BIOLOGY AND MANAGEMENT OF BLACK LEG DISEASE IN WINTER CANOLA

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Abstract: The fungus *Leptosphaeria maculans* is the causal agent of black leg disease in canola (*Brassica napus*). The best strategy to control this disease is the use of genetic resistance. Two types of resistance are described. Quantitative resistance is controlled by multiple genes and expressed at adult stage. Major-gene resistance is expressed at the seedling stage and controlled by major genes (*Rlm*) genes that interact with avirulence genes (*Avr*) in a gene-for-gene manner. *AvrLm4-7* is a unique avirulence gene that is recognized by two major genes (*Rlm4* and *Rlm7*). The genetic variability of the *AvrLm4-7* gene among *L. maculans* isolates with known *Avr* genes was investigated by high resolution melting analysis (HRM). Two sets of newly designed primers and one set of previously reported primers were used to amplify the *AvrLm4-7* gene and generate different melting profiles. Virulent and avirulent isolates could be differentiated by HRM. In addition, two melting profiles that identified avirulent isolates towards *Rlm4* and *Rlm7* (A4A7) and five different melting profiles that identified avirulent isolates only for *Rlm7* (a4A7) were found. Well characterized *L. maculans* isolates with different *Avr* profiles (*AvrLm6,7*; *AvrLm1,6,7*; and *AvrLm1,2,4,7,S*) were used to infer the presence of major-gene resistance in canola breeding lines (n=119), cultivars (n=7) and hybrids (n=17) by cotyledon inoculation. More than 80% of the breeding lines were resistant in response to race *AvrLm1,2,4,7,S* and susceptible to the other races. The presence of gene *Rlm4* was inferred in these entries. The hybrids Dimension, Visby, Artoga, DK-Sensei, DK-Extorm, DK-Imiron, the cultivar Claremore, and four of the breeding lines were resistant to all three races possibly due to the presence of resistance gene *Rlm1, Rlm4, Rlm6* and/or *Rlm7*. Finally, field experiments were conducted to assess the disease and yield responses of resistant and susceptible winter canola genotypes to fungicide application for control of black leg. Resistant genotypes produced the highest yields and the lowest levels of disease compared with the susceptible genotypes. Significant reductions were observed for disease incidence and severity in the plots that were treated with the fungicide. However, there is no significant effect of fungicide application on yield. Results provide information on the genetic interaction between *L. maculans* and *B. napus* that is essential for selecting effective resistance to control black leg, a strategy that was more effective than fungicide application.

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INTRODUCTION

Canola (*Brassica napus*) is a type of rapeseed that belongs to one of the most cultivated family of plants worldwide, the Brassicaceae. Because of their high oil content, canola seeds have been used as a source of vegetable oil, protein meal, and biodiesel (Daun et al., 2011). Europe, China and Canada are the main countries that produce canola. The United States produces 767.00 MT per year and ranks sixth after India and Japan. North Dakota and Oklahoma are the states that produce the largest amount of canola in the U.S (USDA-ERS, 2017).

There are several diseases that can affect canola crops. However, black leg is one of the most significant worldwide. This disease is caused by the fungus *Leptosphaeria maculans*. This hemibiotrophic ascomycete infects the plant by airborne ascospores, which are produced from pseudothecia that are present on infested stubble from the previous crop. The ascospore infections produce leaf spots, the first visible symptoms on the leaves, within which dark spots or pycnidia develop that produce conidia. From the foliar infections, hyphae grow systemically down the petioles and eventually into the basal stem. The stem infection causes the typical dark basal stem canker during the ripening stage of crop development. The canker phase of the disease can cause a dramatic decrease of canola yield when stems are girdles prior maturity (Mazáková et al., 2017; West et al., 2001).

Management practices can successfully reduce the risk of black leg infection. Crop rotation, the use of certified pathogen-free seed, fungicide seed treatment, application of fungicides to foliage, and the use of resistant varieties are effective methods against black leg (Marcroft & Bluett, 2008; Markell et al., 2008). Although there are several ways to manage black leg, the use of genetic resistance is the most effective and least expensive strategy to minimize the impact of this disease (Kutcher et al., 2010). There are two types of resistance against black leg in *Brassica* species. Minor-gene resistance or adult plant resistance conferred by multiple minor resistance genes, and major-gene resistance or seedling resistance that is conferred by single resistance genes (Zhang et al., 2017).

For each major resistance gene in canola (*Rlm*) there is a corresponding avirulence gene in the pathogen (*AvrLm*). In canola, there are 18 major resistance genes identified conferring resistance to *L. maculans* (Dilantha et al., 2018; Marcroft et al., 2012). *L. maculans* isolates can be classified into races according their genetic variation or their avirulence genotype. Currently, there are 14 avirulence genes identified in *L. maculans*, and six have been cloned (Plissonneau et al., 2016). The avirulence genes *AvrLm1* and *AvrLm6* confer avirulence towards *Rlm1* and *Rlm6* respectively. However, the *AvrLm4-7* gene differs from other avirulence genes by its being recognized by two resistance proteins encoded by the resistance genes *Rlm4* and *Rlm7*. It has been shown that a single nucleotide mutation in the *AvrLm4-7* gene is responsible for the breakdown of *Rlm4* resistance (Carpezat et al., 2013; Plissonneau et al., 2016). For this reason, a rapid detection and discrimination tool for this avirulence gene is needed for monitoring and characterization of pathogen races and race shifts. Conventional polymerase chain reaction (PCR) is used to identify the *AvrLm4-7* gene. High Resolution Melting (HRM), a post PCR technique, has been used as a genotyping tool. HRM analysis allows the detection of single mutation point depending on the melting behavior of each sample (Carpezat et al., 2013; Wittwer, 2009).

The use of major-gene resistance can reduce the risk of black leg at the seedling stage. Therefore, it is essential for the canola industry and farmers to know about plant resistance genes harbored in available varieties and hybrids and which type of resistance will be most effective. Moreover, little is known about the presence of major resistance genes in locally grown winter canola cultivars. Specific races of *L. maculans* can be used to characterize corresponding major gene resistance in local *B. napus* cultivars and breeding lines. Knowing what type of major-gene resistance is present in commercial and breeding line cultivars, is important for the industry to make good decisions about which cultivars will be the best for managing black leg.

Currently, there are several sources for resistance to black leg. Canola hybrids with improved resistance to *L. maculans* are being used throughout Europe, Australia, and North America (Balesdent et al., 2001; Kutcher et al., 2013; Marcroft et al., 2012). Although high levels of genetic resistance are available, this method may not be sufficient to manage black leg in the long term. That is because *L. maculans* may produce new races in the field and some of these races can overcome one or more of the resistance genes. Therefore, it is recommended to rotate canola cultivars with different resistance genes to prevent the development of new races and thereby prolong the durability of the resistance genes (Dilmaghani et al., 2009). Also, the use of foliar fungicides is a valuable tool in providing protection from blackleg infection (Ballinger et al., 1988). However, little is known about the benefits of applying fungicides to control blackleg on resistant and susceptible winter canola cultivars.

This research addresses the host-pathogen interaction between *Brassica napus* and *Leptosphaeria maculans*. A better understanding of the biology, epidemiology, and characterization of the causal agent of black leg is crucial for choosing the best management strategy for this disease. The objectives of this research were: i) to assess the genetic variability of the *AvrLm4-7* gene among *Leptosphaeria maculans* isolates by pathogenicity and High Resolution Melting (HRM); ii) to test canola germplasm for major-gene resistance with known

Leptosphaeria maculans races; and iii) to assess the disease and yield responses of resistant and susceptible winter canola cultivars to fungicide application for control of black leg.

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CHAPTER II

LITERATURE REVIEW

Canola

Canola (*Brassica napus* spp. *napus*), a type of rapeseed, is an herbaceous plant member of the family Brassicaceae which is one of the most cultivated plants around the world. The genus *Brassica* was first cultivated almost 10,000 years ago. Reports indicate that oilseed brassicas were cultivated in India as early as 4000 BC while China and Japan started to cultivate oilseed brassicas almost 2000 years ago. (Friedt & Snowdon, 2009). *Brassica* sp. complex includes six different species; *B. rapa, B. oleracea, B. napus*, *B. nigra, B. juncea*, and *B. carinata* (Roche, 2015). The amphidiploid *Brassica napus* (genome AACC, 2n = 38) was originated from the hybridization between two diploid species *Brassica rapa* (genome AA, 2n = 20) and *Brassica oleracea* (genome CC, 2n = 18). The cultivation of these two parental species in nearby geographical areas, resulted in their spontaneous cross (Friedt & Snowdon, 2009).

Rapeseed (*Brassica sp.*) is grown mainly for the oil content of its seeds, and is considered the world's third leading source of vegetable oil behind soybean and palm oil. Rapeseed oil has been used for industrial purposes such as lubricants and biodiesel production. (Bhardwaj & Hamama, 2003). However, the high contents of erucic acid and glucosinolates, made most of the rapeseed oils unsuitable for human consumption, as well as harmful to meal supplement for livestock (Bhardwaj & Hamama, 2000).

In the 1970's, a breeding program started in Canada with the purpose of developing low glucosinolates and low erucic acid *Brassica napus* varieties. The improved varieties contain less than 2% of erucic acid and less than 30 micromoles of glucosinolates per gram (Roche, 2015). In 1978 the name "Canola" was adopted to represent those new varieties characterized by a very low level of erucic acid and glucosinolates. Canola is an acronym derived from CANadian Oil Low Acid. The low levels of these components allow the oil to be used for swine, poultry, and cattle meals, and human consumption (Hang et al., 2009). Canola oil also contains about half the level of saturated fatty acids present in corn oil, olive oil and soybean oil, making it healthier than other vegetable oils. (Daun et al., 2011; Friedt & Snowdon, 2009; Przybylski et al., 2005).

Agronomically, canola is recognized for its benefit in crop rotations with small grain cereals such as wheat and barley (Brown et al., 2008). For instance, growing a non-host plant such as canola in rotation with wheat, can increase the forage and grain yield of the following wheat crop. Also, levels of some soil-borne diseases can be decreased by reducing pathogen population to levels at which significant yield losses does not occur. In Oklahoma, a 15% increase in yield has been reported when wheat is planted the next season after canola (Bushong, 2016; Kutcher et al., 2013).

Also, many winter weed species can be reduced using winter canola in rotation with cereal crops. The rapid establishment and broadleaf plant habit that the Brassicas possess, allow this crop to compete with many weeds, making weed control easier and less expensive compared with other crops (Boyles et al., 2012). Moreover, transgenics in canola have been developed to exhibit special phenotypes such as resistance to diseases or herbicide tolerance (Roche, 2015). By genetic engineering systems, a patented Roundup ready gene developed by Monsanto was inserted in canola varieties, making them glyphosate resistant. Since 1995, Roundup ready herbicide resistant cultivars have been available in Canada. Growers in U.S. have adopted glyphosate transgenic cultivars since 1999 (Phillips, 2003).

Cultivation

The optimal soil conditions for cultivation of *Brassica* species are a pH between 6 and 7 with a wide range of soil textures. Canola crops are adapted to cool temperatures, the range in which this crop can grow varies between 12 to 30°C. Even though canola is considered a cool tolerant crop, temperatures below 3°C may cause damage during the emergence and flowering stage. On the other hand, higher temperatures may result in a late development of the pods reducing seed quantity and quality (Brown et al., 2008).

Depending on the region where canola is planted, spring and winter varieties are available. Spring canola varieties are cultivated in Canada, Australia, India, China, and northern Europe. Winter canola on the other hand is better suited in parts of Europe and China. Winter and spring canola can be cultivated in U.S. However, spring canola is best sown in the northern plains of North Dakota, Minnesota, Montana, and South Dakota. In contrast, the lower temperatures during the winter in Canada and the northern U.S. where spring type of canola is cultivated, are too extreme for survival of winter canola varieties. That is why winter canola is better adapted to the southern Great Plains. Compared with spring varieties, winter varieties require a vernalization period to flower the following spring. Also, winter canola is known to have 20 to 30 percent higher yield than spring varieties (Boyles et al., 2012). In general, winter canola is planted in the fall from late August to mid-October depending on growing region. In Oklahoma, winter canola should be planted at the beginning of fall or at least six weeks before the first freeze date. This typically corresponds to a planting period of September 15th to October 15th. Winter canola has been successfully grown in Oklahoma for the past 13 years (Berglund, 2009; Bushong, 2016; Friedt & Snowdon, 2009).

Production

According to the Food and Agriculture Organization of the United Nations (FAO) Canada, China, India, Germany, France, Australia, and the United States are the major world producers of this crop. The European continent leads the production of canola with 37%, followed by Asia with 34.1%, and the Americas with 24.8% (FAO-STAT, 2017). In the U.S., canola production has increased in the last two decades due to the large demand for oil and meal. In the U.S., canola production is concentrated in the Great Plains states of North Dakota, Oklahoma, Kansas, Colorado, as well as in Washington, Minnesota, Montana, Idaho, and Oregon (Roche, 2015). The acreage in these US. regions have varied during the past five years. In 2017, there were about two million acres of canola harvested in US with an average yield of 1800 pounds per acre. Statistics from the Oklahoma Department of Agriculture, Food and Forestry, and the U.S. Department of Agriculture National Agricultural Statistics Service indicate that Oklahoma is the second largest producer of canola after North Dakota with 135,000 acres of canola harvested with a total yield of 191 million pounds in 2017 (Reese & Coon, 2016; USDA, 2018).

Black leg disease

Several diseases can cause serious losses in canola production. Black leg or phoma stem canker, caused by the fungus *Leptosphaeria maculans* is one of the most important disease of canola worldwide (Grady, 2002; West et al., 2001). *Leptosphaeria biglobosa* is also associated with black leg, however, this specie causes less damage to the host compared with *L. maculans*. Black leg has been reported on rapeseed in widespread regions of production for almost fifty years with the exception of Asia. In Europe, black leg has been reported on winter rapeseed since 1950. After the 1970's, Australia and Canada started to observe this disease in their rapeseed crops (Gugel & Petrie, 1992). In the United States, black leg was first found in North Dakota in

1991 where it caused yield losses in susceptible varieties of canola (Bradley & Hamey, 2005; Brown et al., 2008). In Oklahoma, black leg was first reported on winter canola in October 2009 causing almost a 50% yield reduction in several affected fields (del Río Mendoza et al., 2011).

Symptoms

The first symptoms of black leg in canola are lesions on the cotyledon leaves with gray-green to ash-gray spots. Lesions may also develop on the true leaves, stems, and pods. The size of these lesions can vary between one to two centimeters with a round or irregular margin. The presence of small, round, black spots (pycnidia) in the lesions is a clear sign of black leg. After the leaf infection, the fungus colonizes the rest of the plant through the petioles causing a discoloration on this site. Mycelia extend asymptomatically from the leaves and petioles into the main stem. During the late (ripening) stage of crop development, the pathogen causes cankers at the base of the stem. The stem lesions are usually gray to brown in color with a dark border. Yield loss is associated with the stem cankers. This is because, the rapid necrosis of the vascular tissue at the basal stem caused by the cankers may produce girdling, prematurely ripening, and reduction of the pod fill. *L. biglobosa* can also cause leaf and stem lesions, but those are smaller, with fewer pycnidia, and less damage than *L. maculans*. (Ash, 2000; West et al., 2001).

Causal Agent

Leptosphaeria maculans (Desm.) Ces. & DeNot (anamorph *Phoma lingam*), is the causal agent of black leg disease in canola. This fungus is distributed worldwide and can also cause disease in other cultivated and wild crucifer species such as *Brassica napus, B. oleracea, B. rapa, B. carinata, B. juncea, B. nigra, Raphanus sativus,* and *Thlaspi arvense* (Johnson & Lewis, 1994; T. Rouxel & Balesdent, 2005). *L. maculans* belongs to the phylum Ascomycota, class Dothideomycetes, and order Pleosporales (Howlett et al., 2001). The genus *Leptosphaeria*, includes many species pathogenic on dicotyledonous hosts. The causal agent of black leg on

crucifers was once considered as a single species. However, based on pathogenicity tests, cultural characteristics, and molecular analysis, two different clades were separated from the *L. maculans* species complex. The two species *L. maculans* and *L. biglobosa* are highly and weakly aggressive pathogens respectively (Mendes-Pereira et al., 2003).

L. maculans is known as an hemibiotrophic fungi because in winter canola, it behaves as a saprophyte during the summer, obtaining nutrients from canola debris which was previously infected with black leg. At the primary stages of infection on the cotyledons and true leaves, *L. maculans* becomes necrotrophic causing leaf spots. After the primary infections, the fungus colonizes the stem asymptomatically behaving like a biotrophic organism. During the last stages of the infection, the fungus again becomes necrotrophic, producing basal stem cankers (West et al., 2001).

Epidemiology

After harvest, both *L. maculans* and *L. biglobosa* are able to survive on infested canola stubble as a saprophyte, where fruiting bodies or pseudothecia are formed by sexual reproduction. Ascospores are formed inside the fruiting bodies and serve as the primary inoculum. The timing of ascospore release varies from region to region and the type of canola grown. In regions where winter canola is cultivated, ascospores are discharged from the fruiting bodies and then dispersed by wind or rain during the fall and early winter months after the crop harvest (Hershman & Perkins, 1995). In the northern part of U.S., Canada, and Australia where spring canola is planted, the ascospores are dispersed during May to July. (Rimmer et al., 2007).

After discharge, ascospores land on the cotyledons or leaves and infect the plant by penetrating through stomata and wounds. At this stage the fungus is necrotrophic, producing leaf spots that contain pycnidia where asexual reproduction occurs. Conidia or pycnidiospores are produced in pycnidia. Conidia are splash-dispuised and serve as secondary inoculum. When the

fungus infects leaves, it produces hyphae that grow symptomlessly through vascular tissues and colonizes lower stems. After the flowering stage, *L. maculans* produces cankers in the lower stem and can cause early ripening of the pods. On susceptible varieties, the stem may be girdled enough to cause premature death of the plant, which causes further yield loss (Gajula, 2014; Rouxel & Balesdent, 2005; West et al., 2001).

Disease Management

Black leg has the potential to cause significant yield loss if it is not managed effectively. Black leg disease can be controlled by preventing infection from occurring. Management practices can successfully reduce the risk of black leg infection. Crop rotation, the use of certified pathogen-free seed, fungicide seed treatment, application of fungicides to foliage, and the use of resistant varieties are effective methods against black leg (Marcroft & Bluett, 2008; Markell et al., 2008).

Cultural control

Sanitation is an essential practice to control pests and to prevent the entrance of new pests to in the field. By cleaning tools and machinery, the inoculum can be reduced and the risk of having the disease is reduced (West et al., 2001). Also, conventional tillage can be used to reduce the infested canola stubble by covering it with soil. However, conventional tillage promotes soil erosion. If it is necessary, burning the stubble can help to eliminate the primary inoculum of this disease (Olson et al., 2012).

Crop rotation is considered a primary method to manage black leg. Crop rotation is the practice of growing different crops in succession on the same area. This is an essential control strategy for residue-borne plant diseases like black leg. The main purpose of rotating the crops is to reduce the pathogen inoculum to a level significantly low so that crop damage is reduced to

manageable economic levels. Canola is a crop that is usually grown in a rotation with wheat. (Bushong, 2016; Kutcher et al., 2013).

The use of certified pathogen-free seed is a strategy used to prevent introduction of the fungus into a field and is important when canola is introduced into a new area (Berglund, 2009). Unfortunately, certified black leg-free seed is not available in the U.S. Alternatively, fungicide seed treatments are applied to seeds to reduce and hopefully eliminate seedborne black leg. (Damicone, personal communication, December, 2016).

Chemical Control

Fungicides have been developed to control plant diseases by killing or inhibiting the growth of the pathogen (Shurtleff et al., 2016). The use of fungicide seed treatment is useful to prevent seedborne black leg and another soilborne diseases. Also, a fungicide combined with an insecticide is commonly used as a seed coat to prevent insect pest infestations. Fungicides such as azoxystrobin, carboxin, ipconazole, penflufen, trifolxystrobin, and metalaxyl; and insecticides such as thiamethoxam and clothianidin are registered for use on canola in various commercial formulations (Oklahoma-Cooperative-Extension-Service, 2017).

Foliar applied fungicides on canola can reduce the levels of black leg and provide yield protection in susceptible varieties. Fungicides such as prothioconazole, azoxystrobin, picoxystrobin, and pyraclostrobin are registered to control black leg in Oklahoma. Scouting is very important during the emergence, second leaf, fourth leaf, and sixth leaf stages of the crop. If any sign or symptom of black leg is observed during these stages, application of fungicides might be beneficial. (Kandel & Knodel, 2005; Oklahoma-Cooperative-Extension-Service, 2017).

Biological control

Plant diseases must be controlled to prevent yield losses and to keep good crop quality. A common practice in agriculture for pest control is the use of pesticides. However, possible environmental pollution and the public health effects are some concerns of many consumers nowadays. In order to reduce the use of pesticides in agriculture, researchers have investigated biological alternatives to pest management (Pal & Gardener, 2006).

In the past two decades, a biocontrol agent has been sought to combat the causal agent of black leg disease. Diverse microorganisms isolated from canola fields have been studied for suppression against *Leptosphaeria maculans* (Beatty & Jensen, 2002; Ramarathnam & Dilantha Fernando, 2006; Ramarathnam et al., 2011). Different endophytic bacteria, which are involved in plant disease suppression, have been found. *Pseudomonas chlororaphis*, *Pseudomonas aurantiaca*, and three *Bacillus cereus* strains show antagonism against growth of *Leptosphaeria maculans.* Moreover, some genes involved in fungal suppression are present in these microorganisms, especially genes that encode pyrrolnitrin, which is an antifungal antibiotic (Ramarathnam & Dilantha Fernando, 2006). In addition, an alternative antifungal microorganism was identified as a potential biocontrol agent. The microorganism *Paenibacillus polymyxa* produces antifungal peptides which are related to fungal antagonism. This antifungal component called PKB1 was extracted and purified (Beatty & Jensen, 2002). Even though these antifungal proteins have been tested only in laboratory, these agents are good candidates to develop a biocontrol strategy that can be applied in the field.

Genetic Resistance

Genetic resistance is the putative ability some plant genotypes have, to retard or suppress the development of a pathogen (Hammond-Kosack & Jones, 1997). The use of genetic resistance is one of the most effective, inexpensive, and environmental friendly methods to reduce the

impact of black leg (Kutcher et al., 2010; Plissonneau et al., 2016). There are two types of genetic resistance; multigenic and major gene resistance.

Multigenic resistance:

Multigenic resistance is also called quantitative, horizontal, field, or adult-plant resistance. This resistance depends upon multiple genes known as non-race-specific genes. These genes are in the plant host and they are expressed at the adult plant stage with each gene having a small or moderate phenotypic effect (Corwin & Kliebenstein, 2017; Kaur et al., 2009). An advantage of this type of resistance is that the resistance is durable and difficult to overcome by new races of the pathogen. Although foliar lesions may appear during the early stages of the plant, quantitative resistance reduces the development of the stem canker at the adult stage (Hubbard & Peng, 2018). However, there is not much information about the genes that confer adult plant resistance in *B. napus* against *L. maculans*. Moreover, this type of resistance is non-race specific, it is only partially effective, and it is difficult to screen for during the seedling stage (Marcroft et al., 2012; Tuzun, 2001). Measuring multigenic resistance in canola requires assessing canker incidence and severity in the field at harvest (Damicone, personal communication, December, 2016).

Alternatively, the quantitative trait locus (QTL) mapping is an effective molecular approach for analyzing and identifying a complex group of genes with a related phenotypic response such as morphological and quality traits, abiotic stress, or disease resistance. A combination of molecular markers such as SSRs, AFLPs, and SNPs are commonly used for QTLs genetic mapping. The difference in the genetic polymorphisms between susceptible and resistant cultivars allow the identification of genomic regions that control black leg resistance. Several genetic maps have been constructed for *Brassica species* related with black leg resistance using different molecular genetic marker systems. Also, some QTLs that control disease resistance

against *L. maculans* have been assigned. However, inconsistences in the exact location of QTLs have been reported. The diverse genetic background of different canola cultivars, the high polymorphism of the pathogen, and environmental interaction with gene expression are possible causes of these discrepancies (Kaur et al., 2009; Kole, 2007).

Major gene resistance:

Major gene resistance is also known as vertical or seedling resistance. This type of resistance is monogenically controlled giving a qualitative expression in level of resistance (Parlevliet & Zadoks, 1977). The host-pathogen interaction between *L. maculans* and *B. napus* is known to follow a gene-for-gene relationship. For each major resistance gene in the plant (*Rlm*) there is a corresponding avirulence gene in the pathogen (*AvrLm*) (Marcroft et al., 2012). Gene-for gene interactions between *B. napus* and *L. maculans* have led to an intensive study of the mechanisms related with the host defense against the pathogen. The molecular characterization and the determination of the exact location of the genes in the genome by cloning, allow the identification of DNA sequences which encode plant resistance proteins (*Rlm*) or avirulence effector proteins (*Avr*) from the pathogen. (Jones & Dangl, 2006; Rouxel & Balesdent, 2017).

In canola, there are 18 major resistance genes identified conferring resistance *to L. maculans.* These include: *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, Rlm11, RlmS, LepR1, LepR2, LepR3, LepR4, BLMR1*, and *BLMR2.* From these resistance genes only two have been cloned, *Rlm2* and *LepR3* (Dilantha et al., 2018; Marcroft et al., 2012). Alternatively, there are 14 avirulence genes identified in *L. maculans*, and seven have been cloned. These include *AvrLm1, AvrLm2, AvrLm3, AvrLm5* (previously known as *AvrLmJ1*), *AvrLm4-7*, *AvrLm6*, and *AvrLm11*. (Dilantha et al., 2018; Marcroft et al., 2012; Plissonneau et al., 2016).

The main advantage of using major gene resistance in canola crops is the capacity of the plant to disrupt the primary infection of the pathogen, thus avoiding the development of leaf spots and preventing the systemic infection in the plant (Balesdent et al., 2001). However, this type of resistance is effective just on those *L. maculans* races which have the corresponding *Avr* genotype. Moreover, seedling resistance can be overcome if the pathogen population evolves, changing their *Avr* loci. The dual life cycles of sexual and asexual reproduction that *L. maculans* possesses, contributes to the pathogen's capacity for overcoming the resistance in the plant through mutations and selection pressure. This resistance breakdown has been reported in several regions (Liban et al., 2016). The most notable cases occurred in France and Australia, where the pathogen overcame the resistance of cultivars carrying *Rlm1* and *LepR3* genes respectively (Li et al., 2003; Rouxel et al., 2003).

Race specific resistance genes:

Leptosphaeria maculans isolates can be classified into races according their genetic variation or their avirulence genotype. A set of differential cultivars with known resistance genes can be used to infer the presence of specific avirulence genes in *L. maculans* by pathogenicity tests on seedling cotyledons (Balesdent et al., 2002). Also, the use of polymerase chain reaction (PCR) is useful to identify specific avirulence alleles in the pathogen (Dilantha et al., 2018). Currently, specific primers have been designed to identify those avirulence genes previously cloned. In Oklahoma, *L. maculans* was characterized using a combination of pathogenicity tests on differential cultivars harboring resistance genes and PCR. Westar without any resistant gene, Glacier (harboring *Rlm2* and *Rlm3*), Quinta (harboring *Rlm1*, and *Rlm3*), and Jet Neuf (harboring *Rlm4*) were used. PCR was also used to amplify the avirulence genes *AvrLm1*, *AvrLm4-7*, and *AvrLm6*. Avirulence alleles *AvrLm1, AvrLm2, AvrLm3, AvrLm6, AvrLm4-7*, and *AvrLm7* were identified in the *L. maculans* population. *AvrLm6* and *AvrLm7* alleles were the most prevalent. In

addition, the race classification, consisting of the combined avirulence genes in an isolate into two races Av1,6,7 and Av6,7 which are predominant in Oklahoma (Diaz, 2015).

A good understanding of the pathogen population and their Avr genotype is fundamental to select the best source of resistance against black leg. That is why, the identification of resistance genes in canola cultivars and breeding lines is needed to select which cultivars should be planted or which breeding lines should be developed. Additionally, field experiments are necessary to evaluate the benefits planting black leg resistant cultivars in the Southern Great Plains using fungicide application as a tool to measure yield loss from the disease.

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CHAPTER III

GENETIC VARIABILITY OF THE AVIRULENCE GENE *AvrLm4-7* AMONG *Leptosphaeria maculans* ISOLATES BY HIGH RESOLUTION MELTING ANALYSIS

ABSTRACT

The fungal pathogen, *Leptosphaeria maculans,* causes black leg disease of canola (*Brassica napus*) in Oklahoma and worldwide. Major-gene resistance involves the interaction between avirulence effectors in the pathogen and their corresponding resistance genes in the plant. *AvrLm4-7* is an important avirulence effector that is recognized by two resistance proteins encoded by the genes *Rlm4* and *Rlm7*. A rapid detection, and discrimination tool for these effectors is needed for characterization of *L. maculans* races. Previously reported primers AvrLm7Up1 and AvrLm7LOW2 were used for PCR and high resolution melting (HRM) analysis of an internal region of *AvrLm4-7* gene. Melting temperatures profiles were determined for *L. maculans* isolates with different avirulence allele profiles. Melting temperatures (Tm) and melting curves obtained using the primers for the internal region were different from another Tm previously reported. Three HRM melting profiles were detected for isolates with a functional *AvrLm4-7* (C0) or *AvrLm7* (C1 and C2) effectors, an additional melting curve (C3) was detected on virulent isolates (*avrLm4-7*). To analyze the entire *AvrLm4-7* region, reference nucleotide sequences were retrieved from NCBI-Genbank and aligned using MEGA 6. Two sets of external primers were designed using the software Primer3 (AvrLm47A and AvrLm47B). A total of five melting profiles were found using the primers AvrLm47A. Melting profiles were similar among

isolates with a functional *AvrLm7* gene (A1, A2, and A3), a different HRM profile was observed for those isolates with a functional *AvrLm4-7* gene (A0), and virulent isolates *avrLm4-7* showed a unique melting profile (A4). A total of three HRM profiles with minor variations were detected in the external region using AvrLm47B primers. *AvrLm4-7* or *AvrLm7* (B1 and B2) and avrLm4-7 (B0). The obtained melting profiles allows the examination of the allelic variability among *L. maculans* isolates. HRM analysis allows the identification and discrimination between *AvrLm4-7* from *AvrLm7* isolates.

INTRODUCTION

The hemibiotrophic fungi *Leptosphaeria maculans* is an ascomycete that causes black leg, one of the most important diseases in Brassica crops (West et al., 2001). The damage caused by this disease results in serious yield losses. Therefore, managing black leg is essential to reduce the impact of this disease. Genetic resistance is known to be an effective way to control this disease (Kutcher et al., 2010). The resistance mechanism that the plant uses to combat pathogenic microorganisms is based on the innate immune system of each cell, which recognizes systemic signals produced at the infection sites (Hammond-Kosack & Jones, 1997). The first layer of immunity is based on the recognition of pathogenic molecular signals called pathogen associated molecular patterns (PAMPs) by plant receptors also called pattern recognition receptors (PRRs). This first plant defense layer occurs extracellularly and are known as PAMP-triggered immunity (PTI) (Jones & Dangl, 2006). However, *L. maculans* has the ability to overcome the first plant immunity layer or PTI (Blondeau et al., 2015). It is in the second layer of immunity of the plant, which is based on intracellular nucleotide binding and leucine rich repeat domains (NB-LRR) proteins encoded by resistance genes (R), that small proteins (SSPs) secreted by the pathogen, also known as effectors, are recognized. When an effector is recognized by a specific NB-LRR protein, this recognition results in effector-triggered immunity (ETI). The ETI is highly related

with the major-gene resistance and hypersensitive response (HR) or cell death at the infection cite preventing the dissemination of the pathogen through the rest of the plant (Jones & Dangl, 2006).

In *L. maculans*, 14 effectors encoded by avirulence genes have been identified, and seven genes have been cloned. The cloned genes include *AvrLm1, AvrLm2, AvrLm3, AvrLm5, AvrLm4-7, AvrLm6,* and *AvrLm11*. Some of these avirulence genes reside in two separate clusters consisting of, *AvrLm1-2-6* and *AvrLm3-4-7-9-LepR1* (Balesdent et al., 2002; Dilantha et al., 2018; Ghanbarnia et al., 2018; Marcroft et al., 2012; Plissonneau et al., 2016).

In canola, 18 major resistance genes have been identified. These include *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, Rlm11, RlmS, LepR1, LepR2, LepR3, LepR4, BLMR1,* and *BLMR2*. From these resistance genes only two have been cloned, *Rlm2* and *LepR3* (Dilantha et al., 2018; Marcroft et al., 2012).

The effector encoded by the gene *AvrLm4-7* is unique in the plant-pathogen interaction because *AvrLm4-7* confers dual recognition to resistance genes *Rlm4* and *Rlm7* in the plant (Parlange, 2009). A single mutation caused by a change of a glycine to an arginine residue in the protein encoded by the *AvrLm4-7* gene is responsible for the breakdown of resistance produced by *Rlm4*. This mutation does not affect *Rlm7* recognition (Blondeau et al., 2015; Parlange, 2009). The gene *AvrLm4-7* also has a significant role in the virulence of others *L. maculans* effectors. For example, the resistance gene *Rlm3* is unable to recognize *AvrLm3* in the presence of *AvrLm4-7* because of their related linkage (Plissonneau et al., 2016). The avirulence genes *AvrLm9* and *AvrLepR1* are also closely related with *AvrLm4-7*, because those genes form a group located in supercontig 12 of the *L. maculans* genome that is different from other *Avr* genes (Ghanbarnia et al., 2018; Plissonneau et al., 2016).

A clear understanding of the population biology of the *Leptosphaeria maculans* effectors, and the interaction with their corresponding resistance genes in the plant provides a better view of

the pathogen virulence and how plant immunity works (Blondeau et al., 2015; Jones & Dangl, 2006). Knowledge of the virulence-avirulence genotypes of the pathogen is essential to monitor the impact of the disease regionally. Identifying those specific avirulence genotypes, allows the selection of the best source of resistance in canola (Van de Wouw et al., 2018). According to Diaz (2015), the avirulence allele tests *AvrLm4-7* is present in 100% of the *L. maculans* population in Oklahoma. However, pathogenicity on the cultivar Jet Neuf that harbors *Rlm4* revealed that nearly all had the *avrLm4 – AvrLm7* allele of this gene. Therefore, a rapid detection of this avirulence allele is essential in this region. Pathogenicity tests consisting of inoculation of a fungal isolate suspension on *Brassica* differentials that harbor either the resistance gene *Rlm4* or *Rlm7*, and the amplification of the *AvrLm4-7* gene by PCR are currently required for identification and characterization of *AvrLm4-7*. However, a single PCR is not able to discriminate those isolates that have lost the *Rlm4* recognition (Carpezat et al., 2013). More sensitive and rapid techniques are needed to identify and discriminate virulent and avirulent alleles of the *AvrLm4-7* gene towards the resistance genes *Rlm4* and *Rlm7*.

High Resolution Melting (HRM) is a post PCR technique that discriminates PCR amplicons based on their melting temperature measured when the DNA is denaturing from double stranded to single stranded by an increase in temperature (Wittwer, 2009). Based on the GC content, length, and DNA strand structure of an amplicon, the melting temperature can be determined. This technique has been applied for genotyping and mutation scanning with a high sensitivity (Erali et al., 2008; Wittwer et al., 2003).

Carpezat et al. (2013) developed a HRM technique to identify the *AvrLm4-7* gene *from L. maculan*s isolates. A set of internal primers (AvrLm7UP1/AvrLm7LOW2) that amplified a fragment (493 bp) of the *AvrLm4-7* gene was used for the *Avrlm4-7* characterization. A total of four different HRM profiles were identified among isolates with different virulence/avirulence genotypes. However, almost 50% of the entire *AvrLm4-7* allele (433 bp) was not covered in

Carpezat's study. That is why two sets of external surrounding primers were designed to amplify these fragments of the *AvrLm4-7* allele. The new surrounding primers do not overlap the region amplified by the reported primers AvrLm7UP1/AvrLm7LOW2. The objective of this study was to assess the genetic variability of the *AvrLm4-7* gene among *Leptosphaeria maculans* isolates using pathogenicity and High-Resolution Melting (HRM) analysis.

MATERIALS AND METHODS

Fungal isolates:

A set of 15 *Leptosphaeria maculans* isolates with known avirulence genotypes were used for this study. Two isolates were previously collected and purified from *Brassica napus* leaves with black leg symptoms in Oklahoma. The other 13 isolates are part of the International Blackleg Crucifer Network collection, provided by Dr. A. Van de Wouw, School of Botany, The University of Melbourne, Australia (Table S1). The avirulence genotypes of each isolate were previously identified by cotyledon tests at seedling stage using *Brassica napus* cultivars with known resistance genes. Also, avirulence genes were previously confirmed by PCR amplification for *AvrLm1*, *AvrLm6*, and *AvrLm4-7* using a specific set of primers (Diaz, 2015; Marcroft et al., 2012). Isolates were stored in small freezer vials filled with desiccated filter paper discs colonized by the fungus at -4 °C.

Pathogenicity tests:

To confirm the presence of the avirulence gene *AvrLm4-7*, 10 uL of a conidial suspension at 10^6 mL^{-1} of each isolate were inoculated onto cotyledons of 7-day-old *Brassica* seedlings of the differential Jet Neuf that has *Rlm4* and Westar, a susceptible check with no *Rlm* genes. Eight plants were inoculated for each isolate and cultivar combination. After inoculation, plants were

kept under 100% relative humidity in the dark in a dew chamber at 25 ^{0}C for 48 hours. After high humidity incubation, plants were further incubated in a growth chamber at 24 ^{0}C, RH 80%, with a 12 h light/dark cycle. Disease severity was evaluated 11 days after inoculation using the IMASCORE rating scale which contains six infection classes (IC), IC1 to IC3 are resistance responses while IC4 to IC6 describes susceptibility. IC1 is a typical hypersensitive response (HR), IC2 is denoted by a dark lesion less than 3 mm in diameter, and IC3 represents a larger lesion surrounded by a dark necrotic margin. IC4 to IC5 represent large gray-green lesions. IC4 lesions lack a dark margin and pycnidial formation, IC5 lesions have a few pycnidia while IC6 lesions produce abundant pycnidial formation and tissue necrosis (Balesdent et al., 2001).

DNA extraction:

DNA was isolated from cultures grown in V8 broth for three weeks at room temperature. Mycelial samples (0.7 grams) were collected into 2 mL screw cap tubes containing 5 mm stainless steel beads. The capped tubes were first cooled with liquid nitrogen and then homogenized in a Cole-Parmer Mini bead beater instrument for 20 seconds. Bead beating was repeated twice. Genomic DNA was extracted using the DNeasy 96 Plant Kit, following the specifications of the manufacturer (Qiagen S.A., Germantown, MD, USA).

PCR amplification of the *AvrLm4-7* gene:

The *AvrLm4-7* gene was amplified using a set of specific primers (Avr47extUp3 and Avr47extLo) described by Parlange (2009) (Table 3.1). The polymerase chain reaction (PCR) mix consisted of 2 uL of DNA (10ng/uL), 6 uL of autoclaved water, 1 uL of primers forward and reverse (5 uM), and 10 uL of GoTaq® Green Master Mix (Promega Co. Road Madison, WI, U.S.A). PCR amplifications were performed using the model Eppendorf™ Mastercycler™ pro PCR System (Thermo Fisher Scientific, Grand Island, NY, USA) under the following conditions: 3 min at 95 °C, followed by 30 cycles of 45 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, with a

final extension of 5 min at 72 °C. PCR products were visualized by electrophoresis on a 1.5% agarose gel (60 min at 100V).

Table 3.1. Primer sequences used to amplify the *AvrLm4-7* gene from *Leptosphaeria maculans* in this study.

Primer code	Primer Sequence (5'-3')	Amplicon (bp)	Length (bp)	References
AvrLm7UP1	AACATGCCACTATCCCTC	493	18	Carpezat et al., 2013
AvrLm7LOW2	ACCTCCGTATCTTTAGTC		18	
Avr47extUp3	AACCCTGCTAGATAGGTAAGCT	788	22	Parlange et al., 2009
Avr47extLo	GATGGATCAACCGCTAACAA		20	

Primer design:

The sequence for the *AvrLm4-7* gene (GenBank: AM998638.1) was retrieved from the National Center for Biotechnology Information (NCBI). The sequences of the internal primers (AvrLm7UP1/AvrLm7LOW2) developed by Carpezat et al. (2013) were used to identify the amplicon region in the *AvrLm4-7* gene using the software Primer3. Two sequences from the surrounding external regions upstream and downstream from the internal primers were selected to design two sets of external primers named AvrLm47-A and AvrLm47-B (Fig. 3.1). Primer 3 was used to design the two set of external surrounding primers (Table 3.2). The web interface mFold was used to predict *in silico* the thermodynamics, self-dimer formation, and internal structures of each primer.

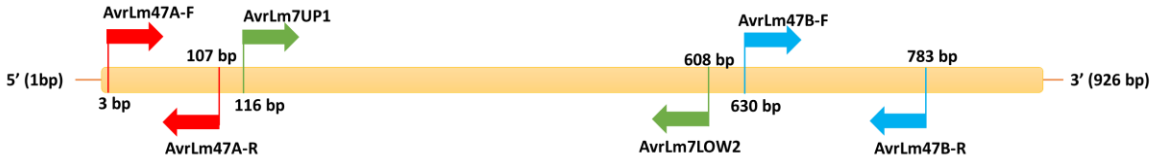

Figure 3.1. Graphical representation showing the locations of the external surrounding primer sets designed for High Resolution melting analysis in the *AvrLm4-7* sequence (GenBank: AM998638.1). Primer sets were AvrLm47A-F/R (105 bp), AvrLmUP1/LOW2 (493 bp), and AvrLm47B-F/R (154 bp).

Table 3.2. Sequences and main features of the primers that were designed for High Resolution Melting analysis.

Primer code	Primer Sequence (5'-3')	Amplicon size (bp)	Primer length (bp)	Tm[a]	GC%[b]	ANY[c]	3'[d]
AvrLm47A-F	GGGTTACAACGACAAGCTTATTTAAC	105	26	54.45	33.33	6	2
AvrLm47A-R	TCAAAGGGTTTATACTTGGTG		21	54.46	38.1	4	0
AvrLm47B-F	ACCGTCTTTGTTAGCGGTTG	154	20	60.17	50	4	0
AvrLm47B-R	ATTTTCAACCAGACCCACCA		20	60.21	45	3	0

[a] Tm: Melting temperature calculated by Primer3.
[b] GC%: Percentage of cytosine and guanine calculated by Primer3.
[c] ANY: tendency to anneal to itself or form secondary structures calculated by Primer3.
[d] 3': tendency of primer to form a primer dimer with itself on the 3' terminal calculated by Primer3.

High Resolution Melting:

The HRM mix was composed of 1 uL of genomic DNA (10 ng/uL), 1 uL of autoclaved water, 1 uL of primers forward and reverse (each at 5 uM), 1 uL of LCGreen (BioFire Defense, Salt Lake City, UT, USA), and 5 uL of Hot-Start DNA Polymerase (Thermo Fisher Scientific, Grand Island, NY, USA). PCR amplification and HRM was performed using a Rotor-GeneTM 6000 real-time rotary analyzer equipment (Qiagen S.A., Germantown, MD, USA). The reaction was done with the following conditions: an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. HRM curves were analyzed using the Rotor-Gene Q series software (Qiagen S.A., Germantown, MD, USA).

AvrLm4-7 sequence analysis:

The *AvrLm4-7* gene was amplified using the primers sets Avr47extUp3/ Avr47extLo described by Parlange (2009). PCR products were cleaned using the QIAquick Gel Extraction Kit (Qiagen S.A., Germantown, MD, USA) and sent to the Oklahoma State University Recombinant

DNA/Protein Resource Facility for sequencing. The sequences were aligned using the Molecular Evolutionary Genetics Analysis software (MEGA 7) and compared to identify polymorphic sites.

RESULTS

AvrLm4-7 pathogenic and PCR amplification tests:

A set of 15 *Leptosphaeria maculans* isolates were characterized for the avirulence/virulence phenotype reaction towards the *Brassica napus* cultivar Jet Neuf that harbors the resistance gene *Rlm4*. The isolates D2, D3, D4, D5, D13, D8, and D17 were avirulent on Jet Neuf (Table 3.3). The isolates D9, 102, and 124 were virulent on Jet Neuf. The avirulence phenotype of the isolates D6, D7, D10, D14, and D16 could not be determined due to a low conidial production. The information about virulence/avirulence towards *Rlm7* was taken from Marcroft et al. (2012) except for the isolates 102 and 124. Finally, the isolates D3, D6, D7, and D10 showed no amplification with the Avr47extUp3/ Avr47extLo set of primers. The rest of the isolates showed the expected PCR amplification (788 bp) of the *AvrLm4-7* gene.

Table 3.3. Characterization of the avirulence gene *AvrLm4-7* based on pathogenicity tests and PCR.

Isolate	Interaction phenotype[a]		PCR[b]	*Avr4-7* Genotype[c]
	Rlm4	*Rlm7*[d]	*AvrLm4-7*	
D2	A	V	+	*A4A7*
D3	A	V	-	*a4a7*
D6	n.d.	V	-	*a4a7*
D7	n.d.	V	-	*a4a7*
D10	n.d.	V	-	*a4a7*
D4	A	A	+	*A4A7*
D5	A	A	+	*A4A7*
D13	A	A	+	*A4A7*
D8	A	A	+	*A4A7*
D9	V	A	+	*a4A7*
D14	n.d.	A	+	*a4A7*
D16	n.d.	A	+	*a4A7*
D17	A	A	+	*a4A7*
102	V	n.d.	+	*a4A7*
124	V	n.d.	+	*a4A7*

[a] Interaction phenotype: V= virulent, A= avirulent, based on resistant and susceptible interaction between the isolate and the differential respectively, n.d.= not determined. [b] + presence or – absence of the *AvrLm4-7* gene, based on the PCR amplification. [c] Inferred *AvrLm4-7* genotype of *L. maculans* isolates, avirulence towards *Rlm4* and *Rlm7* (*A4A7*), virulence towards *Rlm4* and avirulence towards *Rlm7* (*a4A7*), virulence towards *Rlm4* and *Rlm7* (*a4a7*). [d] Data described in Marcroft et al. (2012).

High resolution melting analysis:

The diversity of HRM melting curve profiles of the three segments of the 926 bp fragment of the avirulence gene *AvrLm4-7* was analyzed in 15 *Leptosphaeria maculans* isolates. The first upstream fragment corresponds to an amplicon of 105 bp. It was amplified using the primers set AvrLm47A-F/R. Five different HRM curve profiles were observed (A0, A1, A2, A3, and A4). The expected amplicon for isolates D6 and D7 were not amplified using an end point PCR. However, HRM curves were obtained from these isolates. Isolates D2, D6, D7, and D10 generated symmetric HRM curves with similar melting temperatures (74.7 °C). The HRM profile

A4 was assigned for these isolates. Isolates D4 and D5 share the A0 HRM profile with a melting temperature (Tm) of 75 °C. Isolates D13, D14, D16, and D17 have the same HRM profile A2 with a Tm of 75.2 °C. The HRM profile A1 was assigned to isolates D8 and D9 with a Tm of 75.1 °C. The isolates 102 and 124 showed a different HRM profile (A3) with the highest Tm (75.5 °C). (Table 3.4).

The central fragment corresponded to an amplicon of 493 bp amplified with primers AvrLm7UP1/LOW2. The four HRM profiles obtained for this *AvrLm4-7* gene fragment are C0, C1, C2, and C3. Similar, to the first external fragment of the gene, the expected amplicon for isolates D6 and D7 was not amplified using end point PCR. But they generated HRM curves, with similar Tm and melting profiles (C3). In addition, isolates D2, D6, D7, and D10 displayed the same (C3) melting profile. Isolates D13, D8, D9, D14, D16, and D17 had similar Tm and melting profiles (C1). The isolate D5 and D4 showed a few differences in the HRM profile (C0) if compared with the HRM profile C1. Finally, the isolates 102 and 124 displayed a clear difference in the HRM profiles (C2) compared from the other isolates (Table 3.4). As a consequence of the large amplicon (493 bp), most of the isolates displayed double melting domains (peaks) in the derivative melting curve graph (Figure 3.2-B).

The last fragment corresponded to an amplicon of 154 bp. A total of three HRM profiles were obtained from the amplicon of the AvrLm47B-F/R set of primers (B0, B1, and B2) (Figure 3.2). The isolates D4, D5, D8, D9, D13, D14, D16, and D17 showed the same melting profile (B2). The isolates 102 and 124 had a different melting temperature and variations in the melting curve shapes displayed the HRM profile B1. The isolate D2 showed a unique melting profile (B0).

Isolate D3 did not show any PCR nor HRM amplification in any of the three regions analyzed within the gene *AvrLm4-7*. The isolates D6, D7, and D10 did not display any HRM

profiles for the third region because there was no amplification with the AvrLm47B-F/R set of primers (Table 3.4).

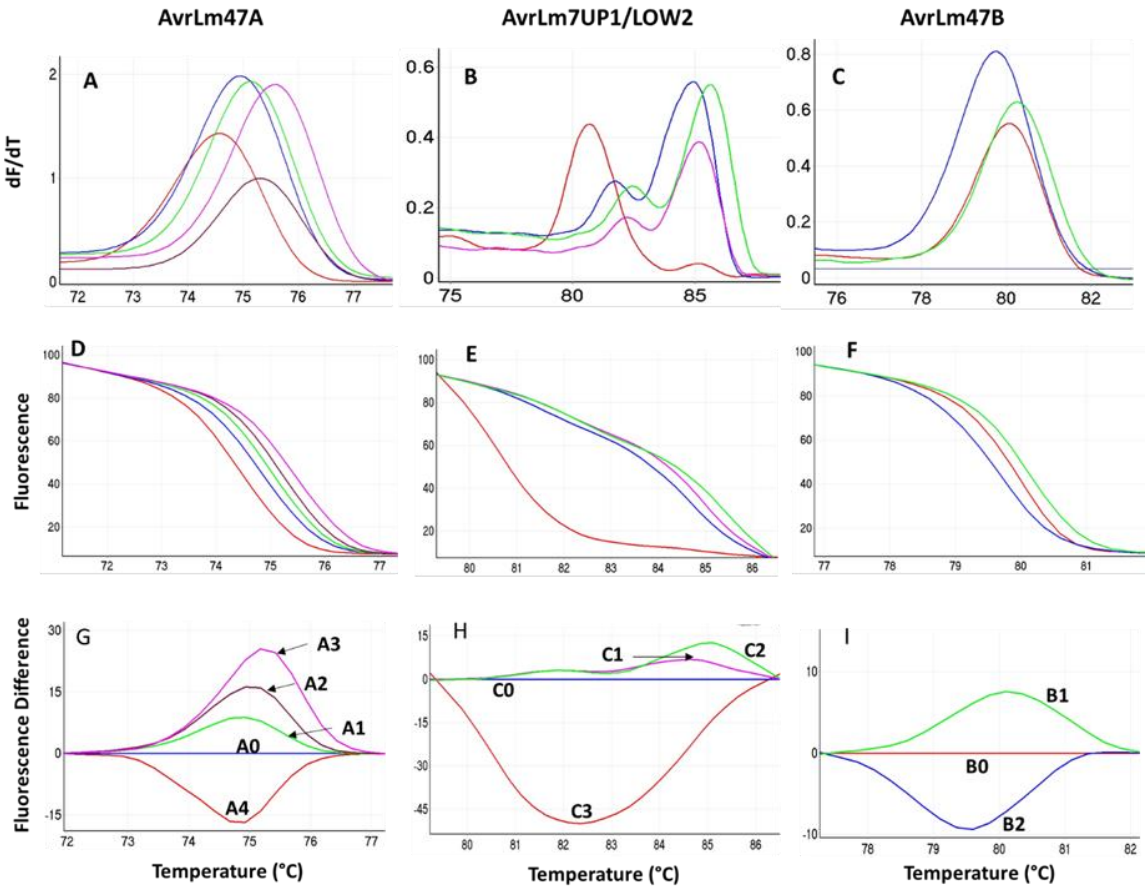

Figure 3.2. High resolution melting (HRM) curves obtained from three PCR amplified segments of the *AvrLm4-7* gene (AvrLm47A, AvrLm7UP1/LOW2, and AvrLm47B). A, B, and C represent the derivative melting curves. D, E, and F represent the loss of fluorescence normalized curves plotted against increasing temperature. G, H, and I are difference graphs derived from the normalized data in which groups A0, C0 and B0 were subtracted. G, melting profiles obtained using the set of primers AvrLm47A. A0 (A4A7), A1, A2, and A3 (a4A7), A4 (a4a7). H, melting profiles obtained using the primers AvrLm7UP1/LOW2, C0 (A4A7), C1 and C2 (a4A7), and C3 (a4a7). I, represent the fluorescence difference profiles obtained using the set of primers ArvLm47B, B0 (a4a7), B1 (a4A7), B2 (A4A7 or a4A7).

Table 3.4. PCR amplification and high resolution melting profiles of the *AvrLm4-7* gene in *Leptosphaeria maculans* isolates.

Isolate	*Avr* Genotype[a]	End point PCR[b]			Quantitative PCR + HRM (Tm °C)[c]				
		AvrLm47A	AvrLm7UP/LOW	AvrLm47B	AvrLm47A	AvrLm7UP/LOW		AvrLm47B	HRM curve profiles[d]
D2	*A4A7*	+	+	+	74.5	80.7		80.1	A4, C3, B0
D3	*a4a7*	-	-	-	-	-	-	-	-
D6	*a4a7*	-	-	-	74.7	81.3		-	A4, C3
D7	*a4a7*	-	-	-	74.8	81.5		-	A4, C3
D10	*a4a7*	+	+	-	74,8	80	81.7	-	A4, C3
D4	*A4A7*	+	+	+	75	81.8	85	79.7	A0, C0, B2
D5	*A4A7*	+	+	+	74.9	81.7	84.9	79.7	A0, C0, B2
D13	*A4A7*	+	+	+	75.3	82	85.3	79.9	A2, C1, B2
D8	*A4A7*	+	+	+	75.1	82	85	79.9	A1, C1, B2
D9	*a4A7*	+	+	+	75.1	82	85	79.9	A1, C1, B2
D14	*a4A7*	+	+	+	75.3	82.3	85.1	79.9	A2, C1, B2
D16	*a4A7*	+	+	+	75.2	82	85	79.9	A2, C1, B2
D17	*a4A7*	+	+	+	75.2	82	85.1	79.8	A2, C1, B2
102	*a4A7*	+	+	+	75.5	82.4	85.6	80.2	A3, C2, B1
124	*a4A7*	+	+	+	75.5	82.5	85.6	80.2	A3, C2, B1

[a] Avirulence genotype of *Leptosphaeria maculans* isolates, A4A7, avirulent towards *Rlm4* and *Rlm7*; a4A7, virulent and avirulent towards *Rlm4* and *Rlm7* respectively; a4a7, virulent towards *Rlm4* and *Rlm7*. [b] PCR amplification of the three fragments of the *AvrLm4-7* allele; +, PCR amplification, - no PCR amplification. [c] Melting temperature of each isolate. [d] HRM curve diversity. Letters represent the set of primers used to analyze the melting curve profiles. A, AvrLm47A; C, AvrLm7UP/LOW; and B, AvrLm47B.

Sequence analysis:

A segment of 660 bp corresponding to the reference *AvrLm4-7* gene was sequenced using the set of primers Avr47extUp3/Lo. The isolates D3, D6, D7, and D10 did not generate amplification using PCR. After sequencing, the alignment of other 11 *L. maculans* isolates showed five polymorphic sites. A change of a Thymine to Adenine at base 80 was observed in the isolates D8 and D17. The isolates 102 and 124 showed a change of a Thymine to Cytosine at base 251. The isolates D2, D8, D9, D14, D16, and D17 showed a change of a Guanine to Adenine at base 373. A change of a Guanine to Cytosine at base 475 was observed in the isolates D2, D8, D9, D14, D16, D17, 102, and 124. Isolate D2 showed two insertions, the first was the addition of a Guanine after the base 604, the second insertion was observed after the base 611 with the addition of a Thymine.

DISCUSSION

This study describes the use of PCR coupled to high resolution melting for the evaluation of the avirulence gene *AvrLm4-7* based on the DNA melting analysis of reference isolates of *Leptosphaeria maculans* which have different virulence/avirulence alleles of the *AvrLm4-7*. High resolution melting analysis is a molecular technique that allows discrimination based on the melting variability of DNA samples. The melting temperature is the temperature in which 50% of the DNA amplicon is double stranded and the other half is denatured and single stranded. When the DNA becomes single stranded it releases a fluorescence dye that was bound to it during PCR amplification. The data generated by the loss of fluorescence during the HRM analysis, can be interpreted based on the melting point (°C) or the shape of the melting curves. This technique allows the discrimination of different genotypes based on the type and number of nucleotides and the GC content of each sample (Carpezat et al., 2013; Erali et al., 2008). Three melting plots were

obtained after HRM analysis. The original HRM derivative curve provides the melting temperature of each isolate. Also, the normalization of the fluorescence during the melting transitions allows differentiation of the melting profiles. However, the differences between genotypes becomes clear by evaluating the fluorescence difference graph where one melting curve is selected as a reference and subtracted for normalization, becoming a horizontal line (zero) in the fluorescence difference plot. The florescence of the reference genotype is subtracted from each sample generating different melting profiles that are easier to identify (Wittwer et al., 2003).

To develop a complete HRM analysis of the *AvrLm4-7* gene, we determined that a fragment used by Carpezat et al. (2013) in a previously analyzed HRM study only covers the central fragment in the region of the *AvrLm4-7* gene. This fragment encompasses a region of only 493 bp of a total of 926 bp that comprises the entire gene. That is why two separated fragments were analyzed in this study to cover the rest of the *AvrLm4-7* allele. A total of five melting profiles were observed in the first fragment of the gene, four melting profiles for the central portion of the gene, and three melting profiles for the third section of the *AvrLm4-7* gene.

Primers used to analyze the three regions within the *AvrLm4-7* gene allowed the discrimination between virulent (a4a7) and avirulent (A4A7 / a4A7) isolates. With four HRM profiles, primers set AvrLm47A and AvrLm7UP1/LOW2 allowed the discrimination of avirulent isolates towards Rlm4 and Rlm7 (A4A7) and virulent and avirulent isolates towards *Rlm4* and *Rlm7,* respectively (a4A7). The melting profiles A4 and C3 describe the virulent isolates towards *Rlm4* and *Rlm7* (a4a7). Isolate D2 displayed the combination of melting profiles A4 and C3 which represent virulence towards *Rlm4* and *Rlm7.* The melting profile that showed D2 as a virulent isolate agrees with the avirulence genotype described by Marcroft et al. (2012). However, D2 displayed avirulence towards *Rlm4* based on the pathogenicity tests and it was positive for

PCR amplification of the *AvrLm4-7* gene. The insertions mutations into the DNA sequence of this isolate would be the cause of this melting temperature differences.

The melting profiles A0 and C0 that were identified in the isolates D4 and D5 indicates avirulence towards *Rlm4* and *Rlm7* (A4A7). There were three different melting profiles (A1, A2, and A3) that were identified for isolates that were virulent and avirulent on *Rlm4* and *Rlm7* respectively (a4A7). The melting profiles C1 and C2 also differentiated isolates that only were avirulent towards *Rlm7*.

On the other hand, the primers set AvrLm47B did not discriminate a4A7 isolates from A4A7 isolates. This may be due the polymorphic sites that may be responsible of the virulence towards *Rlm4*, which are located between the base 80 and 595. However, virulent isolates (a4a7) were differentiated from avirulent isolates (A4A7 / a4A7) for avrLm7.

With the exception of isolate D8, the pathogenicity tests agree with the results obtained from the alignment of the sequences of the isolates D8, D9, D14, D16, D17, 102 and 124. The polymorphic sites that were found in these isolates could be responsible of the virulence towards *Rlm4*. According to Parlange (2009), a single mutation is responsible of the escape from recognition by the resistance gene *Rlm4*. The lack of the PCR amplification of the *AvrLm4-7* gene in the isolate D3 with the primers set reported by Parlange (2009), and Carpezat et al. (2013), and the set of primers designed in this study (AvrLm47A and AvrLm47B), could have corresponded to a complete deletion of the *AvrLm4-7* gene (Daverdin et al., 2012).

Monitoring the frequency of the virulent/avirulent genotype of the *L. maculans* population is essential to develop an effective management strategy by deploying the appropriate resistance genes in the field. There are some techniques that allow for the identification of the avirulence gene *AvrLm4-7*. However, pathogenicity tests are useful for the identification of the avirulence/virulence alleles of *L. maculans* isolates by cotyledon inoculation on *Brassica napus*

differentials harboring either the resistance genes *Rlm4* or *Rlm7*. Pathogenicity tests require a minimum of 20 days for complete identification of the avirulence/virulence phenotype profile. Molecular tools such as sequence analysis of the gene is an effective method. Therefore, PCR coupled with HRM is a rapid and less expensive detection tool that can be used to monitor the virulence and avirulence *AvrLm4-7* allele frequency in *L. maculans* population with a high degree sensitivity.

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CHAPTER IV

IDENTIFICATION OF MAJOR-GENE RESISTANCE TO *Leptosphaeria maculans* IN WINTER CANOLA CULTIVARS AND BREEDING LINES

ABSTRACT

Black leg caused by *Leptosphaeria maculans*, is the most important disease of winter canola (*Brassica napus*) in Oklahoma and worldwide. Genetic resistance is the most effective and economical strategy to reduce the impact of this disease. Identifying major-resistance genes in winter canola is important to make decisions on which cultivars to plant and what breeding lines to develop. A collection of 7 canola cultivars, 17 hybrids, and 119 breeding lines were screened for seedling resistance by using a gene-for-gene interaction with the *L. maculans* races *AvrLm6,7*; *AvrLm1,6,7*; and *AvLm1,2,4,7, S*. Seedling responses were resistant if >80% of plants showed a resistance response, susceptible if >80% of plants showed a susceptible response, and heterogeneous when the cultivar-isolate interaction showed that 20% to 80% of the plants were resistant. *Rlm1, Rlm2, Rlm4, Rlm6, Rlm7* and *RlmS* resistance genes could be inferred in this study. Of the canola cultivars and hybrids, 33% were resistant to all three races, while only 7.5% of the breeding lines were resistant to all three races. Most (83%) of the breeding lines were susceptible to races *AvrLm6,7* and *AvrLm1,6,7*; but were resistant to race *AvrLm1,2,4,7,S*. Less than 10% of the breeding lines and just two cultivars showed a susceptible response to all three

races. Among the breeding lines inoculated with the local races *AvrLm1,6,7* and *AvrLm6,7,* 89% were susceptible, 10% were heterogeneous, and just 1% were resistant. Conversely, more than 80% of the breeding lines were resistant or heterogeneous in response to race *AvrLm1,2,4,7,S* from Australia. These results indicate that *Rlm2* and *Rlm4* were the most common resistance genes in breeding lines. The presence of genes *Rlm1, Rlm2, Rlm6, Rlm7* and/or *RlmS* was inferred in the cultivars, hybrids or breeding lines that had resistance response to all three races. *Rlm7* is known to occur in 41% of the hybrids evaluated.

INTRODUCTION

Canola (*Brassica napus*) is one of the most important oilseed crops in Europe, China, and Canada. In 1988, canola was first introduced in U.S. after its cultivation in Canada (De Block et al., 1989). To date, this crop has become the world's third largest source of vegetable oil after soybean and palm oil (Bhardwaj & Hamama, 2003). An estimated two million acres of canola were harvested in the U.S. in 2017. Oklahoma has become the second largest producer of canola behind North Dakota (USDA-ERS, 2017). However, diseases such as black leg, Sclerotinia stem rot, powdery mildew, alternaria black spot, and aster yellows can damage canola grown in the state (Boyles et al., 2017). Black leg, caused by the ascomycete *Leptosphaeria maculans*, is considered the most severe fungal disease of canola (West et al., 2001). Black leg occurs world-wide and causes severe yield losses in canola when stem cankers are produced that gridle stems prior to maturity (Fitt et al., 2006).

The life cycle of this hemibiotrophic fungus starts every year on infested canola stubble where ascospores are produced via sexual reproduction that serve as a primary inoculum. Airborne ascospores are deposited onto canola leaves where they germinate through stomata and cause infections. Gray to pale green leaf spots develop which become necrotic and produce

pycnidia. Conidia from pycnidia are splash dispersed and secondary infections on more leaves. Leaf spots can occur at any stage of plant development. The pathogen then grows endophytically from infected leaves through petioles and into the crown where hyphae colonizes the base stem until ripening stages (Damicone et al., 2015; Plissonneau et al., 2017). After flowering, *L. maculans* produces cankers in the lower stem during pod ripening. On susceptible varieties that are infected early in development, the stem may be girdled enough to cause premature death of the plant. Because of the slow progression of disease, stem cankers are most severe on plants infected early in their development. When winter canola is infected in the spring, stem cankers are often superficial and less damaging (Gajula, 2014; T. Rouxel & Balesdent, 2005; West et al., 2001).

There are management strategies that can reduce the impact of this disease. Crop rotation, stubble management, chemical control by using seed treatments and foliar fungicides, and genetic resistance are currently used against black leg disease (McCredden et al., 2017). There are two types of genetic resistance. Quantitative or multigenic resistance is expressed at the adult stage of the plant reducing the development of stem cankers. Major-gene or seedling resistance is expressed at early stages of plant development by restricting foliar infections (Corwin & Kliebenstein, 2017; West et al., 2001). Major-gene resistance follows the gene-for-gene theory in which a resistance gene (*R*) in the plant and an avirulence gene (*Avr*) in the pathogen must both to be present to produce phenotypic resistance in the host. Conversely, if the plant lacks the resistant gene (*R*) or the pathogen does not have the avirulence gene (*Avr*), then a compatible reaction or occurs to produce disease (Campbell et al., 2002). To date, 18 major resistance genes have been identified in *Brassica species* (*Rlm1* to *Rlm11, RlmS, LepR1* to *LepR4, BLMR1*, and *BLMR2*), and 14 avirulence genes have been described for *L. maculans* of which seven have been cloned and sequenced (*AvrLm1, AvrLm2, AvrLm3, AvrLm5, AvrLm4-7, AvrLm6*, and *AvrLm11*). (Balesdent

et al., 2002; Dilantha et al., 2018; Gout et al., 2006; Marcroft et al., 2012; Parlange, 2009; Plissonneau et al., 2016).

The use of genetic resistance combining adult plant resistance and seedling resistance in canola cultivars can significantly decrease the impact of black leg disease (Brun et al., 2010). However, changes in the frequency of the avirulence/virulence genes in the pathogen can lead to major-gene resistance being overcome when the same resistance genes are deployed year after year as this increases the selection pressure for virulence may result in a breakdown of the resistance (Marcroft et al., 2012). For example, cultivars harboring the "sylvestris" resistance gene (*RlmS*) were successfully introduced in Australia in 2000 but were overcome by the pathogen three years later causing serious economic losses (Sprague et al., 2006). A similar case occurred in France when the resistance gene *Rlm1*, present in commercial cultivars, was overcome three years after it was introduced (Rouxel et al., 2003).

While breeding programs can improve genetic resistance, rotating canola cultivars with different resistance gene profiles may reduce selection pressure, resulting in a durable protection against black leg (Brun et al., 2010; Marcroft et al., 2012). In the interest of applying genetic resistance as a strategy to control black leg, it is necessary to have knowledge of the resistance genes present in canola cultivars. Major-gene resistance in *Brassica napus* germplasm to *L. maculans* has been described in different canola producing regions. For example, Marcroft et al. (2012) screened diverse Australian *Brassica* cultivars based on their interaction with known *L. maculans* races, inferring the presence of one or more resistance genes in over 60% of the cultivars. Zhang et al. (2017) characterized seedling and adult plant resistance from a collection of *B. napus* cultivars from China. At least four resistance genes were identified in 40% of the cultivars tested, with the most common major-genes being *Rlm3* and *Rlm4*.

Although studies have been conducted to date on identifying resistance genes to *L. maculans* in different *Brassica species*, little is known about major resistance genes present in local winter canola cultivars, hybrids and breeding lines grown in the Oklahoma and the Southern Great Plains. There is a need for canola producers and canola breeders to know about presence of resistance genes in canola varieties and which ones are the most effective against black leg. The use of major-gene resistance as a strategy to manage this disease can reduce the infection of growing plants and resulting canker development in older plants. By inoculating races of *L. maculans* that harbor different avirulence genes on seedling cotyledons, a corresponding resistance gene in the plant can be inferred. The objective of this study was to test canola cultivars, hybrids, and breeding lines for major-gene-resistance to known *Leptosphaeria maculans* races.

MATERIALS AND METHODS

Fungal isolates

Isolates 102 and 124 were isolated from symptomatic canola leaves previously collected in Oklahoma and characterized for avirulence alleles (Table 4.1) using pathogenicity on differential cultivars harboring resistance genes *Rlm1, Rlm2, Rlm3,* and *Rlm4*; and by polymerase chain reaction (PCR) amplification for the avirulence alleles *AvrLm1, AvrLm6,* and *AvrLm4-7* (Diaz, 2015). These isolates represented races *AvrLm1,6,7* (102) and race *AvrLm6,7* (124) which were broadly virulent on the differentials and represented over 90% of the isolates characterized. These isolates sporulated well and were highly virulent. Isolates from Australia were provided by Dr. A. Van de Wouw, School of Botany, The University of Melbourne, Australia. These isolates are part of the International Black leg of Crucifers Network (IBCN), and they were previously

characterized for avirulence alleles. An Australian isolate with different avirulence profile (D5) was selected (Table 4.1).

Isolate storage and inoculum preparation

Isolates were stored on filtered paper colonized by the fungi at -4 °C. Fungal cultures were grown on SV8 juice agar medium (For 1 L: 200 ml of V8 juice [clarified with 3g/L CaCO3], 16g of agar, 100 mg of streptomycin). The plates were incubated under artificial light at room temperature (22-25°C). After 7-8 days of growth, conidia were harvested from a single pycnidium and streaked on YPS agar plates (For 1L: 1g of yeast, 1g of peptone, 1g of dextrose, 17g of agar, 100mg of penicillin, 100mg of streptomycin) using a sterile loop. YPS plates were incubated for 8 to 10 days under artificial light at room temperature and conidial suspensions were harvested in sterile water and dispensed into 15ml conical centrifuge tubes (Thermo Fisher Scientific Inc.). The spore suspensions were filtered through autoclaved cheesecloth to remove excess mycelia and adjusted to 10^6 spores per mL using a hemocytometer.

Table 4.1. *Leptosphaeria maculans* isolates used for cotyledon inoculations.

Isolate code	Isolate name	Country of origin	*Avr* genotype	Reference
102	CO-13-F1-1	U.S.	*AvrLm1,6,7*	Diaz, 2015
124	GR-13-F10-1	U.S.	*AvrLm6,7*	Diaz, 2015
D5	IBCN18	Australia	*AvrLm1,2,4,7,S*	Marcroft et al., 2012

Characterization of seedling resistance in Brassica napus:

A total of 7 cultivars, 17 hybrids, and 119 breeding lines from the Kansas State University canola breeding program were obtained from Michael Stamm at Department of Agronomy, Kansas State University, and screened for seedling resistance to the three races. Seeds were germinated on a moist paper towel placed in a plastic zip bag for 48 hours. Germinated seeds were transplanted into plastic cell pack trays containing Sunshine® soilless media (Canadian sphagnum peat moss, vermiculite, and dolomitic limestone). Trays were kept in a

growth chamber at 24^0C, RH 80%, a 12 h light/dark cycle, and were watered every two days. Cotyledons of 7-d old seedlings were wounded by perforating each cotyledon with a 200 uL micropipette tip (four wounds per plant) and inoculated with a droplet of 10 uL of spore suspension adjusted at 10^6 spores per mL. Eight plants were inoculated for each isolate and cultivar combination. After inoculation, seedlings were kept under 100% relative humidity in the dark in a dew chamber at 25 ^{0}C for 48 hours and then the plants were returned to a growth chamber. Disease severity was scored 11 days after inoculation following the IMASCORE rating scale which contains six infection classes (IC), IC1 to IC3 are resistant and IC4 to IC6 are susceptible. IC1 is denoted by a hypersensitive response (HR), IC2 represents a dark lesion less than 3 mm in diameter, and IC3 denotes a larger lesion surrounded by a dark necrotic margin. IC4 to IC5 represent large gray-green lesions. IC4 lesions lack a dark margin and pycnidial formation, IC5 lesions have a few pycnidia while IC6 lesions produce abundant pycnidial formation and tissue necrosis (Balesdent et al., 2001). A resistant phenotype implies the presence of the corresponding resistance gene *Rlm* in the plant, whereas a susceptible reaction implies the absence of (*Rlm*) gene in the plant. Seedling responses were resistant if >80% of plants showed a resistance response, susceptible if >80% of plants showed a susceptible response, and heterogeneous when the cultivar-isolate interaction showed that 20% to 80% of the plants were resistant (Rouxel et al., 2003). The susceptible cultivar Westar, which has no *Rlm* genes, was used as susceptible check for each cultivar and isolate combination. Inoculations were repeated at least twice.

RESULTS

A total of 15 *Leptosphaeria maculans* isolates were evaluated for avirulence alleles based on phenotype interaction on *Brassica napus* differentials. The isolate 102 was virulent on Westar,

Glacier and Jet Neuf but avirulent on Quinta. While the isolates 124 and D9 were virulent on all four differentials. The isolates from Australia D2, D4, D5, and D13 were avirulent on Quinta, Glacier and Jet Neuf. The isolates D3 and D17 were avirulent on Quinta and Jet Neuf but susceptible on Glacier. The isolate D8 was virulent on Quinta and Glacier but avirulent on Jet Neuf. Finally, the avirulence alleles could not be determined for isolates D6, D7, D10, D14 and D16 from Australia by phenotype interaction due to their low conidia production (Table 4.2).

Table 4.2. Evaluation of *Leptosphaeria maculans* isolates for interaction phenotype with *Brassica napus* differentials.

Isolate code	Isolate name	Interaction phenotype[a]				Inferred race[b]
		Westar	Quinta (*Rlm1,3 or 4*)	Glacier (*Rlm2,3*)	Jet Neuf (*Rlm4*)	
102	CO-13-F1-1	V	A	V	V	*AvrLm1*
124	GR-13-F10-1	V	V	V	V	none
D2	IBCN15	V	A	A	A	*AvrLm1,2,4-7*
D3	IBCN16	V	A	V	A	*AvrLm1,4-7*
D4	IBCN17	V	A	A	A	*AvrLm1,2,4-7*
D5	IBCN18	V	A	A	A	*AvrLm1,2,4-7*
D6	IBCN75	n.d.	n.d.	n.d.	n.d.	n.d.
D7	IBCN76	n.d.	n.d.	n.d.	n.d.	n.d.
D8	----------	V	V	V	A	*AvrLm4-7*
D9	----------	V	V	V	V	none
D10	PHW1223	n.d.	n.d.	n.d.	n.d.	n.d.
D13	----------	V	A	A	A	*AvrLm1,2,4-7*
D14	----------	n.d.	n.d.	n.d.	n.d.	n.d.
D16	----------	n.d.	n.d.	n.d.	n.d.	n.d.
D17	----------	V	A	V	A	*AvrLm1,4-7*

[a]Inoculation phenotype: V= virulence, A= avirulence, resistant and susceptible interaction between the isolate and the differential, respectively, n.d.= not determined. [b]Race: indicates the possible avirulence alleles present in each isolate based on pathogenicity.

The results obtained with the pathogenicity tests showed that the Australian isolate D5 was the only one that matched the results previously reported by Marcroft et al. (2012). The agreement with the avirulence genotype already reported and the presence of an effective

avirulence effector *AvrLm4* which is not present in local isolates, made the isolate D5 (*AvrLm1,2,4,7,S*) to be selected for seedling characterization in *Brassica napus* entries.

Races *AvrLm6,7*; *AvrLm1,6,7*; and *AvrLm1,2,4,7,S* were used for major-gene resistance identification. Of the 24 canola cultivars and hybrids, the hybrids DK Sensei, Dk Imiron, and the cultivar Claremore were resistant to all three races, while the hybrids Dimension, Visby, DK Extorm, DK Impression, Garou, and Artoga were partially resistant (heterogeneous) to all three races (Table 4.3). Just the cultivar Star 915W and the hybrids Chrome and Raffiness were susceptible to all three races. The breeding lines KSR073525, KS3018, KS3350, KS3068, KS3132, KS3254, KS4475, 4145, and KS4763 showed a resistant or heterogeneous response to all three races. The hybrids Safran, Ligora, Liquanta, MH10L23, and MH10G11, and the breeding lines KS10156-7, KS4749, KS4754, and KS4673 were susceptible to only the race *AvrLm6,7.* Most (83%) of the breeding lines were susceptible to the local races *AvrLm6,7* and *AvrLm1,6,7,* but resistant to the race *AvrLm1,2,4,7,S.* Less than 10% of the breeding lines showed a susceptible response to all three *L. maculans* races. Of 122 breeding lines inoculated with the race *AvrLm6,7,* 89% were susceptible, 10% were partially resistant or heterogeneous, and only the breeding line KS3350 was resistant. Similar responses were observed for the breeding lines inoculated with the race *AvrLm1,6,7*. Most of the breeding lines (87%) were susceptible and 13% were heterogeneous. Alternatively, 11% of the breeding lines were susceptible, 49% heterogeneous, and 40% resistant to the race *AvrLm1,2,4,7,S*. The resistance genotype of the cultivars, hybrids and breeding lines were identified based on the phenotypic reaction with the three *L. maculans* races. The presence of resistance genes *Rlm1, Rlm2, Rlm4, Rlm7,* and *RlmS* were inferred for those entries that showed resistance to all three races, and 37% of the cultivars and hybrids, and 7% of the breeding lines were broadly resistant. The resistance genes *Rlm1, Rlm2, Rlm4,* and *RlmS* were inferred for those entries that were resistant to the races *AvrLm1,6,7*; and *AvrLm1,2,4,7,S*; but susceptible to the race *AvrLm6,7*. The genes *Rlm2* and *Rlm4* were

identified in most (85%) of the breeding lines that showed homogeneous or heterogeneous resistance only for race *AvrLm1,2,4,7,S*. Finally, no major resistance genes were found in 11 breeding lines due to their susceptibility to the three races (Table 4.3).

Table 4.3. List of inferred major resistance genes of *Brassica napus* breeding lines, hybrids, and cultivars given by their reaction to known avirulence genotypes of *Leptosphaeria maculans.*

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	***AvrLm1,6,7***	***AvrLm 1,2,4,7,S***	
Westar	Susceptible check	4.72[d]	5.02	4.60	None
Dimension	Hyb	(72%) 3.30	(77%) 2.97	(67%) 2.89	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
Hornet	Hyb	4.78	4.27	(35%) 3.75	*Rlm2* (H), *Rlm4* (H)
Safran	Hyb	4.45	(72%) 2.92	(42%) 3.50	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *RlmS* (H)
Visby	Hyb	(38%) 3.80	(60%) 3.22	(54%) 3.06	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
DK Sensei	Hyb	(>80%) 1.96	(>80%) 1.94	(>80%) 1.91	*Rlm1, Rlm2, Rlm4, Rlm7, RlmS*
DK Extorm	Hyb	(>80%) 2.48	(77%) 2.37	(>80%) 2.85	*Rlm1* (H), *Rlm2, Rlm4, Rlm7, RlmS*
DK Imiron	Hyb	(>80%) 2.33	(>80%) 2.30	(>80%) 2.17	*Rlm1, Rlm2, Rlm4, Rlm7, RlmS*
DK Impression	Hyb	(25%) 4.13	(23%) 3.96	(60%) 2.73	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
Claremore	Cultivar	(>80%) 2.84	(>80%) 2.66	(>80%) 1.84	*Rlm1, Rlm2, Rlm4, Rlm7, RlmS*
VSX-4	Cultivar	4.08	4.58	(64%) 3.34	*Rlm2* (H), *Rlm4* (H)
Star 915W	Cultivar	4.98	4.89	4.44	None
Raffiness	Hyb	4.03	4.50	4.29	None
Garou	Hyb	(63%) 2.71	(27%) 3.73	(>80%) 2.64	*Rlm1* (H), *Rlm2*, *Rlm4*, *Rlm7* (H), *RlmS*
Artoga	Hyb	(64%) 3.32	(>80%) 2.52	(61%) 2.86	*Rlm1*, *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
Alabaster	Hyb	4.50	4.36	(73%) 2.60	*Rlm2* (H), *Rlm4* (H)
KSR07363	B-line	4.24	(48%)3.91	4.50	*Rlm1*(H), *Rlm6* (H)
KSR073525	B-line	(39%) 3.75	(44%) 3.86	(31%) 3.77	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
MH10L23	Hyb	4.18	(55%) 3.34	(>80%) 2.39	*Rlm1* (H), *Rlm2*, *Rlm4*, *RlmS*
MH10G11	Hyb	4.78	(72%) 3.16	(>80%) 2.50	*Rlm1* (H), *Rlm2*, *Rlm4*, *RlmS*
Eurol	Cultivar	4.82	5.31	(50%) 3.78	*Rlm2* (H), *Rlm4* (H)
Bristol	Cultivar	5.00	5.33	(27%) 3.92	*Rlm2* (H), *Rlm4* (H)
KS2185	B-line	4.32	4.56	(23%) 4.03	*Rlm2* (H), *Rlm4* (H)

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	*AvrLm1,6,7*	*AvrLm 1,2,4,7,S*	
KS3018	B-line	(42%)3.62[d]	(46%) 3.39	(61%) 3.09	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H) *Rlm7* (H), *RlmS* (H)
KS3350	B-line	3.27	(64%) 3.35	(>80%) 2.77	*Rlm1* (H), *Rlm2*, *Rlm4*, *Rlm7*, *RlmS*
KS3068	B-line	(59%) 3.30	(57%) 3.38	(>80%) 2.57	*Rlm1* (H), *Rlm2*, *Rlm4*, *Rlm7* (H), *RlmS*
KS4085	B-line	(33%) 3.83	(67%) 3.20	4.03	*Rlm1* (H), *Rlm6* (H)
KS3077	B-line	4.12	4.31	(>80%) 2.38	*Rlm2*, *Rlm4*
KS3132	B-line	(59%) 3.61	(50%) 3.54	(>80%) 2.42	*Rlm1* (H), *Rlm2*, *Rlm4*, *Rlm7* (H), *RlmS*
KS3254	B-line	4.13	4.31	(>80%) 2.04	*Rlm2*, *Rlm4*
KS4475	B-line	(52%) 3.70	(35%) 3.75	(66%) 3.04	*Rlm1* (H), *Rlm2* (H), *Rlm4* (H), *Rlm7* (H), *RlmS* (H)
KS4083	B-line	(21%) 4.07	4.16	(50%) 3.44	*Rlm2* (H), *Rlm4* (H), Rlm6 (H), Rlm7 (H)
KS4549	B-line	4.55	4.30	(60%) 3.13	*Rlm2* (H), *Rlm4* (H)
KS1701	B-line	4.37	4.80	(53%) 3.40	*Rlm2* (H), *Rlm4* (H)
Plainsman	Cultivar	4.38	4.39	(65%) 3.30	*Rlm2* (H), *Rlm4* (H)
KS4430	B-line	4.66	4.52	(46%) 3.52	*Rlm2* (H), *Rlm4* (H)
Ligora	Hyb	4.90	(24%) 4.28	(>80%) 2.73	*Rlm1* (H), *Rlm2*, *Rlm4*, *RlmS*
Liquanta	Hyb	4.81	(71%) 3.31	(>80%) 2.67	*Rlm1* (H), *Rlm2*, *Rlm4*, *RlmS*
KSNT08	B-line	4.47	4.82	(77%) 2.72	*Rlm2* (H), *Rlm4* (H)
KSNT46	B-line	4.45	4.13	(52%) 3.28	*Rlm2* (H), *Rlm4* (H)
KSNT114	B-line	4.67	4.22	(72%) 3.00	*Rlm2* (H), *Rlm4* (H)
KSNT09	B-line	4.38	5.05	(>80%) 2.31	*Rlm2*, *Rlm4*
KSNT32	B-line	4.82	5.39	4.07	None
KSNT22	B-line	4.55	5.06	(77%) 2.77	*Rlm2* (H), *Rlm4* (H)
4025	B-line	4.20	5.03	(>80%) 2.73	*Rlm2*, *Rlm4*
4080	B-line	4.23	5.08	(21%) 3.83	*Rlm2* (H), *Rlm4* (H)

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	*AvrLm1,6,7*	*AvrLm 1,2,4,7,S*	
4082	B-line	4.14[d]	4.69	(70%) 3.00	*Rlm2* (H), *Rlm4* (H)
5037	B-line	4.16	4.46	(72%) 2.56	*Rlm2* (H), *Rlm4* (H)
4006	B-line	4.28	4.83	(73%) 2.89	*Rlm2* (H), *Rlm4* (H)
KS08044-19-5-1-3	B-line	4.61	5.00	4.02	None
KSUR1204	B-line	4.45	4.73	3.81	None
KSUR1206	B-line	4.09	4.55	(29%) 3.56	*Rlm2* (H), *Rlm4* (H)
KS4643	B-line	4.36	4.71	(60%) 3.19	*Rlm2* (H), *Rlm4* (H)
4145	B-line	(40%)3.67	(44%) 3.71	(>80%) 2.31	*Rlm1* (H), *Rlm2*, *Rlm4*, *Rlm7* (H), *RlmS*
4130	B-line	4.51	4.89	(54%) 3.58	*Rlm2* (H), *Rlm4* (H)
4176	B-line	4.15	4.32	(>80%) 2.58	*Rlm2*, *Rlm4*
KS4666	B-line	4.14	5.01	(>80%) 2.73	*Rlm2*, *Rlm4*
KS4719	B-line	4.51	4.90	(>80%) 2.70	*Rlm2*, *Rlm4*
KS08044-19-5-5	B-line	4.28	4.89	4.02	None
KS08295a-5-4-5-3	B-line	4.45	4.65	(>80%) 1.94	*Rlm2*, *Rlm4*
KS08237-2-2-1-3	B-line	4.36	4.66	(>80%) 2.08	*Rlm2*, *Rlm4*
KS08278a-5-2-2	B-line	4.28	4.30	(62%) 3.04	*Rlm2* (H), *Rlm4* (H)
KS08282b-1-4-1	B-line	4.22	4.41	(64%) 3.08	*Rlm2* (H), *Rlm4* (H)
KS10107-7	B-line	4.43	4.78	(>80%) 2.63	*Rlm2*, *Rlm4*
KS10122-1	B-line	4.05	4.55	(68%) 2.83	*Rlm2* (H), *Rlm4* (H)
KS10122-5	B-line	4.44	4.47	(>80%) 3.03	*Rlm2*, *Rlm4*
KS10122-8	B-line	4.48	4.67	(56%) 3.63	*Rlm2* (H), *Rlm4* (H)
KS10122-9	B-line	4.48	4.53	(28%) 4.02	*Rlm2* (H), *Rlm4* (H)
KS10137-2	B-line	4.22	4.38	(>80%) 2.91	*Rlm2*, *Rlm4*

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	***AvrLm1,6,7***	***AvrLm 1,2,4,7,S***	
KS10147-2	B-line	4.19[d]	4.80	(48%) 3.55	*Rlm2* (H), *Rlm4* (H)
KS10147-8	B-line	4.16	4.39	(66%) 3.36	*Rlm2* (H), *Rlm4* (H)
KS10152-9	B-line	4.44	4.41	(>80%) 2.98	*Rlm2*, *Rlm4*
KS10156-2	B-line	4.11	4.78	(62%) 3.31	*Rlm2* (H), *Rlm4* (H)
KS10156-7	B-line	4.20	(30%) 3.67	(>80%) 3.14	*Rlm1* (H), *Rlm2*, *Rlm4*, *RlmS*
KS10159-10	B-line	4.30	4.72	(>80%) 2.66	*Rlm2*, *Rlm4*
KS10247-8	B-line	4.32	5.00	(>80%) 3.13	*Rlm2*, *Rlm4*
KS10247-9	B-line	4.55	4.84	(>80%) 3.05	*Rlm2*, *Rlm4*
Chrome	Hyb	4.30	4.25	4.33	None
KSUR1211	B-line	4.33	4.82	(57%) 3.20	*Rlm2* (H), *Rlm4* (H)
KSR4652	B-line	4.64	5.18	(53%) 3.32	*Rlm2* (H), *Rlm4* (H)
KS4675	B-line	4.64	5.18	(69%) 3.53	*Rlm2* (H), *Rlm4* (H)
KSR4724S	B-line	4.57	4.84	(75%) 2.94	*Rlm2* (H), *Rlm4* (H)
KSR4706S	B-line	4.57	4.87	(>80%) 3.05	*Rlm2*, *Rlm4*
KSR4723	B-line	4.69	5.04	(47%) 3.52	*Rlm2* (H), *Rlm4* (H)
KSR4764	B-line	4.50	4.75	(46%) 3.58	*Rlm2* (H), *Rlm4* (H)
KSR4765	B-line	4.46	4.52	(>80%) 2.25	*Rlm2*, *Rlm4*
KSR4766	B-line	4.64	5.03	(75%) 3.07	*Rlm2* (H), *Rlm4* (H)
KSR4767	B-line	4.48	4.90	(75%) 3.08	*Rlm2* (H), *Rlm4* (H)
KSR4769	B-line	4.38	4.76	(>80%) 2.61	*Rlm2*, *Rlm4*
KSR4773	B-line	4.88	4.80	(>80%) 2.88	*Rlm2*, *Rlm4*
KSR4774	B-line	4.59	4.67	(78%) 3.11	*Rlm2* (H), *Rlm4* (H)
KSR4775	B-line	4.43	4.87	(>80%) 3.09	*Rlm2*, *Rlm4*

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	***AvrLm1,6,7***	***AvrLm 1,2,4,7,S***	
KS4665	B-line	4.63[d]	4.54	(>80%) 2.85	*Rlm2, Rlm4*
KS4668	B-line	4.35	4.52	3.94	None
KS4684	B-line	4.13	4.25	(78%) 3.30	*Rlm2* (H), *Rlm4* (H)
KS4685	B-line	4.23	4.16	(60%) 3.15	*Rlm2* (H), *Rlm4* (H)
KS4687	B-line	4.25	4.17	4.00	None
KS4707	B-line	4.36	4.48	4.00	None
KS4709	B-line	4.28	4.21	(25%) 3.78	*Rlm2* (H), *Rlm4* (H)
KS4713	B-line	4.41	4.58	4.06	None
KS4716	B-line	4.41	4.69	(26%) 3.69	*Rlm2* (H), *Rlm4* (H)
KS4718	B-line	4.37	4.65	(>80%) 2.75	*Rlm2, Rlm4*
KS4720	B-line	4.16	4.33	(47%) 3.60	*Rlm2* (H), *Rlm4* (H)
KS4721	B-line	4.21	4.13	(56%) 3.09	*Rlm2* (H), *Rlm4* (H)
KS4722	B-line	4.18	4.52	4.15	None
KSP4701	B-line	4.67	4.39	(67%) 3.28	*Rlm2* (H), *Rlm4* (H)
KS4725	B-line	4.23	4.57	(50%) 3.65	*Rlm2* (H), *Rlm4* (H)
KS4730	B-line	3.98	4.58	(68%) 2.95	*Rlm2* (H), *Rlm4* (H)
KS4733	B-line	4.39	4.46	3.98	None
KS4734	B-line	4.31	4.75	4.00	None
KS4737	B-line	4.38	5.04	(37%) 3.83	*Rlm2* (H), *Rlm4* (H)
KS4738	B-line	4.33	4.48	(66%) 3.25	*Rlm2* (H), *Rlm4* (H)
KS4739	B-line	4.19	4.43	(59%) 3.30	*Rlm2* (H), *Rlm4* (H)
KS4740	B-line	4.13	4.33	(>80%) 2.36	*Rlm2, Rlm4*
KS4741	B-line	4.27	4.57	(73%) 2.95	*Rlm2* (H), *Rlm4* (H)

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	*AvrLm1,6,7*	*AvrLm 1,2,4,7,S*	
KS4742	B-line	4.52[d]	4.67	(46%) 3.50	*Rlm2* (H), *Rlm4* (H)
KS4745	B-line	4.43	4.86	(56%) 3.25	*Rlm2* (H), *Rlm4* (H)
KS4746	B-line	4.36	4.00	(72%) 2.71	*Rlm2* (H), *Rlm4* (H)
KS4747	B-line	4.25	4.20	(>80%) 2.59	*Rlm2, Rlm4*
KS4748	B-line	3.58	4.54	(>80%) 2.75	*Rlm2, Rlm4*
KS4749	B-line	4.50	(29%) 3.92	(>80%) 2.53	*Rlm1* (H), *Rlm2, Rlm4, RlmS*
KS4750	B-line	4.20	4.05	(>80%) 2.93	*Rlm2, Rlm4*
KS4753	B-line	4.23	4.07	(22%) 3.83	*Rlm2* (H), *Rlm4* (H)
KS4754	B-line	4.21	(25%) 4.16	(>80%) 3.10	*Rlm1* (H), *Rlm2, Rlm4, RlmS*
KS4755	B-line	4.25	3.88	(>80%) 2.88	*Rlm2, Rlm4*
KS4763	B-line	3.98	(36%) 3.92	(>80%) 2.17	*Rlm1* (H), *Rlm2, Rlm4, Rlm7* (H), *RlmS*
KS4626	B-line	4.10	3.89	(>80%) 2.66	*Rlm2, Rlm4*
KS4628	B-line	4.33	4.17	(>80%) 2.83	*Rlm2, Rlm4*
KS4634	B-line	4.06	4.38	(50%) 3.35	*Rlm2* (H), *Rlm4* (H)
KS4660	B-line	4.32	4.32	(27%) 3.83	*Rlm2* (H), *Rlm4* (H)
KS4662	B-line	4.13	4.38	(37%) 3.63	*Rlm2* (H), *Rlm4* (H)
KS4663	B-line	4.30	(25%) 4.31	(58%) 3.21	*Rlm1 (H), Rlm2* (H), *Rlm4* (H), *RlmS* (H)
KS4669	B-line	4.53	5.00	(>80%) 3.03	*Rlm2, Rlm4*
KS4670	B-line	4.72	5.00	(27%) 4.07	*Rlm2* (H), *Rlm4* (H)
KS4673	B-line	4.28	(>80%) 2.95	(>80%) 2.31	*Rlm1, Rlm2, Rlm4, RlmS*
KS4676	B-line	4.34	4.50	(>80%) 2.58	*Rlm2, Rlm4*
KS4677	B-line	4.39	4.42	(>80%) 2.94	*Rlm2, Rlm4*
KS4683	B-line	3.96	4.50	(>80%) 2.89	*Rlm2, Rlm4*

Table 4.3. (continued)

Entries	Type[b]	Races[a]			Resistance genotype[c]
		AvrLm6,7	*AvrLm1,6,7*	*AvrLm 1,2,4,7,S*	
KS4688	B-line	4.75[d]	4.91	(78%) 3.21	*Rlm2* (H), *Rlm4* (H)
KSNT149	B-line	4.33	4.86	(>80%)2.43	*Rlm2, Rlm4*
KSP4698	B-line	4.17	5.28	(>80%)2.42	*Rlm2, Rlm4*
KSUR1209	B-line	4.41	4.67	(>80%)2.78	*Rlm2, Rlm4*
KSUR1212	B-line	4.30	4.48	(>80%)2.55	*Rlm2, Rlm4*

[a] Race is the genetic avirulence profile in the pathogen (*L. maculans*). [b] *Brassica napus* types: cultivars, hybrids (Hyb), or breeding lines (B-line). [c]Inferred major resistance genes based on the reaction of *B. napus* to races of *L. maculans*. (H), heterogeneus resistance where 20% to 80% of each cultivar or breeding line showed resistant response. [d] Black leg severity scale: <4 resistant; ≥4 susceptible; each value represents the mean of 64 observations. The value in parentheses represents the percentage of resistance.

DISCUSSION

The present study identified seedling resistance genes in *Brassica napus* germplasm. Seedling resistance or major-gene resistance is a plant cell response that recognizes the pathogen and suppresses its infection resulting in a resistance response also called the hypersensitive response (Jones & Dangl, 2006). This type of resistance follows a gene-for-gene interaction where each avirulence gene present in the pathogen (*Avr*) corresponds to a resistance gene present in the plant (*Rlm*) producing a resistance reaction (Balesdent et al., 2002). Based on the gene-for-gene concept, the resistance genes in the plant can be identified by inoculation with a set of well characterized pathogens. For this study, three *Leptosphaeria maculans* races with defined avirulence genotypes were used to infer the presence or absence of major resistance genes in different *B. napus* cultivars and breeding lines. Based on the plant and pathogen interactions, the presence of the resistance genes *Rlm1, Rlm2, Rlm4, Rlm6, Rlm7* and *RlmS* could be inferred, providing an overview of the genetic resistance profile of winter canola cultivars and hybrids that are cultivated in the southern Great Plains as well as the experimental breeding lines developed by the Kansas State University canola breeding program. Homogeneous and heterogeneous resistance were identified in this study. Heterogeneous resistance represents those cultivar-isolate interactions that displayed a resistance response in a range of 20% to 80%, whereas a homogeneous resistance is considered if a plant-isolate interaction shows a resistance reaction in 80% or more of the cases. This variation could have happened due to the plant genotype heterogenicity (Balesdent et al., 2002; Rouxel et al., 2003).

Three hybrids (Dimension, Visby, and DK Impression), two cultivars (Garou and Artoga), and eight breeding lines (KSR073525, KS3018, KS3350, KS3068, KS3132, KS3254, KS4475, 4145, and KS4763) exhibited a heterogeneous resistance to the three races. Only the hybrid DK Sensei and the cultivar Claremore had a homogeneous resistance to all three races. There is a possibility that the hybrids, cultivars, and breeding lines that exhibited a homogeneous

or heterogeneous resistance to all three races, harbor at least one of the six genes described for this study (*Rlm1, Rlm2, Rlm4, Rlm6, Rlm7*, and *RlmS*). Cultivar descriptions provided by DEKALB-Monsanto in the European Union indicate that moderate polygenic resistance and *Rlm7* provide effective and durable protection against black leg on winter canola (DEKALB/Monsanto, 2014), that is why, it is likely that the commercial hybrids developed by this company such as DK Sensei and DK Impression are known to harbor the resistance gene *Rlm7*. This assumption can also be applied to the hybrids DK Extorm and DK Imiron which exhibited a resistance response to the local races *AvrLm6,7* and *AvrLm1,6,7*. Currently, deploying a combination of *Rlm7* and quantitative resistance has been durable in Europe (Brun et al., 2010; Plissonneau et al., 2016).

The hybrids Safran, MH10L23 and MH10G11, and six breeding lines displayed resistance to the race *AvrLm1,6,7*; heterogeneous resistance to the race *AvrLm1,2,4,7,S* but they had a susceptible reaction to the race *AvrLm6,7*. Based on these interactions, the presence of *Rlm1*, *Rlm2, Rlm4*, and/or *RlmS* is likely.

The presence of the gene *Rlm6* was inferred for two breeding lines KSR07363 and KS4085, due to their reactions against the *L. maculans* races. A heterogeneous resistance to the local races and susceptible response to the Australian race indicated the possible presence of the gene *Rlm6*. However, according to Kutcher et al. (2010) and Brun et al. (2010) the resistance genes *Rlm5* and *Rlm6* were first identified in *Brassica juncea*, being introgressed into a few *B. napus* varieties which are now being used only for experimental purposes. (Balesdent et al., 2002). This is because the recombinant line "MX" produced by interspecific crosses between *B. napus* and *B. juncea* that carries the resistance gene *Rlm6* is not durable, resulting in a rapid loss of *Rlm6* resistance by the adapted pathogen containing *avrLm6* (Brun et al., 2001; Somda et al., 1999). Therefore, it is more likely that the heterogeneous resistance exhibited in these two breeding lines is due to the resistance genes *Rlm1* or *Rlm7*.

Of the 122 breeding lines screened, 89% of them showed a susceptible reaction to the local races *AvrLm6,7* and *AvrLm1,6,7*; and a mixture of homogeneous and heterogeneous resistance to the race *AvrLm1,2,4,7,S*. The cultivars, Hornet, Alabaster, Eurol, and Bristol displayed susceptibility and a heterogeneous resistance to the two local and the Australian race respectively. The presence of the major resistance genes *Rlm2* and *Rlm4* were inferred on these breeding lines and cultivars. Eurol and Bristol are winter type canola cultivars developed by Semences Cargill in France in the 1990s. According to Balesdent et al. (2005), Bristol carries the resistance genes *Rlm2* and *Rlm9* which agrees with the identification of *Rlm2* in this study.

The possible presence of the resistance genes *Rlm2* and *Rlm4* in the 89% of the breeding lines that were screened is likely due to the genetic selection of the parental lines that were used in the development of new cultivars. In 1999, a winter canola variety called Wichita was developed by the Kansas State University canola breeding program using as a parental line the French cultivar Jet Neuf which carries *Rlm4*. Wichita has been used as a parental line to develop and release more cultivars such as Kiowa, Riley, and Griffin (Balesdent et al., 2001; Rife & Shroyer, 2000; Stamm et al., 2012).

Finally, as it was expected, the spring cultivar Westar used as a susceptible check, did not have any resistance to any of the races. The cultivar Star 915W which is distributed by Star Specialty Seed, also displayed susceptibility to all three races. Even though the company claims that this winter canola cultivar has an excellent emergence and early vigor, no information is provided about black leg resistance (Star Specialty Seed, 2018). The hybrid Chrome showed a susceptible reaction to the three races indicating the absence of major resistance genes. However, the company that produces Chrome and other MH hybrids (Momont), describes their hybrids as having polygenic resistance.

These findings are of considerable importance since they provide evidence of the genetic resistance profile in different cultivars and breeding lines. However, to confirm the presence of these major resistance genes, molecular techniques such as mapping, and cloning are required (Marcroft et al., 2012). In addition, based on the avirulence allele frequency of *Leptosphaeria maculans* in Oklahoma which indicates that *AvrLm7*, *AvrLm6*, and to a lesser degree *AvrLm1* are predominant in this region (Diaz, 2015), the incorporation of their corresponding resistance genes *Rlm1*, *Rlm6*, and *Rlm7* in new cultivars and breeding lines can generate extra protection against Black leg disease. The presence of the resistance genes *Rlm2* and/or *Rlm4* in most of the breeding lines will not provide an effective protection against black leg based on the pathogen avirulence profiles in the Southern Great Plains. However, it is necessary to rotate different canola cultivars with different genetic resistance genes to prevent or delay the loss of resistance caused by pathogen virulence alleles.

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CHAPTER V

DISEASE AND YIELD RESPONSES OF RESISTANT AND SUSCEPTIBLE WINTER CANOLA CULTIVARS TO FUNGICIDE APPLICATION FOR CONTROL OF BLACK LEG

ABSTRACT.

The ascomycete *Leptosphaeria maculans* is the causal agent of black leg, a serious disease of canola (*Brassica napus*) in United States and worldwide. Black leg disease and yield were assessed in field experiments established in 2016 and 2017 using canola cultivars and hybrids with different levels of major-gene and quantitative resistance. Fungicide was applied twice during the fall to keep the disease pressure low. Treated and non-treated plots were arranged in a two-factor randomized complete block design with six replications. Each plot was evaluated for winter survival, black leg disease, and yield. The high quantitative resistance in the hybrids DK-Sensei and Chrome and the presence of the major resistance gene *Rlm7* in DK-Sensei produced the highest yields and the lowest levels of disease. The susceptible cultivars Eurol and Bristol had the lowest yields despite the presence of the resistance genes *Rlm2* and *Rlm9* in Bristol. The low percentage of winter survival and aphid damage also contributed to the low yields of Eurol and Bristol. Disease severity and yield were not significantly different between the cultivar HC-115W and the breeding line KSR07363. Yields of HC-115W and KSR07363 were lower compared to the resistant hybrids DK-Sensei and Chrome, but higher compared to the susceptible cultivars

Bristol and Eurol. Finally, the effects of the fungicide application on disease incidence and severity were significant. However, the effect of fungicide application on yield was not significant. These results indicate the importance of cultivating winter canola varieties with effective resistance to black leg in reducing yield loss to the disease.

INTRODUCTION

Leptosphaeria maculans (Desm.) Ces. & DeNot (anamorph *Phoma lingam*), is the causal agent of black leg, one of the most severe diseases of canola (*Brassica napus*) (West et al., 2001). The main symptoms of this disease are the gray circular spots on the foliage and basal black to gray stem cankers that can gridle plans causing premature ripening and reduced pod fill (Damicone et al., 2015). This hemibiotrophic fungi is able to survive and produce ascospores by sexual reproduction on canola stubble after harvest. Airborne ascospores serve as a primary inoculum landing on canola leaves and infecting the plant by penetrating through stomata or wounds. Fruiting bodies (pycnidia) develop within leaf spots and produce conidia. These asexual spores serve as a secondary inoculum infecting nearby plants and plant parts by rain splash. Hyphae grow symptomlessly from the leaf infections through the petioles into the stem base where cankers develop during the ripening stages of crop development (Damicone et al., 2015; West et al., 2001).

Since the late 1950s, black leg has been reported on oilseed rape in Europe, Australia, and Canada (Gugel & Petrie, 1992). The United States has had this disease since 1991 when serious yield losses occurred in canola fields in North Dakota (Bradley & Hamey, 2005). The first report of black leg in Oklahoma was in 2009 when several canola fields were affected (del Río Mendoza et al., 2011). Therefore, effective management practices are needed to reduce the

impact caused by black leg. The combination of cultural practices such as crop rotation, the use of certified pathogen-free seeds, fungicide seed treatment, the use of foliar fungicides, and using resistant varieties are effective against black leg (Marcroft & Bluett, 2008; Markell et al., 2008). Chemical control by the application of foliar fungicides has the potential to reduce the levels of black leg and provide yield protection in susceptible varieties. Foliar fungicide application is recommended if any sign or symptom of black leg is observed from emergence through the early foliar stages of the plant (Kandel & Knodel, 2005; Oklahoma-Cooperative-Extension-Service, 2017).

There are two types of genetic resistance against black leg. Multigenic resistance, also called quantitative, or adult-plant resistance is expressed at the adult stage of the plant when canker severity is restricted. Multigenic resistance is controlled by many genes that have been poorly characterized. This type of resistance is durable and difficult to overcome by the pathogen (Corwin & Kliebenstein, 2017; Kaur et al., 2009). The other type of resistance is major-gene resistance, also known as vertical or seedling resistance. This resistance is race-specific and controlled by the gene-for-gene interaction between the host and the pathogen. Each resistance gene in the plant (*Rlm*) recognizes its correspond avirulence gene in the pathogen (*Avr*) producing a resistant or hypersensitive response (Rouxel & Balesdent, 2017; West et al., 2001). A total of 18 major resistance genes have been described in canola (*Rlm1* to *Rlm11, RlmS, LepR1* to *LepR4, BLMR1, and BLMR2*), and 14 avirulence genes have been identified in *L. maculans* of which seven have cloned (*AvrLm1, AvrLm2, AvrLm3, AvrLm5, AvrLm4-7, AvrLm6,* and *AvrLm11*) (Dilantha et al., 2018; Marcroft et al., 2012; Plissonneau et al., 2016). The main advantage of using major-gene resistance is that it has the capacity to disrupt the primary infection of the pathogen, preventing the development of leaf spots (Balesdent et al., 2001). However, major-gene resistance often lacks durability in the field, because mutations or deletions in avirulence genes that confer virulence are selected when canola varieties with the same resistant genes are

cultivated year after year. These events lead to a rapid defeat of the plant resistance, rendering it no longer effective (Campbell et al., 2002; Marcroft et al., 2012). In Europe and Australia, significant losses occurred after the deployment of the resistance genes *Rlm1* and *RlmS* in canola cultivars respectively (Rouxel et al., 2003; Sprague et al., 2006). The combination of major-gene and adult plant resistance in canola cultivars can significantly decrease the impact of black leg disease. Moreover, the presence of multigenic resistance can improve the durability of major-gene resistance (Brun et al., 2010; Marcroft et al., 2012; Zhang et al., 2017).

Back leg resistance as well as yield potential, winter survival, oil content, and herbicide tolerance are important traits to be considered when choosing a canola cultivar or hybrid. Therefore, is essential for the canola industry and farmers to know which type of cultivar will be the most efficient to work with (Boyles et al., 2012). That is why a clear understand of the relationship between black leg resistance in cultivars or hybrids and yield is fundamental to determine the most effective and economic method to control black leg disease. By using fungicide application as a tool to keep black leg pressure low, it should be apparent whether or not resistance is having an impact on disease and yield. The hypothesis is fungicides should only reduce disease and increase yield of susceptible cultivars. Therefore, the objective of this study was to assess the disease and yield responses of resistant and susceptible winter canola cultivars to fungicide application for control of black leg.

MATERIALS AND METHODS

Field Experiments:

The experiments were established at the Entomology and Plant Pathology Research Station Farm in Stillwater, OK for two consecutive harvest years of 2017 and 2018. Fertilizer

(56-37-0 kg/ha N-P-K) and herbicide Treflan 4E at 0.84 kg /ha were incorporated into the soil prior to planting the genotypes Eurol, Bristol, Chrome, HC-115W, KSR07363, and DK-Sensei at a rate of at 3.5 g of seeds per plot/cultivar using a grain drill. Canola genotypes were chosen based on their genetic resistance profiles against black leg (Table 5.1). Plots consisted of six 7.62-m-long rows spaced 0.19 m apart. The experiment was arranged in a two-way factorial randomized complete block design with six replications. In 2016, plots were planted on 28 Sept, but stand establishment was poor and plots were reseeded on 12 Oct. In 2017, the planting date was 17 Oct. At the early rosette stage, each plot was inoculated with *Leptosphaeria maculans* by spreading one handful of canola stubble from a previously infested field and 45 ml of oat kernels colonized by the fungus. Proline 480 SC fungicide (Prothioconazole, 0.41 kg a.i. L^{-1}) was broadcast twice during the fall at 0.2 kg a.i. ha^{-1}, through flat-fan nozzles spaced 46 cm apart using a CO_2-pressurized wheelbarrow sprayer. Plots of each genotype had two fungicide treatments, treated and untreated with six replications. Fungicide application dates in 2016 were on 18 Nov. and 5 Dec while in 2017 the application dates were on 12 Nov. and 12 Dec. The precipitation during the cropping season from 28 Sept. 2016 to 31 May 2017 totaled 731.8 mm while the precipitation during the cropping season from 17 Oct 2017 to 31 May 2018 totaled 422.1 mm. Plots were harvested with a small-plot combine, and the yield was adjusted to 10 % of moisture.

Table 5.1. List and details of the winter canola genotypes used for this study.

Entry	Type	Origin/Distributor	Genetic resistance	
			Major-gene	Quantitative
Eurol	Cultivar	INRA France	None	Low
Bristol	Cultivar	INRA France	*Rlm2*, *Rlm9*	Low
Chrome	Hybrid	MOMONT	None	High
HC-115W	Cultivar	Croplan Genetics	None	Moderate
KSR07363	Breeding line	Kansas State University	*Rlm1* and/or *Rlm7*	High
DK-Sensei	Hybrid	Monsanto/DEKALB	*Rlm7*	High

Plot assessments:

Plots were evaluated for winter survival by estimating the percentage of live foliage in each plot in late winter on 27 Feb in 2017, 2018. The percentage of dead plants was evaluated before swathing by estimating the dead foliage. Black leg and winter decline syndrome were assessed on the stubble after swathing on 31 May 2017 and 4 Jun 2018. Black leg severity, incidence, and winter decline syndrome incidence were assessed by arbitrarily selecting 10 plants per each plot and examining the basal cross section of each stem. Black leg severity was evaluated by visually assessing the level of internal stem decay using a scale from 0 to 5, where 0 = no disease; 1 = less than 25% of decay; 2 = 25 to 50% of decay; 3 = 51 to 75% of decay; 4 = 76 to 100% decay; and 5 = plant dead. Black leg incidence was estimated by evaluating the percentage of plants with stem cankers. Incidence of winter decline syndrome was evaluated by estimating the percentage of plants with internal crown discoloration, deterioration, and formation of a hollow cavity.

Data analysis:

The data were analyzed using the software JMP 13 (SAS Institute Inc., Cary, NC, USA). Analysis of variance were performed on a randomized complete block design with two treatment factors (genotype and fungicide). The effects of genotype, fungicide treatment, and their interaction (genotype x fungicide treatment) were considered fixed effects, and the response variables were winter survival, black leg incidence and severity, and yield. The means were compared following the Student's t test by calculating the least significant difference between two means (LSD). Any difference larger than the LSD value was considered significant at P=0.05.

RESULTS

The analysis of variance showed a significant effect of genotypes on winter survival ($p<0.05$). The cultivars Eurol and Bristol, and the hybrid Chrome had low winter survival (Table 5.2). Winter survival for the hybrid DK-Sensei, the cultivar HC-115W, and the breeding line KSR07363, on the other hand, was greater compared with the other three entries. The effect of fungicide application did not have a significant effect on winter survival percentage. Finally, the interaction between genotypes and fungicide applications did not have a significant impact on winter survival (Table 5.2). Even though the percentage of winter survival was significantly greater in 2018 compared to 2017, the averaged over years effects were significant for genotypes.

Winter decline syndrome incidence was less than 20% in most of the treatments except for the hybrid Chrome which had an incidence of 27% winter decline. However, there were not significant differences among genotypes or between fungicide application ($p>0.05$).

Table 5.2. Winter survival percentage and winter decline syndrome incidence of canola genotypes Eurol, Bristol, Chrome, HC-115W, KSR07363, and DK-Sensei and their reaction to fungicide application.

Genotypes	Winter survival (%)[a]			Winter decline syndrome (%)[b]		
	Fungicide		Mean	Fungicide		Mean
	(+)	(-)		(+)	(-)	
Eurol	62.1	52.5	57.3 b	16.7	16.0	16.3 a
Bristol	62.5	53.3	57.9 b	14.4	18.0	15.7 a
Chrome	65.8	68.3	67.1 b	27.1	16.7	22.3 a
HC-115W	81.3	86.3	83.7 a	18.0	16.7	17.5 a
KSR07363	83.7	81.7	82.7 a	15.7	15.0	15.4 a
DK-Sensei	87.9	79.6	83.7 a	12.0	14.30	13.3 a
Mean	73.9 a	70.3 a		17.5 a	16.0 a	

[a] Percentage of plot with live foliage. [b] Percentage of plants with winter decline syndrome. Means values in a row or column followed by same letter are not significantly different ($p=0.05$).

Black leg incidence was lower for the hybrids DK-Sensei and Chrome compared with the other entries ($p<0.05$) (Table 5.3). The cultivar Bristol had the highest disease incidence. The difference in disease severity between Bristol, HC-115W, and KSR0363 was not significant. Fungicide application resulted in a significant reduction in black leg incidence. However, the interaction between genotypes and fungicide application did not have a significant effect on black leg incidence (Table 5.3).

The analysis of variance performed on black leg severity indicated that there was a significant difference between genotypes (Table 5.3). The cultivar Bristol and the breeding line KSR07363 had the highest levels of black leg while the hybrids Chrome and DK-Sensei had the lowest levels of disease severity. Fungicide application had a significant effect on the black leg severity ($p<0.05$). However, the interaction between genotypes and fungicide applications was not a significant.

Finally, the analysis of variance applied to the yield data showed significant differences among genotypes (Table 5.3). The hybrids Chrome and DK-Sensei had the highest yield while the cultivars Eurol and Bristol had the lowest yields. However, the differences in yield between treated and untreated plots were not statistically significant for any of the genotypes ($p>0.05$). Over all entries and fungicide treatments regression analysis indicated a negative relationship between yield and black leg incidence where the disease incidence increased, yield decreased. The relationship between yield and black leg severity followed a similar pattern (Figure 5.1).

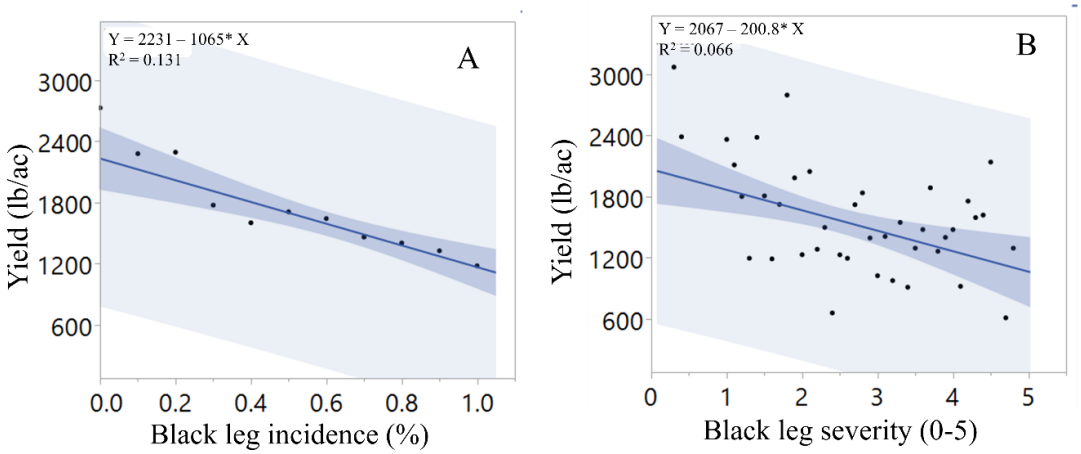

Figure 5.1 Regression analysis between yield of winter canola, and A, black leg incidence; and B, black leg severity.

Table 5.3. Reaction of winter canola genotypes to fungicide application for control of black leg disease.

Genotypes	Black leg								
	Incidence (%)[a]			Severity (0-5)[b]			Yield (lb/ac)		
	Fungicide		Mean	Fungicide		Mean	Fungicide		Mean
	(+)	(-)		(+)	(-)		(+)	(-)	
Eurol	60.1	65.0	62.9 b	2.4	2.40	2.4 b	913	798	855 c
Bristol	71.6	83.3	77.5 a	2.7	3.3	3.0 a	746.00	602	674 c
Chrome	36.6	48.3	42.5 c	1.5	2.1	1.8 c	2219	2271	2245 a
HC-115W	66.6	79.2	72.9 ab	2.5	3.1	2.8 ab	1615	1702	1658 b
KSR07363	65.8	82.5	74.1 ab	2.8	3.4	3.1 a	1811	1571	1691 b
DK-Sensei	37.5	45.0	41.3 c	1.5	1.9	1.7 c	2348	2272	2310 a
Mean	56.5 b	67.2 a		2.2 b	2.7 a		1609 a	1536 a	

[a] Percentage of stems with basal cankers. [b] Internal stem decay caused by black leg on a (0 – 5) scale where 0 = no disease, 1 = 25% stem decay, 2 = 26-50% stem decay, 3 = 51-75% stem decay, 4 = >75% stem decay, 5 = 100% stem decay. Mean in a column or row followed by the same letter are not significantly different at p=0.05.

DISCUSSION

Plant – pathogen interaction is linked by epidemiological and physiological factors such as the disease severity which is the amount of disease or disease intensity. The effect the disease can have an impact on plant growth and development resulting in yield loss. The use of resistant varieties has given an effective control against black leg disease (Gaunt, 1995; West et al., 2001). The present study evaluated the disease and yield responses of resistant and susceptible winter canola cultivars and hybrids to fungicide application for control of black leg. The hypothesis was that fungicide application should reduce disease and increase yield of susceptible but not resistant genotypes.

The resistant genotypes DK-Sensei and Chrome showed significantly higher yield and lower disease severity compared with the susceptible cultivars Eurol and Bristol. The positive impact of planting resistant cultivars is evidenced in the lowest values of black leg incidence and severity for DK-Sensei and Chrome (Assefa et al., 2014). The hybrid DK-Sensei had the highest yield and the lowest values of disease severity. The presence of the major resistance gene *Rlm7* in DK-Sensei (DEKALB/Monsanto, 2014) likely was responsible reduced disease. The higher yields of DK-Sensei in this study agrees with the report of the National Winter Canola Variety Trial for 2017 which showed that the hybrid DK-Sensei was the second highest yielding entry in the trial (Stamm, 2017). Even though the hybrid Chrome lacks major resistance genes, it also had low levels of disease severity. The quantitative resistance in Chrome is likely reason for reduced disease for this genotype. Chrome and DK-Sensei also had higher yields compared with the susceptible cultivars Eurol and Bristol. The genetic resistance present in these hybrids likely had an impact on yield. The cultivar HC-115W which has moderate quantitative resistance, but no major-gene resistance and the breeding line KSR07363 that likely harbor the major resistance gene *Rlm1* or *Rlm7* and high quantitative plant resistance did not differ in black leg incidence and severity. Levels of black leg severity varied between 2.5 and 3.5 on HC-115W and KSR07363,

and the yield values did not differ between these genotypes. The possible presence of the major gene *Rlm1* or *Rlm7* in the breeding line KSR07363, and the moderate quantitative resistance of the cultivar HC-115W was likely ineffective in reducing levels of black leg and increasing yield.

The cultivars Bristol and Eurol had high levels of black leg severity, and the lowest levels of yield. Low percentages of winter survival and the lack of quantitative resistance in these cultivars may have contributed to higher disease and low yield. While it is recommended to release cultivars that harbor more than one major resistance genes to prevent selection pressure on the pathogen (Gladders et al., 2006), the presence of the major resistance genes *Rlm2* and *Rlm9* in the cultivar Bristol did not offer an effective protection against black leg. This is probably because the pathogen population in Oklahoma is mainly composed of the avirulence genes *AvrLm1, AvrLm6, and AvrLm7* (Diaz, 2015). Therefore, the major resistance genes present in the European cultivar Bristol are not effective to reduce black leg disease. Another factor that contributed to the plant mortality for these cultivars was the aphid damage during the spring of 2018 which also contributed to yield loss. However, a significant reduction in plant mortality was observed at the end of the season on the cultivar Bristol that was treated with the fungicide. In addition, Hwang et al. (2016) reported that there is a negative relationship between the yield and the disease severity where there is a reduction in yield for each increase in the disease. This correlation explains the highest yields on the resistant hybrids that had low levels of black leg severity, and the susceptible cultivars that showed higher levels of disease severity and low yields.

Even though a significant reduction on black leg incidence and black leg severity was observed in plots that were treated with fungicide compared with the plots that were not sprayed, an improvement in yield was not observed. In this case, the resistance in the genotypes DK-Sensei and Chrome had a significant effect on yield. Based on these findings, the hypothesis that

was proposed was rejected. The effects of fungicide application contributed to reduced disease, but not increased yield of the susceptible genotypes.

Overall, this study evaluated black leg reaction of resistant and susceptible varieties. As expected, the disease severity was significantly lower, and the yield was higher in those cultivars that have effective genetic resistance against the disease. This information is important for canola growers to make decisions on which cultivar or hybrid is the best plant for black leg management. Good agronomic practices to reduce the impact of black leg also involve the choice of an effective source of resistance, and the use of cultural practices such a rotating the sources of resistance to help prevent the selection of virulence that overcomes the resistance

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APPENDICES

Table S1. List and details of the *Leptosphaeria maculans* isolates used in this study.

Isolate code	Isolate name	Country of origin	*Avr* genotype	Reference
102	CO-13-F1-1	U.S	*AvrLm1,6,7*	
124	GR-13-F10-1	U.S.	*AvrLm6,7*	Diaz, 2015
D2	IBCN15	Australia	*AvrLm5,6,8,S*	
D3	IBCN16	Australia	*AvrLm5*	
D4	IBCN17	Australia	*AvrLm4,5,6,7,8,S*	
D5	IBCN18	Australia	*AvrLm1,2,4,7,S*	
D6	IBCN75	Australia	*AvrLm1,5,6,8,S*	
D7	IBCN76	Australia	*AvrLm1,3,5,6,8,S*	
D8	----------	Australia	*AvrLm5,7 (8)*	
D9	----------	Australia	*AvrLm5,6,7 (8)*	
D10	PHW1223	Australia	*AvrLm5,6,8,9,S*	
D13	----------	Australia	*AvrLm4,6,7 (5,8)*	
D14	----------	Australia	*AvrLm1,7,S (5,8)*	
D16	----------	Australia	*AvrLm5,6,7,S (8)*	
D17	----------	Australia	*AvrLm5,6,7,S*	Marcroft et al., 2012

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