Evaluation of Fungicides for Management of Blackleg Disease on Canola and QoI-fungicide Resistance in *Leptosphaeria maculans* in Western Canada

By

Chang Liu

A Thesis

Submitted to the Faculty of Graduate Studies of

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ABSTRACT

Liu, Chang. M.Sc., University of Manitoba, June 2014. Evaluation of Fungicides for Management of Blackleg Disease on Canola and QoI-fungicide Resistance of *Leptosphaeria maculans* in Western Canada. Major Professor: Dr. W.G.D. Fernando.

Blackleg (phoma stem canker), caused by the fungus *Leptosphaeria maculans* [(Desmaz.) Ces. & De Not.], is one of the most important diseases in canola crops worldwide. Severe epidemics often occur, which causes substantial losses of seed yields and quality. In western Canada, blackleg disease is controlled with integrated management practices that include use of resistant cultivars and four-year crop rotations. However, the variation of avirulence genes in the pathogen population and increasingly intensive crop rotation have led to the increase of pathogen inoculum, the development of virulent races, and the loss of cultivar resistance . Timely and efficient fungicide application is an important disease management strategy, but little is known about the efficacy of fungicides against the disease under western Canadian conditions and possible impact on fungicide sensitivity of *L. maculans* after repeated uses. . The present study was conducted to determine the efficacy of registered fungicides with different modes of action in controlling blackleg disease in canola.

The results of a three-year field study showed that fungicides generally reduced the blackleg incidence and severity on the susceptible canola cultivar Westar, and that the fungicides (pyraclostrobin and azoxystrobin) with the action mode of Quinone outside inhibitor (QoI) were more effective than the fungicides (propiconazole) of

Demethylation inhibitor (DMI). The fungicide treatment reduced the disease level on resistant and moderately resistant cultivars. Early fungicide application at about 2-4 leaf stage provided a small yield improvement on the susceptible cultivar but not on the resistant (45H29) – or moderately resistant (43E01) cultivars. The early application was more effective than a later application just prior to bolting but two applications (at the 2-4 leaf stage and prior to bolting) did not reduce the disease severity or yield further compared to one application at the early stage. .

Assessment of *L. maculans* isolates collected from field plots treated with QoI fungicides showed variation in sensitivity to QoI- fungicides. This baseline *in vitro* sensitivity may be used for fungicide resistance monitoring for a longer term. Typical point mutation was not found in any of these QoI-resistant *L. maculans* field isolates and polymorphism correlating with the QoI-resistant phenotype was not observed either in the sequence of cytochrome *b* gene among the *L. maculans* isolates.

This research highlights that an early fungicide application may provide a benefit against blackleg when cultivar resistance is lost or unavailable. Continued monitoring of *L. maculans* for fungicide sensitivity and identification of typical point mutations are warranted for risk assessment and early detection of fungicide resistant of *L. maculans* populations under field conditions. More in-depth research may be required to better understand the behaviors of those fungicides and their possible shift towards reduced sensitivity in *L. maculans* populations in order to establish an effective management strategy for optimized blackleg control in canola.

FOREWORD

This thesis is written in manuscript style. A general introduction and review of literature precedes manuscripts that comprised the main part of the thesis. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion. A general discussion and conclusions, a list of references cited and a list of appendices follow the manuscripts.

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CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 General introduction

Canola, also known as rapeseed, is cultivated largely throughout the world due to its use in agriculture (Daun, 2011; Gugel and Petrie, 1992). It is the second largest crop for production of oilseeds worldwide and accounts for 13% in total, next to soybeans (Source: U.S. Department of Agriculture (Anon., 2010)). In Canada, canola has been the largest crop since 2010 and grown mainly in Prairie Provinces of Manitoba, Saskatchewan and Alberta, with small amounts in British Columbia and Ontario (Canola Council of Canada -CCC, 2013). Because of the low levels of glucosinolates and erucic acids, and least amount of saturated fat, canola is widely used as a source of edible cooking oil and also as meal, and to a lesser extent, as biodiesel (CCC, 2013).

Phoma stem canker (blackleg) is one of the most economically important and destructive fungal diseases on canola worldwide, causing reduced yield and quality losses across canola growing regions in Europe, Australia and North America, except for China (West *et al.*, 2001; Howlett, 2004). The phytopathogenic fungus, *Leptosphaeria maculans* and *L. biglobosa*, were described as highly and weakly causative agents of blackleg. The proportions of two *Leptosphaeria* species vary within different regions leading to the differences in epidemiology of blackleg disease between continents (Mendes-Pereira *et al.*, 2003; Howlett, 2004; Williams and Fitt, 1999). The isolates of *L. maculans* were first reported in Manitoba in 1984 with low levels on oilseed debris (Platford, 1985). Currently, blackleg is often the most

prevalent disease across the Prairie Provinces and resistant cultivars and extended crop rotation have been recommended as key strategies for blackleg management in western Canada (Kutcher *et al.*, 2011; West *et al.*, 2001).

Deployment of genetic resistance in canola varieties against *L. maculans* is the most effective and least expensive management tool among the disease control strategies (Kutcher *et al.*, 2011; Fitt *et al.*, 2006). However, the major gene resistance could be broken down due to the change in populations of *L. maculans* under the strong selection pressure exerted (Liban *et al.*, 2012; Zhang *et al.*, 2012; Kutcher *et al.*, 2010; Rouxel *et al.*, 2003; Balesdent *et al.*, 2006). Cultural practices such as burning and burying of infested residues or deep plowing of stubbles have been used to reduce the risk of infection by ascospores and pycnidiospores (Turkington *et al.*, 2000; Barbetti and Khangura, 1999). A four-year crop rotation coupled with non-host crops could also decrease the disease incidence and severity by deprivation of the pathogen populations on host plants or limiting the pathogen inoculum to a low level (Guo *et al.*, 2005). Conversely, canola crops can be severely infected by blackleg disease if poor management strategies or practices are used, such as the use of susceptible varieties, short rotations (such as canola-wheat or canola-canola), no variety (or gene) rotations, poor volunteer control and weed management, lack of scouting and record-keeping of history of blackleg in the field, poor agronomic practices such as poor fertility.

Chemical control including seed treatment, application of fungicides to the soil, and foliar fungicide spray programs has been used to prevent disease epidemics and

reduce disease severity (West *et al.*, 2001). In western Canada, two classes of fungicides, Quinone outside inhibitor (QoI) and Demethylation inhibitor (DMI), are registered recently for blackleg control on canola (Kutcher *et al.*, 2011). However, both chemicals are considered to be at high risk for the pathogen to develop resistance due to their site-specific modes of action. Therefore, fungicide efficacy should be investigated further for application timing and multi-application strategies using different chemical groups to optimize disease control and reduce the risk for the pathogen to develop the resistance.

To assess the risk of developing reduced sensitivity to QoI fungicides, it is essential to establish the baseline sensitivity to fungicides and detect possible shifts of sensitivity in *L. maculans* populations. High frequencies of developing resistance to QoI fungicides have been reported in a wide variety of phytopathogenic fungi on many host crops worldwide (Ma and Michailides, 2005; Deising *et al.*, 2008). Several point mutations in the cytochrome *b* gene are known to confer resistance to strobilurin fungicides in plant pathogens, but the level of resistance varies with each amino acid change (Sierotzki *et al.*, 2000; Luo *et al.*, 2010). Therefore, understanding the molecular mechanisms of resistance to QoI fungicides in *L. maculans* is beneficial for developing rapid and efficient tools to identify resistant pathogen genotypes (Ma and Michailides, 2005; Young *et al.*, 2010; Torriani *et al.*, 2009).

In view of the above background, the major objectives of the present study were to evaluate the efficacies of fungicides with different modes of action against blackleg

disease on canola, to determine the effectiveness of disease control in relation with cultivar choice, application timing and fungicide intensity (multiple applications during the infection period), to establish a base-line sensitivity in the *L. maculans* population and detect possible shifts in the insensitivity, and to investigate the underlying molecular mechanisms of resistance to QoI fungicides in field populations of *L. maculans*.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Host

2.1.1 Introduction

Canola, also known as rapeseed, belongs to genus *Brassica* of *Brassicaceae* (or *Cruciferae*) family, which are grown widely throughout the world due to its morphologic diversity and use in agriculture (Daun, 2011). There are nearly 340 genera and about 3200 species in the *Brassicaceae* family. The genus *Brassica* in *Cruciferae* family encompasses nearly 100 species (Johnston *et al.*, 2005; CCC, 2013).

Canola or rapeseed is taxonomically related to several other *Brassica* spp. Crops including cabbage, kale, cauliflower and oriental mustard. Small round seeds mature in pods but the morphology, seed color and size, as well as chemical composition may vary (Daun, 2011; CCC, 2013). The relationship among several major *Brassica* species known as the "Triangle of U" was named after a Japanese scientist and is illustrated in Figure 2.1.

Canola is defined as *brassica* species having a seed oil that must contain less than 2% erucic acid, and the solid component of the seed must contain less than 30 μmol per gram of air-dry, oil-free solid of any one or a mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate (CCC, 2013). Due to low amount of saturated fat and low levels of gluconsinate, canola is usually used as the source of oil for human consumption and meal for animal feed, as well as for biodiesel (CCC, 2013). The oil

of rapeseed may be used as edible oils, fine chemicals, industrial fuels, and steam lubricants. Recently, canola oil has become a popular cooking oil and increasingly incorporated into food products because of its low level of saturated fats and zero trans-fat. The biodiesel produced from canola oil is used as an alternative low emission substitute fuel compared with the conventional fossil-based diesel fuel.

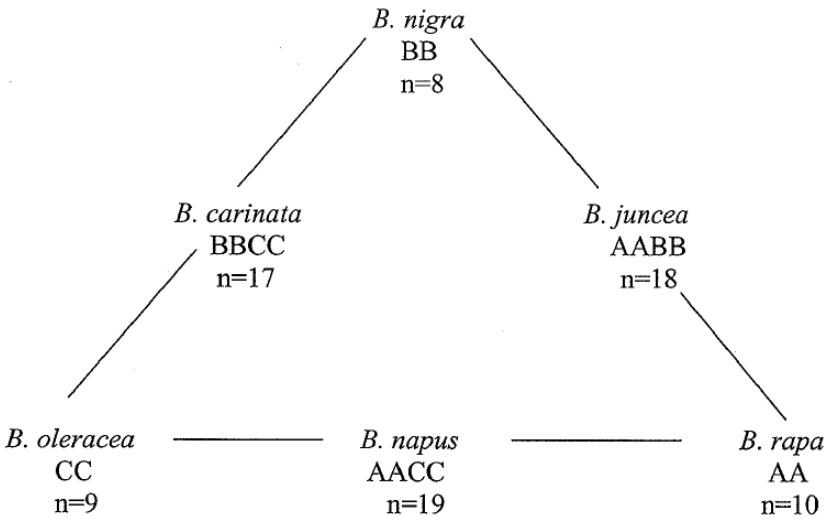

Figure 2.1 The "Triangle of U" showing the genetic relationship between the different brassica species (A, B, C =genome; n=chromosome number) (Savchuk, 2002).

2.1.2 Origin of Canola

The plants of *Brassica* species have been cultivated as food crops for thousands of years in forms of vegetables, oilseeds, feed and fodder, green manure, even condiments. The rapeseed crops were initially planted in India dating back to 2300

BC (Prakash, 1980), and then introduced into China and Japan 2000 years ago (CCC, 2013).

The name rape came from the Latin word ‘rapum’， which means turnip. The oil of *Brassica* crops was initially used for burning lamps and cooking oil (Gupta and Pratap, 2007). In the early middle Ages, crops of *B. rapa* was initially cultivated as turnip rape crop in Europe. In a later period of time, people used this crop to produce vegetable oils for both cooking and lighting because of the smokeless white flame it produces (Appelquist and Ohlson, 1972; CCC, 2013). The serious shortage of rapeseed oil due to the Second World War in Europe and Asia led to the increase of Canadian rape seed production. Due to the development of steam power, the rape oil was found to be an excellent lubricant for the steam engines of military and merchant ships (Gupta and Pratap, 2007).

In the early 1970s, major canola cultivars in western Canada were mostly *B. rapa*. Then *B. napus* was gradually established in the Prairie Provinces and both species accounted for most of the canola acreages in Canada. The growth cycle of canola consists of eight stages. However, the length of each stage is affected or depends on several factors including humidity, temperature, soil conditions and canola varieties (CCC, 2013).

The fatty acid profile of rapeseed oil is the key factor for use of the vegetable oil (Stringam *et al.*, 2003; CCC, 2013). The rapeseed oil contains several different types of fatty acids. Some acids such as linoleic are important to human health and could

not be synthesized by the human body; however, certain fatty acids such as erucic acids were reported to have negative effects for the human body in many studies during 1960s, especially associated with heart abnormalities (Downey an Harvey, 1963; Gupta and Pratap, 2007). In addition, the high content of glucosinolates in rapeseed meal raised another problem in the expansion of markets because the glucosinolates caused pungent odour and a biting taste for rapeseed meal (Krzymanski and Downey, 1969). Therefore, during 1960s and 1970s, plant breeders made efforts on high-quality rapeseed development through genetic modification using traditional plant breeding for both human diet and consumption of livestock (Shahidi, 1990).

In 1968, the first low erucic acid plant of *B. napus* was developed and in 1971 the first *B. campestris* (*B. rapa*) variety was developed. In 1967, the genetic material with low glucosinolate content was also identified in the *B. rapa* variety Bronowski (CCC, 2013). In 1974, Dr. Baldur Stefansson, a plant breeder at University of Manitoba developed the first ‘double low’ rapeseed variety Tower with low erucic acid and low glucosinolate. In 1977, the first ‘double low’ *B. rapa* variety, Candle, was developed by AAFC, Saskatoon (CCC, 2013). This greatly improved rapeseed meeting specific quality standards for human diet and livestock consumption and was considered to be the highly desirable rapeseed product of oil-yielding plants worldwide.

2.1.3 Economic importance of canola

Canola plants are cultivated widely throughout the world due to the advantages of adaptation of oilseed rape to extreme weather conditions (Oplinger *et al.*, 1989). At present, canola crops are grown in Europe, North America, Australia and Asia. However, depending on the natural conditions and plant varieties, the growth cycle and yield can differ substantially. For instance, farmers in Australia normally plant higher yielding winter crop in autumn and plants don't normally bolt until early spring and harvesting occurs during the summer. In contrast, canola in Canada is seeded in May and the crop matures at the end of August, approximately 90-100 days after planting.

Canola is the second largest crop for oilseeds production worldwide and accounts for 13% in total, next to soybeans (Source: Anon. U.S. Department of Agriculture, 2010). In Canada, canola contributes $19.3 billion to the Canadian economy each year from 2009 to 2013 and the crop is grown mainly in the prairie provinces Saskatchewan, Manitoba and Alberta, with small volumes in Ontario and British Columbia (CCC, 2013) (Figure 2.2).

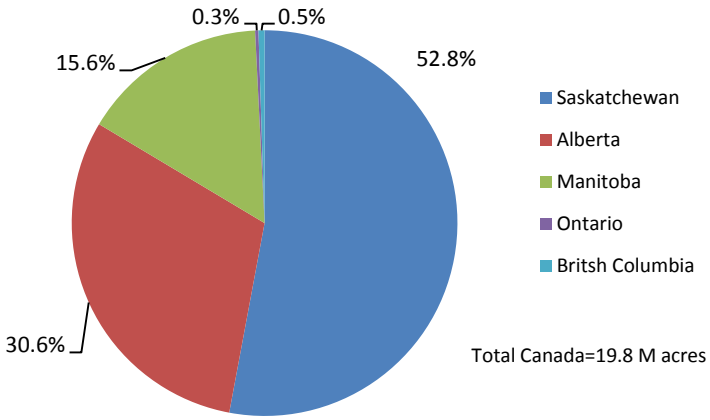

Figure 2.2 Harvest acres share of canola by provinces for 2013. Source: CANSIM Table 001-0010 Statistic Canada

Due to the development of plant breeding programs and the demand of international trade and domestic consumption, the canola seeded areas have reached 19.8 million acres in 2013 and increased by approximately 60% since 2003. In both Saskatchewan and Alberta, the canola seeded areas doubled during the last ten years, which account for the 83% of canola areas in total in Canada. However, the canola seeded areas in Manitoba, Ontario and British Columbia increased only slightly between the years 2003 and 2013 (CCC, 2013).

With the release of hybrid varieties and expansion of canola seeded areas, the production of canola have also increased significantly since 2003. The new canola hybrids were proven to be more robust than cross-pollenated varieties after experiences of drought and excessive moisture in 2010. The international commodity price and volume of output with a steady rate of increase also attributed to the large increase of canola production during the last decade. Canola yields also tripled during

the last ten years. Especially in Saskatchewan and Alberta, the yield tripled since 2003 and accounts for two thirds of production of canola in Canada. The yield of canola also doubled in Manitoba and British Columbia from the period spanning 2003–2013 (CCC, 2013). At present, Saskatchewan still remains the largest canola production province in Canada (Figure 2.3).

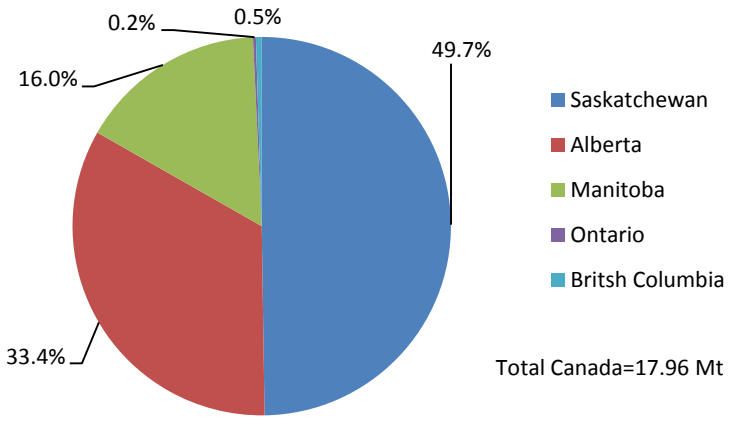

Figure 2.3 Canola production share of canola by provinces for 2013 (Source: CANSIM Table 001-0010 Statistic Canada)

Some canola-related industries such as oilseed processing and international canola products have also benefited from the expansion of canola-seeded areas and growing canola oil consumption domestically in Canada and throughout the world. Currently there are 13 modernized plants for oilseed processing existing in Canada and they receive and provide edible oil and protein meal with energy efficient and labor saving technology. During 2012 – 2013, the volume of canola seed crushing was 6.7 Mt with the annual capacity tripling during the past ten years. In 2013,

approximately 60% of canola production was for protein meal while oil production accounted for over 40% in total (CCC, 2013).

The United States is the largest importer of Canadian canola. In 2012, the export of canola oil to the United States, including both crude and refined, reached 1.4 Mt compared with a small decline in volume from 2011. China is the second largest consumer of Canadian canola oil. The export to China has sharply increased during the period of 2008 to 2013 and this export accounts for 38% of total canola trade. The United States is also the largest consumer of Canadian canola meal. Meal export reached 3.1 Mt and this account for approximately 90% of total Canadian meal production. During the last decade, canola meal production has tripled and reached 3.4 Mt in 2013 (CCC, 2013).

2.1.4 Main diseases on canola crops in western Canada

Several diseases are of concerns on canola crops in western Canada, with some occurring more often and severely.

1) Clubroot

Clubroot, caused by obligate parasite *Plasmodiophora brassicae*, is an economically important disease in cruciferous plants worldwide, and is becoming an emerging threat to canola production in western Canada in recent years (Peng *et al.*, 2011; Hwang *et al.*, 2012). The clubroot disease was first reported in the commercial

fields in Alberta in 2003 (Tewari *et al.*, 2005), since then the disease spread largely and infection by *P. brassicae* was detected in more than 560 fields in Alberta by 2010 (Strelkov *et al.*, 2011). Clubroot causes severe damage on canola crops and yield losses can be up to 90% in severely infected areas (Pageau *et al.*, 2006). The seed quality was also influenced by the *P. brassicae* infection in terms of oil content and 1000 seed weight (Hwang *et al.*, 2012). The symptoms of clubroot is characterized by the gall formation on infected roots, leading to the malfunction of water and nutrient uptake and thus wilting, stunting and premature ripening of the above-ground organs (Hwang *et al.*, 2012). The disease management strategies of clubroot include clubroot-resistant cultivars, crop rotations, use of amendment with lime to increase soil pH, whenever required, and application of fungicides (Gossen *et al.*, 2010; Donald and Porter, 2009).

2) Aster Yellows

The causal agent of Aster Yellows, *Candidatus Phytoplasma asteris*, can attack canola and other crops (Olivier and Galka, 2008). The symptom of Aster Yellow is plant stunting, leaf yellowing or purpling, and pods with small misshapen seeds (Bailey *et al.*, 2003). Although Aster Yellows was reported in western Canada since 1950s, the diseases has been considered to be of less economic importance, with overall incidences lower than 1% in most canola fields (Olivier and Galka, 2008). In 2007, an increased disease incidence up to 12% was reported in Saskatchewan with a provincial average of 2% (Olivier *et al.*, 2008). Currently, there is no resistant variety

to aster yellows available in the market and early scouting for leafhoppers in the field is important for aster yellows disease management.

3) *Sclerotinia* stem rot

Sclerotinia stem rot, incited by the pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, is a major threat to canola production in western Canada (Fernando *et al.*, 2007). The severe outbreaks of the stem rot can result in a yield loss up to 100% (Manitoba Agriculture, 2002). Recently, *Sclerotinia* stem rot is controlled mainly by the fungicide application and crop rotation due to the lack of commercially available resistant cultivars (del R ó *et al.*, 2007). Fungicide application during flowering stage of canola is a common method for stem rot control and has been reported to greatly reduce the disease epidemics and maintain crop yield (Bradley *et al.*, 2006; Kutcher and Wolf, 2006). In addition, biological control has been reported to be an alternative strategy for the control of *Sclerotinia* stem rot on Canola (Fernando *et al.*, 2007).

4) Blackleg

Blackleg (phoma stem canker), caused by the fungus *Leptosphaeria maculans*, is one of the most important diseases in canola crops worldwide (West *et al.*, 2001; Howlett, 2004). Severe epidemics often occur, which result in substantial losses of seed yields and quality in western Canada (Kutcher *et al.*, 2011; Fitt *et al.*, 2006). The main source of inoculum of *L. maculans* often arises from airborne ascospores produced in pseudothecia on the infested canola residues. Pycnidiospores are considered to be the secondary inoculum and spread by rain splash (Howlett *et al.*,

2001). During the last two decades, blackleg disease has been effectively controlled with integrated management practices that include the selection of blackleg resistant cultivars, adoption of crop rotations, and application of improved agronomic practices along with fungicide treatments (Kutcher *et al.*, 2011).

2.2 Blackleg Pathogens

2.2.1 Introduction

Blackleg (phoma stem canker) is one of the most destructive fungal diseases on canola worldwide, causing severe yield losses in canola-growing regions of Europe, North America and Australia (West *et al.*, 2001; Howlett, 2004). The disease is usually caused by a complex of two *Leptosphaeria* species, *L. maculans* and *L. biglobosa* (Howlett, 2004), but the proportions of two species may vary in different regions and result in differences of epidemics and severity of blackleg disease between continents (Fitt *et al.*, 2006).

Historically, the two *Leptosphaeria* species were described as highly or weakly virulent groups of a single *L. maculans* pathogen based on the pathogenicity to oilseed rape stems (Taylor *et al.*, 1991; Williams and Fitt, 1999; Mendes-Pereira *et al.*, 2003). They have also been divided into two groups termed as aggressive and nonaggressive (Hammond and Lewis, 1987), Tox1 and Tox0 (Balesdent *et al.*, 1992), or A and B groups (Johnson and Lewis, 1994). Two methods were previously used to identify the complex of *Leptosphaeria* species: based on the presence of a common phytotoxin, sirodesmin PL, in fungal culture filtratesusing RFLP technique which correlates the pigment production and pathogenicity of isolate with different molecular marker patterns (Koch *et al.*, 1991).

In 1990s, the causal agent of blackleg disease was considered to be only *Leptosphaeria maculans*. Based on the differences of reactions on canola cotyledons

of *B. napus* cultivars Quinta, Westar and Glacier, the pathogen was divided into different pathogenicity groups (PGs) (Koch *et al.*, 1991). PG1 was weakly virulent, causing less damage on oilseed rape plants, whereas PG2, PG3, PG4 and PGT were highly virulent on canola. Because crossing between single ascospores of A and B group isolates has not be successful, *L. biglobosa* was separated from the *L. maculans*. Both species can infect spring and winter canola, leading to seedling death, as well as lodging and basal stem canker under a wide range of weather conditions and agriculture practices, although the genotype, metabolite production and disease severity caused by the two species are different (Taylor *et al.*, 1991; Balesdent *et al.*, 1992; Johnson and Lewis, 1994).

2.2.2 Distribution of blackleg

The worldwide distribution of *L. maculans* and *L. biglobosa* may have arisen from the transmission in oilseed rape of *Brassica napus*, *B. rapa* or other *Brassica* crops (West *et al.*, 2001). The disease could have also been initiated by the release of airborne ascospores by wind or pycnidia spores by rainfall or rain splash within neighboring canola growing regions (Ghanbarnia *et al.*, 2009; Guo and Fernando, 2005). In western Canada, *L. biglobosa* on adult oilseed rape plants was first isolated in 1961 (Vanterpool, 1961; Petrie and Vanterpool, 1965). Since this fungus generally causes limited damage only on the upper stem of canola plants, it was not economically important in most years. *Leptosphaeria maculans* was first discovered

in Saskatchewan in 1975 with low levels on canola debris. From 1976 to 1981 the disease prevalence increased ten-fold in Saskatchewan and up to 50% yield losses were reported in some individual fields (Petrie, 1978; Gugel and Petrie, 1992). The occurrence of blackleg caused by *L. maculans* was first reported in Alberta in 1983 fields very close to the Saskatchewan border (Gugel and Petrie, 1992). *Leptosphaeria maculans* was first reported in Manitoba in 1984 (Platford, 1985).

Currently, blackleg is the most prevalent disease on canola across the Prairie Provinces. In 2011 and 2012, blackleg basal cankers were found in up to 70% of the crop surveyed in Manitoba with the disease incidence of approximately 9 to 16%. In China, only *L. biglobosa* has been found on oilseed rape crops. However, because highly susceptible rapeseed and vegetable *Brassica* cultivars are grown on large acres there, strict quarantine standards have been established to avoid the entrance of *L. maculans* (West *et al.*, 2001). In Europe, blackleg was first reported in 1950. The disease occurred in several rapeseed fields with disease incidence of 50-80% found near Pairs, France (Gugel and Petrie, 1992). In the following decades, blackleg spread quickly and by 1968 was present in most rapeseed growing regions in France with severe damage caused yearly. Blackleg disease also occurred on winter rapeseed in England during the late 1970s (Gladders and Musa, 1979). In Australia, a severe epidemic occurred in 1971 leading to a rapid decrease of acreage sown to rapeseed (West *et al.*, 2001). Although both *Leptosphaeria* species were present, the pathogen population consisted almost entirely of *L. maculans* (Plummer *et al.*, 1994).

2.2.3 Host range

Leptosphaeria maculans and *L. biglobosa* mainly attack *Brassica* crops including *B. rapa, B. napus, B. juncea, B. oleracea*, and other genera in the crucifer family (West *et al.*, 2001). In addition, some wild cruciferous weeds such as *Thlaspi spp., Sisynbrium spp., Descurainia spp., Capsella spp., Raphanus spp.,* and *Erusimum spp.* can also be infected by these fungal pathogens (Williams and Fitt, 1999).

2.2.4 Life cycle and epidemiology

The spread of blackleg disease generally starts with the release of airborne ascospores produced in pseudothecia on the infested residue (Hall, 1992; Mahuku *et al.,* 1997). Some cruciferous crops can also be the alternative host of fungus *L. maculans* and serve as the source of inoculum of blackleg disease (Figure 2.4). Ascospores are usually released after rainfall and coincide with the seedling period. Ghanbarnia *et al.* (2011) reported a high level of blackleg incidence and severity without the apparent presence of ascospores, demonstrating that pycnidiospores could also be a main inoculum source in western Canada. After spores land on the cotyledons or leaves of young crops, the pathogen normally invades the leaves via the wounds or stomatal pores (Chen and Howlett, 1996; Hammond *et al.*, 1985). The pathogen then produces hyphae in the intercellular spaces between mesophyll cells. During this stage, wetness is more important than temperature for infection (Hall, 1992). After the colonization of leaf tissues, the pathogen moves within the plant via

xylem vessels and forms lesions. Lesions close to the ground on the lower stem are often referred to as basal stem cankers or phoma stem cankers and are more associated with plant lodging, which often cause plant death and sever yield losses (Hammond *et al.*, 1985).

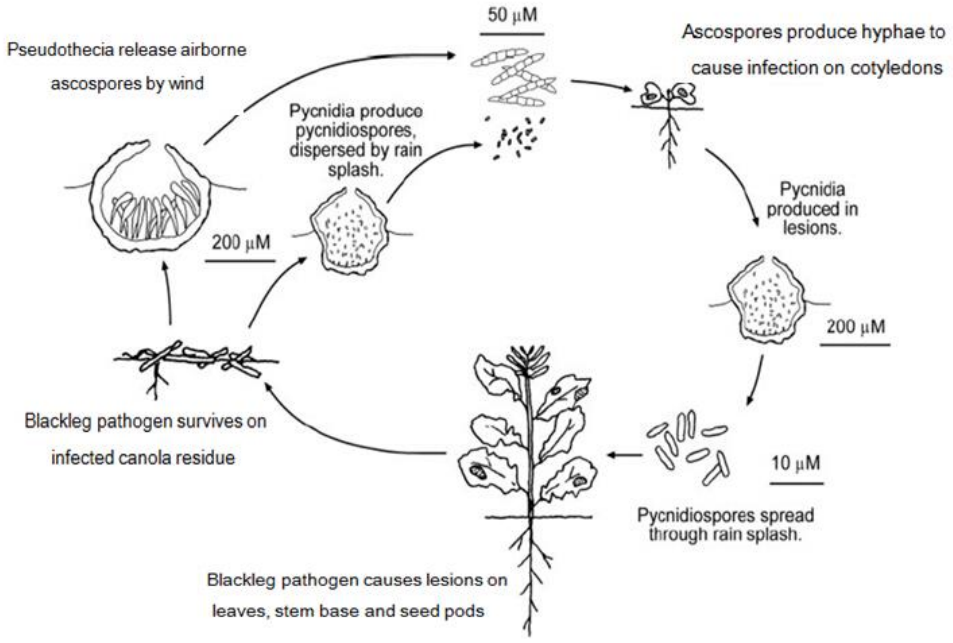

Figure 2.4 Life cycle of *Leptosphaeria maculans* on *Brassica napus* (Sources: Howlett *et al.*, 2001).

2.3 Blackleg control strategies

Blackleg disease management on canola may combine some or all of genetic, cultural, and chemical approaches based on disease epidemiology and economic importance of oilseed production in different regions. Best disease-management practices call for an integrated strategy using genetic resistance, proper agronomic

practice, four-year crop rotation and chemical treatment.

2.3.1 Genetic resistance

The development of resistant canola cultivars is the most effective and economical management tool against blackleg disease. Basically, there are two types of resistance: qualitative or major gene resistance and quantitative or adult plant resistance. Qualitative resistance was conferred by single dominant resistance genes and operates at the initial infection on cotyledons or leaves. This race-specific resistance may last several years under field conditions if the pathogen isolates harboring the corresponding avirulent gene are dominant in the pathogen population (Balesdent *et al.*, 2002; Rouxel *et al.*, 2003). Quantitative resistance is conferred by several genes with relatively smaller effects and its expression can be influenced by weather conditions. It normally operates at the adult plant stage when the pathogen infects the stem tissues (West *et al.*, 2001). Adult plant resistance is considered to be more durable than race-specific resistance and is a good resistance source for plant breeding programs (Delourme *et al.*, 2006).

In 1971, the first stem canker resistant rape cultivar, Major, which carried the resistance gene *Rlm4* was registered and widely used in Europe (Delourme *et al.*, 2006). Another resistant hybrid cultivar, Jet Neuf, which was introduced into the market in 1978, combined both specific (*Rlm4*) and quantitative resistance to *L. maculans* (Delourme *et al.*, 2006). It was grown widely across Europe during the

following decade and was an important source of resistance to plant breeding programs.

At present, the employment of cultivars with resistance to *L. maculans* has become the major strategy for blackleg control. Deployment of resistant genes in commercial canola varieties was successfully applied in western Canada and other oilseed rape growing regions worldwide. However, the major gene resistance could be broken down due to the change in populations of *L. maculans* under strong selection pressure exerted. For instance, cultivars with high levels of major gene resistance to stem canker were released in Australia in 1999 and showed few blackleg disease symptoms initially (Li and Cowling, 2003). However, in 2002, a serious epidemic of blackleg occurred on these cultivars in two individual canola growing fields (Li *et al.*, 2005). A survey in France found a rapid decrease for *L. maculans* isolates harboring *AvrLm1*in the pathogen population between 1997 (>83%) and 1999 (<13%) due to large-scale use of *Rlm1* cultivars (Rouxel *et al.,* 2003). Additionally, the large-scale cropping of resistant canola cultivars also led the farmers to shorten the crop rotation and reduce tillage, which resulted in the increase of disease inoculum pressure.

In order to use blackleg resistance effectively, understanding shifts of virulence in the *L. maculans* population is needed to provide better information for deployment of effective sources of resistance and to enhance the durability of resistant varieties. Several approaches have been developed to address the resistance durability issue, including the mixture of cultivars (Mundt, 2002), multiline employment (Kutcher *et*

al., 2011a), and spatio-temporal deployment of different resistances (Holt and Chancellor, 1999).

2.3.2 Cultural practice

Burning or burying of infested residues is recommended to decrease the risk of infection by primary inoculum from ascospores and pycnidiospores (Barbetti and Khangura, 1999). The 2 or 3 years old canola stubbles were usually considered to be the viable primary source for infection, especially under western Canadian conditions where the pathogen may survive longer on infested residues due to relatively slow decomposition of stubble (Kharbanda and Tewari, 1996; Kutcher *et al.*, 2011a). Deep plowing may speed up the decomposition process (Guo *et al.*, 2005; Blenis *et al.*, 1999), but the majority of crop field in western Canada use very little tillage.

The emergence of new races and long-distance dispersal of *L. maculans* ascospores could reduce the impact of a crop rotation strategy (Kharbanda and Tewari, 1996). A good buffer distance or isolation of new canola crops from adjacent fields with infested stubbles was recommended to alleviate the impact by the movement of ascospores. In Australia, the highest blackleg severity was observed in a canola field which was just adjacent to 6-month-old canola stubble, and the disease severity declined sharply within the distance of first 100 meters. An effective buffer distance of more than 100 m and preferably 500 m was suggested for the new canola sown (Marcroft *et al.*, 2004). In western Canada, the ascospores of *L. maculans* were

trapped within a distance of 25 m and pycnidiospores as secondary infection were detected up to 45 m from the inoculum source. Therefore, an appropriate buffer distance of at least 50 to 100 m was recommended for isolating a canola crop from the previous year's stubble (Guo and Fernando, 2005).

Altering the seeding date was considered as one of the approaches for disease control (Kharbanda and Tewari, 1996). Usually, canola plants at the 2- to 6-leaf stage are highly susceptible to infection (McGee and Petrie, 1979). Changing the sowing time could evade the high level of inoculum (ascospores) or favorable weather conditions for disease development at seedling stage (West *et al.*, 1999; Kharbanda and Tewari, 1996). For instance, in France the early crop establishment has been used as one of the strategies in managing blackleg infection because an early establishment of the crop allows canola plants sufficient number of leaves and canopy with better capacity in dealing with potential infection (West *et al.*, 1999). On the contrary, postponing the seeding date was recommended in western Canada to avoid the period when most ascospores are released coinciding with the sensitive seedling stage of canola (McGee and Emmett, 1977). However, changing seeding dates have not shown any effect on the reduction of blackleg in Canada due to the dissemination of ascospores throughout the whole growing season (Kharbanda and Tewari, 1996).

Crop rotation was considered one of the most important and effective tools of blackleg control strategies. A rotation with non-host crops could reduce disease incidence and severity by depriving of the pathogen population or limiting the

population to a low level when the host plants are seeded again. Field studies in Manitoba indicated that a 4-year crop rotation of canola with wheat, or with flax, under both conventional and zero till systems reduced the incidence and severity of blackleg (Guo *et al.,* 2005). The increased length of rotation from two to four years has been shown to lower blackleg incidence and severity significantly for both blackleg resistant and susceptible cultivars (Kutcher *et al.,* 2013).

2.3.3 Chemical control

Fungicide treatments through seed treatment, soil application and foliar sprays are employed world-wide depending on the disease severity and economic importance of canola or oilseed rape crops (West *et al.,* 2001). Seed treatment was first used in Canada in 1978 and applied even in some fields where blackleg was not found in seed lots (Guo and Fernando, 2005). Fungicides thiram and iprodione were applied in parts of Europe and iprodione was also recommended as the seed treatment in Australia (West *et al.,* 2001). Seed treatment could effectively limit the survival of seedborne inoculum but not protect the infection from ascospores coinciding with seedling emergence (CCC, 2013). In Europe where high yields are normally obtained, foliar fungicide sprays were often used in consideration of economic importance. Foliar sprays generally did not show any economic benefit for growers in western Canada (West *et al.,* 2001; Kutcher *et al.,* 2011a). Although some site-specific fungicides such as triazole or strobilurins were introduced in western Canada in the early 1990s, yield

increases in canola were only observed on susceptible varieties (Kutcher *et al.*, 2011b). For optimal application of foliar fungicides, an accurate forecasting system is needed to provide early warnings based on the correlation between infection and rainfall, temperature and peak release of ascospores.

2.4 Fungicides

2.4.1 The history of fungicides

Fungicides are either chemicals or biological agents that kill, repel or prevent the growth of fungi or fungal pathogens (Russell, 2005). Application of fungicides to protect plants from fungal attack is one of the major disease control strategies in agriculture. More than 200 years ago, Prevost described that bunt of wheat was caused by a fungus, *Tilletia caries* and could be controlled to some degree by copper sulphate (cited from Russell, 2005). With the increased availability of fungicides to growers, especially to small-scale farmers in developing countries, the use and impact of fungicides have increased significantly. Up to 1940, most users prepared their own fungicides according to basic recipes and high rates of fungicide (typically in the 10–20 a.i. kg ha^{-1} range) and there was generally a lack of awareness on the environmental impact. .

Proprietary products were also available at that time but were in their infancy. A number of new chemistry classes of fungicides were introduced between the 1940s to the 1970s, especially the use of the more active, less phytotoxic fungicides such as dithiocarbamates and phthalimides. Dithiocarbamates, the most widely used group of organic fungicides was introduced by Horsfall (Horsfall, 1975). The rise of the chemical crop protection industry occurred during 1940s and 1960s. The introduction of an antibiotic, blasticidin S, in 1955, specifically for rice blast control, is a notable addition to fungicides. During 1960-1970, a considerable expansion in chemical crop protection and rapid market growth was witnessed. After 1970, several important fungicide classes were introduced, including benzimidazole, morpholine, piperazines, lmidazoles, pyrimidines, triazoles, anilides and strobilurins.

For control of blackleg on canola, fungicides may be used as the last resort (Kutcher *et al.*, 2011a). When disease pressure is high and genetic resistance is inadequate, it is necessary to apply a foliar fungicide (Elliott *et al.*, 2011; Kutcher *et al.*, 2011a). It has been shown that a single fungicide application can be effective on canola varieties susceptible to blackleg.

However, the application of fungicide is not without problems. Health/safety and environmental issues of fungicide applications are of primary concerns with the public.

2.4.2 The classification of fungicide

There are several ways to classify fungicides, such as the fungicide's origin, mobility in the plant, role in protection of plants, breadth of activity, mode of action, chemical group, as well as classification by the Fungicide Resistance Action Committee (FRAC) group.

Fungicides can be classified into two major groups according to the origin of the fungicides: chemically based fungicides and biologically based fungicides (bio-fungicides). Chemically based fungicides are synthesized from organic and inorganic chemicals. Biologically based fungicides, on the other hand, are biological agents containing living microorganisms (bacteria, fungi) that are antagonistic to the pathogens that cause disease. Most of the fungicides used today are chemically based fungicides.

Fungicides can be classified as either contact fungicide or systemic fungicide, based on mobility in the plant. Contact fungicides remain on the surface of the plant after foliar application but do not go deeper. This type of fungicide requires repeated applications to protect new growth of the plant. Systemic fungicide is absorbed into plant tissue and can move upward, locally, or freely throughout the plant.

According to protection activities, fungicides can be assigned into three categories: preventative activity, early-infection activity and anti-sporulant activity (Mueller and Bradley, 2008). Preventative activity fungicide can be a protective barrier to prevent infection from occurring. Early-infection activity fungicide can

penetrate the plant and stop the pathogen in the plant tissues, usually most effective at early stage of the infection. Anti-sporulant activity fungicide can prevent spores from being produced. In this case, the amount of inoculum available to infect surrounding plants is reduced by way of reducing spore production (Mueller and Bradley, 2008).

Single-site fungicides and multi-site fungicides can be classified based on the number of infection sites. Single-site fungicides affect one single critical enzyme or protein necessary to the pathogenicity of the fungus or only one point/function in one of the metabolic pathways of a fungus. These fungicides often have systemic properties. Multi-site fungicides, on the other hand, target several different metabolic sites within the fungus.

2.4.3 Mode of action of fungicide

Mode of action is how a fungicide affects a target fungus, which is specific to the biochemical process of target affected by a fungicide. The fungicide can have impact on different organelles and biochemical processes of the fungus. They can damage the fungal cell membranes; inhibit the synthesis of lipids, sterol and other membrane components of fungi, or nucleic acid, amino acid and protein synthesis; inactivate critical enzymes or proteins. They can also block fungal signal transduction, respiration, mitosis and cell division. Fungicide can affect specific sites or multiple sites (Yang *et al.*, 2011). The mode of action of fungicides, fungicide chemical groups and common names can be found listed in Table2.1

2.4.4 FRAC code

An organization named Fungicide Resistance Action Committee (FRAC) was developed to address the issue of fungicide resistance. A code of numbers and letters has been made by this organization that can be used to distinguish the different groups based on the mode of action, known as the FRAC code.

Table 2.1 Fungicide Mode of Action Table (Sorted by: FRAC)

FRAC Group	Mode of Action	Chemical Family (Group)	Active Ingredients
1	Mitosis and cell division	benzimidazoles	thiabendazole
1		thiophanates	thiophanate-methyl
2	Respiration		iprodione vinclozolin
3	Sterol synthesis	imidazoles	Imazilil
3		piperazines	Triforine
3		pyrimidines	Fenarimol
3		triazoles	bitertanol cyproconazole difenoconazole fenbuconazole flusilazole ipconazole metconazole myclobutanil propiconazole prothioconazole tebuconazole tetraconazole triadimefon triadimenol triticonazole

4	Nucleic acid synthesis	acylalanines	metalaxyl metalaxyl-M (=mefenoxam)
7	Respiration		boscalid carboxin flutolanil
9	Protein synthesis		cyprodinil
11	Respiration	methoxyacrylates	azoxystrobin picoxystrobin
11		methoxycarbamates	pyraclostrobin
11		oximinoacetates	kresoxim-methyl trifloxystrobin
11		oxazolidinediones	famoxadone
11		dihydrodioxazines	fluoxastrobin
11		imidazolinones	fenamidone
12	Signaling		fludioxonil
13	Signaling		quinoxyfen
14	Lipids and membranes		chloroneb dicloran quintozene (PCNB)
14		1,2,4-thiadiazoles	etridiazole
17	Sterol synthesis		fenhexamid
19	Cell wall synthesis	peptidyl pyrimidine nucleoside	polyoxin
21	Respiration	cyanoimidazole	cyazofamid
22	Cell division		zoxamide
24	Protein synthesis		kasugamycin
25	Protein synthesis		streptomycin
27	Unknown		cymoxanil
28	Cell membrane permeability		propamocarb
29	Respiration	2,6-dinitro-anilines	fluazinam
30	Respiration	tri phenyl tin compounds	fentin hydroxide
33	Unknown	ethyl phosphonates	fosetyl-Al

33			phophorous acid and salts
40	Cell wall synthesis	cinnamic acid amides	dimethomorph
40		mandelic acid amides	mandipropamid
41	Protein synthesis		oxytetracycline
P	Host plant defense induction	benzo-thiadiazole BTH	acibenzolar-S-methyl
M	Multi-site contact activity	inorganic	copper
		inorganic	sulphur
		Dithiocarbamates andrelatives	ferbam mancozeb maeb metiram thiram ziram
		phthalimides	captan
		chloronitriles (phthalonitriles)	chlorothalonil
		guanidines	dodine
NC	Not classified	diverse	mineral oils, organic oils, potassium bicarbonate

2.5 Fungicide resistance

2.5.1 Introduction

The application of fungicide plays a major role in disease management strategies for a range of economically important crops, eEspecially when highly resistant cultivars are not available (i.e *Sclerotinia* in canola; *fusarium* head blight in cereals). In these cases, a fungicide treatment is considered the most effective approach for disease control. They are most commonly employed for the maintenance of healthy

crops and high-quality agricultural products by reducing pathogen population sizes and delaying the epidemics (Brent, 1995). Failure of disease control with fungicides may be caused by many factors such as insufficient rates, improper application timing or low fungicide efficacy, but the occurrence of fungicide resistance has been considered a major issue for reduced efficacy and limited lifetime with single-site fungicides. Before 1970s, nearly all fungicides available in agriculture were multi-site inhibitors which affected several different biochemical processes within the fungus, so rarely did fungicide resistance appear in spite of their intensive use (Ma and Michailides, 2005). However, since the registration and widespread use of systemic fungicides, also known as single-site fungicides in the late 1960s, fungicide resistance in pathogen populations became apparent and many cases of field resistance were reported with several plant diseases.

Fungicide resistance is an inheritable genetic adjustment by fungus resulting in reduced sensitivity to a fungicide and usually caused by various possible mechanisms. However, the most common resistance is conferred by single or multiple gene mutations associated with the target site of fungicide (Brent, 1995). Different mechanisms could confer the resistance to phytopathogenic fungus including; 1) a changing of target site which limits the binding of the fungicide; 2) the synthesis of alternative enzyme which substitute the target enzyme; 3) over expression of the target gene of a fungicide; 4) an active efflux or reduced uptake of the fungicide; and 5) a metabolic breakdown of the fungicide (Ma *et al.*, 2003; Gisi *et al.*, 2002).

In most cases, the presence of less sensitive or resistant strains was due to a low frequency of genetic mutation, and this event is less affected or not controlled by selection pressure exerted by the fungicide. Since the fungicide could effectively inhibit the increase of the sub-population of sensitive strains, resistant strains build up and subsequently dominate the pathogen population, therefore the failure to control disease finally occurs after a few years of selection pressure imposed by repeated use of fungicides (Gisi *et al.*, 2000). The fungicide-resistant isolates sometimes have less ecological fitness than sensitive strains, and in this case the resistant sub-population may decrease in frequency as long as fungicide applications are ceased. In most instances, however, the resistant isolates can fit as well as sensitive strains and survive for a long time in the fields even without fungicide use due to the selections of both resistance and high ecologic fitness in the same individuals (Baraldi *et al.*, 2003).

Many factors attribute to the build-up of resistance in the field, so it is difficult to accurately predict the rate and severity of fungicide resistance. The occurrence of fungicide resistance is mainly influenced by the fungicide type, mode of action, specific interaction of the chemicals with targets and potential mechanisms of resistance. Additionally, the properties of fungal pathogens, such as the number of life cycles, rate of natural mutation and sexual recombination type, are also important for the fungicide resistance process, with some of certain fungal species are thought to be more prone to the development of resistance than others. The agronomic practices may also affect the emergence of fungicide resistance. In the areas with intensive and exclusive fungicide treatments applied due to the high disease pressure, the frequency

of evolution for fungicide resistance is much higher than in areas of lower disease pressure . Therefore, to reduce the risk of selecting for fungicide resistance, properties of the fungicide, biology of the pathogen and cropping system should be taken into account for resistance risk assessment.

2.5.2 Demethylation inhibitors fungicide (DMIs)

The Demethylation inhibitors (DMI) fungicides inhibit the sterol C-14 a-demethylation of 24-methylene dihydrolanosterol (eburicol), which are the substrates for the cytochrome P450-dependent14-a demethylase in the biosynthesis of fungal sterols such as ergosterol (Brent, 1995; Gisi *et al.*, 2000). DMI fungicides were first introduced commercially in the early 1970s and comprise many effective chemical products. However, the development of resistance to DMI fungicide under practical conditions has occurred in a range of important phytopathogenic fungi since the 1980s (Delye *et al.*, 1998; Ma and Michailides, 2005).

Several single point mutations in the target gene (14-a demethylase gene, *CYP51 or ERG11*) conferred resistance to DMIs (Sanglard *et al.*, 1998). Single point mutation in the target gene results in low level of resistance whereas the combined amino acid (aa) alteration could increase the resistance factor at very high levels. Sanglard (1998) identified point mutations located at aa position 129,132,405 and 466 in the target gene of phytopathogenic fungus *Candida albicans* which was responsible for DMI resistance. The point mutation in the *CYP51* gene at codon position 136

leading to a substitution of phenylalaninefor tyrosine (Y136F) was also described in the field isolates of fungus *uncinul anecator* and *erysiphe graminis* (Delye *et al.*, 1998). The over expression level of *CYP51* gene was another important mechanism associated with the resistance to DMI fungicides. The increase of *CTP51* gene expression could rise from several different ways including an increase in copy number of the *CYP51* (Marichal, *et al.*, 1997) or unique 126 repeats in the promoter region of *CYP51* gene (Hamamoto, *et al.*, 2000).

2.5.3 Quinone outside inhibitor (QoI)

QoI fungicide is one of the most important classes of agricultural fungicides and derived from a group of natural fungicidal derivatives of *b*-methoxy-acrylic acid (Bartlett *et al.*, 2002). The QoIs that have protective and curative characteristics could control a broad spectrum of plant diseases via systemic action against a specific target site (Hirooka and Ishii, 2013). There are six chemical groups sharing a common mode of action classified within the Qo inhibitors based on the structural similarities and the cross resistance tested in different assays such as in-planta tests, spore germination, mycelia growth and cell-free enzyme tests, although their spectra and intrinsic levels of biological activity are quite different (Bartlett *et al.*, 2002). Based on the similarity of active ingredients, seven commercially available chemicals, including azoxystrobin, picoxystrobin, pyraclostrobin, kresoxim-methyl, trifloxystrobin, metominostrobin and dimoxystrobin, are classified as strobilurin fungicides (Fernández-Ortuño *et al.*, 2008).

Strobilurins are natural substances extracted mainly from mushrooms (*basidiomycetes*) and the name is derived from the mushrooms genera *Strobilurus* (Balba, 2007). Due to the chemical distinction from the strobilurins but in the same cross resistance group, famoxodone, fenamidone are also classified into QoI fungicide group (Bartlett *et al.*, 2002). QoIs fungicides were introduced into the market in 1996. Azoxystrobin and pyraclostrobin replaced kresoxim-methyl, and triflroxystrobin is currently among the top three QoI fungicides in the market (Hirooka *et al.*, 2013). World sales of strobilurin and related fungicides for crop use totaled approximately $9.91 billion in 2010 with an increase of 6.5% annually since 1999 (McDougall, 2011).

QoI fungicides are generally at high risk of developing resistance in phytopathogenic fungi and the resistance to QoIs was detected in many important plant pathogens shortly after their use (Bartlett *et al.*, 2002). Chemicals in this class have a single-site mode of action and they inhibit mitochondrial respiration of the fungus by binding to the Qo site of the cytochrome bc_1 enzyme complex (complex III), and thus interrupting electron transportation in the respiration pathway and leading to the deficiency of energy due to a lack of ATP (Bartlett *et al.*, 2002). The first case for the field resistance to strobilurins was reported in 1998 in northern Germany on wheat powdery mildew (*Blumeria graminis f. sp. tritici*) (Heaney, *et al.*, 2000). Since then, resistance to strobilurins has been reported in most pathogen species. Resistance to strobilurins was also detected only two years after application for controlling powdery mildew (*Podosphaera fusca*) and downy mildew (*Pseudoperonospora cubensis*) in Japan, even though many growers followed the recommendation for the fungicide

application (Ishii, *et al.*, 2001).

The cytochrome *bc1* enzyme is encoded by the cytochrome *b* (*cyt b*) gene located in the mitochondria. In most cases, resistance to QoI fungicides was conferred by at least eleven single or combined mutations with single nucleotide polymorphisms (SNPs) in two regions of cytochrome b (*cyt b*) gene causing single amino acid (aa) changes in the Qo centre at aa positions from 127 to 147 and from 275 to 296 in different organisms (Gisi *et al.*, 2000; Ma and Michailides, 2005). Similar to the cases of resistance to other site-specific fungicides, QoI-resistant isolates with mutations at different codons present different levels of resistance. For instance, single point mutations leading to an amino acid change from phenylalanine to leucine at codon 129 (F129L), or from glycine to alanine at codon 143 (G143A) of the *cyt b* gene can result in high resistance factors without any negative effects on enzyme activity and thus on fitness of individuals (Bartlett *et al.*, 2002). Some alternative mechanisms were also described to be responsible for QoI resistance. In addition, the activation of alternative oxidase (AOX) was also assumed to confer the resistance to field isolates of *M. graminicola* which were not controlled effectively with a low application rate of an azoxystrobin fungicide (Miguez, *et al*., 2004).

2.5.4 Detection and monitoring of fungicide resistance

The fungicide resistance can be detected based on bioassay measurements or the molecular genetic detection of mutations (Ma and Michailides, 2005). The

conventional methods refer to the pure isolation of the pathogen and subsequent plating on the medium amended with gradient concentrations of fungicide or inoculating plants treated with the fungicides, such procedures are usually labor-intensive and time-consuming and especially for the monitoring of fungicide resistance on large numbers of phytopathogen isolates (Ma and Michailides, 2005). Several PCR based molecular techniques have provided the possibility for rapid fungicide resistance detection once the mechanisms of fungicide insensitivity were elucidated at molecular levels (Fontaine *et al.*, 2009, Fernández-Ortuño *et al.*, 2008). Currently, PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR and real-time PCR have been employed successfully to detect genotypes of many phytopathogenic fungi and assess the risk of fungicide resistance under practical conditions (Sierotzki *et al.*, 2007; Banno *et al.*, 2009; Kim *et al.*, 2003).

1) Conventional PCR detection

PCR amplification is a revolutionized molecular technique for the rapid detection of fungicide-resistant isolates in plant pathogen populations. In one study on *Podosphaera fusca* powdery mildews, one primer set was designed to amplify the coding sequence spanning the Qo site (amino acid positions 120–280) based on published sequences of the mitochondrial *cyt b* gene of *P. fusca*, using this PCR method, the QoI-resistant isolates of *P. fusca* can be clearly differentiated from the

sensitive isolates within 4-5 hours (Fernández-Ortuño *et al.*, 2008). In *Blumeria graminis* associated with resistance to DMI fungicides, a primer combination which yielded a reliable read of approximately 700bp fragment of *CYP51* gene from all isolates of *B. graminis* was developed and DMI-resistant isolates correlated with specific point mutation in the *CYP51* gene was detected based on the analysis of DNA sequence data, resulting in an effective assay for monitoring DMI-resistant isolates of *B. graminis* (Wyand *et al*., 2005).

2) PCR-restriction fragment length polymorphism (PCR-RFLP)

PCR amplification followed by restriction enzyme analysis is another rapid and effective molecular technique to detect the point mutation which alters a restriction enzyme site (Ishii, *et al.*, 2001; Gisi *et al.*, 2002). In a case on azoxystrobin-based fungicide resistance on *Alternaria* late blight in some California pistachio orchards, resistant isolates had a point mutation at the amino acid position 143 in the cytochrome *b* gene, causing an amino acid change from glycine to alanine. Based on the sequences of two regions from *Alternaria* species which were identified as unique from published *cyt b* sequences of other fungal species using BLAST of NCBI/GenBank, a pair of PCR primers specific to *Alternaria spp.* was developed to amplify a 226-bp DNA fragment of the *cyt b* gene including the point mutation site from all three *Alternaria* species but not from 30 other fungal species frequently isolated from pistachio. The restriction endonuclease *Fnu*4HI recognized the sequence

GCTGC at codon 143 and 144 of the cyt b gene from resistant isolates only and digested the 226-bp PCR product at the position 177 from resistant isolates to produce two bands (177 and 49-bp) separately on agarose gels (Ma *et al.*, 2003). The PCR-RFLP approach has also been applied for detection of benzimidazole-resistant *Monilinia laxa* isolates and strobilurins-resistant *Erysiohe graminis f. sp. Tritici* field isolates within a few hours (Ma *et al.*, 2003; Sierotzki, *et al.*, 2000).

3) Allele-specific PCR

Allele-specific PCR (termed As-PCR) is another powerful and reliable tool for rapid detection of occurrence of pathogen strains resistant to fungicide. Usually, one of two PCR primers used in allele specific PCR is designed to generate target gene fragments by matching the desired allele and mismatching the other allele at the 3' end of primer (Ma and Michailides, 2005; Gisi *et al.*, 2002). In a study on resistance of *Penicillium digitatum* to azoxystrobin, the highly resistant UV mutants had a target-site mutation referred to as an amino acid change of glycine to alanine at position 143 in the mitochondrial cytochrome *b* (*cyt b*) gene, based on the point mutation at code 143 (from GGT to GCT), two modified forward primers cytbMF2 and cytbMF3 were designed to match the target site of the *cyt b* gene in azoxystrobin-resistant isolates only, using the primer pair cytbMF2/cytbR1, a 400-bp DNA fragment was consistently amplified from highly resistant isolates but not from any sensitive or low resistant isolates of *P. digitatum* isolates (Zhang *et al.*, 2009). A

multiplex AsPCR assay was also developed to detect the occurrence of fungicide resistance in *Plasmopara viticola* populations causing downy mildew of grapes, using this assay, a typical G143A mutation of the *P. viticola* in the *cyt b* gene, which conferred QoI fungicide resistance, and a G1105S substitution of the *P. viticola* in the cellulose synthase gene PvCesA3, which conferred CAA fungicide resistance, were detected simultaneously within two hours (Aoki *et al.*, 2013).

CHAPTER 3

3.0 EFFICACY OF QoI AND DMI FUNGICIDES AGAINST BLACKLEG OF CANOLA CAUSED BY *Leptosphaeria maculans*

Abstract

Blackleg, caused by the fungus *Leptosphaeria maculans*, is a serious disease on canola crops worldwide, and it causes significant losses in seed yield and quality. In western Canada, the variation of avirulence genes in the pathogen population and the trend to more intensive crop rotation systems have led to the increase of disease despite the fact that current cultivars are generally resistant to blackleg. Little is known about the effectiveness of using a combination of genetic resistance and fungicide strategies in controlling this disease on canola. In this study, we determined (i) the effectiveness of fungicides from different chemical groups (varying in the mode of action) against blackleg disease; and (ii) the efficacy of disease control in relation with cultivar choice, fungicide application timing, and fungicide intensity (multiple applications). Field experiments were conducted at Carman, Manitoba between 2011 and 2013. Each year, a total 12 treatments were arranged in a randomized, complete block design with four replicates. Averaged across all treatments, fungicide treatments reduced blackleg disease incidence by 42, 25 and 17% in 2011, 2012, and 2013, respectively, and decreased disease severity by 37, 40 and 36% in the three years, as compared to the unsprayed control. The QoI (pyraclostrobin and azoxystrobin) fungicides decreased disease severity more than DMI (propiconazole) fungicide on the susceptible cultivar 'Westar'. The application of pyraclostrobin at 2-4 leaf stage decreased disease severity by 61.5% and reduced disease incidence by 46.3% relative to the same fungicide applied prior to bolting. Significant yield increase with fungicide application was obtained only on Westar in 2013 when QoI (pyraclostrobin

and azoxystrobin) fungicide treatments increased seed yield by 185%, and a mix of propiconazole and azoxystrobin increased seed yield by 118% compared to the unsprayed check. QoI fungicide application at the 2-4 leaf stage followed by an application of DMI prior to bolting had the same positive effect on yield as a single QoI fungicide treatment at the 2-4 leaf stage on Westar. Moderately or highly resistant cultivars showed less response to the fungicide treatments; disease incidence and severity were reduced slightly but no yield benefit was observed.

3.1 Introduction

Blackleg disease (Phoma stem canker), caused by the fungus *Leptosphaeria maculans*, is one of the most economically important and devastating diseases on canola/rapeseed worldwide, including Australia (Marcroft *et al.*, 2002), the American continent (Dilmaghani *et al.*, 2009), and Europe (Stonard *et al.*, 2009). In western Canada, blackleg disease has been one of the major threats to canola production, especially in the 1970s and 1980s when disease-resistant cultivars were not readily available (Howlett, 2004). Up to 50% of yield losses were reported in some canola fields on the prairies in the 1980s (Gugel and Petrie, 1992). In Manitoba, blackleg disease was found in 77% of the canola fields surveyed provincially in 2012, with the disease prevalence up to 90% in the central Manitoba region (McLaren *et al.*, 2012).

The epidemics and severity of blackleg disease are associated with many factors, including race structure of the pathogen population, types of resistant cultivars, weather conditions, agronomic practices and the interactions among these factors (Delourme *et al.*, 2006; Petrie, 1978; West *et al.*, 2001). During the last two decades, the disease has been effectively controlled in western Canada by using blackleg resistant cultivars, adoption of 3- or 4-year crop rotations, and best agronomic practices coupled with fungicides (Kutcher *et al.*, 2013; Kutcher *et al.*, 2011). However, Change in frequencies of certain avirulence genes in the pathogen population has also been reported, consequently making the corresponding resistance genes ineffective against the pathogen population. In Australia, breakdowns of major

resistance genes have resulted in losses in previously resistant cultivars (Marcroft *et al.*, 2012). To mitigate the risk of resistance breakdown efforts in blackleg resistance breeding have been on going. Additionally, fungicides are considered another tool under the threat of widespread resistance erosion (Ma and Michailides, 2005).

Fungicides may be applied as a seed treatment, into the soil or to the foliage for blackleg control on canola (West *et al.*, 2001). In western Canada, two groups of fungicides, Quinone outside inhibitors (QoI) and Demethylation inhibitors (DMI), are registered to control blackleg disease on canola (Kutcher *et al.*, 2011a). QoI fungicides block mitochondrial respiration by binding to the cytochrome *bc1* enzyme complex at the Qo site, which causes the depletion of adenosine triphosphate (ATP) and inhibits spore germination as a result of an energy deficiency (Bartlett *et al.*, 2002). DMI fungicides inhibit demethylation at position 14 of lanosterol or 24-methylene dihydrolanosterol, which are the precursors of sterols in fungi (Gisi *et al.*, 2000). However, strobilurin fungicides are considered to be at high risk for plant pathogens to develop resistance because of their site-specific modes of action. Thereby, a combination of fungicide products from different chemical groups may reduce the risk for fungicide resistance to develop. The timing for fungicide application was not well understood; although ascospores of *L. maculans* can be released throughout most of the growing season (Kharbanda and Tewari, 1996), early infection that is more related to the basal stem canker later on can be most detrimental to canola yield, Therefore, an earlier fungicide application may protect the crop more effectively against the disease than a later application. .

The objectives of this study were to (i) evaluate the effectiveness of fungicides from different chemical groups (varying in the mode of action) against blackleg disease on canola; (ii) determine the efficacy of disease control in relation with the level of cultivar resistance, fungicide application timing and fungicide intensity (multiple applications at the infection period); and (iii) detect possible variation and changes in the sensitivity to QoI and DMI fungicides in the *L. maculans* population.

3.2 Materials and methods

3.2.1 Fungal inoculum

In 2010, ten strains of *L. maculans* were collected from canola fields infected by blackleg pathogen in Carman, MB, and these strains were used as the source of inoculum for the 2011 field experiment. Similarly, ten isolates used in the 2012 field experiment were collected from a diseased canola field in Carman in 2011. In the 2013 growing season, however, the canola plants were infected by natural inoculum from canola stubble in adjacent fields. After two years of canola and high levels of infection, the experimental site had a sufficient amount of inoculum for infection in 2013. In each year, these isolates were confirmed as *L. maculans* on the basis of their colony morphology (West *et al.*, 2002) coupled with multiplex PCR determination (LmacF: 5'- C T T G C C C A C C A A T T G G A T C C C C T A - 3'; LmacR: 5'- G C A A A A T G T G CT G C G C T C CA G G - 3'; LbigAll2: 5'- C T A T C A G A G G A T T G G T G T C A G G - 3'; provided by Canadian Food Inspection Agency).

3.2.2 Inoculation

L. maculans pycnidia were produced on V8 juice (Campbell Soup Company, Toronto, ON) agar amended with streptomycin sulfate at 0.1 g liter^{-1}. After 12 days of incubation at room temperature (22-24℃), pycnidiospores were harvested by flooding the fungal cultures with distilled sterilized water, filtered into 50-ml sterile centrifuge tubes (Fisher Scientific, Pittsburgh, PA), and counted using a hemocytometer (Hausser Scientific Company, Horsham, PA). The tubes were kept on ice during transportation to the field site. The spore suspension was diluted to 1 $\times 10^7$ spores ml^{-1} in distilled water. Tween 20 was added into spore suspensions at the ratio of 1ml Tween per L of spore suspension. In the field, inoculation was performed by using a CO_2 powered back-pack sprayer at 2- to 4-leaf stage of canola plants.

3.2.3 Fungicides

In the study, we tested four commercially registered fungicides - Headline® (pyraclostrobin, BASF Canada Inc., Mississauga, ON), and three other products - Quadris® (azoxystrobin), Tilt® (Propiconazole,) and Quilt® (propiconazole + azoxystrobin) were from Syngenta Canada Inc. (Guelph, ON). Details on those fungicides and their application rates are summarized in Table 3.1.

Table 3.1 Information on the fungicide used in field experiments at Carmon, Manitoba (2011-2013)

Fungicide trade name	Common name	Active ingredient (a.i.) concentration (g L^{-1})	Rate of application (recommended)
Headline EC	Pyraclostrobin	250	250 g L^{-1}
Quadric	Azoxystrobin	250	494 ml ha^{-1}
Tilt	Propiconazole	250	494 ml ha^{-1}
Quilt	Azoxystrobin + Propiconazole	75 Azoxystrobin +125 Propiconazole	1.0 L ha^{-1}

3.2.4 Experimental design

Three canola (*Brassica napus L.*) cultivars 'Westar' (highly susceptible (S) to blackleg disease), '43E01' (moderately resistant (MR)) and '45H29' (resistant (R)), were used in the study. The experiments were carried out at the Ian Morrison Research Station of the University of Manitoba, Carman, Manitoba, in 2011, 2012, and 2013. In each year, a total of 12 treatments were arranged in a randomized complete block design with four replicates (Table 3.2). Plot size was 2 m by 8 m. Seeding rates were 9.8 kg ha^{-1} for 'Westar', 8.1kg ha^{-1} for '43E01' and 7.2 kg ha^{-1} for '45H29'; these seeding rates were based on the seed size, germination rates and an

estimated field emergence rate, to target a plant stand of 100 plants m^{-2}.

Table 3.2 Treatment structure of the field experiment conducted at Carmon, Manitoba, 2011-2013.

Treatment number	Cultivar and resistance	Fungicide	Application timing
1	45H29 (R)	Control	
2	45H29 (R)	Pyraclostrobin	2-4 leaf stage
3	43E01 (MR)	Control	
4	43E01 (MR)	Pyraclostrobin	2-4 leaf stage
5	Westar (S)	Control	
6	Westar (S)	Pyraclostrobin	2-4 leaf stage
7	Westar (S)	Azoxystrobin	2-4 leaf stage
8	Westar (S)	Propiconazole	2-4 leaf stage
9	Westar (S)	Pyraclostrobin	prior to bolting
10	Westar (S)	1)Propiconazole 2)Pyraclostrobin	2-4 leaf stage prior to bolting
11	Westar (S)	1)Pyraclostrobin 2)Propiconazole	2-4 leaf stage prior to bolting
12	Westar (S)	Mix of propiconazole and azoxystrobin	2-4 leaf stage

Each year, the inoculum was applied initially at the cotyledon stage and a second inoculation carried out five days later. In 2013, no artificial inoculation was applied because the plots contained sufficient amounts of remnant canola stubble pieces infected by blackleg disease from the previous years. The fungicide application was performed at the 2-4 leaf stage using a CO_2 powered back-pack sprayer equipped with a six nozzle boom at 20 cm spacing between nozzles. Ten days after the first fungicide application or near the canola bolting stage, plots designed for the different application timings were sprayed, using the similar spraying method described above. The check plots were sprayed with distilled water.

3.2.5 Disease and yield evaluation

Blackleg disease incidence and severity were assessed at the crop growth stage of 5.2 to 5.3 according to the scale of Harper and Berkenkemp (1975). Plants in a l m x l m quadrat in the center of each plot were uprooted and the rating scale recommended by Western Canada Canola and Rapeseed Recommending Committee (WCC/RRC) was used to evaluate disease severity. Each plant was cut through the stem base and the area of diseased tissues in the cross-section was estimated. Disease incidence was expressed as the percentage of the number of infected plants divided by the total number of plants sampled. The disease severity was evaluated with a 0 to 5 scale in which 0 = no infection, 1 = blackened area less than 25% of the cross-section of the crown, 2 = 25 to 50%, 3 = 51 to 75%, 4 = 76 to 100% girdled with weak stem,

and 5= tissue dry and brittle, and plant dead (WCC/RRC, 2009). At maturity, an area of 1 x 1 m within each plot was harvested using a plot combine for seed yield.

Weather data were obtained from an Environment Canada weather station near the experimental site (Table 3.3).

Table 3.3 Growing season mean temperatures and precipitation for 2011-2013 at the Ian Morrison Research Station, Carman, Manitoba.

	Mean air temperature (℃)					**Precipitation (mm)**				
Year	May	June	July	Aug	Mean	May	June	July	Aug	Total
2011	10.2	16.7	20.3	19.3	16.6	72.0	59.2	37.6	12.2	181.0
2012	12.2	17.7	21.9	19.0	17.7	60.5	86.2	27.8	47.2	221.7
2013	10.4	17.7	18.6	18.7	16.4	111.0	50.6	49.0	59.4	270.0

Source: National Climate Data and Information Archive, Environment Canada.

3.2.6. Statistical analysis

Analysis of variance (ANOVA) for blackleg disease incidence, disease severity and seed yield was performed using the "PROC MIXED" procedure of the SAS software (SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). An overall analysis for all the 12 treatments across the three growing seasons was performed for each variable separately. In these analyses, cultivars and fungicides were considered as fixed effect; replicate, years, and their interactions with cultivar and fungicide as

random effect. The model statement used in the analysis for the comparison of fungicide efficacy on susceptible cultivar was ‘variables=fungicide year fungicide*year’. The model statement used in the analysis for the comparison of fungicide efficacy on different cultivars was ‘variables=fungicide cultivar year fungicide*cultivar fungicide*year cultivar*year fungicide*cultivar*year’. The model statement used in the analysis for the comparison of efficacy of fungicide with different application timing was ‘variables=apptiming year apptiming*year’. Initial analyses revealed significant treatment by year interactions for most of the variables evaluated; this suggested that treatment effects differed significantly from year to year. Therefore, a separate ANOVA was performed to determine the treatment effects for each year, and thus, the results were presented primarily by each year.

In order to evaluate the efficacy of fungicide on blackleg variables and their relation with fungicide application timing and cultivar choices, more detailed analysis was performed using the SAS Mixed model to determine the significant differences between the four fungicides and the unsprayed check, between the susceptible, moderately-resistant and resistant cultivars, and between application timing for each year.

3.3 Results

There were significant fungicide treatment by year interactions for disease incidence and seed yield (Table 3.4), suggesting that efficacy for fungicide treatments on these two variables varies from year to year. In contrast, the fungicide treatment by year interaction for the disease severity was not significantly different, suggesting that the effect of fungicide application on blackleg severity can be constant regardless of environmental conditions encountered in different years. Furthermore, both year and treatment had significant effects on all the variables evaluated, showing that the blackleg disease severity and incidence, and canola seed yield can differ with fungicide application, and the magnitude of the differences varies with years. Based on the ANOVA results, the effects of treatment (cultivars, fungicides, application timing) are presented and discussed separately for each year in the following paragraphs. The results relevant to blackleg severity are summarized in Table 3.5 (A, B, C), disease incidence in Table 3.6 (A, B, C) and seed yield in Figure 3.1 (A, B, C).

Table 3.4 Summary of ANOVA and the significance of the effects on blackleg disease incidence, severity and seed yield for canola at Carmon, Manitoba, 2011-2013.

Source of Variation	Df	Mean square values		
		Disease Severity	**Disease Incidence (%)**	**Seed Yield (kg ha^{-1})**
Treatment	11	5.184**[1]	0.269**	9297256**
Year	2	9.789**	1.472**	4939194**
Treatment*Year	22	0.392ns[1]	0.030**	1057720**
Rep	3	0.257ns	0.026ns	891231*
Residual	105	0.307	0.014	306669
Fungicide	4	7.474**	0.390**	1910969**
Fungicide*Year	8	0.483ns	0.032**	473287*
Cultivar	2	1.449**	0.013ns	31225807**
Cultivar*Year	4	0.442ns	0.005ns	1993228**
Cultivar*Fungicide	2	1.078*	0.050*	1339641*
Cultivar*Fungicide *Year	4	0.279ns	0.019ns	701653ns
Application Timing	4	6.229**	0.346**	1698317**
Application Timing*Year	8	0.183ns	0.020ns	435041ns

[1] Significant at $P \leq 0.05$; ** Significant at $P \leq 0.01$; ns= not significant.

3.3.1 Blackleg disease severity

The ANOVA revealed that year, cultivar, fungicide, application timing, and cultivar by fungicide interaction all had significant effects on disease severity, with no significant interaction for fungicide by year, cultivar by year or application timing by year in the two-way ANOVA or the three-way interaction of cultivar by fungicide by year (Table 3.4). Averaged across all fungicide treatments, canola had significantly lower disease severity in 2011 (1.17) as compared to 2012 (1.93) and 2013 (1.98).

Among the four fungicides applied to the susceptible cultivar Westar, QoI (pyraclostrobin and azoxystrobin) fungicide treatments resulted in significantly lower disease severity in each of the three years (averaging 0.49, 1.26 and 1.36) as compared to the unsprayed control (1.83, 2.92 and 2.95 respectively) (Table 3.5A). However, no significant difference in disease severity was observed between the mix of azoxystrobin with propiconazole fungicide and the unsprayed check in 2011, or between propiconazole and unsprayed checks in all years.

Each year, pyraclostrobin was applied to each of the three cultivars - S (Westar), MR (43E01), and R (45H29) at the 2-4 leaf stage, and it showed a highly significant fungicide effect and also cultivar by fungicide interaction in affecting disease severity (Table 3.4). Pyraclostrobin application decreased disease severity significantly in all three years as compared to unsprayed checks on each of the three cultivars (Table 3.5B). The lowest blackleg disease severity (0.35) was obtained on the R cultivar 45H29 sprayed with pyraclostrobin. As expected, the blackleg severity across was

significantly higher for Westar compared to the corresponding treatments on moderately resistant (43E01) and resistant (45H29) cultivars (Table 3.5B). There were a few cases in which no significant differences were observed between the pyraclostrobin treatment and unsprayed checks on the resistant (45H29) cultivar in 2012 or in 2013 (Table 3.5B)

Timing of foliar fungicide application had an inconsistent effect on disease severity between the study years (Table 3.5C). In 2011, the application of pyraclostrobin at the 2-4 leaf stage significantly decreased disease severity compared to the application made just prior to bolding stage of Westar, but in 2012 and 2013, this timing of application did not reduce disease severity significantly relatively to controls. Also, multi-applications (i.e., pyraclostrobin at the 2-4 leaf stage plus propiconazole prior to bolting) significantly decreased disease severity compared to the unsprayed check, but the efficacy was not significantly different from that of pyraclostrobin just prior to bolting.

Table 3.5A Mean values of blackleg disease severity on the susceptible cultivar 'Westar' in response to different fungicides applied at the 2-4 leaf stage in 2011-2013.

Fungicide	Disease Severity		
	2011	2012	2013
Control	1.83 ab[1]	2.92 a	2.95 a
Pyraclostrobin	0.37 c	0.93 c	1.47 bc
Azoxystrobin	0.60 c	1.59 bc	1.25 c
Propiconazole	2.69 a	2.33 ab	2.89 a
Mix of azoxy+ propi	0.98 bc	2.14 b	1.91 b

[1]Values followed by the same letter in a column are not significantly different based on the LSD test at $P<0.05$.

Table 3.5B Mean values of blackleg disease severity on the different cultivars in response to pyraclostrobin applied at the 2-4 leaf stage in comparison with the non-treated control in 2011-2013

Cultivar	Fungicide Treatment	Disease Severity		
		2011	2012	2013
45H29(R)	Control	1.71 a[1]	1.81 bc	1.53 b
45H29(R)	Pyraclostrobin	0.35 b	1.16 c	1.17 b
43E01(MR)	Control	1.77 a	2.68 ab	2.46 a
43E01(MR)	Pyraclostrobin	0.42 b	1.55 c	1.16 b
Westar (S)	Control	1.83 a	2.92 a	2.95 a
Westar (S)	Pyraclostrobin	0.37 b	0.93 c	1.47 b

[1]Values followed by the same letter in a column are not significantly different based on the LSD test at $P<0.05$.

Table 3.5C Mean values of blackleg disease severity on the susceptible cultivar 'Westar' in response to the timing of fungicide application and multi-applications in 2011-2013

Fungicide	Application Timing	Disease Severity		
		2011	2012	2013
Control	2-4 leaf	1.83 a[1]	2.92 a	2.95 a
Pyraclostrobin	2-4 leaf	0.37 d	0.93 b	1.47 b
Pyraclostrobin	Prior to bolting	1.55 ab	2.26 a	2.90 a
Propiconazole	2-4 leaf	1.16 bc	2.57 a	2.53 a
Pyraclostrobin	Prior to bolting			
Pyraclostrobin	2-4 leaf	0.65 cd	1.18 b	1.54 b
Propiconazole	Prior to bolting			

[1] Values followed by the same letter in a column are not significantly different based on LSD test at $P<0.05$.

3.3.2 Blackleg disease incidence

During the 2011-2013 growing seasons, the year factor had a significant effect on blackleg incidence and a significant difference was also found in the two-way interaction of fungicide by year (Table 3.4). On average, the mean disease incidence was lower in 2011 than that in 2012 and 2013. However, there was no significant difference in cultivar by year, or application timing by year, or the three way

interaction of cultivar by fungicide by year, in affecting disease incidence.

In 2011 and 2013, the mix of propiconazole and azoxystrobin resulted in the lowest disease incidence, while in 2012, the lowest disease incidence was obtained on the canola crop treated with pyraclostrobin alone (Table 3.6A). The significant fungicide by year interaction in affecting disease incidence was also reflected on the control efficacy of different fungicides applied in different years. In 2012, spray of pyraclostrobin alone significantly decreased disease incidence than the spray of azoxystrobin alone, but in the other two study years, these two fungicide treatments resulted in an equal level of disease incidence.

Comparing all fungicide treatments applied in the field experiment, the lowest mean blackleg incidence was observed in the R cultivar (45H29) with pyraclostrobin applied at the 2-4 leaf stage treatment (averaging at 17%). QoI (pyraclostrobin) fungicide had a significant effect on all three cultivars, with the blackleg incidence across all fungicide treatments being higher in Westar than in the moderately resistant (43E01) and resistant (45H29) cultivars (Table 3.6B).

There was a significant difference between 2-4 leaf stage application timing and unsprayed check, and significant difference was also found in both multi-application treatments compared with unsprayed check (Table 3.6C). Fungicide application prior to bolting stage did not show a significant effect on blackleg incidence as compared to the application at the 2-4 leaf stage.

Unlike 2011 and 2013, only QoI (pyraclostrobin and azoxystrobin) treatments

were significantly different from the unsprayed control in disease incidence and no significant difference was observed between the mixture treatments and unsprayed check in 2012 (Table 3.6A). In 2012 and 2013, similar to 2011, the QoI (pyraclostrobin) treatment had a significant effect on highly susceptible cultivar Westar and the moderately resistant cultivar (43E01), but not on the resistant (45H29) cultivar (Table 3.6B). In 2012, comparing all treatments, the lowest mean blackleg incidence was given by the S cultivar (Westar) - pyraclostrobin- application at the 2-4 leaf stage interaction (42%) and highest was S cultivar (Westar) – unsprayed control interaction (86%) (Table 3.6A, Table 3.6B). In 2013, the lowest mean blackleg incidence was observed in the highly susceptible cultivar (Westar) - azoxystrobin-application at the 2-4 leaf stage interaction (63%) and highest disease incidence was observed in the moderately resistant cultivar (43E01) – unsprayed control interaction (95%) (Table 3.6A, Table 3.6B).

Table 3.6A Mean values of blackleg incidence on the susceptible cultivar 'Westar' in response to different fungicides applied at the 2-4 leaf stage, in 2011-2013.

Fungicide	Disease Incidence (%)		
	2011	2012	2013
Control	69.97 a[1]	85.95 a	89.66 a
Pyraclostrobin	18.00 c	41.83 c	66.26 c
Azoxystrobin	27.50 bc	63.65 b	63.18 c
Propiconazole	77.20 a	78.67 a	91.50 a
Mix of azoxy + propi	40.75 b	73.05 ab	78.14 b

[1] Values followed by the same letter in a column are not significantly different based on the LSD test at $P<0.05$.

Table 3.6B Mean values of blackleg disease incidence on the different cultivars in response to pyraclostrobin applied at the 2-4 leaf stage in comparison with the control, in 2011-2013.

Cultivar	Fungicide Treatment	Disease Incidence (%)		
		2011	2012	2013
45H29(R)	Control	62.77 a[1]	73.53 ab	83.49 bc
45H29(R)	Pyraclostrobin	17.15 b	59.36 b	78.00 c
43E01(MR)	Control	74.82 a	78.91 a	94.91 a
43E01(MR)	Pyraclostrobin	22.42 b	61.12 b	65.19 d
Westar (S)	Control	69.97 a	85.95 a	89.66 ab
Westar (S)	Pyraclostrobin	18.00 b	41.83 c	66.26 d

[1] Values followed by the same letter in a column are not significantly different based on the LSD test at $P<0.05$.

Table 3.6C Mean values of blackleg disease incidence on the susceptible cultivar 'Westar' in response to the timing and intensity of fungicide application in 2011-2013

Fungicide	Application Timing	Disease Incidence (%)		
		2011	2012	2013
Control	2-4 leaf	69.97 a[1]	85.95 a	89.66 a
Pyraclostrobin	2-4 leaf	18.00 c	41.83 b	66.26 c
Pyraclostrobin	Prior to bolting	62.05 a	72.05 a	89.84 a
1)Propiconazole	2-4 leaf	44.02 b	75.43 a	84.06 ab
2)Pyraclostrobin	Prior to bolting			
1)Pyraclostrobin	2-4 leaf	25.20 c	50.38 b	71.13 bc
2)propiconazole	Prior to bolting			

[1]Values followed by the same letter in a column are not significantly different based on the LSD test at $P<0.05$.

3.3.3 Seed yield

The effects of year, fungicide, cultivar, application timing, and the two-way interactions on seed yield were all significant (Table 3.4). There was a significant interaction between fungicide and year in affecting seed yield (Figure 3.1A). In 2011and 2012, cv. Westar treated with the foliar fungicides produced seed yields similar to the unsprayed check; i.e., foliar fungicide application did not influence seed

yield at all. In 2013, however, the canola crop treated with pyraclostrobin or azoxystrobin significantly increased seed yield as compared to the unsprayed check. The propiconazole showed insignificant effect.

Cultivar and fungicide interaction was significant in affecting canola seed yield (Table 3.4). In each of the three years, the resistant cultivar 45H29 receiving no fungicide had significantly higher seed yield compared to the moderate resistant cultivar 43E01 and the susceptible cultivar Westar (Figure 3.1B). A close examination of the effect of fungicide application on seed yield revealed that the pyraclostrobin treatment had no effect on the seed yield for 43E01 or Westar, with the only significant difference found between the pyraclostrobin treatment and unsprayed control on 45H29. Fungicide application timing affected canola seed yield significantly (Table 3.4). A significantly higher yield at 2617 kg ha^{-1} was obtained in the multi-application treatments (pyraclostrobin at the 2-4 leaf stage plus propiconazole prior to bolting) (Figure 3.1C). Within all treatments, the highest mean yield of 4507 kg ha^{-1} was obtained on resistant (45H29) cultivar and the lowest 1307 kg ha^{-1} was observed on Westar after pyraclostrobin treatment. The 2011 growing season was excellent for canola crops, resulting in seed yields much greater than the long-term average in western Canada. Fungicide treatments at the 2-4 leaf stage or prior to the bolting stage had no significant effect on seed yield. However, multi-applications (i.e., pyraclostrobin at the 2-4 leaf stage plus propiconazole prior to bolting) significantly increased the yield on Westar in 2011 and 2013 (Figure 3.1C).

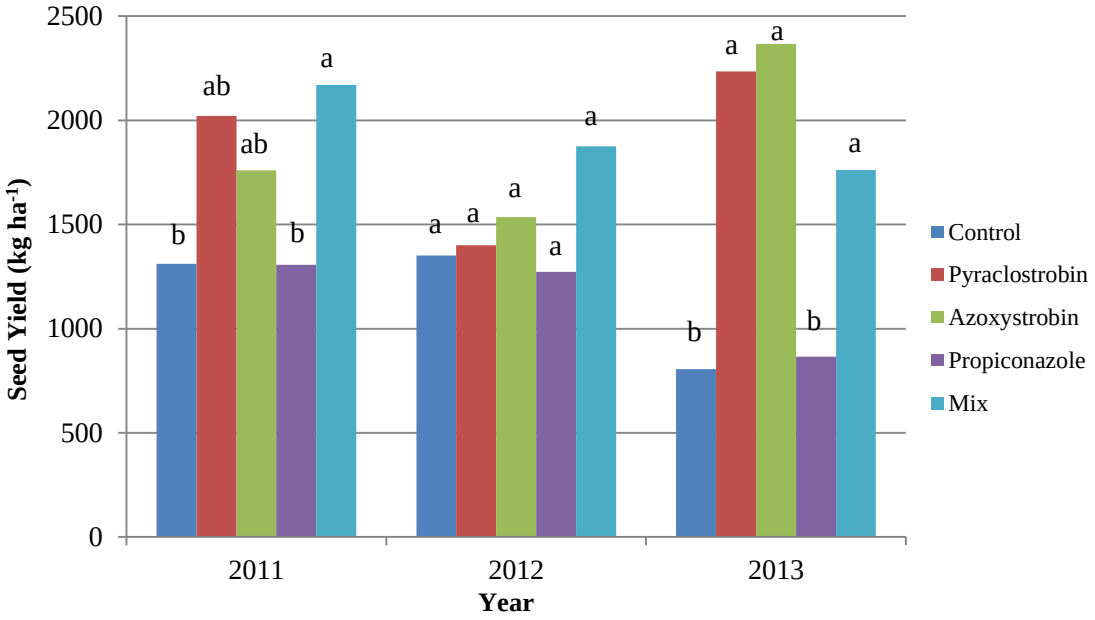

Figure 3.1A Mean values of seed yield on the susceptible cultivar 'Westar' in response to different fungicides applied at the 2-4 leaf stage, in 2011-2013.

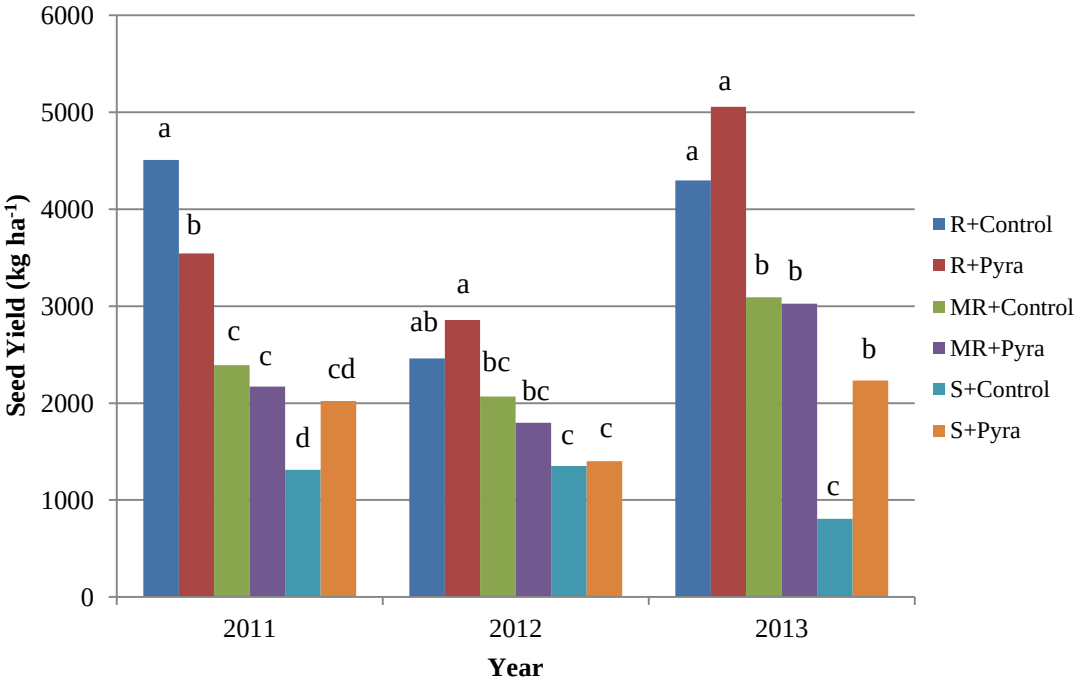

Figure 3.1B Mean values of seed yield on the different cultivars in response to pyraclostrobin applied at the 2-4 leaf stage in comparison with the control, in 2011-2013.

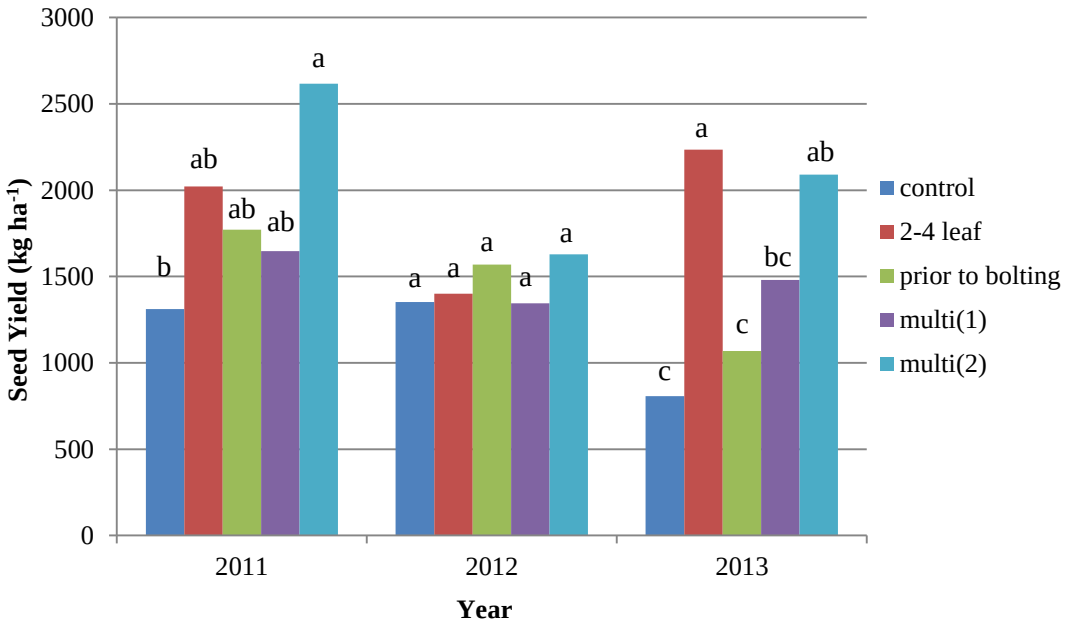

Figure 3.1C Mean values of seed yield on the susceptible cultivar 'Westar' in response to the timing and intensity of fungicide application, in 2011-2013.

3.4 Discussion

This was the first field experiment conducted in Manitoba in determining the efficacy of fungicide strategies against blackleg disease caused by the fungus *L. maculans* on canola. During the 2011-2013 field seasons, the environmental conditions at the experimental site (Carman, Manitoba) were favourable to the development of blackleg disease; the precipitation was generally higher than the long-term average and temperature was close to normal. These conditions resulted in moderate to moderately high levels of disease severity, incidence, and yield impact for the assessment of fungicide strategies. The fungicides used in this study included DMI (propiconazole) which was introduced into western Canada in the early 1990's, and azoxystrobin and pyraclostrobin (strobilurins) which were available in the early 2000's and 2010, respectively. The field experiment was conducted on the same field during 2011 to 2013 growing seasons, allowing more natural inoculum available for pathogen infection and also to increase the risk of resistance evolution in pathogen populations.

The QoI fungicide treatments significantly reduced blackleg disease incidence and severity and generally showed a positive effect on seed yield compared to the unsprayed check on the susceptible cultivar Westar. Also, fungicide application at the 2-4 leaf stage had a significant effect on both blackleg disease incidence and severity on the susceptible cultivars, but the foliar fungicide treatment often showed little or no efficacy on cultivars with moderate and high resistance levels. Brown *et al.*, (1976)

reported that fungicide treatments on weakly-resistant cultivars have variable but generally poor control of blackleg disease under field conditions in Australia. The efficacy of blackleg management may vary with fungicide products or the growing conditions (Kutcher and Brandt, 2008; Kharbanda *et al.*, 1999).

The results of this study also showed that the choice of active ingredient is important for blackleg control. The QoI fungicides (pyraclostrobin and azoxystrobin) showed a significantly higher capability for reducing the disease severity compared to DMI (propiconazole) fungicide on the susceptible cultivar Westar. However, QoI fungicides did not consistently increase yield on Westar and had no significant effect on seed yield in moderately resistant (43E01) or resistant (45H29) cultivars. Since the development of resistance to strobilurin fungicides has been a major concern for many crops (Ma *et al.*, 2003; Gisi *et al.*, 2002), the rotation of fungicides with different modes of action is crucial for the management of fungicide insensitivity, especially in regions where single-site fungicides were applied intensively and exclusively (Gisi *et al.*, 2002). In western Canada, however, rotation between different fungicide families will be a challenge against blackleg because limited number of registered families and poor efficacy of the DMI fungicide.

Previous studies have shown that the efficacy of fungicide treatments is variable, even on a susceptible cultivar. This is likely due to a relatively long infectivity period in canola (Kutcher *et al.*, 2011a). The causal agent *L. maculans* can infect the crop from the cotyledon stage to flowering stage, consequently leading to severe epidemics

by maturity. It has been hypothesized that the multi-application strategy may be more effective than a single application of fungicide during this lengthy period. However, the results of the present study showed that a single application at the 2-4 leaf stage was comparable to two applications (i.e., at the 2-4 leaf stage and prior to bolting). Also, improved disease suppression was achieved with the less effective fungicide propiconazole sprayed at the 2-4 leaf stage as opposed to prior to bolting or even multi applications. The second application at bolting did not show any benefit in terms of seed yield. It seems that timing the application at the early crop stage is more effective for blackleg control with fungicide.

In the study, propiconazole fungicide treatments were generally ineffective, did not contribute further under multi applications strategies. Bailey *et al.*, (2000) used multiple fungicide applications for blackleg and sclerotinia in canola, and found that multi applications with two systemic fungicides did not provide better disease control or increase yield compared to a single application or an untreated control. The minor yield decrease may result from the mechanical damage to the crop caused by the two separate fungicide treatments with different application timings. The results from the present study and others show that the selection of active ingredients plays a major role in chemical control of blackleg on canola.

Based on this study, and in support of research done elsewhere, it is clear that optimal application timing of fungicide is also a key factor for blackleg management in canola. Application timing is critical in reference to the onset of disease epidemics,

rain events, and the growth stage of crops. Generally, protectant fungicides need to be applied before rain events and systemic fungicides within a short window after a rain event (Banniza *et al.*, 2011). In our study, a 2-4 leaf stage fungicide application was more effective for blackleg control, and a prior to bolting stage application was less successful as infection had already been established in plants by that time. Also, spraying foliar fungicide based on symptoms visible in the field may not be effective since initial symptoms under the canopy can be easily overlooked.

Disease forecasting models for early detection of *L. maculans* spores might be useful in western Canada to predict critical periods of infection (Banniza *et al.*, 2011, Kutcher *et al.*, 2011a, Salam *et al.*, 2007). In Poland, an early warning model has been developed to determine the optimum application timing (Jedryczka *et al.*, 2008). Since airborne *L. maculans* spores represent the primary source of initial inoculum, the seasonal and diurnal patterns of spore dispersal related to the growth stage of the crop and weather conditions are forecasted using models. Often, the period of ascospores and pycnidiospores dispersal by *L. maculans* may coincide with the susceptible growth stages under favorable weather conditions (Guo *et al.*, 2005). Thus, weather-based models may be developed and used to predict the date of onset of seasonal ascospores release from canola stubble (Salam *et al.*, 2007).

In the present study, the choice of cultivars plays an important role in controlling blackleg disease, and the level of resistance in cultivar has an ultimate effect on the efficacy of chemical control for blackleg. The strobilurins fungicides consistently

reduced disease incidence and severity among all the cultivars, but the yield increase was only observed in the susceptible cultivar Westar in one of the three study years when the disease pressure was high. No significant yield increase was observed in the moderately resistant (43E01) and resistant (45H29) cultivars, suggesting a limited impact of fungicide treatments on the resistant canola cultivars. A similar field study done by Kutcher *et al.* (2013) has also shown that fungicide treatments did not benefit blackleg-resistant cultivars and only a small yield improvement was recorded on susceptible cultivars. The current findings under Manitoba conditions are in agreement with observations in Saskatchewan and with other studies on barley and wheat in western Canada and the Great Plains of the USA (Bailey *et al.*, 2000; Stover *et al.*, 1996) in which cultivar resistance generally provides higher economic returns than fungicides. Unlike in Western Europe where greater yields are obtained and expensive fungicide spray treatments can be justified, the crop yield is generally lower in western Canada due to short growing seasons (West *et al.*, 2001). In this case, additional fungicide applications for disease control would increase production costs and are uneconomical unless the increase of seed yield or quality offsets the fungicide treatment cost. Therefore, the economic costs must be considered by canola growers in western Canada when applying fungicides for blackleg control.

The plots were established using artificial inoculum in 2011 and 2012, whereas the source of inoculum in 2013 was from the infected canola residues from the previous years. A significant increase of blackleg severity was observed in 2013 from the two previous years, suggesting that infected canola residue can be substantial in

increasing disease pressure, especially under the continuous monoculture in the same location over years. Guo *et al.* (2008) reported that the continuous canola cultivation had a higher disease incidence than that in 4-year rotations of canola with wheat and flax in Manitoba.

The environment plays an important role in disease severity of many foliar diseases and thus influences the economic benefit of fungicide application (Bailey *et al.,* 2001). Some crops, including barley, are generally responsive to fungicide treatments; a 15-20% yield increase can be obtained with fungicide application in Saskatchewan (Kutcher *et al.,* 2011b). Bailey *et al.* (2000) also reported that fungicide treatment on net blotch susceptible barley cultivar consistently reduced disease severity and returned economically higher yields (average 23%) and seed quality (13% in 1000-kernel seed weight and 25% plumpness) in their study. However, the benefit of fungicide use is always variable among years for most other crops, most likely due to differences in environments (Bailey *et al.*, 2000). Fungicide applications generally benefit the production of wheat and canola in years of normal precipitation, but dry conditions or even less precipitation may have a negative effect on the benefit of chemical control and no economic benefit is obtained for these crops (Kutcher *et al.*, 2011b). In the current study, the yield increase was only observed on susceptible canola cultivar with strobilurin fungicides in only one of the three years, suggesting that the fungicide application in canola benefits the yield of a susceptible canola cultivar also variably depending on the year and disease pressure.

In summary, the results of this study revealed that fungicides can be one of the management tools against blackleg on canola but the efficacy highly depends on the cultivar resistance, active ingredients of fungicides, and the application timing. The QoI (pyraclostrobin and azoxystrobin) fungicides showed a significantly higher capability to reduce disease incidence and severity than DMI (propiconazole) fungicide on Westar in all three field seasons. A 2-4 leaf stage application reduced the disease significantly in all field trials, indicating that early protection is very important for the management of blackleg. Although fungicide treatments reduced the disease incidence and severity also on moderately resistant (43E01) and resistant (45H29) cultivars, the yield benefit was generally absent. This study clearly demonstrated that the level of resistance in the canola cultivar influences the benefit of fungicide treatments for control of blackleg.

CHAPTER 4

4.0 OCCURRENCE AND MOLECULAR CHARACHTERIZATION OF QoI-INSENSITIVE *Leptosphaeria maculans* ISOLATES IN Canola

Abstract

Strobilurin fungicides, including pyraclostrobin and azoxystrobin, are site-specific fungicides that may be used for control of blackleg under certain conditions in western Canada. In vitro bioassays with strobilurins at 0.001, 0.01, 0.1, 0.5, 0.7, 1.0 and 5.0 μg ml^{-1} amended into V8 juice agar medium were used to evaluate the sensitivity of 172 isolates sampled from infested canola residues at fungicide study sites in Manitoba, Saskatchewan and Alberta. The sensitivity levels among *L. maculans* isolates were assessed by mycelia growth inhibition against controls. For baseline-sensitivity, isolates collected in 2011 showed the mean 50% effective concentration (EC_{50}) values for pyraclostrobin, azoxystrobin, propiconazole and mix of propiconazole and azoxystrobin were 0.198, 0.525, 0.706 and 0.550 μg ml^{-1}, respectively; the mean EC_{50} values for *L. maculans* isolates from the same field sites in 2012 were 1.924, 0.695, 0.439 and 1.164 ug ml^{-1}, respectively. Eight isolates exhibited significantly (P=0.05) lower *in vitro* sensitivity to QoI fungicides than the mean EC_{50} values of baseline strains. The underlying mechanism of resistance to strobilurins was investigated by comparing amino acid sequences of the cytochrome *b* (*CYTB*) gene from baseline and less sensitive *L. maculans* isolates. The result of sequence analysis revealed that the G143A and F129L substitutions were not found in any of the 13 QoI-insensitive *L. maculans* field strains. Moreover, no polymorphism correlating with the QoI-resistant phenotype was observed in sequenced part of cytochrome *b* gene among the *L. maculans* field isolates, and this suggests that the observed lower sensitivity to QoI fungicides was not caused by genetic mutation at

these loci.

4.1 Introduction

Fungicide use remains an important component in the integrated approach for blackleg management in canola. In western Canada, several fungicides have been registered and they are applied to susceptible canola cultivars to reduce blackleg disease severity (Bailey *et al.*, 2000; Kutcher *et al.*, 2011a). Site-specific fungicides, triazole and strobilurin, were first introduced to western Canada for blackleg control in the early 1990s and 2000s, respectively. Since then, several other fungicides have been registered and applied for registration for use on canola, including azoxystrobin (Quadris, BASF Canada Inc., CA) and pyraclostrobin (Headline, BASF Canada Inc., CA). Azoxystrobin is also marketed as premixed fungicide with propiconazole (Quilt, Syngenta Canada Inc., CA). However, with the increased use of strobilurin fungicides (i.e., QoI fungicide), pathogen resistance has been found extensively in many pathogen species, including *Mycosphaerella fijiensis* (Sierotzki *et al.*, 2000), *Pyricularia grisea* (Vincelli and Dixon, 2002), *Colletotrichum cereale* (Young *et al.*, 2010) and *Monilinia fructicola* (Amiri and Brannen, 2010). Owing to the associated risk of reduced sensitivity to respiration inhibitor fungicides, it is essential to establish a baseline sensitivity to strobilurin fungicides and detect possible shifts of sensitivity in *L. maculans* populations.

QoI fungicide inhibits the respiration of fungi by binding the quinol oxidation

site of cytochrome *bc1* enzyme complex (also known as complex III) in the mitochondrial respiration chain (Bartlett *et al.*, 2002; Bradley and Pedersen, 2011). The binding process blocks the electron transport between cytochrome *b* and cytochrome *c1* in respiration pathway, resulting in an energy deficiency in fungal cells by halting the adenosine triphosphate (ATP) production (Luo *et al.*, 2010; Bartlett *et al.*, 2002).

The chemical Salicylhydroxamic acid (SHAM) has been shown to disturb the alternative respiration pathway of the fungus, which can bypass the QoI fungicide binding site in vitro, leading to the small amount of ATP synthesis for respiration (Ziogas *et al.*, 1997; Vinvelli and Dixon, 2002). Because the alternative oxidation has only been reported in pathogenic fungi *in vitro* (Bradley and Pedersen, 2011; Wise *et al.*, 2008; Olaya and Koller, 1999), it may not play an important role in the sensitivity of *L. maculans* isolates *in vivo*; possibly the release of host flavones during the infection could interfere with the induction of pathway and only limited amount of energy is provided by the alternative oxidation (Olaya and Koller, 1999; Ziogas *et al.*, 1997).

There are eleven single or combined point substitutions in the mitochondrial cytochrome *b* gene (*cytb*) involved with resistance to QoI fungicides in two extra-membrane regions of complex III: at amino acid positions from 127 to 147 and from 275 to 296 (Gisi *et al.*, 2002). The two typical mutations, a change from glycine to alanine at position 143 (G143A) and a substitution from phenylalanine to leucine at

position 129 (F129L), have been found to confer a high level of resistance to QoI in a wide variety of phytopathogenic fungi (Gisi *et al.*, 2000; Gisi *et al.*, 2002; Ma and Michailides, 2005). Also, an alternative respiration pathway associated with QoI resistance has been reported in *Mycosphaerella graminicola* and *Pyricularia grisea,* where the *in vitro* sensitivity to QoI fungicides has been shown to be reduced (Miguez *et al.*, 2004; Vincelli and Dixon, 2002).

The objectives of this study were to: 1) establish baseline sensitivities of *L. maculans* isolates in western Canada to pyraclostrobin, azoxystrobin, propiconazole and mix of propiconazole and azoxystrobin fungicides, 2) monitor any change in sensitivity of *L. maculans* isolates to fungicides within the canola growing regions, and 3) determine and characterize the typical point mutation in the cytochrome *b* gene of *L. maculans* isolates, which confer a high level of resistance to QoI fungicides in many phytopathogenic fungi.

4.2 Materials and Methods

4.2.1 Collection and isolation of *L. maculans* isolates

During the 2011 and 2012 growing seasons, 172 *L. maculans* strains originating from four experimental sites of western Canada (Carman and Brandon in Manitoba, Vegreville in Alberta, and Melfort in Saskatchewan) were single spored. These sites represent major canola production regions in western Canada. Canola stems with

blackleg symptoms were collected from field plots treated with strobilurin or propiconazole fungicide, surface sterilized by immersing them in 0.5% sodium hypochlorite solution for 1 min and air dried on sterile filter paper in a laminar flow hood. The sterilized stem pieces were excised from darker inner lesion areas of each stem and transferred (five per plate) onto each V8 juice (Campbell Soup Company, Toronto, ON, Canada) agar media amended with $CaCO_3$ at 0.75 g L^{-1}(Fisher Scientific, Fair Lawn, NJ) and streptomycin sulfate at 0.1 g L^{-1}(Sigma-Aldrich, St. Louis, MO, USA).

After incubation at room temperature (approximately 22^{o}C) with fluorescent light for 3 to 5 days, pycnidiospores from single pycnidia were transferred onto new V8 juice agar media to produce pure culture of *L. maculans* isolates. Sterile distilled water was added on *L. maculans* cultures and glass slides were used to dislodge pycnidia to make a pycnidiospore suspension. The spore suspension was filtered through a double layer of sterile cheesecloth for the removal of mycelia debris. The final concentration of suspension was adjusted to approximately 1 x 10^7 sporeml^{-1} and 200 µl of spore suspension was spread using bent glass rod on petri dishes (100mm in diameter) containing V8 juice agar media for mycelia growth assays. Mono conidial isolates of *L. maculans* were kept at -20 ^{o}C for long-term storage on air-dried, sterilized filter paper discs (Fisher Scientific, Pittsburg, PA). The colonized paper discs were plated on V8 juice agar media and harvested into 1.5ml centrifuge tubes (Fisher Scientific, Pittsburg, PA) for molecular study.

4.2.2 Fungicides

Commercial formulations of azoxystrobin (Quadris, 22.9% or 250 g L^{-1} active ingredient<a.i.>; Syngenta Canada Inc., Guelph, ON, Canada), pyraclostrobin (Headline EC, 250 g L^{-1}a.i.; BASF Canada Inc., Mississauga, Ontario, Canada), propiconazole (Tilt 250 EC, 250 g L^{-1}a.i.; Syngenta Canada Inc., Guelph, ON, Canada), and mix of propiconazole and azoxystrobin (Quilt, 7.0%azoxystrobin and 11% propiconazole or 75 g $L^{-1}$and 125 g L^{-1} a.i.; Syngenta Canada Inc., Guelph, ON, Canada) were used in this study. For the biomass assays, the fungicides were dissolved and stored in sterile distilled water at room temperature and adjusted to the concentrations of 1 mg a.i. ml^{-1}. The stock solution of fungicides was added into the autoclaved V8 juice agar media when it cooled to approximately 50^{o}C to deliver the desired concentrations for the study.

4.2.3 Determination of *in vitro* sensitivity of *L. maculans* isolates to fungicides

Fungicides were added into autoclaved V8 juice agar media when it cooled to approximately 50 ^{o}C to obtain the final concentrations of 0.01, 0.1, 0.3, 0.7, 1.0, 5.0 and 10.0µg a.i. ml^{-1}. Salicylhydroxamic acid (SHAM; Sigma-Aldrich, St. Louis, MO, USA) at 100 µg ml^{-1} was dissolved in distilled water and added to pyraclostrobin-, azoxystrobin- and the mix of propiconazole + azoxystrobin-amended V8 juice agar media. Preliminary results suggested no effect of SHAM on the inhibition of the alternative oxidase respiration of propiconazole on mycelia growth of *L. maculans*

isolates (data not shown); Hence, SHAM was not added into propiconazole-amended V8 juice agar media.

To initiate mycelial growth, a hyphal plug (7 mm in diameter) was taken from the advancing margins of a 7-day-old *L. maculans* culture and placed, mycelium-side down, in the center of fungicide-amended and non-amended media. Three petri dish replicates of each fungicide concentration were assessed for each strain. Petri dishes were incubated at the room temperature (approximately 22 ^{o}C) with fluorescent light for 5 days before evaluation. The diameter of colony was measured daily from day 6 to day 9 and in two perpendicular directions from the edge of each plug. The mean colony diameter was used to calculate percent reduction of growth relative to the non-amended control treatment.

4.2.4 Data analysis

The effective concentration required to achieve 50% inhibition of mycelia growth on fungicide-amended media (EC_{50}) was calculated. For each of the replicated plates, the mycelia growth was converted using the equation:

$\mathrm{GI} = \left(1 - \frac{\mathrm{Gf}}{\mathrm{Gc}}\right) * 100\%$, in which growth inhibition (GI) is the percent relative inhibition compared with non-amended control, Gf is the percent growth of colony on fungicide-amended media is , and Gc is the percent growth colony on non-amended media. The values of percent growth inhibition were plotted against $\log_{10}$-transformed

fungicide concentrations and EC_{50} values (µg/ml) were calculated for each isolate subjected to regression procedure (PROC REG) in SAS (version 9.2; SAS Institute, Inc., Cary, NC). The parameter estimates of the regression equation, intercept (b_0) and slope (b_1) were obtained and the EC_{50} value for each isolate was calculated using the equation:$EC_{50-fungicide} = e^{\frac{50-b_0}{b_1}}$. Where, b_0 is regressions intercept, b_1 is regression slope, and e is the natural log. Data from in vitro biomass assays was analyzed using the general linear model procedure (PROC GLM), and the least significant difference tests at a level of 0.05 were performed to compare the mean EC_{50} values of *L. maculans* isolates in 2011 and 2012 field seasons.

4.2.5 Isolates for molecular characterization of resistance

The *L. maculans* isolates collected from three experimental sites were previously screened for *in vitro* sensitivity to QoI and DMI fungicides. A total of 17 isolates of *L. maculans* representing a range of *in vitro* sensitive diversity response to QoI fungicides were used to confirm the molecular mechanisms of QoI resistance in the present study (Table 4.1).

Table4.1 Molecular characterization of *L. maculans* isolates collected from canola grown in western Canada. The EC_{50} value of 1.98 μg ml^{-1} of pyraclostrobin and 5.49 μg $ml^{-1}$of mixture fungicide (ten-fold mean EC_{50} value of baseline isolates) are used to separate the 'insensitive' from 'sensitive' fungal populations.

Isolate **code**	**Location**	**EC_{50} value(μg ml^{-1})**	**QoI sensitivity**
C6-2	***Carman***	***2.413***	***insensitive***
C6-10	***Carman***	***6.940***	***insensitive***
M6-2	***Melfort***	***2.210***	***insensitive***
M6-4	***Melfort***	***9.197***	***insensitive***
M6-16	***Melfort***	***2.132***	***insensitive***
C6-42	Carman	0.510	sensitive
M6-11	Melfort	0.801	sensitive
M6-17	Melfort	0.595	sensitive
V6-8	Vegreville	0.411	sensitive
V6-23	Vegreville	0.730	sensitive
C7-22	Carman	2.630	sensitive
M7-25	Melfort	1.726	sensitive
C7-32	Carman	0.368	sensitive
V7-18	Melfort	0.403	sensitive
C12-21	Carman	4.916	sensitive
V12-25	***Vegreville***	***8.519***	***resistant***
V12-11	Vegreville	0.271	sensitive

4.2.6 DNA extraction

The *L. maculans* isolate used for genomic DNA (gDNA) extraction was cultured on V8 juice agar medium at room temperature (approximately 22℃) under

fluorescent light for 10 days. Fresh mycelium and pycnidiospores were harvested and transferred into a 1.5 ml microcentrifuge tube containing ceramic beads. Four hundred microliters of TES buffer (100 mM Tris, 10 mM EDTA and 2% sodium dodecyl sulphate) was added and pulverized in the homogenizer for 45 sec. Then, 140 µl 5M NaCl was added and followed by 70 µl 10% CTAB (CTAB needs to be warmed to 65 ℃ before starting). The microcentrifuge tubes were incubated at 65 ℃ for 20 min, mixed 3-4 times by inverting. To remove proteins, 300 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution and mixed for 1 min by inverting gently. The materials were centrifuged at 10,000 rpm for 15 min and then the top aqueous layer was transferred into a clean tube. The former step was repeated on the transferred aqueous layer in the second set of tubes, approximately 400 µL of the second aqueous layer were transferred into the last set of labeled tubes. And then 80 µL of 5 M NaCl and 1,000 µl (2.5 vol) of 100% ethanol were added to precipitate DNA. The materials were mixed by inverting gently and centrifuged at 13,000 rpm for 5 min. The supernatant was removed and DNA pellet was washed with 200 µl ice-cold 95% ethanol carefully, without disturbing the pellet. The microcentrifuge tubes were tipped over on paper towel and dried for 20 min. The pellet was re-suspended in 200 µl sterile water at 65 ℃. The gDNA samples were kept at 4 ℃ overnight for complete resuspension. To remove RNA, 2 µl RNase (1 mg ml^{-1}) was added to the solution and the tubes were incubated at 37 ℃ for 30 min. DNA samples were kept at -20 ℃ until further use. The DNA was quantified using 1% agarose gel for electrophoresis. The concentration of DNA samples was measured using

NanoDrop2000 (Thermo Fisher Scientific Inc.).

4.2.7 PCR amplification

The two sets of primers LmCB-P2 (LmCB-P2f: 5'-A C C T G G A A A A A G C C A G G T G T T-3' and LmCB-P2r: 5'-G T A A C C C A G T C T G T A G C G A C C-3'), LmCB-P3 (LmCB-P3f: 5'-C C T G G A A A A A G C C A G G T G T T-3' and LmCB-P3r: 5'- T A A C C C A G T C T G T A G C G A C C-3') and LmCB-P5 (LmCB-P5f: 5'-T A C C T G G A A A A A G C C A G G T G T-3' and LmCB-P5r: 5'-A C C C A G T C T G T A G C G A C C A-3'), based on previous partial sequences of the cytochrome *b* gene of *L. maculans* isolates were designed to produce the expected regions (Rouxel *et al.*, 2011).

Polymerase chain reaction (PCR) assays were performed in the thermal cycler using 20 ng of fungal DNA in a total reaction volume of 20µl containing 10 mM Tris HCl (pH 8.1), 2.0mM MgCl2, 2.5 mM dNTPs, 1U Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 10 µM from each primer. The PCR amplifications of LmCB-P2 and LmCB-P3 primer sets included an initial step at 94 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 1 min, 58 ℃ for 1 min and 72 ℃ for 1 min, then a final extension at 72 ℃ for 5 min before cooling down to 4 ℃. The amplicon were separated by 1.0% agarose gel electrophoresis, stained with Redsafe at a final concentration of 4.0 µl ml^{-1} and visualized under UV light to verify the amplicon corresponded to the expected size of 452 bp.

4.2.8 Sequencing of cytochrome *b* gene

A collection of the 5 QoI-resistant isolates and 12 QoI-susceptible *L. maculans* isolates were used for sequencing. The PCR products of selected isolates were sequenced at Macrogene Corporation (Macrogene Corp., USA). The sequence data were analyzed using the Bioinformatics software Chromas 2.4.1 (Technelysium Pty. Ltd., AU). The multiple sequence alignments were performed using ClustalW (http://www.genome.jp/tools/clustalw/).

4.3 Results

4.3.1 Establishment of baseline EC_{50} values of *L. maculans* isolates (2011)

In 2011, the range of EC_{50} values for *L. maculans* isolates exposed to pyraclostrobin was between 0.046 and 0.463 μg ml^{-1}, with the mean value of 0.198 μg $ml^{-1}$and the median 0.179 μg ml^{-1} (Figure 4.1). The distribution of pyraclostrobin EC_{50} values was normal. For azoxystrobin, the EC_{50} values were ranged between 0.084 μg $ml^{-1}$and 1.984 μg ml^{-1} with the mean and median values being 0.525 μg ml^{-1} and 0.462 μg ml^{-1}, respectively (Figure 4.2). The azoxystrobin EC_{50} values were not normally distributed. For propiconazole, the EC_{50} values ranged between 0.329 and 1.611 μg ml^{-1} with the mean and median values being 0.706 μg ml^{-1} and 0.578 μg ml^{-1}, respectively (Figure 4.3). The propiconazole EC_{50} values were not normally distributed either. The range of EC_{50} values for *L. maculans* isolates exposed to the

mix of azoxystrobin and propiconazole was between 0.178 and 0.549 µg ml^{-1}, with the mean of 0.549 µg ml^{-1} and the median 0.420 µg ml^{-1} (Figure 4.4).

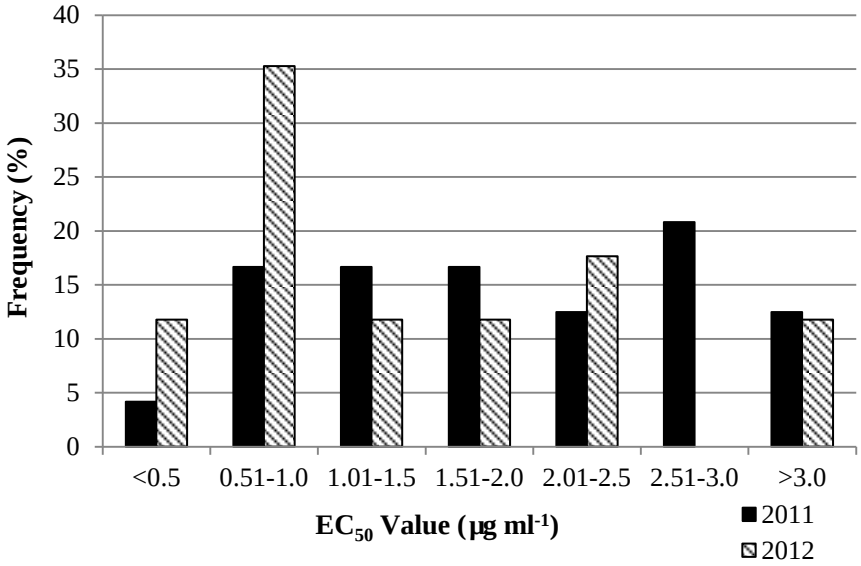

Figure 4.1 Sensitivity (EC_{50} value) distribution of *L. maculans* isolates collected in 2011 and 2012 to pyraclostrobin fungicide based on mycelial growth.

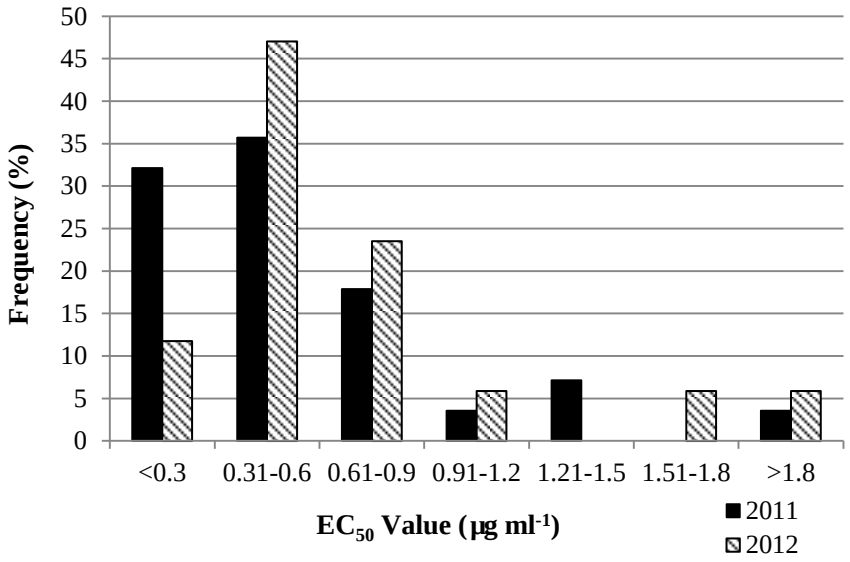

Figure 4.2 Sensitivity (EC_{50} value) distribution of *L. maculans* isolates collected in 2011 and 2012 to azoxystrobin fungicide based on mycelial growth.

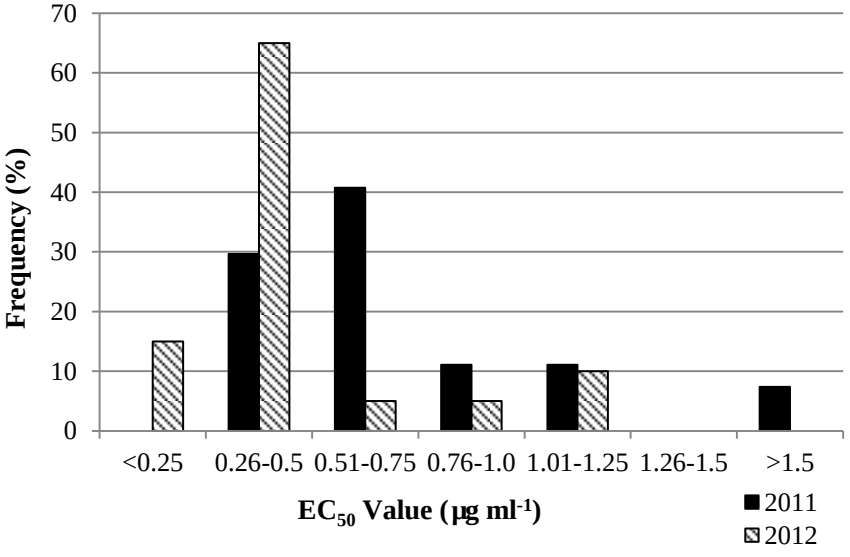

Figure 4.3 Sensitivity (EC_{50} value) distribution of *L. maculans* isolates collected in 2011 and 2012 to propiconazole fungicide based on mycelial growth.

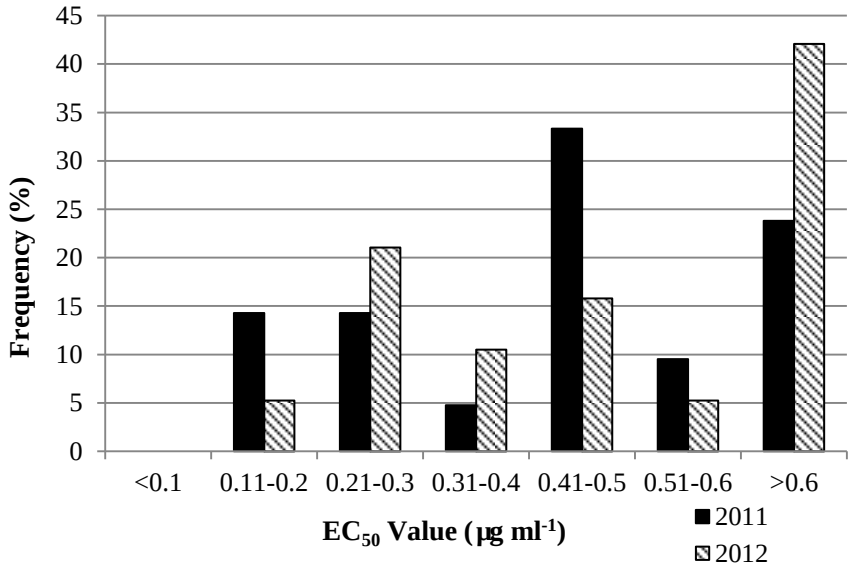

Figure 4.4 Sensitivity (EC_{50} value) distribution of *L. maculans* isolates collected in 2011 and 2012 to mix of propiconazole and azoxystrobin fungicide based on mycelial growth.

4.3.2 Monitoring of fungicide sensitivity in the *L. maculans* population (2012)

In 2012, the EC_{50} values obtained by mycelial growth inhibition for pyraclostrobin ranged from 0.128 to 9.197 µg ml^{-1} with the mean and median values of 1.924 µg ml^{-1} and 1.053 µg ml^{-1}, respectively (Figure 4.5). The range of EC_{50} values for *L. maculans* isolates exposed to azoxystrobin was from 0.241 to 2.629 µg $ml^{-1}$and the mean and median values were 0.695 µg ml^{-1} and 0.465 µg ml^{-1}, respectively (Figure 4.6). For propiconazole, the range of EC_{50} values was from 0.142 to 1.098 µg ml^{-1} with the mean and median 0.439 µg ml^{-1} and 0.342 µg ml^{-1},

respectively (Figure 4.7). The range of EC_{50} values for *L. maculans* isolates exposed to the mix of propiconazole and azoxystrobin was from 0.173 to 8.519 μg ml^{-1} and the mean and median values were 1.164 μg ml^{-1} and 0.466 μg ml^{-1}, respectively (Figure 4.8).

The *L. maculans* isolates showed different levels of *in vitro* sensitivity to the fungicides and baseline sensitivity of *L. maculans* populations varied among the four study sites. The mean EC_{50} value of *L. maculans* populations in 2012 was significantly higher than that of 2011 for pyraclostrobin, but no differences were found between the two growing seasons for the other fungicides (Figure 4.8). Two highly resistant isolates were detected to pyraclostrobin fungicide with the EC_{50} values of 6.940 μg ml^{-1} and 9.167 μg ml^{-1}, respectively (Figure 4.9). In contrast, the response of the isolates to azoxystrobin and propiconazole was similar between the two growing seasons. Although the mix of fungicides propiconazole with azoxystrobin resulted in a greater EC_{50} value in 2012 compared to 2011, they did not differ statistically. Overall, the highest EC_{50} values based upon mycelial growth inhibition tests were 2.630 μg ml^{-1}, 1.098 μg ml^{-1}, 8.519 μg ml^{-1}, respectively, to azoxystrobin, propiconazole and the mix of azoxystrobin with propiconazole in 2012. The variation of EC_{50} values of *L. maculans* populations was higher response to pyraclostrobin and the mix of azoxystrobin with propiconazole compared with azoxystrobin and propiconazole alone in the 2012 field season.

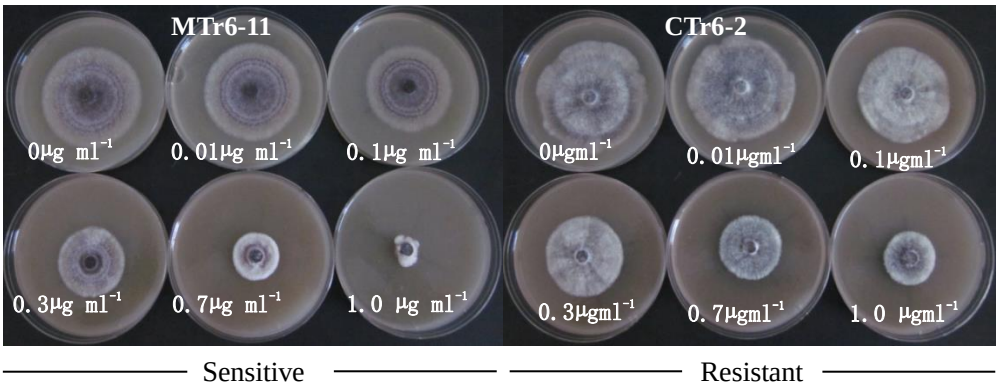

Figure 4.5 Comparison of mycelial growth of MTr6-11 (sensitive) and CTr6-2 (resistant) *L. maculans* isolates on V8 juice agar media amended with pyraclostrobin at different concentrations. The EC_{50} value of 1.98 μg ml^{-1} of pyraclostrobin (ten-fold mean EC_{50} value of baseline isolates) is used to separate the 'resistant' from 'sensitive' fungal populations.

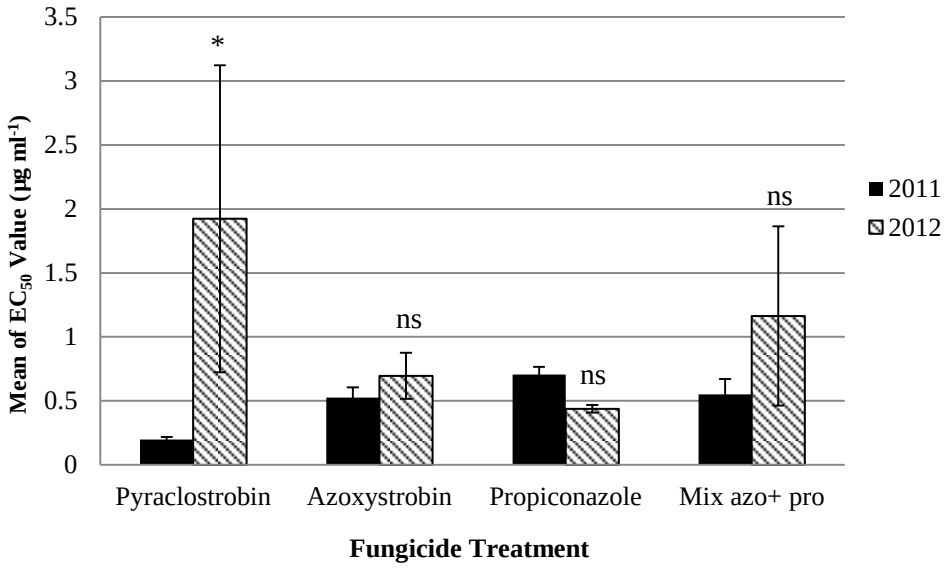

Figure 4.6 Comparison of mean EC_{50} values of *L. maculans* isolates response to fungicides in 2011 (black bars) and 2012 (oblique line bar). * means significant difference ($p<0.05$) between the two growing seasons; ns means no significant difference.

4.3.3 Molecular characterization of *L. maculans* isolates with different sensitivity to strobilurin fungicides

The molecular mechanism of resistance to QoI fungicide in *L. maculans* population was determined by comparing amino acid sequences of the cytochrome *b* protein. A total of 17 field isolates (Table 4.1) exhibiting different levels of sensitivity to strobilurins were screened, of which 5 were QoI resistant. The amplified PCR products were 452 bp in size surrounding the F129L and G143A mutation sites in cytochrome *b* gene using primer pairsLmCB-P2, LmCB-P3 and LmCB-P5 (Figure 4.7, 4.8, 4.9). The comparison of protein sequence between QoI-sensitive and QoI-resistant isolates of *L. maculans* and ClustalW sequence alignment analysis revealed that amino acid components of cytochrome *b* gene were highly conserved independently of the QoI resistance phenotype and no polymorphism was present among the 17 field isolates (Figure 4.10). The typical substitution F129L and G143A conferring high level of resistance to QoI fungicide in many phytopathogenic fungi was not observed in any of 5 QoI-resistant isolates of *L. maculans* which were screened.

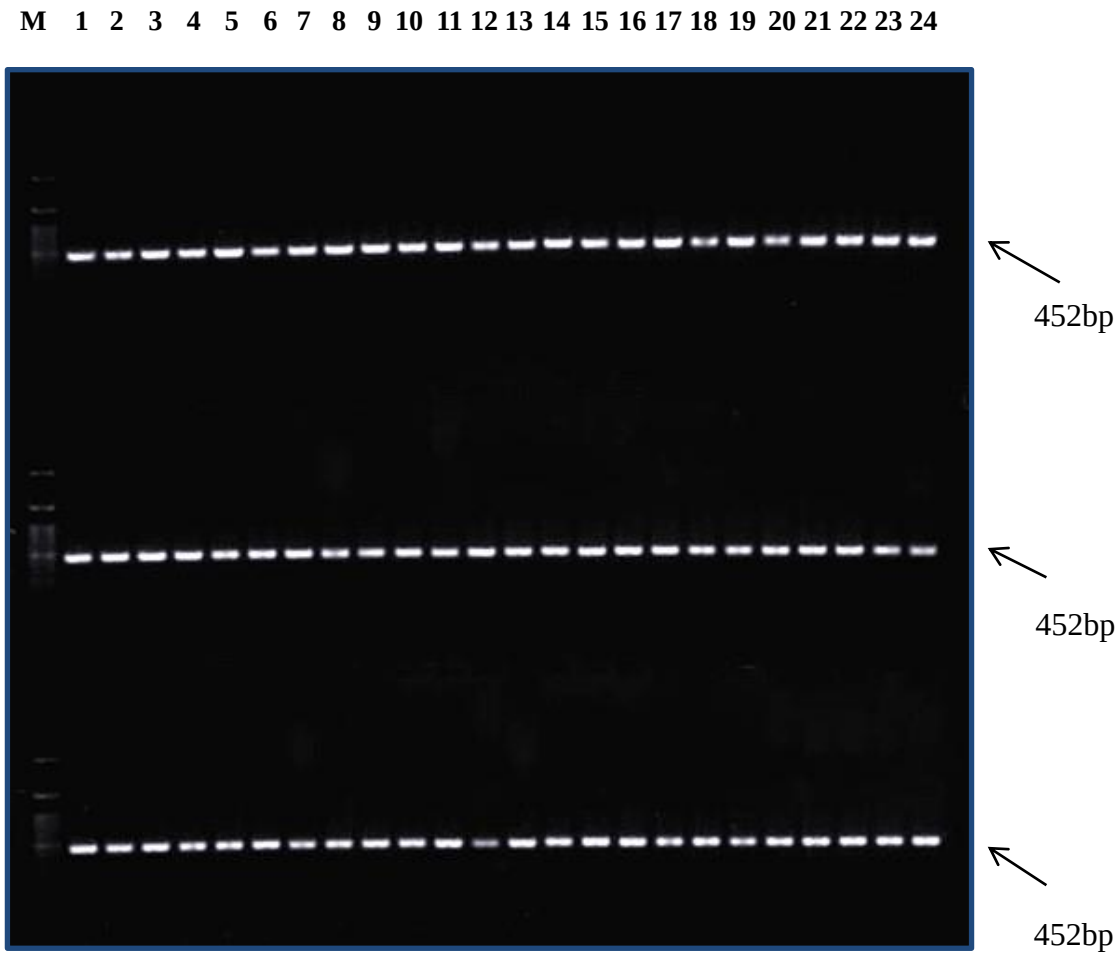

Figure 4.7 Gradient PCR assay to determine the optimum concentration of $MgCl_2$ and annealing temperature for amplification of 452 base pair fragment of cytochrome *b* gene in *L. maculans*. Shown are the agarose gel electrophoresis patterns of PCR products of *L. maculans* isolate M6-2 amplified using 3 pairs of primers (LmCB-P2, LmCB-P3 and LmCB-P5) at different concentrations of $MgCl_2$ with annealing temperatures ranging from 53°C to 63°C. Lanes M: 1kb plus ladder marker; Lanes 1 to 8: Conc. ($MgCl_2$) =1.5mM μl^{-1}; Lanes 9 to 16: Conc. ($MgCl_2$) = 2.0mM μl^{-1}; Lanes 17 to 24: Conc. ($MgCl_2$) =2.5mM μl^{-1}.

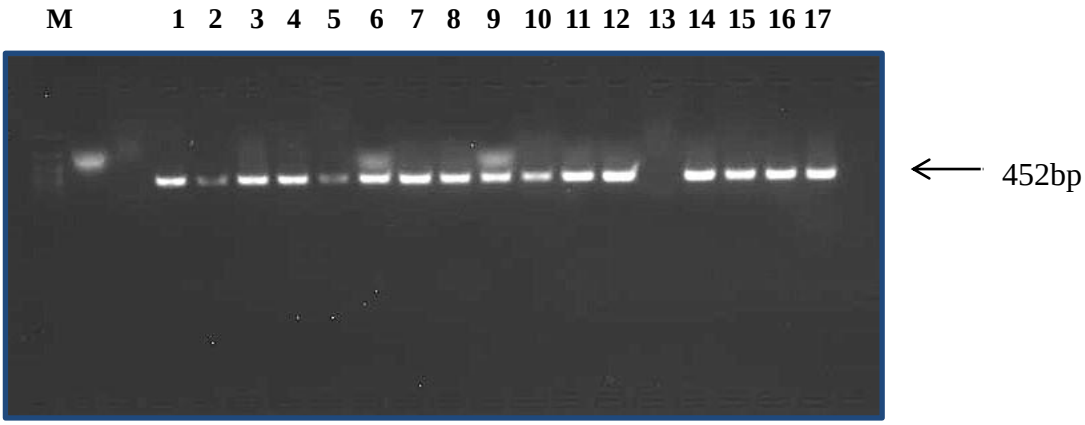

Figure 4.8 PCR assay to detect possible point mutations in cytochrome *b* gene fragment of *L. maculans* isolates using primer pair LmCB-P2. Shown are the agarose gel electrophoresis patterns of PCR products of amplified *L. maculans* isolates. Lanes M: 1kb plus ladder marker; Lane 1 to 17: M6-4, C6-10, C6-2, M6-2, M6-16, M6-11, V6-23, C6-42, V6-8, C7-22, M7-25, V7-8, M7-11, C7-32, V12-25, C12-21 and V12-11.

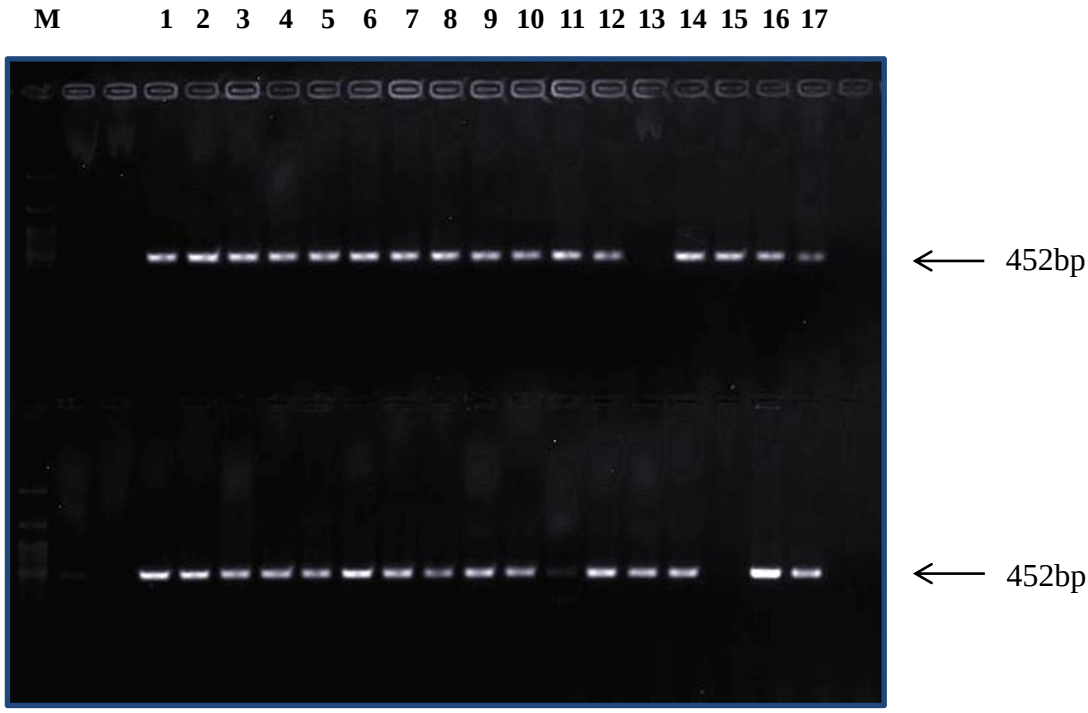

Figure 4.9 PCR assay to detect possible point mutations in cytochrome *b* gene fragment of *L. maculans* isolates using primer pairs LmCB-P3 and LmCB-P5. Shown are agarose gel electrophoresis patterns of amplified PCR products of *L. maculans* isolates; Lanes M: 1kb plus ladder marker; Lane 1 to 17: M6-4, C6-10, C6-2, M6-2, M6-16, M6-11, V6-23, C6-42, V6-8, C7-22, M7-25, V7-8, M7-11, C7-32, V12-25, C12-21 and V12-11.

```
C6-2          AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
C6-10         AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
C6-42         AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
M6-4          AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
C7-22         AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
C7-32         AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
V12-25        AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
V12-11        AGGTAGAATCATTGTCATTAGCTTTATTTACTAAGTTTGGTATAAAAAGTACATTACATA 60
              ************************************************************

C6-2          AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
C6-10         AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
C6-42         AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
M6-4          AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
C7-22         AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
C7-32         AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
V12-25        AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
V12-11        AAAACAATTCAAATTTCCAATTATATATTAAACAAGAATCTATCTCTTTATTAAAAGAAT 120
              ************************************************************

C6-2          TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
C6-10         TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
C6-42         TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
M6-4          TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
C7-22         TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
C7-32         TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
V12-25        TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
V12-11        TGGTGTTACCTTATATGATTCCTAGTATGCATTACAAATTAGGATTATAAATATATATAA 180
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C6-2          GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
C6-10         GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
C6-42         GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
M6-4          GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
C7-22         GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
C7-32         GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
V12-25        GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
V12-11        GTAGTCGTGAGATTAAATATTTAATCTCTAAGTTTCCTACCGGATACTTAAATTATAATA 240
              ************************************************************

C6-2          CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
C6-10         CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
C6-42         CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
M6-4          CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
C7-22         CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
C7-32         CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
V12-25        CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
V12-11        CTTACCCCCCCCCTCTATGGGGACTATCCCTTTTGAGATTGTAGAAAACATTTTGTAAGTT 300
              ************************************************************

C6-2          ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
C6-10         ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
C6-42         ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
M6-4          ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
C7-22         ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
C7-32         ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
V12-25        ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
V12-11        ATTATGGTTTGGCAATGCCAGTAAAAACGGTTAAGATATTAACATATCGCTTAATATTAG 360
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C6-2          ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
C6-10         ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
C6-42         ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
M6-4          ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
C7-22         ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
C7-32         ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
V12-25        ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
V12-11        ACCGTCGGTCATTTTAATATTAGACTAGTCTAATACATGGTCGCTACAGA 410
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Figure 4.10 Comparison of the deduced amino acid sequence of the cytochrome *b* gene fragment of sensitive and insensitive isolates of *L. maculans* isolates. Asterisk indicates the identity of the amino acid in the compared strains.

4.4 Discussion

The study describes the *in vitro* sensitivities of *L. maculans* populations and their reactions to fungicides, and identifies potential changes in sensitivity of the isolates to QoI and DMI fungicides. To the best of our knowledge, this is the first report on QoI and DMI fungicides *in vitro* sensitivity study for *L. maculans* isolates.

The sensitivity of both baseline and fungicide-treated *L. maculans* isolates increased when the chemical SHAM was added to QoI fungicides amended medium. The result suggests that *L. maculans* isolates may utilize the alternative respiration pathway to circumvent complex III in the mitochondrial pathway and germinate in the

presence of high levels of QoI fungicides (Ziogas*et al.*, 1997; Wise *et al.*, 2008). Many pathogenic fungi have also been reported to use the alternative respiration pathway to overcome mycelial growth inhibition caused by QoI fungicides, including *Cercosporazeae-maydis*, *Ascochytarabiei*, *Venturiainaequalis* (Bradley and Pedersen, 2011; Wise *et al.*, 2008; Olaya and Koller, 1999). Ziogas *et al.* reported the presence of alternative respiration pathway in both mutant and wild-type isolates of *Septoria tritici*, suggesting that the strains may not need to be previously exposed to QoI fungicide to be capable of utilizing the alternative oxidation (Ziogas *et al.*, 1997). This is in agreement with the result of this study in which the alternative respiration pathway observed in *L. maculans* strains were never exposed to QoI fungicides. Therefore, it is necessary to include SHAM in QoI fungicide *in vitro* assays to inhibit alternative oxidation of *L. maculans* isolates.

The ranges in the sensitivity of *L. maculans* baseline isolates to pyraclostrobin and propiconazole were relatively narrowed (approximately 10-fold or less). This is similar to the range of *in vitro* baseline sensitivity of other pathogenic fungi previously reported (Pasche *et al.*, 2004; Wong and Wilcox, 2002; Wise *et al.*, 2008). The sensitivity range of *Ascochyta rabiei* baseline isolates to pyraclostrobin was 3-fold whereas the sensitivity range of *Monilinia fructicola* baseline isolates to pyraclostrobin was 9-fold (Wise *et al.*, 2008; Amiri *et al.*, 2010). The isolates of *L. maculans* also exhibited a narrow range of EC_{50} values for a mixture of azoxystrobin and propiconazole, ranging from 0.18 to 0.55 μg ml^{-1}. Similarly, in *Monilinia fructicola*, EC_{50} values for baseline isolates for a mixture of pyraclostrobin and

boscalid ranged from 0.07 to 0.31 μg ml^{-1} (Amiri *et al.*, 2010). Few studies of *in vitro* sensitivities of pathogenic fungi to propiconazole are available. In *Sclerotinia homoeocarpa*, EC_{50} values of isolates exposed to propiconazole ranged from 0.020 to 0.048 μg ml^{-1} (Hsiang *et al.*, 2007). Miller *et al.* (2002) reported the range of EC_{50} value for *Sclerotinia homoeocarpa* isolates was 0.005–0.057 μg ml^{-1}, with a mean of 0.028 μg ml^{-1}.

In the current research, the mycelial growth of *L. maculans* isolates was more inhibited by pyraclostrobin or azoxystrobin compared with propiconazole. Isolates were approximately 4 times more sensitive to pyraclostrobin than to propiconazole when the mean EC_{50} values were compared. This is consistent with other studies in which the strains of *Uncinula necator* were approximately 3 times more sensitive to pyraclostrobin than to trifloxystrobin (Wong and Wilcox, 2002). Among *Ascochyta rabiei* strains, Wise *et al.* (2008) also reported higher sensitivity to pyraclostrobin (10 times) than azoxystrobin, which was taxonomically similar to *L. maculans* isolates tested in the current study. The results of this study revealed the difference in the intrinsic activities of QoI fungicide and the intrinsic activity of pyraclostrobin is always greater than trifloxystrobin or azoxystrobin. Bradley *et al.* (2011) reported that no differences were present between pyraclostrobin and azoxystrobin on gray leaf spot control in field trials suggesting that intrinsic activity of different QoI fungicides may not have a direct correlation with efficacy of fungicides *in vivo* (Bradley *et al.*, 2011; Bradley and Ames, 2009). This is in agreement with our field study which confirmed that there was no significant difference in blackleg incidence and severity

of canola treated with pyraclostrobin and azoxystrobin. However, Amiri *et al.* (2010) reported the higher brown rot incidence was observed when stone fruits were inoculated with *Monilinia fructicola* isolates having higher EC_{50} values, suggesting that the EC_{50} values of plant pathogens may provide a good indication of fungicide efficacy in detached fruit assays.

The significantly higher EC_{50} values to pyraclostrobin were observed for fungicide-treated *L. maculans* isolates compared with the baseline strains collected in 2011 across all study sites, suggesting that a shift toward reduced sensitivity of *L. maculans* populations to QoI might have occurred within the span of this study. However, the presence of less sensitive *L. maculans* isolates in the present study has not noticeably altered the efficacy of strobilurins on blackleg disease control in the field trials conducted. It is possible that repeated applications of site-specific fungicides may cause emerging resistance problems leading to failure of chemical control in the future (Amiri *et al.*, 2010).

Several factors should be kept in mind when considering chemical control of blackleg disease on canola in western Canada. Firstly, fungicide application needs to be employed only when necessary (Brent and Hollomon, 2007; Hewitt, 1998). Several site-specific fungicide products have been registered and marketed for use on canola, including Quadris, Headline and Tilt. The frequent use of a single site-specific fungicide may increase the fungicide selection pressure on less sensitive pathogen populations, leading to the failure of disease control with fungicides (Bradley and

Petersen, 2011). Currently, several non-disease related properties of QoI fungicides have been well used in greenhouse and field practices, including delay of senescence, increased activity of anti-oxidative enzymes and alteration of plant hormone amounts (Grossman *et al.*, 1999; Wu and von Tiedemann, 2002). Secondly, a regional fungicide resistance monitoring program may enable growers to determine and evaluate fungicide management strategies (Ma and Michailides, 2005). For example, the low frequency of azoxystrobin resistant *Alternaria* isolates in pistachio orchards were detected using the allele-specific real-time PCR method and alternative registered fungicides in a different class were applied for control of *Alternaria* late blight (Ma and Michailides, 2005). Thirdly, alternation of fungicides with different mode of actions and fungicide mixture rather than a stand-alone product are recommended to reduce the fungicide selection pressure on pathogen populations (Amiri *et al.*, 2010).

Plant pathogenic fungi could generate variation via sexual recombination leading to an increased risk of development of resistance to fungicide (Brent and Hollomon, 2007; Bradley *et al.*, 2011). Recently, *L. maculans* is known to have a polycyclic disease cycle and sexual recombination occurs in *L. maculans* ascospore release (Li *et al.*, 2006). Although airborne ascospores are considered to be the primary inoculum of blackleg disease, *L. maculans* pycnidiospores as secondary inoculum are the major target of QoI and DMI fungicide application to inhibit the repeated infection. Philion reported that the *in vitro* sensitivity of *Venturia inaequalis* ascospores were higher to flusilazole than that of conidia (Philion, 2007). However, the *in vitro* fungicide

sensitivities of *L. maculans* pycnidia and ascospores were not compared in our study. Moreover, Pasche *et al.* (2004) reported that a shift towards decreased sensitivities of *Alternaria solani* isolates suggests reduced sensitivities in plant fungal pathogens without a known teleomorphic stage.

Due to the biological properties of *L. maculans* isolates and high risk of resistance development for QoI fungicides, establishing baseline *in vitro* sensitivity and detection of shifts towards reduced sensitivity in pathogen populations in our research will be important in a fungicide resistance monitoring program and will provide a more accurate evaluation for fungicide spray strategies to be developed.

The high frequencies of resistance to site-specific fungicides have been reported in a wide variety of phytopathogenic fungi from many host crops worldwide (Ma and Michailides, 2005; Deising *et al.*, 2008). To investigate whether the typical substitutions F129L and G143A in mitochondrial cytochrome *b* (*CYTB*) gene was the mechanism conferring resistance to QoI in western Canadian pathogen populations of *L. maculans*, a collection of 17 field isolates obtained from different study sites, of which 5 were QoI resistant and were screened. In contrast to molecular studies reporting the presence of G143A in many fungal pathogen populations including *Ascochyta rabiei* (Delgado *et al.*, 2013), *Erysiphe graminis* (Sierotzki *et al.*, 2000), *Cercospora beticola* (Bolton *et al.*, 2013) and *Erysiphe necator* (Miles *et al.*, 2012), the G143A mutation was not found in any of the 5 QoI-resistant *L. maculans* field isolates. Moreover, no polymorphism correlating with the QoI-resistant phenotype

was observed in the sequenced part of cytochrome *b* gene among the *L. maculans* field isolates.

Several point mutations in the *CYTB* gene can confer resistance to strobilurin fungicides in plant pathogen populations, but the level of resistance varies with each amino acid change (Luo *et al.*, 2010). The substitution G143A is known to confer complete resistance to respiration inhibitors at the Qo center among phytopathogenic fungi in the literature (Ma and Michailides, 2005; Sierotzki *et al.*, 2000), however, In the study reported by Fernandez-Ortuno *et al.*, none of the F129L and G143A mutation was observed in QoI-resistant field isolates, suggesting that the mechanism of resistance to QoI fungicides in *Podosphaera fusca* isolates were not linked with G143A and F129L amino acid changes in the cytochrome *b* gene (Fernandez-Ortuno *et al.*, 2008). Steinfeld *et al.* reported that substitution G143A was found in some of QoI resistant *Venturia inaequalis* field isolates but not in others (Steinfeld *et al.*, 2002). Similar result has also been reported for the fungus *Podosphaera fusca* where G143A substitution was present only in two QoI-resistant isolates but not observed in the third one (Ishii *et al.*, 2001).

The F129L point mutation in cytochrome *b* gene, which confers moderate resistance, was also observed in many phytopathogenic fungi, including *Colletotrichum cereale* (Young *et al.*, 2010), *Pyricularia grisea* (Kim *et al.*, 2003) and *Alternaria solani* (Pasche *et al.*, 2013). Leiminger *et al.* reported that the occurrence of F129L mutation was investigated in a collection of 203 *Alternaria solani* field

isolates in Germany, of which 74 strains contained the F129L substitution in the *CYTB* gene (Leiminger *et al.*, 2013). In the study reported by Young *et al.*, the *in vitro* assay conducted on fungicide-amended media was consistent with the molecular characterization showing a moderate resistance to QoI fungicide caused by F129L mutation in the cytochrome *b* gene in the isolates of *Colletotrichum cereale* (Young *et al.*, 2010). The substitution G137R can also confer comparatively low level of resistance to QoI fungicide (Sierotzki *et al.*, 2007). This point mutation was detected in two strains out of 250 isolates of *Pyrenophora tritici-repentis*, indicating a very low level of frequency for G137R mutation (Sierotzki *et al.*, 2007).

Data derived from ClustalW sequence alignment analysis of fragment of cytochrome *b* gene in *L. maculans* isolates revealed that no amino acid polymorphism was observed between QoI-resistant and QoI-sensitive isolates, indicating a high degree of conservation for the sequenced region of *CYTB* gene. The amino acid sequence of cytochrome *b* gene was reported to be highly conserved within a single species; little variation was observed between different species (Sierotzki *et al.*, 2007; Torriani *et al.*, 2009). However, in the study reported by Fernandez-Ortuno *et al.*, QoI-resistant *Podosphaera fusca* field strains contained more amino acid polymorphisms than QoI-sensitive isolates and majority of sensitive isolates were closely related to each other, forming an independent cluster separated from the most of QoI-resistant isolates (Fernandez-Ortuno *et al.*, 2008). The high degree of genetic variation at the nucleotide level can cause the accumulation of mutations in *CYTB* gene alleles from resistant strains, leading to development of resistance to QoI

fungicide at a higher frequency because of the electron transport inhibition by respiration inhibitor fungicides and occurrence of mutants under selection pressure (Fernandez-Ortuno *et al.*, 2008; Bohr and Anson, 1999).

A final confirmation of the linkage between the resistance mechanism to strobilurins in *L. maculans* isolates and the typical point mutations in the cytochrome *b* gene is still pending and may be achieved with the further characterization of gene fragments which includes all positions which are considered to affect the sensitivity to Qo center inhibitors (Ma *et al.*, 2002). The presence or absence of point mutation in *CYTB* gene is consistent with the selection pressure action on this gene, demonstrating the importance of the gene in cellular respiration and leading to the rapid increase of frequency of resistance to respiration inhibitors fungicide in pathogen populations when mutations occur (Gisi *et al.*, 2005; Torriani *et al.*, 2009). If resistance to QoI fungicide emerges, molecular methods will be very useful and efficient to detect the mechanisms of resistance in phytopathogenic fungi and optimize the chemical control strategies for growers (Torriani *et al.*, 2009; Ma and Michailides, 2005).

CHAPTER 5

5.0 AGGRESSIVENESS OF *Leptosphaeria maculans* ISOLATES INOCULATED ON CANOLA UNDER GREENHOUSE CONDITIONS

Abstract

Leptosphaeria maculans isolates causing blackleg disease on canola were collected from three experimental sites (Carman, Melfort and Vegreville) in western Canada in the 2012 field season. Eleven *L. maculans* isolates representing a range of *in vitro* sensitive diversity response to strobilurin fungicides were characterized for their aggressiveness on the sensitive genotype Westar and on the moderately resistant genotype 43E01 under controlled environmental conditions. The aggressiveness of *L. maculans* isolates was determined based on blackleg disease severity by estimating the area of diseased tissue in the cross-section at the pod-filling stage. Significant differences were observed between the two canola genotypes, with Westar having significant higher disease severity than 43E01. Also, there were significantly high variations in blackleg severity among the isolates, ranging from 1.44 to 4.29 for those isolates on Westar, and from 0.12 to 3.13 for the isolates on 43E01. No significant correlation was detected between aggressiveness and *in vitro* fungicide sensitivity in *L. maculans* populations, suggesting that resistant strains may not suffer from a significant aggressiveness cost.

5.1 Introduction

Blackleg (phoma stem canker), caused by the fungus *Leptosphaeria maculans*, is the most important disease on canola. Severe epidemics often occur and that causes substantial yield losses in many regions of the world, including Europe, Australia and North America (Dilmaghani *et al.*, 2009; Howlett, 2004; Fitt *et al.*, 2006). In western Canada, *Leptosphaeria maculans* isolates were first isolated from oilseed rape in Saskatchewan in 1975 and since then the pathogen has been widespread across the major canola growing areas of the Prairie Provinces (Gugel and Petrie, 1992). Yield losses have been reported as high as 50% in commercial fields on susceptible varieties (Gugel and Petrie, 1992).

Phoma stem canker is generally a polycyclic fungal disease and its epidemic is usually established by air-borne *L. maculans* ascospores as the main inoculum and rain-splashed conidia as secondary infection (Hall, 1992; Mahuku *et al.*, 1997). However, Ghanbarnia *et al.* (2011) reported a high level of disease incidence and severity of infected canola plants without the presence of ascospores, demonstrating that pycnidiospores could be considered as a main inoculum for disease infection in western Canada. The integrated management strategies for blackleg control are recommended to canola growers in western Canada, including planting disease resistant varieties (Dusabenyagasani and Fernando, 2008), rotating canola with other crop species (Kutcher *et al.*, 2013), using pathogen free seed and seed treatment (Rempel and Hall, 1995), and adopting tillage practice as well as foliar fungicide

application during the growing season (Fitt *et al.*, 2006; West *et al.*, 2001; Kutcher *et al.*, 2011).

Studies on genetic resistance of host plants and especially the assessment of varietal variations in disease responses have led us to better understand the principals of susceptibility, and such knowledge has been used to rank canola varieties for their resistance or susceptibility (Delourme *et al.*, 2006; Rouxel *et al.*, 2003). The release of a number of genetic resistant varieties over the years in western Canada has improved the effectiveness of blackleg management in canola. However, the breakdown of race-specific resistance due to genetic changes in pathogen populations has been reported in western Canada as well as in the other regions of the world (Li *et al.*, 2003; Rouxel *et al.*, 2003; Kutcher *et al.*, 2010).

Timely and effective fungicide treatments still remain a major approach in disease management (Kutcher *et al.*, 2013; Rempel and Hall, 1995). For blackleg control on canola, two classes of fungicides have been registered during the recent two decades. However, the increased use of site-specific fungicides may pose a high risk of resistance development in many fungal species (Bradley and Pedersen, 2011; Sierotzki *et al.*, 2000). In response to the challenge of the development of blackleg resistance, a monitoring program has been conducted to detect the shift of *in vitro* sensitivity of *L. maculans* isolates to QoI and DMI fungicides in western Canada.

Aggressiveness, defined as a quantitative component of pathogenicity without specific relevance to host genotypes (Van der Plank, 1968), is the key characteristic

being considered in plant epidemiology, particularly the host-pathogen interaction and their evolution for dynamics of disease (Pariaud *et al.*, 2009). Several quantitative traits of pathogens are often used as the criteria of the aggressiveness, including disease severity, sporulation rate, the number of lesions, lesion size, and lesion latent period (Boedo *et al.*, 2012; Pariaud *et al.*, 2009). However, little is known about how the aggressiveness of *L. maculans* isolates may be correlated with the reduced *in vitro* fungicide sensitivity in canola crops.

The objectives of this study were to determine 1) whether the development of pathogen resistance to QoIs is associated with fitness costs, expressed by the aggressiveness of QoI-resistant and QoI-sensitive *L. maculans* isolates; and 2) whether the canola cultivars with different resistance levels to blackleg perform similarly to QoI-resistant and QoI-sensitive *L. maculans* isolates under the Canadian prairie conditions.

5.2 Materials and Methods

5.2.1 Fungal isolates

Seven QoI-resistant and four QoI-sensitive isolates of *L. maculans* were used for the assessment of aggressiveness in this study. All the isolates were collected from three experimental sites (Carman in Manitoba; Melfort in Saskatchewan; and Vegreville in Alberta) in 2012, and blackleg strains were isolated from the collected

stubble material. Single spore isolations were carried out. The methods of isolation and *in vitro* sensitivity tests have been described in chapter three above. Details on the *in vitro* sensitivity to strobilurins of isolates and the locations are given below (Table 5.1).

Table 5.1 Levels of *in vitro* sensitivity of *L. maculans* isolates to QoI fungicide at the different western Canada locations in 2012.

Isolate code	Location	EC_{50} value(μg ml^{-1})[1]	QoI sensitivity[2]
C6-2	Carman	2.413	resistant
C6-10	Carman	6.940	resistant
M6-2	Melfort	2.210	resistant
M6-4	Melfort	9.197	resistant
M6-17	***Melfort***	***0.595***	***sensitive***
V6-23	***Vegreville***	***0.730***	***sensitive***
C7-22	Carman	2.630	resistant
M7-11	**Melfort**	***0.432***	***sensitive***
V12-11	**Vegreville**	***0.271***	***sensitive***
V12-25	Vegreville	8.519	resistant
C12-21	Carman	4.916	resistant

[1]EC_{50} value μg ml^{-1}-QoI, based on inhibition of mycelia growth

[2]QoI sensitivity of isolate refer to baseline *in vitro* sensitivity of isolates in 2011

5.2.2 Plant materials and experimental design

The study was conducted in a greenhouse facility at the Department of Plant Science, University of Manitoba, in 2013. The canola genotypes 43E01 (with moderate blackleg resistance) and Westar (with high blackleg susceptibility) were used. The canola plants were grown in plastic pots (18 cm diameter x 65 cm in height)

containing a 2:2:1 mix of sand, peat and manure. Six seeds were hand-planted in each pot, and at the 3-leaf stage seedlings were thinned to 3 per pot. Plants were grown under day/night temperatures of 23/16 ℃ for the period of 16/8 hours, and relative humidity was at 80±10%. Photosynthetic photon flux density was at 500 ±12 µmol m^{-2} sec^{-1} on the canopy level. Plants were watered every another day with the same amount of water for all pots.

A total of 24 treatments including 11 *L. maculans* isolates and untreated control were arranged in a completely randomized block design with seven replicates. There were three plants in each pot for each of the seven replicates. Canola plants were artificially inoculated at the cotyledon stage by injecting a 10µl spore suspension (2 x 10^7conidia ml^{-1}) at the inoculation point. For the control plants, the cotyledons were inoculated with sterilized distilled water.

5.2.3 Disease evaluation

Severity of blackleg disease was assessed at the crop growth stage 5.2 to 5.3 according to the scale of Harper & Berkenkemp (1975). Plants were carefully uprooted, washed with tap water and the Western Canada Canola and Rapeseed Recommending Committee (WCC/RRC) scale was used to evaluate disease severity. Tap roots were cut and the area of diseased tissue in the cross-section was estimated using the set scale by WCC/RRC. Stem disease severity was evaluated using a 0 to 5 scale in which 0=no infection, 1=lesion area less than 25% of the cross-section area of

the crown, 2=25 to 50%, 3=51 to 75%, 4=76 to 100% girdled stem, and 5= tissue dry and brittle, plant was dead (Western Canada Canola/Rapeseed Recommending Committee (WCC/RRC), 2009).

5.2.4 Statistical analysis

Analysis of variance (ANOVA) for blackleg disease severity was performed using the "PROC MIXED" procedure of the SAS software (SAS version 9.2, SAS Institute, Inc., Cary, USA). Isolate and cultivar were considered as fixed effects, and the treatment effect was compared using Fisher's least significant difference (LSD) at the probability level of 5%. Also, the relationship among cultivars, blackleg disease severity and EC_{50} values was determined using the PROC CORR procedure in SAS (version 9.2) software package.

5.3 Results

There was a significant isolate by cultivar interaction for the disease severity (Table 5.2); therefore, the disease response to the various isolates was presented separately for each of the two distinct genotypes.

On the susceptible cultivar Westar, all plants inoculated with *L. maculans* isolates expressed significantly higher blackleg disease severity than the control plants (Figure 5.1). Little or no symptoms were detected on the control plants. There was a large

variation in disease severity among the isolates evaluated, with C6-1, M6-2, M7-11, and C12-21g having the greatest severity values, averaging 4.1, and the C6-10 and M6-17 the lowest, averaging 1.8. There was no obvious difference between the four sensitive isolates and the seven resistant isolates in affecting the disease severity on the susceptible cultivar.

Similarly, on the moderately-resistant genotype 43E01, there were significant differences between the isolates, with the plants inoculated with the isolates of C6-2, M6-2, and V12-25 having the highest disease severity (averaging 2.8) among the isolates assessed (Figure 5.2). The plants inoculated with the isolates M6-10, M6-4, M6-17, and M7-11 had the lowest disease severity, the same as the non-inoculated control.

Across the two genotypes, the aggressiveness among the isolates displayed a continuous change with the severity values ranging from 1.44 to 4.29 for the sensitive genotype Westar and from 0.12 to 3.13 for the resistant genotype 43E01 (Figures 5.1 versus 5.2). On average, the disease severity was greater for Westar (3.01) than for 43E01 (1.29). The mean values of disease severity between the two genotypes were statistically significant (Table 5.1). A significant negative correlation (R^2=0.875) was observed between the aggressiveness and EC_{50} value of *L. maculans* isolates on moderately resistant canola cultivar (43E01), but there was no significant difference found on Westar (data not presented).

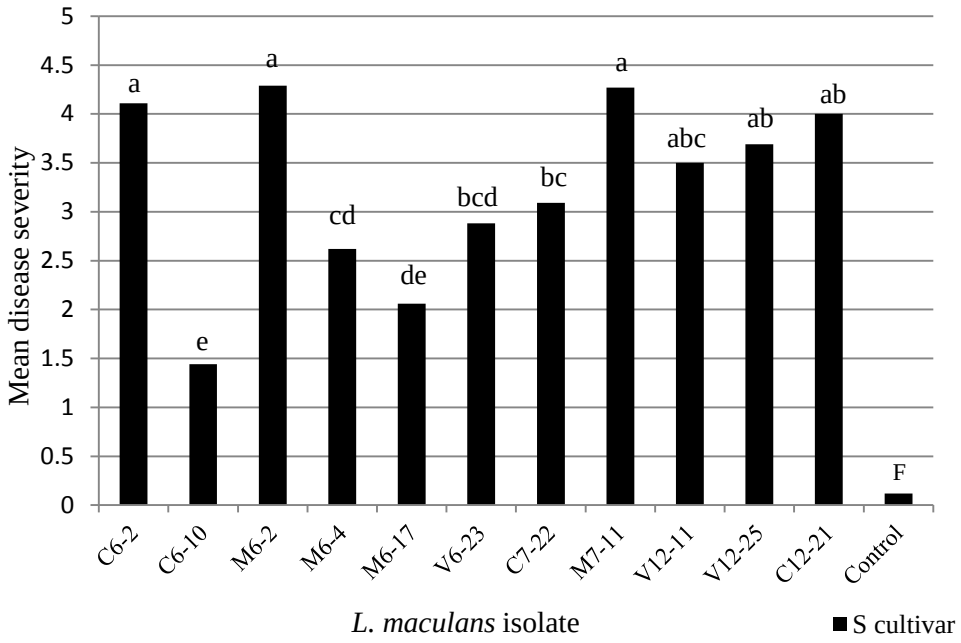

Figure 5.1 Comparison of disease severity on susceptible canola plants inoculated with QoI-sensitive and QoI-insensitive *L. maculans* isolates. Bars with different letters are significantly different (P<0.05) based on Fisher's Least Significant Difference test.

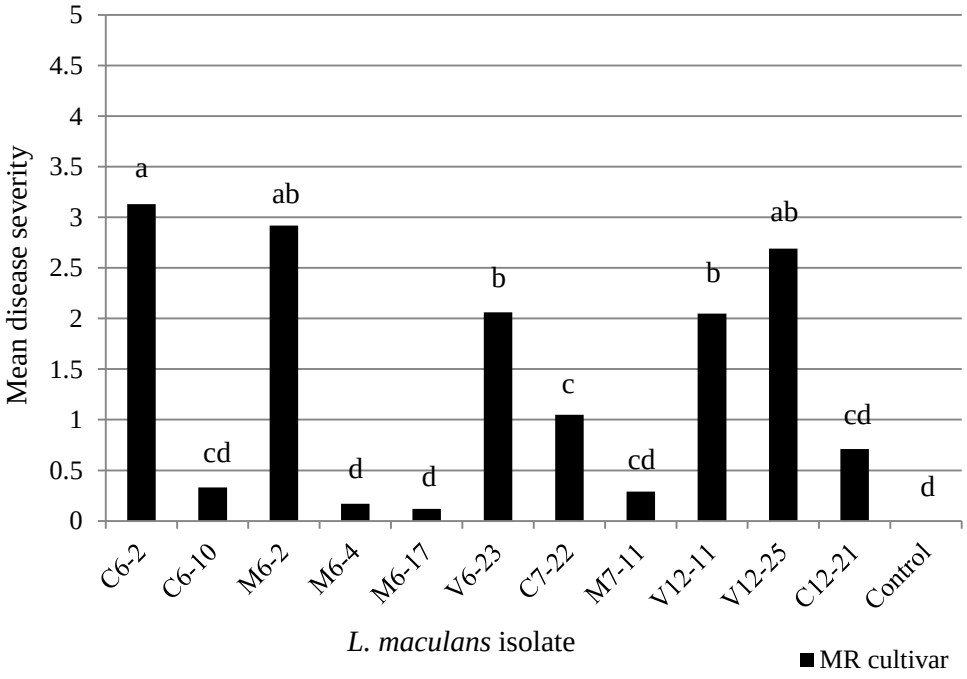

Figure 5.2 Comparison of disease severity on moderately resistant canola plants inoculated with QoI-sensitive and QoI-insensitive *L. maculans* isolates. Bars with distinct letters are significantly different ($P<0.05$) based on the Least Significant Difference test.

Table 5.2 Analysis of variances for the effect of isolates, cultivars and their interaction for the mean disease severity of blackleg on canola inoculated with QoI-sensitive and QoI-resistant *L. maculans* isolates.

		Mean disease severity			
Source of variance	DF	Sums of square	Mean square	*F Value*	Pr>F
Isolate	11	457.50	41.59	25.84	<0.0001
Cultivar	1	254.26	254.26	157.97	<0.0001
Isolate*Cultivar	11	101.50	9.23	5.73	<0.0001
Residual	359	577.83	1.61	……	……

5.4 Discussion

To evaluate variations in *L. maculans* aggressiveness, we selected a wide range of isolates from the major canola growing areas of western Canada. This effort allowed the detailed assessment of the isolate ranging from sensitive to resistant to their in vitro sensitivity to strobilurin fungicides. Many approaches may be used to evaluate in vitro sensitivity to fungicides in field crops, but the aggressiveness, defined as the relative ability of an organism to colonize and cause damage to host plants without distinguishing between quantitative and qualitative aspects (Shurtleff and Averre, 1997), has been shown to provide an adequate assessment on canola crops. Therefore, in the present study, aggressiveness which is evaluated with disease severity was employed to determine the differences between isolates and between genotypes in their sensitivity to the strobilurin fungicides. Previous studies have shown that the susceptible cultivar Westar has no major resistance genes with all *L.*

maculans isolates being virulent, according to gene-for-gene interaction model. In the present study, significantly large intra-species variations in aggressiveness were found among the *L. maculans* isolates on Westar under controlled environmental conditions.

Several studies have reported the variability in aggressiveness among strains belonging to the same pathotype or the pathotype sharing similar genotype. Carlisle *et al.* (2002) found highly significant variations in aggressiveness referring to lesion size, sporulation capacity and latent period within 15 *Phytophthora infestans* strains with the same genetic background. Flier and Turkensteen (1999) tested the potential correlations of aggressiveness variability among 36 *P. infestans* isolates collected from the potato (*Solanum tuberosum L.*) growing regions of Netherlands; they found high variations in aggressiveness (infection efficiency, latent period and maximal growth rate) among isolates under controlled conditions. Significant variations were also observed for the aggressiveness parameters of incubation period, frequency of lesion and latent period among 43 *Cercospora arachidicola* isolates (Tuggle *et al.*, 1999). However, in our study, the correlation between blackleg disease and in vitro fungicide sensitivity of *L. maculans* strains was not significant on Westar, suggesting that disease severity may not be the best parameter for the assessment of in vitro sensitivity of pathogen isolates to strobilurin fungicides on susceptible canola genotypes. Similar to our results, it was previously documented that no significant correlation was observed between the in intro fungicide sensitivity and aggressiveness of *Alternaria dauci* isolates (Rogers and Stevenson, 2010).

The moderately resistant genotype 43E01 carries blackleg resistance gene *Rlm2* and *Rlm3* (data not presented). In the present study, a significantly high blackleg severity was observed with *L. maculans* isolates containing the avirulent gene *AvrLm2*, as shown by the isolates C6-2, M6-2. In contrast, a significantly low severity was found among isolates of *L. maculans* with the avirulent gene *AvrLm3* alone as shown by the isolates M6-4, M6-17 or a combination of *AvrLm2* and *AvrLm3* as shown by the isolates C7-22 and M7-11. The cost of qualitative virulence has been reported to the decreased pathogen aggressiveness resulting from a mutation from avirulence to qualitative virulence (Pariaud *et al.*, 2009). However, it may be difficult to elaborate the correlation between aggressiveness cost and virulence gene (Menzies and MacNeil, 1987). Although some studies have been conducted to estimate this cost in pathogen populations, the results are usually inconclusive especially with indirect measurements (Grant and Archer, 1983). The present aggressiveness analysis did not reveal any direct evidence of virulence penalty among *L. maculans* isolates because the impact of genetic background cannot be easily explained by the avirulence gene alone.

Fitness, defined as the capability to survive and produce as compared to other isolates under the same environmental conditions, is an essential quantitative and frequently evolved trait linked with the concept of aggressiveness in plant disease epidemiology (Galvani, 2003). Pathogen fitness is considered to play a key role in the evolution of resistance to fungicides in pathogen populations and many studies have been done investigating the correlations between fitness and survival ability in

pathogen populations (Skylakakis, 1987; Karaoglanidis *et al.*, 2001). Under fungicide-treatment conditions, resistant strains may arise at a low natural rate through single or multiple genetic mutations; when combined with selection pressure from fungicides, these isolates may become dominant in pathogen populations over time. Since it has been assumed that fungicide-resistant isolates are less ecologically fit than the sensitive strains, a decrease of resistance frequency may occur in the absence of fungicide treatment (Karaoglanidis *et al.*, 2001; Ma and Michailides, 2005). However, the contradictory evidences on sensitivity related fitness cost have been observed among many fungal species. Some studies show a fitness penalty associated with development of resistance to fungicides (Holmes and Eckert, 1995), while in other research no fitness reduction is noted in fungicide resistant isolates (Peever and Milgroom, 1994; Karaoglanidis *et al.*, 2001).

CHAPTER 6

6.0 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

Fungicide application plays an important role in integrated blackleg control strategies in many canola-growing regions worldwide (Fitt *et al.*, 2006; West *et al.*, 2001). In western Canada, two groups of site-specific fungicides, Quinone outside inhibitor (QoI) and Demethylation inhibitor (DMI), are registered for blackleg disease control (Kutcher *et al.*, 2011). In the present study, fungicides generally reduced the blackleg incidence and severity on susceptible canola cultivars and significant difference in disease severity was observed between QoI (pyraclostrobin and azoxystrobin) fungicide treatments and unsprayed checks in each of three years, demonstrating the effectiveness of strobilurins for blackleg control. However, no significant difference in seed yield was found with any of the fungicide treatments on Westar in 2011 and 2012, suggesting that foliar fungicide application produces variable benefits to canola seed yield on susceptible canola cultivar. This variation was possibly related to disease pressure and the yield benefit was noticeable when disease severity was high. This is in agreement with the study reported by Bailey *et al.* (2000), indicating that fungicide application with four-year crop rotation often decreased the disease severity, but did not increase the seed yield and quality.

In the present study, a 2-4 leaf stage fungicide application was more effective for blackleg control than an application just prior to bolting, demonstrating that the optimal application timing of fungicide against blackleg in canola is one of the key practices. Banniza *et al.* (2011) reported that protectant fungicides need to be applied

before rain events and systemic fungicides within a short window after a rain event. Therefore, Application timing is critical in reference to the onset of a disease epidemic, the growth stage of crops and the rain events. The results of this study showed that a single application at the 2-4 leaf stage was comparable to two applications (i.e., at the 2-4 leaf stage and prior to bolting), suggesting that multiple applications did not show added benefit in terms of seed yield.

The results in the present study revealed that QoI fungicides did not consistently increase yield on Westar and had no significant effect on seed yield in moderately resistant (43E01) and resistant (45H29) cultivars, suggesting that level of resistance in cultivar has an ultimate effect on the efficacy of chemical control for blackleg. These findings were consistent with a similar field study done by Kutcher *et al.* (2013) in Saskatchewan, indicating that fungicide application was not necessary on blackleg-resistant cultivars and only a small yield improvement was observed on susceptible canola cultivars in one of the three years. Additional fungicide applications for disease management increase input expenses and are only economical when the increase of seed yield or quality can offset this additional cost.

The studies in chapter 4 established a baseline *in vitro* sensitivity of *L. maculans* isolates and detected *L. maculans* isolates with potential decreased fungicide sensitivity in the pathogen population. The ranges in sensitivities of *L. maculans* baseline isolates to fungicides were relatively narrowed and the mycelial growth of *L. maculans* isolates was more inhibited by pyraclostrobin or azoxystrobin

relative to propiconazole. This is similar to the range of *in vitro* baseline sensitivity of other pathogenic fungi previously established (Bradley and Pedersen, 2011; Pasche *et al.*, 2004; Amiri *et al.*, 2010). The present study also proposed that *L. maculans* isolates could utilize the alternative respiration pathway to overcome mycelial growth inhibition by QoI fungicides, which has been reported in several phytopathogenic fungi (Wise *et al.*, 2008; Bradley and Pedersen, 2011). The significantly increased EC_{50} values to QoI fungicides recorded in *L. maculans* populations in 2012 across study sites, suggest a possible shift towards reduced sensitivity for some of the *L. maculans* strains. However, the emergence of less sensitive *L. maculans* isolates in this study has not distinctively altered the efficacy of strobilurins against blackleg disease in the field trial.

Molecular characterization based on the comparison of amino acid sequences of the cytochrome *b* protein among *L. maculans* strains showed that the G143A mutation was not found in any of the 13 QoI-resistant *L. maculans* field isolates. Moreover, no polymorphism correlating with the QoI-resistant phenotype was observed in the sequenced part of cytochrome *b* gene among the *L. maculans* field isolates. In the study reported by Fernandez-Ortuno *et al.* (2008), none of the F129L and G143A mutation was observed in QoI-resistant field isolates, suggesting that the mechanism of resistance to QoI fungicides in *Podosphaera fusca* isolates may not be linked with G143A and F129L amino acid changes in the cytochrome *b* gene.

The aggressiveness test in chapter 5 revealed the significant differences between

the two canola genotypes, with Westar having significant higher disease severity than 43E01. Also, there was significantly high variation in blackleg severity among the isolates, but no significant correlation was observed between aggressiveness and *in vitro* fungicide sensitivity in *L. maculans* isolates. These results suggest that resistant strains may not suffer from a significant aggressiveness cost. Carlisle *et al.* (2002) also reported highly significant variations in aggressiveness referring to lesion size, sporulation capacity and latent period among 15 *Phytophthora infestans* isolates with the same genetic background. Similar to our results, Rogers and Stevenson (2010) documented that no significant correlation was found between the *in vitro* fungicide sensitivity and aggressiveness of *Alternaria dauci* isolates.

In summary, the studies described in this thesis attempted to evaluate the performance on several aspects with currently registered fungicides for blackleg disease control on canola under Manitoba field conditions. In addition, fungicide resistance monitoring program for *L. maculans* populations based on *in vitro* sensitivity and molecular identification of typical point mutations are crucial for the detection of fungicide insensitive *L. maculans* isolates under selection pressure by site-specific fungicides. Therefore understanding the behaviors of these fungicides and possible shift towards reduced sensitivity in *L. maculans* populations is important in integrated blackleg disease management and optimization of chemical control strategies for canola growers in western Canada.

LITERATURE CITED

Amiri, A., Brannen, P., & Schnabel, G. (2010). Reduced sensitivity in *Monilinia fructicola* field isolates from South Carolina and Georgia to respiration inhibitor fungicides. Plant Disease, 94(6), 737-743.

Aoki, Y., Hada, Y., & Suzuki, S. (2013). Development of a multiplex allele‐specific primer PCR assay for simultaneous detection of QoI and CAA fungicide resistance alleles in *Plasmopara viticola* populations. Pest management science, 69(2), 268-273.

Appelqvist, L.-A., & Ohlson, R. (1972). Rapeseed: cultivation, composition, processing and utilization: Elsevier.

Avila-Adame, C., Olaya, G., & Köller, W. (2003). Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin-related QoI fungicides. Plant Disease, 87(12), 1426-1432.

Bailey, K., Gossen, B., Gugel, R., & Morrall, R. (2003). Diseases of field crop crops in Canada: APS Press, Canada.

Bailey, K., Johnston, A., Kutcher, H., Gossen, B., & Morrall, R. (2000). Managing crop losses from foliar diseases with fungicides, rotation, and tillage in the Saskatchewan Parkland. Canadian Journal of Plant Science, 80(1), 169-175.

Balba, H. (2007). Review of strobilurin fungicide chemicals. Journal of Environmental Science and Health Part B, 42(4), 441-451.

Balesdent, M., Attard, A., Kühn, M., & Rouxel, T. (2002). New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology, 92(10), 1122-1133.

Balesdent, M.-H., Gall, C., Robin, P., & Rouxel, T. (1992). Intraspecific variation in soluble mycelial protein and esterase patterns of *Leptosphaeria maculans* French

isolates. Mycological research, 96(8), 677-684.

Banniza, S., Armstrong-Cho, C., Gan, Y., & Chongo, G. (2011). Evaluation of fungicide efficacy and application frequency for the control of *ascochyta* blight in chickpea. Canadian Journal of Plant Pathology, 33(2), 135-149.

Banno, S., Yamashita, K., Fukumori, F., Okada, K., Uekusa, H., Takagaki, M., Kimura, M., Fujimura, M. (2009). Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome *b* gene. Plant Pathology, 58(1), 120-129.

Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., & Pratella, G. (2003). Studies on thiabendazole resistance of *Penicillium expansum* of pears: pathogenic fitness and genetic characterization. Plant Pathology, 52(3), 362-370.

Barbetti, M. J., & Khangura, R. K. (1999). Managing blackleg in the disease-prone environment of Western Australia. Paper presented at the Proceedings of the 10th international rapeseed congress.

Bartett, D. W., Clough, J. M., Godfrey, C. R., Godwin, J. R., Hall, A. A., Heaney, S. P., & Maund, S. J. (2001). Understanding the strobilurin fungicides. Pesticide Outlook, 12(4), 143-148.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr‐Dobrzanski, B. (2002). The strobilurin fungicides. Pest management science, 58(7), 649-662.

Blenis, P. V., Chow, P. S., & Stringam, G. R. (1999). Effects of burial, stem portion and cultivar on the decomposition of canola straw. Canadian Journal of Plant Science, 79(1), 97-100.

Boedo, C., Benichou, S., Berruyer, R., Bersihand, S., Dongo, A., Simoneau, P., Lecomte, M., Briard, M., Le Clerc, V., Poupard, P. (2012). Evaluating aggressiveness

and host range of *Alternaria dauci* in a controlled environment. Plant Pathology, 61(1), 63-75.

Bokor, A., Barbetti, M., Brown, A., MacNish, G., & Wood, P. M. (1975). Blackleg of rapeseed. Journal of Agriculture, Western Australia, 16(1), 7.

Bolton, M. D., Rivera, V., & Secor, G. (2013). Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. Pest management science, 69(1), 35-39.

Bradley, C., & Ames, K. (2009). Evaluation of foliar fungicides on corn in Auburn, Illinois, 2008. Plant Disease Management Reports, 3.

Bradley, C., Lamey, H., Endres, G., Henson, R., Hanson, B., McKay, K., Halvorson, M., LeGare, D., Porter, P. (2006). Efficacy of fungicides for control of *Sclerotinia* stem rot of canola. Plant Disease, 90(9), 1129-1134.

Bradley, C., & Pedersen, D. (2011). Baseline sensitivity of *Cercospora zeae-maydis* to quinone outside inhibitor fungicides. Plant Disease, 95(2), 189-194.

Brent, K. J., & Hollomon, D. W. (1995). Fungicide resistance in crop pathogens: how can it be managed? : Citeseer.

Brown, A., Barbetti, M., & Wood, P. (1976). Effect of benomyl on blackleg disease of rape in Western Australia. Animal Production Science, 16(79), 276-279.

Canola council of Canada, (2013). http://www.canolacouncil.org

Carlisle, D., Cooke, L., Watson, S., & Brown, A. (2002). Foliar aggressiveness of Northern Ireland isolates of *Phytophthora infestans* on detached leaflets of three potato cultivars. Plant Pathology, 51(4), 424-434.

Chen, C.-Y., & Howlett, B. J. (1996). Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves of Indian mustard (*Brassica juncea*) inoculated

with avirulent isolates of *Leptosphaeria maculans*. Physiological and Molecular Plant Pathology, 48(2), 73-81.

Daun, J. K., Eskin, N. A. M., & Hickling, D. (2011). Canola: chemistry, production, processing, and utilization: AOCS Press.

Deising, H. B., Reimann, S., & Pascholati, S. F. (2008). Mechanisms and significance of fungicide resistance. Brazilian Journal of Microbiology, 39(2), 286-295.

Del R ó, L., Bradley, C., Henson, R., Endres, G., Hanson, B., McKay, K., Halvorson, M., Porter, P., Le Gare, D., Lamey, H. (2007). Impact of *Sclerotinia* stem rot on yield of canola. Plant Disease, 91(2), 191-194.

Delgado, J., Lynnes, T., Meinhardt, S., Wise, K., Gudmestad, N., Bradley, C., Markell, S., Goswami, R. (2013). Identification of the mutation responsible for resistance to QoI fungicides and its detection in *Ascochyta rabiei* (teleomorph *Didymella rabiei*). Plant Pathology, 62(3), 688-697.

Delourme, R., Chevre, A., Brun, H., Rouxel, T., Balesdent, M., Dias, J., Salisbury, P., Renard, M., Rimmer, S. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). European Journal of Plant Pathology, 114(1), 41-52.

Delye, C., Bousset, L., & Corio-Costet, M.-F. (1998). PCR cloning and detection of point mutations in the eburicol 14a-demethylase (CYP51) gene from *Erysiphe graminis f. sp. hordei*, a "recalcitrant" fungus. Current genetics, 34(5), 399-403.

Dilmaghani, A., Balesdent, M., Didier, J., Wu, C., Davey, J., Barbetti, M., Li, H., Moreno‐Rico, O., Phillips, D., Despeghel, J. (2009). The *Leptosphaeria maculans*–*Leptosphaeria biglobosa* species complex in the American continent. Plant Pathology, 58(6), 1044-1058.

Dilmaghani, A., Gladieux, P., Gout, L., Giraud, T., Brunner, P., Stachowiak, A.,

Balesdent, M., Rouxel, T. (2012). Migration patterns and changes in population biology associated with the worldwide spread of the oilseed rape pathogen *Leptosphaeria maculans*. Molecular ecology, 21(10), 2519-2533.

Donald, E., Jaudzems, G., & Porter, I. (2008). Pathology of cortical invasion by *Plasmodiophora brassicae* in clubroot resistant and susceptible *Brassica oleracea* hosts. Plant Pathology, 57(2), 201-209.

Downey, R. K., & Harvey, B. L. (1963). Methods of breeding for oil quality in rape. Canadian Journal of Plant Science, 43(3), 271-275.

Dusabenyagasani, M., & Fernando, W. (2008). Development of a SCAR marker to track canola resistance against blackleg caused by *Leptosphaeria maculans* pathogenicity group 3. Plant Disease, 92(6), 903-908.

Elliott, V., & Marcroft, S. (2011). Foliar fungicide for blackleg control.

Fernández-Ortuño, D., Pérez-García, A., López-Ruiz, F., Romero, D., De Vicente, A., & Torés, J. (2006). Occurrence and distribution of resistance to QoI fungicides in populations of *Podosphaera fusca* in south central Spain. European Journal of Plant Pathology, 115(2), 215-222.

Fernández‐Ortuño, D., Tores, J. A., de Vicente, A., & Pérez‐García, A. (2008). Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome *b* gene. Pest management science, 64(7), 694-702.

Fernández-Ortuño, D., Torés, J. A., De Vicente, A., & Pérez-García, A. (2010). Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology, 11(1), 1-9.

Fernando, W., Nakkeeran, S., Zhang, Y., & Savchuk, S. (2007). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on

canola petals. Crop Protection, 26(2), 100-107.

Fitt, B. D., Brun, H., Barbetti, M., & Rimmer, S. (2006). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*) Sustainable strategies for managing *Brassica napus* (oilseed rape) resistance to *Leptosphaeria maculans* (phoma stem canker) (pp. 3-15): Springer.

Flier, W., & Turkensteen, L. (1999). Foliar aggressiveness of *Phytophthora infestans* in three potato growing regions in the Netherlands. European Journal of Plant Pathology, 105(4), 381-388.

Fontaine, S., Remuson, F., Fraissinet‐Tachet, L., Micoud, A., Marmeisse, R., & Melayah, D. (2009). Monitoring of *Venturia inaequalis* harbouring the QoI resistance G143A mutation in French orchards as revealed by PCR assays. Pest management science, 65(1), 74-81.

Ghanbarnia, K., Fernando, W., & Crow, G. (2009). Developing rainfall-and temperature-based models to describe infection of canola under field conditions caused by pycnidiospores of *Leptosphaeria maculans*. Phytopathology, 99(7), 879-886.

Ghanbarnia, K., Fernando, W., & Crow, G. (2011). Comparison of disease severity and incidence at different growth stages of naturally infected canola plants under field conditions by pycnidiospores of Phoma lingam as a main source of inoculum. Canadian Journal of Plant Pathology, 33(3), 355-363.

Gisi, U., Chin, K., Knapova, G., Küng Färber, R., Mohr, U., Parisi, S., Sierotzki, H., Steinfeld, U. (2000). Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Protection, 19(8), 863-872.

Gisi, U., Sierotzki, H., Cook, A., & McCaffery, A. (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest management science, 58(9), 859-867.

Gladders, P., & Musa, T. (1980). Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. Plant Pathology, 29(1), 28-37.

Gossen, B., McDonald, M., Hwang, S., Kalpana, K., & Peng, G. (2009). Managing seeding date to minimize clubroot (*Plasmodiophora brassicae*) damage in canola and vegetable *brassicas*. Paper presented at the Proceedings of International Clubroot Workshop 2009.

Grant, M., & Archer, S. (1983). Calculation of selection coefficients against unnecessary genes for virulence from field data. Phytopathology, 73(4), 547-551.

Grasso, V., Sierotzki, H., Garibaldi, A., & Gisi, U. (2006). Characterization of the cytochrome *b* gene fragment of *Puccinia* species responsible for the binding site of QoI fungicides. Pesticide biochemistry and physiology, 84(2), 72-82.

Grossmann, K., Kwiatkowski, J., & Caspar, G. (1999). Regulation of Phytohormone Levels, Leaf Senescence and Transpiration by the Strobilurin Kresoxim-methyl in Wheat (*Triticum aestivum*). Journal of Plant Physiology, 154(5), 805-808.

Gugel, R., & Petrie, G. (1992). History, occurrence, impact, and control of blackleg of rapeseed. Canadian Journal of Plant Pathology, 14(1), 36-45.

Guo, X., & Fernando, W. (2005). Seasonal and diurnal patterns of spore dispersal by *Leptosphaeria maculans* from canola stubble in relation to environmental conditions. Plant Disease, 89(1), 97-104.

Guo, X., Fernando, W., & Entz, M. (2005). Effects of crop rotation and tillage on blackleg disease of canola. Canadian Journal of Plant Pathology, 27(1), 53-57.

Guo, X., Fernando, W., & Entz, M. (2008). Dynamics of infection by *Leptosphaeria maculans* on canola (Brassica napus) as influenced by crop rotation and tillage. Archives of Phytopathology and Plant Protection, 41(1), 57-66.

Gupta, S., & Pratap, A. (2007). History, origin, and evolution. Advances in Botanical

Research, 45, 1-20.

Gupta, S. K. (2009). Biology and breeding of crucifers: CRC Press.

Hall, R. (1992). Epidemiology of blackleg of oilseed rape. Canadian Journal of Plant Pathology, 14(1), 46-55.

Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y. J., Makizumi, Y., Akutsu, K., & Hibi, T. (2000). Tandem Repeat of a Transcriptional Enhancer Upstream of the Sterol 14α-Demethylase Gene (CYP51) in*Penicillium digitatum*. Applied and environmental microbiology, 66(8), 3421-3426.

Hammond, K. E., & Lewis, B. (1987). Variation in stem infections caused by aggressive and non−aggressive isolates of *Leptosphaeria maculans* on *Brassica* napus *var. oleifera*. Plant Pathology, 36(1), 53-65.

Hammond, K. E., Lewis, B., & Musa, T. (1985). A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathology, 34(4), 557-565.

Harper, F., & Berkenkamp, B. (1975). Revised growth-stage key for *Brassica campestris* and *B. napus*. Canadian Journal of Plant Science, 55(2), 657-658.

Heaney, S., Hall, A., Davies, S., & Olaya, G. (2000). Resistance to fungicides in the Qol-STAR cross-resistance group: current perspectives. Paper presented at the The BCPC Conference: Pests and diseases, Volume 2. Proceedings of an international conference held at the Brighton Hilton Metropole Hotel, Brighton, UK, 13-16 November 2000.

Hirooka, T., & Ishii, H. (2013). Chemical control of plant diseases. Journal of General Plant Pathology, 79(6), 390-401.

Holmes, G., & Eckert, J. (1995). Relative fitness of imazalil-resistant and-sensitive biotypes of *Penicillium digitatum*. Plant Disease, 79(10), 1068-1073.

Holt, J., & Chancellor, T. (1999). Modelling the spatio-temporal deployment of resistant varieties to reduce the incidence of rice tungro disease in a dynamic cropping system. Plant Pathology, 48(4), 453-461.

Horsfall, J. G. (1975). Fungi and Fungicides The Story of a Nonconformist. Annual review of phytopathology, 13(1), 1-14.

Howlett, B. J. (2004). Current knowledge of the interaction between *Brassica* napus and *Leptosphaeria maculans*. Canadian Journal of Plant Pathology, 26(3), 245-252.

Howlett, B. J., Idnurm, A., & Pedras, M. S. C. (2001). *Leptosphaeria maculans*, the Causal Agent of Blackleg Disease of *Brassicas*. Fungal Genetics and Biology, 33(1), 1-14.

Hsiang, T., Liao, A., & Benedetto, D. (2007). Sensitivity of *Sclerotinia homoeocarpa* to demethylation−inhibiting fungicides in Ontario, Canada, after a decade of use. Plant Pathology, 56(3), 500-507.

Hwang, S., Strelkov, S. E., Feng, J., Gossen, B. D., & Howard, R. J. (2012). *Plasmodiophora brassicae*: a review of an emerging pathogen of the Canadian canola (*Brassica napus*) crop. Molecular plant pathology, 13(2), 105-113.

Ishii, H., Fraaije, B., Sugiyama, T., Noguchi, K., Nishimura, K., Takeda, T., Amano, T., Hollomon, D. (2001). Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology, 91(12), 1166-1171.

Ishii, H., Yano, K., Date, H., Furuta, A., Sagehashi, Y., Yamaguchi, T., Sugiyama, T., Nishimura, K., Hasama, W. (2007). Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant fungal pathogens. Phytopathology, 97(11), 1458-1466.

Jedryczka, M., Kaczmarek, J., Dawidziuk, A., & Brachaczek, A. (2008). System for

Forecasting Disease Epidemics–aerobiological methods in Polish agriculture. Aspects of Applied Biology, 89, 65-70.

Johnson, D., Hulbert, S., & Demoz, B. (2012). Manitoba Regional Meeting, 2012/Réunion régionale de l'Manitoba, 2012. Paper presented at the Abstracts–Manitoba Regional Meeting.

Johnson, R., & Lewis, B. (1994). Variation in host range, systemic infection and epidemiology of *Leptosphaeria maculans*. Plant Pathology, 43(2), 269-277.

Johnston, J. S., Pepper, A. E., Hall, A. E., Chen, Z. J., Hodnett, G., Drabek, J., Lopez, R., Price, H. J. (2005). Evolution of genome size in *Brassicaceae*. Annals of botany, 95(1), 229-235.

Karaoglanidis, G., Thanassoulopoulos, C., & Ioannidis, P. (2001). Fitness of *Cercospora beticola* field isolates–resistant and–sensitive to demethylation inhibitor fungicides. European Journal of Plant Pathology, 107(3), 337-347.

Kharbanda, P., & Tewari, J. (1996). Integrated management of canola diseases using cultural methods. Canadian Journal of Plant Pathology, 18(2), 168-175.

Kharbanda, P., Yang, J., Beatty, P., Jensen, S., & Tewari, J. (1999). Biocontrol of *Leptosphaeria maculans* and other pathogens of canola with *Paenibacillus polymyxa* PKB1. Paper presented at the Proceedings of the 10th International Rapeseed Congress. Canberra, Australia. http://www. regional. org. au/au/gcirc.

Kim, Y.-S., Dixon, E. W., Vincelli, P., & Farman, M. L. (2003). Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome *b* gene. Phytopathology, 93(7), 891-900.

Koch, E., Song, K., Osborn, T., & Williams, P. (1991). Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Molecular Plant-Microbe Interactions, 4(4), 341-349.

Köller, W., Parker, D., Turechek, W., Avila-Adame, C., & Cronshaw, K. (2004). A two-phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoxim-methyl and trifloxystrobin. Plant Disease, 88(5), 537-544.

Krzymanski, J., & Downey, R. (1969). Inheritance of fatty acid composition in winter forms of rapeseed, *Brassica napus*. Canadian Journal of Plant Science, 49(3), 313-319.

Kutcher, H., & Brandt, S. (2008). Optimizing canola production: Pest implications of intensive canola rotations. Final Canola Agronomic Research Program Report to the Canola Council of Canada.

Kutcher, H., Brandt, S., Smith, E., Ulrich, D., Malhi, S., & Johnston, A. (2013). Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. Canadian Journal of Plant Pathology, 35(2), 209-221.

Kutcher, H., Fernando, W., Turkington, T., & McLaren, D. (2011a). Best management practices for blackleg disease of canola. Prairie Soils and Crops eJournal, 4, 122-134.

Kutcher, H., Johnston, A., Bailey, K., & Malhi, S. (2011b). Managing crop losses from plant diseases with foliar fungicides, rotation and tillage on a Black Chernozem in Saskatchewan, Canada. Field Crops Research, 124(2), 205-212.

Kutcher, H., & Wolf, T. (2006). Low-drift fungicide application technology for *sclerotinia* stem rot control in canola. Crop Protection, 25(7), 640-646.

Leiminger, J., Adolf, B., & Hausladen, H. (2013). Occurrence of the F129L mutation in *Alternaria solani* populations in Germany in response to QoI application, and its effect on sensitivity. Plant Pathology.

Li, C. X., & Cowling, W. (2003). Identification of a single dominant allele for resistance to blackleg in *Brassica napus* 'Surpass 400'. Plant Breeding, 122(6), 485-488.

Li, H., Barbetti, M., & Sivasithamparam, K. (2005). Hazard from reliance on cruciferous hosts as sources of major gene-based resistance for managing blackleg (*Leptosphaeria maculans*) disease. Field Crops Research, 91(2), 185-198.

Li, H., Tapper, N., Dean, N., Barbetti, M., & Sivasithamparam, K. (2006). Enhanced pathogenicity of *Leptosphaeria maculans* pycnidiospores from paired co-inoculation of *Brassica napus* cotyledons with ascospores. Annals of botany, 97(6), 1151-1156.

Liban, S., Cross, D., Fernando, W., Peng, G., & Kutcher, H. (2013). Characterization of *Leptosphaeria maculans* race structure in Western Canada. Can. J. Plant Pathol., 35(2), 271.

Luo, C. X., Hu, M. J., Jin, X., Yin, L. F., Bryson, P. K., & Schnabel, G. (2010). An intron in the cytochrome *b* gene of *Monilinia fructicola* mitigates the risk of resistance development to QoI fungicides. Pest management science, 66(12), 1308-1315.

Ma, Z., Felts, D., & Michailides, T. J. (2003). Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. Pesticide biochemistry and physiology, 77(2), 66-74.

Ma, Z., & Michailides, T. J. (2005). Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection, 24(10), 853-863.

Ma, Z., Yoshimura, M. A., Holtz, B. A., & Michailides, T. J. (2005). Characterization and PCR‐based detection of benzimidazole‐resistant isolates of *Monilinia laxa* in California. Pest management science, 61(5), 449-457.

Mahuku, G. S., Goodwin, P. H., Hall, R., & Hsiang, T. (1997). Variability in the highly virulent type of *Leptosphaeria maculans* within and between oilseed rape fields. Canadian Journal of Botany, 75(9), 1485-1492.

Marcroft, S., Sprague, S., Pymer, S., Salisbury, P., & Howlett, B. (2004). Crop

isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans*) severity of canola (*Brassica napus*) in south-eastern Australia. Animal Production Science, 44(6), 601-606.

Marcroft, S., Wratten, N., Purwantara, A., Salisbury, P., Potter, T., Barbetti, M., Khangura, R., Howlett, B. (2002). Reaction of a range of *Brassica* species under Australian conditions to the fungus, *Leptosphaeria maculans*, the causal agent of blackleg. Animal Production Science, 42(5), 587-594.

Marcroft, S. J., Elliott, V. L., Cozijnsen, A. J., Salisbury, P. A., Howlett, B. J., & Van de Wouw, A. P. (2012). Identifying resistance genes to *Leptosphaeria maculans* in Australian *Brassica napus* cultivars based on reactions to isolates with known avirulence genotypes. Crop and Pasture Science, 63(4), 338-350.

Marichal, P., Bossche, H. V., Odds, F. C., Nobels, G., Warnock, D. W., Timmerman, V., Van Broeckhoven, C., Fay, S., Mose-Larsen, P. (1997). Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. Antimicrobial agents and chemotherapy, 41(10), 2229-2237.

Mathre, D., Kushnak, G., Martin, J., Grey, W., & Johnston, R. (1997). Effect of residue management on barley production in the presence of Net Blotch Disease. Journal of production agriculture, 10(2), 323-326.

McGee, D., & Emmett, R. (1977). Blackleg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.) of rapeseed in Victoria: crop losses and factors which affect disease severity. Crop and Pasture Science, 28(1), 47-51.

McGee, D., & Petrie, G. (1979). Seasonal patterns of ascospore discharge by *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology, 69(6), 586-589.

Mendes-Pereira, E., Balesdent, M.-H., Brun, H., & Rouxel, T. (2003). Molecular phylogeny of the *Leptosphaeria maculans-L. biglobosa* species complex. Mycological

research, 107(11), 1287-1304.

Menzies, J., & MacNeill, B. (1987). Effect of 'unnecessary' genes for virulence on six components of parasitic fitness in *Erysiphe graminis f. sp. tritici*. Canadian Journal of Plant Pathology, 9(3), 214-217.

Miguez, M., Reeve, C., Wood, P. M., & Hollomon, D. W. (2004). Alternative oxidase reduces the sensitivity of *Mycosphaerella graminicola* to QOI fungicides. Pest management science, 60(1), 3-7.

Miles, L., Miles, T., Kirk, W., & Schilder, A. (2012). Strobilurin (QoI) resistance in populations of *Erysiphe necator* on grapes in Michigan. Plant Disease, 96(11), 1621-1628.

Miller, G. L., Stevenson, K. L., & Burpee, L. L. (2002). Sensitivity of *Sclerotinia homoeocarpa* isolates to propiconazole and impact on control of dollar spot. Plant Disease, 86(11), 1240-1246.

Morrall, R. (1997). Evolution of lentil diseases over 25 years in western Canada. Canadian Journal of Plant Pathology, 19(2), 197-207.

Mueller, D. S., Bradley, C. A., & Nielsen, J. (2008). Field crop fungicides for the north central United States: Agricultural Experiment Station, Iowa State University.

Mundt, C. (2002). Use of multiline cultivars and cultivar mixtures for disease management. Annual review of phytopathology, 40(1), 381-410.

Olaya, G., & Köller, W. (1999). Diversity of kresoxim‐methyl sensitivities in baseline populations of *Venturia inaequalis*. Pesticide Science, 55(11), 1083-1088.

Olivier, C., & Galka, B. O. 47-Consequences of phytoplasma infection on canola crop production in the Canadian prairies.

Olivier, C., Galka, B., Rott, M., & Johnson, R. (2008). First report of molecular

detection of '*Candidatus* Phytoplasma asteris'-related strains in seeds of *Brassica napus* in Saskatchewan, Canada. Cruciferae newsletter, 27, 22-23.

Olivier, C., Galka, B., & Séguin-Swartz, G. (2010). Detection of aster yellows phytoplasma DNA in seed and seedlings of canola (*Brassica napus* and *B. rapa*) and AY strain identification. Canadian Journal of Plant Pathology, 32(3), 298-305.

Oplinger, E., Hardman, L., Gritton, E., Doll, J., & Kelling, K. (1989). Alternative field crops manual, canola (rapeseed). Ext. Bull. Nov. University of Wisconsin, Madison, WI. Alternative field crops manual, canola (rapeseed). Ext. Bull. Nov. University of Wisconsin, Madison, WI.

Pageau, D., Lajeunesse, J., & Lafond, J. (2006). Impact de l'hernie des crucifères [*Plasmodiophora brassicae*] sur la productivité et la qualité du canola. Canadian Journal of Plant Pathology, 28(1), 137-143.

Pariaud, B., Ravigné, V., Halkett, F., Goyeau, H., Carlier, J., & Lannou, C. (2009). Aggressiveness and its role in the adaptation of plant pathogens. Plant Pathology, 58(3), 409-424.

Pasche, J., Wharam, C., & Gudmestad, N. (2004). Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. Plant Disease, 88(2), 181-187.

Peever, T. L., & Milgroom, M. G. (1994). Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. Phytopathology, 84(5), 515-519.

Peng, G., McGregor, L., Lahlali, R., Gossen, B., Hwang, S., Adhikari, K., Strelkov, S., McDonald, M. (2011). Potential biological control of clubroot on canola and crucifer vegetable crops. Plant Pathology, 60(3), 566-574.

Petrie, G. (1978). Occurrence of a highly virulent strain of blackleg (*Leptosphaeria maculans*) on rape in Saskatchewan (1975-77). Canadian Plant Disease Survey, 58(2),

21-25.

Petrie, G. (1995). Patterns of ascospore discharge by *Leptosphaeria maculans* (blackleg) from 9- to 13-month-old naturally-infected rapeseed/canola stubble from 1977 to 1993 in Saskatchewan. Canadian Plant Disease Survey, 75(1), 35-43.

Petrie, G., & Vanterpool, T. (1965). Diseases of rape and cruciferous weeds in Saskatchewan in 1965. Can. Plant Dis. Surv, 45, 111-112.

Philion, V. (2007). A comparison of fungicide resistance monitoring techniques currently in use for *Venturia inaequalis*. Paper presented at the Phytopathology.

Platford, G. (1985). 1985 Manitoba canola survey. Paper presented at the Report to the Western Committee on Plant Disease Control, 10th Annual Meeting, Winnipeg.

Plummer, K., Dunse, K., & Howlett, B. (1994). Non-aggressive strains of the blackleg fungus, *Leptosphaeria maculans*, are present in Australia and can be distinguished from aggressive strains by molecular analysis. Australian journal of botany, 42(1), 1-8.

Prakash, S. (1980). Cruciferous oilseeds in India. Brassica crops and wild allies.[I]. 151-163.

Rempel, C., & Hall, R. (1995). Effects of time and rate of application of triazole fungicides on incidence and severity of blackleg and growth and yield of canola. Canadian Journal of Plant Science, 75(3), 737-743.

Rogers, P., & Stevenson, W. (2010). Aggressiveness and fungicide sensitivity of *Alternaria dauci* from cultivated carrot. Plant Disease, 94(4), 405-412.

Rouxel, T., Grandaubert, J., Hane, J. K., Hoede, C., van de Wouw, A. P., Couloux, A., Dominguez, V., Anthouard, V., Bally, P., Bourras, S. (2011). Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. Nature communications, 2, 202.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., Gout, L., Delourme, R., Schmit, J., Balesdent, M.-H. (2003). A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the Rlm1 resistance gene of oilseed rape. European Journal of Plant Pathology, 109(8), 871-881.

Russell, P. (2005). A century of fungicide evolution. The Journal of Agricultural Science, 143(01), 11-25.

Salam, M., Fitt, B. D., Aubertot, J. N., Diggle, A., Huang, Y., Barbetti, M., Gladders, P., Jędryczka, M., Khangura, R., Wratten, N. (2007). Two weather–based models for predicting the onset of seasonal release of ascospores of *Leptosphaeria maculans* or *L. biglobosa*. Plant Pathology, 56(3), 412-423.

Sanglard, D., Ischer, F., Koymans, L., & Bille, J. (1998). Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrobial agents and chemotherapy, 42(2), 241-253.

Savchuk, S. C. (2002). Evaluation of biological control of *Sclerotinia sclerotiorum* on canola (*Brassica napus*) in the laboratory, in the greenhouse, and in the field.

Shahidi, F. (1990). Canola and rapeseed: production, chemistry, nutrition, and processing technology: Springer.

Shurtleff, M. C., & Averre, C. W. (1997). Glossary of plant-pathological terms: APS Press St. Paul, MN, USA.

Sierotzki, H., Frey, R., Wullschleger, J., Palermo, S., Karlin, S., Godwin, J., & Gisi, U. (2007). Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici‐repentis* and implications for QoI resistance. Pest management science, 63(3), 225-233.

Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., & Gisi, U. (2000). Mode

of resistance to respiration inhibitors at the cytochrome *bc1* enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest management science, 56(10), 833-841.

Skylakakis, G. (1987). Changes in the composition of pathogen populations caused by resistance to fungicides. Population of plant pathogens: their dynamics and genetics/edited for the British Society for Plant Pathology by MS Wolfe and CE Caten.

Sprague, S. J., Balesdent, M.-H., Brun, H., Hayden, H. L., Marcroft, S. J., Pinochet, X., Rouxel, T., Howlett, B. J. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. European Journal of Plant Pathology, 114(1), 33-40.

Steinfeld, U., Sierotzki, H., Parisi, S., & Gisi, U. (2002). Comparison of resistance mechanisms to strobilurin fungicides in *Venturia inaequalis*. Modern Fungicides and Antifungal Compounds III, 167-176.

Stonard, J. F., Latunde-Dada, A. O., Huang, Y.-J., West, J. S., Evans, N., & Fitt, B. D. (2010). Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans*/*L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). European Journal of Plant Pathology, 126(1), 97-109.

Stover, R. W., Francl, L. J., & Jordahl, J. G. (1996). Tillage and fungicide management of foliar diseases in a spring wheat monoculture. Journal of production agriculture, 9(2), 261-265.

Strelkov, S., Manolii, V., Rennie, D., Xiao, Q., Cui, D., & Hwang, S. (2011). The occurrence of clubroot on canola in Alberta in 2010. Can. Plant Dis. Surv, 91, 109-111.

Taylor, J. L., Borgmann, I., & Séguin-Swartz, G. (1991). Electrophoretic karyotyping of *Leptosphaeria maculans* differentiates highly virulent from weakly virulent isolates.

Current genetics, 19(4), 273-277.

Tewari, J., Strelkov, S., Orchard, D., Hartman, M., Lange, R., & Turkington, T. (2005). Identification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. Canadian Journal of Plant Pathology, 27(1), 143-144.

Torriani, S. F., Brunner, P. C., McDonald, B. A., & Sierotzki, H. (2009). QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. Pest management science, 65(2), 155-162.

Tuggle, J., Smith, O., & Starr, J. (1999). Variation in aggressiveness and virulence among isolates of *Cercospora arachidicola*. Peanut Science, 26(2), 95-98.

Turkington, T., Clayton, G., & Woods, D. (2000). The impact of soil incorporation of canola residues and stubble application of chemicals on decomposition and inoculum production by *Leptosphaeha maculans*. Canadian Journal of Plant Pathology, 22(2), 155-159.

Van der Plank, J. (1968). Disease resistance in plants. New York and London, 125.

Vanterpool, T. (1961). Rape diseases in Saskatchewan in 1961. Can. Plant Dis. Surv, 41, 372-373.

Vincelli, P., & Dixon, E. (2002). Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Disease, 86(3), 235-240.

West, J., Balesdent, M. H., Rouxel, T., Narcy, J., Huang, Y. J., Roux, J., Steed, J., Fitt, B., Schmit, J. (2002). Colonization of winter oilseed rape tissues by A/Tox+ and B/Tox0 *Leptosphaeria maculans* (phoma stem canker) in France and England. Plant Pathology, 51(3), 311-321.

West, J., Biddulph, J., Fitt, B. D., & Gladders, P. (1999). Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. Annals of Applied Biology, 135(2), 535-546.

West, J., Kharbanda, P., Barbetti, M., & Fitt, B. D. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathology, 50(1), 10-27.

Williams, R., & Fitt, B. D. (1999). Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathology, 48(2), 161-175.

Wise, K., Bradley, C., Pasche, J., & Gudmestad, N. (2009). Resistance to QoI fungicides in *Ascochyta rabiei* from chickpea in the Northern Great Plains. Plant Disease, 93(5), 528-536.

Wise, K., Bradley, C., Pasche, J., Gudmestad, N., Dugan, F., & Chen, W. (2008). Baseline sensitivity of *Ascochyta rabiei* to azoxystrobin, pyraclostrobin, and boscalid. Plant Disease, 92(2), 295-300.

Wong, F. P., & Wilcox, W. F. (2002). Sensitivity to azoxystrobin among isolates of *Uncinula necator*: Baseline distribution and relationship to myclobutanil sensitivity. Plant Disease, 86(4), 394-404.

Wu, Y.-x., & von Tiedemann, A. (2002). Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare L.*) exposed to ozone. Environmental Pollution, 116(1), 37-47.

Wyand, R., & Brown, J. (2005). Sequence variation in the *CYP51* gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. Fungal Genetics and Biology, 42(8), 726-735.

Yang, C., Hamel, C., Vujanovic, V., & Gan, Y. (2011). Fungicide: Modes of action and possible impact on nontarget microorganisms. ISRN Ecology, 2011.

Young, J. R., Tomaso-Peterson, M., Tredway, L. P., & de la Cerda, K. (2010). Occurrence and molecular identification of azoxystrobin-resistant *Colletotrichum*

cereale isolates from golf course putting greens in the Southern United States. Plant Disease, 94(6), 751-757.

Yu, F., Lydiate, D., & Rimmer, S. (2005). Identification of two novel genes for blackleg resistance in *Brassica napus*. Theoretical and applied genetics, 110(5), 969-979.

Zhang, X., Fernando, W. G. D., Peng, G., & Kutcher, H. R. (2013). Identifying major resistance genes against blackleg in Canadian canola germplasm. Can. J. Plant Pathol., 35(2), 272.

Zhang, Z., Zhu, Z., Ma, Z., & Li, H. (2009). A molecular mechanism of azoxystrobin resistance in *Penicillium digitatum* UV mutants and a PCR-based assay for detection of azoxystrobin-resistant strains in packing-or store-house isolates. International journal of food microbiology, 131(2), 157-161.

Ziogas, B. N., Baldwin, B. C., & Young, J. E. (1997). Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. Pesticide Science, 50(1), 28-34.

APPENDICES

Appendix 1: Experimental canola plots for fungicide study at Carman, Manitoba, 2012

Appendix 2: Experimental canola pots for aggressiveness test in the greenhouse at Department of Plant Science, University of Manitoba, 2013

Appendix 3: Lesions caused by fungus Leptosphaeria maculans on canola plants on a lower leaf at the mid-flowering stage

Appendix 4: Blackleg symptoms on basal stems of clipped canola plants showing varying degrees of severity of infection