ANALYSIS OF THE *Leptosphaeria maculans* RACE STRUCTURE AND IDENTIFICATION OF MAJOR-GENE RESISTANCE TO BLACK LEG IN WINTER CANOLA

By

CLAUDIA ISABEL DIAZ PROAÑO

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Universidad de las Fuerzas Armadas (ESPE)

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Thesis Approved:

Dr. John Damicone

Thesis Adviser

Dr. Robert Hunger

Dr. Carla Garzon

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Abstract: Black leg, caused by the fungus *Leptosphaeria maculans*, is a widespread disease of winter canola (*Brassica napus*) in Oklahoma. Major resistance genes (*Rlm*) are expressed in seedlings and interact with avirulence genes in *L. maculans* (*AvrLm*) in a gene-for-gene manner. Little is known about the avirulence genes and race structure of the pathogen population in the southern Great Plains. Likewise, there is limited information about the presence of resistance genes in cultivars and hybrids grown in the region. The presence of avirulence alleles and the race structure of the *L. maculans* population were determined using a combination of pathogenicity tests on differential cultivars harboring resistance genes *Rlm1* and *Rlm2,3* and PCR amplification of avirulence alleles *AvrLm1*, *AvrLm4-7*, *AvrLm6*. Avirulence alleles *AvrLm6* and *AvrLm4-7* were prevalent (100%) in the local population (N=95), whereas *AvrLm1* and *AvrLm2,3* presence was 38% and 9%, respectively. Four races (*Av1,2,3,6,4-7; Av1,6,4-7; Av2,3,6,4-7; Av6,4-7*) were identified in the population. Races *Av6,4-7* (56%) and *Av1,6,4-7* (35%) were the most predominant and were further characterized for *AvrLm4, AvrLm5 and AvrLm6* based on the phenotype interaction on differential cultivars harboring *Rlm4* and *Rlm5,6*. This characterization resulted in a reclassification into three races *Av1,6,7,(5); Av1,4,6,(5,7)* and *Av6,7,(5)* which were used to screen 53 winter canola cultivars, hybrids and breeding lines for seedling resistance. Most (62%) entries were susceptible to all three races and lacked specific resistance genes. Several (23%) conventional (non-glyphosate tolerant) cultivars and hybrids were heterogeneous in resistance to one or more races. Glyphosate-tolerant entries currently grown in the region generally lacked major resistance genes, except for DKW46-15 which had heterogeneous resistance from *Rlm4* and *Rlm7*. The hybrids Dimension, Safran, Visby, DK Sensei, and the rapeseed cultivar Rossini (9%), were resistant to all races possibly due to the presence of *Rlm6* and/or *Rlm7*. Unknown resistance was found in 6% of the entries, which suggested the presence of other resistance genes not assessed in this study. Understanding the race structure of the pathogen population will be useful for development of resistance and effective deployment to control black leg in winter canola. There is a need to grow cultivars or hybrids with effective major gene resistance in Oklahoma and surrounding states.

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CHAPTER I

INTRODUCTION

Canola (*Brassica napus* L.), is a special type of rapeseed which was bred to reduce the concentration of erucic acid and glucosinolates and to produce 40% oil content in the seeds (Raymer, 2002). Canola is an economically important oilseed crop in many countries (Downey, 1971; Rakow, 2004) and it has become the second largest source of vegetable oil in the world after soybean (USDA ERS 2012). Canola is highly desirable because it produces one of the healthiest edible oils and the highest protein content for animal feed (U.S. Canola Association n.d.).

Canola is extensively cultivated in Europe, Asia, Australia and Canada and to a lesser extent in the United States. In the U.S., canola is a relatively new crop and its production has steadily increased over the past three decades. In 2014, the national acreage planted to canola was 1.7 million of which 270,000 acres were planted in Oklahoma (USDA NASS 2014). Oklahoma has been growing winter-type canola for the past ten years and is now the second leading canola producing state after North Dakota.

Black leg, caused by *Leptosphaeria maculans* (Desm.) Ces & DeNot, is a fungal disease that attacks several *Brassica* species. The disease has the potential to severely damage canola, causing a reduction in yield of up to 50% in fields planted with susceptible cultivars and where disease pressure is high (Damicone et al. 2012; Kutcher et al. 2010). Canola is very susceptible to black leg infection at the seedling stage, however symptoms

can be observed during all stages of plant development (Rimmer et al. 2007). The disease first appears as leaf spot lesions on the leaves. Stem cankers develop later during ripening stages of crop development. Stem cankers may girdle stems and reduce yields by causing lodging and premature ripening. Leaf spots are circular, greyish in color and produce numerous dark fruiting bodies that impart a speckled appearance within the spots. Stem cankers that usually form at the base of stems, are oval in shape, greyish to tan in color, surrounded by a dark-brown margin, and often contain fruiting bodies (Rimmer et al. 2007).

Leptosphaeria maculans is a member of the class Dothideomycetes, order Pleosporales, which includes several important plant pathogens (Berbee 2001). In the past, the fungi causing black leg on canola were considered a single species. Variability was then observed in virulence and the species was divided into highly and weakly aggressive strains. However due to additional polymorphisms observed in cultural characteristics and genetics, it was reclassified into two closely related species, *L. maculans* and *L. biglobosa* (Rouxel and Balesdent 2005). *L. maculans* is the virulent species which causes damaging cankers. *L. biglobosa* is a weakly virulent species that produces smaller leaf spots with less sporulation and only superficial stem cankers.

L. maculans is a hemi-biotrophic pathogen on *B. napus*. Necrotrophic infection of leaves is often followed by periods of symptomless endophytic colonization, during which the mycelia grows through the vascular system of the petiole and into the stem. Finally, the fungus turns necrotrophic at the base of the stem and the upper root causing a stem canker, which may girdle the stem and lodge the plant (Rimmer et al. 2007).

Severe epidemics of black leg in other *Brassica* crops have occurred since the 19th century (Henderson 1918). However, black leg became a major concern after the expansion of canola as one of the major oilseed crops in the mid-20th century (Rouxel and Balesdent 2005). Today, black leg is endemic in most oilseed rape and canola growing regions, including

Oklahoma. It can potentially damage the crop when the genetic resistance of the cultivars is inadequate and the environmental conditions are favorable (high humidity and cool temperatures) for the production of ascospores which are the primary inoculum of the disease (Rouxel and Balesdent 2005). Airborne ascospores result from the colonization of stubble from the previous canola crops by the fungus. Because farmers widely practice minimum tillage, stubble is left on the soil surface providing a readily available inoculum source. In the U.S., black leg of canola was first identified in 1989 when an epidemic caused by a pathogenicity group 4 (PG-4) strain developed in southern Kentucky (Mengistu et al. 1990). In North Dakota, black leg was identified in 1991 on spring-type canola caused by weakly virulent strains (Lamey and Hershman 1993). However in 2003, new surveys revealed that the more virulent strains were common in North Dakota and Canada (Chen and Fernando 2006). In Oklahoma, the disease was first identified in 2009 on winter-type canola and both the highly virulent and weakly virulent species were present in the state (del Rio Mendoza et al. 2011). Sexual recombination of the fungus on the stubble each year may have led to the development of new pathotypes.

Strategies for management of the disease include application of fungicides and deployment of resistant cultivars or hybrids. Genetic resistance is considered the most efficient way to manage the disease. Major-gene resistance, also known as single-gene or race-specific resistance, is expressed from the seedling to the adult stage of the plant by one or a few specific resistance genes (Delourme et al. 2004). Resistance genes (*Rlm*) in *B. napus* interact with avirulence (*AvrLm*) genes in *L. maculans* in a gene-for-gene manner. The interaction results in a resistant reaction, or no disease, whenever a dominant resistance gene in the plant and its corresponding dominant avirulence gene in the pathogen are present (Balesdent et al. 2005). Conversely, the lack of a *Rlm* gene in the plant or a recessive gene for virulence (*avrlm*) in the pathogen results in a susceptible reaction or the expression of disease.

Resistance genes in *B. napus* have been characterized based on the genetic identification of their corresponding avirulence gene *AvrLm* in *L. maculans* (Rouxel and Balesdent 2005). Deployment of *B. napus* resistance genes have proven to be very effective in *L. maculans* populations that possess the corresponding avirulence genes. However, the repeated cropping of cultivars with specific resistance genes in extended areas has created a high selection pressure on the pathogen population (Rouxel et al. 2003a). The breakdown of *Rlm1* in France and development of 'Surpass' resistance in Australia caused important economic losses when virulent populations of *L. maculans* became prevalent (Li et al. 2003; Balesdent et al. 2006; Rouxel et al. 2003a; Rouxel and Balesdent 2005). The pathogen may adapt to resistance genes and overcome resistance in as few as three to four years after their first introduction (Dilmaghani et al. 2009).

The pathogen should be regularly monitored to assess variation in the virulence structure of the population in geographic locations where specific resistance has been deployed (Kutcher et al. 2010). Knowledge of the virulence structure is crucial to detect races with the ability to overcome specific resistance genes, and to choose the best resistance sources to be used locally (Dilmaghani et al. 2009). To date, studies describing the avirulence patterns and race structure of the pathogen population have been done in few countries in Europe, Australia, and the Americas (Balesdent et al. 2005; Balesdent et al. 2006; Dilmaghani et al. 2009). However there is no information on the race structure of *L. maculans* in Oklahoma or the southern Great Plains. Knowledge of the frequency of the avirulence (*Avr*) alleles and the common races of the pathogen is needed for identifying and developing canola cultivars resistant to black leg. This will be helpful for canola breeders to develop new canola cultivars with specific *Rlm* genes based on the avirulence *Avr* alleles currently present in a region. Selecting effective resistance genes to deploy would contribute to the effective management of black leg in the region.

Isolates of *L. maculans* characterized for avirulence alleles can be used to infer the presence of resistance genes by inoculating *B. napus* cultivars whose resistance is unknown. European and Canadian oilseed rape cultivars have been characterized for resistance genes (Rouxel et al. 2003b; Kutcher et al. 2010); however, there is a lack of information on the specific resistance *Rlm* genes present in commercial or conventional cultivars of winter canola grown in Oklahoma and the southern Great Plains. Screening winter canola cultivars, hybrids and breeding lines with local *L. maculans* races to identify or infer the presence of resistance *Rlm* genes is critical for deployment of resistant cultivars and management of the disease. Breeders and seed companies will know which if any *Rlm* genes are present in the cultivars they are developing, which *Rlm* genes are lacking in their cultivars, and whether the *Rlm* genes in their cultivars would be effective or not in the region. Selecting cultivars with known *Rlm* genes effective against the local *Avr* alleles in the pathogen population by canola growers will facilitate control of the disease in an environmentally friendly manner, reduce the application of fungicides, and increase yield and profits.

The objectives of this thesis research were: i) to determine the frequency of avirulence (*Avr*) alleles in the local *L. maculans* population in order to define its race structure; and ii) to use predominant and broadly virulent races to identify or infer the presence of resistance genes in winter canola cultivars grown in the region or breeding lines under development.

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CHAPTER II

LITERATURE REVIEW

Canola

Canola (*Brassica napus* L.), a member of the family Brassicaceae related to rapeseed, mustard, cabbage, and oilseed radish, is an economically important oilseed crop in many countries around the world (Downey 1971; Rakow 2004). Canola is a special type of rapeseed that was first bred in Canada to reduce the concentration of undesirable components to ≤ 2% erucic acid in the oil and 3 macromoles of aliphatic glucosinolates in the meal (Raymer 2002). The name canola is a registered trademark of the Canola Canada Association which stands for "CANadian Oil Low Acid" (Raymer 2002). In Europe, where the term canola is not broadly accepted, the terms "double low" or "00" oilseed rape are used.

Canola seed at maturity contains about 40% oil. The oil consists of about 6% saturated fat, which is the lowest concentration among other vegetable oils and high concentration of mono-unsaturated fat (oleic acid). The low production of erucic acid, aliphatic glucosinolates and saturated fat have ranked canola as one of the healthiest edible oils and among the top three oilseed crops worldwide. Canola meal obtained after crushing the seeds is highly desirable as a high protein supplement for livestock. It contains about 38% crude protein and 11% crude fiber (U.S. Canola Association, n.d.).

Canola oil differs considerably from traditional rapeseed oil in quality characteristics. Specialty canola oil refers to canola cultivars with improved edible oil profiles, which are low in erucic acid, have high temperature stability, and improved shelf life. Whereas, industrial rapeseed oil, comes from oilseed rape cultivars that produce oil with 45% or more erucic acid and meals with low protein content. Industrial rapeseed oil is used in industry as lubricants and in hydraulic fluids, but not for edible purposes (Raymer 2002).

Canola quality oilseed rape has been developed in three different *Brassica* species. *Brassica napus*, also known as Argentine rape, Swede rape, colza or rapeseed; *Brassica rapa* (formerly known as *Brassica campestris*) commonly called Polish canola, turnip rape, or field mustard; and *Brassica juncea* which is the canola quality brown mustard (Downey 1971; Rimmer et al. 2007). Seeds of these species commonly contain 40% or more oil and produce meals with 35 to 40% protein (Raymer 2002).

Brassica napus L. is the most common canola grown and is widely adapted to cool seasons. *B. napus* is an annual or biennial plant, normally 0.5 to 1.3 m tall with branching stems, yellow flowers and globular black seeds produced in elongated pods. The basal rosette leaves are usually blue-green, lobed, and short-petiolate. The stem leaves are smaller and usually entire. Yellow flowers are arranged as a raceme with 4 petals, 1 to 12 ovules and 6 stamens each. Canola is primarily self-pollinated, but has entomophilous flowers that are capable of being both self and cross-pollinated (Williams et al. 1986). The blooming period differs depending on the type of variety. Spring types bloom without vernalization, but winter types rely on a vernalization period for flowering. Generally only half of the canola flowers form seed pods. Seeds are arranged in pods, they are nearly spherical and they can be reddish-brown, brown or black (Gulden et al. 2003).

Origin and taxonomy

Brassica is one of the oldest genera of cultivated plants. Based on the distribution of the wild species, it is believed that the genus *Brassica* originated in the Mediterranean-Middle Eastern region. China is considered a secondary center of origin since *Brassica* species were introduced into the region thousand years ago (Rimmer et al. 2007).

The brassica crops comprise six economically important and interrelated species with genetic and morphological diversity. The interrelation between the six species has been described by Morinaga in 1934 and confirmed by U in 1935 (Morinaga 1934; Raymer 2002; U 1935). Three species are diploids and include *B. rapa* (genome AA, N=10), *B. nigra* (genome BB, N=8) and *B. oleracea* (genome CC, N=9). The remaining three species are amphidiploids resulting from combining a set chromosomes from each parental taxa and include *B. juncea* (genome AABB, N=18), *B. carinata* (genome BBCC, N=17) and *B. napus* (genome AACC, N=19) (Rimmer et al. 2007).

Cultivation

Canola grows best on well-drained soils with a pH between 5 and 6. Canola has high nitrogen requirements like most non-legumes. It has a low requirement of phosphorous similar to that of wheat. Good levels of potassium will help increase the oil content in the seed (Rimmer et al. 2007).

Canola can be seeded in fall or spring depending on the type. For winter-type canola, timing of planting in fall is important. The presence of six true leaves at the beginning of winter is optimal for survival. Planting spring-type canola should start as soon as the soil texture and weather conditions permit. Winter-type canola requires 700 to 800 hours of chilling temperature before bolting. Spring type canola needs very little or no winter chill for bolting (Buntin et al. 2013). Water requirement through the growing season is 41 to 46 cm. Oilseed rape is susceptible

to shattering, so it is often harvested by swathing when about one third of the seed have turned dark. The crop is then windrowed for about 10 days prior to harvesting with a combine (Rimmer et al. 2007).

Canola production

Canola is the second largest source of vegetable oil in the world after soybean. In 2008 and 2009, the world production of rapeseed/canola was 15 percent of the total world oil crop production (USDA ERS 2012). Canola is extensively cultivated in Europe, Canada, Asia, and Australia and to a lesser extent in the United States. *Brassica napus*, *B. rapa* and *B. juncea* are the most commonly commercialized species. Spring-type canola is mainly produced in Canada, northern Europe, Australia and northern and southeastern parts of the United States. Winter-type canola is grown in most of Europe, parts of China, and the southern Great Plains of the U.S., including Oklahoma (Raymer 2002).

In the United States, canola is considered a relatively new crop, yet its production has steadily increased over the past three decades after the FDA recognized canola oil as a safe edible oil. According to the U.S. Department of Agriculture, over 1.3 million acres of canola were planted in 2013 nationwide, and increased up to 1.7 million acres in 2014. Canola is a new crop introduced in Oklahoma about ten years ago. Acreage increased to 205,000 acres in 2013 and 270,000 acres in 2014 (USDA NASS 2015). Oklahoma is now the second leading canola producing state after North Dakota. However, the U.S continues importing canola just to meet the increasing consumer demand for healthier edible oils (Boyles and Sanders, 2009).

U.S. canola production is concentrated in the northern Plains where a drier, shorter growing season makes corn and soybean production less attractive (USDA ERS 2012). However, canola is also well-adapted to the southern Great Plains. Both spring and winter types of canola are grown in the United States. Winter canola has a greater yield potential than spring types.

Spring canola has a shorter grain-filling period because it flowers one month later than the winter type after vernalization. Winter-type cultivars are the most commonly grown in the southern Great Plains (Boyles and Peeper n.d.). The production of winter canola has been well accepted in Oklahoma and the Great Plains because it has shown to be a viable and profitable rotational crop with winter wheat. Wheat has been traditionally grown for decades in the region. Thus, the monoculture practice and the lack of crop rotation has increased the appearance of weeds and diseases that have significantly reduced wheat yields. Most importantly, grassy winter weeds that are resistant to most herbicides build up in continuous wheat fields. Rotating fields with glyphosate-tolerant winter canola facilitates effective weed control in contaminated fields. Therefore, winter canola has been adopted as a sustainable rotational crop with winter wheat in the region, since it has the same growing season and it has been shown to increase wheat yields in the following season (Boyles and Peeper n.d.). It is recommended to plant canola only once every three to four years on the same field.

Black leg disease

Black leg (also known as Phoma stem canker), caused by the ascomycete fungus *Leptosphaeria maculans* (Desm.) Ces. & DeNot, is an economically important disease of canola (*Brassica napus* L.) in most regions of the world where the crop is grown (Rouxel and Balesdent 2005). This disease attacks several *Brassica* species such as cabbage, cauliflower, and broccoli; and has the potential to severely damage canola. Black leg can cause a reduction in yield of up to 50 percent in fields planted with susceptible cultivars where disease pressure is high (Damicone et al. 2012; del Rio Mendoza et al. 2012; Kutcher et al. 2010; West et al. 2001). Yield losses attributed to black leg have been reported in Europe, Australia, Canada and some parts in the United States (Balesdent et al. 2005; Hayden et al. 2007; Mengistu et al. 1991).

History of Black leg

Severe epidemics of black leg or stem canker in cabbage, cauliflower, and rapeseed have occurred in Europe, Australia and North America during the 19th century and into the 20th century (Henderson 1918). Black leg became a major concern after the expansion of oilseed rape and canola as major oilseed crops in the mid-20th century (Rouxel and Balesdent 2005). Winter rapeseed has been severely affected by this disease in Europe since 1950. Similarly, the rapeseed industry almost vanished in western Australia in 1972, two years after introducing Canadian spring rapeseed cultivars to the area (Gugel and Petrie 1992). In Canada in 1975 a virulent strain of the fungus was discovered in Saskatchewan and now it is widespread in most regions that produce the crop, often causing important economic losses (Gugel and Petrie 1992). Today, black leg is endemic in most oilseed rape growing regions, except Asia. It can potentially damage the crop when the genetic resistance of the cultivars is inadequate and the environmental conditions are favorable (high humidity and cool temperatures) for the production of ascospores which are the primary inoculum of the disease (Rouxel and Balesdent 2005).

L. maculans isolates were originally classified into pathogenicity groups, based on a standardized pathogenicity test on cotyledons of differential cultivars (Mengistu et al. 1991). The cotyledon assay permitted classification of isolates of the pathogen into four pathogenicity groups (PGs) from the highly virulent PG-4 to the weakly virulent PG-1 (Mengistu et al. 1991). In the United States, black leg was first identified in 1989 when an epidemic caused by a PG-4 strain developed in southern Kentucky (Mengistu et al. 1990). In 1991, black leg was identified on spring canola in North Dakota caused by strains belonging to the weakly virulent PG-1 and PG-2 isolates (Lamey and Hershman 1993). However, in 2003, a survey of isolates in the state revealed for the first time the presence of the more virulent PG-3 and PG-4 strains which were previously found in western Canada (Bradley et al. 2005; Chen and Fernando 2006; Fernando and Chen 2003). To date, PG-4 is the predominant pathogenicity group in Canada and North Dakota (Nepal

et al. 2014). In Georgia, the disease was first observed on winter canola late in the 1992-1993 cropping season at several locations (Buntin et al. 2013). In Oklahoma, the disease was first identified in 2009 on winter type canola caused by strains belonging to weakly virulent (PG-1) and highly virulent pathogenicity groups (PG-4) (del Río Mendoza et al. 2011). An increase in the incidence and prevalence of black leg caused by highly virulent strains of the fungus has important implications to canola breeding programs, management of black leg and the canola industry in the U.S. Therefore, research has focused on the characterization of the strains of the pathogen, identifying genetic resistance, assessment of fungicides, and yield loss studies in the region (Damicone et al. 2012).

Symptoms

L. maculans is a hemibiotrophic pathogen on *B. napus* and in most brassica hosts. In winter canola *L. maculans* oversummers as a saprophyte on infested stubble (crop residue), where it undergoes sexual recombination and forms pseudothecia and ascospores (sexual spores). Airborne ascospores are the primary inoculum for black leg epidemics. Airborne ascospores result from the colonization of stubble from the previous canola crops by the fungus. Because farmers widely practice minimum tillage, stubble is left on the soil surface providing a readily available inoculum source. Sexual recombination of the fungus on the stubble each year has led to the development of new pathotypes. Ascospores can be transported in air currents up to 5 km (Hall 1992) where they can land on cotyledons or true leaves and cause infections via stomata or wounds (Rimmer et al. 2007). Ascospores can be discharged from infected stubble anytime from one week to more than a year after harvest (Hershman and Perkins 1995). Previous studies reported that *L. maculans* could survive and discharge ascospores for at least 3 to 5 years after harvest (Alabouvette and Brunin 1970; McGee and Petrie, 1979; Petrie 1978). Discharge of ascospores is common during fall and the beginning of winter, which coincides with the time at which fall-seeded canola is the most susceptible to infection (Hershman and Perkins 1995).

After leaf infection, the fungus becomes necrotrophic and causes leaf spots. Lesions are round or irregularly shaped, becoming necrotic and greyish with age. Lesions may have a dark margin and produce pycnidia in the center of the lesions, which appear as black specks. Under moist and warm conditions, single-cell conidia (asexual spores) are produced in a pink ooze exuded by pycnidia which are dispersed at short distances by rain-splash (Hayden et al. 2007; Rimmer et al. 2007).

After leaf penetration, the fungus endophytically colonizes the plant. Mycelia grow in the vascular tissue from the site of entry on the leaves to the petiole, and continue into the basal stem (Hayden et al. 2007). During the ripening stages of crop development near the end of the growing season, the fungus turns necrotrophic at the base of the stem and the upper root, causing a stem canker. Stem lesions are usually oval, with a grey to tan colored center and dark margin, and internally the xylem is decayed and blackened. Pycnidia often form in stem cankers. Severely infected stems become malformated and brown to grey in color as decay of the basal stem advances. Finally, the stem becomes girdled, plants ripen prematurely and the crop is more likely to lodge (Rimmer et al. 2007). Seed stalks and pods can also be infected by spores that land on upper plants surfaces. Symptoms are gray to tan lesions, often with pycnidia, and surrounded by a dark border (Rimmer et al. 2007).

Causal Agent

Leptosphaeria maculans (Desm.) Ces. & De Not. (anamorph *Phoma lingam* Tode) was first described by Tode in Germany in 1791, who reported black leg on dried red cabbage stems (*Brassica oleracea*) and named the pathogen *Sphaeria lingam* (Henderson 1918). In France in 1849, Desmazieres recognized the fungus on living cabbage plants and named the pathogen *Phoma lingam* (Henderson 1918; Rouxel and Balesdent 2005). In 1956, Smith first discovered the perfect (sexual) stage of *P. lingam* and named it *Leptosphaeria napi*, later changing it to

Leptosphaeria maculans (Smith and Sutton 1964). At present, *L. maculans* is a member of the class Dothideomycetes, order Pleosporales which includes other important plant pathogens from the genera *Pleospora*, *Alternaria*, *Venturia*, *Cochliobolus*, among others (Berbee 2001).

In the past, the fungus causing stem canker or black leg on crucifers, more specifically on oilseed rape and canola, was considered a single species. Variability was then observed in virulence and the species was divided into highly and weakly aggressive strains. However due to additional polymorphisms observed in cultural characteristics and genetics, it was reclassified into two closely related species, *L. maculans* and *L. biglobosa* (Rouxel and Balesdent 2005). This species complex resulted in a division into two groups, highly aggressive and weakly aggressive, or also termed as virulent and avirulent, group A and group B, or Tox^{+} and Tox^{0} (West et al. 2001). These species may infect the same host, sometimes the same individual. However, they exhibit differences in cultural characteristics, genetics, toxin (sirodesmin) production, and the symptoms they cause on hosts (Kaczmarek and Jedryczka 2011).

Leptosphaeria maculans (group A) is a highly virulent fungus that can attack plants at all ages causing damaging stem cankers and significantly affecting yield, while *Leptosphaeria biglobosa* (group B) is recognized as a weakly aggressive pathogen that and generally affects plants later in the season by causing stem lesions that minimally impact yield (Shoemaker and Brun 2001).

Leptosphaeria maculans morphology

L. maculans is a heterothallic species and displays some variability in cultural characteristics and pathogenicity. The fungus produces a range of phytotoxins known as sirodesmins. According to Boerema et al. (2004), *L. maculans* produces two types of pycnidia *in vivo*. Pycnidial type I which develops in leaf spots, stems, pods, and seed, is variable in shape from subglobose to flask-shaped with broad base and variable in size from 150 to 400 µm in

diameter. At maturity, pycnidial type I usually develops one distinct black poroid papilla which may grow into a long neck. Pycnidial type II, present in woody parts of old crop residue, is highly variable in shape and size, mostly suglobose with an irregular flat base. Type II pycnidia are relatively large and range in size from 200 to 1000 µm in diameter, non-papillate or only slightly papillate, with a narrow pore or opened by rupture. Conidia are ellipsoidal, occasionally with 2 small polar gutulles 2.5-5 x 1-2 µm. Pseudothecia develop in the subepidermis of stems while oversummering and can reach a size of 600 µm in diameter. Asci are 100-150 x 12-16 µm, 8-spored, quadriseriate above and biseriate below. Ascospores are 35-70 x 4.5-8 µm, narrowly fusiform, 5 septate, and yellowish-brown with guttules (Boerema et al. 2004).

In vitro, L. maculans colonies are slow growers on oatmeal agar reaching 1.5 to 2.5 cm diameter after 7 days with regular and irregular border, usually with abundant aerial mycelium varying in color from white, grey, green or brown. Pycnidial type I is abundant in or on agar, mostly globose-papillate, black and relatively small at 150 to 250 µm in diameter. In old cultures, thickened pycnidial walls are common (Boerema et al. 2004).

Leptosphaeria biglobosa morphology

Boerema et al. (2004), classified *L. biglobosa* under the unnamed phoma-anamorph of *Leptosphaeria sensu lato* group. *In vitro, L. biglobosa* produces thin walled pycnidia on and in the agar that are globose papillate, 150 to 400 µm in diameter, black or greyish brown in color. The conidial matrix is reddish brown. Conidia are subcilindrical, straight biguttulate, hyaline, and 4-5 x 1.5-2 µm. Pseudothecia possess a long neck that is swollen on the upper part (Boerema et al. 2004). Colonies of *L. biglobosa* are fast growers on oatmeal agar, (5 to 7 cm in 7 days) are white or grey in color and characterized by a yellow-brown discoloration on the agar which can vary from pale straw to cinnamon color (Boerema et al. 2004)

Epidemiology

Environmental conditions can influence the production of ascospores. Ascospores are discharged during the months when temperatures range from 8^{o} to 15^{o}C and relative humidity is high. In the northern part of the United States and Canada, ascospores are dispersed during May to July when spring type canola is most susceptible (Rimmer et al. 2007). In France and regions of the U.S. where winter type canola is grown, the period of ascospore dispersion occurs during fall and the first months of winter, when winter canola is most susceptible to infection (i.e plants that are at or before 6 leaf stage) (Alabouvette and Brunin 1970; Hershman and Perkins 1995). Minor ascospore discharges occur in the spring from February to April in the year after the crop was planted (Hershman and Perkins 1995). Ascospore biology has been modeled and used to predict disease outbreaks in areas where canola is grown.

Temperature influences the appearance of symptoms on infected leaves. Above 20^{o}C, lesions mature rapidly and temperature is the optimal pycnidial development on leaf spots, and for pycnidial and pseudothecial development on stems cankers. At temperatures at or below 10^{o}C, colonization is symptomless in the plant (Rimmer et al. 2007).

Disease Management

Strategies to manage black leg on brassica crops include cultural practices, application of fungicides, and deployment of resistant cultivars or hybrids. Cultural practices such as crop rotation, management of stubble (old crop residue), and use of certified seeds help decreasing the risk of infection. Rotation with non-host crops effectively reduces the pathogen population. The length of an effective crop rotation varies from 3 to 4 years between host crops. A shorter rotation results in an increased amount of infested stubble in the field (West et al. 2001). Infested stubble on the soil surface available for ascospore production can be reduced by sanitation, removal or deep tillage. Buried residues decompose faster and the soil interfers with spores release, however,

deep tillage is not a recommended practice anymore because it increases soil erosion and quality (West et al. 2001). In Europe, Australia and North America a greater use of minimum tillage is recommended (West et al. 2001). Flooding soils for 6 to 10 days can effectively eliminate the pathogen from the residues (Rimmer et al. 2007). In China and India, black leg is not considered an important problem because the entire plant is removed at harvest and fields are flooded during rice production the next season. The utilization of disease-free seed is recommended in areas where black leg has not been reported to prevent introducing the pathogen or new races of the pathogen to new areas. Adjusting the planting date is also a strategy to reduce the risk of infection by released ascospores. In France, early sowing is recommended to allow the crop to have a sufficient number of leaves in order to evade or survive the infection by ascospores at its most sensitive stage (Le Page 1995; West et al. 2001).

Combinations of fungicide treatments such as seed treatments and foliar sprays are used for management of black leg. Seed treatment with fungicides such as benomyl, flutriafol, thiram, and iprodione are used to eradicate the pathogen from the seed in Canada, Europe, Australia (West et al. 2001). Foliar sprays with the fungicide benomyl in combination with a cultivar with a low level of resistance or no resistance was not effective against stem canker in Australia (Brown et al. 1976; West et al. 2001). Similarly, in Canada, propiconazole has not provided adequate levels of stem canker protection. In Europe, a combination of foliar sprays with difenoconazole plus carbendazim or flusilazole plus carbendazil, has been effective for the control of stem canker. The higher yields obtained in Europe justify the application of fungicides (West et al. 2001). In the U.S., several fungicides have been registered for black leg control including azoxystrobin, picoxystrobin, prothioconazole, pyraclostrobin, and a combination of pyraclostrobin and fluxapyroxad (Damicone 2015). Seed-treatment fungicides registered for control of black leg include azoxystrobin, trifloxystrobin, difenconazole, fludioxanil, carboxin and metalaxyl (Damicone 2015).

However, fungicides are effective only for a limited period of time, due to degradation, leaf expansion, and growth of new untreated leaves. Therefore it is important to correctly time applications (West et al. 2000; West et al. 2001). In addition, after a certain growth stage, some authors suggest that it is unnecessary to apply foliar fungicides because there would not be sufficient time for the pathogen to cause severe stem cankers that affect yield (Hammond and Lewis 1986; McGee and Petrie 1979). Others believe that the critical growth stage for applying a fungicide is difficult to identify because it varies depending on the environmental conditions. Therefore, use of forecasting systems to optimize the use of fungicides for black leg control are recommended (Fitt et al. 1997).

The most effective strategy to manage black leg disease is the planting of cultivars with genetic resistance. Using resistant cultivars harboring different specific resistance genes has been suggested for sustainable management of black leg. At present, both vertical and horizontal types of resistance have been identified in *B. napus* and have been used to develop resistant cultivars. Cultivars with moderate to high resistance are being used in Australia, Canada, and Europe (West et al. 2001). However, the breakdown of race-specific resistance has been reported in different countries as a consequence of planting single gene resistance where the inoculum pressure is high. The increase of inoculum pressure is due to the short period of rotations between oilseed rape crops, in combination with use of minimum tillage of the fields. The pathogen undergoes sexual recombination each year on stubble left on the soil surface which may favor the rapid ability of the fungus to adapt to new resistance genes.

Genetic resistance

Genetic resistance is considered the most environmentally friendly and efficient method of control of black leg (Balesdent et al. 2001). Two types of genetic resistance have been distinguished in canola, polygenic and single gene resistance. Polygenic resistance, also known as

quantitative, horizontal, or durable resistance occurs at the adult stage of the plant and confers partial resistance to the disease. It is mediated by a combination of multiple, generally uncharacterized, resistance genes which confer a moderate level of resistance that is broadly effective (Balesdent et al. 2001). Single-gene or major-gene resistance, also known as qualitative, vertical or race-specific resistance is expressed from the seedling to the adult stage of the plant by a single or a few specific resistance genes (Delourme et al. 2004; Marcroft et al. 2012). Adult-plant resistance is determined by evaluating the severity of stem and crown cankers in the field at the end of the season; while seedling resistance is assessed by evaluating leaf spot severity on cotyledons or young leaves under controlled conditions (Balesdent et al. 2001). Race-specific resistance has great potential for avoiding the formation of stem cankers because it prevents the pathogen from entering the plant, which otherwise would grow systemically through the leaf, petiole, and then stem to cause a stem canker (Balesdent et al. 2001; Hammond et al. 1985). Screening for the latter type of resistance is the main focus of this research.

L. maculans has a gene for gene interaction with *B. napus*, where the outcome of the infection (resistance or susceptibility) depends on the presence of a major gene for resistance (*Rlm*) in the plant and a corresponding avirulence (*Avr*) gene in the pathogen (Balesdent et al. 2005; Marcroft et al. 2012). Conversely, the lack of a dominant resistance gene in the plant or a recessive gene for virulence (*avrLm*) in the pathogen results in a susceptible reaction or the expression of disease. For example, resistance gene *Rlm1* interacts with *AvrLm1*, resulting in an incompatible reaction (no disease) between the plant harboring the *Rlm1* and the isolate harboring the *AvrLm1* by inhibiting infection from the germinated ascospores or conidia and subsequent development of leaf lesions (Fitt et al. 2006).

To date, 17 major resistance genes have been identified (*Rlm1* to *Rlm11*, *LepR1* to *LepR4*, *BLMR1*, and *BLMR2*) in *Brassica* species conferring resistance to *Leptosphaeria maculans* (Marcroft et al. 2012; Van de Wouw et al. 2014b). Although only one, LepR3, has been

cloned (Larkan et al. 2013). Most of these genes have been positioned on *B. napus* linkage maps and have shown to be organized in clusters (Delourme et al. 2006; Delourme et al. 2004). *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* were shown to belong to linkage group 10 (Howlett 2004). Of the corresponding avirulence genes in *L. maculans,* five have been cloned and sequenced (*AvrLm1, AvrLm4-7, AvrLm6, AvrLm11* and *AvrLmJ1*) (Fudal et al. 2007; Gout et al. 2006; Parlange et al. 2009; Balesdent et al. 2013; Van de Wouw et al. 2014a). Balesdent et al. (2002) reported the clustering of several *Avr* genes into two main clusters, "*AvrLm1-AvrLm2-AvrLm6*" and "*AvrLm3 - AvrLm4 - AvrLm7*", which have shown to be genetically linked at specific loci.

Dominant major specific genes have been identified through genetic studies involving different oilseed rape cultivars/lines and different characterized *L. maculans* isolates. *Rlm1* is in *B. napus* cv. Quinta and in cv. Lirabon having resistance to PG-3 (Ansan-Melayah et al. 1998). *Rlm2* is in cv. Glacier controlling resistance to PG-2 (Ansan-Melayah et al. 1998). *Rlm3* is a single dominant allele derived from Glacier that confers resistance to European races (Delourme et al. 2004). *Rlm4* controls resistance in cv. Jet Neuf, and it is closely linked to *Rlm1* (Balesdent et al. 2002). *Rlm5* and *Rlm6* have been identified in *B. juncea* (Indian mustard) cvs. Picra and Aurea (Balesdent et al. 2002). *Rlm7* was identified in *B. napus* and is linked to *Rlm3* (Balesdent et al. 2002). *Rlm8* is present in *B. rapa* (Balesdent et al. 2002). *Rlm9* is in *B. napus* cv. Darmor (Balesdent et al. 2002; Delourme et al. 2004). *Rlm10* and *Rlm11* are from *B. nigra* (Chevre et al. 1997).

Race specific resistance genes

A standardized pathogenicity test which uses inoculation of cotyledons has been developed to evaluate the interaction phenotype (resistant or susceptible reaction) on sets of *Brassica* differentials (Mengistu et al. 1991). This assay was the first step towards classification

and genetic analysis of the specificity of the interaction between *B. napus* and *L. maculans* (Mengistu et al. 1991; Williams et al. 1979).

L. maculans isolates were at first categorized into pathogenicity groups (PG) based on the interaction phenotype of the isolate when inoculated on the cotyledons of three *B. napus* differential cultivars Westar (susceptible control), Glacier (*Rlm2*, *Rlm3*) and Quinta (*Rlm1*) (Mengistu et al. 1991). Using this set of *B. napus* differential cultivars, three pathogenicity groups (PG) could be discriminated in a *L. maculans* population. PG-4 isolates were virulent on Westar, Glacier and Quinta. PG-3 were virulent on Westar and Glacier but avirulent in Quinta, and PG-2 were virulent only on Westar but avirulent on Quinta and Glacier (Balesdent et al. 2005; Mengistu et al. 1991). PG-1 was avirulent on all three cultivars and was classified as a weakly aggressive isolate, now recognized as *L. biglobosa* (Shoemaker and Brun, 2001). Chen and Fernando (2005) added PGT, a new pathogenicity group, which is virulent on Westar and Quinta, but not Glacier. Originally, PG-2 was prevalent in western Canada, whereas PG-3 and PG-4 were mainly present in Europe and Australia. However, from 2002 to 2004, PG-3 and PG-4 isolates were found in western Canada and North Dakota (Chen and Fernando, 2005).

Badawy et al. (1991) proposed a second set of differentials, replacing spring type *B. napus* cv. Westar with winter cv. Lirabon and adding cv. Jet Neuf (*Rlm4*) to Glacier and Quinta (Badawy et al. 1991; Balesdent et al. 2005; Fitt et al. 2006). This new set of differential cultivars allowed the subdivision of each PG-2, PG-3 and PG-4 into 2 groups and classified the isolates of *L. maculans* into a six corresponding PGs termed A1 to A6 (Badawy et al. 1991; Balesdent et al. 2005; Koch et al. 1991).

The classification of *Leptosphaeria maculans* isolates based on pathogenicity groups gives ambiguous information of all the possible avirulence (*Avr*) genes existing in an isolate. A single isolate may possess more than one *Avr* gene. Therefore isolates having different

combination of avirulence genes could be found in the same pathogenicity group (PG) (Rouxel and Balesdent 2005). In other words, the isolates belonging to the same PG could be polymorphic at the *Avr* loci (Chen and Fernando 2006). In order to avoid this confusion among pathogenicity groups and avirulence genes, the terminology to classify *Leptosphaeria maculans* has changed from pathogenicity groups into races. The race terminology names the avirulence genes present in the isolate indicating all *Avr* loci for which the isolate is avirulent, preceeded by the letters *'Av'* (Balesdent et al. 2005; Rouxel and Balesdent 2005). Thus, the race of an isolate harboring avirulence alleles *AvrLm1*, *AvrLm2* and *AvrLm4* but lacking *AvrLm3* would be named *Av 1,2,4* using the race terminology. One benefit of using the race terminology is that it directly provides the information necessary to know which corresponding resistance genes may be used to manage the disease in places where the pathogen population has been characterized.

L. maculans has adapted to new and different major resistance genes. Mutation or deletions in the coding regions of the avirulence genes due to selection pressure imposed by the host, and changes in the frequency of avirulent to virulent isolates in the pathogen population can lead to major gene resistance being overcome (Gout et al. 2006; Gout et al. 2007; Kutcher et al. 2007; Marcroft et al. 2012; Parlange et al. 2009; Van de Wouw et al. 2010). In Europe, Canada, and Australia, several resistant cultivars have been released followed by a breakdown of race-specific resistance as a result of the rapid adaption of *L. maculans* populations (Delourme et al. 2006). In France, resistance gene *Rlm1* was overcome causing important economic losses when virulent strains became prevalent within three growing seasons (Balesdent et al. 2006). A similar case occurred in Australia when the *B. napus* cultivar Surpass400 harboring the *LepR3* resistance gene became susceptible after only three years of being released to the market, resulting in 90% yield losses (Hayden et al. 2007; Li et al. 2003; Van de Wouw et al. 2010). Both are examples of the breakdown of major-gene resistance due to strong selection pressure on the pathogen population by resistant cultivars. The pathogen may adapt to resistance genes and overcome

resistance in a little as three to four years (Dilmaghani et al. 2009). Virulence changes in *L. maculans* populations in short period of time have been documented also in the U.S. (Chen and Fernando 2006; del Rio Mendoza et al. 2012; Li et al. 2003). In western Canada and North Dakota in 2002, most of the cultivars planted were considered resistant or partially resistant to PG-2 strains. However, one year later, strains from PG-3 and PG-4 were found in canola residues from the same area (Chen and Fernando 2006; del Rio Mendoza et al. 2012).

Knowledge of the PGs and the virulence alleles involved in black leg epidemics is key in breeding for black leg resistance. Information on the occurrence of avirulence alleles in the population and the race structure of the pathogen is useful for breeders attempting to develop canola cultivars that have effective black leg resistance. Avirulence alleles and race structure of *L. maculans* populations have been analyzed in Germany, France, Chile, Mexico, Canada, Australia and southeastern United States (Dilmaghani et al. 2009). However, there is no information on the race structure of *L. maculans* in Oklahoma or the southern Great Plains regions of winter canola production. Likewise, having an understanding of the genetic basis of the resistance in canola is strategically important for identification and deployment of resistant cultivars. The presence of major resistance genes in unknown *B. napus* germplasm has been inferred using *L. maculans* isolates with known genotype in several studies (Marcroft et al. 2012; Rouxel et al. 2003). It is important to know which major genes are present in the common cultivars and hybrids grown in Oklahoma and the southern Great Plains so that resistant types can be recommended and planted.

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CHAPTER III

ANALYSIS OF THE RACE STRUCTURE OF *Leptosphaeria maculans* IN OKLAHOMA

ABSTRACT

Black leg, caused by the fungus *Leptosphaeria maculans*, is a widespread disease of winter canola (*Brassica napus* L.) in Oklahoma. Strategies to manage the disease are cultural practices, application of fungicides, and deployment of resistance genes. Resistance genes (*Rlm)* in canola interact with avirulence genes in the fungus (*AvrLm*) in a gene-for-gene manner. Little is known about the diversity and local distribution of pathogenicity groups (PGs), frequency of avirulence (*Avr*) genes in the pathogen population, and race structure in Oklahoma. A collection of 107 isolates of *Leptosphaeria* spp. from different counties was first assigned to four pathogenicity groups PGs based on the phenotype interaction on cotyledons of the differential cultivars Westar, Glacier (*Rlm2, Rlm3*) and Quinta (*Rlm1*). PG-4, virulent on all differentials, indicated that the virulence alleles *avrLm1, avrLm2* and *avrLm3* were common in 49% of the population. PG-3, avirulent on Quinta, revealed that the avirulence allele *AvrLm1* was present in 22% of the isolates. PG-2 avirulent on Glacier and Quinta represented either *AvrLm1,2,3; AvrLm1,2 or AvrLm1,3* in 8% of the isolates, and PG-1 the weakly virulent group and avirulent on the three differentials was found at 21%. Amplification of the ITS region specific for the highly virulent group (HV) and mating type locus (MAT) further classified the local pathogen collection as mostly highly virulent *L. maculans* isolates (N=95) and fewer weakly virulent *L. biglobosa* isolates (N=12). *L. maculans* isolates were characterized for avirulence alleles *AvrLm1*, *AvrLm2,3*, *AvrLm4-7* and *AvrLm6* based on phenotype interaction on differentials harboring

Rlm1, *Rlm2* and *Rlm3* and amplification of *AvrLm1*, *AvrLm4-7*, *AvrLm6* alleles by polymerase chain reaction (PCR). Avirulence alleles *AvrLm4-7* and *AvrLm6* were predominant (100%) in the local *L. maculans* population. *AvrLm1* was found in 38% of the population, and *AvrLm2,3* at only 9%. Four distinct races (combination of avirulence alleles) were identified in the collection (*Av1,2,3,6,4-7; Av1,6,4-7; Av2,3,6,4-7; Av6,4-7*). Races *Av6,4-7* (56%) and *Av1,6,4-7* (35%) were predominant in Oklahoma. Resistance genes *Rlm6* and *Rlm4 and/or Rlm7* should be broadly effective resistance genes in Oklahoma. *Rlm1, Rlm2* and *Rlm3* are not expected to be effective against the corresponding avirulence alleles that occured at low frequency. Understanding the avirulence frequency and race structure in the pathogen population will be useful for the identification and development of resistant cultivars and hybrids to control black leg disease of winter canola in Oklahoma.

INTRODUCTION

Canola (*Brassica napus* L.) is a relatively new crop in the United States. Oklahoma has been growing winter-type canola for the past ten years and is now the second leading canola producing state after North Dakota (USDA NASS 2014). Black leg disease, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. & DeNot, is an economically important disease of oilseed rape and canola in most regions of the world where crop is grown (Rouxel and Balesdent, 2005). Canola is susceptible to black leg infection at seedling stage; however, symptoms can be observed during all stages of crop development (Rimmer et al. 2007). Primary infection during the early stages appear as leaf spots. Stem cankers that girdle stems and may kill the plant, develop during ripening stages, which occur near the end of the season (Rimmer et al. 2007). The disease is associated with a complex of two closely related species, *L. maculans* and *L. biglobosa* (Mendes-Pereira et al. 2003; Shoemaker and Brun 2001); which were previously classified as highly virulent and the weakly virulent forms of the pathogen respectively (Cunningham, 1927; Petrie, 1978; Shoemaker and Brun 2001). These species may share the same ecological niche,

have similar epidemiology and infection strategies; however, they differ in symptoms, severity of the disease and genetics (West et al. 2002). *L. maculans* has been found to be a highly specialized pathogen interacting in a gene-for-gene manner with *B. napus*. By contrast, *L. biglobosa* has not exhibited these specialized interactions (Vincenot et al. 2008).

Major gene resistance has been widely used to control black leg (Delourme et al. 2006). Major gene resistance occurs as a gene-for-gene interaction between *B. napus* and *L. maculans*, where the resistant phenotype depends on the presence of a major gene for resistance (*Rlm*) in the plant and a corresponding avirulence (*AvrLm*) gene in the pathogen (Balesdent et al. 2005; Marcroft et al. 2012). Conversely, the lack of a resistance gene in the plant or a recessive gene for virulence (*avrlm*) in the pathogen results in a susceptible reaction and the expression of disease. To date, at least 17 major resistance genes have been identified (*Rlm1* to *Rlm11, LepR1* to *LepR4, BLMR1* and *BLMR2*) in *Brassica* species conferring resistance to *Leptosphaeria maculans* (Marcroft et al. 2012; Van de Wouw et al. 2014). Although only one, *LepR3,* has been cloned (Larkan et al. 2013). Most of these genes have been positioned on *B. napus* linkage maps and have shown to be organized in clusters (Delourme et al. 2006; Delourme et al. 2004). Of the corresponding avirulence genes in *L. maculans* conferring host specificity, five have been cloned and sequenced (*AvrLm1, AvrLm4-7, AvrLm6, AvrLm11* and *AvrLmJ1*) (Fudal et al. 2007; Gout et al. 2006; Parlange et al. 2009; Balesdent et al. 2013; Van de Wouw et al. 2014).

For a better understanding of the variation of virulence and genetic interaction between *B. napus* and *L. maculans*, the phenotype interaction on a set of *B. napus* differential cultivars Westar, Glacier (*Rlm2,3*) and Quinta (*Rlm1*), was originally used to classify *L. maculans* isolates into pathogenicity groups (PGs) (Mengistu et al. 1991). PG-4 isolates were virulent on Westar, Glacier and Quinta; PG-3 were avirulent on Quinta; PG-2 were avirulent on Glacier and Quinta. Avirulence on Westar discriminated *L. biglobosa* (PG-1) from *L. maculans*. This PG system was initially valuable to detect changes in pathogen population, however it is limited only to four

pathogenicity groups based on two differential cultivars (Kutcher et al. 2010a). In order to overcome this limitation, a race system was proposed as a new system of classification (Balesdent et al. 2005). Race is designated as the *Avr* allele composition of each isolate by listing all the *Avr* loci for which an isolate has been characterized and is avirulent, preceded by the letters 'Av'. The *Avr* loci for which a characterization is not possible, due to the unavailability of differentials with single resistance genes, is indicated in parenthesis (Balesdent et al. 2005).

Resistance genes in *B. napus* have been characterized based on the genetic identification of corresponding avirulence alleles (*AvrLm*) in *L. maculans* (Rouxel and Balesdent 2005). Deployment of *B. napus* resistance genes have proven to be very effective in *L. maculans* populations that display the corresponding avirulence genes. However, the repeated planting of the cultivars with specific resistance genes in extended areas has created high selection pressure on the pathogen population (Rouxel et al. 2003). In as few as three to four years the pathogen may adapt to resistance genes and overcome resistance (Dilmaghani et al. 2009). In France, resistance gene *Rlm1* was overcome after three growing seasons causing important economic losses (Balesdent et al. 2006; Rouxel et al. 2003). Similarly, the resistance gene *LepR3* declined three years after deployment in Australia (Hayden et al. 2007; Li et al. 2003).

The pathogen should be monitored at regular intervals to assess changes in the *Avr* structure of the population in different geographic locations to effectively employ specific resistance (Kutcher et al. 2010b). Knowledge of the race structure is crucial to detect races with the ability to overcome current specific resistance genes, and to choose the best resistance sources to be used locally (Dilmaghani et al. 2009). To date, studies describing the avirulence patterns and race structure of the pathogen population have been done in few countries such as Germany and France in Europe; Chile in South America; Mexico, Canada and the southeastern United States in North America, and in Australia (Balesdent et al. 2006; Balesdent et al. 2005; Dilmaghani et al. 2009) using different sets of differential cultivars. However there is no

information on the race structure of the *L. maculans* population in Oklahoma and surrounding states in the southern Great Plains where winter canola is grown.

The objective of this study was to determine the race structure of *L. maculans* in Oklahoma using a combination of pathogenicity tests on *B. napus* differential cultivars (Westar, Glacier and Quinta) and amplification of avirulence genes (*AvrLm1, AvrLm4-7, AvrLm6*) by polymerase chain reaction (PCR). Understanding the diversity of avirulence *Avr* alleles and the race structure of *L. maculans* in Oklahoma is necessary in order to identify effective *Rlm* genes to deploy for black leg control.

MATERIALS AND METHODS

Pathogen sampling, isolation and inoculum production

The pathogen collection consisted of 107 single-pycnidial isolates of *Leptosphaeria* spp. that were obtained from *Brassica napus* leaves with leaf spot symptoms. From 2009 to 2013, samples were collected from nine counties in Oklahoma and one county in Kansas. Pycnidia were isolated and cultured on clarified V8 juice agar amended with streptomycin (100ppm), hyphal-tip purified at least twice, and incubated for 12 to 15 days at 24^0C and under continuous fluorescent light until sporulation (Mengistu et al. 1991). For long-term storage, the fungus was grown on V8 juice agar overlaid with sterile filter paper. Small pieces of filter paper colonized with mycelia were stored dry at 4^0C in sterile vials.

Inoculum of *L. maculans* was produced by collecting conidia from sporulating cultures on clarified V8 juice agar as described by Mesgistu et al. (1991). Conidial suspensions were prepared by flooding plates with sterile distilled water, gently rubbing culture surfaces with a sterile spreader and then straining the suspensions through cheesecloth. Sporulating cultures were produced by spreading conidial suspensions over the surface of YPS agar or V8 juice agar and incubating at room temperature under continuous white fluorescent light. For pathogenicity tests, conidia were

collected from 10 to 15-day-old sporulating cultures presenting a dense lawn of mature pycnidia with little mycelium (Balesdent et al. 1998). Spore suspensions were adjusted to 3 x 10^6 spores ml^{-1} using a hemocytometer. Aliquots of the spore suspensions, were centrifuged and kept frozen until needed to produce new sporulating cultures (Mengistu et al. 1991).

Pathogenicity tests

Seeds of *Brassica napus* cvs. Westar (spring type) with no *Rlm* genes, Glacier (winter type) harboring *Rlm2* and *Rlm3* and Quinta (winter type) harboring *Rlm1* (Balesdent et al. 2002; Balesdent et al. 2001) were obtained from the USDA Germplasm Resources Information Network (GRIN) and increased in the greenhouse. Seeds were sown in plastic flats fitted with 72 celled packs containing growing media (vermiculite, canadian sphagnum peat moss, coarse perlite, dolomitic limestone). Seedlings were maintained for seven days in a growth chamber at 24^0C and RH 80%, with continuous fluorescent light. Plants were watered as needed and nutrients were supplied by applying fertilizer (24-8-16 g/L N/P/K respectively) within the first week.

Seven days after sowing, seedlings were wounded by puncturing each half of a cotyledon with a 200 µl micropipette tip. Each wound was inoculated with a 5 µl droplet of conidial suspension adjusted to 10^6 spores ml^{-1}. Two wounds were made per cotyledon resulting in four wounds per plant. Inoculated seedlings were placed in a dew chamber for 2 days at 24^0C, 100% RH and no light. Seedling were transferred back to the growth chamber for another 8 days. Emerging true leaves were removed every two to three days by pinching the growing tip of the seedlings to help the cotyledons remain green until assessing disease severity (Mengistu et al. 1991). Each isolate was inoculated onto eight plants, and experiments were repeated at least twice (Balesdent et al. 2005).

The phenotype interactions were scored ten days after inoculation, by assessing disease severity using the IMASCORE rating scale comprised of six infection classes (IC) proposed by Volke (1999) and used by Balesdent et al. (2001). Infection classes IC1 to IC3 correspond to avirulent isolates that exhibit a resistant reaction, and IC4 to IC6 correspond to virulent isolates which produce susceptible reactions. IC1 is the hypersensitive response, IC2 represents a larger (1.5 to 3mm) dark necrotic lesion, and IC3 is a non-sporulating lesion that is sharply delimited by a dark necrotic margin. IC4 to IC6 are characterized by spreading, gray-green lesions with no dark margin. IC4 has no sporulation, IC5 has a few pycnidia, and IC6 has abundant sporulation. The result of each isolate-cultivar interaction was averaged and classified as resistant or susceptible. A resistant phenotype interaction implied the presence of the corresponding avirulence (*Avr*) allele in the pathogen, whereas a susceptible reaction implied the presence of a virulence *(avr)* allele.

Race was designated as proposed by Balesdent et al. (2005) which describes the *Avr* allele composition of each isolate by listing all the *Avr* loci for which the isolate has been characterized and is avirulent, preceded by the letters 'Av'. The Avr loci for which the characterization was not possible, because differentials with single resistance genes were unavailable, were indicated in parenthesis. For example, race *Av 1,4,6,(7)* is composed of isolates containing *AvrLm1*, *AvrLm4,* and *AvrLm6,* and can either be avirulent *AvrLm7* or virulent *avrLm7*.

PCR amplification of avirulence alleles

Leptosphaeria spp. isolates were characterize for mating type, virulence group, and avirulence alleles *AvrLm1*, *AvrLm6* and *AvrL4-7* (confers dual recognition of *Rlm4* and *Rlm7*), by polymerase chain reaction (PCR). Genomic DNA was extracted from semi-dried mycelia of the

107 isolates cultured in clarified V8 juice broth with the DNeasy 96 Plant Kit (Qiagen S.A) following the manufacturer's recommendations and amplified using specific primers.

Leptosphaeria maculans was identified using the primer pair HV17 and HV26C which amplified the ITS region (377 bp) specific for highly virulent isolates and *Leptosphaeria biglobosa* was identified using the primer pair WV17 and 5.8C (237 bp) specific for the weakly virulent isolates (Mahuku et al. 1995; Xue et al. 1992). The mating type amplification was used as a control for DNA quality and to confirm the identification of *L. maculans* species. Specific primers for the two alternate forms of the mating type (*MAT)* locus were used in a multiplex polymerase chain reaction (PCR) (Cozijnsen and Howlett 2003). A PCR fragment of 686 bp was amplified for *MAT1-1* isolates, and a 443 bp fragment was amplified for *MAT1-2* isolates.

The frequency of avirulence alleles *AvrLm1*, *AvrLm4-7* and *AvrLm6* was determined for *L. maculans* isolates. Primers pair *AvrLm-F* and *AvrLm1-R* was used to amplify *AvrLm1* gene (695 bp) (Van de Wouw et al. 2010). The pair of primers *Avr47ext-Lo* and *Avr47ext-Up3* amplified the gene *AvrLm4-7* (788 bp) (Parlange et al. 2009). Finally, primers *AvrLm6-F* and *AvrLm6-R* amplified *AvrLm6* (751 bp) (Van de Wouw et al. 2010). PCR amplifications were carried out in 25 µl reactions. PCR reactions for each gene were performed in a *Techne TC-4000* Thermal Cycler as described previously (Cozijnsen and Howlett 2003; Mahuku et al. 1995; Parlange et al. 2009; Van de Wouw et al. 2010). The PCR products were separated by electrophoresis in 1.2% agarose gels in TAE buffer.

RESULTS

Pathogenicity grouping of isolates according to PG

Inoculation of differential cultivars harboring specific resistance genes Westar, Glacier (*Rlm2, Rlm3*) and Quinta (*Rlm1*), allowed the classification of the isolates into pathogenicity groups PG-1 to PG-4 according to the phenotype interaction. Half of the population was PG-4, a

virulent group that had virulence alleles *avrLm1*, *avrLm2* and *avrLm3* (Table3.1). PG-3 isolates were avirulent on Quinta and had the avirulence allele *AvrLm1* represented 22% of the isolates. Only a few isolates (8%) were classified as PG-2, avirulent on Glacier and Quinta, and had *AvrLm1,2,3; AvrLm1,2 or AvrLm1,3*. PG-1 isolates were avirulent on Westar and were found at a frequency of 21%. PG-1 isolates, were considered the weakly virulent group classified as *L. biglobosa* (Kutcher et al. 2010a; Shoemaker and Brun 2001).

TABLE 3.1. Frequency of pathogenicity groups (PG)[a] and corresponding avirulence and virulence alleles in the local *Leptosphaeria maculans* population.

PG	Avirulence and virulence alleles	No. of isolates	Frequency (%)
PG-4	*avrLm1,2,3*	52	49
PG-3	*AvrLm1,avr2,3*	24	22
PG-2	*AvrLm1,2,3 or AvrLm1,2 avr3 or AvrLm1,3 avr2*	9	8
PG-1		22	21
Total		107	100

[a] Pathogenicity group: PG-4 isolates virulent on Westar, Glacier and Quinta; PG-3 avirulent on Quinta; PG-2 avirulent on Glacier and Quinta; PG-1 avirulent on all cutivars and classified as *L. biglobosa*.

Avr allele frequencies in the collection

Amplification of the ITS region specific for the highly virulent (HV) group and mating type (MAT) locus differentiated *L. maculans* isolates from *L. biglobosa* isolates in the local collection. Of the 107 isolates, 12 failed to amplify the ITS region specific for highly virulent isolates and the mating type locus using *L. maculans* specific primers. In addition, the ITS region specific for weakly virulent isolates or *L. biglobosa* was amplified with specific primers for these 12 isolates. These isolates produced small necrotic spots and chlorosis on Westar that were different from the susceptible symptoms observed when inoculated with *L. maculans*. *L. biglobosa* isolates were found in Kiowa and Major Counties in 2012, and in Kingfisher County in 2011. The rest of the isolates (N=95), positive for the highly virulent group and for the *MAT* locus, were classified as *L. maculans*. Amplification and sequencing of few isolates (N=5 of 95)

using general ITS primers (ITS1 and ITS4) confirmed the species as *L. maculans*. *MAT1-1* isolates (49%) and *MAT1-2* (51%) isolates occurred at similar frequencies in the population. However, amplification of the ITS region of the highly virulent (HV) group and mating type (MAT) locus demonstrated that 10 of the 22 isolates avirulent on Westar were actually *L. maculans* and had been misclassified as PG-1 and *L. biglobosa* based on pathogenicity.

Leptosphaeria maculans isolates (N=95) were characterized for avirulence alleles *AvrLm1*, *AvrLm2,3*, *AvrLm4-7* and *AvrLm6* based on phenotype interaction on differentials harboring *Rlm1*, *Rlm2* and *Rlm3* and PCR amplification of *AvrLm1*, *AvrLm4-7*, *AvrLm6* (Table 3.2). The frequency of *AvrLm1* (Fig. 3.1) was determined by the resistant phenotypic interaction on differential cultivar Quinta and by PCR. The frequencies of isolates that gave a resistant phenotype on Quinta (35%) and positive for the amplification of the *AvrLm1* (38%) were similar. Avirulence alleles *AvrLm2,3* determined by a resistant phenotype on Glacier were the lowest frequency found (9%) (Fig. 3.1). Avirulence alleles *AvrLm4-7* and *AvrLm6* were present in all isolates of the population (Fig. 3.1). The frequency of *AvrLm4-7* and *AvrLm6* was similar for years and geographic location (Fig. 3.2 and Fig. 3.3).

TABLE 3.2. Characterization of isolates of *Leptosphaeria maculans* for interaction phenotype with host differentials, pathogenicity groups (PG), avirulence (*Avr*) alleles and races.

			Interaction phenotype[b]				**Genotype *AvrLm*[d]**				
Isolate Name	**Year**	**County[a]**	**Westar**	**Glacier (*Rlm2,3*)**	**Quinta (*Rlm1*)**	**PG [c]**	**1**	**2,3**	**4-7**	**6**	**Race [e]**
CV-12-3	2012	Major	V	V	V	4	-	-	+	+	*Av6,4-7*
K-VT-12-4	2012	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
K-VT-12-5	2012	Kiowa	V	V	V	4	+	-	+	+	*Av1,6,4-7*
K-VT-12-6	2012	Kiowa	V	V	V	4	+	-	+	+	*Av1,6,4-7*
K-VT-12-7	2012	Kiowa	V	V	V	4	-	-	+	+	*Av6,4-7*
K-HC125-12-1	2011	Kiowa	V	V	V	4	-	-	+	+	*Av6,4-7*
K-HC125-12-2	2011	Kiowa	A	A	A	1	-	-	+	+	*Av6,4-7*
K-HC125-3	2011	Kiowa	V	V	V	4	-	-	+	+	*Av6,4-7*
Lahoma11	2011	Garfield	A	A	A	1	-	-	+	+	*Av6,4-7*
Lahoma19	2011	Garfield	V	V	V	4	-	-	+	+	*Av6,4-7*

BL-CC-2012-1	2012	Caddo	V	V	V	4	-	-	+	+	*Av6,4-7*
BL-CC-2012-2	2012	Caddo	V	V	V	4	-	-	+	+	*Av6,4-7*
BL-CC-2012-3	2012	Caddo	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
BL-CC-2012-4	2012	Caddo	V	V	V	4	-	-	+	+	*Av6,4-7*
BL-CC-2012-5	2012	Caddo	V	V	V	4	-	-	+	+	*Av6,4-7*
BL-CC-2012-7	2012	Caddo	A	A	A	1	+	-	+	+	*Av1,6,4-7*
BL2-2010-3	2010	Unknown	A	A	A	1	-	-	+	+	*Av6,4-7*
CS5	2009	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
C1C5	2009	Kiowa	V	V	V	4	+	-	+	+	*Av1,6,4-7*
C5A-2C8	2009	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
K-12-14	2012	Kiowa	V	V	V	4	+	-	+	+	*Av1,6,4-7*
C3	2009	Kiowa	V	V	V	4	-	-	+	+	*Av6,4-7*
CS3	2009	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
C5B-C2	2009	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
K-VT-12-8	2012	Kiowa	V	V	A	3	+	-	+	+	*Av1,6,4-7*
Lahoma 12-2	2011	Garfield	V	A	A	2	+	+	+	+	*Av1,2,3,6,4-7*
BL-2010#2	2010	Unknown	V	V	V	4	-	-	+	+	*Av6,4-7*
Lahoma 13-2	2011	Garfield	V	V	A	3	+	-	+	+	*Av1,6,4-7*
CS 1	2009	Kiowa	A	A	A	1	-	-	+	+	*Av6,4-7*
STW-2012-1	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
STW-2012-2	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
STW-2012-3	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
OK-1-2013 1	2013	Cotton	V	V	A	3	+	-	+	+	*Av1,6,4-7*
OK-1-2013 2	2013	Cotton	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F1-2013-6	2013	Cotton	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
F1-2013-4	2013	Cotton	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F1-2013-7	2013	Cotton	A	A	A	1	+	-	+	+	*Av1,6,4-7*
F2-2013-6	2013	Canadian	A	A	A	1	-	-	+	+	*Av6,4-7*
F2-2013-13	2013	Canadian	A	A	A	1	-	-	+	+	*Av6,4-7*
F2-2013-10	2013	Canadian	V	V	V	4	-	-	+	+	*Av6,4-7*
F3-2013-1	2013	Blaine	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F3-2013-2	2013	Blaine	V	V	V	4	-	-	+	+	*Av6,4-7*
F3-2013-3	2013	Blaine	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F5-2013-4	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F5-2013-6	2013	Grant	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
F5-2013-9	2013	Grant	V	V	V	4	+	-	+	+	*Av1,6,4-7*
F6-2013-9	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F6-2013-7	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F7-2013-4	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F7-2013-6	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F8-2013-2	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F8-2013-3	2013	Grant	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
F8-2013-4	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*

F9-2013-4	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F10-2013-1	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F10-2013-2	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F10-2013-5	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F11-2013-6	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F12-2013-9	2013	Garfield	V	V	V	4	+	-	+	+	*Av1,6,4-7*
F13-2013-2	2013	Garfield	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F13-2013-4	2013	Garfield	V	V	V	4	-	-	+	+	*Av6,4-7*
F6-2013-6	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F11-2013-4	2013	Grant	V	V	V	4	-	-	+	+	*Av6,4-7*
F11-2013-5	2013	Grant	V	V	A	3	-	-	+	+	*Av6,4-7*
F14-2013-2	2013	Noble	V	V	V	4	-	-	+	+	*Av6,4-7*
F14-2013-3	2013	Noble	V	V	V	4	-	-	+	+	*Av6,4-7*
F15-2013-4	2013	Noble	V	V	V	4	-	-	+	+	*Av6,4-7*
F15-2013-5	2013	Noble	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
F9-2013-6	2013	Grant	V	A	A	2	+	+	+	+	*Av1,2,3,6,4-7*
F9-2013-7	2013	Grant	V	V	A	3	+	-	+	+	*Av1,6,4-7*
F15-2013-1	2013	Noble	A	A	A	1	-	-	+	+	*Av6,4-7*
F14-2013-1	2013	Noble	V	V	V	4	-	-	+	+	*Av6,4-7*
F12-2013-6	2013	Garfield	V	V	V	4	-	-	+	+	*Av6,4-7*
F6-2013-5	2013	Grant	V	A	A	2	+	+	+	+	*Av1,2,3,6,4-7*
F6-2013-1	2013	Grant	V	A	A	2	-	+	+	+	*Av2,3,6,4-7*
F13-2013-1	2013	Garfield	V	V	A	3	+	-	+	+	*Av1,6,4-7*
STW2013F1-1	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
STW2013F2-1	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
STW2013F3-1	2013	Payne	V	V	V	4	+	-	+	+	*Av1,6,4-7*
STW2013F4-1	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
STW2013F5-1	2013	Payne	V	V	V	4	+	-	+	+	*Av1,6,4-7*
STW2013F6-1	2013	Payne	V	V	V	4	-	-	+	+	*Av6,4-7*
ER-2013F-1-1	2013	Canadian	V	V	V	4	-	-	+	+	*Av6,4-7*
ER-2013F-2-1	2013	Canadian	V	V	V	4	-	-	+	+	*Av6,4-7*
ER-2013F-5-1	2013	Canadian	V	V	V	4	-	-	+	+	*Av6,4-7*
ER-2013F-6-1	2013	Canadian	V	V	A	3	+	-	+	+	*Av1,6,4-7*
ER-2013F-7-1	2013	Canadian	V	V	A	3	+	-	+	+	*Av1,6,4-7*
CV-2013F-1-1	2013	Major	V	V	V	4	-	-	+	+	*Av6,4-7*
CV-2013F-2-1	2013	Major	V	V	V	4	-	-	+	+	*Av6,4-7*
CV-2013F-3-1	2013	Major	V	V	V	4	-	-	+	+	*Av6,4-7*
CV-2013-4-1	2013	Major	V	V	V	4	-	-	+	+	*Av6,4-7*
CV-2013F-6-1	2013	Major	V	V	V	4	+	-	+	+	*Av1,6,4-7*
CV-2013F-7-1	2013	Major	V	V	A	3	-	-	+	+	*Av6,4-7*
C1	2009	Kiowa	A	A	A	1	-	-	+	+	*Av6,4-7*
CS6	2009	Kiowa	V	V	V	4	-	-	+	+	*Av6,4-7*

[a] Kiowa county in Kansas and counties in Oklahoma where isolate was collected.
[b] Differential cultivars were Westar (no *Rlm* genes), Glacier (*Rlm2,3*), Quinta (*Rlm1*). V= virulence, resistant interaction between isolate and cultivar, A = avirulence, susceptible interaction between the isolate and the differential.
[c] PG = pathogenicity groups. PG-4 isolates virulent on Westar, Glacier and Quinta; PG-3 avirulent on Quinta; PG-2 avirulent on Glacier and Quinta; PG-1 avirulent in all cutivars.
[d] Genotype *AvrLm*: + = presence of the avirulence allele (*Avr)*, - = presence of the virulence allele (*avr*). *AvrLm1* was determined by pathogenicity tests on Quinta (*Rlm1*) and by PCR. *AvrLm2-3* was determined by pathogenicity tests on Glacier (*Rlm2,3). AvrLm4-7* and *AvrLm6* were determined by PCR
[e] Race nomenclature indicates the avirulence *Avr* alleles present in the isolate.

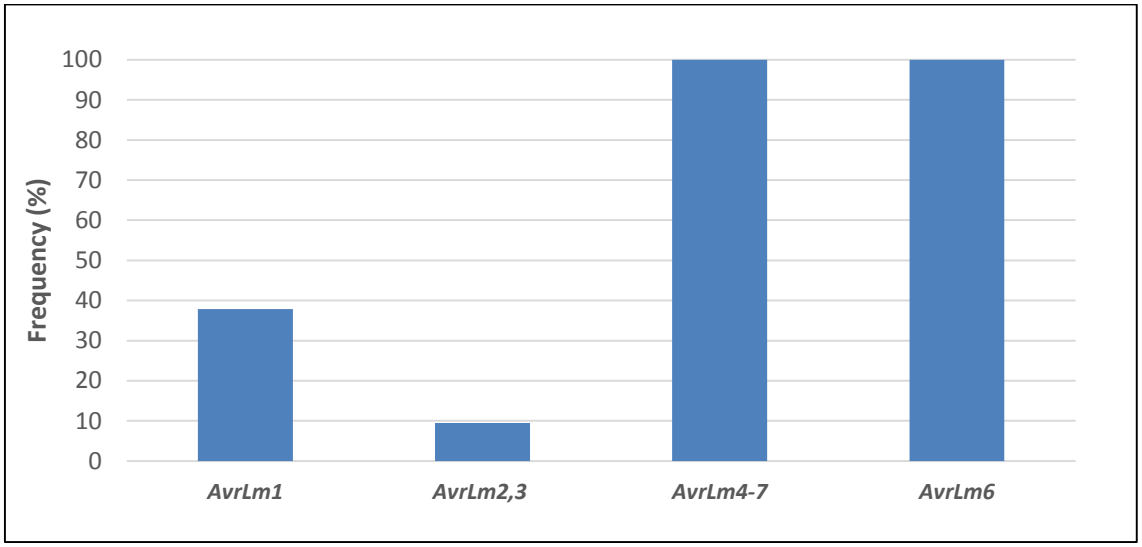

Fig. 3.1. Frequency of avirulence alleles in the *L. maculans* population from Oklahoma and Kansas (N=95). *AvrLm1* was determined by pathogenicity tests on Quinta (*Rlm1*) and by PCR. *AvrLm2-3* was determined by pathogenicity tests on Glacier (*Rlm2,3). AvrLm4-7* and *AvrLm6* were determined by PCR.

Depending on the *Avr* locus and the county of origin of the isolate, the frequency of *Avr* ranged from 0 to 1. The frequency of loci *AvrLm4-7* and *Avrlm6* was the same (100%) for every county. In contrast, site-to-site variation was observed in *AvrLm1* (0-80%) and *AvrLm2,3* (0-23%) (Table 3.3). Significant differences were observed between counties for loci *AvrLm1* and *AvrLm2,3* according to the Fisher's exact test (Table 3.3).

TABLE 3.3 Number of races and frequencies of avirulence (*Avr*) alleles in the population of *Leptosphaeria maculans* by county of isolate origin.

	Number of		Frequency of Avr alleles			
Origin	**Isolates**	**Races**	**AvrLm1**	**AvrLm2,3**	**AvrLm4-7**	**AvrLm6**
Blaine, OK	3	2	0.67	0.00	1	1
Caddo, OK	6	3	0.17	0.17	1	1
Canadian, OK	8	2	0.25	0.00	1	1
Cotton, OK	5	2	0.80	0.20	1	1
Garfield, OK	9	3	0.56	0.11	1	1
Grant, OK	22	4	0.36	0.23	1	1
Kiowa, KS	18	2	0.56	0.00	1	1
Major, OK	7	2	0.14	0.00	1	1
Noble, OK	6	2	0.000	0.17	1	1
Payne, OK	9	2	0.22	0.00	1	1
Total	93	4	0.38	0.10	1	1
p-values [a]			0.04	<0.01	0.5	0.5

[a] Probability that *AvrLm* allele frequency differed according to the Fisher's exact test.

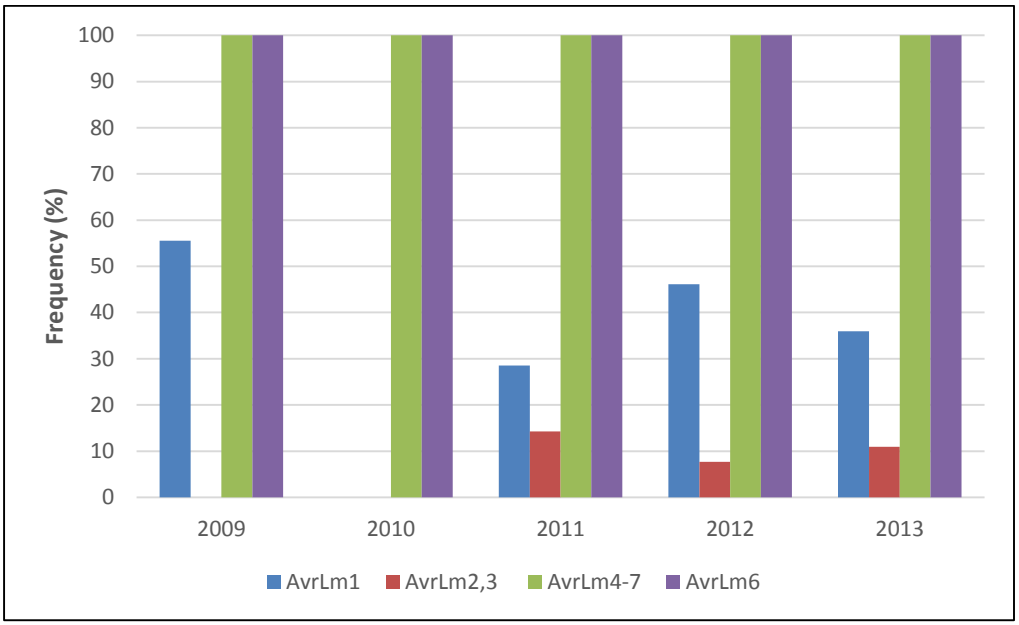

Fig. 3.2. Avirulence (*Avr*) allele frequency by year from 2009 to 2013.

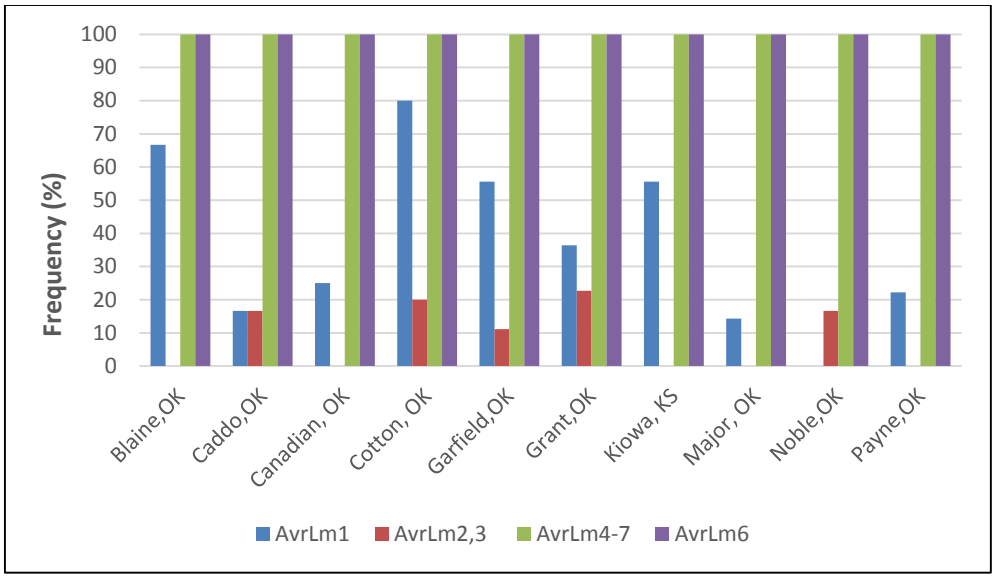

Fig. 3.3. Avirulence (*Avr*) allele frequency by county. Isolates were collected from nine counties in Oklahoma and one in Kansas.

Race structure of *L. maculans*

The race structure of the *L. maculans* population (N=95) was determined based on the avirulence alleles identified from the phenotypic interactions on differential cultivars harboring *Rlm1*, *Rlm2* and *Rlm3* and the amplification of *AvrLm1*, *AvrLm4-7*, *AvrLm6* (Table 3.2). There were four single or combined avirulence alleles that could be identified in the collection *AvrLm1, AvrLm2,3, AvrLm4-7,* and *AvrLm6.* In theory, a total of 16 different races (2^4 combinations) could exist in the population. The combination of *Avr* alleles identified in each isolate resulted in the presence of four of the 16 possible races, which included *Av 1,2,3,6,4-7; Av 1,6,4-7; Av 2,3,6,4-7;* and *Av 6,4-7* (Fig. 3.4). Race *Av 6,4-7* was the most prevalent race occurring at a frequency of 56%, followed by *Av 1,6,4-7* at 35%. The least frequent were *Av 2,3,6,4-7* at 6% and race *Av 1,2,3,6,4-7,* which has all of the avirulence genes assessed in this research, at only 3% of isolates. These four races also contained a combination of 0 to 3 of the virulence (*avrLm)* alleles in the population.

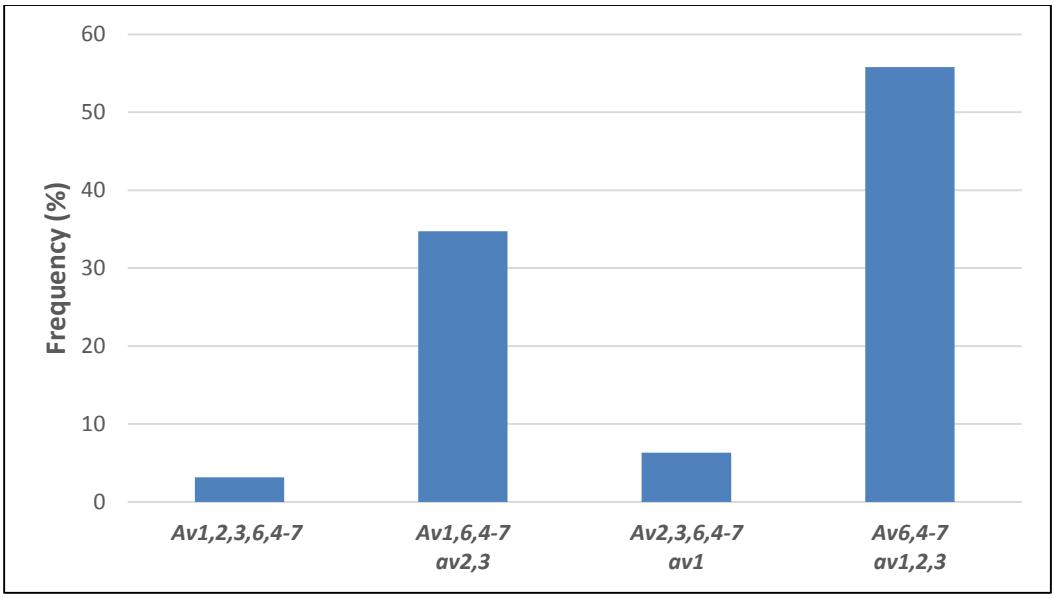

Fig. 3.4. Races of *Leptosphaeria maculans* identified in Oklahoma.

DISCUSSION

Understanding the diversity of avirulence alleles and the race structure of *L. maculans* in Oklahoma is necessary to develop both integrated and durable management strategies to control black leg disease by selecting specific resistance genes for deployment. To date, studies describing the avirulence patterns and race structure of the pathogen population have been done in Europe, North America, and Australia (Balesdent et al. 2006; Balesdent et al. 2005; Dilmaghani et al. 2009; Mengistu et al. 1991; Rouxel et al. 2003) using different sets of differential cultivars and systems. The present study is the first to determine the avirulence alleles and race structure of a collection of *L. maculans* isolates obtained from production areas of the southern Great Plains.

Both species of the *Leptosphaeria* complex, *L. maculans* and *L. biglobosa*, were identified in the local population. According to the old pathogenicity group system, results of isolate classification showed that the highly virulent isolates of *L. maculans* (PG-2, PG-3 and PG-4); and the weakly virulent isolates (PG-1), now recognized as *L. biglobosa* (Shoemaker and Brun

2001) were present in canola fields in Oklahoma and southern Kansas. There was no relationship observed between PG and geographic location (data not shown), which may be due to the close proximity between sampled sites (Dilmaghani et al. 2009). Amplification of the mating type locus suggested sexual recombination is possible since both mating types (*MAT1-1* and *MAT1-2*) were identified at similar frequencies in the population (Cozijnsen and Howlett 2003). These results support the observations of ascospore production on stubble left on the soil surface following canola production.

Identification of the *Avr* alleles in the local *L. maculans* population provided relevant information about the frequency of *Avr* alleles in the state and the *Rlm* genes that would be the most effective in managing black leg in the region. The frequency of the avirulence alleles in the population was determined based on the phenotype interaction on a set of *B. napus* differentials Westar, Glacier and Quinta and on the amplification of *AvrLm1*, *AvrLm4-7* and *AvrLm6* alleles. However, inconsistencies were observed for some isolates when classified by pathogenicity or PCR amplification, mostly related to the interaction of *Rlm1* and *AvrLm1*. Some isolates showed avirulence on Quinta but were PCR negative for *AvrLm1*; others where virulent on Quinta and PCR positive for *AvrLm1*. Therefore, the accuracy of the resulting phenotype interactions between differential Quinta and local isolates was not always supported by the amplification of the corresponding *AvrLm1* avirulence gene.

The avirulence allele *AvrLm1* was found in 38% (N=36) of the local population of *L. maculans* when determined by PCR. However, 17 of the 36 isolates showed discrepancies when classified by pathogenicity to Quinta and PCR amplification of *AvrLm1*. Two isolates classified as PG-3 and six isolates classified as PG-2 were avirulent on Quinta, but were PCR negative for the *AvrLm1* allele. Nine isolates classified as PG-4 and virulent on Quinta, were positive for *AvrLm1* by PCR. These inconsistencies related to *Rlm1* and *AvrLm1* may have resulted from differential cultivar Quinta being a heterogeneous genotype. Others have reported that Quinta

harbors *Rlm1* (Ansan-Melayah et al. 1995) and, depending on the seed lot origin, it is probable that Quinta also carries *Rlm3* (Kutcher et al. 2010a), or *Rlm4* as linked resistance genes (Balesdent et al. 2001). It is also possible that some of the isolates avirulent on Quinta and classified as PG-2 and PG-3 were actually *avrLm1* virulent and *AvrLm3* or *AvrLm4* avirulent. Thus, Quinta is probably not a good differential cultivar used for characterization of *L. maculans* isolates. Instead, other available differential cultivars that harbor *Rlm1* in combination with another *Rlm* gene, like cvs. Columbus (*Rlm1, Rlm3*) (Balesdent et al. 2002), or Cooper *(Rlm1, Rlm4*) (Dilmaghani et al. 2009) should be used. However, these differentials were not available for this study. Also, cases where resistant phenotypes were observed on Quinta in the absence of amplification of *AvrLm1,* could be due to the presence of a different *Rlm* not assessed in this study or the presence of a novel *Rlm* not discovered or described yet (Dilmaghani et al. 2009). Another possibility could be that the *L. maculans* population in Oklahoma may have a non-functional *AvrLm1* (PCR positive but virulent), or DNA sequence variation at the primer annealing sites (PCR negative but avirulent).

Both avirulence genes *AvrLm2 and AvrLm3,* identified only by phenotypic interaction on cultivar Glacier, were found at the lowest frequency (9%). This result indicates that resistance genes *Rlm2* and *Rlm3*, separate or combined, are not expected to be completely effective in this region. Similar to the cultivar Quinta, Glacier has more than one genotype, it contains simultaneously resistance genes *Rlm2* and *Rlm3* (Dilmaghani et al. 2009). If this cultivar were extensively grown in the region its resistance would be expected to be overcome quickly by the virulence alleles *avrLm2* and *avrLm3* present in the population due to selective advantage in favor of pathogen genotypes carrying these alleles.

In contrast, avirulence alleles *AvrLm4-7* and *AvrLm6,* identified by PCR, represented the highest frequency (100%) in all the sampled counties. Since *AvrLm4-7* and *AvrLm6* are fixed in the population, cultivars with the corresponding resistance gene *Rlm4-7* or *Rlm6* should be

broadly effective if deployed in Oklahoma and surrounding states at this time. Resistance gene *Rlm6* has been introgressed into *B. napus* from *B. juncea* (Chèvre et al. 1997). Therefore, the fact that all isolates from the collection carry avirulence allele *AvrLm6* and are avirulent to *Rlm6* is expected as this resistance gene had been mostly used in experimental research (Chèvre et al. 1997; Kutcher et al. 2010b) To date, it is only present in a few canola cultivars in Europe designated by a "MX" trademark. Therefore, it is unlikely that the local population of *L. maculans* in Oklahoma had been exposed to *Rlm6.* Similarly, *AvrLm7* is expected to be present in a high frequency because *Rlm7* has been widely used in European germplasm (Mathew Clarke, personal communication), but is not common in North American cultivars. Conversely, avirulence gene *AvrLm4* is expected to be found, at a lesser extent in Oklahoma because resistance gene *Rlm4* has been present in the southern Great Plains since winter canola was first grown in the region 15 years ago. *Rlm4* was incorporated from the French *B. napus* cultivar Jet Neuf (*Rlm4*) into the U. S. winter canola germplasm, and used as a parental line to develop new cultivars and breeding lines that are now commonly grown in the southern Great Plains (Rife and Shroyer 2000; Stamm et al. 2012). Unfortunately, at this point of the study, there are no results on the independent frequencies of *AvrLm4* and *AvrLm7.*

It would be ideal to work with an available set of differential cultivars where each cultivar possesses only one specific resistance *Rlm* gene, and if not possible, work with an extended number of differentials with complementary combinations of *Rlm* genes to assess all the possible *Avr* alleles that exist in the population (Balesdent et al. 2005). In this case, it would be convenient if cultivars harboring only one resistance gene *Rlm2, Rlm3, Rlm4* or *Rlm7* were available in order to discriminate the presence of *AvrLm2, AvrLm3, AvrLm4* or *AvrLm7* in the local collection of *L. maculans* isolates. The *B. napus* cultivar Jet Neuf (*Rlm4*) (Balesdent et al. 2001) and *B. juncea* cultivar Aurea (*Rlm5,6*) (Balesdent et al. 2002) were recently added to the set of differentials used to screen the isolates from the present study. Consequently, more

information could be obtained about the frequency of *AvrLm1, AvrLm2,3, AvrLm4, AvrLm5, AvrLm6* and *AvrLm7* and theoretically 64 races could be differentiated.

Until this study, there was little known about the *L. maculans* race structure in Oklahoma or the southern Great Plains region. Surveys to determine the race structure of *L. maculans* in the United States have been reported from Georgia in the southeastern U.S. and North Dakota in the northern part of the country (Chen and Fernando 2006; Dilmaghani et al. 2009). This study, although lacking a set of differential cultivars with specific resistance genes for every *Avr* locus, provides the first overview of the *L. maculans* race structure in Oklahoma and Kansas. The avirulence alleles for four single or grouped *Avr* genes (*AvrLm1; AvrLm2 and AvrLm3; AvrLm4 and/or AvrLm7; AvrLm6*) were confirmed in Oklahoma *L. maculans* population. Although potentially 16 races (2^4) could be present, the combination of *Avr* alleles identified in each isolate revealed the presence of only four races in the local pathogen population. Furthermore only two major races, *Av6,4-7* and *Av1,6,4-7,* predominated. All local isolates had the avirulence alleles *AvrLm6* and *AvrLm4-7.* The virulence alleles a*vrLm1, avrLm2, avrLm3* were also common in the population. *L. maculans* race structure in Oklahoma might actually be more diverse than expected. Race diversity could be better assessed if *AvrLm2, AvrLm3, AvrLm4,* and *AvrLm7* could be analyzed individually, and if additional *Rlm* and *AvrLm* genes were added to the analysis. It should be noted that there were large differences in the number of isolates collected from each county and from each year. Extended sample sizes might provide more robust information about *Avr* allele variation over counties and years.

The avirulence allele and race structure of the *L. maculans* population in Oklahoma, showed features similar to those reported by Dilmanghani et al. (2009) in other parts of the world. Isolates from Chile and Georgia in the U.S showed lack of avirulence allelles *AvrLm2, AvrLm3, and AvrLm9;* and a prevalence of avirulence alleles *AvrLm6* and *AvrLm7*. Isolates from Europe also lacked *AvrLm2 and AvrLm9; AvrLm3, AvrLm1 and AvrLm4* to a lesser extent, while *AvrLm6*

and *AvrLm7* were prevalent (Balesdent et al. 2006). Similarly, the Oklahoma and Kansas population had a low frequency of avirulence alleles *AvrLm2* and *AvrLm3,* and high prevalence of *AvrLm6* and most likely *AvrLm7.* Frequencies of the individual alleles *AvrLm4* and *AvLm7* are still being studied. Isolates from Oklahoma and Kansas were not characterized for *AvrLm9* in this study. The population from Oklahoma and Kansas differed from those in Australia and Canada, where only frequencies of the avirulence alleles *AvrLm6* and *AvrLm7* were similar among populations. Avirulence alleles *AvrLm3* and *AvrLm7* were common in isolates from western Australia. Ontario's predominant avirulence alleles were *AvrLm1* and *AvrLm6.* By contrast, isolates from central Canada and North Dakota were predominantly PG-2 harboring *AvrLm2* and/or *AvrLm3* and *AvrLm1* and/or *AvrLm4* (Chen and Fernando 2006). Likewise, the most prevalent *Avr* alleles in Mexico were *AvrLm1* and *AvrLm2* (Moreno-Rico et al. 2001). PG-2 isolates were not common in Oklahoma.

As in other studies, results from this research showed that more than one race can be classified as the same pathogenicity group when evaluating the phenotype interaction between *L. maculans* and *B. napus* (Balesdent et al. 2005). PG-4, PG-3 and PG-2 included two races each. PG-1 which should not include any race because it is considered *L. biglobosa,* also contained two different races. The races included in PG-1 can be explained by the 10 *L. maculans* isolates that were misclassified as PG-1 group for being avirulent on Westar. Poor sporulation of these ten isolates may had led to inconsistent results from the inoculations on differentials (Balesdent et al. 2005). This demonstrates the need to classify the isolates based on the *Avr* allele combinations for each isolate instead the arbitrary system of pathogenicity groups. Thus, based on the race system, information is provided about the characterized avirulence *Avr* genes, the *Avr* genes not characterized, and the avirulence and the virulence alleles present in the isolates. Classification of isolates by the *Avr* allele pattern, rather than by a low or high virulent pathogenicity status, is more informative and specific for deployment of resistance genes.

The results presented in this study were limited by a set of differential cultivars that were insufficient to identify all the possible *Avr* alleles present in the *L. maculans* local population. Nevertheless, this study provides significant knowledge of the avirulence allele variation and race structure of *L. maculans* population in Oklahoma. There is now need to provide a more extensive overview of race structure population using additional informative differential cultivars to cover all the possible *Avr* alleles in the population or to develop PCR primers to amplify more *Avr* alleles. Knowledge of avirulence gene frequency and race structure of *L. maculans* across Oklahoma and in other canola growing regions of the U.S. will allow pathologists, breeders, growers and the canola industry to develop efficient strategies to manage and maintain specific resistance.

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CHAPTER IV

SCREENING WINTER CANOLA FOR MAJOR-GENE RESISTANCE TO BLACK LEG

ABSTRACT

Black leg disease, caused by the fungus *Leptosphaeria maculans*, is a common disease of winter canola (*Brassica napus*) in Oklahoma. Major resistance genes (*Rlm*) are expressed in seedlings and interact with avirulence genes in *L. maculans* (*AvrLm*). The presence of avirulence alleles and the race structure of the *L. maculans* population were determined by inoculating differential cultivars harboring *Rlm1* and *Rlm2,3* and PCR amplification of avirulence alleles *AvrLm1*, *AvrLm4-7*, *AvrLm6*. Avirulence alleles *AvrLm6* (100%) and *AvrLm4-7* (100%) were prevalent in the local population (N=95), whereas *AvrLm1* and *AvrLm2* and/or *AvrLm3* presence was only 38% and 9%, respectively. Four races (*Av1,2,3,6,4-7; Av1,6,4-7; Av2,3,6,4-7; Av6,4-7*) were identified in the population. Races *Av 6,4-7* (56%) and *Av 1,6,4-7* (35%) were predominant in Oklahoma and Kansas and were further characterized for *AvrLm4, AvrLm5* and *AvrLm6* based on phenotype interaction on differential cultivars harboring *Rlm4* and *Rlm5,6*. This characterization resulted in a reclassification into three races *Av1,6,7,(5); Av1,4,6,(5,7)* and *Av6,7,(5)* which were used to screen 53 winter canola cultivars, hybrids, and breeding lines for seedling resistance. Most (62%) cultivars and breeding lines were susceptible to all three races and lacked specific resistance genes. Several (23%) conventional (non-glyphosate tolerant) cultivars and hybrids were heterogeneous in resistance to one or more races. Glyphosate tolerant (Roundup-Ready) entries currently grown in the region generally lacked major resistance genes, except for DKW46-15 which appeared to have heterogeneous resistance from *Rlm4* and possibly

Rlm7. The conventional hybrids Dimension, Safran, Visby, DK Sensei, and the rapeseed cultivar Rossini (9%), were resistant to all races possibly due to the presence of resistance gene *Rlm6* and/or *Rlm7*. Unknown resistance was found in 6% of the entries, which suggested the presence of other resistance genes not assessed in this study. There is a need to grow cultivars or hybrids with effective major gene resistance in Oklahoma and surrounding states.

INTRODUCTION

Canola (*Brassica napus* L.) is a broadleaf crop relatively new in the United States, yet its production has steadily increased over the past three decades. Winter canola cultivars adapted to the southern Great Plains have been developed within the past fifteen years and the crop has been commercially grown in Oklahoma for about ten years (Boyles and Sanders, 2009). Oklahoma is the second leading canola-producing state in the U.S. after North Dakota (USDA NASS 2014). Black leg disease, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. & DeNot, is an economically important disease of oilseed and canola in most regions of the world where the crop is grown (Rouxel and Balesdent 2005). Symptoms of black leg are leaf spots and stem cankers. The stem canker phase of the disease can severely affect the plant by girdling the stem and killing the plant. Black leg has the potential to severely damage canola causing a reduction in yield up to 50% in fields planted with susceptible cultivars (Damicone et al. 2012).

Major gene resistance, also known as seedling resistance because it is expressed at the seedling stage of the plant, has been widely used to control black leg (Delourme et al. 2006). Major gene resistance consists of a gene-for-gene interaction between *B. napus* and *L. maculans*, where the resistant phenotype depends on the presence of a dominant major gene for resistance (*Rlm*) in the plant and a dominant corresponding avirulence (*Avr*) gene in the pathogen (Balesdent et al. 2005; Marcroft et al. 2012a). Conversely, the lack of a dominant resistance gene in the plant

or a recessive gene for virulence (*avr*) in the pathogen results in a susceptible reaction and the expression of disease.

To date, at least 17 major resistance genes have been identified (*Rlm1* to *Rlm11, LepR1* to *LepR4, BLMR1,* and *BLMR2*) in *Brassica* species conferring resistance to *Leptosphaeria maculans* (Marcroft et al. 2012a; Van de Wouw et al. 2014b). *LepR3*, is the only *Rlm* gene that has been cloned (Larkan et al. 2013). Most of these genes have been positioned on *B. napus* linkage maps and have shown to be organized in clusters (Delourme et al. 2006; Delourme et al. 2004). Of the corresponding avirulence genes in *L. maculans* conferring host specificity, (*AvrLm1, AvrLm4-7, AvrLm6, AvrLm11* and *AvrLmJ1*) have been cloned and sequenced (Fudal et al. 2007; Gout et al. 2006; Parlange et al. 2009; Balesdent et al. 2013; Van de Wouw et al. 2014a). Balesdent et al. (2002), reported the clustering of several *Avr* genes into two main clusters, "*AvrLm1 - AvrLm2 - AvrLm6*" and "*AvrLm3 - AvrLm4 - AvrLm7*", which have been shown to be genetically linked at specific loci.

Breeding programs and crop management practices can improve durability of genetic resistance. Rotation of cultivars harboring different major resistance genes has been shown to improve the durability of seedling resistance (Marcroft et al. 2012b). In order to implement this strategy, knowledge of the resistance genotype in sown cultivars is required. However very few cultivars have been characterized for major resistance genes. Rouxel et al. (2003) genotyped Australian cultivars for resistance genes *Rlm1, Rlm2* and *Rlm4* based on phenotype interaction with *L. maculans* isolates with known *Avr* genotypes. *B. napus* accessions from Canada and France also have been screened for major genes resistance (Rouxel et al. 2003). Similarly, major resistance genes against black leg were characterized in Canadian canola germplasm by Zhang et al. (2013). Studies that have characterized major resistance genes have indicated that cultivars that were thought to have one resistance gene actually have more resistance genes. For example, the 'sylvestris resistance' in cultivar 'Surpass400' was initially thought to be from resistance gene

LepR3 (Li and Cowling 2003) and later it was found to also involve *Rlm1* and *RlmS* (Van de Wouw et al. 2009). Unfortunately, there is no information about the major resistance genes contained in winter canola cultivars commonly sown in Oklahoma and surrounding states, or in breeding lines used in the development of winter canola cultivars.

Major gene resistance has been assessed on seedling cotyledons in growth chamber pathogenicity experiments (Ansan-Melayah et al. 1998), because this type of resistance is effective at site of infection on leaves of young plants (Kutcher et al. 2010). The cotyledon test has become the accepted method for determining gene-for-gene interaction (Williams et al. 1979) between *Brassica* spp. and *L. maculans* and has been used in numerous studies to screen for corresponding resistance genes or novel resistance genes (Rimmer and Van den Berg 1992; Williams et al. 1979). However due to the difficulty of obtaining isolates harboring a single avirulence allele or at least a few well-defined avirulence alleles, these studies usually used isolates with more than one *Avr* allele which makes the identification of the corresponding resistance genes difficult (Rouxel et al. 2003). Most of the field isolates characterized harbor numerous *Avr* alleles (Balesdent et al. 2002).

The analyses of the *L. maculans* race structure in Oklahoma (Chapter 3) enabled us to identify four races (*Av1,2,3,6,4-7; Av1,6,4-7; Av2,3,6,4-7; Av6,4-7*) in the population. Races *Av6,4-7* (56%) and *Av1,6,4-7* (35%) were predominant in the population. The objective of this study was to use predominant races of *L. maculans* to identify or infer the presence of resistance genes present in winter canola cultivars grown in the region. *L. maculans* isolates representing the two common and broadly virulent races in Oklahoma were used to discriminate seedling resistance genes *Rlm1, Rlm4, Rlm6* and *Rlm7* in winter *B. napus* commercial cultivars, hybrids and breeding lines.

MATERIALS AND METHODS

Brassica napus germplasm

The *B. napus* cultivar Westar, which is the susceptible control; the *B. napus* differential cultivars Glacier, Quinta, Jet Neuf; and the *B. juncea* cultivar Aurea were used to characterize *L. maculans* isolates from Oklahoma (Table 4.1). These were obtained from the USDA Germplasm Resources Information Network (GRIN) and increased in the greenhouse and/or field. In addition, a total of 53 entries were analyzed for major resistance genes against black leg. The *B. napus* germplasm consisted of glyphosate-tolerant (Roundup Ready) and conventional (non-glyphosate tolerant) cultivars and hybrids. Glyphosate-tolerant cultivars and hybrids are grown on over 90% of the acreage in the southern Great Plains. The entries were part of the National Winter Canola Variety Trial (NWCVT) which is a regional and national testing system that evaluates the performance of experimental and commercial cultivars (Stamm and Dooley 2013), and were obtained from M. Stamm at Kansas State University.

Characterization of the avirulence genotype of *L. maculans* isolates

Races were characterized based on the combination of *Avr* alleles expressed in each isolate from the phenotype interaction on differential cutivars harboring the resistance genes *Rlm1*, *Rlm2* and *Rlm3* (Table 4.1) and amplification of avirulence alleles *AvrLm1*, *AvrLm4-7*, and *AvrLm6* by polymerase chain reaction (See Chapter 3). Three isolate representatives of the two common and broadly virulent races, *AvrLm6,4-7*, which was virulent on Westar, Glacier and Quinta, and *AvrLm1,6,4-7* virulent on Westar and Glacier, were selected for further characterization of the avirulence alleles *AvrLm4* and *AvrLm5* using the newly acquired differential *B. napus* cv. Jet Neuf (*Rlm4*) and *B. juncea* cv. Aurea (*Rlm5, Rlm6*).

Cotyledons of 7-day old seedlings were wounded (four wounds per plant) and inoculated with conidial suspension (10^6 conidia per droplet) of each isolate (Marcroft et al. 2012a;

Mengistu et al. 1991). Eight plants were inoculated with each isolate-cultivar combination. After inoculation, seedlings were kept under high humidity and darkness in a dew chamber at 25^0C for two days and then returned to a growth chamber where conditions were 24^0C, RH 80% and continuous light. Phenotype interactions (disease severity) were scored 10 days after inoculation using the IMASCORE rating scale described by Volke (1999) and used by Balesdent et al. (2001) which consisted of 6 infection classes (IC). Infection classes IC1 to IC3 correspond to resistant reactions, where IC1 is the hypersensitive response, IC2 is a larger (1.5 to 3mm) dark necrotic lesion, and IC3 is a non-sporulating lesion that is sharply delimited by a dark necrotic margin. IC4 to IC6 correspond to susceptible reactions and are characterized by spreading, gray-green lesions with no dark margin. IC4 has no sporulation, IC5 has a few pycnidia, and IC6 has abundant sporulation. Mean IC values were determined from the 32 inoculation sites and values below 4 were considered resistant reactions and, values equal or above 4 were considered susceptible (Marcroft et al. 2012a). Experiments with each isolate and cultivar combination were repeated at least twice.

Race terminology as proposed by Balesdent et al. (2005) was used to describe the *Avr* allele composition of each isolate by listing all the *Avr* loci for which an isolate was avirulent, preceded by the letters 'Av'. The Avr loci for which the isolates were not characterized due to the unavailability of differentials with single resistance genes were indicated in parenthesis (Balesdent et al. 2005). The races with known avirulence genotypes were then used to screen canola cultivars, hybrids, and breeding lines for seedling resistance.

TABLE 4.1. Cultivars with specific resistance *(Rlm)* genes used to characterize *Leptosphaeria maculans*

Plant genotype	**Resistance gene**	**Reference**
Westar	None	Balesdent et al. 2002
Glacier	*Rlm2, Rlm3*	Balesdent et al. 2002
Quinta	*Rlm1,* (*Rlm3 or Rlm4)*	Balesdent et al. 2001, 2002
Jet Neuf	*Rlm4*	Balesdent et al. 2001
Aurea*	*Rlm5, Rlm6*	Balesdent et al. 2002

* *B. juncea* cultivar.

Characterization of major resistance genes in *Brassica napus*

Brassica napus cultivars, hybrids, and breeding lines were characterized for major resistance genes by inoculating them with isolates with known avirulence genotypes. Based on the phenotype interaction of each race - cultivar combination, the absence or presence of the corresponding *Rlm* genes was inferred (Rouxel et al. 2003). Phenotype interactions were scored ten days after inoculation, using the IMASCORE rating scale describe above. Each race was inoculated onto eight plants and four cotyledons per plant, and experiments were repeated at least twice. A resistant phenotype interaction implied the presence of the corresponding resistance gene in the plant, whereas a susceptible reaction implied the absence of an *Rlm* gene in the plant.

A major resistance gene was considered present if more than 80% of the plants of a particular entry exhibited a resistant reaction towards the isolate harboring the corresponding avirulence allele. A major resistance gene was considered absent if more than 80% of the plants of a specific cultivar exhibited a susceptible reaction when inoculated with an isolate with a particular avirulence gene (Marcroft et al. 2012a). The race and cultivar interaction was heterogeneous when 20% to 80% of plants showed resistance to a particular avirulence allele (Rouxel et al. 2003). The susceptible cultivar Westar, which has no *Rlm* genes, was used as positive control for each cultivar and isolate combination.

RESULTS

The addition of the winter *Brassica napus* cultivar Jet Neuf (*Rlm4*) and the *Brassica juncea* cultivar Aurea (*Rlm5,6*) allowed further characterization of three isolates from the two common races, *Av1,6,4-7* and *Av6,4-7*. Based on phenotype interaction on differential cultivars harboring specific resistance genes *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5 and Rlm6* and PCR amplification of *AvrLm1, AvrLm4-7, and AvrLm6* alleles, there were three races *Av1,6,7,(5), Av1,4,6,(5,7)* and *Av6,7,(5)* represented in the three isolates (Table 4.2). The reclassification of these races was based on differences in the virulence alleles *avrLm1* and *avrLm4*.

Table 4.2. Avirulence genotypes of *Leptosphaeria maculans* isolates based on response phenotype of inoculated *Brassica* cultivars and amplification of *Avr* genes.

	Differential phenotype interaction [a]					**PCR** [b]			
Isolate	**Westar** No ***Rlm***	**Glacier** ***Rlm2,3***	**Quinta** ***Rm1***	**Jet Neuf** ***Rlm4***	**Aurea** ***Rlm5,6***	***AvrLm1***	***AvrLm4-7***	***AvrLm6***	**Avr genotype**[c]
102	V	V	A	V	A	+	+	+	*Av 1,6,7,(5)*
165	V	V	V	A	A	+	+	+	*Av 1,4,6,(5,7)*
124	V	V	V	V	A	-	+	+	*Av 6,7,(5)*

[a] Phenotype interaction on differentials, Westar (no *Rlm* genes), Glacier (*Rlm2,3*), Quinta (*Rlm1, Rlm3 or Rlm4*); Jet Neuf (*Rlm4*), Aurea (*Rlm5,6*). A= avirulence, resistant interaction between isolate and cultivar, V = virulence, susceptible interaction between the isolate and the differential.

[b] PCR + = presence of the avirulence (*Avr*) allele, - = presence of the virulence (*avr*) allele.

[c] Race indicates the avirulence *Avr* loci for which the isolate is avirulent; numbers in parentheses indicates *Avr* loci for which the allele has not been determined (Balesdent et al. 2005).

In order to identify effective resistance genes to the three races, 53 *Brassica napus* winter canola cultivars, hybrids and breeding lines were screened for major-gene resistance to *L. maculans* (Tables 4.3 and 4.4). Most (62%) entries were susceptible to the three races and lacked any resistance genes. Several (23%) conventional (non-glyphosate tolerant) cultivars and hybrids were heterogeneous in resistance to one or more races. For these entries, only 20 to 80% of the seedlings contained the expected major resistance gene (Rouxel et al. 2003). Most of the heterogeneous resistance was observed in open pollinated cultivars. Glyphosate tolerant (Roundup-Ready) entries currently grown in the region generally lacked major resistance genes,

except for DKW46-15 which appeared to have heterogeneous resistance from *Rlm4* and possibly *Rlm7*. The conventional hybrids Dimension, Safran, Visby, DK Sensei, and the rapeseed cultivar Rossini (9%), were resistant to all races possibly due to the presence of resistance gene *Rlm6* and/or *Rlm7*. A few entries (6%) including KS4506, Hornet and Linglandor, exhibited unknown resistance which could not be associated with the *Rlm* genes assessed in the study.

Table 4.3. Interaction phenotypes of *Brassica napus* cultivars determined by their response to *Leptosphaeria maculans* isolates (races) with known avirulence genotypes.

		Races[b]			
Cultivar	**Type[a]**	***AvrLm1,6,7,(5)***	***AvrLm1,4,6,(5,7)***	***AvrLm6,7,(5)***	**Resistance genotype[c]**
Westar		S	S	S	None
Monsanto /Dekalb					
DKW41-10[d]	OP	S	S	S	None
DKW44-10[d]	OP	S	S	S	None
DKW46-15[d]	OP	R/S	R/S	R/S	Rlm6(H),Rlm7(H),Rlm4?(H)
DKW47-15[d]	OP	S	S	S	None
DK Sensei	Hyb	R	R	R	Rlm6, Rlm7
DK ExStorm	Hyb	R	R/S	S	Rlm1(H), Rlm4?(H)
DK Imiron Cl	Hyb	R/S	R/S	S	Rlm1(H), Rlm4?(H)
DK ImpressionCl	Hyb	S	S	S	None
CROPLAN by WinField					
HC125W[d]	OP	S	S	S	None
HC115W[d]	OP	S	S	S	None
Kansas State University					
Kiowa	OP	R/S	R/S	R	Rlm6(H),Rlm7(H),Rlm4?(H)
Riley	OP	S	S	S	None
Sumner	OP	S	R/S	R/S	Rlm4(H), Unknown
Wichita	OP	R/S	R/S	R/S	Rlm6(H),Rlm7(H),Rlm4?(H)
Griffin/KS4022	OP	S	S	S	None
KS4410	OP	S	S	S	None
KS4426	OP	R/S	R/S	R	Rlm6(H),Rlm7(H),Rlm4?(H)
KS4428	OP	R/S	R	R/S	Rlm4, Rlm6(H), Rlm7(H)
KS4564	OP	R/S	R	R/S	Rlm4, Rlm6(H), Rlm7(H)
KSUR21	OP	S	S	S	None
KS4506	OP	S	S	R/S	Unknown
KSR073525[d]	OP	S	S	S	None
KSRO7363[d]	OP	S	S	S	None

Virginia State University					
Virginia	OP	S	S	S	None
VSX-3	OP	S	S	S	None
DL Seeds Inc. / Rubisco Seeds LLC					
Dimension	Hyb	R	R	R	Rlm6, Rlm7
Safran	Hyb	R	R	R	Rlm6, Rlm7
Visby	Hyb	R	R	R	Rlm6, Rlm7
Hornet	Hyb	S	S	R/S	Unknown
Sitro	Hyb	S	S	S	None
Mercedes		S	S	S	None
Popular		S	S	S	None
MOMONT, France					
Chrome	Hyb	R/S	S	R/S	Rlm7(H)
MH09E3		S	S	S	None
Technology Crops International					
Rossini (rapeseed)	OP	R	R	R	Rlm6, Rlm7
Syngenta					
NK Techni	Hyb	S	S	S	None
NK Petrol	Hyb	S	S	S	None
SY Marten	Hyb	S	S	S	None
SY Saveo	Hyb	S	S	S	None
DuPont Pioneer					
46W94[d]	Hyb	S	S	S	None
46W99[d]	Hyb	S	S	S	None
X12W377C	Hyb	S	S	S	None
X12W447C	Hyb	S	S	S	None
High Plains Crop Development					
HPX 501	OP	S	S	S	None
Limagrain					
Albatros	Hyb	S	R/S	S	Rlm4(H)
Miscellaneous Entries					
TT11	OP	S	S	S	None
Zhongyou	OP	S	S	S	None
Synia	OP	S	S	S	None
Linglandor	OP	R/S	S	S	Unknown
Lindora-00	OP	S	S	S	None
MAR	OP	S	S	S	None
PI649127	OP	S	S	S	None
Liradonna	OP	S	R/S	S	Rlm4(H)

[a] Type: OP = Open pollinated, Hyb = Hybrid

[b] Phenotype interaction with races: S = susceptible reaction, absence of the major resistance gene; R = resistant reaction, presence of the major resistance gen; R/S = heterogeneous resistance where a percentage of 20% to 80% of plants of an entry contain the major resistance gene.

[c] Resistance genotype describes the major resistance genes that are present in a cultivar; Unknown refers either to the presence of a new resistance gene or the combination of resistance genes that cannot be distinguished. None refers to the absence of all resistance genes tested in the current study. Other seedling resistance genes may be present. ? =possible presence of a major resistance gene.
[d] Glyphosate tolerant.

The presence or absence of major resistance genes *Rlm1, Rlm4, Rlm6* and *Rlm7* was determined in winter canola germplasm based on reactions with the three races of *L. maculans* with characterized avirulence genotypes (Table 4.4). Known major resistance genes (*Rlm1, Rlm4, Rlm6, Rlm7*) were inferred in 17 entries (32%). Twelve of those entries had heterogeneous resistance. None of the major resistance genes assessed in this study were identified in 33 entries (62%). Three entries (6%) were classified as having unknown resistance, that might have additional major resistance genes not considered in this study or new uncharacterized resistance genes. Within 17 cultivars that had at least one major resistance gene, it was inferred that 12 entries contained *Rlm6* and/or *Rlm7,* 8 entries contained *Rlm4*, and 2 entries contained *Rlm1*.

Table 4.4. Major resistance genes present in lines and cultivars of *Brassica napus* determined by their response to *L. maculans* isolates with known avirulence genotypes.

	Resistance genes[a]								
Cultivar	***Rlm1***	***Rlm2***	***Rlm3***	***Rlm4***	***Rlm5***	***Rlm6***	***Rlm7***	**Other**	**Resistance genotype**
Westar	-	n.d	n.d	-	n.d	-	-		None
Monsanto /Dekalb									
DKW41-10	-	n.d	n.d	-	n.d	-	-		None
DKW44-10	-	n.d	n.d	-	n.d	-	-		None
DKW46-15	H	n.d	n.d	H	n.d	H	H		Rlm6(H),Rlm7(H),Rlm4?(H)
DKW47-15	-	n.d	n.d	-	n.d	-	-		None
DK Sensei	-	n.d	n.d	-	n.d	+	+		Rlm6, Rlm7
DK ExStorm	H	n.d	n.d	H?	n.d	-	-		Rlm1(H), Rlm4?(H)
DK Imiron Cl	H	n.d	n.d	H?	n.d	-	-		Rlm1(H), Rlm4?(H)
DK Impression Cl	-	n.d	n.d	-	n.d	-	-		None
CROPLAN by WinField									
HC125W	-	n.d	n.d	-	n.d	-	-		None
HC115W	-	n.d	n.d	-	n.d	-	-		None
Kansas State University									
Kiowa	-	n.d	n.d	H?	n.d	H	H		Rlm6(H),Rlm7(H),Rlm4?(H)

Riley	-	n.d	n.d	-	n.d	-	-		None
Sumner	-	n.d	n.d	H	n.d	-	-	+	Rlm4(H), Unknown
Wichita	-	n.d	n.d	H?	n.d	H	H		Rlm6(H),Rlm7(H),Rlm4?(H)
Griffin/KS4022	-	n.d	n.d	-	n.d	-	-		None
KS4410	-	n.d	n.d	-	n.d	-	-		None
KS4426	-	n.d	n.d	H?	n.d	H	H		Rlm6(H),Rlm7(H),Rlm4?(H)
KS4428	-	n.d	n.d	+	n.d	H	H		Rlm4, Rlm6(H), Rlm7(H)
KS4564	-	n.d	n.d	+	n.d	H	H		Rlm4, Rlm6(H), Rlm7(H)
KSUR21	-	n.d	n.d	-	n.d	-	-		None
KS4506	-	n.d	n.d	-	n.d	-	-	+	Unknown
KSR073525	-	n.d	n.d	-	n.d	-	-		None
KSRO7363	-	n.d	n.d	-	n.d	-	-		None
Virginia State University									
Virginia	-	n.d	n.d	-	n.d	-	-		None
VSX-3	-	n.d	n.d	-	n.d	-	-		None
DL Seeds Inc. / Rubisco Seeds LLC									
Dimension	-	n.d	n.d	-	n.d	+	+		Rlm6, Rlm7
Safran	-	n.d	n.d	-	n.d	+	+		Rlm6, Rlm7
Visby	-	n.d	n.d	-	n.d	+	+		Rlm6, Rlm7
Hornet	-	n.d	n.d	-	n.d	-	-	+	Unknown
Sitro	-	n.d	n.d	-	n.d	-	-		None
Mercedes	-	n.d	n.d	-	n.d	-	-		None
Popular	-	n.d	n.d	-	n.d	-	-		None
MOMONT, France									
Chrome	-	n.d	n.d	-	n.d	-	H		Rlm7(H)
MH09E3	-	n.d	n.d	-	n.d	-	-		None
Technology Crops International									
Rossini (rapeseed)	-	n.d	n.d	-	n.d	+	+		Rlm6, Rlm7
Syngenta									
NK Techni	-	n.d	n.d	-	n.d	-	-		None
NK Petrol	-	n.d	n.d	-	n.d	-	-		None
SY Marten	-	n.d	n.d	-	n.d	-	-		None
SY Saveo	-	n.d	n.d	-	n.d	-	-		None
DuPont Pioneer									
46W94	-	n.d	n.d	-	n.d	-	-		None
46W99	-	n.d	n.d	-	n.d	-	-		None
X12W377C	-	n.d	n.d	-	n.d	-	-		None
X12W447C	-	n.d	n.d	-	n.d	-	-		None
High Plains Crop Development									
HPX 501	-	n.d	n.d	-	n.d	-	-		None
Limagrain									
Albatros	-	n.d	n.d	H	n.d	-	-		Rlm4(H)
Miscellaneous Entries									

TT11	-	n.d	n.d	-	n.d	-	-		None
Zhongyou	-	n.d	n.d	-	n.d	-	-		None
Synia	-	n.d	n.d	-	n.d	-	-		None
Linglandor	-	n.d	n.d	-	n.d	-	-	+	Unknown
Lindora-00	-	n.d	n.d	-	n.d	-	-		None
MAR	-	n.d	n.d	-	n.d	-	-		None
PI649127	-	n.d	n.d	-	n.d	-	-		None
Liradonna	-	n.d	n.d	H	n.d	-	-		Rlm4 (H)

[a] + = Presence of the major resistance gene; - = absence of the major resistance gene; n.d. = resistance gene whose presence or absence could not be determined; H = cultivars with heterogeneous resistance, where 20% to 80% of the plants contain the major resistance gene.
[b] Resistance genotype describes the major resistance genes that are present in a cultivar; Unknown refers either to the presence of a new resistance gene or the combination of resistance genes that cannot be distinguished. None refers to the absence of all resistance genes tested in the current study. Other major resistance genes may be present. ? =possible presence of a major resistance gene.

DISCUSSION

Based on cultivar and isolate interactions, three isolates with known avirulence genotype representative of three *L. maculans* races present in Oklahoma were used to characterize major resistance genes in winter-type *Brassica* germplasm. This study, although lacking of a set of *L. maculans* isolates harboring single avirulence alleles (*Avr*), allowed us to infer the presence of resistance genes *Rlm1*, *Rlm4*, *Rlm6* and *Rlm7* and to provide an overview of the major resistance genes common in winter canola cultivars and hybrids grown in the southern Great Plains. However, for total confirmation of the presence of major resistance genes, mapping and cloning are required (Marcroft et al. 2012a).

Of the 53 winter canola cultivars, hybrids, and breeding lines analyzed, 17 (32%) had major resistance to one or more races including homogeneous and heterogeneous resistance. The most common major resistance genes (71%) within the entries with seedling resistance were *Rlm6* and/or *Rlm7* (12 entries). The hybrids Dimension, Safran, Visby, DK Sensei, and the cultivar Rossini had homogeneous resistance reactions to all races, likely due to resistance gene *Rlm6* and/or *Rlm7*. The cultivars DKW 46-15, Kiowa and Wichita; the breeding line KS4426 and the hybrids Chrome and possibly DK ExStorm had heterogeneous resistance from *Rlm6* and/or *Rlm7*. The resistance gene *Rlm6* has been introgressed into *B. napus* from *B. juncea* (Chèvre et al.

1997). According to Kutcher et al. (2010), the resistance gene *Rlm6* has been mostly used in experimental research and it is only present in few canola cultivars in Europe with a "MX" trademark. Therefore, it is unlikely that winter canola cultivars developed for seeding in the U.S. harbor resistance gene *Rlm6.* Consequently, it can be assumed that the resistant reaction exhibited in these 12 entries is more likely to be due to resistance gene *Rlm7.* The finding of *Rlm7* in DK Sensei and possibly in DK ExStorm, both commercial DEKALB hybrids from Monsanto, was consistent with the information in its advertisements which indicates that *Rlm7* is present in combination with polygenic resistance to provide durable protection against black leg (DEKALB/Monsanto, 2014). The hybrids DK Imiron Cl and DK Impression Cl, which are for exclusive use with Cleranda herbicide, also combine *Rlm7* with polygenic resistance against black leg (DEKALB/Monsanto, 2014). However, *Rlm7* was not identified in these two hybrids when screened with the three characterized *L. maculans* races.

The presence of resistance gene *Rlm4* was found to a lesser extent (41%) in 7 of 17 entries exhibiting resistance. Entries KS4428, KS4564 showed homogeneous resistance to *Av 1,4,6,7,(5)* which harbors *AvrLm4,* and heterogeneous resistance to *Av1,6,(5,7)* and *Av6,7,(5)* which suggests the presence of *Rlm4*, *Rlm6* and *Rlm7.* The hybrid Albatros and cultivar Liradonna exhibited heterogeneous resistance to *Av1,4,6,(5,7),* but susceptible reaction to *Av 1,6,7,(5)* and *Av6,7,(5)* which infers that these two entries may contain heterogeneous seed with *Rlm4* and a lack of *Rlm6* and/or *Rlm7*. The cultivar Kiowa and breeding line KS4426 indicated heterogeneous resistance to *Av1,4,6,(5,7)* and could possibly harbor *Rlm4* besides *Rlm6* and/or *Rlm7*. The cultivar Sumner exhibited heterogeneous resistance to *Av1,4,6,(5,7)* and *Av6,7,(5),* but it was susceptible to *Av1,6,7,(5)* which suggested that the cultivar may contain heterogeneous seeds with *Rlm4* and some other *Rlm* gene that was not assessed in this study.

In the late 1990s, Australia began incorporating European winter canola germplasm, especially from France, to expand and diversify their breeding programs (Marcroft et al. 2012a).

Similarly in the U.S., the cultivar Wichita from Kansas State University (KSU), was developed from the French *B. napus* cultivar Jet Neuf (*Rlm4*) and released in 1999 as a new winter canola cultivar to be used in the southern Great Plains (Rife and Shroyer 2000). Since then, other cultivars including Kiowa and Riley have been released and breeding lines developed by the KSU breeding program using Wichita as a parental line (Stamm et al. 2012). This probably reflects the presence of *Rlm4* in the breeding lines KS4428 and KS4564, and the cultivars Sumner and Kiowa developed by KSU. Conversely, in this study the cultivar Wichita exhibited heterogenous resistance and Riley was susceptible to *Av1,4,6,(5,7)* which harbors *AvrLm4*. Results from rating scores (% of plants with resistant reaction to a corresponding Avr allele) for both cultivars were 25% and 17% resistance, near to the borderline (20%) that would be considered as heterogeneous resistance.

Resistance gene *Rlm1* was found only in 12% of the entries with major resistance genes. The hybrids DK ExStorm and DK Imiron Cl exhibited heterogeneous resistance to *Av1,6,7,(5)* and *Av1,4,6,(5,7)*, both harboring *AvrLm1, AvrLm6* and *AvrLm7*. Similarly, it is probable that both hybrids contain heterogeneous seed for *Rlm6* and/or *Rlm7*.

Finally, an unknown pattern of resistance was observed in three entries (6%). Unknown resistance suggests the presence of other seedling resistance whose corresponding *Avr* genes were absent in the selected races and not assessed in this screening. Likewise, it is possible that entries that exhibited a major resistance gene *Rlm1, Rlm4, Rlm6* and *Rlm7* could possibly contain other resistance genes in their genetic background. Therefore, it is desirable to have a set of isolates with single *Avr* alleles that correspond to known *Rlm* genes to completely assess resistance genes in winter canola genotypes.

Heterogeneous resistance was common within the screened entries. By definition, entries with heterogeneous resistance had only 20 to 80% of the seedlings exhibiting a resistant

phenotype (Rouxel et al. 2003). Most of the heterogeneous resistance was observed in open pollinated cultivars. These results may reflect a lack of selection for *Rlm* genes in breeding programs or low disease pressure for identification of resistance. Crosspollination by insect vectors may be an additional source of heterogeneous seeds. In Australia, changes in breeding practices were made to overcome heterogeneity in seeds, and now plants are being bagged when in flower to ensure self-pollination. Additionally hybrids and double haploids are being produced which facilitates the uniform insertion of traits (Marcroft et al. 2012a).

The rating scale used to qualitatively assess each isolate/entry interaction, as incompatible or resistant (more than 80% of resistant plants with an *Rlm* gene), compatible or susceptible (more than 80% of susceptible plants without an *Rlm* gene), or heterogeneous (20% to 80% of plants showing resistance) has been applied in previous studies that screened for resistance genes to *L. maculans* in *B. napus* accessions from France, Australia and Canada (Rouxel et al. 2003; Marcroft et al. 2012a). However, the interaction of some isolates and entries falls into intermediate categories between resistance and heterogeneous resistance (70 to 80 %) and susceptible and heterogeneous resistance (20% to 30%). Imprecise reaction assessments may have contributed to heterogeneous reactions of the DK hybrids and cultivar Wichita, which were expected to have resistance from *Rlm7* and *Rlm4,* respectively. Different results for isolate and entry interactions were sometimes observed when the interactions were recorded by the average of the IC index values (1-6). Mean IC values that were less than 4 were considered resistant reactions and greater than or equal to 4 were considered susceptible (Marcroft et al. 2012a). Assessing reactions using mean IC values fit better with the isolate/entry interaction that showed unexpected results. For example, the hybrid DK Exstorm which is reported to have *Rlm7,* was resistant to *Av1,6,7,(5),* heterogeneous to *Av1,4,6,(5,7),* and susceptible to *Av6,7,(5)* because 81% of the plants exhibited susceptible phenotypes. Similarly, the *Rlm7* hybrid DK Imiron Cl, showed heterogeneous resistance to *Av1,6,7,(5)* and *Av1,4,6,(5,7),* but was susceptible to *Av6,7,(5)*

because 84% of the plants had susceptible symptoms. However, when rated by the average of IC score, the hybrid DK Exstorm and DK Imiron Cl were resistant to the three races with mean IC values less than 4. Similarly, cultivar Wichita which is suspected to have resistance gene *Rlm4*, showed heterogeneous resistance to race *Av1,4,6,(5,7),* but was considered resistant by the average IC score (3.6).

This research demonstrated that most of the entries exhibiting major resistance genes contained resistance gene *Rlm7* and *Rlm4* which should be useful in management of black leg, because of the high frequency of avirulence alleles *AvrLm6*, *AvrLm4* and/or *AvrLm7* in the regional population of *L. maculans*. There is a need to include a more diverse set of isolates that harbor single avirulence *Avr* alleles to improve and complete the screening of cultivars and hybrids and detect all the possible major resistance genes. The identification of major resistance genes to black leg disease in winter *B. napus* enables the development of resistant cultivars and hybrids. Cultivar selection is one of the most important decisions made by canola growers. Producers should carefully review cultivar characteristics to make effective choices. In addition to yield, several traits to consider when selecting a winter canola include winter survival, sulfonylurea residual tolerance, herbicide resistance, pod shattering resistance, oil quantity and quality, and black leg resistance (Boyles et al. 2012). However, growers in the southern Great Plains are not currently planting cultivars with effective resistance genes. They are mostly planting cultivars that are glyphosate tolerant (Roundup-Ready) because they prefer that convenience. Currently, more than 90% of the acreage in the southern Great Plains regions is planted to glyphosate-tolerant HyClass and DeKalb cultivars, and Pioneer Hybrids (John Damicone, personal communication). Knowledge about the resistance genes present in the cultivars and hybrids commonly grown in the region should help growers better manage the disease, avoid having to spray a fungicide for disease control and to avoid selecting a cultivar with a defeated (ineffective) resistance gene. For example, a selection of *Rlm1* would only be

effective against about 35% of the local *L. maculans* population. It would be useful for breeders to determine what experimental lines should be released as new cultivars and where those cultivars can be effectively deployed to resist black leg disease based on a previous knowledge of the pathogen race structure.

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VITA

Claudia Isabel Diaz Proaño

Candidate for the Degree of

Master of Science

Thesis: ANALYSIS OF THE *Leptosphaeria maculans* RACE STRUCTURE AND IDENTIFICATION OF MAJOR-GENE RESISTANCE TO BLACK LEG IN WINTER CANOLA

Major Field: Entomology and Plant Pathology

Biographical:

Personal data:

Born in Quito, Ecuador, February 11, 1987.

Education:

Completed the requirements for the Master of Science in Entomology and Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in May, 2015.
Completed the requirements for the Bachelor of Science in your Biotechnology at Universidad de las Fuerzas Armadas (ESPE), Sangolquí, Ecuador in 2012.

Experience:

Graduate Research Assistant, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, Oklahoma, January 2013 to May, 2015.
Undergraduate Research Assistant. Department of Biotechnology, Universidad de las Fuerzas Armadas (ESPE), Sangolquí, Ecuador, February 2011 to July 2012

Professional Memberships:

American Phytopathological Society (APS)
Southern Division of the American Phytopathological Society
Honor Society of Phi Kappa Phi