC_{50} was used to screen the full collection of 117 single-spore isolates of *L. maculans*. Isolates were cultured and growth medium was prepared as described previously. The medium contained pyraclostrobin at 6.25 mg L^{-1} and SHAM at 100 µg mL^{-1}. The treatments were arranged in a completely randomized design, with eight replicates (Petri dishes) per isolate. There were eight control replicate plates per isolate that were amended with SHAM at 100 µg mL^{-1}, but which did not contain any fungicide. The plates were placed on a table under fluorescent lighting at 20°C ±

2°C. The radial growth of each isolate was measured as previously described, and converted to a percentage of radial growth of the un-amended control. Isolates with a growth inhibition <50% were designated as insensitive to pyraclostrobin (Wise et al. 2009).

2.2.4.2 Microtiter Assay

A subset of 41 isolates was evaluated for sensitivity to pyraclostrobin via a microtiter plate assay. Briefly, after the isolates were cultured, the spores were collected and immersed in YBA medium (composition per L of deionized water: 20 g yeast extract, 20 g Bacto peptone, 40 g sodium acetate) to increase spore density. The spore concentration was adjusted to 20000 spores mL^{-1}. Since YBA medium was used, the addition of SHAM was not necessary, as this relatively poor nutrient medium prevents the fungus from gaining enough energy to undergo alternative respiration, while providing enough nutrients for sufficient growth (Spiegel and Stammler 2006). Fifty µL of the spore suspension, containing about 1000 spores, was transferred into each well of a 96-well microtiter plate. Formulated Headline 250 EC fungicide was diluted to achieve end concentrations of 0 mg L^{-1} (control), 0.003 mg L^{-1}, 0.010 mg L^{-1}, 0.030 mg L^{-1}, 0.100 mg L^{-1}, 0.300 mg L^{-1}, 1.000 mg L^{-1}, and 3.000 mg L^{-1} of the active ingredient. Fifty µL of fungicide solution was added to each well and mixed with the spore suspension. There were 4 replicate wells for each isolate and fungicide concentration. Four replicate wells containing only fungicide and YBA medium also were included for each fungicide concentration and served as blanks. The plates were incubated for 7 days at 18°C in darkness. Pycnidiospore germination and subsequent growth, as indicated by absorbance, was measured with a spectrophotometer at a wavelength of 405 nm. For each isolate and concentration, growth inhibition was calculated using the equation: [(*average absorption with spores and fungicide – average absorption of*

blanks) × 100] / *average absorption of control without fungicide*. Two isolates of *L. maculans* with known sensitivity to pyraclostrobin were used as sensitive controls. The growth inhibition of the sensitive controls was determined in the same manner as for the other 41 isolates.

2.2.5 Data Analysis

The normality of the growth inhibition residuals of the 13 "baseline" sensitivity isolates in the absence and presence of SHAM were analysed visually and with the Shapiro-Wilk test using the statistical procedure/package shapiro.test/stats in R: A Language and Environment for Statistical Computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013). The homogeneity of variance also was analysed visually and tested using the Bartlett test (bartlett.test/stats). If residual data visually appeared to be normal and homogeneous, no transformations were applied. Outliers were identified with an outlier test (outlierTest/car). If the outlier test reported values with a Bonferroni p-value < 0.05, the values were removed from the data set. Analysis of variance of the transformed growth inhibition data was carried out using a fit linear mixed-effect model (lmer/lmerTest). The model included $\log_{10}$ fungicide concentration, isolate, and $\log_{10}$ fungicide concentration × isolate interaction. Log_{10} fungicide concentration and isolate were considered fixed, while replication was random. If the effect of isolate was significant on growth inhibition, or if the variance across isolates was not normal, EC_{50} values were calculated separately for each isolate, and averaged to find the baseline EC_{50}.

The EC_{50} value for each of the 13 isolates (in the absence and presence of SHAM) in the growth plate assay and the 41 isolates in the microtiter assay was determined by analysing the growth inhibition data using probit regression analysis. The analysis was done using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0, IBM Corporation, Armonk, NY, USA). A base-10 logarithm ($\log_{10}$) transformation of the fungicide concentrations was used

to linearize the growth inhibition data. The resulting linear regression equation was used to estimate the fungicide concentration at which the radial growth was inhibited by 50% (EC_{50}) for each isolate. The EC_{50} values were displayed in a histogram and the normality of the residuals was analysed visually and tested using the Shapiro-Wilk test in R. The mean EC_{50} value of the isolates was calculated for each assay.

The mean EC_{50} values of each of the 13 "baseline" sensitivity isolates in the presence and absence of SHAM were compared using a two-sided paired T-test (t.test/stats). For the microtiter assay, a one-sided t-test (t.test/stats) was used to determine if the EC_{50} values of the 41 isolates were greater than the two sensitive controls.

The mean growth inhibition values of all of the 117 isolates in the growth plate assay were displayed in a histogram and the normality was analysed visually and tested using the Shapiro-Wilk test (shapiro.test/stats). The relationship of the EC_{50} values determined from the microtiter assay and the growth inhibition from the mycelial growth plate assay of the 41 isolates was tested using Pearson's product-moment correlation (cor.test/stats).

2.3 RESULTS

2.3.1 Determination of EC_{50} Values of Baseline Isolates

Thirteen isolates of *L. maculans* were randomly selected from different locations in Alberta to serve as baseline isolates. The EC_{50} values of the 13 individual isolates ranged from 0.01 mg L^{-1} to 0.28 mg L^{-1}, with a mean (± standard deviation) of 0.11 mg L^{-1} (± 0.09 mg L^{-1}) (Figure 2.1A; Table 2.2). The distribution of the residual data was normal (P = 0.5866).

2.3.2 Effect of SHAM and Pyraclostrobin on Mycelial Growth

The effect of including SHAM in addition to pyraclostrobin in the growth medium was evaluated on the same 13 baseline isolates. The EC_{50} values of the individual isolates ranged from 0.02 mg L^{-1} to 0.24 mg L^{-1}, with a mean of 0.09 mg L^{-1} (± 0.07 mg L^{-1}) (Figure 2.1B; Table 2.2). The distribution of the residual data was normal (P = 0.8588). The result of the two-sided pairwise T-test indicated that there was no significant difference between the EC_{50} values of each isolate tested in the presence or absence of SHAM (P = 0.3882).

2.3.3 Sensitivity of a Collection of *L. maculans* Isolates From Alberta

Pyraclostrobin sensitivity in a collection of 117 *L. maculans* isolates from Alberta was assessed in a conventional growth plate assay and a microtiter assay. In the growth plate assay, growth inhibition of the 117 isolates ranged from 66.6% to 100.0% with a mean of 84.3% (± 8.05%), relative to a control treatment in which no pyraclostrobin was included. None of the isolates had a growth inhibition of < 60.0%; eight exhibited a growth inhibition of 60.0% to < 70.0%; 24 exhibited a growth inhibition of 70.0% to < 80.0%; 53 exhibited a growth inhibition of 80.0% to < 90.0%; 31 exhibited a growth inhibition of 90.0% to < 100.0%; and a single isolate exhibited a growth inhibition of 100.0% (Figure 2.2). The residuals of the growth inhibition data were not normally distributed ($P \leq 0.05$) and were negatively skewed.

In the microtiter assay, the EC_{50} values of the two pyraclostrobin-sensitive controls included in the test were 4.1×10^{-3} mg L^{-1} and 5.7×10^{-3} mg L^{-1}. The EC_{50} values of the *L. maculans* isolates from Alberta ranged from 1.0×10^{-4} mg L^{-1} to 7.4×10^{-3} mg L^{-1}, with a mean of 3.6×10^{-3} mg L^{-1} (± 2.0×10^{-3} mg L^{-1}) (Figure 2.3), and were not significantly different from either control (P = 0.9974; P = 1.000). The distribution of the residual EC_{50} values was not normal (P < 0.05) and was positively skewed. No significant correlation (P = 0.1549) was found between

the EC_{50} values from the microtiter assay and percent growth inhibition from the growth plate assay for the subset of 41 isolates evaluated by both methods.

2.4 DISCUSSION

Many studies have examined the sensitivity of various plant pathogens to pyraclostrobin. For example, pyraclostrobin sensitivity was demonstrated in isolates of *Phytophthora cactorum* Lebert and Cohn from infected strawberries in seven states of the U.S.A. (Rebollar-Alviter et al. 2007), and in isolates of *P. tritici-repentis* from infected wheat in North Dakota, U.S.A (Patel et al. 2012). In contrast, isolates of *Alternaria alternata* Fr. collected from infected pistachio (*Pistacia vera* L.) in California, U.S.A (Avenot et al. 2008), and *Ascochyta rabiei* Pass. from infected chickpea in Alberta (Chang et al. 2007), were found to be insensitive to pyraclostrobin. To our knowledge, however, there have been no reports published on the pyraclostrobin sensitivity of *L. maculans* isolates from Alberta.

The first step in evaluating the sensitivity of *L. maculans* to pyraclostrobin was to determine the EC_{50} of a set of baseline isolates. Typically, isolates that have never been exposed to a fungicide are used as the baseline in a sensitivity study. For this study, however, there were no sets of isolates available that had been collected prior to the registration and use of pyraclostrobin in Alberta; the isolates analysed were recovered after the registration of this product on canola. Since the sensitivity of the isolates could not be compared with the sensitivity of isolates collected prior to the use of the fungicide, shifts in the sensitivity of the pathogen population could not be determined. Nonetheless, the analysis provided important information on the current status of fungicide sensitivity in *L. maculans* populations from Alberta, which is essential for future monitoring of changes in this sensitivity.

The range of EC_{50} values obtained in the mycelial growth assays was small (0.01 mg L^{-1} to 0.28 mg L^{-1}), with a mean of 0.11 mg L^{-1}. In a recent abstract by Liu et al. (2013), the EC_{50} values of 27 *L. maculans* isolates from 3 fields where pyraclostrobin and azoxystrobin had been sprayed were determined to range from 0.126 mg L^{-1} to 0.477 mg L^{-1}, with an average of 0.312 mg L^{-1}. While those values are similar to the ones obtained for *L. maculans* in the current study, it is impossible to compare how the sensitivity of the two isolate collections may have shifted from previous years, since the isolates of Liu et al. (2013) had previous exposure to pyraclostrobin and the ones in this report may have been exposed to the fungicide. Differences in fungicide sensitivity also may reflect intrinsic diversity in isolates from different populations of the fungus, and/or varying fungicide spray regimes across western Canada. More frequent or heavier use of fungicides in an area may cause a greater shift in sensitivity relative to areas with less frequent use of the same product (Brent and Hollomon 2007).

There are many sensitivity studies involving pyraclostrobin and other fungal pathogens in the Pleosporales (Ahmed et al. 2014; Avenot et al. 2008; Bowness et al. 2016; Gossen and Anderson 2004; Mondal et al. 2005; Pasche et al. 2004; Patel et al. 2012; Vega and Dewdney 2014; Vega et al. 2012). However, due to natural variation in the fungicide sensitivity of species, different EC_{50} values are to be expected (Vega and Dewdney 2014). For example, Ahmed et al. (2014) determined the EC_{50} of *Didymella rabiei* Kovatsch to pyraclostrobin to be 1.0 mg L^{-1} with a range of 0.3 mg L^{-1} to 3.8 mg L^{-1}, which is much greater than the values observed for *L. maculans* in both the current study and the abstract of Liu et al. (2013). The EC_{50} values for the same species in response to different fungicides within the strobilurin group also may be highly variable, given the different chemical properties of each fungicide. Studies involving various

strobilurin fungicides have resulted in different EC_{50} values when tested on the same pathogen species (Patel et al. 2012).

In the current study, a comparison of the EC_{50} values obtained in the presence or absence of SHAM for the 13 baseline isolates indicated that this compound did not have any effect, strongly suggesting that the baseline isolates did not use alternative respiration to bypass the effects of pyraclostrobin. To our knowledge, there are no other reports on the effect of pyraclostrobin and SHAM on *L. maculans*. However, there have been many reports indicating the effect of SHAM on other pathogens. Ahmed et al. (2014) reported no effect of SHAM on spore germination in *D. rabiei* in the presence or absence of pyraclostrobin. In contrast, Patel et al. (2012) found that isolates of *P. tritici-repentis* were able to undergo alternative respiration when exposed to pyraclostrobin in the absence of SHAM. Seyran et al. (2010) reported that in the presence of SHAM, but absence of azoxystrobin fungicide, the growth of *Fusicladium effusum* Wint. isolates was reduced, suggesting some toxicity of SHAM towards this fungus. Wise et al. (2008) conducted a study on the baseline sensitivity of *A. rabiei* to various QoI fungicides, including pyraclostrobin. They found that some isolates of the fungus had higher EC_{50} values when exposed to azoxystrobin in the absence of SHAM, indicating that individual isolates may be capable of alternative respiration. Therefore, despite the absence of an effect of SHAM on any of the 13 baseline isolates tested in the present study, SHAM was added to the medium as a precaution when screening the full collection of *L. maculans* isolates.

The EC_{50} of the baseline isolates in the presence of SHAM and pyraclostrobin was 0.09 mg L^{-1}. The discriminatory dose used to screen the isolates was 6.25 mg L^{-1}, a concentration 69 times greater than the EC_{50} and 4 times the recommended application rate of Headline fungicide. Changes in the sensitivity to strobilurin fungicides are qualitative in nature (Gisi et al. 2002),

resulting in almost complete insensitivity to treatment with the chemical (Georgopoulos and Skylakakis 1986). When the mechanism of fungicide insensitivity is known and involves a point mutation in a single gene, as is the case with pyraclostrobin insensitivity in *L. maculans*, a high discriminatory dose can be used to clearly detect insensitive isolates (Russell 2004). Indeed, insensitive isolates can tolerate high levels of strobilurin fungicides, often at doses > 10 mg L^{-1} (Mondal et al. 2005). Wise et al. (2009) reported that two *A. rabiei* isolates had EC_{50} values 704 times greater than the mean sensitivity of baseline isolates, indicating insensitivity to pyraclostrobin. In an assessment of the fungicide sensitivity of *A. alternata* isolates, Vega and Dewdney (2014) determined the EC_{50} of insensitive populations to be 5.507 µg ml^{-1}, which represented a 239-fold decrease in sensitivity relative to the baseline isolates (0.023 µg/ml). The results of the growth plate assay in the current study suggest that there were no isolates insensitive to pyraclostrobin in the collection from Alberta, since no isolates with a < 50% reduction in colony growth were identified. Nevertheless, while the discriminatory dose used in the current study was appropriate for the detection of qualitative changes in the insensitivity to pyraclostrobin, it did not allow for the identification of isolates exhibiting tolerance or intermediate levels of fungicide insensitivity. The distribution of the sensitive isolates was negatively skewed, suggesting that a majority of the isolates had greater sensitivity relative to the entire collection.

Since conidia are more sensitive to QoI fungicides, the EC_{50} values obtained from microtiter assays generally will be much lower than those from mycelial growth plate assays (Wise et al. 2008). In the current study, the mean EC_{50} of the baseline isolates in the growth plate assay was almost 18-fold greater than the mean EC_{50} of the two sensitive control isolates in the microtiter assay. Nevertheless, there were no significant differences between the EC_{50} values

of the sensitive controls and the isolates being screened in the microtiter assay. The results both from the growth plate and microtiter assays support the conclusion that all of the *L. maculans* isolates tested from Alberta are sensitive to pyraclostrobin. However, these isolates represented a relatively small number of fields. Additional evaluation of a larger number of fields and isolates collected over a wider geographic area will be required to confirm this conclusion and for subsequent monitoring of shifts in sensitivity.

The isolates in this study were collected in the fall after the first season of registered use of pyraclostrobin on canola. Pyraclostrobin (as the commercial formulation Headline EC, BASF) may be applied 1 to 3 times on canola in a single growing season: once at the 2-4 leaf stage for blackleg management, and twice at the early pod stage for the control of *Alternaria brassicae* Berk., *A. alternata*, and *Alternaria raphani* J.W. Groves & Skolko (BASF 2003). While a single year of exposure would not likely result in the development of insensitivity, there have been cases where insensitivity has developed within a very short period. For example, Wise et al. (2009) identified fungicide-insensitive isolates of *A. rabiei* in North Dakota after only two years of exposure to pyraclostrobin. In Alberta, pyraclostrobin is also applied to other field crops, including cereals and pulses (BASF 2003). Since *L. maculans* can survive saprophytically on canola residues, it is possible that the fungus could be unintentionally exposed to pyraclostrobin when it is applied to other crops, especially if volunteer canola was present and was infected by *L. maculans*. Nonetheless, the failure to identify any *L. maculans* isolates insensitive to pyraclostrobin suggests that whatever exposure the fungus has had to this fungicide, it has not decreased its fungicide sensitivity substantially. It is possible that continued exposure to pyraclostrobin could change this situation, especially with the repeated applications within and between growing seasons. Pyraclostrobin, along with other strobilurin fungicides, provide plant

health benefits independent of disease control such as maintenance of green leaf area, delay of leaf senescence, increase in photosynthetic activity, and increase in yield (Bartlett et al. 2002). These properties may encourage growers to apply pyraclostrobin fungicides regardless of the disease situation. However, the number of applications per season should be limited and fungicide used only when necessary, in order to avoid selection pressure for insensitive isolates (Brent and Hollomon 2007). The judicious use of pyraclostrobin as part of an integrated blackleg management strategy, along with continued monitoring of *L. maculans* populations for changes in fungicide sensitivity, will be important in ensuring the continued efficacy of this product in the management of blackleg of canola.

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2.6 TABLES

Table 2.1 Origin of a collection of 117 *Leptosphaeria maculans* isolates from Alberta, Canada, tested for sensitivity to pyraclostrobin fungicide.

Location	Number of isolates tested
Camrose	40
Lacombe	4
Lethbridge	13
Ponoka	37
Strathcona	22
Wetaskiwin	1

Table 2.2 The effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 13 isolates of *Leptosphaeria maculans* in the absence or presence of salicylhydroxamic acid (SHAM). These isolates were used to assess baseline sensitivity to the fungicide. [z]

	EC_{50} (mg L^{-1})	
Isolate	Without SHAM	With SHAM
33	0.01	0.07
104	0.07	0.04
139	0.03	0.02
144	0.06	0.04
166	0.17	0.13
228	0.10	0.08
269	0.09	0.04
325	0.03	0.04
385	0.09	0.03
394	0.03	0.13
538	0.18	0.17
545	0.28	0.17
626	0.23	0.24
Mean	0.11	0.09

[z] A two-sided paired t-test indicated no significant difference between EC_{50} values of individual isolates in the absence or presence of SHAM (P = 0.3882).

2.8 FIGURES

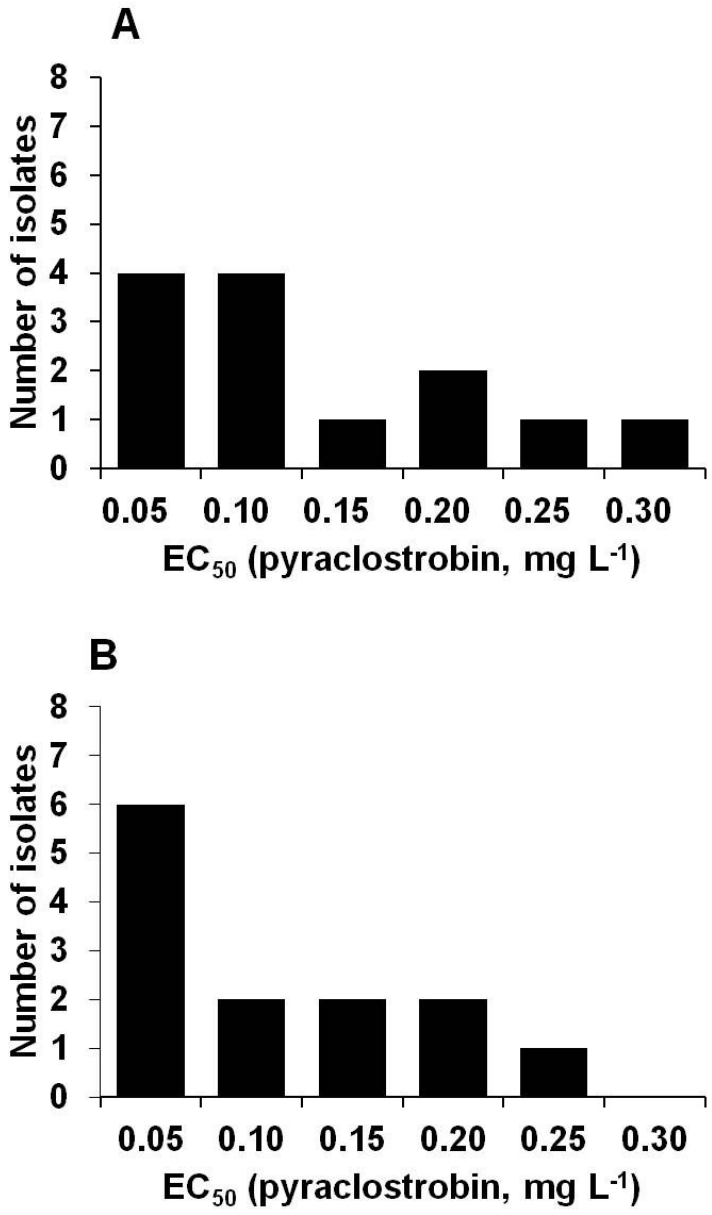

Figure 2.1 Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 13 isolates of *Leptosphaeria maculans* from Alberta, Canada, in the absence (A) or presence (B) of salicylhydroxamic acid (SHAM) at 100 µg mL^{-1}. Individual isolates are grouped in class intervals of 0.05 mg L^{-1}.

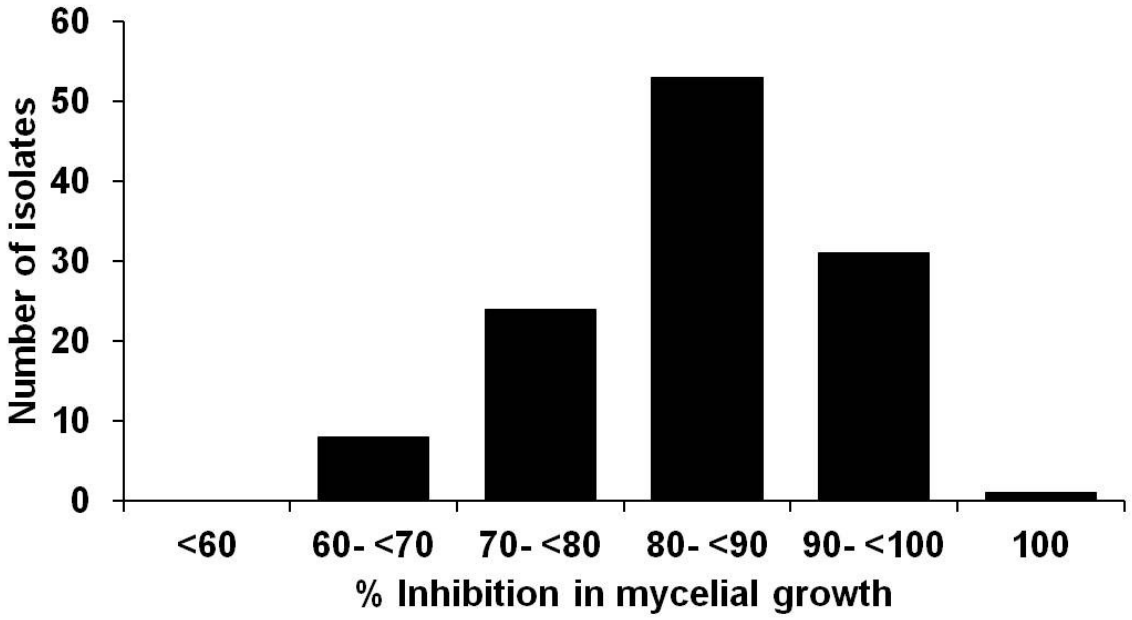

Figure 2.2 Frequency distribution of the inhibition of mycelial growth in 117 *Leptosphaeria maculans* isolates in response to inclusion of pyraclostrobin (6.25 mg L^{-1}) in V8 growth medium. Inhibition of colony growth is expressed as a percentage relative to a control treatment in which no pyraclostrobin was included. Assays were conducted in the presence of salicylhydroxamic acid (SHAM) at 100 µg mL^{-1}.

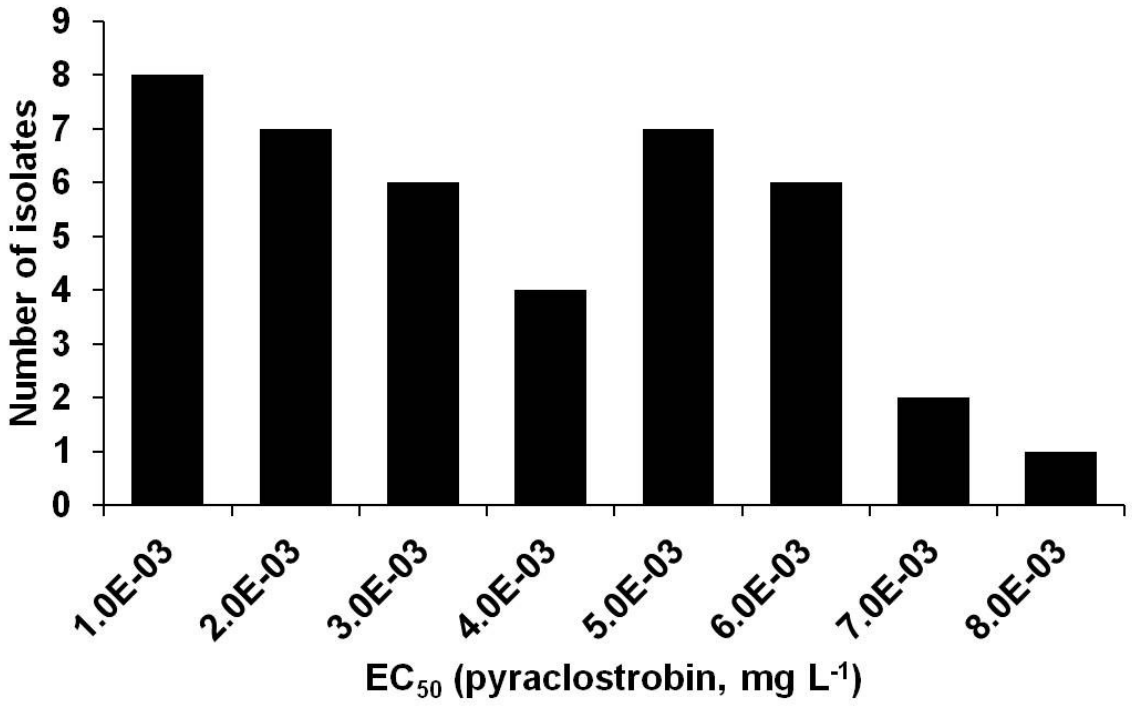

Figure 2.3 Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 41 *Leptosphaeria maculans* isolates from Alberta, Canada. The EC_{50} values were determined for each isolate via a microtiter assay, and were based on the inhibition of pycnidiospore germination and mycelial growth as measured photometrically at 405 nm. Individual isolates are grouped in class intervals of 1×10^{-3} mg L^{-1}.

3 CHAPTER 3: EVALUATION OF PYRACLOSTROBIN AS A SEED AND FOLIAR FUNGICIDE FOR THE MANAGEMENT OF BLACKLEG (*LEPTOSPHAERIA MACULANS*) OF CANOLA (*BRASSICA NAPUS*)

3.1 INTRODUCTION

Blackleg or phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.), is an important disease of canola (oilseed rape; *Brassica napus* L.) (Kutcher et al. 2010). Blackleg development results in yield losses that are generally below 10%, but which can reach 30% to 50% under high disease pressure (West et al. 2001). Grain quality also can be reduced; McCartney et al. (1999) reported losses in seed oil content of up to 30% in inoculated canola plots. There is a correlation between early infection of seedlings/young leaves by *L. maculans* and the incidence of stem cankers in mature plants (Hall 1992). Therefore, protection early during plant growth is critical for the prevention of major losses.

Seed and foliar fungicides are tools that can be useful in managing blackleg. Several studies have examined the efficacy of fungicide treatments for controlling blackleg in canola, which have given variable results. For example, Khangura and Barbetti (2002) found that applying a fertilizer coated with flutriafol, a demethylation inhibitor (DMI), as an in-furrow treatment was effective at reducing the severity of stem infection and improving yield in canola. Marcroft and Potter (2008) also found a reduction in the severity of internal stem infection and plant mortality with the use of fluquinconazole (DMI) as a seed treatment. Steed et al. (2007) evaluated the efficacy of flusilazole (DMI) in combination with carbendazim, a methyl benzimidazole

carbamate (MBC), and reported a decrease of phoma leaf spots, a reduction in stem canker severity, and an increase in yield. Huang et al. (2011) found similar results with respect to phoma leaf spot incidence and stem canker severity, while yield response varied in certain years. Results from a study by Khangura and Barbetti (2004) suggest a potential for the use of seed treatments in combination with foliar fungicides in managing blackleg disease in canola.

The strobilurins, also known as quinone outside inhibitors (QoIs), represent the most commonly used class of fungicides worldwide (Balba 2007). Many properties of the strobilurins make them popular: they are effective against most major fungal pathogens, they have a novel target specificity and selective toxicity, and they provide good coverage due to translaminar activity and the ability to bind tightly to the leaf cuticle (Balba 2007). In addition to effective disease control, strobilurin fungicides are known to cause beneficial physiological changes in crops, such as enhanced photosynthetic activity, delayed leaf senescence, and an increased tolerance to abiotic stressors (Vera et al. 2014). Kutcher et al. (2013) investigated the effect of azoxystrobin (QoI) on blackleg disease of canola and found that a foliar fungicide treatment was effective at reducing disease incidence in a susceptible and resistant cultivar. However, the magnitude of disease reduction was greater for the susceptible cultivar. The results also showed a small yield increase in the susceptible cultivar compared with the untreated control, with no yield benefit in the resistant cultivar. While the fungicide had little effect on oil content in the resistant cultivar, there was an increase in oil content in the susceptible cultivar, but only when it was sown in a continuous rotation. The results of the study suggest that the use of fungicides may be valuable to canola growers only in situations where genetic resistance to *L. maculans* is not effective (Kutcher et al. 2013).

Pyraclostrobin is a strobilurin fungicide that has been recently registered for the control of blackleg on canola. The fungicide was developed in 2000 by BASF and was first registered as a foliar treatment (Headline EC) on canola in 2012. In 2014 another fungicide, Priaxor (pyraclostrobin and fluxapyroxad; BASF), was registered on canola to control blackleg in western Canada. Priaxor is a product with two effective modes of action designed to reduce the risk of selecting fungicide insensitive strains within fungal populations. There have been limited reports on the use of pyraclostrobin for blackleg control in Alberta. A recent study showed that an early application (2-4 leaf stage) of pyraclostrobin (Headline) resulted in a significant reduction of disease, but there was no increase in yield regardless of cultivar resistance level (Peng et al. 2012).

The objective of the current study was to evaluate the efficacy of various combinations of seed and foliar fungicides, some of which contain pyraclostrobin, for the management of blackleg disease of canola. Treatments were evaluated under field conditions in central Alberta and in greenhouse experiments under controlled environmental conditions. The results of this study could be useful for developing more sustainable blackleg management strategies, taking into consideration factors such as cultivar resistance and fungicide mode-of-action, in order to reduce disease levels and maximize canola yields in Alberta. It was hypothesized that susceptible and moderately resistant canola cultivars treated with a combination of seed and foliar fungicides containing pyraclostrobin would provide the best control of blackleg and improve yield.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Two canola cultivars were used. These included Westar, which is highly susceptible to *L. maculans* and is consequently no longer available for commercial use, and Delkab 73-15 RR (Monsanto Canada Inc.), which is a commercial cultivar grown in Alberta with moderate resistance to *L. maculans*. Seeds of the cultivar 73-15 were obtained from a collection stored at the Crop Diversification Centre North (CDCN), Alberta Agriculture and Forestry, while seed of Westar was provided by Dr. G. Peng (Saskatoon Research Centre and Development Centre, Agriculture and Agri-Food Canada, Saskatoon, SK).

3.2.2 Inoculum preparation

To increase disease levels, all experiments were inoculated with pycnidiospores and mycelia of a local isolate of *L. maculans* recovered from central Alberta. Pycnidiospores were maintained as glycerol stocks at -86°C, and plated on 10-cm diameter Petri dishes filled with solid V8 medium as needed. Cultures were grown at room temperature (RT) under fluorescent lighting (16 h light/8 h dark). Grain inoculum was added to potting mix or soil at the time of seeding to simulate soil-borne inoculum in greenhouse and field trials. Grain inoculum was produced based on a method modified from Hwang et al. (2014). Briefly, cultures of *L. maculans* were incubated for 7 days on V8 medium as described above. After incubation, 7 mm plugs were excised from each colony and placed mycelial side down on fresh V8 medium. The cultures were maintained under the above conditions for 21 days to encourage pycnidiospore production. At that time, each culture was cut into many small pieces (each about 0.5 cm^2) and mixed with 1 L of water-soaked, sterilized wheat grains in a sterilized polyethylene bag. The bag was sealed with a foam plug and plastic ring mechanism. The inoculated grain was

maintained RT for 21 days and then placed into a drier for 3 days. After drying, the grain was mechanically ground into a powder (about the consistency of sand). The grain inoculum was stored at 4°C until needed (Hwang et al. 2014). Sand (1:10 w:w) was mixed thoroughly with the grain inoculum in order to encourage movement through the seeder.

A pycnidiospore suspension also was prepared for direct application to canola foliage, in order to induce leaf and stem infection in the greenhouse and field trials. To prepare the foliar inoculum, *L. maculans* cultures were prepared as described above, but rather than sectioning the cultures into small pieces to inoculate grain, each Petri dish was flooded with 10 mL of sterile distilled (sd)-water. The surface of the agar was gently scraped with the edge of a glass microscope slide to dislodge the spores, with the resulting suspension carefully decanted and filtered through cheesecloth to remove any large fragments or clumps. The pycnidiospore concentration in the filtrate was determined with a hemocytometer and adjusted to final concentration of at least 1×10^6 spores/mL with sd-water. To ensure the spores were dispersed evenly throughout the solution and did not clump, 1 drop of Tween 80 (polyoxyethylenesorbitan monooleate; Sigma Ultra, Saint Louis, MO) was added per 1 L of volume. The pycnidiospore suspensions were kept on ice prior to use, and were applied on the same day that they were prepared.

3.2.3 Fungicide treatments

The foliar fungicides assessed included Priaxor (fluxapyroxad 37.6g ai ha^{-1}, and pyraclostrobin 74.9 g ai ha^{-1}; BASF Corporation, Research Triangle Park, NC) and Tilt (propiconazole 125g ai ha^{-1}; Syngenta Crop Protection Canada Inc., Guelph, ON). The foliar treatments were applied at a volume of 100 L ha^{-1} with a manually pressurized backpack sprayer

between the 4-6 leaf stage. Tilt and Priaxor were applied at 0.500 L/ha and 0.225 L/ha, respectively. The active ingredients of Priaxor were applied as a pre-mixed solution.

The seed treatment fungicides included BAS 720 F (fluxapyroxad 10g ai 100 kg^{-1} seed, pyraclostrobin 10g ai 100 kg^{-1} seed, and metalaxyl 8g ai 100 kg^{-1} seed) and Prosper FX (Clothianidin insecticide 400g ai 100kg^{-1} seed, carbathiin 70g ai 100 kg^{-1} seed, trifloxystrobin 10g ai 100 kg^{-1} seed, and Metalaxyl 7.5g ai 100 kg^{-1} seed; Bayer CropScience Inc., Calgary, AB). Each treatment was applied at the recommended rate directly to the seed. With the exception of those treated with Prosper FX, all seeds were treated with Allegiance FL (metalaxyl, 317g L^{-1}; Gustafson Partnership, Calgary, AB) for *Pythium* control and Poncho 600 (clothianadin, 600g L^{-1}; Bayer Crop Science Inc., Calgary, AB) for flea beetle control.

Seven treatments were evaluated: 1) uninoculated, untreated with fungicide, 2) inoculated, untreated, 3) inoculated, treated with BAS 720 F (half rate: 10g ai 100 kg^{-1} seed), 4) inoculated, treated with BAS 720 F (full rate; 20g ai 100 kg^{-1} seed), 5) inoculated, treated with Prosper FX (80g ai 100 kg^{-1} seed), 6) inoculated, treated with BAS 720 F (20g ai 100 kg^{-1} seed) + Priaxor (112g ai ha^{-1}), and 7) inoculated, treated with BAS 720 F (20g ai 100 kg^{-1} seed) + Tilt (125g ai ha^{-1}). Other than the inoculated control, all treatments were inoculated with grain and foliar inoculum.

3.2.4 Greenhouse experiments

The efficacy of the foliar fungicide treatments was examined in greenhouse experiments that were repeated independently in separate greenhouses (Trial 1 and Trial 2). Each experiment also was repeated twice on different benches within the same greenhouse at the same time. Experiments were arranged in a completely randomized block design with 4 replicates per

treatment. A single pot represented 1 replicate with 7 seeds each. The plants were grown under a light/dark regime of 16/8h at 21/18°C ± 1°C, although at certain times the temperature fluctuated up to 25°C due to heat from sunlight. The pots were 15 cm in diameter and 18 cm deep, and were lightly packed with 2.5 L of potting mix (Sunshine Mix #4 Aggregate Plus, Sun Gro Horticulture Canada, Saint-Mathieu, Quebec). Seven seeds were evenly placed on the surface of the potting mix. In Trial 1, the seeds were covered with 200 mL of potting mix to a depth of approx. 1 cm, and 10 mL of grain inoculum was added on top of this layer. In Trial 2, 10 mL of grain inoculum was sprinkled evenly over the seeds before the top layer of potting mix was added, based on the method of Hwang et al. (2014). The pots were watered daily and fertilized weekly with 20-20-20 fertilizer (The Scotts Company LLC, Mississauga, Ontario, Canada).

Emergence counts began when the first seedlings began to emerge. Counts were conducted at 3, 7 and 14 days after planting (DAP). When the majority of the canola plants were at the 4-leaf stage (BBCG growth stage 14 (Lancashire et al. 1991)), the foliar fungicides were applied. The method of foliar fungicide and inoculum application was similar to that of Kharbanda (1992). The fungicides were mixed with water at the appropriate rate and evenly applied to the point of run-off using a hand- held spray bottle. Four days after applying the foliar fungicides, the foliar pycnidiospore suspension was applied using a separate spray bottle. The plants were sprayed until just before run-off where the solution formed beads on the leaf surfaces. To encourage disease establishment, the humidity around the plants was increased by placing the pots inside large clear plastic bags for 3 days.

Five days after inoculation, when symptoms started to appear, the leaves were evaluated for disease severity (percentage of leaf area with lesions) and incidence. Evaluations were

conducted 3 times, 7 days apart. In Trial 1, every leaf per pot was evaluated. In Trial 2, two leaves were evaluated at the lower, middle, and top canopy of each plant, for a total of 6 leaves per plant. The area under the disease progress curve (AUDPC) was calculated for leaf disease severity and incidence using the equation (Simko and Piepho 2012):

$$AUDPC = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})}{2} \times (t_{i+1} - t_i)$$

Where: y_i is the disease severity or incidence at the i^{th} observation, n is the total number of observations on which the severity or incidence was recorded, and t_i is the number of days at the i^{th} observation.

After about 12 weeks from seeding, the plants were cut at the soil line, and the height and fresh weight of each plant were recorded. Using pruning shears, the plants were then cut between the root and crown to evaluate the severity and incidence of stem necrosis. Severity was determined by visually assessing the percentage of the stem cross-section that displayed cankers and the characteristic black staining of the disease (Peng et al. 2012). To determine yield, the pods were removed from the stems and placed in paper bags. The pods were allowed to dry at RT, and once completely dry, they were manually threshed and the seeds were cleaned and weighed. Plant survival was taken as the percentage of plants still alive at harvest relative to the total number of plants alive at141 days after planting (DAP) for each treatment (Marcroft and Potter 2008). In Trial 2, stem disease severity and yield also were determined for each individual plant in order to evaluate the correlation between stem infection severity and yield.

3.2.5 Field experiments

Field experiments were conducted over 4 site years in central Alberta at Camrose and Edmonton in 2012, and Namao and Edmonton in 2013. The Edmonton experiments were conducted in a blackleg nursery at the Crop Diversification Center North (CDCN), Alberta Agriculture and Forestry. The experiments in Camrose and Namao were conducted in field plots where blackleg disease already was prevalent. The experimental plots in Camrose and Namao were not tilled and contained naturally infested canola stubble from previous canola crops, while the soil at the CDCN was tilled before seeding. At the CDCN site in 2013, canola stubble collected from an infested canola field in Namao was scattered on the surface of each plot (except the un-inoculated control) after seeding, in order to increase the amount of inoculum.

Weather data for each site and year were obtained from the nearest weather station identified on the Current and Historical Alberta Weather Station Data Viewer (http://agriculture.alberta.ca/acis/alberta-weather-data-viewer.jsp). For Camrose, data were collected from the Camrose City Airport weather station, while data for CDCN and Namao were collected from the Oliver Agricultural Drought Monitoring Station (AGDM).

The experiments were arranged as a completely randomized block design and contained 4 replicates of each treatment. Each plot represented 1 replicate. The dimensions of the plots in 2012 were 1.5 m x 6 m and contained 6 rows (two of which served as guard rows) that were approximately 22 cm apart. In 2013, only 4 rows were seeded for each plot to give a total dimension of approximately 1.2 m x 6 m per plot. In 2012, the experiments were seeded on May 28th and May 31st at CDCN and Camrose, respectively. In 2013, the experiments were seeded on June 4th and June 6th at CDCN and Namao, respectively. The plots were planted using a cone seeder. Each row was seeded with 0.75g of seed at a depth of 1.5-2 cm and simultaneously

inoculated with grain inoculum (Hwang et al. 2014). The amount of grain inoculum in 2012 was limiting. Therefore, the plots at CDCN were inoculated with 7 g of grain inoculum per row, while the plots at Camrose were inoculated with 4 g per row. In 2013, each row received about 16 g of grain inoculum per row at both CDCN and Namao. In-crop broadleaf and grassy weeds were controlled with one application of herbicide between the 4-6 leaf stage, and by hand weeding at later plant growth stages. In those plots in which the canola cv. 73-15 was planted, Roundup (Glyphosate; Monsanto Canada, Winnipeg, MN) was used as the herbicide, while in plots of the canola cv. Westar, Muster (ethametsulfuron methyl; E.I. DuPont Canada Company, Mississauga, ON) and Equinox EC (tepraloxydim, BASF Canada Inc., Mississauga, ON) were applied.

Emergence counts began when the first seedlings began to emerge, which was about 7 to 10 days after seeding. Counts were conducted three times, 7 days apart. However, only two counts were conducted in 2012 because the plants emerged sooner than expected.

The foliar fungicide treatments were applied at a volume of 100 L ha^{-1} with a manually pressurized backpack sprayer, when the plants reached the 4-6 leaf stage. Four days after the fungicide application, all plots were inoculated with the foliar pycnidiospore suspension using a separate backpack sprayer. Plots were inoculated in the early evening to provide conditions conducive for disease development. In the morning, if there was no natural dew on the leaves, the plants were misted lightly with water using a manually pressurized backpack sprayer to create moist conditions for the pathogen.

When disease symptoms began to appear, about 30 days after inoculation, evaluations for leaf disease severity and incidence began. Three evaluations were conducted, 7 days apart, for

each site year. In 2012, a meter stick was randomly placed along one of the inside rows of each plot, and every leaf on every plant along the meter stick was assessed for blackleg. In 2013, 30 leaves were sampled from each plot: 10 from the lower canopy, 10 from the middle, and 10 from the top. Evaluations for disease severity in 2012 were based on visual estimates while in 2013 disease severity was measured using Assess 2.0: Image Analysis Software for Plant Disease Quantification (American Phytopathological Society, 2008). Leaves were stacked and placed in paper bags for transport to the lab for image analysis, where they were gently wiped clean with a wet sponge prior to scanning in order to remove any debris that might interfere with the analysis. The mean disease severity, expressed as the percent of the leaf area that was covered in lesions, was averaged for each plot in each year. Leaf disease incidence was determined by calculating the percent of infected leaves per plot.

When pods began to ripen, about 16 weeks after seeding, the plots were harvested with a small plot combine. The trials were harvested on Sept. 18 (CDCN) and Sept. 26 (Camrose) in 2012, and on Sept. 17 (Namao) and Sept. 25 (CDCN) in 2013. The seeds were dried, cleaned, and weighed to obtain yield. A seed sample from each plot was sent to BioVision Seed Labs (Sherwood Park, Alberta) to determine oil and protein content along with the incidence of *L. maculans*-infected seed. The day after harvest, 25 stems were pulled from each plot to evaluate severity and incidence of stem necrosis. The cross-section of each stem was evaluated as described above for the greenhouse experiments. To determine the relationship between severity of stem necrosis and yield, 10 mature plants were pulled from each plot before harvest from each of the experimental sites in 2013. For each individual plant, the stems were evaluated for severity and the pods were dried in paper bags, manually threshed, and the seeds were cleaned and weighed, to determine a seed yield parameter.

3.2.6 Data analysis

Statistical analyses were performed with R: A Language and Environment for Statistical Computing (R Core Team, R Foundation for Statistical Computing, Vienna Austria, 2013). Residual data were analyzed visually and tested for normality using the Shapiro-Wilk test in R shapiro.test stats. The homogeneity of variance also was analyzed visually and tested using the Bartlett test bartlett.teststats. Transformations were applied to achieve or improve normality and equal variance where appropriate. The analysis of variance indicated that the different environments for each experiment (i.e., greenhouse Trials 1 and 2, and field locations and years) contributed to large variation in many of the parameters examined. Therefore, all experiments and trials were analyzed separately.

All main effects were analyzed further with pair-wise comparisons of means using Tukey Honest Significant Difference TukeyHSD stats. For greenhouse Trial 2 and the 2013 field experiments, the relationship between the severity of internal blackleg stem infection and yield was analyzed using Pearson's product-moment correlation coefficient cor.teststats. Differences were considered significant at $P \leq 0.05$.

3.3 RESULTS

3.3.1 Greenhouse Trial 1

In general, fungicide treatments had no effect on the emergence rates for either canola cultivar at 3, 7 or 14 DAP (Table A3.1). The final emergence counts at 14 DAP ranged from 88% to 97% for Westar, and from 97% to 100% for 73-15. Final emergence in the inoculated, untreated controls was 98% for Westar and 97% for 73-15. The only significantly lower emergence rates, relative to the inoculated controls, were observed for Westar treated with BAS

720 F at 7 and 14 DAP (88%), and for Westar treated with Prosper FX at 14 DAP (93%). No other differences were significant.

Foliar disease incidence and severity, expressed as the AUDPC, was greater for Westar than for 73-15 (Table A3.2). The incidence and severity of blackleg ranged from 66 to 95 and from 3 to 4, respectively, for 73-15. No significant differences were observed among any of the treatments for this cultivar. For Westar, the incidence and severity of blackleg ranged from 97 to 167 and from 5 to 13, respectively. The half rate of BAS 720 F and the combination of BAS 720 F + Tilt resulted in no reduction in incidence or severity compared with the inoculated control (150 and 9 for each parameter, respectively). The combination of BAS 720 F + Priaxor significantly reduced blackleg severity (5) in Westar, relative to the half rate of BAS 720 F (13). The incidence and severity of stem necrosis were higher in Westar than in 73-15 (Figure 3.1). In the fungicide treatments, the incidence and severity ranged from 75% to 98% and 28% to 56% in 73-15, respectively, and from 95% to 100% and 59% to 82% in Westar. There were no significant differences in either parameter (incidence or severity) between any of the treatments or the inoculated, untreated controls.

Plant height and weight for 73-15 ranged from 63.6 cm to 67.1 cm and 6.1 g to 7.1 g respectively (Table A3.3). There were no significant differences in plant height or weight for 73-15. For Westar, treatment with BAS 720 F, BAS 720 F + Priaxor, and BAS 720 F + Tilt resulted in significantly greater heights (53.0 cm to 54.6 cm) relative to the inoculated control (26.0 cm) and to Prosper FX (30.4 cm). For Westar, weight ranged from 4.2 g (Prosper FX) to 9.6 g (BAS 720 F + Priaxor). There were no significant differences in weight for Westar.

Plant survival for the fungicide treatments ranged from 91% to 96% for 73-15 and from 45% to 70% for Westar (Table A3.3). There were no significant differences in plant survival between treatments or controls for either cultivar.

Overall, 73-15 yielded greater than Westar (Figure A3.1). Yield ranged from 10.0 g to 11.0 g per pot for 73-15, but there were no significant differences between treatments or the inoculated control. Yields for Westar ranged from 0.1 g to 2.7 g per pot. Only Priaxor with BAS 720 F (2.7 g) significantly increased yield relative to the inoculated control (0.1 g) and the Prosper FX treatment (0.3 g).

3.3.2 Greenhouse Trial 2

The final emergence rates for the fungicide seed treatments in the case of 73-15 and Westar ranged between 68% to 80% and 59% to 70%, respectively, at 14 DAP. There were no significant differences in emergence for either cultivar.

Overall, the AUDPC for foliar disease incidence and severity was greater in Trial 2 than Trial 1 and greater for Westar than for 73-15 (Table A3.2). Foliar disease incidence in the fungicide treatments ranged from 121 to 218 for 73-15 and from 253 to 366 for Westar. There were no significant differences in foliar disease incidence for Westar. All fungicide treatments resulted in a significant decrease in severity compared with the inoculated control (53), but there were no differences between fungicide treatments. In the case of 73-15, treatment with a combination of BAS 720 F + Priaxor resulted in a significantly lower disease incidence (121) than treatment with Tilt + BAS 720F (218). There were no differences in disease severity for 73-15.

Relative to Trial 1, the incidence and severity of stem necrosis in Trial 2 was similar for Westar, but lower for 73-15 (Figure 3.2). Incidence and severity of stem infection was greater in Westar than in 73-15. The incidence of stem necrosis ranged from 35% to 48% in 73-15, and from 69% to 100% in Westar, in the fungicide treatments. While there were no significant differences between fungicide treatments for 73-15, all treatments significantly reduced the incidence of internal stem infection compared with the inoculated control (79%). In the case of Westar, BAS 720 F + Priaxor (16%) significantly reduced the incidence of stem necrosis relative to the seed treatments and inoculated control (100%), while BAS 720 F + Tilt (18%) significantly reduced incidence relative to Prosper FX and the inoculated control. The severity of stem necrosis for the fungicide treatments ranged from 4% to 10% in 73-15, and from 16% to 28% in Westar. While there were no significant differences between fungicide treatments, all fungicides significantly reduced incidence relative to the inoculated control in both cultivars.

Unexpectedly, for 73-15, the inoculated control had significantly greater individual plant weight than the un-inoculated control (26.9 g), the half rate of BAS 720 F (28.1 g), and Prosper FX (30.3 g) (Table A3.3). In the inoculated control, many of the plants in the pots died off, leaving more room for the others to grow. The remaining canola plants were able to branch out and fill the empty space in the pots, resulting in the higher weights. These two fungicide treatments, along with the inoculated control, also had among the lowest plant counts and highest levels of blackleg. There were, however, no significant differences in weight among fungicide treatments. As for Westar, there were no significant differences in plant weight.

In the case of Westar, treatment with Prosper FX (109.0 cm) and the combination of BAS 720 F + Tilt (106.1 cm) resulted in a significantly greater plant height than in the inoculated control (84.3 cm) (Table A3.3). However, there were no significant differences in height for 73-

15. There were also no increases in height or weight between BAS 720 F + Priaxor and BAS 720 F alone for either cultivar.

Plant survival in Trial 2 was lower than in Trial 1 (Table A3.3). In the fungicide treatments, survival rates ranged treatments ranged from 68% to 73% for 73-15 and from 48% to 68% for Westar. In the case of 73-15, there were no significant differences between fungicide treatments and the inoculated control. In Westar, treatment with BAS 720 F at the full rate, BAS 720 F + Priaxor, or BAS 720 F + Tilt significantly increased plant survival (64% - 68%) compared with the inoculated control (29%). The combination of BAS 720 F + Priaxor did not result in an increase in plant survival compared with BAS 720 F alone for either cultivar.

Yields in Trial 2 were generally higher than in Trial 1 (Figure A3.2). Overall, the yield of 73-15 was greater than that of Westar. In the fungicide treatments, yield ranged from 8.16 g to 12.26 g per pot for 73-15 and from 6.58 g to 7.40 g per pot for Westar. There were no significant differences in yield between fungicide treatments for 73-15. In the case of Westar, all fungicide treatments resulted in a significant increase in yield relative to the inoculated control (2.67 g), but there were no significant differences between fungicide treatments.

There was no correlation (-0.06) between stem disease severity and yield for 73-15 (Table A3.4). Conversely, for Westar, there was a slight negative correlation (-0.26) that was significant ($P \leq 0.05$).

3.3.3 Field experiment 2012

At Camrose, the average precipitation from May to September 2012 was 18 mm below normal, while the average temperature was 1ºC higher than normal (Table A3.5). At CDCN, precipitation levels over the same period were below normal for all months except July, when

precipitation was 48 mm above normal. Just as at Camrose, the average temperature at CDCN was 1ºC above normal from May to Sept. Mean monthly temperatures at CDCN were slightly higher than at Camrose, while the number of daily rainfall events for each month was greater in Camrose. In the two-week period following inoculation of the field plots, there were 10 rainfall events totaling 38.7 mm at Camrose, and eight rainfall events totaling 52.4 mm at CDCN (Table A3.6).

Emergence counts at Camrose ranged from 109 to 121 and 116 to 122 seedlings per plot for 73-15 and Westar, respectively (Table A3.7). At CDCN, emergence counts ranged from 104 to 119 seedlings per plot for 73-15 and 118 to 145 seedlings per plot for Westar. None of the fungicide treatments resulted in a significant increase in emergence at either location or for either cultivar.

In Camrose, the AUDPC for foliar blackleg incidence ranged from 271 to 314 (73-15) and from 309 to 364 (Westar) for the fungicide treatments (Table A3.8). Blackleg severity ranged from 9 to 10 and from 16 to 19 for 73-15 and Westar, respectively. At the CDCN site, blackleg incidence for the fungicide treatments ranged from 123 to 150 and 211 to 299, respectively, for 73-15 and Westar, and severity ranged from 2 to 7 and 7 to 21. There were no significant differences in foliar disease incidence or severity for either cultivar at either location. It was observed that the older un-treated leaves on the lower canopy tended to drop quicker, particularly in the case of Westar, while the healthier leaves remained attached to the plant at both locations.

The incidence of stem necrosis was greater on Westar (65% to 82%) than on 73-15 (57% to 71%) at Camrose (Figure 3.3). Similarly, the severity of stem necrosis also was generally higher on Westar (11% to 20%) than on 73-15 (8% to 14%) (Figure 3.4). At the Camrose site,

treatment with BAS 720 F + Priaxor (65%) significantly reduced stem blackleg incidence on Westar relative to the inoculated control (86%). This same treatment (Priaxor + BAS 720 F) also resulted in a significant decrease in severity (11%) on Westar compared with the full rate of BAS 720 F (20%) alone, or with the inoculated control (24%). In the case of 73-15, there were no significant differences in incidence or severity at Camrose. At the CDCN site, the incidence of the stem symptoms ranged from 26% to 37% and from 38% and 92%, respectively, on 73-15 and Westar (Figure 3.3). The severity of stem blackleg ranged from 2% to 3% on 73-15 and from 6% to 30% on Westar (Figure 3.4). There were no significant differences in incidence for 73-15 at the CDCN site. However, treatments containing the full rate of BAS 720 F (2% to 3%) resulted in significantly lower disease severity compared to the inoculated control (5%). On Westar, treatment with BAS 720 F + Priaxor also resulted in the lowest incidence and severity of blackleg relative to all other treatments and the inoculated control, and in this case the values were significantly lower.

At Camrose, seed yield for the fungicide treatments ranged from 415.0 kg ha^{-1} g to 555.0 kg ha^{-1} per plot for 73-15 and from 183.9 kg ha^{-1} to 382.2 kg ha^{-1} per plot for Westar (Figure 3.5). There were no significant differences in yield for either cultivar at Camrose. At the CDCN site, yield for fungicide treatments ranged from 2774.4 kg ha^{-1} to 3207.2 kg ha^{-1} and 1808.9 kg ha^{-1} to 2146.7 kg ha^{-1}, respectively, for 73-15 and Westar (Figure 3.5). On Westar, treatment with BAS 720 F + Priaxor (2146.7 kg ha^{-1}) resulted in a significant increase in yield compared with the inoculated control (1638.3 kg ha^{-1}).

There were no significant differences in protein or oil content for either cultivar at either location (Table A3.9), although the highest oil content usually was associated with the lowest protein content. At Camrose, protein content for the fungicide treatments ranged from 24.16% to

24.83% for 73-15, and from 23.82% to 24.89% for Westar. The oil content ranged from 47.91% to 48.57% (73-15) and from 45.35% to 46.76% (Westar) at that site. At CDCN, protein content was higher and oil content was lower relative to Camrose, and ranged from 26.45% to 26.95% for 73-15 and from 27.36% to 27.89% for Westar. Oil content ranged from 45.09% to 45.65% and from 42.60% to 42.95% for 73-15 and Westar, respectively, in the fungicide treatments.

In 2012, a large proportion of the seed samples were contaminated with *Rhizopus* sp. (black mold), which prevented the quantification of *L. maculans*-infected seed at either location in that year.

3.3.4 Field experiment 2013

Precipitation was below normal at Namao and CDCN in May, July, August and September 2013 (Table A3.5). In June, it was 57 mm above normal at both sites. The mean temperatures were above normal in May, August, and September, and below normal in June and July. The average temperature over the entire May – September period was 0.7°C above normal at both sites. The number of daily rainfall events was greatest in June and July (Table A3.6). In the 2 weeks following inoculation of the experimental plots at CDCN, there were 11 rainfall events totaling 66.3 mm. At Namao, there were eight rainfall events totaling 18.1 mm.

No significant differences in emergence were observed between any of the treatments at either location (Table A3.10). At CDCN, the final plant counts for the fungicide treatments ranged from 59 to 69 per plot for 73-15, and from 53 to 62 per plot for Westar. At Namao, the final plant counts ranged from 104 to 120 per plot (73-15) and from 115 to 120 per plot (Westar).

The AUDPC for foliar blackleg incidence and severity was greater at CDCN than at Namao (Table A3.11). At CDCN, foliar disease incidence for the fungicide treatments ranged from 689

to 730 and from 632 to 837, respectively, for 73-15 and Westar. At the CDCN site, treatment of Westar with Prosper FX significantly reduced disease incidence (632) relative to treatment with BAS 720 F at the full rate (837) or BAS 720 F + Tilt (807), but there was no impact on incidence in the remaining treatments for both cultivars at this location. At Namao, foliar blackleg incidence ranged from 395 to 611 and from 420 to 618 for 73-15 and Westar, respectively. In the case of 73-15, treatment with BAS 720 F + Priaxor resulted in a significant decrease in disease incidence (395) compared with treatment with BAS 720 F at the full rate (611) or with Prosper FX (586). There were no significant differences in incidence for Westar at Namao. At the CDCN site, foliar blackleg severity ranged from 7 to 14 and from 15 to 26 for 73-15 and Westar, respectively. The combination of BAS 720 F + Priaxor resulted in a significantly lower disease severity (7) on 73-15 compared with the inoculated control (15), and compared with the half-rate of BAS 720 F (14) or BAS 720 F + Tilt (14). There were no significant differences in severity for Westar at that location. At Namao, foliar blackleg severity ranged from 3 to 13 and from 6 to 14 for 73-15 and Westar, respectively. At that location, BAS 720 F + Priaxor significantly reduced foliar blackleg severity (6) on Westar compared with the inoculated control (16.0). The same treatment on 73-15 (3) significantly reduced disease severity compared with the full rate of BAS 720 F.

Overall, the incidence of stem necrosis was greater at CDCN than at Namao (Figure 3.6). At the CDCN site, the incidence for the fungicide treatments ranged from 51% to 79% for 73-15, and from 62% to 92% for Westar. Treatment with the combination of BAS 720 F + Priaxor resulted in a significant decrease in incidence compared with all other fungicide treatments and the inoculated control for both cultivars at CDCN. At Namao, the incidence of stem necrosis ranged from 29% to 55% and from 39% to 50% on 73-15 and Westar, respectively, in the

fungicide treatments. Treatments containing the full rate of BAS 720 F resulted in a significant decrease in incidence (38 to 47) for 73-15 compared with the inoculated control (50). There was no impact of the fungicide treatments on stem disease incidence on Westar at Namao. The severity of stem necrosis at CDCN was greater than at Namao (Figure 3.7) and ranged from 8% to 24% on 73-15 and from 18% to 48% on Westar. At CDCN, disease severity following treatment of 73-15 with BAS 720 F + Priaxor was significantly lower (8%) than the severity on the inoculated control (20%), as well as being significantly lower than all other fungicide treatments except the half rate of BAS 720 F. Similarly, treatment of Westar with BAS 720 F + Prixaor also was associated with significantly lower stem blackleg severity (18%), relative to the inoculated control (47%) and all other fungicide treatments. At Namao, the severity of blackleg on the stem ranged from 3% to 8% (73-15) and 6% to 10% (Westar) in the fungicide treatments. In the case of Westar, however, none of the fungicide treatments reduced severity relative to the inoculated control. For 73-15, significant decreases were observed following treatment with BAS 720 F (5%), BAS 720 F + Priaxor (3%), or BAS 720 F + Tilt (5%). There were no significant differences in severity on Westar at Namao.

Seed yield was greater at CDCN than at Namao (Figure 3.8). Yield for the fungicide treatments at CDCN ranged from 3009.0 kg ha^{-1} to 3214.8 kg ha^{-1} per plot for 73-15 and from 995.1 kg ha^{-1} to 2041.7 kg ha^{-1} per plot for Westar. For Westar, treatment with BAS 720 F + Priaxor (2041.7 kg ha^{-1}) resulted in a significantly higher yield compared with the inoculated control (893.8 kg ha^{-1}) and all seed treatments (995.1 kg ha^{-1} to 1330.6 kg ha^{-1}). Westar treated with BAS 720 F + Tilt (1495.8 kg ha^{-1}) resulted in significantly higher yield than the inoculated control. In the case of 73-15 at CDCN, there were no significant differences in yield. Seed yield for the fungicide treatments at Namao ranged from 759.7 kg ha^{-1} to 1070.8 kg ha^{-1} per plot and

from 409.7 kg ha^{-1} to 595.8 kg ha^{-1} per plot for 73-15 and Westar, respectively. However, there were no significant differences in yield for either cultivar at that location.

Just as in 2012, the highest oil content usually was associated with the lowest protein content for both cultivars at each location (Table A3.12). Overall, oil content was greater at Namao than at CDCN. At the latter site, oil content for the fungicide treatments ranged from 50.06% to 50.32% for 73-15, and from 44.55% to 45.75% for Westar, while protein ranged from 20.74% to 21.08% and from 22.67% to 23.78%, respectively, for the cultivars. The differences in oil and protein content were not significant for 73-15 at CDCN. Treatment with BAS 720 F + Priaxor (45.74%) resulted in a significant increase in oil content relative to the half-rate of BAS 720 F (44.70%) or Prosper FX (44.5%) for Westar. Conversely, treatment of Westar with BAS 720 F + Priaxor also resulted in significantly lower protein content (22.67%) relative to the half-rate of BAS 720 F (23.78%). At Namao, oil content for the fungicide treatments ranged from 51.44% to 52.98% and from 47.82% to 48.97%, respectively, for 73-15 and Westar. Protein content ranged from 16.64% to 17.88% and from 19.43% to 20.52% for these cultivars. Fungicide treatments did not result in an increase in oil content for Westar at Namao. In the case of 73-15, the BAS 720 F + Priaxor treatment (52.80%) resulted in a significant increase in oil content relative to the inoculated control. There were no significant differences in protein content for either cultivar at Namao.

The incidence of *L. maculans*-infected seed was greater at CDCN than at Namao (Table A3.13). At the CDCN site, the incidence of infected seed ranged from 5×10^{-2} % to 2×10^{-1} % for 73-15 and from 1×10^{-1} % to 3×10^{-1} % for Westar. At Namao, 3×10^{-2} % to 8×10^{-2} % of seeds of 73-15 and 3×10^{-2} % to 2×10^{-1} % of Westar seeds were infected with *L. maculans*.

None of the fungicide treatments resulted in a significant decrease in infected seed for either cultivar at both locations.

There were no significant correlations between stem disease severity and yield at Namao for either cultivar (Table A3.14). At CDCN for Westar, there was a small negative correlation (-0.17) which was significant ($P < 0.05$).

3.4 DISCUSSION

Blackleg has been an important disease of canola in western Canada since the mid-1970s, when it was recognized as a threat to yields (Kutcher et al. 2011). Growers depend heavily on blackleg resistant cultivars to manage blackleg. However, with tight canola rotations and an increase in inoculum levels, there is a risk that *L. maculans* will overcome cultivar resistance (Kutcher et al. 2011). Many studies have reported that the largest benefits of fungicide application occur under high disease pressure and/or when growing cultivars with low to moderate resistance, and that there is little benefit to fungicide treatment in cultivars with higher levels of resistance (Huang et al. 2011; Khangura and Barbetti 2002; Kutcher et al. 2005; Marcroft and Potter 2008). Blackleg disease levels and yield also are influenced by the timing of fungicide application, inoculum release, and environmental conditions (Huang et al. 2011; Kharbanda 1992; Steed et al. 2007; Wherrett et al. 2003). In this study, the efficacy of combining seed and foliar fungicides with two effective modes of action was evaluated for blackleg control in Alberta.

Significant differences between the inoculated and uninoculated controls for many of the parameters under both field and greenhouse conditions indicate the utility of applying inoculum to initiate disease. While ascospores are more efficient at infecting leaves, pycnidiospores may

serve as primary inoculum if ascospores are not present in the environment (Ghanbarnia et al. 2011). Soil-borne pycnidiospores could play an important role in the epidemiology of *L. maculans*, since they have been shown to germinate in soil and infect young canola seedlings (Li et al. 2007). Overall, stem disease levels were greater in the greenhouse, while foliar disease levels were greater under field conditions. Under field conditions in western Canada, ascospores are released from canola residues between May to August (West et al. 2001). Therefore, in addition to the application of pycnidiospore inoculum, plants in the field trials were likely exposed to natural sources of inoculum, explaining why low to moderate levels of blackleg were observed on the uninoculated controls. In greenhouse Trial 1, the uninoculated controls developed some leaf and stem disease as a result of being placed on the same bench as the inoculated pots and, consequently, the pathogen spread to them. To avoid this occurring in greenhouse Trial 2, the uninoculated controls were placed on different benches.

The development of blackleg disease depends on the number of rainfall events, amount of moisture and humidity, and temperature (West et al. 2001). Plants were watered daily in the greenhouse and, as a result, were exposed to moisture and water splash. At each field location, there was a rainfall event within 24 hours after inoculation. The greatest number of rainfall events within 2 weeks after inoculation occurred at the CDCN site, while Namao received the least. The amount of precipitation received is reflected to some extent in the blackleg levels observed at each location, with those sites receiving the most moisture having higher levels of disease.

The number of emerged seedlings was counted to evaluate the efficacy of seed treatment fungicides. Marcroft and Potter (2008) found the application of fluquinconazole as a seed treatment reduced plant mortality in susceptible cultivars sown in soils with high inoculum loads.

An alternative to assess seed treatment efficacy is to evaluate the number of infected seedlings after emergence. Kharbanda (1992) assessed seed treatment fungicides, some of which included pyraclostrobin. The results showed effective suppression of seed-borne and external inoculum with iprodione and prochloraz treatment, based on the number of healthy seedlings. However, these effects were not observed in the field. There can also be positive effects with respect to seedling infection from application of the in-furrow product Impact (flutrifol) (Khangura and Barbetti 2002, 2004). In the current study, fungicide seed treatments did not increase seedling emergence under field or greenhouse conditions. It is likely that disease pressure was not sufficiently high to have a negative impact on emergence. Perhaps inoculating the canola seed with pycnidiospores before applying the seed treatment would have increased the disease pressure on germinating and emerging seedlings. In greenhouse Trial 2, there was a significant difference between the uninoculated and inoculated controls, while this was not observed in Trial 1. In Trial 1 the grain inoculum was applied on top of the potting mix rather than below it. Thus, the seeds may not have been exposed to the inoculum on the 'soil' surface prior to germination. Interestingly, there was a significant decrease in the percent emergence of Westar following treatment with Prosper FX or BAS 720 F at the full rate in Trial 1, perhaps due to lower seed vigor.

There are reports that in experiments under controlled environmental conditions, infection by pycnidiospores is only possible when plant parts have been wounded. Under conducive conditions, however, only 1 or 2 pycnidiospores are needed to produce a lesion (West et al. 2001). In the greenhouse trials, foliar disease was initiated without the need to injure or wound the seedlings. Leaf disease severity was evaluated either visually or with computer imaging software, in order to determine the efficacy of fungicide treatments on lesion development.

Marcroft et al. (2005) found no significant difference in the incidence of foliar lesions 14 days after inoculation, regardless of the stage at which the plants were inoculated. Nevertheless, those plants inoculated at the cotyledon and 3-leaf stage had significantly greater stem canker incidence than plants inoculated at the 5-leaf stage, suggesting that plants inoculated after the 3-leaf growth stage do not develop severe stem canker infection (Marcroft et al. 2005). In greenhouse Trial 2, there were no signs or symptoms of infection on young leaves prior to bolting. However, after bolting, mycelial growth could be seen on adult leaves, which later developed into lesions. Contrary to previous studies, the development of foliar lesions on adult leaves still resulted in severe stem necrosis in Westar in Trial 2, although it is possible that this may have resulted from the presence of soil inoculum that directly infected the stem tissue.

West et al. (2001) stated that there is sometimes a poor relationship between leaf lesions and stem disease, particularly if *L. maculans* has been isolated from leaves without the presence of lesions. Despite the low concentration of pycnidiospores in the inoculum applied in greenhouse Trial 1, the incidence and severity of stem necrosis were still high. Although foliar disease severity was low, it is likely that the grain inoculum placed on the surface of the potting mix contributed to stem disease, since girdled stems and pycnidia were observed on the surface of the crowns.

Ijaz and Honermeier (2012) evaluated the effect of various triazole and strobilurin fungicides applied at the floral bud and pod development stages on green area index (GAI). They found that the strobilurin treatments had a positive effect on GAI due to their ability to delay senescence. In the current field and greenhouse trials, disease severity was greater on the lower leaves compared with leaves in the middle and top canopy, where almost no disease was present. The lower leaves in the inoculated but untreated controls were not protected and

therefore would senesce and drop more quickly than the lower leaves of fungicide-treated plants, leaving the healthier leaves behind. The inoculated controls would have been expected to have higher levels of foliar disease than the treated plants, but the remaining healthier leaves of the inoculated plants brought the average severity levels down. Conversely, the infected lower leaves on the treated plants would stay attached to the plant longer than on the untreated plants. This could be explained by the properties of strobilurin fungicides, which are known to delay senescence by lowering ethylene concentrations (Ijaz and Honermeier 2012).

In some cases, under both field and greenhouse conditions, the combination of BAS 720 F with foliar fungicides resulted in lower foliar blackleg incidence and severity than the application of seed treatments alone. This effect was expected, since the foliar fungicides would be protecting new leaves, while the seed treatments only protected newly emerged seedlings via their systemic activity. The combination of BAS 720 F + Priaxor often resulted in the lowest foliar disease levels among all of the fungicide treatments. In contrast, at some field locations, the application of Tilt did not result in any benefit relative to BAS 720 F alone.

Severe stem necrosis is associated with infection before the 3-5 leaf stage of canola (Marcroft et al. 2005). It was expected that the systemic seed treatments in this study would result in a reduction in stem disease levels, since seedlings would be protected from soil-borne and airborne inoculum at the most vulnerable growth stage. However, the systemic activity of seed treatment fungicides is limited to the cotyledon stage or first true leaves, and once new leaves grow, they are no longer protected from airborne *L. maculans* inoculum. Fungicide seed treatments have been shown to reduce internal infection in cultivars which are susceptible or partially resistant to blackleg under high disease pressure (Khangura and Barbetti 2004; Marcroft and Potter 2008). In the current study, the effect of seed treatment on stem disease severity

varied by trial. The seed treatments did not always lead to a decrease in stem disease compared with the control. Nonetheless, some significant differences were observed. For instance, at CDCN in 2012 and at Namao in 2013, the full rate of BAS 720 F resulted in a significant decrease in stem disease for the canola cv. 73-15 relative to the inoculated, untreated control. Similarly, all of the seed treatments in greenhouse Trial 2 resulted in a significant reduction in stem disease severity for both 73-15 and Westar.

Peng et al. (2012) evaluated various foliar fungicides, including Headline (pyraclostrobin) and Tilt (propiconazole), for blackleg control in Westar at various timings. They also tested Headline on a moderately resistant and resistant cultivar at the 2-4 leaf stage. At one location, Headline significantly reduced stem disease severity and incidence at the 2-4 leaf stage on all cultivars compared with the controls, while Tilt had no effect on Westar. The application of Headline on Westar at the rosette stage did not result in a significant decrease in blackleg incidence or severity. In the current study, Tilt often had little to no effect on stem disease under field conditions, while the Priaxor treatment most often led to a decrease in stem disease severity, especially for the susceptible cultivar Westar.

There are numerous reports suggesting that older canola plants cannot be protected from blackleg by seed treatments alone (Gugel and Petrie 1992). It was expected that the combination of seed and foliar treatments would lead to the greatest reduction in disease, since seed treatments only protect young seedlings while foliar treatments protect new leaves after the seedling stage. Khangura and Barbetti (2004) found that while all of the treatments they tested reduced blackleg disease, combining a seed treatment with single or multiple foliar applications reduced blackleg disease compared with a seed treatment or foliar spray alone. The combination of BAS 720 F and Priaxor often resulted in lower stem disease severity compared with the seed

treatments alone, especially for Westar. Nonetheless, in certain trials, the blackleg levels following treatment with BAS 720 F alone and in combination with foliar treatments were the same, suggesting that there was a benefit from the seed treatment and not the foliar treatment. In contrast, in certain trials there was only a reduction in stem disease with the addition of a foliar treatment, suggesting there was no benefit from the seed treatment alone. This result may be attributed to the source of inoculum (soil-borne vs. airborne) or the timing of disease establishment. To confirm this assumption, future studies should include an evaluation of the foliar fungicides as solo treatments.

Cultivar resistance plays an important role in managing blackleg in canola. Overall, the moderately resistant cv. 73-15 developed lower levels of disease and had higher yields than the susceptible cv. Westar under both greenhouse and field conditions. This was expected, given the resistance rating and yield potential of each cultivar. While stem disease severity was lower at Camrose and Namao than at CDCN in both years of the field experiment, there was less difference in disease severity between the two cultivars. Since the experimental plots at Camrose and Namao were not tilled before seeding, there was an abundance of canola volunteers which may have impacted the results for 73-15 at these locations. The greatest differences in stem disease levels were seen when fungicide treatments were applied to the susceptible Westar. Marcroft and Potter (2008) found that the untreated controls of cultivars with higher resistance ratings generally performed better than more susceptible cultivars with fungicide treatments. In the current study, the moderately resistant 73-15 almost always developed a lower stem disease severity regardless of fungicide application, which is consistent with the results of Marcroft and Potter (2008). Nonetheless, Marcroft and Potter (2008) also suggested that there was an additive effect of using a moderately resistant cultivar with the fungicide fluquinconazole, resulting in

blackleg levels similar to those of a resistant cultivar. Similar results were observed in the current study with 73-15. At CDCN in 2013, 73-15 treated with BAS 720 F + Priaxor developed a disease severity that was lower than in the absence of the fungicides.

Khangura and Barbetti (2002) found a significant negative linear relationship between stem infection and yield for susceptible to moderately resistant canola cultivars. The results of the current study are similar in that there was a correlation between stem disease and yield for the susceptible cv. Westar at CDCN in 2013 and in greenhouse Trial 2. However, there was no correlation for 73-15 in any of the trials. There also was no correlation for Westar at Namao in 2013. In Namao, the seed bed was not tilled and it was difficult to control volunteer canola in the plots containing Westar, since this cultivar is not herbicide-tolerant. It is likely that the volunteers were a confounding factor in these plots, since there was no effect of fungicide on stem disease. As noted above, several studies have suggested that the greatest benefit of fungicides occurs when they are applied to cultivars with lower resistance and/or under high disease pressure (Bailey et al. 2000; Khangura and Barbetti 2002, 2004; Marcroft and Potter 2008). Consistent with these suggestions, significant differences were observed for stem disease on 73-15 and Westar in some trials, but there was only a significant yield response for the susceptible cv. Westar. While results varied across experiments and cultivars, the application of a seed treatment (BAS 720 F) in addition to a foliar treatment generally resulted in a higher yield relative to a seed treatment fungicide alone. This was expected since applying a seed treatment would only protect emerging seedlings from soil-borne inoculum, but the addition of a foliar fungicide would also protect young canola plants from air-borne inoculum.

Ijaz and Honermeier (2012) reported no effect of triazole and strobilurin foliar fungicides on morphological traits, such as height and branching, in canola. Similarly in the current study,

there was very little to no benefit of fungicide application on height or weight for either cultivar under greenhouse conditions.

Overall, plant survival under greenhouse conditions was greater for 73-15 than for Westar, which was expected due to the higher blackleg resistance rating of the former. In Trial 2, treatments containing the full rate of BAS 720 F resulted in an increase in plant survival, but no differences were observed in Trial 1. It is possible that the placement of the grain inoculum above and below the potting mix surface, respectively, in Trial 1 and Trial 2 may have impacted the disease pressure on growing seedlings.

While the most important source of blackleg inoculum is from infested canola residues, protecting canola against infected seed is critical to prevent the spread of the disease into regions free of *L. maculans* (West et al. 1999). Reports of the percent incidence of infected seed per sample is usually <5% (West et al. 1999). In the current study, *L. maculans*-infected seed remained below 0.5%. Seed infection was higher at CDCN than at Namao in 2013, possibly due to higher disease pressure. Overall, the percentage of *L. maculans*-infected seed was higher for Westar than for 73-15. This was expected, since Westar is susceptible to *L. maculans* and generally developed higher levels of blackleg than 73-15. Nevertheless, there was no significant effect of fungicide treatment on seed infection for either cultivar.

McCartney et al. (1999) highlighted the importance of protecting canola from disease for ensuring consistent quality for processing. The results of their study suggested a negative linear correlation between canola seed oil content and protein content and blackleg severity; seed from heavily infected plots had high protein levels, and when there was high protein there was an equal decrease in oil content (McCartney et al. 1999). A correlation between protein and oil

content was evident in the current study. It was often found that the treatments with the lowest protein content had the highest oil content and vice versa. The cv. Westar usually had higher protein and lower oil content level than 73-15, possibly due to higher disease levels in Westar or an improved varietal profile for 73-15. However, few differences in seed oil and protein content were observed as a result of any of the fungicide treatments within the specific cultivars, and it can be concluded the treatments provided little benefit to seed quality in these experiments.

Fungicide stewardship is important in order to preserve the effectiveness of fungicides. As described in Chapter 2, no isolates of *L. maculans* from Alberta were found to be insensitive to pyraclostrobin. In that study, the average concentration of pyraclostrobin required to effectively inhibit mycelial growth by 50% (EC_{50}) was 0.09 mg L^{-1}, which was 11% lower than the rate of pyraclostrobin used in this chapter as a foliar treatment. Based on these results, it appears that the recommended rate of Priaxor is still effective at reducing the mycelial growth of *L. maculans* isolates from Alberta.

The fungicide treatments did not always decrease blackleg or improve yield; the results varied depending on weather conditions, disease pressure, inoculum timing, and cultivar. Nonetheless, the combination of BAS 720 F seed treatment with Priaxor was most effective in reducing the levels of blackleg. While the fungicide treatments reduced blackleg incidence and severity in both the susceptible moderately resistant cultivars, there was a greater yield benefit in treating blackleg in the susceptible cultivar Westar. Based on these results, it appears that the use of pyraclostrobin as an active ingredient in a seed treatment and in combination with a foliar fungicide provides protection against blackleg disease in susceptible and moderately resistant canola cultivars under moderate to high disease pressure, which in turn may lead to a greater yield potential.

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3.6 FIGURES

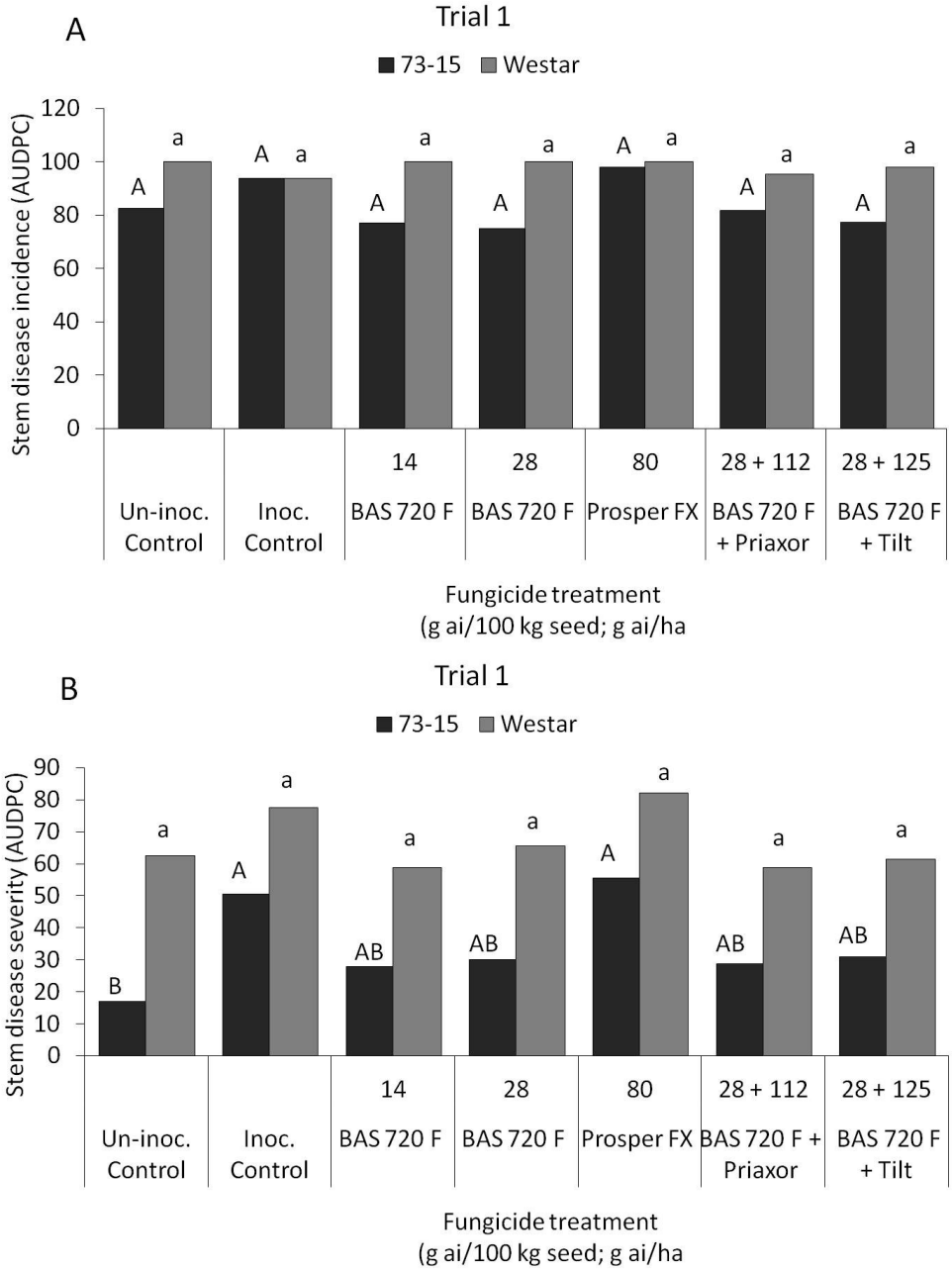

Figure 3.1 Effect of fungicide treatments on blackleg stem disease incidence (A) and severity (B) in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under greenhouse conditions (Trial 1). Bars with the same lowercase letter indicate means (n = 8 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to both data sets before the analysis. Untransformed means are presented. Rates include fungicide components only.

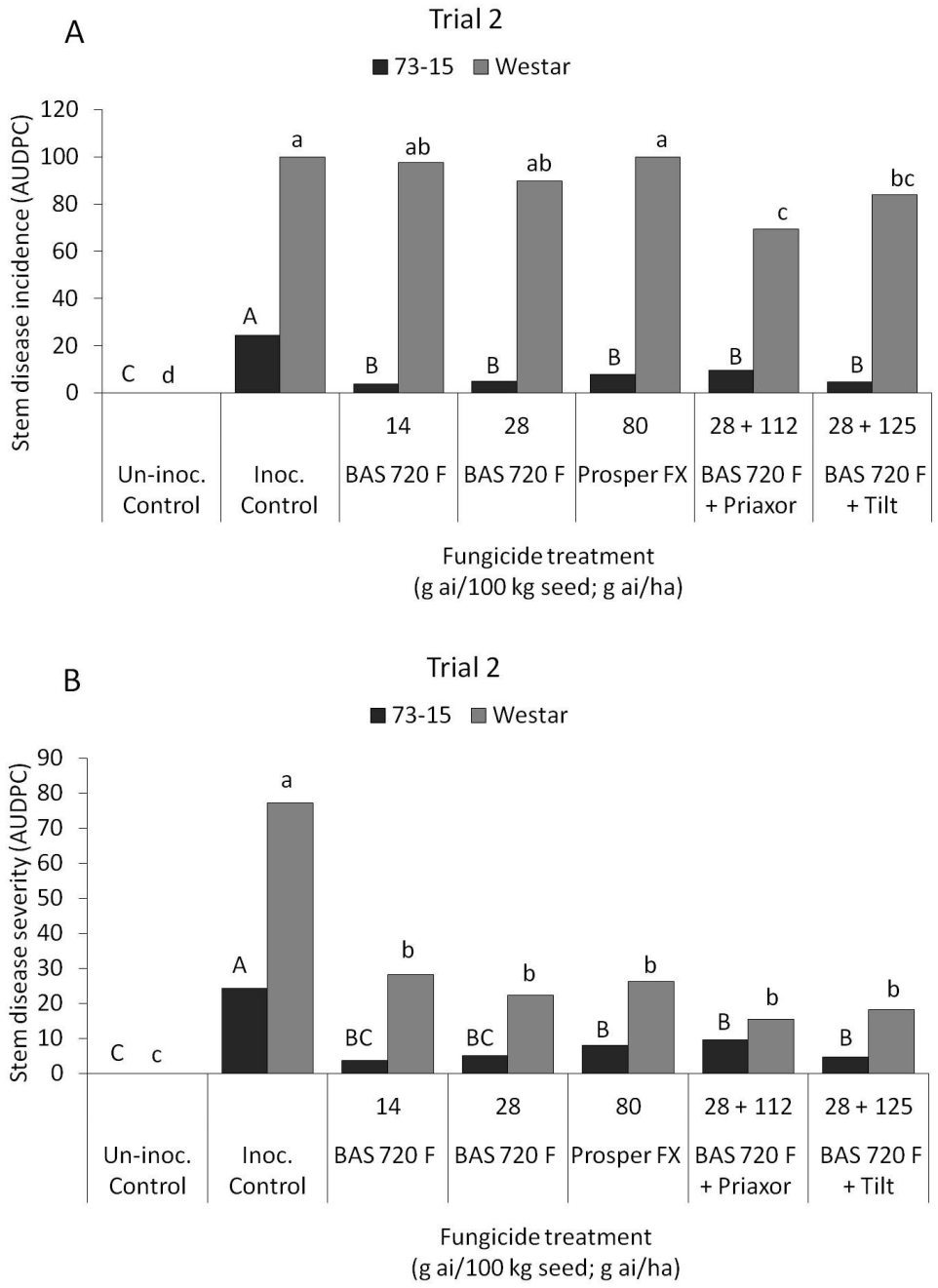

Figure 3.2 Effect of fungicide treatments on blackleg stem disease incidence (A) and severity (B) in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under greenhouse conditions (Trial 2). Bars with the same lowercase letter indicate means (n = 8 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to both data sets before the analysis. Untransformed means are presented. Rates include fungicide components only.

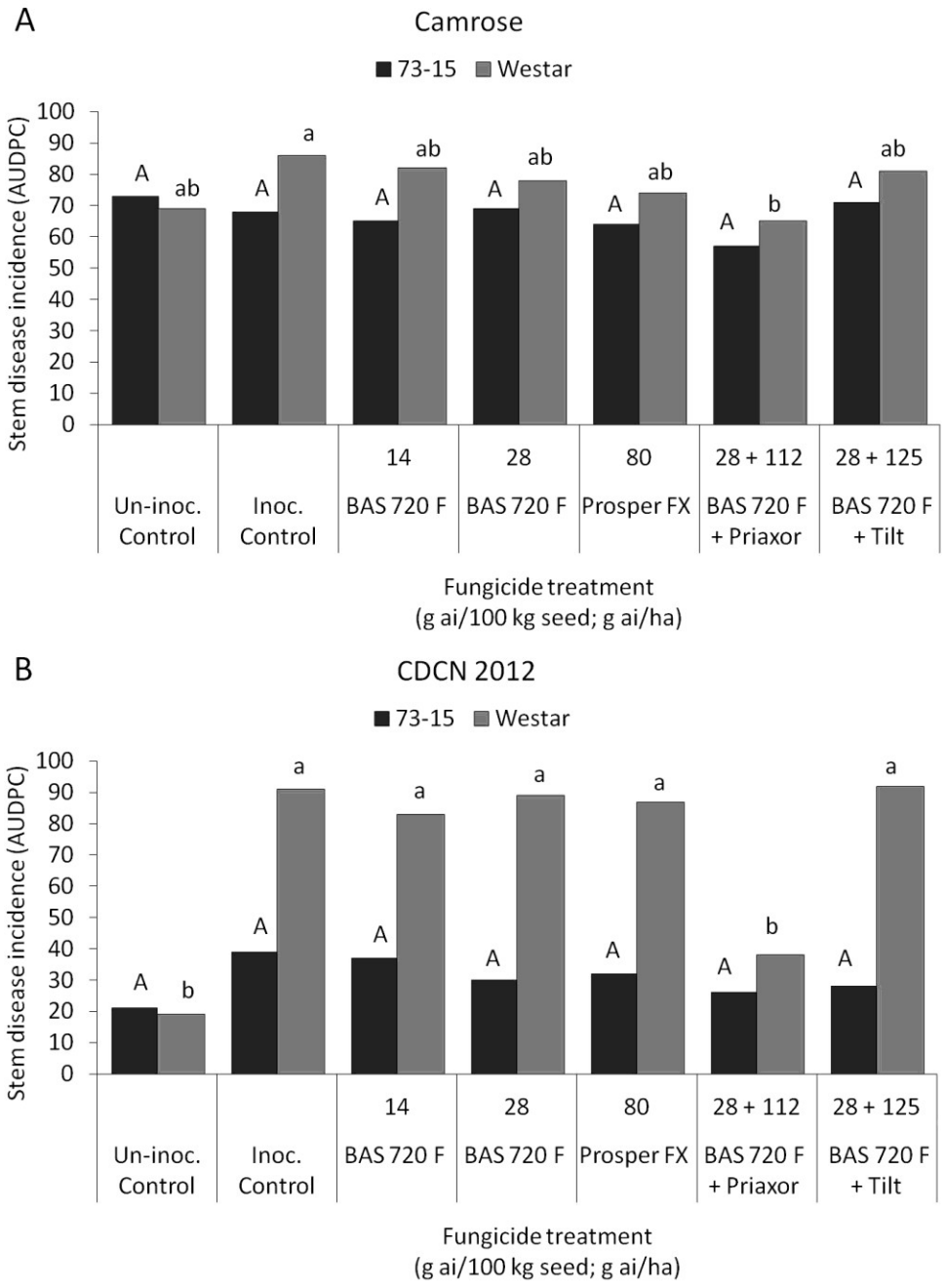

Figure 3.3 Effect of fungicide treatments on blackleg stem disease incidence in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at A) Camrose and B) the Crop Diversification Centre North (CDCN), Edmonton, Alberta, in 2012. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to the data before the analysis. Untransformed means are presented. Rates include fungicide components only.

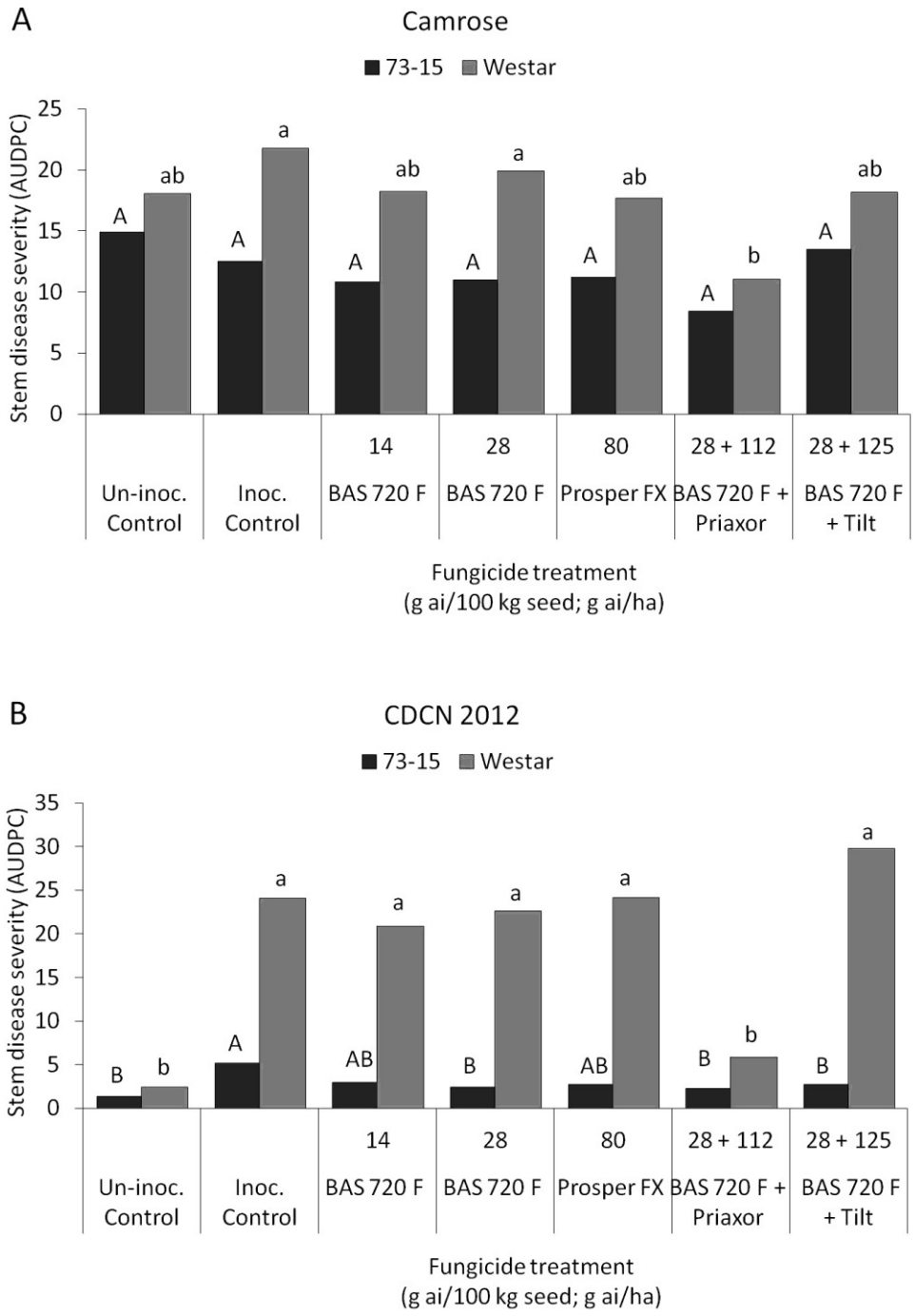

Figure 3.4 Effect of fungicide treatments on blackleg stem disease severity in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at A) Camrose and B) the Crop Diversification Centre North (CDCN), Edmonton, Alberta, in 2012. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to the data before the analysis. Untransformed means are presented. Rates include fungicide components only.

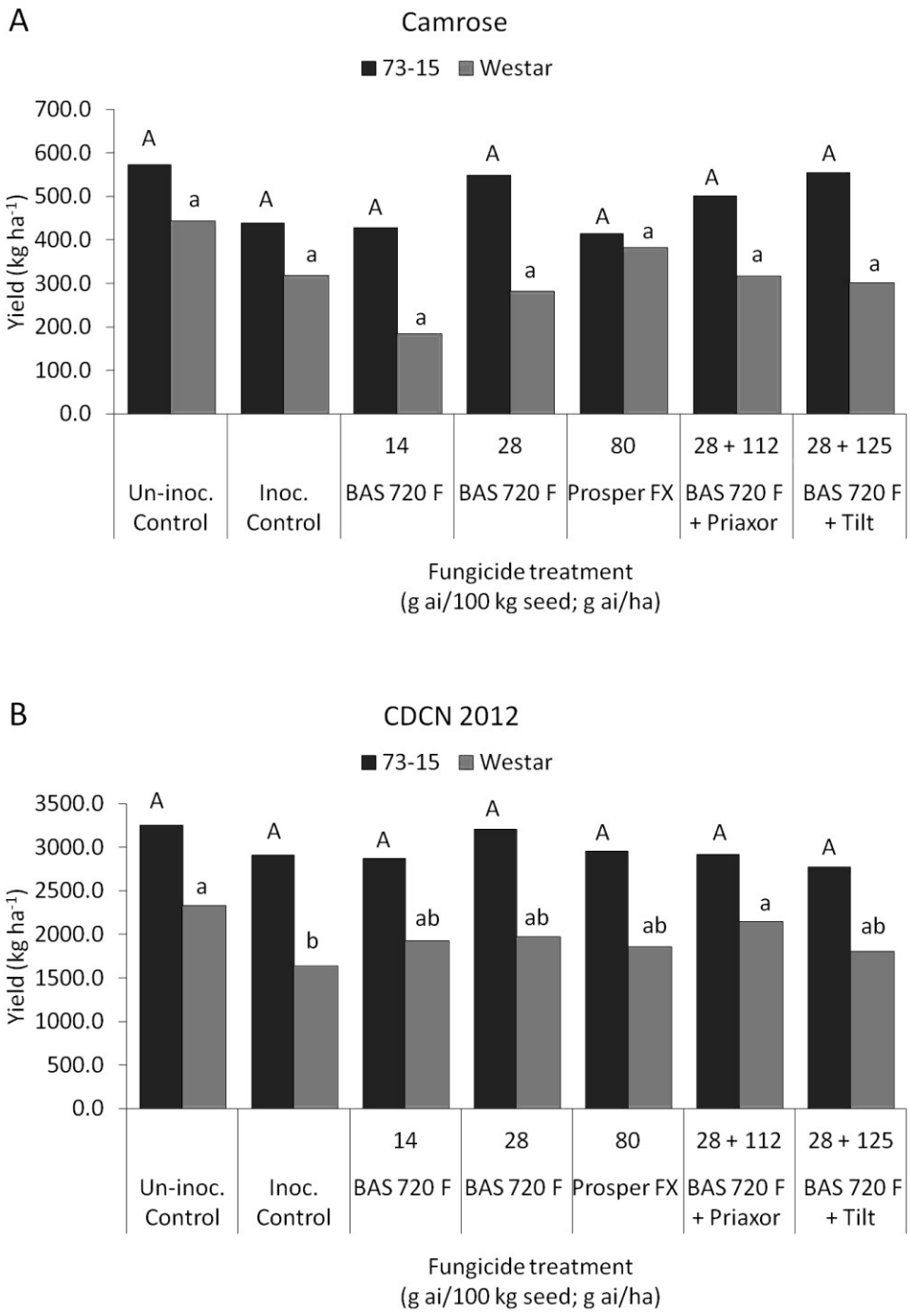

Figure 3.5 Effect of fungicide treatments on yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at A) Camrose and B) the Crop Diversification Centre North (CDCN), Edmonton, Alberta in 2012. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. A square-root transformation was applied to the data before the analysis. Untransformed means are presented. Rates include fungicide components only.

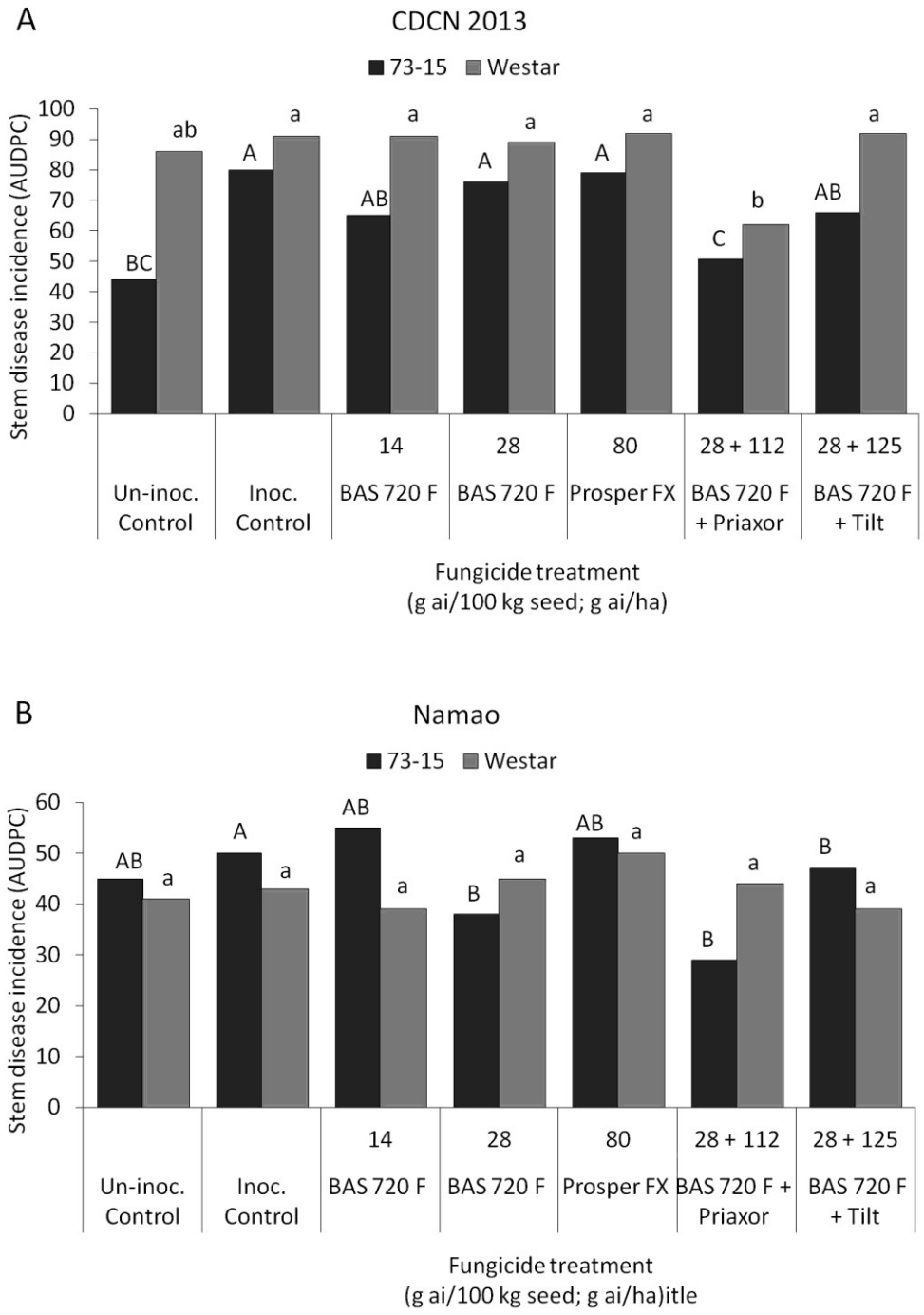

Figure 3.6 Effect of fungicide treatments on blackleg stem disease incidence in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in 2 repetitions under field conditions at A) the Crop Diversification Centre North (CDCN), Edmonton, and B) Namao, Alberta, in 2013. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to the data before the analysis. Untransformed means are presented. Rates include fungicide components only.

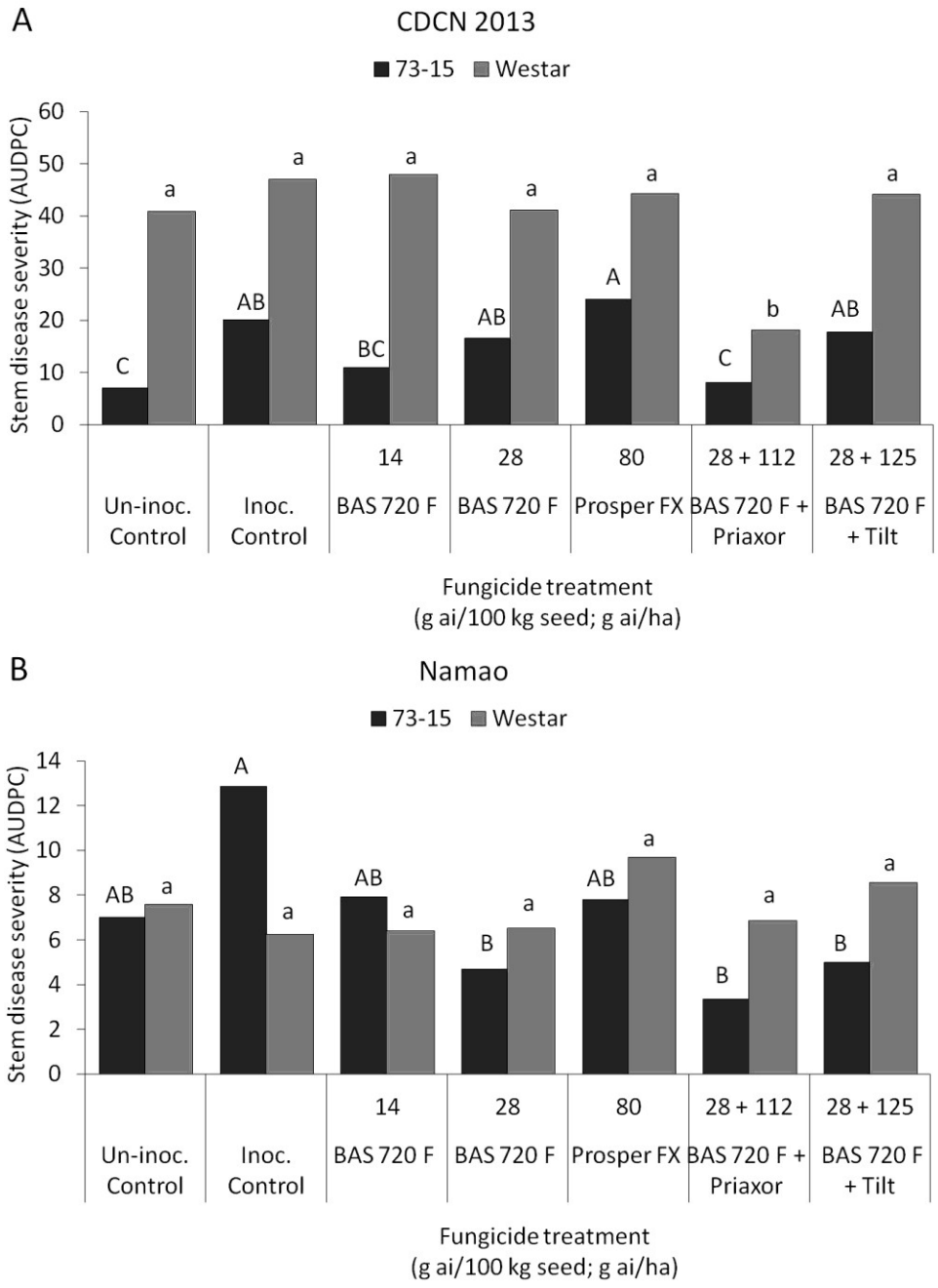

Figure 3.7 Effect of fungicide treatments on blackleg stem disease severity in two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at A) the Crop Diversification Centre North (CDCN), Edmonton, and B) Namao, Alberta, in 2013. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. An arcsine square-root transformation was applied to the data before the analysis. Untransformed means are presented. Rates include fungicide components only.

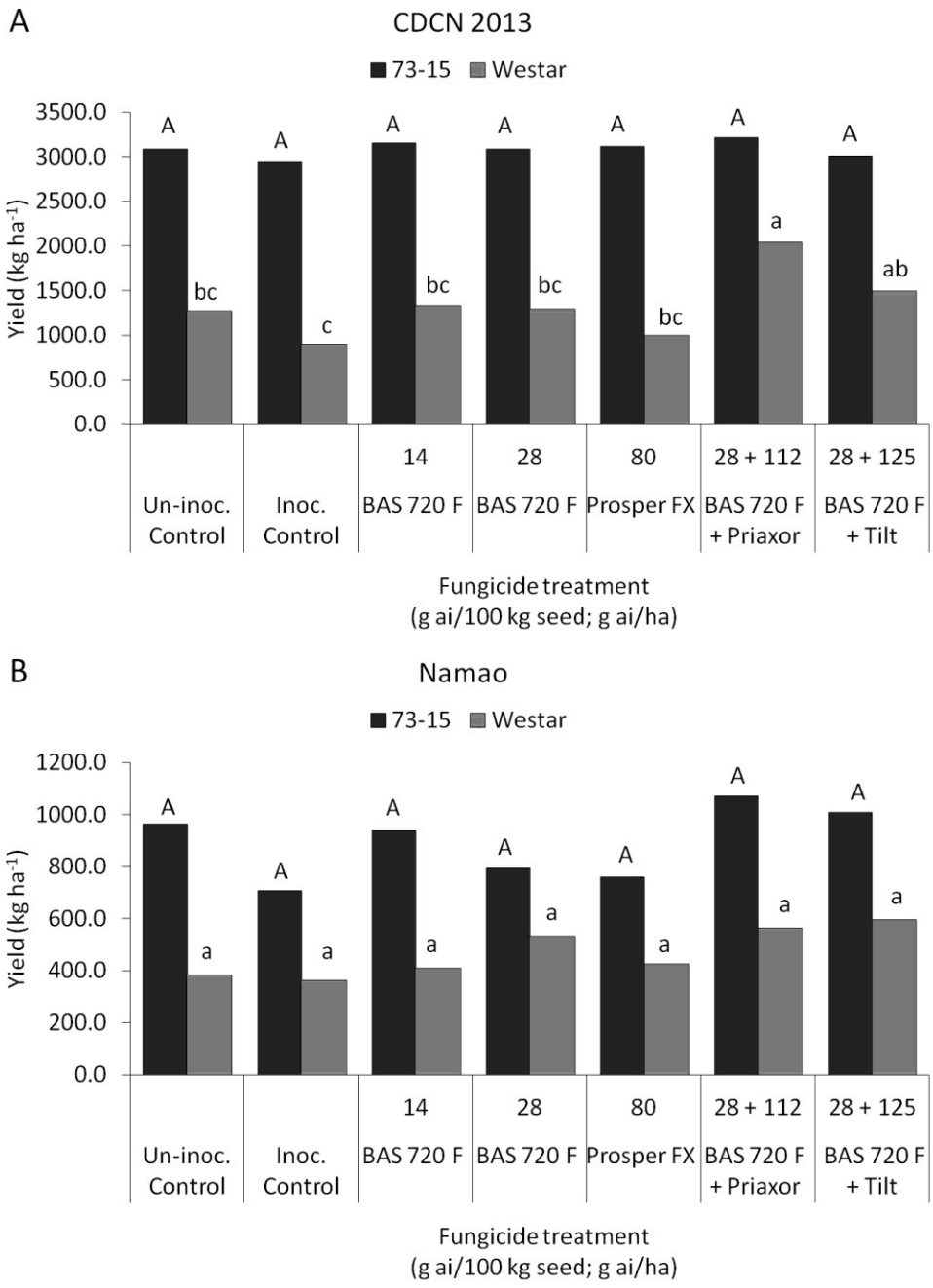

Figure 3.8 Effect of fungicide treatments on yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in 2 repetitions under field conditions at A) the Crop Diversification Centre North (CDCN), Edmonton, and B) Namao, Alberta, in 2013. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. A square-root transformation was applied to the yield data before the analysis. Untransformed means are presented. Rates include fungicide components only.

4 GENERAL CONCLUSIONS

Numerous strategies have been shown to be effective for blackleg management, but each has its own limitations. Therefore, it is best to include a variety of strategies in an integrated pest management program. A rotation of canola once every 4 years will allow some time for infected canola residues to decompose, reducing the amount of inoculum and size of pathogen populations, and consequently reducing the incidence and severity of blackleg (Kutcher et al. 2013). While this strategy decreases the amount of inoculum in a field, it will not eradicate the disease from the field; *L. maculans* can still be introduced into a field through infected seed or be blown in from surrounding canola fields up to several kilometers away (Hall 1992). Moreover, *L. maculans* can survive on volunteer canola and susceptible weeds (Kutcher et al. 2011), as well as on infected residues that have not fully decomposed (Kutcher et al. 2011).

Ploughing or deep cultivation of residues was a common practice in western Canada (Gugel and Petrie 1992), and in part was practiced to bury infested crop debris in the soil. However, there have been inconsistent findings regarding the effectiveness of stubble management as a blackleg reduction technique. Burying infested stubble increases the rate of decomposition, but this rate also depends on soil moisture levels (Gugel and Petrie 1992). While tillage may increase stubble decomposition, it was found that burying stubble actually initiated the sexual stage of *L. maculans* (production of pseudothecia and ascospores), which may in turn increase inoculum levels if buried stubble is brought back to the surface by subsequent tillage operations (Turkington et al. 2000).

The introduction of blackleg resistant canola cultivars in western Canada, combined with an increased market for canola, growers saw an opportunity to shorten canola rotations (Gugel and Petrie 1992). The planting of resistant cultivars has become the primary method of blackleg

management (Peng et al. 2012). However, with shortened rotations, increased inoculum levels, and larger and more diverse pathogen populations, there is an increased selection pressure on *L. maculans* isolates to overcome genetic resistance (Gugel and Petrie 1992; Kutcher et al. 2013; Kutcher et al. 2011; Peng et al. 2012). That being said, under current cropping practices in western Canada, a reliance on resistance as the only blackleg management tool may not be sustainable. Other than genetic resistance, fungicides are a direct control method to protect canola plants against blackleg. With very few fungicidal active ingredients available for blackleg control in Alberta (Alberta Government 2015), growers are limited in their choices of fungicide products. Intensive use of fungicides of the same class may eventually lead to the selection and development of insensitive pathogen populations (Brent and Hollomon 2007). Strobilurin fungicides, such as pyraclostrobin, are labeled as high risk for insensitivity development (Fungicide Resistance Action Committee 2014) because of their single site-specific mode of action (Mueller et al. 2013). Pyraclostrobin is a fairly new active ingredient; it was developed in 2000 (Bartlett et al. 2002) and was first registered for use on canola in Alberta in 2010. Developing a sustainable fungicide resistance management strategy for pyraclostrobin is critical in order to conserve its effectiveness in controlling blackleg on canola. In order to do this, two questions needed to be addressed: (1) are current *L. maculans* populations sensitive or insensitive to pyraclostrobin?; and (2) what is the best way to apply fungicides to increase their efficacy while maintaining fungicide resistance stewardship?

There are no known published studies on the sensitivity of *L. maculans* populations from Alberta to pyraclostrobin, and very few studies on the efficacy of pyraclostrobin on blackleg. Therefore, the objectives of this thesis study were to: (1) evaluate pyraclostrobin sensitivity of *L. maculans* populations in Alberta via growth plate and microtiter assays; and (2) examine the

efficacy of an experimental seed treatment containing pyraclostrobin with and without various foliar fungicides under greenhouse and field conditions.

Determining the sensitivity of *L. maculans* populations will improve understanding of the potential of pyraclostrobin as a chemical control method, and help determine if it is a sustainable solution for blackleg management. Examining the performance of fungicides will provide knowledge on the most effective way to manage blackleg through chemical control. With this knowledge, there is potential to decrease blackleg disease and improve canola yields in Alberta.

4.1 FUNGICIDE SENSITIVITY STUDY

In the sensitivity study (Chapter 2), 117 single-spore isolates of *L. maculans* were collected from infected canola stubble from different fields in various counties in Alberta. These isolates were collected in 2011, after the first season that pyraclostrobin was used on canola and, therefore, and therefore do not represent true baseline isolates (i.e., isolates that have never been exposed to the fungicide). A total of 13 isolates were selected from the entire collection to determine the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}). This was done via a radial growth plate assay with plates amended with pyraclostrobin. The EC_{50} of the 13 isolates was similar, but slightly lower than reported by Liu et al. (2013) in a preliminary analysis (abstract) of the sensitivity of a different collection of isolates in western Canada.

In order to detect highly insensitive isolates within the collection, all 117 isolates were exposed to pyraclostrobin at a level 69 times higher than the EC_{50} (and 4 times the recommended rate of Headline fungicide). Isolates were considered insensitive if their radial growth was inhibited by less than 50% of the control (Chang et al. 2007). The radial growth of all 117 isolates was inhibited by more than 50% and, therefore, it was appears that none of the isolates

were insensitive to pyraclostrobin. To confirm the results of the growth plate assay, a subset of the isolates was subjected to a microtiter test. Since pyraclostrobin inhibits spore germination (Bartlett et al. 2002), the microtiter test represents a more accurate method to detect insensitivity, since it involves exposing pycnidiospores, as opposed to mycelium, direct to the fungicide. The EC50 values of the isolates were then compared to two isolates with known sensitivity that served as controls. No significant differences were found in the pyraclostrobin sensitivity of the Alberta isolates versus the sensitive controls, confirming that all of the isolates tested from Alberta were sensitive to pyraclostrobin. The results of the microtiter test were consistent with the results of the growth plate assay. The results of the sensitivity studies were not unexpected, since the isolates were assumed to have had very little or no previous exposure to pyraclostrobin. Therefore, it was unlikely that insensitivity could develop in such a short time period. Nonetheless, there have been cases where other fungal species have developed insensitivity to pyraclostrobin after only two years of exposure (Wise et al. 2009).

The results of this study may provide valuable information to growers, as they suggest that there are no insensitivity issues with pyraclostrobin in Alberta, at least at the present time. Any previous possible exposure to pyraclostrobin did not seem to affect the sensitivity of isolates tested. Therefore, pyraclostrobin has significant potential to be an effective blackleg management tool, but only if fungicide stewardship practices are maintained. While there were no insensitive isolates detected, it is important to continue to monitor *L. maculans* populations to identify any changes in the future. Pyraclostrobin is very effective at managing a broad spectrum of diseases on various crops (Fernández-Ortuño et al. 2010), and in order to preserve its effectiveness, a proper use pattern needs to be developed.

4.2 FUNGICIDE EFFICACY STUDY

In Chapter 3, various combinations of seed and foliar fungicides were evaluated for their performance under greenhouse and field conditions. Treatments consisted of the half and full rate of an experimental seed treatment BAS 720 F (pyraclostrobin and fluxapyroxad), Prosper FX (carbathiin and trifloxystrobin), and the foliar fungicides Priaxor (pyraclostrobin and fluxapyroxad) and Tilt (propiconazole), which were each applied with BAS 720 F. All treatments were applied to the cultivars Westar and 73-15, which are susceptible and moderately resistant to blackleg, respectively. Seed treatments were applied to the seed before sowing, and foliar fungicides were applied the 4-6 leaf stage. Other than the non-treated control, all plants were inoculated with pycnidiospores in the form of a grain inoculum at seeding and with a foliar spore suspension 4 days after foliar treatment with fungicides. Field studies were conducted in 2012 at Camrose and at the Crop Diversification Centre North (CDCN), Edmonton, and in 2013 at Namao and CDCN.

Various parameters were evaluated including emergence, leaf disease, stem disease, plant height and weight, seed yield, and seed oil and protein content. Seed treatments did not improve emergence counts under field or greenhouse conditions. Blackleg disease levels were low to moderate in both the greenhouse and field trials. Overall, leaf and stem disease levels were greater for the susceptible cultivar Westar than the moderately resistant cultivar 73-15, as was expected. While results were not always consistent between trials, applying the fungicide BAS 720 F in combination with Priaxor often led to the lowest leaf and stem disease severities in both cultivars. Where there were significant differences in stem disease, it was usually reflected in the yield results, but only for Westar. Most often, the greatest treatment differences with respect to disease levels and yield were observed when blackleg disease pressure was higher, such as at

CDCN in 2012 and 2013. There was a weak but significant negative correlation for stem disease severity and yield in Westar under field and greenhouse conditions, but not for 73-15. It is suspected that disease levels were not sufficiently high to have a severe impact on yield. Under greenhouse conditions, fungicide treatments did not result in an increase in weight and height in either cultivar. The levels of *L. maculans*-infected seed were very low for both field locations in 2013, and were higher in Westar than in 73-15. There was no benefit of fungicide treatments on infected seed, possibly because over-all disease pressure was only low to moderate, and the infection of siliques is typically rare (West et al. 2001). Since blackleg negatively affects seed oil content, it is important to protect canola plants, such as with fungicides, to improve seed quality (McCartney et al. 1999). In the current study, high protein levels were often associated with low oil content, especially between cultivars and site-years. While not consistent, combining BAS 720 F with Priaxor often resulted in the highest oil content in both cultivars, with significant differences for Westar at CDCN in 2013, and 73-15 at Namao.

Many factors affect the performance of fungicides in controlling blackleg. These include weather, cultivar resistance, timing of fungicide application, and timing and source of inoculum. The results of this study suggest the greatest benefit of using fungicides is obtained with a susceptible cultivar. Nevertheless, when a moderately resistant cultivar is used, additional disease protection can be achieved with fungicides. A combination of seed and foliar treatments, specifically BAS 720 F and Priaxor, gave the best blackleg disease protection and improvements in oil content, with the greatest benefit when disease pressure was moderately high versus in years when it was low. Nonetheless, the only yield benefit was observed for Westar. Differences between the performance of the foliar fungicides Priaxor and Tilt may have been based on the properties of each active ingredient; while fluxapyroxad and propiconazole are

locally systemic, the pyraclostrobin in Priaxor adheres to the waxy leaf cuticle and has translaminar activity for additional coverage and protection on the leaf surfaces (Balba 2007). A fungicide that contains two active ingredients, such as with Priaxor, could be more efficient than Tilt, since it affects more than one target site within the pathogen. The combination of a seed treatment and foliar fungicide with two effective built-in modes of action will also help growers maintain resistance stewardship in their integrated pest management plan.

4.3 FUTURE OUTLOOK FOR PYRACLOSTROBIN IN ALBERTA

The results presented in Chapters 2 and 3 suggest that using a fungicide containing pyraclostrobin has merit as a blackleg management tool in Alberta. It is effective at decreasing blackleg disease and improving canola yield, and there is currently no indication of pyraclostrobin insensitivity in *L. maculans* populations from this province. By combining two effective modes of action, BAS 720 F and Priaxor are tools that can improve resistance stewardship and ultimately will reduce the risk of the development of pyraclostrobin insensitive pathogen populations in the field. The rate of pyraclostrobin used in the foliar application of Priaxor in Chapter 3 was 11% higher than the mean EC_{50} value of the baseline isolates in Chapter 2; this indicates that even at a rate below that recommended for pyraclostrobin, mycelial growth by *L. maculans* would still be controlled. However, farmers are always advised to follow the recommended fungicide application rates to ensure the best efficacy of the product. The frequency of avirulence alleles are changing within populations of *L. maculans* populations in western Canada, due to intensive cropping of canola cultivars with the same single resistance genes, which is reducing the effectiveness of canola resistance (Liban et al. 2016; Zhang et al. 2015). Therefore, growers need to be equipped with additional blackleg management tools aside from genetic resistance. As long as resistance stewardship practices are implemented, fungicide

products containing pyraclostrobin will remain an effective tool for growers looking to improve yield and protect seed quality.

4.4 FURTHER RESEARCH

In this thesis study, I examined the sensitivity of *L. maculans* populations in Alberta to pyraclostrobin and the efficacy of pyraclostrobin as a blackleg management tool. However, further research is required to understand the long term potential of this active ingredient. While no pyraclostrobin insensitive isolates were detected in the current study, it is important to continually monitor populations over time. While there is no known sensitivity of *L. maculans* to pyraclostrobin in Alberta, this pathogen possesses some qualities that put it at risk for developing fungicide insensitivity. These qualities include sexual recombination in the lifecycle, frequent dispersal of spores over time, and an ability of the spores to travel relatively long distances (FRAC 2013). With the intensive and continued use of high risk strobilurin fungicides, pathogens such as *L. maculans* have the potential to develop pyraclostrobin insensitivity (Brent and Hollomon 2007). Monitoring *L. maculans* in Alberta and other canola growing regions will help identify any developing insensitivity issues, and if help determine if current resistance management strategies are effective (Brent and Hollomon 2007). In future studies, it would also be beneficial to compare current *L. maculans* isolates to those which have been collected earlier (before the introduction of pyraclostrobin fungicides), in order to determine if there have been any shifts in sensitivity over time. Similarly, the use of molecular methods to detect fungicide insensitivity would allow for a greater number of isolates to be screened in a shorter time frame. Assessment of the sensitivity of *L. maculans* to multiple strobilurin fungicides would help determine if there was is cross-resistance present within pathogen populations.

The application of fungicides only when necessary and at the proper time (i.e., preventatively) will help reduce selection pressure for insensitive isolates (Brent and Hollomon 2007) and improve the efficiency of the fungicidal products. Comparing different timings of fungicide and inoculation application, such as before and after the 3 leaf stage, may help to identify the optimum timing of pyraclostrobin application for control of blackleg of canola. In addition, examining other parameters, such as disease incidence in seedlings under greenhouse and field conditions, may provide a better understanding the impact of seed treatment fungicides on stem canker severity and yield. Exploring other methods of fungicide treatment, such as in-furrow applications, would broaden the spectrum of tools available to growers to manage blackleg. Previous research by Khangura and Barbetti (2002, 2004) suggested good potential for the management of blackleg with an in-furrow fungicide product containing flutriafol. While there are in-furrow fungicides registered for corn and many horticultural crops in Canada, it would be worth investigating the performance of pyraclostrobin as an in-furrow application in canola. Obtaining information from Alberta growers on their fungicide use patterns and cropping practices may help us to better interpret the results of the current studies, while also allowing an assessment of the potential for the development of fungicide insensitivity issues in *L. maculans*.

4.5 LITERATURE CITED

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APPENDIX: SUPPLEMENTARY DATA FOR CHAPTER 3

Table A3.1 Effect of fungicide seed treatments on seedling emergence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in two trials under greenhouse conditions

			Seedling emergence (%)			
			Trial 1		Trial 2	
Days after planting	Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed)	73-15	Westar	73-15	Westar
3	Un-inoculated control	–	46a[yx]	32a	70a	73a
	Inoculated control	–	55a	41a	25b	38b
	BAS 720 F	14	43a	34a	32b	25b
	BAS 720 F	28	41a	37a	27b	29b
	Prosper FX	80	43a	25a	29b	20b
7	Un-inoculated control	–	95a	98a	88a	91a
	Inoculated control	–	95a	98a	71b	69b
	BAS 720 F	14	97a	95ab	82b	64b
	BAS 720 F	28	95a	86b	71b	71ab
	Prosper FX	80	95a	97ab	77b	64b
14	Un-inoculated control	–	98a	100a	88a	91a
	Inoculated control	–	97a	98a	64b	59b
	BAS 720 F	14	97a	97ab	80ab	59b
	BAS 720 F	28	97a	88b	68ab	70b
	Prosper FX	80	100a	93b	77ab	62b

[z] Rates include fungicide components only.
[y] Means (n = 8 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.
[x] An arcsine square-root transformation was applied to all emergence data before the analysis. Un-transformed means are presented.

Table A3.2 Effect of fungicide treatments on phoma leaf spot severity and incidence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in two trials under greenhouse conditions

Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; g ai ha^{-1})	Leaf disease incidence (AUDPC)		Leaf disease severity (AUDPC)	
		73-15	Westar	73-15	Westar
Trial 1					
Un-inoculated control	–	46a	58b	2a[yx]	3c
Inoculated control	–	80a	149a	3a	9abc
BAS 720 F	14	95a	167a	4a	13a
BAS 720 F	28	64a	126a	3a	8abc
Prosper FX	80	100a	122a	4a	7abc
BAS 720 F + Priaxor	28 + 112	66a	97ab	3a	5bc
BAS 720 F + Tilt	28 + 125	75a	131a	3a	13ab
Trial 2					
Un-inoculated control	–	3c	8b	0b	0c
Inoculated control	–	212a	349a	8a	53a
BAS 720 F	14	150ab	366a	6a	21b
BAS 720 F	28	187ab	282a	6a	21b
Prosper FX	80	138ab	357a	7a	24b
BAS 720 F + Priaxor	28 + 112	121b	253a	3a	13b
BAS 720 F + Tilt	28 + 125	218a	274a	8a	18b

[z] Rates include fungicide components only.
[y] Means (n = 8 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.
[x] A square-root transformation was applied to all AUDPC data before the analysis. Un-transformed means are presented.

Table A3.3 Effect of fungicide treatments on plant height, weight and survival of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* over two trials under greenhouse conditions

		Plant height (cm)		Plant weight (g)		Plant survival (%)	
Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; g ai ha^{-1})	73-15	Westar	73-15	Westar	73-15	Westar
Trial 1							
Un-inoculated control	–	59.8a[y]	59.8a	6.0a[x]	7.5a	98a[w]	77a
Inoculated control	–	70.7a	26.0c	6.2a	4.4a	87a	43a
BAS 720 F	14	63.6a	43.6abc	6.3a	7.1a	91a	57a
BAS 720 F	28	66.7a	53.0ab	6.5a	8.9a	96a	59a
Prosper FX	80	64.0a	30.4bc	7.1a	4.2a	91a	45a
BAS 720 F + Priaxor	28 + 112	66.5a	53.3ab	6.5a	9.6a	96a	57a
BAS 720 F + Tilt	28 + 125	67.1a	54.6ab	6.1a	6.1a	96a	70a
Trial 2							
Un-inoculated control	–	87.1a	90.6ab	26.9b	27.2a	86a	89a
Inoculated control	–	92.3a	84.3b	58.7a	35.9a	48b	29c
BAS 720 F	14	82.1a	103.9ab	28.1b	59.2a	73ab	48bc
BAS 720 F	28	85.2a	102.1ab	39.8ab	38.8a	68ab	68ab
Prosper FX	80	83.8a	109.0a	30.3b	55.1a	73ab	50bc
BAS 720 F + Priaxor	28 + 112	85.5a	98.7ab	36.2ab	38.5a	70ab	64b
BAS 720 F + Tilt	28 + 125	90.4a	106.1a	43.0ab	36.6a	70ab	66ab

[z] Rates include fungicide components only.
[y] Means (n = 8 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.
[x] A square-root transformation was applied to all weight data before the analysis. Un-transformed means are presented.
[w] An arcsine square-root transformation was applied to all plant survival data before the analysis. Un-transformed means are presented.

Table A3.4 Correlation of stem disease severity and yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under greenhouse conditions for greenhouse trial 2

				Confidence interval (95%)	
Cultivar	Correlation coefficient (r)	df	t-value	Lower	Upper
Trial *2*					
73-15	-0.06	271	-1.05	-0.18	0.06
Westar	-0.26*	230	-4.10	-0.38	-0.14

*Significant at P = 0.05

Table A3.5 Environmental data for 2012 at Camrose and Edmonton (CDCN), and for 2013 at Namao and Edmonton, Alberta from May 1st to September 30th [z]

Month	Total precipitation (mm)	Normal precipitation (mm)	Mean Temperature (°C)	Normal temperature (°C)
Camrose 2012				
May	40.9	42.2	9.6	10.4
June	28.8	77.3	14.7	14.4
July	59.6	82.5	18.2	16.6
Aug.	63.8	58.6	16.8	15.6
Sept.	15.7	37.3	12.8	10.5
Season average	41.8	59.6	14.4	13.5
CDCN 2012				
May	46.7	43.2	10.4	10.8
June	46.2	77.7	15.5	14.8
July	136.5	89.0	18.4	16.9
Aug.	57.1	58.7	17.1	15.9
Sept.	16.4	36.9	12.9	10.8
Season average	60.6	61.1	14.9	13.8
CDCN & Namao 2013				
May	29.3	43.2	12.7	10.8
June	134.6	77.7	14.6	14.8
July	77.1	89.0	15.6	16.9
Aug.	49.8	58.7	16.5	15.9
Sept.	7.4	36.9	13.0	10.8
Season average	59.6	61.1	14.5	13.8

[z] Data from Alberta Agriculture and Forestry. http://agriculture.alberta.ca/acis/alberta-weather-data-viewer.jsp. Camrose data obtained from Camrose City Airport weather station. CDCN and Namao data obtained from Oliver ADGM weather station.

Table A3.6 Mean temperature, amount of precipitation, and number of rainfall events the weeks and hours surrounding the date of foliar inoculation of field trials in 2012 and 2013.

				Number of daily rainfall events (weeks after inoculation)			Total precipitation (mm) weeks after inoculation			Total precipitation (mm) X hours after inoculation			
Site - Year	Application date	Mean temperature (°C)	Daily total precipitation (mm)	-1-0	0-1	1-2	-1-0	0-1	1-2	Hours until rain	24	48	72
Camrose 2012	04-Jul	11.9	3.7	2	5	5	17.4	11.2	27.5	24	3.7	0.0	0.0
CDCN 2012	25-Jun	18	0.2	3	5	3	7.1	22.0	30.4	24	0.2	0.0	0.0
CDCN 2013	10-Jul	19.6	18	6	5	6	2.0	53.0	13.3	24	17.5	0.0	0.0
Namao 2013	20-Jul	17.7	4.5	5	6	2	34.0	17.7	0.4	24	4.5	0.0	0.0

Table A3.7 Effect of fungicide seed treatments on seedling emergence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Camrose and Edmonton (CDCN), Alberta in 2012

			Emergence count (# of plants)			
			Camrose 2012		CDCN 2012	
Days after planting	Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed)	73-15	Westar	73-15	Westar
7	Un-inoculated control	–	77a[y]	76a	98a	95a
	Inoculated control	–	81a	87a	65b	79a
	BAS 720 F	14	87a	91a	69b	99a
	BAS 720 F	28	85a	85a	85ab	84a
	Prosper FX	80	82a	75a	89ab	96a
14	Un-inoculated control	–	104a	116a	135a	121a
	Inoculated control	–	109a	125a	99b	93a
	BAS 720 F	14	121a	116a	104b	145a
	BAS 720 F	28	119a	122a	117ab	118a
	Prosper FX	80	109a	118a	119ab	127a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.8 Effect of fungicide treatments on phoma leaf spot severity and incidence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Camrose and Edmonton (CDCN), Alberta in 2012

Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; g ai ha^{-1})	Leaf disease incidence (AUDPC)		Leaf disease severity (AUDPC)	
		73-15	Westar	73-15	Westar
Camrose 2012					
Un-inoculated control	–	259a	312a	9a[y]	18a
Inoculated control	–	276a	341a	9a	19a
BAS 720 F	14	288a	364a	9a	19a
BAS 720 F	28	294a	321a	9a	17a
Prosper FX	80	271a	309a	10a	17a
BAS 720 F + Priaxor	28 + 112	314a	335a	10a	16a
BAS 720 F + Tilt	28 + 125	288a	336a	10a	16a
CDCN 2012					
Un-inoculated control	–	139a	274a	6a	19a
Inoculated control	–	170a	232a	10a	16a
BAS 720 F	14	123a	270a	7a	19a
BAS 720 F	28	150a	299a	7a	21a
Prosper FX	80	141a	276a	7a	15a
BAS 720 F + Priaxor	28 + 112	126a	241a	2a	7a
BAS 720 F + Tilt	28 + 125	124a	211a	5a	15a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.9 Effect of fungicide treatments on protein and oil content of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Camrose and Edmonton (CDCN), Alberta in 2012

Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; g ai ha^{-1})	Protein content (%)		Oil content (%)	
		73-15	Westar	73-15	Westar
Camrose 2012					
Un-inoculated control	–	24.85a[y]	25.06a	47.95a	45.67a
Inoculated control	–	24.67a	24.29a	48.24a	46.17a
BAS 720 F	14	24.74a	24.59a	48.21a	45.67a
BAS 720 F	28	24.44a	24.49a	48.36a	46.20a
Prosper FX	80	24.83a	24.89a	47.91a	45.35a
BAS 720 F + Priaxor	28 + 112	24.16a	24.50a	48.57a	46.03a
BAS 720 F + Tilt	28 + 125	24.46a	23.82a	48.38a	46.76a
CDCN 2012					
Un-inoculated control	–	26.51a	27.56a	45.49a	42.77a
Inoculated control	–	27.22a	27.01a	44.80a	42.83a
BAS 720 F	14	26.85a	27.55a	45.09a	42.95a
BAS 720 F	28	26.95a	27.61a	45.32a	42.60a
Prosper FX	80	26.93a	27.38a	45.33a	42.94a
BAS 720 F + Priaxor	28 + 112	26.45a	27.89a	45.65a	42.95a
BAS 720 F + Tilt	28 + 125	26.69a	27.36a	45.49a	42.79a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.10 Effect of fungicide seed treatments on seedling emergence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Edmonton (CDCN) and Namao, Alberta in 2013

			Emergence count (# plants)			
			CDCN 2013		Namao 2013	
Days after planting	Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed)	73-15	Westar	73-15	Westar
7	Un-inoculated control	–	79a[y]	71a	30a	20a
	Inoculated control	–	28b	17b	23a	20a
	BAS 720 F	14	33b	22b	25a	17a
	BAS 720 F	28	34b	28b	22a	19a
	Prosper FX	80	28b	24b	24a	19a
14	Un-inoculated control	–	96a	95a	114a	124a
	Inoculated control	–	58b	54b	112a	107a
	BAS 720 F	14	74ab	63b	110a	112a
	BAS 720 F	28	68b	65ab	103a	111a
	Prosper FX	80	65b	53b	111a	117a
21	Un-inoculated control	–	91a	88a	114a	111a
	Inoculated control	–	49b	50b	114a	102a
	BAS 720 F	14	67ab	62ab	104a	115a
	BAS 720 F	28	69b	62ab	112a	119a
	Prosper FX	80	59b	53b	120a	120a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.11 Effect of fungicide treatments on phoma leaf spot severity and incidence of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Namao and Edmonton (CDCN), Alberta in 2013

Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; g ai ha^{-1})	Leaf disease incidence (AUDPC)		Leaf disease severity (AUDPC)	
		73-15	Westar	73-15	Westar
Namao 2013					
Un-inoculated control	–	528ab	604a	6ab[y]	16a
Inoculated control	–	497ab	540a	9ab	16a
BAS 720 F	14	535ab	517a	8ab	14ab
BAS 720 F	28	611a	618a	13a	12ab
Prosper FX	80	586a	514a	9ab	8ab
BAS 720 F + Priaxor	28 + 112	395b	420a	3b	6b
BAS 720 F + Tilt	28 + 125	468ab	479a	9ab	7ab
CDCN 2013					
Un-inoculated control	–	677a	701ab	6c	16a
Inoculated control	–	734a	720ab	15a	16a
BAS 720 F	14	713a	717ab	14a	21a
BAS 720 F	28	689a	837a	12ab	26a
Prosper FX	80	690a	632b	11abc	18a
BAS 720 F + Priaxor	28 + 112	708a	689ab	7bc	15a
BAS 720 F + Tilt	28 + 125	730a	807a	14a	24a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.12 Effect of fungicide treatments on protein and oil content of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Namao and Edmonton (CDCN), Alberta in 2013

		Protein content (%)		Oil content (%)	
Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; a ai ha^{-1})	73-15	Westar	73-15	Westar
Namao 2013					
Un-inoculated control	–	19.13a[y]	19.62a	50.99ab	48.30a
Inoculated control	–	17.03b	19.44a	50.49b	48.75a
BAS 720 F	14	17.74ab	19.72a	51.65ab	48.31a
BAS 720 F	28	16.64b	19.43a	52.98ab	48.10a
Prosper FX	80	17.41ab	19.47a	51.44ab	48.97a
BAS 720 F + Priaxor	28 + 112	17.74ab	20.52a	52.80a	47.82a
BAS 720 F + Tilt	28 + 125	17.88ab	19.65a	52.24ab	48.45a
CDCN 2013					
Un-inoculated control	–	21.65a	23.01ab	49.93a	44.46b
Inoculated control	–	21.16a	23.32ab	50.30a	45.06ab
BAS 720 F	14	21.08a	23.78a	50.06a	44.70b
BAS 720 F	28	20.83a	23.06ab	50.31a	44.75ab
Prosper FX	80	20.89a	23.16ab	50.27a	44.55b
BAS 720 F + Priaxor	28 + 112	20.99a	22.67b	50.32a	45.74a
BAS 720 F + Tilt	28 + 125	20.74a	23.03ab	50.20a	45.30ab

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.

Table A3.13 Effect of fungicide treatments on infected seed of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Namao and Edmonton (CDCN), Alberta in 2013

		L. maculans-infected seed (% incidence)	
Fungicide treatment	Rate[z] (g ai 100 kg^{-1} seed; a ai ha^{-1})	73-15	Westar
Namao 2013			
Un-inoculated control	–	8.E-02a[yx]	3.E-02a
Inoculated control	–	3.E-02a	8.E-02a
BAS 720 F	14	5.E-02a	1.E-01a
BAS 720 F	28	3.E-02a	3.E-02a
Prosper FX	80	3.E-02a	5.E-02a
BAS 720 F + Priaxor	28 + 112	5.E-02a	2.E-01a
BAS 720 F + Tilt	28 + 125	8.E-02a	1.E-01a
CDCN 2013			
Un-inoculated control	–	8.E-02a	2.E-01a
Inoculated control	–	1.E-01a	2.E-01a
BAS 720 F	14	5.E-02a	3.E-01a
BAS 720 F	28	1.E-01a	3.E-01a
Prosper FX	80	2.E-01a	3.E-01a
BAS 720 F + Priaxor	28 + 112	7.E-02a	1.E-01a
BAS 720 F + Tilt	28 + 125	8.E-02a	3.E-01a

[z] Rates include fungicide components only.
[y] Means (n = 4 replications) followed by the same lowercase letter do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$.
[x] A square-root transformation was applied to the *L. maculans*-infected seed data before the analysis. Un-transformed means are presented.

Table A3.14 Correlation of stem disease severity and yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* under field conditions at Namao and Edmonton (CDCN), Alberta in 2013

				Confidence interval (95%)	
Cultivar	Correlation coefficient (r)	df	t value	Lower	Upper
Namao					
73-15	0.10	278	1.69	-0.02	0.22
Westar	0.06	277	0.98	-0.06	0.17
CDCN					
73-15	-0.04	268	-0.63	-0.16	0.08
Westar	-0.17*	276	-2.85	-0.28	-0.05

*Significant at P = 0.05

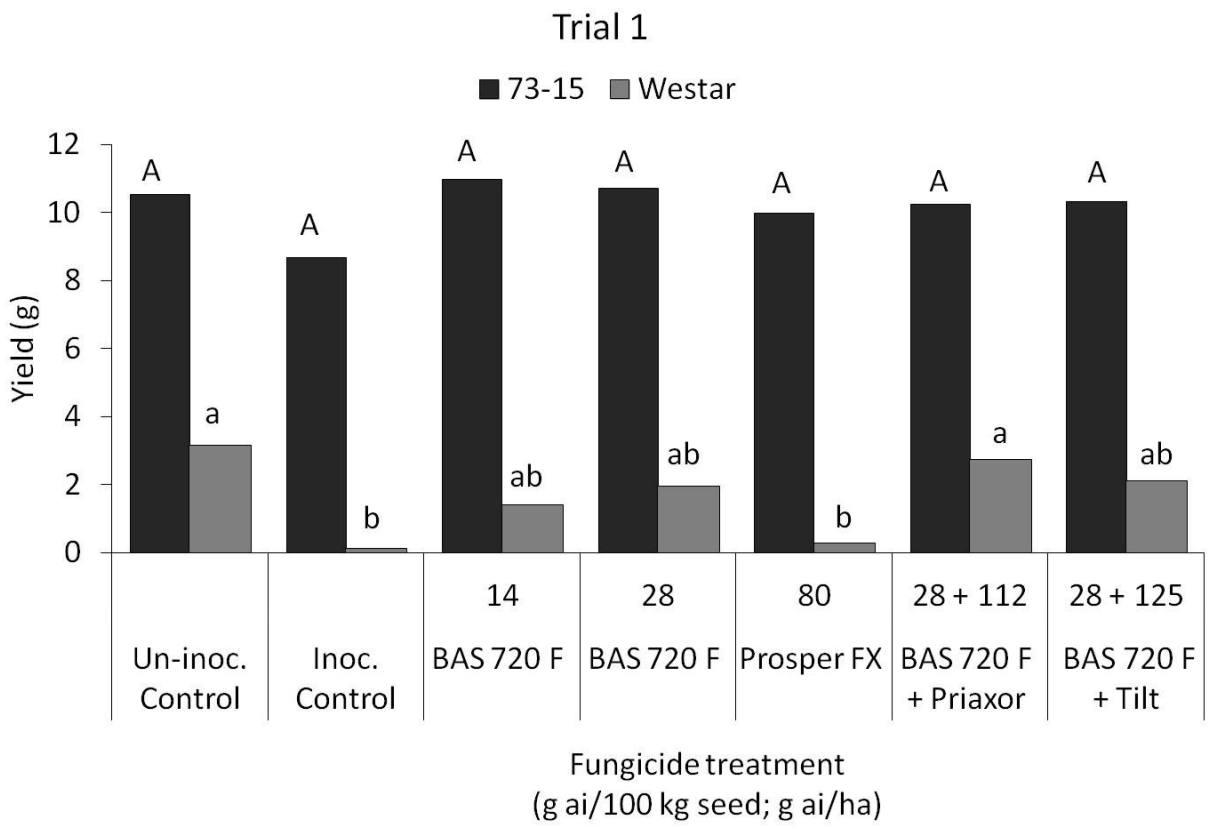

Figure A3.1 Effect of fungicide treatments on yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in Trial 1 under greenhouse conditions. Columns with the same lowercase letter indicate means (n = 8 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. A square-root transformation was applied to all yield data before the analysis. Un-transformed means are presented. Rates include fungicide components only.

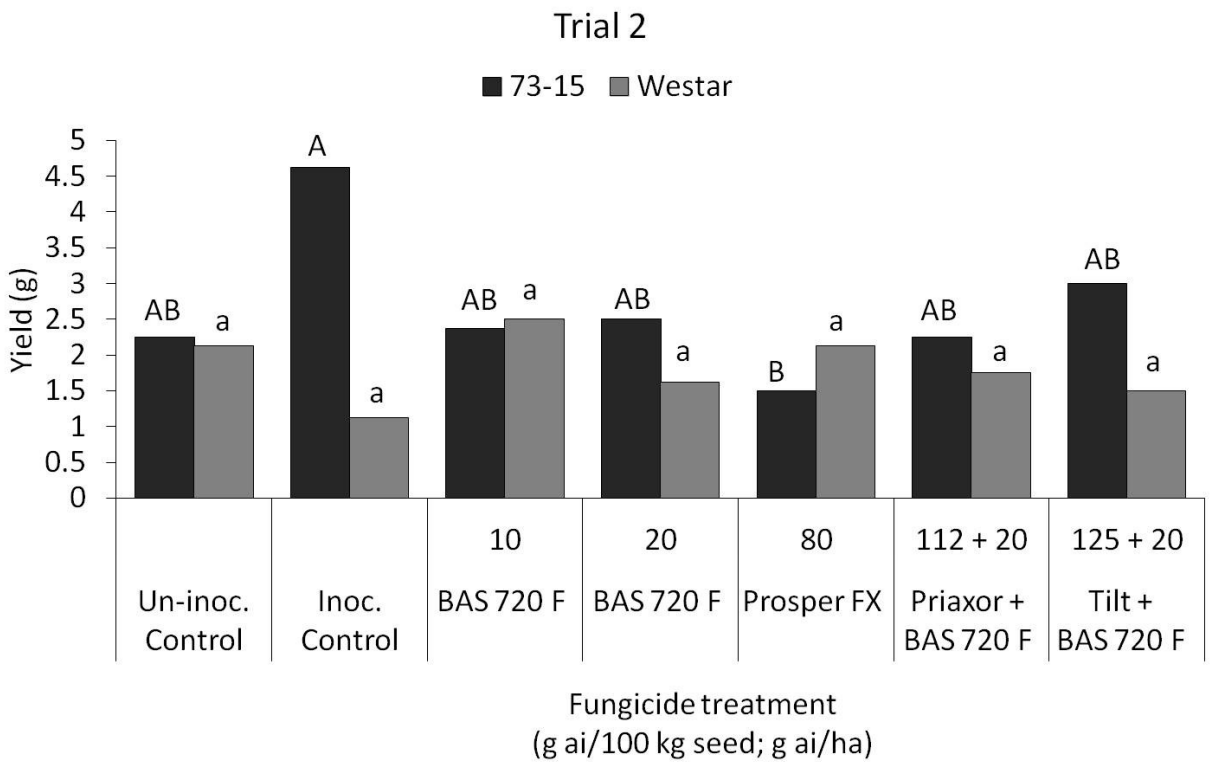

Figure A3.2 Effect of fungicide treatments on yield of two canola cultivars (73-15 and Westar) inoculated with *Leptosphaeria maculans* in Trial 2 under greenhouse conditions. Columns with the same lowercase letter indicate means (n = 4 replications) that do not differ significantly based on Tukey's Multiple Comparison Test at $P \leq 0.05$. A square-root transformation was applied to the data before the analysis. Un-transformed means are presented. Rates include fungicide components only.