Temperature Effects on *Brassica napus* Resistance to *Leptosphaeria maculans* and Race Structure Survey of Blackleg Isolates in Manitoba

By

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Abstract

Lihua, Rong. M. Sc. The University of Manitoba, December 2017. Temperature Effects on *Brassica napus* Resistance to *Leptosphaeria maculans* and Race Structure Survey of Blackleg Isolates in Manitoba. Major Professor: Dr W. G. Dilantha Fernando.

Blackleg, caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & De Not, is an economically important disease in canola (*Brassica napus* L.) production. This study explored how temperature affects the *Rlm* gene-mediated resistance to *L. maculans* in canola. It also investigated the frequency of *AvrLm* genes and race structure of *L. maculans* in High Erucic Acid Rapeseed (HEAR) and canola fields in Manitoba, Canada. This study found that *Rlm1, Rlm2, Rlm3* and *Rlm4* in Topas isogenic lines (ILs) of *B. napus* are not sensitive to temperature, while *Rlm3* in Quantum and *Rlm4* in Falcon broke down at higher temperatures of 25/20 and 30/25 °C. The temperature had a significant effect on lesion size, where a higher temperature resulted in larger lesions in Topas ILs. Temperature also had a significant effect on *L. maculans*, where the optimum temperature was 25 °C for its growth and sporulation. Under different temperature treatments, different genotypes with the same *Rlm* genes responded differently to *L. maculans.* This may indicate that the genetic background is important in single *Rlm* gene-mediated resistance expression. This study also identified and examined 297 isolates of *L. maculans* from stubble collected in HEAR and canola fields in Manitoba in the 2013 and 2014 growing season. *AvrLm6* and *AvrLm5* were present in more than 90% of the population; *AvrLm7*, *AvrLm4*, *AvrLm11*, and *AvrLm2* were present in more than 50% of isolates, while *AvrLm3* and *AvrLm9* had a very low frequency. In total, 85 races were identified, and *AvrLm2-4-5-6-7-11* was identified in 14.8% of the population. *AvrLm9* was not found in Manitoba in the 2014 growing

season. The frequency of *AvrLm* genes and race structures varied over years, regions and cultivars.

Foreword

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

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Chapter 1

General Introduction

1.0 General Introduction

Blackleg, also known as stem canker, is caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & De Not. Blackleg is an economically important disease in canola (*Brassica napus* L.) and other *Brassica* crops. Blackleg causes severe yield loss in almost all growing regions except China (West and Fitt, 2005). The methods used to manage this disease include a four-year rotation with non-*Brassica* crops, the use of resistant cultivars and fungicide application. *Leptosphaeria maculans* has the evolutionary ability to lose avirulence (*Avr*) genes and gain virulence (*avr*) to resistance genes through sexual reproduction under selection pressure, which is often caused by growing cultivars with the same *Rlm* gene over large acres. This ability allows the pathogen to overcome new resistant cultivars within a few years, which has been reported in France, Australia and Canada (Rouxel *et al.*, 2003a; Li *et al.*, 2003; Zhang *et al.*, 2016). Additionally, *L. maculans* can adapt to a wide range of climates and attack both winter and spring types of oilseed rape worldwide (West *et al.*, 2001).

Due to ever-growing concerns about climate change, it is important to protect the yield of major cash crops, such as canola. One factor that requires our attention is temperature because it is implicated in many disease epidemics (Agrios, 2005). For example, research on flax (*Linum usitatissimum* L.) and wheat (*Triticum aestivum* L.) has demonstrated that resistannce genes could be less effective at higher or lower temperatures and the genetic background plays a key role in resistance expression (Luig and Rajaram, 1972; Islam *et al.*, 1989). Currently, there is little research regarding the impact of temperature on *L. maculans* resistance in canola. With potential climate change, it is necessary to discover the temperature effects on resistance expression in different canola genotypes. The *B. napus*-*L. maculans* pathosystem follows a classical gene-for-gene interaction (Flor, 1971; Ansan-Melayah *et al.*, 1998). Approximately 18

resistance genes have been identified in *B. napus*, which are effective against isolates of *L. maculans* with corresponding *Avr* genes (Raman *et al.*, 2013). These cultivars with known *Rlm* genes could be used to test and identify the *AvrLm* genes carried by the pathogen. Disease surveys revealed that the frequency of *AvrLm* genes and race structure in the population of *L. maculans* has changed over time in western Canada (Kutcher *et al.*, 1993; Kutcher *et al.*, 2007; Liban *et al*., 2016; Zhang *et al.*, 2016). It is important to monitor the regional variation of *AvrLm* gene frequency and race structure in the pathogen population to develop enhanced integrated blackleg management strategies in the future.

Chapter 2

Literature Review

2.1 Canola

2.1.1 Family and Species

Canola belongs to the *Cruciferae* family, which includes many important edible and economical vegetable plants and weeds. Canola includes the species of *Brassica napus* L., *B. rapa* L. and *B. juncea* L. (Ratnayake and Daun, 2004). There are spring and winter types in both *B. napus* and *B. rapa*, which are morphologically and physiologically different (Booth and Gunstone, 2004). *Brassica napus* has a slightly higher yield than *B. rapa*, while *B. rapa* can be grown in more northern areas because of the earlier maturity (Downy, 1983). The relationships among the main cultivated *Brassica* species are illustrated in **Figure 2.1**. *Brassica nigra* L., *B. oleracea* L. and *B. rapa* have 8, 9 and 10 chromosomes respectively and form the three corners of the triangle. The hybridization between these species generates three other species, *B. carinata* L. (BBCC), *B. juncea* (AABB) and *B. napus* (AACC), which carry 17, 18 and 19 chromosomes respectively (Nagaharu, 1935).

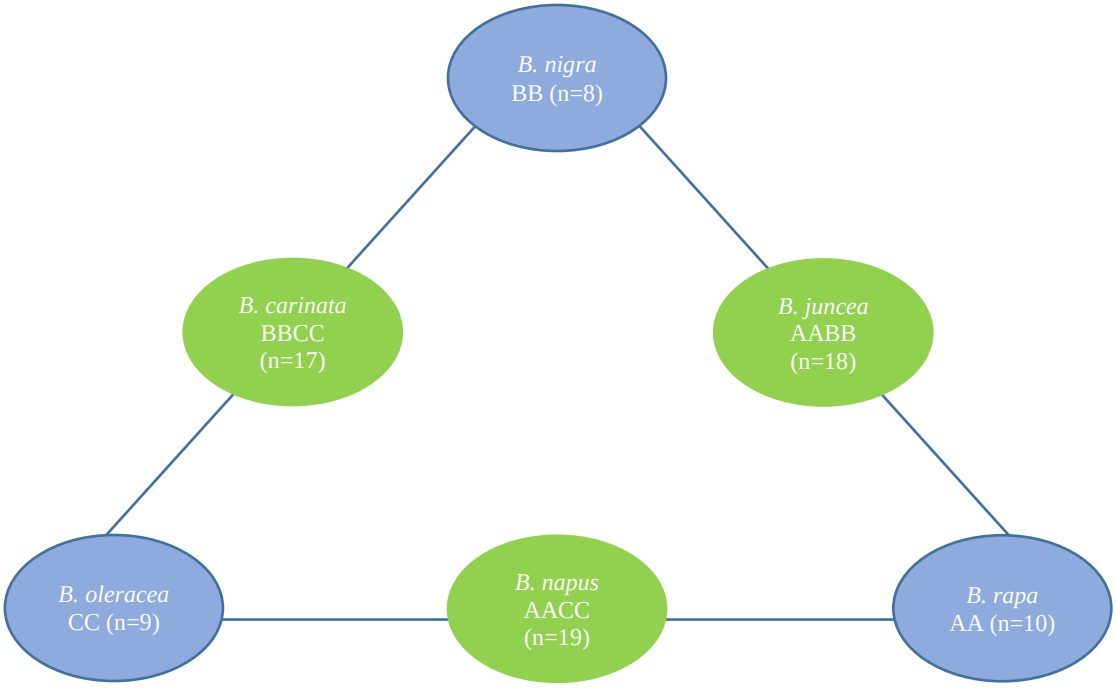

Figure 2.1. The relationship among the *Brassica* species (A, B, C: genome; n: chromosome number).

2.1.2 History

Oilseed rape was one of the earliest plants to be systematically cultivated by man, and this vegetable crucifer was widely cropped as early as 10,000 years ago (Friedt and Snowdon, 2009). The earliest records found in India suggest that oilseed *Brassicas* (probably *B. rapa*) were being used as early as 4000 BC and their use had spread into China and Japan 2000 years ago (Gupta and Pratrap, 2007; Snowdon *et al.*, 2007). In Europe, Swedes (*B. napus* ssp. *napobrassica*) were known at the time of the Romans and were used for oil purposes in the 13th century in northern Europe (Friedt and Snowdon, 2009). By the 16th century, oilseed rape became the major source of lamp oil in Europe (Kimber and McGregor, 1995). By the end of the 19th century, the high-quality nature of oilseed rape fats made it useful as a lubricant in industrial machinery; this guaranteed the continued production of the crop into the 20th century (Canola Council of Canada, 2017a). In Canada, spring type of oilseed rape was first grown in the mid-20th century. The large-scale worldwide production of oilseed rape began in the mid-1970s when the value of rape oil and seed meal was significantly improved (Friedt and Snowdon, 2009; Canola Council of Canada, 2017a).

Oil from early oilseed rape cultivars contained a high quantity of erucic acid (13-docosenoic acid, 22:1 n-9); this is associated with cardiac damage and related health problems in humans (Downy, 1983). Thus, oil from oilseed rape had a poor reputation as food prior to the development of oilseed rape cultivars with zero erucic acid and low glucosinolate content in the 1970s (Stefansson, 1983; Downey and Röbbelen, 1989; Downey, 1990). The first breakthrough came with the initial 0-quality cultivars with erucic acid levels of less than 1%, while the earlier oilseed rape cultivars contained up to 50% erucic acid in the seed oil (Stefansson and Hougen, 1964). The first erucic acid-free variety, derived from a spontaneous mutant of the German

spring oilseed rape cultivar Liho by Dr Stefansson, was released in Canada in early 1970s (Stefansson and Kondra, 1975; Kimber and McGregor, 1995). The seed also contains high quantities of glucosinolates, which made the meal unsuitable as a livestock feed and this suppressed the value of the crop (Kimber and McGregor, 1995). The toxic byproducts released during the digestion of glucosinolates could cause liver and kidney damage along with lymph dysfunction (Kimber and McGregor, 1995). In 1969, the Polish spring rape variety Bronowski was identified with low glucosinolate content, and this polygenic trait was introduced into the high-yielding erucic acid-free material by an international backcrossing program (Stefansson and Kondra, 1975). The first 00-quality spring oilseed rape variety, Tower, was released with zero erucic acid and low glucosinolate content, and from there, oilseed rape (canola) has advanced in the following decades to become one of the most widely grown and most important oil crops in temperate regions (Stefansson and Kondra, 1975; Kimber and McGregor, 1995; Daun *et al.,* 2011; Canola Council of Canada, 2017a).

The name 'Canola' was registered by the Western Canadian Oilseed Crushers' Association, which is now known as the Canadian Oilseed Processors Association (Oplinger *et al.,* 1989). The official definition of canola specifies that the oil must contain less than 2% of erucic acid, and the solid component of the seed must contain less than 30 micromoles of any mixtures of 3-buteny1 glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid (Canola Council of Canada, 2017a). The term canola has generally been accepted to distinguish the newly improved oilseed rape from the old, less desirable seeds and products (Downy, 1983). Since the release of canola, plant breeders have developed many canola cultivars to improve agronomic traits, oil and meal quality, and disease resistance (Canola Council of Canada, 2017a).

All these improvements accelerated the expansion of the Canadian canola industry (Canola Council of Canada, 2017a).

2.1.3 Economic Importance of Canola

The oilseed rape grown in Europe is mainly of the winter type, whereas the spring forms are grown in Canada, northern Europe and Australia, and semi-winter oilseed rape predominates in China (Diederichsen *et al.* 2011). Canola has become a significant agricultural product and cash crop during the past three decades, and it is now the world's third leading source of vegetable oil (after soybean and palm) in 2017/18 and the second major oil meal (after soybean) in 2017 (US Department of Agriculture, 2017). In 2017, the total yield of five major oilseeds was 555.03 million metric tonnes (MMT) throughout the world, with a yield of 348.44 MMT of soybeans and followed by 72.14 MMT of canola (US Department of Agriculture, 2017; USDA Foreign Agricultural Service, 2017). The major contributors to the annual production of oilseed rape are the European Union, Canada, China and India (Canadian Food Inspection Agency, 2017; USDA Foreign Agricultural Service, 2017). The world average yield was 2.71 metric tons per hectare (t/ha) in 2005, varying from 4.14 t/ha in western Europe, 1.81 t/ha in China, 1.63 t/ha in Canada, 1.04 t/ha in Australia and 0.90 t/ha in India (Snowdon *et al.*, 2007). These differences in yield are due to different cultivars (e.g., spring vs. winter type), climate, soil, agricultural inputs (seed quality, fertilizers, and agrochemicals) and agronomic practices (Allen and Morgan 1975).

Canola (*B. napus*) is an important cash crop in Canada, and the canola industry contributed about $26.7 billion to Canadian economy annually (Canola Council of Canada, 2017b). The production of canola continues to expand in Canada with continued plant breeding and varietal research. The acreage of canola in Canada has grown from 6.8 million hectares in 2010 to 9.2 million hectares in 2017, largely due to an extensive breeding program and to the

release of numerous hybrid cultivars with agronomic advantages (Canola Council of Canada, 2017b; Statistics Canada, 2017). Approximately 85% of Canada's production is exported to other countries throughout the world (Canola Council of Canada, 2017b). China is the largest importer of Canadian canola with a total value of $2.7 billion in 2016 (Canola Council of Canada, 2017b); however, one of the challenges is the risk of introducing the *Leptosphaeria maculans* pathogen into China (Fitt *et al.,* 2008). Consequently, an embargo was placed in 2009; this pushed the Canadian government, industry and institutes to advance research on the blackleg pathogen (Canola Council of Canada, 2017b).

2.1.4 Nutrition and Chemical Composition of Canola Oil

Canola contains 40 to 45% oil in the seeds, and the meal is a good source of animal feed due to its rich protein content (Downy, 1983). Canola oil is widely used as cooking oil and salad oil; it is an important ingredient for food products such as margarine, spreads, mayonnaise, salad dressing, baked goods, convenience foods and infant formulas (Trautwein, 1997). It is highly recommended as a healthy oil due to its oil profile with extremely low saturated fatty acid, and is an important source of the monounsaturated oleic acid (18:1 n-9) (Trautwein, 1997). Also, canola oil and canola oil-based margarine are recommended as a major source of α-linolenic acid in the secondary prevention of coronary heart disease (de Lorgeril *et al.,* 2001).

2.1.5 Non-food Uses of Rape Oil and Protein

The value of rape oil lies in its diverse range of uses. Oil from oilseed rape is not only a highly nutritional food oil, but also provides the raw material for an astounding array of products, such as rape methyl ester (biodiesel), industrial lubricant, biodegradable plastics and tensides for detergent and soap production (Daun *et al.*, 2011). Over the whole range of oils and fats, about 80% is used as human food, 6% goes into animal feed and 14% goes to the oleochemical

industry (Lühs and Friedt, 1994). Other than canola cultivars grown for human consumption with a low content of erucic acid, there are also high erucic acid rapeseed (HEAR) and newer cultivars with a modified fatty acid composition that furnish speciality uses and niche markets, such as oleochemistry or frying purposes (Friedt and Lühs, 1998). The breeding of high erucic acid, low glucosinolate oilseed rape started in 1969 at the University of Manitoba (Jacobs, 2001; McVetty *et al*., 2009). HEAR cultivars contain over 50% of erucic acid in the oil and less than 20 μmol glucosinolates per gram of seed at 8.5% moisture (Scarth *et al.*, 1991; Wang *et al.*, 2003). Erucic acid (C22:1) is an important raw material for industry and is used as an additive to lubricants and solvents, a softener for textiles, and an amide derivative in polymer synthesis (Mastebroek and Lange, 1997; Jacobs, 2001; Wang *et al.*, 2003; McVetty *et al*., 2009). In Europe, rape oil with low-erucic acid content is used mainly as industrial raw material. Rape meal also produces protein-based products for non-food markets, such as bioplastic products and adhesives (Snowdon *et al.*, 2007).

Overall, rape oil is a renewable and environmentally beneficial industrial lubricant resource; it has low toxicity towards humans and the ecosystem and does not contribute carbon dioxide to the atmosphere (Graboski and McCormick, 1998; Foglia, 2001).

2.1.6 Diseases of Canola

In Canada, the major diseases in canola are Sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary) and stem canker (aka Blackleg) (Snowdon *et al.* 2007; Zhang and Fernando, 2017); however, clubroot (*Plasmodiophora brassicae* Wor) is a growing concern in western Canada (Tewari *et al.*, 2005). These two major diseases, Sclerotinia stem rot and stem canker are also found widely in Europe. China has the less aggressive species of blackleg, *Leptosphaeria biglobosa* RA Shoemaker & H. Brun, Sclerotinia stem rot and several viral diseases (Fitt *et al.*,

2006). Other less common diseases in Canada include white rust, also known as staghead (*Albugo candida* (Pers.) Kuntze), Alternaria black spot (*Alternaria brassicae* (Berk.) Sacc), damping off and root rot (*Rhizoctonia solani* Kühn, *Fusarium spp.*), downy mildew (*Peronospora parasitica* (Pers. Ex Fr.) Fr), verticillium wilt (*Verticillium dahliae* Klebahn), aster yellows disease (aster yellow phytoplasma) and other less damaging bacterial, viral and fungal diseases (Canola Council of Canada, 2017c).

2.2 The Blackleg Disease

2.2.1 Blackleg Pathogen

Blackleg disease, also known as phoma stem canker, is the most damaging disease of oilseed rape and canola (*B. napus* and *B. rapa*) worldwide (Howlett, 2004; Fitt *et al.*, 2006; Kutcher *et al.*, 2010a). The disease is attributed to a complex of two closely related species, *L. maculans* and *L. biglobosa* (Shoemaker and Brun, 2001), which were previously referred to as highly and weakly virulent forms of *L. maculans*, respectively (Cunningham, 1927; Petrie, 1978). Both species are found in many, but not all, of the countries in which blackleg disease has been identified (Rouxel *et al.*, 2003a). Blackleg disease has been reported in Europe, Australia, USA, Canada, Mexico, Brazil, Argentina, and Kenya (Fitt *et al.*, 2006). In China, only *L. biglobosa* has been reported on oilseed rape crops. It is important and necessary to establish strict quarantine standards to prevent the spread of *L. maculans* to China because the Chinese grow large acres of susceptible oilseed rape as well as an abundance of vegetable *Brassica* cultivars (West *et al.*, 2001; Fitt *et al.*, 2008).

In Canada, blackleg disease was first reported in 1961 (Vanterpool, 1961) and the symptoms were described as superficial lesions and stem lesions on oilseed rape (McGee and Petrie, 1979). The pathogen was isolated on adult oilseed rape plants and later proven to be the

less aggressive pathogen *L. biglobosa* (Vanterpool, 1961; Petrie and Vanterpool, 1965). *Leptosphaeria maculans* was first found in Saskatchewan in 1975 with low levels in canola residue (McGee and Petrie, 1979). After that, the pathogen was reported in Alberta in 1983 and Manitoba in 1984 (Platford, 1985; Gugel and Petrie, 1992). From 1976 to 1981, the disease prevalence increased ten-fold in Saskatchewan, and up to 50% yield loss was reported in individual fields (Petrie, 1978; Gugel and Petrie, 1992). Since *L. biglobosa* generally causes limited damage on the upper stem of canola plants, it was not economically important in most years (West *et al., 2002*). In other parts of the world, blackleg was reported to decimate oilseed rape production in France in the 1950s and 1960s, Australia in the 1970s (Gugel and Petrie, 1992) and the USA in the 1990s (Lamey, 1995).

Currently, blackleg caused by *L. maculans* is the most prevalent disease in canola across the Prairie Provinces (McLaren *et al.*, 2012). In 2011 and 2012, up to 70% of surveyed crops in Manitoba were infected with blackleg basal cankers, with a disease incidence of 9 to 16% (McLaren *et al.*, 2012). This caused significant economic losses in canola growing regions (McLaren *et al.*, 2012). This disease is estimated to be responsible for a loss of $1B per season globally (Fitt *et al.*, 2008).

Many breeding efforts have been applied to manage blackleg disease in canola. In the early 1990s, canola cultivars with high levels of genetic resistance to the disease were introduced and combined with agronomic management practices (Kutcher, 2013). These methods reduced the losses in yield and quality associated with the disease but did not eliminate the pathogen (Kutcher, 2013). By the late 1990s up until recent years, producers have reported high disease severity in previously resistant cultivars (Keri *et al.*, 2001; Zhang *et al.*, 2016). Due to the sexual reproduction in the life cycle and selection pressure under resistant cultivars, *L. maculans* can

lose avirulence (*Avr*) genes and gain virulence (*avr*) to resistance genes (Keri *et al.,* 2001; Zhang *et al.,* 2016). This ability allows the pathogen to overcome newly developed resistant cultivars and cause large economic losses. This situation did occur in France with the 'breakdown' of *Rlm1* cultivars and in Australia with the breakdown of 'sylvestris' resistance (Rouxel *et al.,* 2003a; Li *et al.,* 2003). The same situation happened in western Canada; new races of *L. maculans* have been identified in the prairies (Chen and Fernando, 2006) and the breakdown of *Rlm3* was observed in Manitoba (Zhang *et al.,* 2016). Since *L. maculans* and *L. biglobosa* have similar life cycles, they can adapt to different climates, and thus can cause severe damage on both spring and winter (autumn-sown) types of oilseed rape in different geographic areas (West *et al.,* 2001; Fitt *et al.,* 2008). *Leptosphaeria maculans* would cause considerable damage if it were introduced into an area where only *L. biglobosa* exists, such as China (Fitt *et al.,* 2008). These factors made it more challenging to manage blackleg disease and have driven further research into blackleg, specifically *L. maculans*, aimed at expanding knowledge and finding solutions to manage the disease.

2.2.2 Host Range

Henderson (1918) first identified blackleg disease on cabbage *(B. oleracea)* in Wisconsin. Both *L. biglobosa* and *L. maculans* are known to cause infection on *B. oleracea* (Dilmaghani *et al.,* 2010). Most *B. napus* crops cultivated today have some level of resistance to *L. biglobosa*; however, *L. maculans* has adapted to *B. napus* and has caused severe infection (West *et al.,* 2001). Since the rapid expansion of growth in acreage of oilseed rape in the early 1970s, *L. maculans* displaced *L. biglobosa* as the dominant species in North America and Europe (Fitt *et al.,* 2008).

Leptosphaeria biglobosa is a common pathogen on cabbage (*B. oleracea),* while it is less popular on *B. napus* cultivars today due to resistance derived from *B. rapa* (AA genome) (West *et al.,* 2001). *Leptosphaeria maculans* infects a wide range of *Brassica* species including *B. rapa, B. napus, B. juncea, B. oleracea* and other genera in the crucifer family (West *et al.,* 2001). In addition, some cruciferous weeds, pennycress (*Thlaspi praecox* Wulf.), tall tumble-mustard (*Sisymbrium altissimum* L.), flixweed (*Descurainia sophia* (L.) Webb), Maca (*Lepidium peruvianum* Chacón) and wild mustard (*Sinapis arvensis* L.) can also host *L. maculans* (Sawatsky, 1989). Some *B. napus* genotypes are resistant to *L. maculans* by carrying resistance genes *Rlm1, 2, 3, 4, 7, 9, LepR1, LepR2, LepR3* and *RlmS* (Hayward *et al.* 2011). Most *B. juncea* cultivars are very resistant to *L. maculans* (Chèvre *et al.,* 1997). *Brassica nigra* (BB) is the most resistant species in the *Brassica* triangle and could be a good source of resistance in breeding programs (Eber *et al.,* 2011).

2.3 *Leptosphaeria maculans*

2.3.1 Life Cycle

The life cycle of *Leptosphaeria maculans* on *Brassica napus* is shown in **Figure 2.2**. *Leptosphaeria maculans* can over-winter on residues of canola, such as mycelia, pycnidia and pseudothecia in western Canada, (West *et al.,* 2001). Pseudothecia produce and release ascospores under favourable conditions of temperature, radiation and relative humidity (Howlett *et al.,* 2001). Wind dispersed ascospores are the primary inoculum at the beginning of the growth season (Howlett *et al.*, 2001). Pycnidia produce rain splash-dispersed pycnidiospores, which causes the secondary inoculum during the growth season (Williams, 1992). Ascospores or pycnidiospores land on and adhere to cotyledons or young leaves and penetrate host tissue through stomata or wounds (Hammond and Lewis, 1987). Hyphae grow intercellularly in the

plant tissue and induce cell death and degradation. The necrotic leaf lesion or stem cankers provide resources to support the production of pycnidia on the lesions (Williams, 1992). Pycnidiospores are the dominant source of primary inoculum in western Canada (Guo *et al.*, 2005; Ghanbarnia *et al.*, 2011). The sexual reproduction of *L. maculans* in western Canada is limited by the colder climate (Liban *et al*., 2016) and the lack of ascospores meditated infection (Dilmaghani *et al.*, 2009, 2013). Compared to ascospores, pycnidiospores have limited genetic variation and distance spread (Guo *et al.*, 2005).

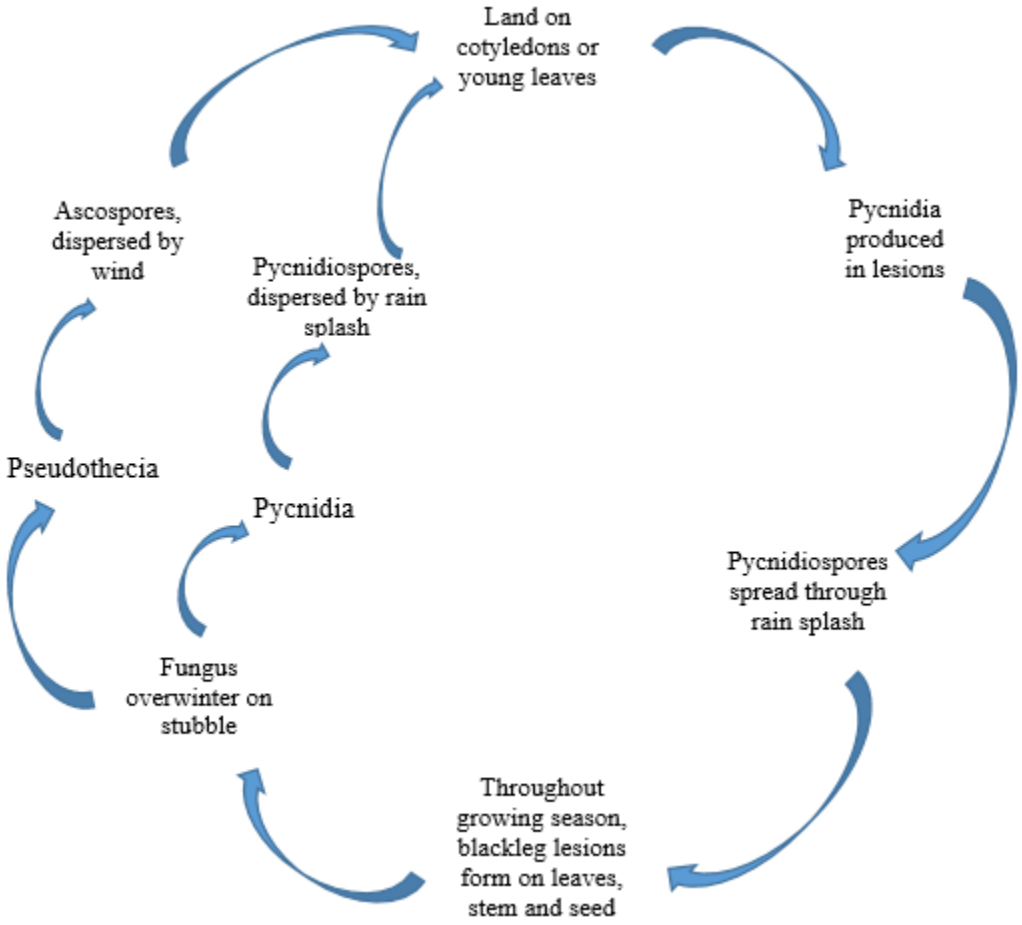

Figure 2.2. The life cycle of *Leptosphaeria maculans* on *Brassica napus*.

2.3.2 Epidemiology of *Leptosphaeria maculans*

2.3.2.1 Initiation of Epidemics

Blackleg is usually a monocyclic disease in Australia and Europe, with epidemics generally initiated by airborne ascospores (Bokor *et al.*, 1975). Infections can also be caused by infected seed (Jacobsen and Williams, 1971; Wood and Barbetti, 1977) from direct contact of infected stubble and by rain-splashed conidia (Hall, 1992; Thürwächter *et al.*, 1999). Other cruciferous hosts of *L. maculans* could also be a possible source of inoculum (Hall, 1992). The main source of primary inoculum, ascospores, produced in pseudothecia on infected canola stubble, is released during the rainfall that coincides with the sowing period (Hall, 1992; Mahuku *et al.*, 1997). Ascospore release occurs shortly after wetting by rain (Pérès *et al.*, 1999) and evening dew (McGee, 1977) and usually coincides with the presence of young, susceptible plants. In western Canada, blackleg is polycyclic because the primary inoculum is asexual conidiospores (pycnidiospores) and they are released continuously during the growing season (Guo *et al.*, 2005). Ascospores are released from residue from May to August after the long, cold winter, and land on the leaves of the new spring oilseed rape crops (McGee and Petrie, 1979; Kharbanda, 1993).

Wood and Barbetti (1977) reported that only one or two ascospores could initiate a lesion under the optimal temperature and wetness conditions. The controlled environment experiments showed that conidia could infect only wounded leaves, petioles and stem (Hammond, 1985) and were unlikely to infect unwounded leaves unless applied at a very high concentration to older leaves (Vanniasingham and Gilligan, 1989).

2.3.2.2 Cotyledon and Leaf Infection

After landing and adhering to the cotyledons and leaves of new crops, ascospores and conidia germinate in humid or wet conditions and produce hyphae (Hammond, 1985). Hyphae invade the hosts mainly via stomatal pores but also via wounds and cause infection (Hammond, 1985; Chen and Howlett, 1996). Hall (1992) reported that ascospores germinated within 4 h at 4–28 ^{o}C in laboratory experiments, suggesting that infection is limited by wetness rather than by temperature. Biddulph *et al.* (1999) also found that 4 h was the minimum wetness period required to produce leaf lesions by ascospores of *L. maculans* and that most lesions were produced with a leaf wetness duration of 48 h at 12–20 °C.

2.3.2.3 Phoma Leaf Spots

Lesions develop in similar ways on the leaves, whether in Australia, Canada or Europe; however, the appearance does vary among host resistances, *L. maculans* group and stages of lesion development (West *et al*., 2001). Lesions begin as pale green spots on the leaves, and then the spots enlarge to 1 to 2 cm in diameter and often turn to pale brown with small dark specks of pycnidia. The centre of the lesion might eventually break or fall out completely as the disease develops (West *et al*., 2001). In western Canada, leaf spots appear on leaves of spring canola in June and July (Kharbanda, 1993). The pycnidiospores can cause secondary infection of nearby plants by rain splashing and/or wind dispersal (West *et al*., 2001).

2.3.2.4 Hypocotyl and Stem Infection

Blackleg infection on hypocotyls causes a constriction in the stem just above the ground and below the first leaves, and the lesion can cause severe seedling blight (West *et al., 2001*). It was reported that up to 70% of seedlings were killed by blackleg lesions in individual crops in

Western Australia (Barbetti and Khangura, 1999). In Canada, Blackleg lesions may sever the tender stem base of seedlings even up to the six-leaf stage (Kharbanda, 1993).

Blackleg lesions at the stem base (crown or root collar) show a distinct dark brown or purple margin and are typically associated with leaf scars, developing from lesions of leaves early in the season (Hammond, 1985). During the pod development and seed ripening stage, these lesions may spread, enlarge or coalesce, and even crack open to form the dry rots or cankers on the stem base, which is called the damaging phase of epidemics (Paul and Rawlinson, 1992). Terms used to describe disease symptoms on the stem base include blackleg, canker, crown canker, collar rot and basal canker (West, *et al*., 2001). The crown canker may girdle the stem and disrupt water transport and cause premature ripening of the pods (Davies, 1986). In severe cases, stems are weakened to cause lodging and death of the crop. Infected pods can cause premature ripening, splitting and yield loss (Petrie and Vanterpool, 1974; Sprague *et al.*, 2017). More importantly, pod infection can spread to the inside of seeds (Wood and Barbetti, 1977) and may introduce the pathogen to a new area through seed.

2.3.2.5 Survival and Disease Spread

After harvest, the degrading stem tissue and residue are rapidly colonized by *L. maculans* and pycnidia are produced abundantly. Additionally, conidia can also colonize stubble saprophytically and may increase inoculum levels and numbers of pseudothecia (West *et al.*, 2001). Pseudothecia develop in these residues if both mating types are present and ascospores are released in an extended period (West *et al.*, 2001). Ascospores from infected residue are the primary cause of new infections in Australia, and they are a potential risk for crops several kilometres away (Bokor *et al.*, 1975; Petrie, 1978; Gladders and Musa, 1980). In Canada,

pycnidiospores are more commonly found on the remaining stubble and are the primary source of inoculum (Guo *et al.,* 2005).

Although seed infection is rare, seed-borne infection can spread the disease to completely new areas (Agostini *et al*., 2013). The mycelium of *L. maculans* can be found in crucifer seed within the seed coat or even within the embryo (Jacobsen and Williams, 1971). Experiments conducted in Canada have shown that the incidence of infected (artificially contaminated) canola at sowing was correlated with the incidence of plants with phoma stem canker and the incidence of infected seed at crop harvest (Hall *et al.* 1996). Hall *et al.* (1996) reported that the incidence of infected seed per sample in Canada was less than 5%. Infected seed from other *Brassica* crops, such as mustard (*B. juncea*), might also help to spread the disease (Gugel and Petrie, 1992).

2.4 Disease Management

According to the 'disease tetrahedron of epidemiology' (**Figure 2.3**), plant disease epidemics are a result of the combination of the following elements: susceptible host plants, a virulent pathogen, and favourable environmental conditions over a long period (Agrios, 2005). Disease management strategies are human activities that interfere or stop the initiation and development of disease epidemics (Agrios, 2005). These human activities include cultivar selection; plant resistance; the density of the plants; and cultural practices, including chemical and biological controls, tillage, rotation and timing of seeding and harvest (Agrios, 2005).

Specific strategies to manage blackleg disease in canola are divided into several categories: selection of resistant cultivars, cultural practices, chemical controls and biological controls, as described below.

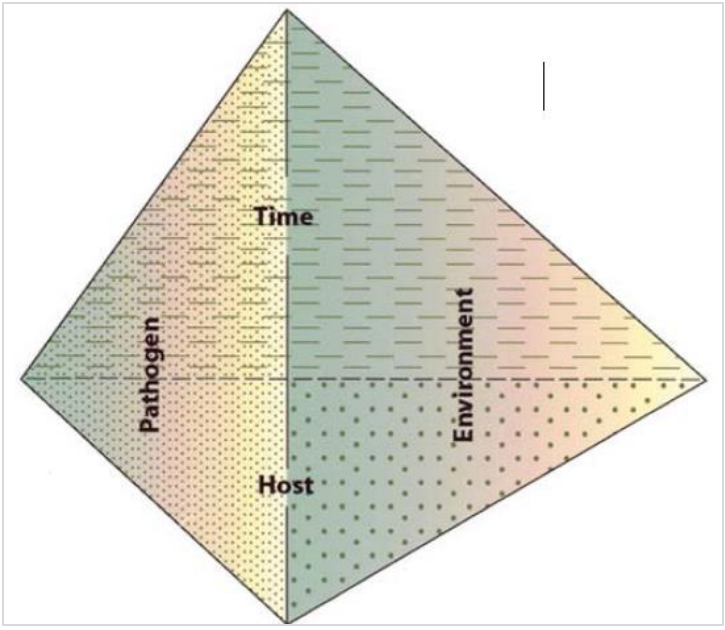

Figure 2.3. The disease tetrahedron (Agrios, 2005). Used with permission, copyright license in **Appendix V**.

2.4.1 Resistant Cultivars

Resistant cultivars are considered to be the most effective management practice for blackleg disease in canola caused by *L. maculans* (Kutcher *et al.,* 2013). There are two types of resistance to the pathogen: race-specific resistance (qualitative) and race-nonspecific resistance (quantitative) (Delourme *et al.,* 2006). Quantitative resistance is controlled by several genes and expressed in the adult stage, and is referred to as adult plant resistance (APR) (Rimmer, 2006; Raman *et al., 2013*). Qualitative resistance is mediated by single resistance genes (*Rlm*) in a gene-for-gene manner (Flor, 1971). Qualitative resistance is effective as early as the cotyledon stage, such as in the *L. maculans* and *Brassica* interaction (Kutcher *et al.,* 2011).

Race-specific *Rlm* genes were first observed in Europe and Australia as an effective practice to manage blackleg disease in oilseed rape (Howlett, 2004). Early in 1971, a blackleg resistant cultivar carried the resistance gene *Rlm4* was developed and widely grown in Europe (Delourme *et al.,* 2006). Another resistant cultivar, Jet Neuf, was registered in Europe in 1978

which combined resistance gene *Rlm4* and quantitative resistance to *L. maculans* (Delourme *et al.,* 2006). Breeders in Australia and Canada developed *B. napus* cultivars with improved resistance to *L. maculans* (Roy and Reeves, 1975; Roy, 1984; Wratten and Mailer, 1989 Canola Council of Canada, 2017d). Most *B. napus* cultivars in Canada were considered resistant to *L. maculans* (Rimmer, 2006). However, resistance efficacy was reduced for several reasons: the long history of blackleg, tighter crop rotations, rapid expansion of acreage, and pathogen population shift by repeatedly using certain single resistance genes (Canola Council of Canada, 2017d). The breakdown of resistant canola cultivars has been reported in several countries in both commercial fields and experimental fields (Rouxel *et al.,* 2003a; Li *et al.,* 2003; Sprague *et al.,* 2006; Brun *et al.,* 2000; Zhang *et al.,* 2016). Breeders are expected to develop cultivars with maximum durable and effective resistance by combining both qualitative resistance genes and quantitative resistance genes (Brun *et al.*, 2010). A potential method to develop cultivars with strong resistance is gene pyramiding by introducing multiple resistance genes expressing resistance against multiple races of the pathogen (Rimmer, 2006). Also, *Rlm* gene rotation is another method to reduce the selection pressure (Kutcher *et al.*, 2013). To maximize blackleg resistance in resistant cultivars, it is necessary to have access to knowledge of host *Rlm* genes diversity, pathogen *AvrLm* gene diversity and the interaction between specific *Rlm* and *AvrLm* genes.

2.4.2 Agronomic Practices

Canola growers use several practices to reduce and limit the risk of blackleg disease and yield loss in the fields. Rotations with non-crucifer crops are highly recommended (Kutcher *et al.*, 2013). The pathogen *L. maculans* overwinters on the stubble and residue of canola crops after harvest and can survive for years on infected residues, as the stubble decomposes slowly due to the long, cold winter in western Canada (Kharbanda and Tewari, 1996; Kutcher *et al.*, 2007). Stubble management strategies, such as burning or burying of infected canola residue and deep ploughing are also helpful in reducing blackleg inoculum (Barbetti and Khangura, 1999; Blenis *et al.*, 1999; Guo *et al.*, 2005).

Stubble decomposition is faster in the soil than on the surface (Blenis *et al.*, 1999). Tillage reduces disease by improving the soil microorganisms and promoting decomposition through burying infected canola residue (Kharbanda *et al.*, 1999). Tillage shortly after harvest or before seeding in the following spring could promote decomposition and prevent the release of ascospores, causing infection in the following growing season (Gladders and Musa, 1980; Canola Council of Canada, 2017d).

Typically, the young seedlings at the 2–6 leaf stage are highly susceptible to *L. maculans* infection, especially when weather conditions favour the release of ascospores (West *et al.*, 2001). Infection can be mitigated by changing the seeding date so that this stage does not coincide with the peak release of ascospores (McGee and Petrie, 1979; Kharbanda and Tewari, 1996; West *et al.*, 1999). Early seeding is recommended in France, while late seeding is recommended in Australia and western Canada (Lepage and Penaud, 1995; McGee and Emmett, 1977). However, there was no evidence to prove that changing the seeding dates can reduce blackleg disease in the growing season in Canada (Kharbanda and Tewari, 1996). On the other

hand, changing the seeding date might impact yield and is only recommended when the expected yield loss from disease is significantly higher than the yield loss caused by early/late seeding.

Crop rotation was recognized as an important and efficient strategy to manage blackleg disease (Kutcher *et al.,* 2013). It is a fundamental method for residue-born plant disease (Curl, 1963). Crop rotation reduces the pathogen population to a level that is low enough to limit its growth, survival and reproduction, thus avoiding significant crop damage (Kutcher *et al.,* 2013). Intensive rotation could increase blackleg disease severity and amount of infected residue even in resistant cultivars, which increase the risk of inoculum carry-over, resistance breakdown and yield loss (Kutcher *et al.,* 2013). Field trials in Manitoba indicated that a 4-year rotation of canola with non-crucifer crops reduces the incidence and severity of blackleg (Guo *et al.*, 2005). One canola crop every 4 years is highly recommended in western Canada (Kutcher *et al.*, 2013).

2.4.3 Chemical Control

Fungicides are used in seed treatments, soil application and foliar sprays worldwide to minimize infection of blackleg disease in canola and other oilseed rape crops in different regions, depending on the epidemiology of the disease and the crop economy (West *et al.,* 2001). In Canada, three fungicides, carbathin, thiram and iprodione, are currently registered for seed treatment (West *et al.*, 2001). Seed treatment could decrease the probability of blackleg surviving on the seed and effectively reduce seed-borne blackleg. Although seed treatment does not protect seedlings from infection by airborne spores, it does protect plants from infected seed. It is important to prevent the disease spread in areas where blackleg is not yet established (Canola Council of Canada, 2017d).

Fungicides are recommended only in crops with high yield potential and high level of inoculum (moderate to severe), and with low resistance cultivars (susceptible or moderate adult

plant resistance to blackleg) (West *et al.,* 2001). In Australia, it has been proven that foliar fungicide application in cultivars with little or no resistance was ineffective for blackleg control (Brown *et al.,* 1976) and it provided only limited damage control of blackleg in resistant cultivars in Western Australia (Sivasithamparam *et al.,* 2005). In Canada, three fungicides, pyraclostrobin (Headline®), propiconazole (Tilt) and azoxystrobin (Quadris), are the main products used to manage blackleg (Canola Council of Canada, 2017d). Kutcher *et al.* (2013) reported that fungicide is effective in reducing blackleg symptoms on the susceptible cultivars, but it is not economically beneficial on resistant cultivars, due to the relatively lower canola yield in Canada. Although propiconazole has occasionally been used as a foliar fungicide, it could not completely control blackleg disease (Kharbanda *et al.,* 1999). The optimum time for foliar fungicide application is during the 2–4 leaf stage (Liu, 2014). Fungicide applications reduce disease incidence and severity effectively, but no evidence has been shown to improve yield on resistant or moderately resistant cultivars in Canada (Kutcher *et al.* 2005; Liu, 2014). However, fungicide application could bring benefit in western Europe due to the higher yield of oilseed rape (Ghanbarnia *et al.,* 2012).

2.4.4 Biological Control

Biological control is more eco-friendly than fungicides, reducing chemical applications. Research found that the bird's nest fungi, *Cyanthus striatus* and *C. olla*, could reduce inoculum production of *L. maculans* by competing for the stubble food base that blackleg spores survive on (Tewari *et al.,* 1997). *Paenibacillus polymyxa* (syn. *Bacillus polymyxa*) strain PKB1 was found to inhibit *L. maculans* by producing two closely related antifungal peptides. *In vitro,* PKB1 significantly reduced germination and germ-tube length of pycnidiospores in culture. In field experiments, PKB1 significantly reduced the formation of pseudothecia and ascospores of

L. maculans and decreased survival of the fungus on *B. napus* cv. Westar stems (Kharbanda *et al.,* 1999). Bacteria isolated from soil, canola stubble and plant parts were found to carry antifungal properties and could suppress blackleg disease on cotyledons and 3–4 leaf stage seedlings significantly (Ramarathnam and Fernando, 2006).

2.5 Interaction between *Leptosphaeria maculans* and *Brassica napus*

2.5.1 Pathogen Avirulence Genes and Race Structure

According to the classical interpretation of the gene-for-gene hypothesis, each resistance gene detects and provides resistance against a pathogen isolate carrying the corresponding *Avr* gene (Flor, 1942). However, resistance genes *Rlm4* and *Rlm7* from *B. napus* detect the *L. maculans AvrLm4-7* gene while both *Rlm1* and *LepR3* recognize the *L. maculans AvrLm1* gene (Parlange *et al.,* 2009; Van de Wouw *et al.,* 2009). This causes more challenges for disease management; two resistance genes in the host could be ineffective due to the loss of one single *Avr* gene in the pathogen (Liban *et al*., 2016). Although the *L. maculans-B. napus* pathosystem does not strictly follow the gene-for-gene interaction model, the genetic relationship between *L. maculans* and *B. napus* has also been suggested as a model system for the study of the host-pathogen interaction between hemibiotrophic pathogens and their hosts (Fitt *et al.,* 2006). Earlier, a set of three host differentials (*B. napus* cvs. Westar, Quinta, and Glacier) was used to classify isolates of *L. maculans* into one of three pathogenicity groups (PGs) (PG2 to PG4) (Koch *et al.,* 1991; Mengistu *et al.,* 1991). As additional resistant cultivars were developed, Jet Neuf was added into the host list to classify isolates of *L. maculans* into six PGs (A1 to A6) (Badawy *et al.,* 1991; Kuswinant *et al.,* 1999). The PG system of classifying isolates has been valuable to detect changes in the pathogen population (Kutcher *et al.,* 2007). Cunningham (1927) was the first to report variability for *avr* in *L. maculans*. Isolates of *L. maculans* collected in the late 1980s in

Manitoba and Saskatchewan were identified as PG2: avirulent on both 'Quinta' and 'Glacier' (Kutcher *et al.,* 1993). In 1998, a new pathotype, PGT, was reported in western Canada, which showed virulent on 'Quinta' but avirulent on 'Glacier' (Keri *et al.,* 2001). In 1999, a single PG3 isolate (avirulent on 'Quinta', virulent on 'Glacier') was isolated from southern Manitoba (Kutcher *et al.,* 2007). Since then, more PG3 isolates have been reported (Fernando and Chen, 2003) and PG4 isolates (avirulent on both 'Quinta' and 'Glacier') were isolated in 2005 (Bradley *et al.,* 2005; Chen and Fernando, 2006).

Approximately 18 resistance genes have been identified among various *Brassica spp*. that are effective against *L. maculans* isolates with corresponding *Avr* genes from the seedling stage to full maturity. These resistance genes are *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, Rlm11, LepR1, LepR2, LepR3, LepR4, LepS, BLMR1* and *BLMR2* (Ferreira *et al.,* 1995; Mayerhofer *et al.,* 1997; Ansan-Melayah *et al.,* 1998; Zhu and Rimmer, 2003; Rimmer, 2006; Delourme *et al.,* 2006). In addition to those above single resistance genes, adult plant resistance (ARP) in *B. napus* is mapped to quantitative trait loci (QTL) within the genome, and ARP provides partial resistance in the adult plants (Hayward *et al.,* 2012).

As more resistant cultivars were developed, a new system consisting of a set of *B. napus* with known resistance genes was proposed to analyze the race structure of *L. maculans* under controlled conditions in terms of *Avr*/*avr* allele frequencies and complexity of races (Balesdent *et al.,* 2005; Delourme *et al.,* 2006). Using those resistance genes known to date, there are potentially 14 *Avr* alleles that could be detected in pathogen isolates (Rimmer, 2007). To date, 14 *Avr* genes have been hypothesized in *L. maculans* based on the phenotypic interaction; these genes are *AvrLm 1, 2, 3, 4-7, 5, 6, 8, 9, 10, 11, LepR1, LepR2, S* and *J1* (Van de Wouw *et al.,* 2014a). Among them, *Avr* genes of *AvrLm 1, 2, 3, 4-7, 6, 9, 11, LepR1* and *AvrLmJ1* have been

mapped and seven *Avr* genes have been cloned: *AvrLm1* (Gout *et al.* 2006), *AvrLm2* (Ghanbarnia *et al.,* 2015), *AvrLm3* (Plissonneau *et al.,* 2016), *AvrLm5/AvrLmJ1* (Van de Wouw *et al.* 2014a), *AvrLm6* (Fudal *et al.* 2007), *AvrLm4-7* (Parlange *et al.* 2009) and *AvrLm11* (Balesdent *et al.* 2013).

Race structure and frequency of *Avr* alleles in the *L. maculans* population can be detected with a differential set of *Brassica* genotypes carrying known resistance genes and the cloned *Avr* genes in the pathogen. Pathogen population surveys have been used to geographically define the composition of *Avr* genes in *L. maculans* (Liban *et al.,* 2016). Knowledge of the host-pathogen interaction between *B. napus* and *L. maculans* is necessary to develop integrated and durable strategies to manage blackleg disease. Surveys to characterize *Avr* genes of *L. maculans* has been conducted in France (Balesdent *et al.,* 2006) and across the oilseed rape growing regions of northern Europe (Stachowiak *et al.,* 2006). A survey in France showed that *AvrLm 1-9* were detected in 65% of the population (Balesdent *et al.,* 2006), although the frequency of each of the *Avr* alleles varied greatly. In the same study, *AvrLm6* and *AvrLm7* were detected in greater than 99% of the 1,787 isolates characterized while *AvrLm2* and *AvrLm9* were not carried by any of the isolates (Balesdent *et al.,* 2006). Similarly, studies conducted in Europe (UK, Germany, Sweden and Poland) showed that there was no detection of *AvrLm2, AvrLm3* or *AvrLm9* among the 603 isolates examined, while the alleles *AvrLm*1 and *AvrLm4* were identified only at low levels (< 10%) (Stachowiak *et al.,* 2006). However, the frequency of alleles *AvrLm5* (86%), *AvrLm6* (100%) and *AvrLm7* (> 99%) was high (Stachowiak *et al.,* 2006). In western Canada, 96 isolates of *L. maculans* were identified, and there were similarities in the frequency of various *Avr* alleles between the European and the Canadian studies, but major differences were also detected. *AvrLm2* was detected in 97% of *L. maculans* population in western Canada, but not

observed in Europe (Stachowiak *et al.*, 2006; Kutcher *et al.*, 2010b). On the other hand, *AvrLm7* was detected at high frequency in European studies but carried by only 25% of the Canadian isolates characterized (Kutcher *et al.*, 2010b). Knowledge of the frequency of *Avr* genes in the pathogen population across the Canadian prairies and in other oilseed rape growing regions of the world will allow pathologists, breeders and farmers to consider specific strategies to managethe pathogen.

2.5.2 Temperature Effects on the Interaction of *Leptosphaeria maculans* and *Brassica napus*

Temperature, cultivar, and plant tissue significantly affect the germination of, hyphal growth from and penetration of ascospores in controlled-environment conditions (Naseri *et al.*, 2008). Germination of ascospores of *L. maculans* began after 2 h at 10–20 °C and after 4 h at 5 °C (Huang *et al.*, 2003; Naseri *et al.*, 2008) and the germination percentage of ascospores was greater at the higher temperatures of 15 and 20 °C than at the lower temperatures of 5 and 10 °C after 24 h on both agar and on plant tissue (Huang *et al.*, 2003). Germination percentage of ascospores was greater on agar-coated slides and cotyledons than on leaves (Naseri *et al.*, 2008). Meanwhile, germination was greater on the leaves or cotyledons of the susceptible cultivars than on the resistant cultivars (Naseri *et al.*, 2008). Elongation of germ tubes was greater on cotyledons than on leaves of the cultivars tested and greater on susceptible cultivars than resistant cultivars after 24 h at 15–20 °C (Naseri *et al.*, 2008). Ascospores produced long, straight hyphae to penetrate the leaves predominantly through stomata at temperatures from 5 to 20 °C (Huang *et al.*, 2003). The percentage of germinated ascospores that penetrated through stomata increased with increasing temperature from 5 to 20 °C (Huang *et al.*, 2003). Temperature also affects the maturation of pseudothecia of *L. maculans* (Toscano-Underwood *et al.*, 2003). The maturation time decreased almost linearly with increasing temperature from 5 to

20 °C under continuous wetness, but was longer in natural conditions, especially in dry weather (Toscano-Underwood *et al.,* 2003). When pycnidiospores are co-inoculated with ascospores, it caused greater disease in *B. napus*, compared to when inoculations consisted of pycnidiospores alone (Toscano-Underwood *et al.,* 2003). Although this effect was significantly influenced by the host resistance of the cultivar, it was independent of the *L. maculans* isolate used in the experiment (Li *et al.,* 2006).

Temperature has been shown affect incubation period, lesion size and lesion numbers of canola inoculated with *L. maculans* (Huang *et al.,* 2006). In the research conducted by Huang *et al*. (2016), the incubation period decreased with increasing temperature from 5 to 25 °C for both susceptible and resistant cultivars. The number of lesions on DarmorMX (*Rlm6*) increased as the temperature increased from 15 to 25 °C. Lesion diameter was greater on DarmorMX at 25 °C than at lower temperature. Also at 25 °C, DarmorMX (*Rlm6*) was susceptible 16 days post-inoculation (DPI) with inoculation of both ascospores and pycnidiospores while it showed resistance at 20 °C 18 DPI (Huang *et al.,* 2006). At the higher temperature of 25 °C, the *Rlm6* mediated resistance in DarmorMX (*Rlm6*) broke down (Huang *et al.,* 2006).

The objectives of this study are: 1) to explore the temperature effects on *Rlm* gene-mediated resistance to *L. maculans* and whether the *Rlm* gene-mediated resistance breaks down at a higher temperature; and 2) to examine and identify the frequency of *AvrLm* genes and race structure of *L. maculans* in Manitoba, Canada.

Chapter 3

Temperature Effects on Resistance Expression and Lesion Development in *Brassica napus* Infected by *Leptosphaeria maculan*

3.0 Abstract

Blackleg disease, caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & De Not, is responsible for severe damage and yield loss in canola (*Brassica napas* L.) production around the world. Temperature is one of the most important environmental factors that affect the development of plant disease epidemics. Resistance to pathogens could be less effective at higher or lower temperatures. Temperatures may even reduce or eliminate the race-specific resistance of host plants under certain situations. This research was conducted to explore how temperature affects *Rlm* genes performance at the seedling stage. The first objective of this study was to examine if *Rlm* gene-mediated resistance is temperature sensitive; the second objective looked at how *Rlm* genes respond to temperature increase. Seven-day-old seedlings of 14 *B. napus* genotypes were inoculated with *L. maculans* pycnidiospores suspension on cotyledons. The plants were grown under four temperatures (15/10, 20/15, 25/20 and 30/25 °C). The lesion size of Topas isogenic lines (ILs) was measured daily with Access 2.0. This study identified four genotypes, Falcon (*Rlm4*), Quantum (*Rlm3*), Surpass400 (*LepR3*, *RlmS*) and Samourai (*Rlm2*, *9*), that became susceptible at higher temperatures (25/20 or 30/25 °C), while Topas *Rlm1* (*Rlm1*), Topas *Rlm2* (*Rlm2*), Topas *Rlm3* (*Rlm3*) and Topas *Rlm4* (*Rlm4*) were resistant to *L. maculans* at all the temperatures. The same *Rlm* genes responded differently to temperatures in different genotypes, indicating that the genetic background is an important factor in resistance response to temperature increase. The lesion development of Topas ILs showed that both genotype ($p < 0.05$) and temperature ($p < 0.05$) had significant effects on lesion size. The lesion size increased as the temperature increased, where Topas *Rlm4* illustrated the largest lesion followed by Topas *Rlm1*, Topas *Rlm2* and Topas *Rlm3*. There were no pycnidiospores produced on the lesions 10 days post inoculation (DPI) and 14 DPI, indicating that there was no resistance breakdown in any of

the Topas ILs at any temperature treatment. Lesion size is highly temperature-dependent; however, the effects of temperature on *Rlm*-mediated resistance appears to depend on the specific genotype, as we did not find similar temperature sensitivities for *Rlm6*- and *Rlm1*-mediated resistance in DarmorMX and Quinta. The *in vitro* growth of three isolates of *L. maculans* showed that both temperature and isolate have significant effects on the growth and sporulation of *L. maculans*. The growth and sporulation of *L. maculans* were restricted by higher or lower temperature, while 25 °C is the optimum temperature for *L. maculans*.

3.1 Introduction

Canola (*Brassica napus* L.) is one of the most important oilseed crops in the world (Fitt *et al.*, 2006). The blackleg disease is caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & De Not, which causes serious damage and yield loss in canola production around the world, particularly in Europe, Australia and North America (Fitt *et al.*, 2006). Host resistance is an effective and environmentally-friendly disease management practice. Both quantitative and qualitative resistance have been identified in *B. napus* cultivars (Pongam *et al.*, 1998; Balesdent *et al.*, 2001; Pilet *et al.*, 1998, 2001; Jestin *et al.* 2011, 2015). Currently, about 18 major *Rlm* genes against *L. maculans* have been identified in *Brassica* species: *Rlm1-11, LepR1-4, RlmS, BLMR1* and *BLMR2* (Ferreira *et al.*, 1995; Mayerhofer *et al.*, 1997; Ansan-Melayah *et al.*, 1995; Zhu and Rimmer, 2003; Rimmer, 2006; Delourme *et al.*, 2006; Balesdent *et al.*, 2002, 2013; Chèvre *et al.*, 1996, 1997; Eber *et al.*, 2011; Yu *et al.*, 2005, 2007a, 2007b, 2013; Van de Wouw *et al.*, 2009; Long *et al.* 2011). Two *Rlm* genes, *LepR3* (which interacts with *AvrLm1*) and *Rlm2* have been cloned (Larkan *et al.* 2013, 2015).

Seven corresponding avirulence (*AvrLm*) genes of *L. maculans* have been cloned: *AvrLm1* (Gout *et al.*, 2006), *AvrLm2* (Ghanbarnia *et al.* 2015), *AvrLm3* (Plissonneau *et al.*, 2016), *AvrLm5* (Van de Wouw *et al.*, 2009), *AvrLm4-7* (Parlange *et al.* 2009), *AvrLm6* (Fudal *et al.* 2007) and *AvrLm11* (Balesdent *et al.* 2013). Sexual recombination may occur in the reproduction of *L. maculans* and the population can adapt to selection pressures rapidly when exposed to resistant cultivars carrying single or major *Rlm* genes (Fitt *et al.*, 2006). This situation increases the frequency of virulent isolates and can cause the breakdown of resistance, often resulting in severe yield loss (Sprague *et al.*, 2006). The breakdown has been observed in France, where *L. maculans* adapted to the *Rlm1* gene within three years with the widely grown *Rlm1*

resistant cultivars (Rouxel *et al.*, 2003a). Similarly, the breakdown of *LepR3* in Australian cultivars led to crop losses of up to 90% (Li *et al.,* 2003). These examples confirm that resistance based on single dominant genes is unlikely to be durable.

In Canada, blackleg caused by *L. maculan*s, remains the most economically significant disease of canola (Kutcher *et al.,* 2011), although all commonly grown cultivars of canola carry moderate to high resistance to *L. maculans* (Zhang *et al.,* 2016). The most recent research has shown that *Rlm3* is the major *Rlm* gene prevalent in Canadian canola cultivars and germplasm, and *Rlm2* and *Rlm1* were detected in a few cultivars only (Zhang *et al.,* 2016). Meanwhile, the survey in the *L. maculans* population indicated that the frequency of *AvrLm3* decreased from 60% in 2005/2006 (Dilmaghani *et al.*, 2009) to 8% in 2010/2011 (Liban *et al*., 2016) in western Canada and 2.7% in 2012/2013 in Manitoba (Zhang *et al.*, 2016). The survey data indicated that the resistance gene *Rlm3* broke down in western Canada from the use of a single resistance gene developed in the 1970s when blackleg disease was first reported in Canada (Gugel and Petrie, 1992; Zhang *et al.,* 2016). To develop disease management strategies, in addition to employing resistant cultivars, it is important to consider other factors, such as cultural practices, climate, *L. maculans* pathogen population, and economic importance of *B. napus* (West *et al.*, 2001). Integrated management strategies involve using resistant cultivars, crop rotation, stubble management, fungicide application and rotation of *Rlm* genes to manage the disease effectively (Kutcher *et al.,* 2011; Marcroft *et al.,* 2012).

Temperature is considered one of the most important environmental factors that affect the development of plant disease epidemics (Agrios, 2005). Higher or lower temperatures relative to the optimum temperature could reduce the effectiveness of resistance in the host plant (Agrios, 2005). Certain temperatures may even reduce or eliminate the race-specific resistance in host

plants by limiting spore germination, host penetration and invasion, and pathogen growth and sporulation (Agrios, 2005).

In each pathosystem, the high or low temperature may have a positive or negative effect on the infection type, and this depends on the interaction between the genes of the host and the genes of the pathogen. There are many examples showing a positive association between higher temperature and susceptibility. In wheat (*Triticum aestivum* L.), the seedlings carrying resistance genes *Sr5*, *Sr8* and *Sr9b* against stem rust (*Puccinia graminis* Pers.f. sp. *ritici* Eriks. & E. Henn) were resistant at lower temperatures of 15, 18, 21 and 24 °C, but became susceptible or semi-susceptible at temperatures of 27 and 30 °C (Luig and Rajaram, 1972). In tobacco (*Nicotiana tabacum* L. *cv Xanthi-nc*), the *N* gene-mediated resistance to *Tobacco mosaic virus* (TMV) showed resistance at temperatures below 27 °C but became susceptible at temperatures above 27 °C and the virus could infect the plant systemically (Wright *et al.*, 2000). In tomato (*Lycopersicon esculentum* L.), in a cultivar from a cross between Moneymaker (MM)-Cf4 and MM-Cf0), the *Cf-4* gene is responsible for mediating resistance against leaf mould *Cladosporium fulvum* (Cooke) (syn. *Fulvia fulva* [Cooke] Cif). Transformed tomato seedlings that expressed both a *Cf* and the matching *Avr* genes died rapidly from systemic necrosis at 20 °C but survived at 33 °C, which indicated the *Cf* gene became ineffective at higher temperatures (de Jong *et al.*, 2002). In other pathosystems, resistance genes lose function at low temperatures. Research in flax (*Linum usitatissimum* L.) showed that resistance genes expressed themselves fully at the higher temperature of 25 °C and susceptibility tended to increase at a lower temperature (Islam *et al.*, 1989). In flax, the isogenic line carrying *Rlm* gene *L10* was susceptible to rust strain 1 (*Melampsora lini* (Ehrenb) Lev.) at 21 ± 3 °C but was resistant at 32 ± 3 °C (Islam *et al.*, 1989). The resistance of downy mildew (*Pseudoperonospora cubensis* (Berk.

Et Curt.)) in muskmelon (*Cucumis melo* L. *cv* Hemed) is also temperature dependent. Resistance genes *Pc-1* and *Pc-2* in cv. PI 124111F were specifically inactive at lower temperatures (12–15 °C) (Balass *et al.,* 1993).

Previous research has been conducted on the temperature effects on the *B. napus-L. maculans* system. When cotyledons of Quinta (carrying *Rlm1*) were inoculated with *L. maculans* race A2 (carrying *AvrLm1*), the seedlings were resistant at 18 °C but susceptible at 27 °C (Badawy *et al.,* 1992). In another study, *B. napus* isogenic line (IL) carrying/lacking resistance gene *Rlm6* were inoculated with both ascospores and conidia on leaves (Huang *et al.,* 2006). Darmor (lacking *Rlm6*) showed susceptibility at all the temperature treatments while DarmorMX (carrying *Rlm6*) showed resistance at 15 and 20 °C but was susceptible at 25 °C 16 days post-inoculation (DPI) (Huang *et al.,* 2006). Authors have also investigated the effects of temperature on the maturation of pseudothecia, ascospores germination and penetration of *L. maculans* and *L. biglobosa* on oilseed rape (Toscano-Underwood *et al.,* 2003; Huang, 2003). Germination and hyphal growth of *L. maculans* ascospores increased with the increase of temperature from 5 to 20 °C after 24 h of incubation on agar-coated slides (Huang *et al.,* 2006). The germination of conidia started 24 h after inoculation, while ascospores started 2 h after inoculation. However, there is no difference in the attachment and germination of ascospores and the penetration of cotyledons between resistant and susceptible cultivars. The difference comes from the disease development after penetration (Li *et al.,* 2006).

As more resistance genes were identified in *B. napus*, whether other *Rlm* genes respond to temperature in the same way as *Rlm1* in Quinta and *Rlm6* in DarmorMX is still unknown; likewise, it is unclear whether the same *Rlm* genes in different genotypes have the same response to temperature. More research is required to examine the interactions between *B. napus* and *L.*

maculans. In this study, 14 *B. napus* genotypes carrying nine *Rlm* genes were inoculated with *L. maculans* carrying corresponding *Avr* genes and treated under four different temperatures to explore temperature effects on the interaction between *Rlm* genes and *Avr* genes, the lesion development on cotyledons and the growth and sporulation of pycnidiospores.

3.2 Materials and Methods

3.2.1 *Brassica napus* Genotypes and Isolates of *Leptosphaeria maculans*

Nine genotypes of *B. napus* including 'Westar'-no *Rlm* (Balesdent *et al.*, 2002), 'Darmor'-*Rlm9* (Delourme *et al.*, 2004), 'MT29'-*Rlm1, Rlm9* (Delourme *et al.*, 2008), 'Forge'-*Rlm6* (Fernando Lab unpublished), 'Falcon'-*Rlm4* (Rouxel *et al.*, 2003b), 'Samourai'-*Rlm2, Rlm9* (Rouxel *et al.*, 2003b), 'Quantum'-*Rlm3* (Larkan *et al.*, 2013), 'Surpass400'-*LepR3, RlmS* (Larkan *et al.*, 2013) and '01.23.2.1'-*Rlm7* (Dilmaghani *et al.*, 2009), each carrying different single or multiple *Rlm* genes (**Table 3.1**), and ILs 'Topas *Rlm1*'-*Rlm1* (Borhan, 2013), 'Topas *Rlm2*'-*Rlm2* (Borhan, 2013), 'Topas *Rlm3*'-*Rlm3* (Borhan, 2013) and 'Topas *Rlm4*'-*Rlm4* (Borhan, 2013) were selected as host genotypes in this study **(Table 3.2)**. Six *L. maculans* isolates, collected and identified in the Fernando Lab, A10-509A (*AvrLm2,3,6*), DM59 (*AvrLm6,7, LepR3*), A11-736 (*AvrLm6,7, LepR1*), Sb1 (*AvrLm2,3,6, LepR1, LepR3*), Sb2 (*AvrLm2,4,6,7*), and R2-7 (*AvrLm1,4,6,7*) were selected as pathogen isolates (**Table 3.1**).

Table 3.1. Nine *Brassica napus* genotypes inoculated with different isolates of *Leptosphaeria maculans*.

Genotype	**Isolate**[***]
Westar (no *Rlm*[*] genes)	A10-509A (*AvrLm*[**]*2,3,6, LepR1, LepR3*)
Darmor (*Rlm9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*)
MT29 (*Rlm1,9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*) DM59 (*AvrLm6,7, LepR3*)
Forge (*Rlm6*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*) A10-509A (*AvrLm2,3,6, LepR1, LepR3*) A11-736 (*AvrLm*6,7, *LepR1*)
Falcon (*Rlm4*)	Sb2 (*AvrLm2,4,6,7*)
Samourai (*Rlm2,9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*) Sb2 (*AvrLm2,4,6,7*)
Quantum (*Rlm3*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)
Surpass400 (*LepR3, RlmS*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)
01.23.1 (*Rlm7*)	DM59 (*AvrLm6,7, LepR3*)

[*]*Rlm* indicates resistant genes in *B. napus* against the pathogen *L. maculans*.

[**]*AvrLm* indicates the avirulent genes in the pathogen *L. maculans* that correspond with *Rlm* genes in *B. napus*.

[***] Isolates of *L. maculans* were collected and identified in the Fernando Lab.

Table 3.2. Seedlings of Five Topas ILs (*Brassica napus*) were inoculated with *Leptosphaeria maculans* isolates with corresponding *AvrLm* genes and treated under different temperatures.

Genotypes	***Rlm* genes**	**Isolate**	***AvrLm* gene**	**Temperature Treatment**	**Relative Humidity**
Topas *DH 16516*	no *Rlm* gene	Sb2	*AvrLm2,4,6,7*	15/10 °C 20/15 °C 25/20 °C 30/25 °C	80–90%
Topas *Rlm1*	*Rlm1*	R2-7	*AvrLm1,4,6,7*		
Topas *Rlm2*	*Rlm2*	Sb1	*AvrLm2,3,6*		
Topas *Rlm3*	*Rlm3*	Sb1	*AvrLm2,3,6*		
Topas *Rlm4*	*Rlm4*	Sb2	*AvrLm2,4,6,7*		

3.2.2 Inoculum Preparation

Isolates were cultured from paper disks on V8 juice agar media [200 mL V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 mL distilled water, 15 g agar and 0.75 g calcium carbonate ($CaCO_3$), 0.1 g streptomycin sulfate] in Petri dishes (Fisher Science, Pittsburgh, PA). Plates were placed on a light bench under cool white fluorescent light (100–150 $E \cdot m^{-2} \cdot sec^{-1}$) at room temperature for 5 to 8 days. When hyphae and conidia of *L. maculans* were present on 2/3 of the juice agar media, 4 mL of sterile distilled water was added to the plates and pycnidiospores were harvested by flooding the *L. maculans* cultures and scraping with microscope slides. Spore suspensions were collected in sterile centrifuge tubes (Fisher Science, Pittsburgh, PA); the upper layer was collected to prepare the inoculum. After spore suspensions were placed in centrifuge tubes, the concentration of spores was estimated by counting spore number on a hemocytometer (Hausser Scientific Company, Horsham, PA) and diluted to a concentration of 1×10^7 $spores \cdot mL^{-1}$ and stored in 10 mL sterile centrifuge tubes at -20 °C until seedlings were ready for inoculation.

3.2.3 Experimental Design

This chapter included four experiments which were in randomized block design with three replications in each. Experiment I was designed to investigate effects of temperature on *Rlm* gene-mediated resistance on the cotyledons of nine *B. napus* genotypes (**Table 3.1)**. Experiment II was designed to investigate effects of temperature on *Rlm* gene-mediated resistance on the cotyledons of five Topas ILs (**Table 3.2)**. In experiments I and II, six seedlings were inoculated in each treatment. Experiment III was designed to investigate effects of temperature on lesion development on the cotyledons of five Topas ILs (**Table 3.2)**. Ten seedlings were inoculated in each treatment. Experiment IV was designed to investigate effects of temperature on growth and

sporulation of *L. maculans* isolates (Sb1, Sb2, and R2-7). Experiments I, II and III were conducted in temperature regulated growth chambers (15/10, 20/15, 25/20 or 30/25 °C) with three replicates and all the experiments were repeated twice. Experiment IV was conducted in temperature regulated incubators (20, 25 or 30 °C) with three replicates and the experiment was repeated twice.

3.2.4 Inoculation on Cotyledons

In Experiment I and II, seeds of the different genotypes of *B. napus* in **Table 3.1** and **Table 3.2** were seeded into flats of 96 cells with Pre-Mix soil (Sun Gro Horticulture Distribution Inc., Agawam, MA). Flats were placed in growth rooms (22 °C/18 °C, 16/8 h light/dark cycles) and watered every two days or as needed. After 7 days, seedlings of each genotype were inoculated with an inoculum suspension listed in **Table 3.1**.and **Table 3.2**. Each half-cotyledon was wounded with a pair of modified fine forceps, and each seedling had 4 wounds. A 10-µL droplet of spore suspension was deposited on each wound on the 7-day-old seedlings (Delwiche and Williams, 1979). The flats were placed on benches with overhead lights until the inoculum droplet was dried and then returned to the growth rooms or growth chambers for temperature treatment.

3.2.5 Temperature Treatments

Flats of seedlings in Experiments I and II were placed in growth chambers after inoculation. There were four temperature treatments, 15/10, 20/15, 25/20 and 30/25 °C, with 16/8 h light/dark cycle under cool white fluorescent light; for example, 15/10 °C means that the seedlings were treated at 15 °C for 16 h of light exposure and followed by 10 °C for 8 h of darkness. Temperature and relative humidity were monitored with RHT10 Humidity and Temperature USB Data-loggers (FLIR Commercial Systems Inc., Nashua, NH). The temperature variance was

controlled to ± 1 °C, and the relative humidity was maintained between 80 to 90%. Seedlings were watered as needed.

3.2.6 Pathogenicity Tests

In Experiments I and II, flats of seedlings were fertilized with 20:20:20 (N:P:K) two days after inoculation and true leaves were trimmed to delay the senescence of cotyledons. Symptoms on cotyledons were scored for interaction phenotype (IP) at 10 DPI and 14 DPI using a rating scale of 0 (no symptom) to 9 (severe) (**Figure 3.1**) based on lesion size, chlorosis or necrosis, and presence of pycnidia as described in **Table 3.3**. Each isolate was inoculated on at least six seedlings of each genotype. The mean score was calculated from the 24 lesions of the six seedlings for each isolate-*Brassica* genotype and was used to classify the IP into two categories: *Avr* = avirulent or resistant (IP 0–4.9) or *avr* = virulent or susceptible (IP 5–9) (**Table 3.3**).

0 1 3 5 7 9

Figure 3.1. Rating Scale (0–9) (Delwiche, 1980) for cotyledons of *Brassica napus* inoculated with pycnidiospores of *Leptosphaeria maculans*, photographed at 10 DPI.

Table 3.3. Interaction phenotypes (IPs) (Delwiche, 1980) resulting from interaction between *Brassica napus* and *Leptosphaeria maculans* on cotyledons.

Host Response	Average IPs	IPs	Symptoms
Resistant	0–4.9	0	No darkening around the wound, as in controls.
		1	Limited blackening around the wound, lesion diameter is between 0.5–1.5 mm, faint chlorotic halo may be present. Sporulation absent.
		3	Dark necrotic lesions, 1.5–3.0 mm, chlorotic halo may be present; sporulation is absent.
Susceptible	5–9	5*	Non-sporulating 3–6 mm lesions, sharply delimited by a dark necrotic margin, may show grey-green tissue collapse as in interaction phenotype 7 and 9 or dark necrosis throughout.
		7	Green tissue collapse 3–5 mm diameter, sharply delimited non-darkened margin.
		9	Rapid tissue collapses at about 10 days, accompanied by profuse sporulation in large (more than 5 mm) lesions with diffuse margins.

*Occasionally, if cotyledons contacted the soil, senesced under humid conditions, or were observed more than 14 DPI, sporulation may be seen in interaction phenotype 5 (Delwiche, 1980).

3.2.7 Lesion Size Measurement

In Experiment III, Topas DH16516, Topas *Rlm1,* Topas *Rlm2,* Topas *Rlm3* and Topas *Rlm4* were seeded in the method described above. Seven-day-old seedlings were inoculated with a suspension of pycnidiospores of isolates Sb1, Sb2 and R2-7 (**Table 3.2)**. Flats were placed on the light bench until the inoculum dried and then moved into the growth chambers for temperature treatment. Ten inoculated cotyledons in each treatment were sampled daily from 2 to 14 DPI, and lesion size was measured with software Assess 2.0 (Image Analysis Software for Plant Disease Quantification, The American Phytopathological Society, USA) (Lamari, 2008). Effects of temperature on the lesion size on cotyledons were analysed by using SPSS 17.0 software (SPSS, an IBM company, and Chicago, IL, USA). The mean lesion size was compared with Duncan's Multiple Range Test ($p = 0.05$). The relationship between the mean lesion size and the DPI was analyzed by linear regression separately for each genotype-temperature treatment (Uysal and Kurt, 2017).

3.2.8 *In Vitro* Growth Rate and Sporulation of *Leptosphaeria maculans*

In Experiment IV, three *L. maculans* isolates, Sb1, Sb2 and R2-7, were cultured on V8 juice agar at room temperature, as described above. Five days later, a single pycnidiospore was taken from the leading edge of actively growing colonies of each isolate and placed in the centre of a Petri dish (60 × 15 mm) (Fisher Science, Pittsburgh, PA) that was filled with 20 mL V8 juice agar. Petri dishes were wrapped with Parafilm (Fisher Science, Pittsburgh, PA) and placed into incubators. Treatments consisted of three temperatures: 20, 25 and 30 °C with continuous white cool fluorescent light (100–150 $E \cdot m^{-2} \cdot sec^{-1}$). Colony diameter was measured along two axes perpendicular to one another at 3, 5 and 7 DPI and the average of the two diameters was recorded as the radial colony diameter. The Parafilm was removed from Petri dishes at 5 DPI to

allow the formation of pycnidiospores. Isolates were harvested as described previously in **Section 3.2.1** after 7 days of incubation. Spore concentration of each treatment was assessed by counting spores on a hemocytometer after they were diluted 10–40 times. The total number of spores of each treatment was calculated using the formula:

$$\text{Total Spores} = \frac{\text{sum of spores counted in five '4x4 squares'}}{80} \times 400 \times 10^4 \times dilution\ factor \times total\ volume$$

To estimate the growth rate, a linear regression of colony diameters and of total number of spores versus incubation time was conducted for each isolate by using SPSS 17.0 software (SPSS, an IBM company, and Chicago, IL, USA). The mean colony diameters and total number of spores were compared with Duncan's Multiple Range Test (p = 0.05). The relationship between the colony diameters and the temperature was analyzed by linear regression separately for each isolate (Uysal and Kurt, 2017).

3.3 Results

3.3.1 Resistance Evaluation at Different Temperatures

The IPs of each genotype-isolate interaction in Experiment I are listed in **Table 3.4**. Four genotypes, Westar, Darmor, MT29 and 01.23.2.1, were susceptible to inoculated isolates at the growth room condition. Westar (no *Rlm*) was susceptible to all the selected isolates at four temperatures and fewer pycnidiospores were observed on the lesion at 30/25 °C. Darmor (*Rlm9*) inoculated with isolate A10-509A (*AvrLm2,3,6, LepR1, LepR3*) was resistant at 15/10 °C but susceptible at the other three temperatures. MT29 (*Rlm1,9*) inoculated with isolate A10-509A (*AvrLm2,3,6, LepR1, LepR3*) was resistant at the lower temperatures of 15/10 and 20/15 °C and the higher temperature of 30/25 °C; but it was susceptible at 25/20 °C. When MT29 (*Rlm1,9*)

Table 3.4. Interaction phenotypes (IPs) between *Rlm* genes in *Brassica napus* and *Leptosphaeria maculans* under different temperatures. Nine genotypes of *B. napus* were inoculated with *L. maculans* and treated in the growth room and growth chamber with different temperature cycles. IPs were recorded for each treatment.

Genotype	Isolates	Growth[1] Room	Growth Chamber[2]			
			15/10 °C	20/15 °C	25/20 °C	30/25 °C
Westar (no *Rlm*)	All	S[3] (9[4])	S (7.2)	S (9)	S (9)	S (9)
Darmor (*Rlm9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*)	S (9)	R[5] (2.3)	S (5.2)	S (9)	S (9)
MT29 (*Rlm1,9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*)	S (8.3)	R (1.6)	R (4.4)	S (9)	R (4.3)
	DM59 (*AvrLm6,7, LepR3*)	S (8)	R (3.3)	S (5.4)	S (9)	R (4.9)
01.23.2.1 (*Rlm7*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)	S (5)	R (1)	R (1.7)	S (9)	S (5.2)
Forge (*Rlm6*)	DM59 (*AvrLm6,7, LepR3*)	R (1)	R (1)	R (1.3)	R (1.4)	R (1.5)
	A10-509A (*AvrLm2,3,6, LepR1, LepR3*)	R (1)	R (1)	R (1)	R (1.9)	R (2.8)
	A11-736 (*AvrLm*6,7, *LepR1*)	R (1.8)	R (2.1)	R (1.4)	R (2.8)	R (2.6)
Falcon (*Rlm4*)	Sb2 (*AvrLm2,4,6,7*)	R (1.3)	R (1)	R (1.2)	S (6.8)	R (4.1)
Samourai (*Rlm2,9*)	A10-509A (*AvrLm2,3,6, LepR1, LepR3*)	R (1.3)	R (1)	R (1.2)	R (3.2)	R (1.4)
	Sb2 (*AvrLm2,4,6,7*)	R (3.1)	R (1.1)	R (1.4)	R (4.8)	R (1.8)
Quantum (*Rlm3*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)	R (2.6)	R (1)	R (1.1)	S (6.1)	S (5.1)
Surpass400 (*LepR3, RlmS*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)	R (2.8)	R (1.2)	R (1.8)	S (5)	R (4.3)

[1] The growth room was set at 22/18 °C with a 16/8 h light/dark cycle and seedlings were scored 10 days post-inoculation (DPI). The score was re-evaluated at 14 DPI if the first score was ambiguous.
[2] There were four temperature treatments in the growth chamber: 15/10, 20/15, 25/20 and 30/25 °C, with 16/8 h light/dark cycle. At the temperature of 15/10 °C, lesions on the cotyledons appeared 9 DPI, and scoring was evaluated on 11 DPI and adjusted on 14 DPI.
[3] The letter "S" indicates that seedlings were susceptible to inoculated *L. maculans*.
[4] The number in brackets indicates the average IPs of six seedlings. Resistant = IP of 0–4.9; susceptible = IP of 5–9.
[5] The letter "R" indicates that seedlings were resistant to inoculated *L. maculans*.

was inoculated with isolate DM59 (*Lm6,7, LepR3*), it was resistant at 15/10 and 30/25 °C but was susceptible at 20/15 and 25/20 °C. Genotype 01.23.2.1 (*Rlm7*) inoculated with isolate DM59 (*AvrLm6,7, LepR3*) was resistant at 15/10 and 20/15 °C but was susceptible at 25/20 and 30/25 °C. The other five genotypes, Forge (*Rlm6*), Falcon (*Rlm4*), Samourai (*Rlm2,9*), Quantum (*Rlm3*) and Surpass400 (*LepR3, RlmS*) were resistant to inoculated isolates under the growth room temperature (22/18 °C). Forge (*Rlm6*) and Samourai (*Rlm2,9*) were inoculated with more than one isolate and showed consistent resistance under all the temperature treatments. Falcon (*Rlm4*) was susceptible to isolate Sb2 at 25/20 °C and was resistant at the other three temperature treatments. Quantum (*Rlm3*) was resistant to the isolate Sb1 at 15/10 and 20/15 °C, but it was susceptible at 25/20 and 30/25 °C. Surpass400 (*LepR3, RlmS*) was susceptible to isolate Sb1 at 25/20 °C and was resistant at the other three temperatures.

Temperature treatments on Topas ILs in Experiment II are listed in **Table 3.5**. Wildtype Topas DH16516 was susceptible at all temperature treatments. However, fewer pycnidiospores were observed on the lesion at 30/25 °C, although it has not been quantified. Resistant ILs, Topas *Rlm1*, Topas *Rlm2*, Topas *Rlm3* and Topas *Rlm4* showed resistance at all the temperature treatments. No pycnidiospores were observed on cotyledons of resistant genotypes (Topas *Rlm1*, Topas *Rlm2*, Topas *Rlm3*, and Topas *Rlm4*) at any temperatures. At 30/25 °C, Topas *Rlm3* had few large lesions on the cotyledons, which were given an IP score of 7; however, the mean IPs of ten seedlings was 4.9, and no pycnidiospores were observed on the lesions. In the other three resistant genotypes (Topas *Rlm1*, Topas *Rlm2* and Topas *Rlm4*) the average IPs were 2.5, 1.3 and 2.3 respectively at 30/25 °C; they showed strong resistance at the high temperature.

Table 3.5. Interaction phenotypes (IPs) between Topas ILs *and Leptosphaeria maculans* under different temperatures. Five Topas ILs, Topas *Rlm1*, Topas *Rlm2*, Topas *Rlm3*, Topas *Rlm4* and Topas DH16516 inoculated with *L. maculans* and treated at different temperatures.

Topas Genotypes	**Isolates**	**Interaction Phenotype**			
		15/10 °C	**20/15 °C**	**25/20 °C**	**30/25 °C**
DH16516 (no *Rlm* gene)	Sb2 (*AvrLm2,4,6,7*)	S[1] (7.6[2])	S (9.0)	S (9.0)	S (9.0)
Topas *Rlm1* (*Rlm1*)	R2-7 (*AvrLm1,4,6,7*)	R[3] (1.0)	R (1.0)	R (1.3)	R (2.5)
Topas *Rlm2* (*Rlm2*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)	R (1.0)	R (1.0)	R (1.2)	R (1.3)
Topas *Rlm3* (*Rlm3*)	Sb1 (*AvrLm2,3,6, LepR1, LepR3*)	R (1.0)	R (1.0)	R (2.1)	R (4.9)
Topas *Rlm4* (*Rlm4*)	Sb2 (*AvrLm2,4,6,7*)	R (1.0)	R (1.0)	R (2.0)	R (2.3)

[1] The letter "S" indicates that seedlings were susceptible to inoculated *L. maculans*.
[2] The number in brackets indicates the average IP of each treatment. Resistant = IP of 0–4.9; susceptible = IP of 5–9.
[3] The letter "R" indicates that seedlings were resistant to inoculated *L. maculans*.

In several genotypes, such as Falcon (*Rlm4*), Quantum (*Rlm3*) and Surpass400 (*LepR3, RlmS*), resistance genes became ineffective at the higher temperatures of 25/20 or 30/25 °C while in the other genotypes, MT29 (*Rlm1,9*), Darmor (*Rlm9*) and 01.23.2.1 (*Rlm7*), the susceptibility was suppressed at the lower temperatures of 15/10 and/or 20/15 °C. However, tests on Topas ILs showed that *Rlm1, Rlm2, Rlm3* and *Rlm4* are not temperature sensitive. The same *Rlm* genes in different genotypes responded differently to temperature, which indicated that other than single *Rlm* genes, there are other factors in the genetic background that affect the interaction between *Rlm* genes and *L. maculans*. Furthermore, fewer pycnidiospores were observed on the lesions of Westar and Topas DH16516 at 30/25 °C, which indicates that higher temperature may restrict the sporulation of *L. maculans*.

3.3.2 Temperature Effects on Lesion Development in Topas ILs

In Experiment III, lesions enlarged in both susceptible and resistant genotypes as temperature increased. **Figure 3.2** illustrates lesions on cotyledons of Topas ILs at 14 DPI under different temperatures. Lesions on Topas DH16516 developed rapidly as the temperature increased, and over 50% of the cotyledons were damaged when the temperature increased to 30/25 °C after 10 DPI. In resistant genotypes, the lesion size was small at the lower temperatures of 15/10, 20/15 and 25/20 °C and enlarged slightly when temperature increased to 30/25 °C (**Appendices I to IV**).

Lesion development over time on resistant Topas ILs is shown in **Figure 3.3** and **Appendices I to IV**. In Topas *Rlm1* (**Figure 3.3 Panel A**), lesion size was less than 0.470 mm^2 at 15/10 and 20/15 °C, 0.850 mm^2 at 25/20 °C, and 0.825 mm^2 at 30/25 °C at 2 DPI; the lesion sizes at higher temperatures (25/20 and 30/25 °C) were double of those at lower temperatures (15/10 and 20/15 °C). Lesion size increased significantly until 7 DPI at 15/10 °C and enlarged to

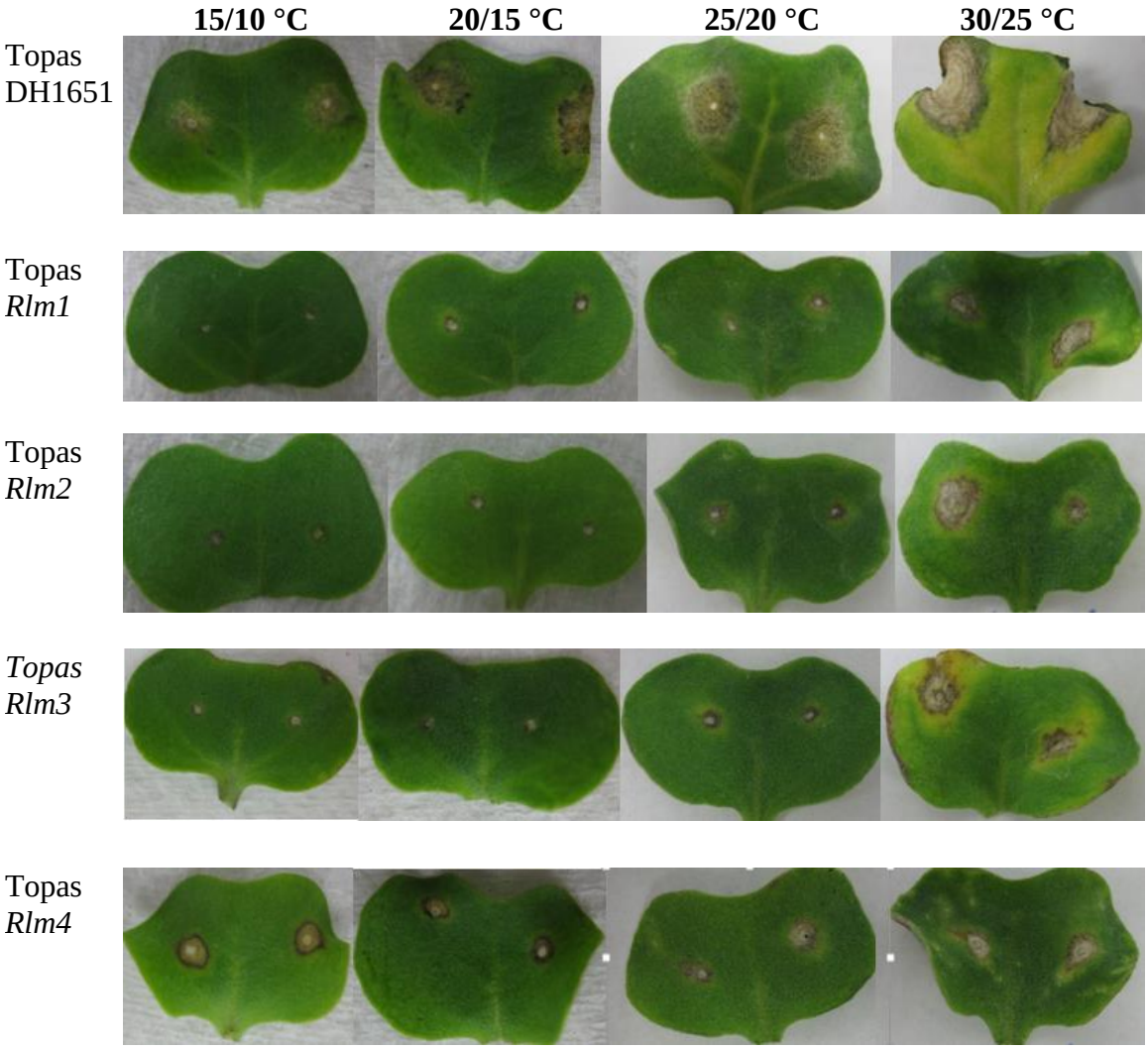

Figure 3.2 Blackleg lesions on cotyledons of Topas ILs at 14 DPI. Five Topas ILs (*Brassica napus*) were inoculated with isolates of *Leptosphaeria maculans* with corresponding *AvrLm* genes and incubated at different temperatures. Pictures were photographed at 14 DPI.

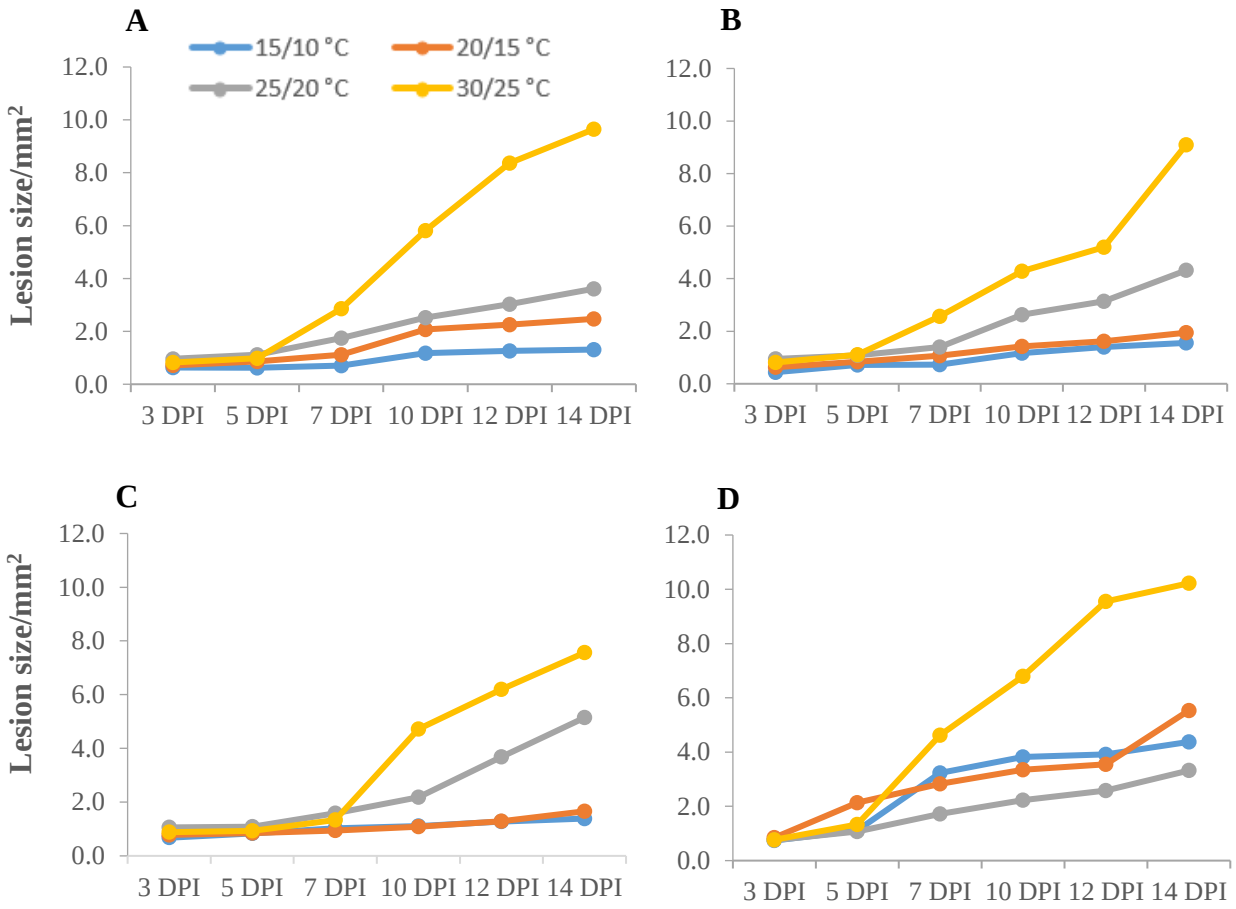

Figure 3.3. Blackleg lesion development on cotyledons of Topas ILs under different temperatures. **Panel A**: Seedlings of Topas *Rlm1* were inoculated with isolate R2-7 (*AvrLm1,4,6,7*). **Panel B**: Seedlings of Topas *Rlm2* were inoculated with isolate Sb1 (*AvrLm2,3,6*). **Panel C**: Seedlings of Topas *Rlm3* were inoculated with isolate Sb1 (*AvrLm2,3,6*). **Panel D**: Seedlings of Topas *Rlm4* were inoculated with isolate Sb2 (*AvrLm2,4,6,7*). Inoculated seedlings were incubated in growth chambers at four different temperatures. Cotyledons were sampled and lesion size was measured at 2–14 DPI.

1.154 mm^2, and was 1.311 mm^2 at 14 DPI. At 20/15 °C, lesion size enlarged significantly to 0.713 mm^2 at 3 DPI, then to 1.264 mm^2 at 8 DPI, and then doubled to 2.472 mm^2 at 14 DPI. At 25/20 °C, the lesion size enlarged significantly to 1.421 mm^2 at 5 DPI, then to 2.047 mm^2 at 7 DPI, and then to 3.613 mm^2 at 14 DPI. At 30/25 °C, lesion size enlarged significantly to 2.244 mm^2 at 5 DPI, and then doubled to 4.722 mm^2 at 7 DPI, then to 9.648 mm^2 at 14 DPI, which is 8-fold larger than the lesion size at 15/10 °C, 4-fold larger than at 20/15 °C and 2.5-fold larger than at 25/20 °C (**Appendix I**).

A similar trend was observed in Topas *Rlm2* (**Figure 3.3 Panel B** and **Appendix II**), Topas *Rlm3* (**Figure 3.3 Panel C** and **Appendix III**), and Topas *Rlm4* (**Figure 3.3 Panel D** and **Appendix IV**). The lesion size was smaller at the lower temperatures of 15/10 and 20/15 °C, and much larger at the higher temperatures of 25/20 and 30/25 °C. Overall, lesion size increased as the temperature increased. Lesions developed slowly at the lower temperatures of 15/10 and 20/15 °C, whereas it developed faster at the higher temperatures of 25/20 and 30/25 °C, which was more obvious on Topas *Rlm2* and Topas *Rlm3*. The largest lesion size was observed on Topas *Rlm 4* (10.2 mm^2) at 30/25 °C at 14 DPI, followed by Topas *Rlm1* (9.648 mm^2), Topas *Rlm2* (9.096 mm^2) and Topas *Rlm3* (7.563 mm^2) at 30/25 °C at 14 DPI.

3.3.3 Temperature Effects on the Growth and Sporulation of *Leptosphaeria maculans*

In Experiment IV, three isolates of *L. maculans*, Sb1, Sb2 and R2-7, were incubated at different temperatures and their colonies were shown in **Figure 3.4**. Sb1 and Sb2 grew faster at 25 °C than at 20 and 30 °C (**Figure 3.5, Panel A and B**). However, the growth of isolate R2-7 increased as temperature increased (**Figure 3.5, Panel C**). Both isolates of Sb1 and Sb2 had an optimum

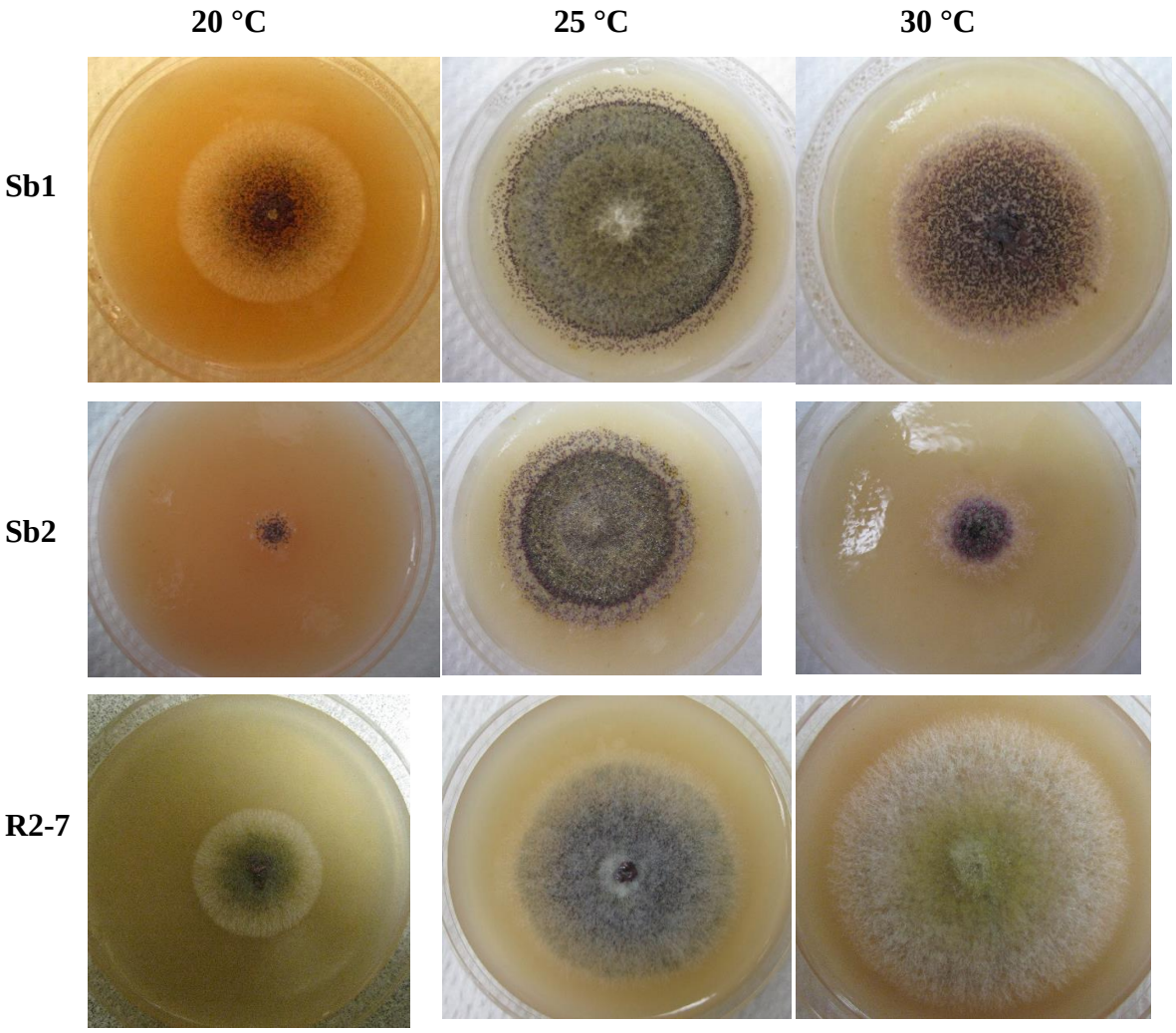

Figure 3.4. The growth of *Leptosphaeria maculans* isolates on V8 juice agar under different temperatures at 7 DPI. Three isolates of *L. maculans* (Sb1, Sb2 and R2-7) were incubated at different temperatures, and the colonies were photographed at 7 DPI.

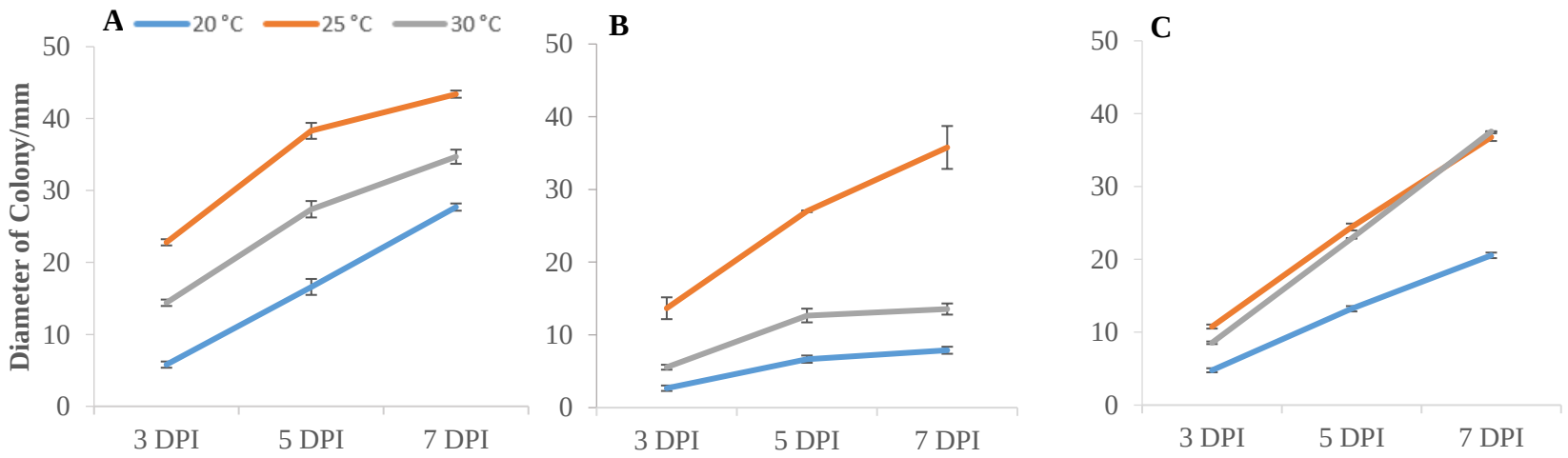

Figure 3.5. Radial colony diameter of *Leptosphaeria maculans* isolates under different temperatures. **Panel A**: Isolate Sb1 was incubated at three different temperatures. **Panel B**: Isolate Sb2 was incubated at three different temperatures. **Panel C**: Isolate R2-7 was incubated at three different temperatures. All colony diameters were measured at 3, 5 and 7 DPI. Bars represent standard error of the mean.

growth temperature of 25 ºC, while isolate R2-7 had an optimum growth temperature of 30 ºC (**Figure 3.5**). The radial colony diameters of Sb1, Sb2 and R2-7 at 25 ºC were 43.44, 35.78 and 36.78 mm, respectively at 7 DPI.

The effects of temperature on sporulation of *L. maculans* isolates were evaluated by the total number of pycnidiospores produced on mycelia from one single pycnidiospore on V8 juice agar. Both isolate and temperature had significant effects on the total number of pycnidiospores, but the interaction between isolate and temperature is not significant ($p < 0.05$). These three isolates produced more pycnidiospores at 25 °C than at 20 and 30 °C (**Figure 3.6**). The total number of pycnidiospores was 4.32×10^9 for Sb1, 3.18×10^9 for Sb2, and 3.4×10^9 for R2-7 at 25 °C. The sporulation was restricted by both lower and higher temperature; however, the lower temperature of 20 °C limited the growth and sporulation more so than the higher temperature of 30 °C. Although isolate R2-7 had the highest radial colony diameter at 30 °C, the total number of pycnidiospores was significantly higher at 25 °C. Overall, the sporulation of *L. maculans* was significantly affected by temperature and isolate. The optimum temperature for the growth and sporulation of *L. maculans* is 25 °C.

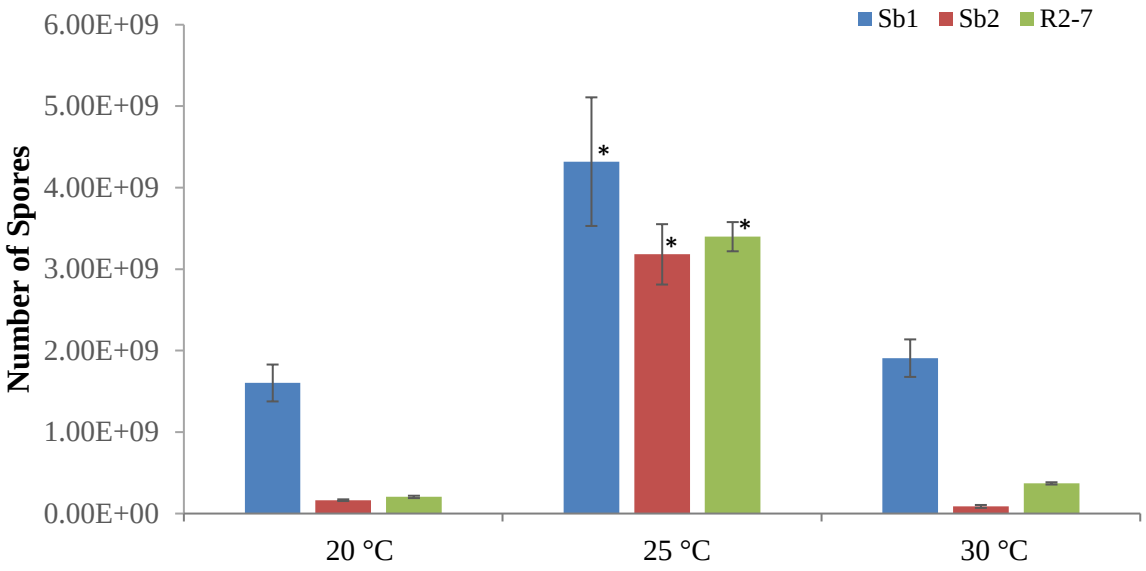

Figure 3.6. The total number of pycnidiospores of *Leptosphaeria maculans* incubating under three different temperatures. The isolates were cultured on V8 juice agar medium and harvested at 7 DPI. The symbol * associated with bars within isolate are significantly different according to the Duncan Multiple Range Test at $p = 0.05$. Bars represent standard error of the mean.

3.4 Discussion

In this study, seedlings of 14 *B. napus* genotypes were inoculated with *L. maculans* isolates with or without corresponding *AvrLm* genes and treated under different temperatures. We found that different *Rlm* genes responded differently to temperature and that the same *Rlm* genes in different genotypes responded differently to temperature. *Rlm1*, *Rlm2*, *Rlm3* and *Rlm4* in Topas ILs and *Rlm6* in *cv* Forge are not sensitive to temperature, and the resistance is still effective at 30/25 °C; there is no resistance breakdown. Meanwhile, *Rlm3* from *cv* Quantum and *Rlm4* from *cv* Falcon are temperature sensitive, where the resistance broke down at higher temperatures of 25/20 °C and 30/25 °C. Our findings are different from previous research, which found that *Rlm1* in Quinta and *Rlm6* in DarmorMX are temperature sensitive (Badawy *et al.*, 1992; Ansan-Melayah *et al.*, 1995; Balesdent *et al.*, 2001; Huang *et al.*, 2006). This research enriched the knowledge and understanding of *Rlm* gene-mediated resistance responding to temperature

changes and shows that the genetic background might play an important role in *Rlm* gene-mediated resistance expression in different *B. napas* genotypes.

In other host-pathogen systems, it has been shown that genetic background has a significant effect on the expression of dominant resistance genes. Flor (1942) found that genetic background is important in determining the degree of dominant resistance gene expression in flax. Research in wheat has indicated that genetic background has a significant effect on the response of infection type (Samborski and Dyck, 1968; Gupta *et al.,* 1984; McIntosh and Dyck, 1975; Roelfs and McVey, 1979). Two *T. aestivum* cultivars, Kanred (*Sr5*) and Reliance (*Sr5*), were immune to stem rust at all temperature treatments, where high temperatures did not influence the resistance expressed by *Sr5* in the genetic background of these two genotypes (Gupta *et al.,* 1984). After *Sr5* was introduced into the background of W2691-W3498, it was tested against stem rust (*P. graminis* f. sp. *tritici*) in the greenhouse at an average temperature of 21 ^{o}C; however, *Sr5*-mediated resistance partially broke down at 30 °C (Gupta *et al.,* 1984). A similar situation occurred for resistance genes *Sr6* and *Sr11*. In other words, when *Sr5* was transferred to the 'susceptible' genetic background of W2691 and W3468, the gene became largely ineffective (Gupta *et al.,* 1984). In this study, *Rlm* genes in certain genotypes are not temperature sensitive, whereas they are ineffective at a higher temperature in other genotypes. The results from this study demonstrate that in the *B. napus-L. maculans* system, genetic background significantly affects the *Rlm* genes expression at different temperature treatments. This study highlights the importance of considering the genetic background in the development of resistant cultivars by introducing *Rlm* genes into existing cultivars or known elite germplasm in breeding programs.

To reduce the interference of genetic background, Topas ILs were used as material to further explore how temperature affects *Rlm* genes-mediated resistance expression and lesion development. Topas ILs were developed by introducing *Rlm* genes (*Rlm1,2,3,4, LepR1, LepR2* and *LepR3*) into a common susceptible *B. napus* background, Topas DH16516 (Borhan, 2013). Four resistant ILs, Topas *Rlm1*, Topas *Rlm2*, Topas *Rlm3* and Topas *Rlm4*, as well as wildtype Topas DH16516, were selected to examine the lesion development under temperature treatments. We found that lesion development was highly temperature related; lesions expanded as the temperature increased in both susceptible and resistant genotypes. Fewer pycnidiospores were produced on lesions of wildtype Topas DH16516 at a higher temperature of 30/25 °C, which revealed that sporulation might be restricted by higher temperature. This has been shown by growth and sporulation tests on three *L. maculans* isolates. Both growth and sporulation of *L. maculans* are restricted by higher and lower temperatures. In certain genotypes of *B. napus* (e.g., Darmor, MT29, and 01.23.2.1), the susceptibility to *L. maculans* was suppressed at lower temperatures; this may also be explained by the limited growth and sporulation of the pathogen at a lower temperature.

Long-term studies have shown that climate change affects both host and pathogen (Frey *et al.*, 1999). Regarding the host, climate change might alter physiological processes (e.g., earlier flowering and fruiting) and increase the risk of springtime frost damage (Puc and Kasprzyk, 2013; Bock *et al.*, 2014; Kasprzyk *et al.*, 2014; Augspurger, 2013). Regarding the pathogen, research on *L. maculans* indicated that pseudothecial development is faster between 5–20 °C (Toscano-Underwood *et al.*, 2003; Aubertot *et al.*, 2006; Kaczmarek and Jedryczka, 2008); similarly, the release of ascospores occurs in the same temperature range (Huang *et al.*, 2005). The formation and maturation rate of fruiting bodies of *L. maculans* changed as temperature

increased, and the subsequent release of ascospores could increase the risk of infection in host plants (Kaczmarek *et al.,* 2016). Research in the UK predicted that climate change could significantly increase economic loss of oilseed rape from blackleg disease, where global warming has increased its range and severity (Kaczmarek *et al.*, 2016). Research has shown that global temperatures continue to rise as each of the last three decades has been successively warmer than any preceding decade since the 1850s (The Science of Climate Change, 2015). Furthermore, warming is not uniform; temperatures in Canada have been increasing at roughly double the global mean rate and the Arctic is warming even faster. Thus a 2 ºC increase globally means a 3 to 4 ºC increase for Canada (The Science of Climate Change, 2015). As a major canola producer in the world, the potential risk of yield loss caused by resistance breakdown at high temperature in Canada could be enormous.

In Manitoba, the recommended seeding date is from May 1st to June 10th for Argentine canola (*B. napus*) and even extends to June 20th for Polish canola (*B. rapa*) (Manitoba Agriculture, 2017). In the past five years, the month of May experienced an average temperature between 10–15 °C, with a maximum temperature of 24.5–35.3 °C in the canola growing regions; in June, the average temperature was 15–19 ºC and the maximum temperature was 28–31 °C (Government Canada, 2017). Here, we set up four temperature treatments that covered the temperature range in the seedling stage of canola in Manitoba. The study provided knowledge of *Rlm* genes temperature sensitivity in multiple canola experimental genotypes and revealed that the genetic background plays an important role in *Rlm* genes resistance expression. This knowledge could also be used in blackleg disease management: when seeding happens late or a hot season is predicted in certain regions, canola cultivars carrying non-temperature sensitive *Rlm* genes are highly recommended to reduce the risk of early infection and potential yield loss.

From this study, more questions arose than were answered. First, how does genetic background affect *Rlm* gene-mediated resistance expression under different temperatures? Second, different genotypes of *B. napus* carrying the same *Rlm* genes respond differently to the same isolate of *L. maculans*. Are there unknown *Rlm* genes in these genotypes or is there an interaction among several *AvrLm* genes in the pathogen isolate? Finally, what breeding efforts in canola are required for the future of Canadian agriculture considering the changing climate that it faces?

Chapter 4

Race Structure Survey of Blackleg in High Erucic Acid Rapeseed and Canola (*Brassica napus*) in Manitoba, Canada

4.0 Abstract

Leptosphaeria maculans (Desm.) Ces. & De Not is responsible for blackleg disease in *Brassica* crops, such as canola and high erucic acid rapeseed (HEAR). Blackleg is the most prevalent disease in western Canada and continues to be a concern for oilseed rape production across the Prairie Provinces. The disease has been managed effectively with genetic resistance and four-year rotations. However, survey results have indicated that blackleg disease incidence and severity are on the rise. Expanding acres of oilseed rape carrying single resistance genes and shorter rotations have placed selection pressure on the pathogen population, which has reduced the efficacy of resistance genes in the host. Furthermore, new races with new virulence genes could be generated during the sexual reproduction of *L. maculans*. Maintaining genetic resistance to blackleg and reducing yield loss are priorities for oilseed rape growers and associated industries. This study examined *L. maculans* isolates derived from HEAR and canola stubble in commercial fields in Manitoba. Infected stubble samples were collected in HEAR and canola fields in 2013 and 2014 growing season across Manitoba to survey the presence and frequency of *AvrLm* genes in the pathogen population. A total of 297 isolates of *L. maculans* were identified for the presence of 12 avirulence (*AvrLm*) alleles: *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2* and *AvrLmS* by using a differential set of *Brassica* genotypes carrying known resistance genes and PCR amplification of *AvrLm5* and *AvrLm11*. The combinations of these 12 *Avr* genes could theoretically generate up to 2^{12} (4096) different races of the fungus. An updated knowledge of the race structure of *L. maculans* is therefore needed to ensure the better use of available major resistance genes for both farmers and breeders. The objective of this study was to characterize the race structure of *L. maculans* population in Manitoba. This study found that several alleles were more prevalent in the

pathogen population, namely *AvrLm6* (100%) and *AvrLm5* (90.91%). Subsequently, *AvrLm7, AvrLm4, AvrLm11* and *AvrLm2* were present in 78.1%, 72.7%, 64.7% and 59.3% of isolates, respectively. *AvrLm3* and *AvrLm9* were detected at a very low frequency (less than 3.0%). Eighty-five races were identified in the population, with one prevalent race, namely *AvrLm2-4-5-6-7-11* (i.e., virulent to *Rlm1*, *Rlm3*, *Rlm9*, *LepR1*, *LepR2* and *RlmS*), representing 14.8% of the population. Races of *AvrLm2-4-5-6-7-11-S, AvrLm5-6, AvrLm1-4-5-6-7-11-(S)* and *AvrLm2-4-5-6-7* represented 3.4–5.7% of the population, while 40 races only had one isolate in each. *AvrLm9* was not detected in 2014 HEAR fields, but it represented 4.1% and 3.8% of the population in 2013 HEAR fields and 2013 canola fields, respectively. Isolates from 2013 canola fields had a higher frequency of *AvrLm1, AvrLm3, AvrLm4, AvrLm5, AvrLm7* and *AvrLm11* than isolates from 2013 and 2014 HEAR fields, while *AvrLepR1* and *AvrRlmS* were present at a lower frequency in both years.

4.1 Introduction

Oilseed rape is the third leading source of edible oil in the world, and canola (*Brassica napus* L.) is one of the major oilseed rape crops produced globally. According to a study released in 2017, canola contributes $26.7 billion to the Canadian economy annually (Canola Council of Canada, 2017b). Other than canola for edible oil production, breeding efforts have also focused on high erucic acid rapeseed (HEAR) for producing industrial erucic acid. Erucic acid (also known as *cis*-13-docosenoic acid, C22:1) is an unbranched, monounsaturated fatty acid with 22 carbon atoms and a double bond at the cis-13 position of the carbon chain (Sanyal *et al.,* 2015). Erucic acid is a valuable renewable resource and raw material for the manufacture of a wide range of industrial products: plastic, nylon 13-13, high temperature lubricant polymers, cosmetics, surfactant detergents, plasticizers and pharmaceuticals (Mikolajczak, 1961; Lazzeri *et al.*, 1994; Bhardwaj and Hamama, 2000; Dyer *et al.*, 2008; Carlsson *et al.*, 2011). Products derived from erucic acid are environmentally-friendly due to its enhanced biodegradability and could be an alternative to petroleum-based products (Coonrod *et al.*, 2008).

Currently, the major source of erucic acid is HEAR oil (Leonard, 1992; Sonntag, 1995; Foglia, 2001). The first HEAR (*Brassica napus* L.) variety was developed by breeders from the Agriculture and Agri-Food Canada Saskatoon Research Centre; however, the meal had a high level of glucosinolate, which made it unsuitable for livestock consumption (Stefansson and Downey, 1995). Breeders from the University of Manitoba developed and registered the second HEAR variety, Reston (*B. napus*) in 1982 (Stefansson and Downey, 1995). Reston seeds contained erucic acid levels as high as 40–48% and had a medium level of glucosinolate (Stefansson and Downey, 1995). Subsequently, more HEAR cultivars have been developed and released by breeders from the University of Manitoba, in which the erucic acid content was as

high as 62–64% (McVetty *et al.*, 2009; Stefansson, 1983; Stefansson and Downey, 1995; Canola Council of Canada, 2017a). Nath *et al.* (2008, 2009) reported the first transgenic SHEAR line with a 72% erucic acid content in the seed oil by co-expression of the rape fatty acid elongase gene (*FAE1*) with the LPAAT gene (Ld-LPAAT) from *Limnanthes douglasii* L.

The fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & De Not is the causal agent of blackleg disease in oilseed rape, including canola and HEAR. Blackleg is the most economically important disease of oilseed rape in North America, Europe, Australia and other growing regions (West *et al.*, 2001; Howlett, 2004). Currently, the use of resistant cultivars is one of the most efficient methods to manage this disease in canola and other oilseed rape (Kutcher, 2013). Race-specific *Rlm* genes were first observed in Europe and Australia as an effective method for managing blackleg disease on oilseed rape (Howlett, 2004). However, populations of *L. maculans* adapted rapidly under selection pressure from the large-scale cultivation of cultivars with certain single resistance genes. The breakdown to resistant cultivars of canola has been reported in several countries: resistance genes *Rlm1* in France (Rouxel *et al.*, 2003a) and *LepR3* in Australia (Li *et al.*, 2003; Sprague *et al.*, 2006) became ineffective within three growing seasons in commercial fields. A similar result was obtained for *Rlm6*, which was overcome in three growing seasons under artificially increased inoculum pressure (Brun *et al.*, 2000). In Canada, the breakdown of *Rlm3* was observed in cultivars carrying the resistance gene *Rlm3,* which was associated with low frequency of *AvrLm3* detected in the population of *L. maculans* (Zhang *et al.*, 2016).

In earlier research, isolates of *L. maculans* were characterized by using the pathogenicity group (PG) (Mengistu *et al.*, 1991), which was a useful tool to recognize variations in the pathogenicity of populations in the early 1990s in western Canada (Kutcher *et al.*, 2010b).

However, as pathogen race surveys were conducted on larger scales, the information obtained from the PG system became limited since this system is based on three or four resistance genes only, while more than 18 major resistance genes have been identified and reported (Kutcher *et al*., 2010b; Zhang *et al.*, 2016). Theoretically, following the gene-for-gene interaction, there would be 2^{18} or 262,144 races of the pathogen if all 18 resistance genes are different from each other. In the race structure survey conducted by Liban *et al.* (2016) in western Canada, 17 different races were identified in a single PG group, PG2, which indicated that the characterization of isolates of *L. maculans* into races based on the *Avr* allele frequencies and patterns (corresponding to known resistance genes in the host) would provide better knowledge and understanding of the host-pathogen interaction and race structure variation than the PG system (Kutcher *et al.*, 2010b; Liban *et al*., 2016). Cotyledon inoculation on oilseed rape cultivars with known *Rlm* genes has been used to evaluate resistance to *L. maculans* (Williams and Delwiche, 1979; Rimmer and van den Berg, 1992; Rouxel *et al.* 2003b; Marcroft *et al.* 2012). Kutcher *et al.* (2010b, 2011) reported the frequency of ten *Avr* alleles (*AvrLm1-7, 9, 10* and *AvrLepR3*) for 96 isolates of *L. maculans* collected in western Canada between 1997 and 2005, and 16 races were identified with seven races accounting for 89.7% of the isolates. Liban *et al.* (2016) identified the frequency of 10 *Avr* alleles (*AvrLm1–4, 6, 7, 9*, *AvrLepR1* and *2*, and *AvrLmS*) for 674 isolates of *L. maculans* collected across western Canada in 2010 and 2011, and 55 races were identified in total. Furthermore, 150 races were identified from 300 isolates collected in Manitoba in 2012 (Zhang *et al.*, 2016). These race surveys and *Avr* allele identifications were conducted on seedlings of a differential set of *Brassica* genotypes with known *Rlm* genes. The survey results reflected that the frequencies of *Avr* alleles changed over time in western Canada. Thus, it is necessary and important to continuously monitor the

pathogen population in terms of the frequency of *Avr* alleles, race structure and regional variation. The information on the frequency of individual *Avr* alleles present in the pathogen population could provide insights into the corresponding resistance genes present in the commercial cultivars and their potential durability for blackleg management. The knowledge helps breeders select resistance sources and develop effective strategies to manage blackleg disease across western Canada. The major objective of this study was to identify the presence and frequency of *AvrLm* genes in the *L. maculans* population in commercial HEAR and canola cultivars across Manitoba.

4.2 Materials and Methods

4.2.1 Sample Collection

High erucic acid rapeseed and canola stubble samples with blackleg symptoms in the basal stem were collected from commercial fields in Manitoba from the 2013 and 2014 growing seasons. In each field, 20 to 40 infected stubble samples were collected and retained in individual paper bags. The maps of sampled fields are shown in **Figures 4.1**.

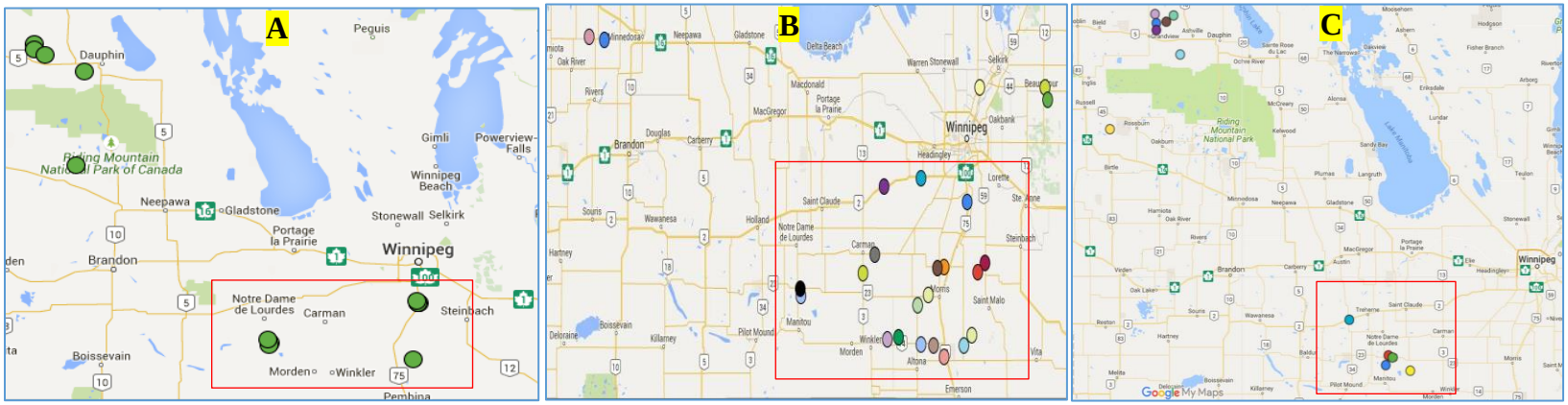

Figure 4.1. Map of locations where HEAR and canola stubble samples were collected across Manitoba in 2013 and 2014. **Panel A**: Fourteen HEAR fields were visited in the 2013 growing season. Nine fields located south of Winnipeg were categorized as the South region of Manitoba (red frame). **Panel B**: Twenty-five canola fields were visited in the 2013 growing season. Twenty fields located south of Winnipeg were categorized as the South region of Manitoba (red frame). **Panel C**: Twelve HEAR fields were visited from the 2014 growing season. Five fields located south of Winnipeg were categorized as the South region of Manitoba (red frame).

4.2.2 Pathogen Isolation

The infected stubble was cut into small pieces and surface sterilized with 30% bleach for 2 min and then dried under the sterile hood for 5 min prior to incubation in Petri dishes (Fisher Science, Pittsburgh, PA) filled with V8 juice agar [200 mL V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 mL distilled water, 15 g agar, 0.75 g calcium carbonate ($CaCO_3$) and 0.1 g streptomycin sulfate]. Petri dishes were placed on the light bench under cool white fluorescent lights (100–150 $E \cdot m^{-2} \cdot sec^{-1}$) at room temperature for 5 to 8 days. When blackleg hyphae and conidia became apparent in the culture media, samples were selected while excluding other fungal growths and transferred onto another fresh V8 juice agar media under a dissecting microscope. After 5 to 7 days, the conidial ooze from single pycnidia was picked and transferred from the edge of the colony to a fresh V8 juice agar media plate by using a fine needle under a dissecting microscope. The new plates were placed on the light bench at room temperature, and single spore isolates were cultured after 5 to 8 days. For each infected stubble sample, one isolate culture was selected. This was the method used to isolate blackleg pathogens from samples collected in the 2013 growing season.

A different method was used to isolate blackleg from samples collected in the 2014 growing season due to the high level of contamination. In the first two steps, water agar media [1000 mL distilled water, 15 g agar, 0.1 g streptomycin sulphate] was used to replace the V8 juice agar media. Colonies that appeared to be blackleg were transferred to the water agar media until no contamination was present. After 5 to 10 days, the conidial ooze from single pycnidia of blackleg was picked with a fine needle under a dissecting microscope and cultured on a fresh V8 juice agar media plate to attain a single spore isolate.

4.2.3 Inoculum Suspension and DNA Samples

Pycnidiospores of blackleg were harvested by flooding the culture plate with 4 mL of sterile distilled water and then scraping the surface with microscope slides. Spore suspensions were collected in two sterile centrifuge tubes (Fisher Science, Pittsburgh, PA); the upper layer was collected for inoculum, and the bottom layer was the residual mixture of hyphae, pycnidia and spores, and collected for DNA extraction. For long-term storage of the isolates, paper disks were saturated with the spore suspension, then dried in the sterile hood, and retained in centrifuge tubes under refrigeration at -20 °C. The inoculum suspension was diluted to 1×10^7 spores·mL^{-1} by estimating spore concentration on a hemocytometer (Hausser Scientific Company, Horsham, PA) and stored in 10 mL sterile centrifuge tubes at -20 °C.

4.2.4 The Plant Differential Set

Both *L. maculans* and *L. biglobosa* could be isolated from infected stubble tissue. These two pathogens were distinguished by inoculating on the cotyledons of canola cv. Westar, which shows resistance to *L. biglobosa* and susceptibility to *L. maculans* (Kutcher *et al.*, 2010b). A differential set of *Brassica* genotypes carrying known different single or multiple *Rlm* genes was used to identify races of *L. maculans*. The differential set consisted of 11 genotypes listed in **Table 4.1** including '*cv*. Westar'-no *Rlm* (Balesdent *et al.*, 2002), 'Topas *Rlm1*'-*Rlm1* (Borhan, 2013), 'Topas *Rlm2*'-*Rlm2* (Borhan, 2013), '02.22.2.1'-*Rlm3* (Gout *et al.*, 2006), 'Jet Neuf'-*Rlm4* (Gout *et al.*, 2006), 'Forge'-*Rlm6* (tested by colleague in Fernando Lab-unpublished), '01.23.2.1'-*Rlm7* (Dilmaghani *et al.*, 2009), 'Darmor'-*Rlm9* (Delourme *et al.*, 2004), '1065'-*LepR1* (Kutcher *et al.*, 2010a), '1135'-*LepR2* (Kutcher *et al.*, 2010a) and 'Surpass400'-*LepR3*, *RlmS* (Larkan *et al.*, 2013).

Table 4.1. *Brassica napus* genotypes used as differentials to identify avirulence genes of *Leptosphaeria maculans* isolates.

Genotype	Blackleg Resistance Genes
Westar	No *Rlm* gene
Topas *Rlm1*	*Rlm1*
Topas *Rlm2*	*Rlm2*
02-22-2-1	*Rlm3*
Jet Neuf	*Rlm4*
Forge	*Rlm6*
01.23.2.1	*Rlm7*
Darmor	*Rlm9*
1065	*LepR1*
1135	*LepR2*
Surpass400	*LepR3, RlmS*

4.2.5 Pathogenicity Tests and Race Structure

The seeds of the plant differential set (**Table 4.1)** were seeded into 96-cell flats with Pre-Mix soil (Sun Gro Horticulture Distribution Inc., Agawam, MA). Flats were placed in growth rooms (22/18 °C, 16/8 h light/dark cycles) and watered every two days or when needed. After 7 days, six seedlings of each genotype were inoculated with the pycnidiospore suspension. Each half-cotyledon was wounded by a pair of modified fine forceps. A 10-µL droplet of spore suspension was deposited on each wound on cotyledons (Delwiche and Williams, 1979). Flats were placed on the light benches until the inoculum droplet was air dried and then moved back to growth rooms. Two days after inoculation, the plants were fertilized with 20:20:20 (N:P:K) and true leaves were trimmed to delay the senescence of the cotyledons. Interaction phenotypes (IPs) were scored at 10 days post-inoculation (DPI) and 14 DPI based on the symptoms on cotyledons. The IP rating scale and description are shown in **Figure 3.1** and **Table 3.3** of the previous chapter. The mean score of 24 lesions (from six seedlings) for each genotype-isolate was used to

classify the IPs into one of two categories: *Avr* = avirulent or resistant (IP 0–4.9) or a*vr* = virulent or susceptible (IP 5–9). The *Avr* alleles of an isolate could be determined from the combinations of IPs for all genotype-isolate pairs. The genotype(s) with multiple resistance genes was deducted from multiple phenotypic reactions to determine which resistance gene was responsible for the resistance response. For instance, 'Surpass400' carries two *Rlm* genes, *LepR3* and *RlmS*. The *AvrLm* gene(s) of *AvrLepR3* and/or *AvrLmS* in the isolates cannot be determined from the reaction of 'Surpass400' alone since either of the two *Rlm* genes can induce resistance against an isolate of *L. maculans* carrying *AvrLepR3* or *AvrLm*S or both. There were limitations with this plant differential set. First, there were no cultivars available to identify *AvrLm5, AvrLm8, AvrLm10, AvrLm11* and *AvrLepR4* in the isolates. *AvrLm*5 and *AvrLm11* could be only determined with polymerase chain reaction (PCR) amplification of the cloned fungal gene using markers. Second, the plant differential set was unable to determine the presence or absence of *AvrLepR3* and *AvrLmS* if the isolate carries *AvrLm1* at the same time; this is because *LepR3* is also able to interact with *AvrLm1* (Larkan *et al.*, 2012). Thus, *AvrLmS* can only be deduced in isolates without *AvrLm1*. The isolates were classified into races based on the *AvrLm* genes they carried, as described by Balesdent *et al.* (2005). For example, the race *AvrLm2-4-5-6-11* is composed of isolates that possess *AvrLm* genes of *AvrLm2, AvrLm4, AvrLm5, AvrLm6* and *AvrLm11*.

4.2.6 DNA Extraction and PCR

The DNA extraction procedure used in this study was a modified version developed by Lee and Taylor (1990). When single spores of blackleg isolates appeared on fresh V8 juice agar media after being cultured for 5 to 8 days, the spores were harvested for DNA extraction. The samples were thawed on ice, centrifuged and reduced to a volume less than 0.5 mL. A lysis buffer

(containing Tris, EDTA, SDS, and NaCl) was added to the samples. The mixture was then lysed with mechanical beads at 5000 RPM for 45 s and incubated at 65 °C for 30 min with a gentle invert every 5 min. Then, it was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated with 5 M NaCl. Following the final centrifugation, the DNA pellet was washed several times with ice-cold ethanol and dried under a fume hood. The DNA was dissolved in 50–100 µL of autoclaved distilled water.

Polymerase chain reactions were performed on DNA samples in 25-µL reactions [buffer 2.5 µL; dNTP 2.5 µL; $MgCl_2$ 3 µL; forward primer 0.25 µL; reverse primer 0.25 µL, Taq 0.2 µL; DNA 2 µL (30–80 ng/µL); water 14.3 µL]. DNA samples of isolates were identified as *L. maculans* or *L. biglobosa* by using the primers targeting the ITS (internally transcribed spacer) region (**Table 4.2**). The identified *L. maculans* isolates were screened for the presence or absence of *AvrLm1* (Gout *et al.,* 2006), *AvrLm2* (Ghanbarnia *et al.,* 2015), *AvrLm5* (Van de Wouw *et al.,* 2014), *AvrLm6* (Fudal *et al.,* 2009), *AvrLm47* (Parlange *et al.,* 2009), and *AvrLm11* (Balesdent *et al.,* 2013), using the primers listed in **Table 4.2**. PCRs were run on the Bio-Rad T100^{TM} thermal cycler with the following program: 3 min at 95 °C; 30 cycles of 15 s at 95 °C, 30 s at 70 °C, 1 min at 72 °C, and finally, 5 min at 72 °C. PCR products of the six cloned *AvrLm* genes were run on 1.5% agar gel under a voltage of 110 V for 30 min. Pictures were taken, and the *Avr* genes were recorded when the corresponding bands showed up on the gels.

Table 4.2. Primers to identify *Leptosphaeria biglobosa* and *Leptosphaeria maculans* and avirulence genes of *L. maculans* by using PCR.

Genes	**Primers (5'-3')**
ITS (for *L. maculans* and *L. biglobosa*)	F 5′-CCG TTG GTG AAC CAG CGG AGG GAT C-3′ R 5′-TCC GCT TAT TGA TAT GCT TAA G-3′
AvrLm1	F 5′-CTA TTT AGG CTA AGG GTA TTC ATA AG-3′ R 5′-GCG CTG TAG GCT TCA TTG TAC-3′
AvrLm2	F 5′-CGT CAT CAA TGC GTT CGG-3′ R 5′CTG GAT CGT TTG CAT GGA-3′
AvrLm4-7	F 5′-TAT CGC ATA CCA AAC ATT AGG C-3′ R 5′-GAT GGA TCA ACC GCT AAC AA-3′
AvrLm5	F 5′-ACA ACC ACT CTT CAC AGT-3′ R 5′-TGG TTT GGG TAA AGT TGT CCT-3′
AvrLm6	F 5′-TCA ATT TGT CTG TTC AAG TTA TGG A-3′ R 5′-CCA GTT TTG AAC CGT AGT GGT AGC A-3′
AvrLm11	F 5′-TGC GTT TCT TGC TTC CTA TAT TT-3′ R 5′-CAA GTT GGA TCT TTC TCA TTC G-3′

4.3 Results

4.3.1 Avirulence Genes Frequency and Race Structure of *Leptosphaeria maculans* Isolates Collected in HEAR Fields in the 2013 growing season

In total, 81 blackleg isolates were identified from HEAR stubble samples collected in the 2013 growing season, which included 74 *L. maculans* isolates and seven *L. biglobosa* isolates. All 12 *Avr* genes (*AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2* and *AvrLmS*) were identified from these 74 isolates of *L. maculans*. Since *LepR3* is also able to interact with *AvrLm1* (*Rlm1* typically interacts with *AvrLm1*), the plant differential set was unable to determine the presence or absence of *AvrLepR3* and *AvrLm*S if the isolate carries *AvrLm1* at the same time (Larkan *et al.*, 2013). *AvrLmS* can only be deduced in isolates without *AvrLm1*. In the entirety of 74 isolates of *L. maculans*, *AvrLmS* was only confirmed in 67 isolates, since *AvrLm1* was present in seven isolates. None of the 12 identified *Avr* genes were absent within the population, although there was a large variation in frequency observed among these genes. The frequencies of *Avr* genes were as low as 1.3% in *AvrLm3* and up to 100% in *AvrLm6*. Six other *AvrLm* genes were tested at low frequencies: *AvrLm9* (5.4%), *AvrLepR2* (8.1%), *AvrLm1* (17.6%), *AvrLepR1* (24.3%), *AvrLmS* (32.4%) and *AvrLm11* (35.1%). Along with *AvrLm6*, four other *AvrLm* genes were tested in more than half of the isolates: *AvrLm2* (62.2%), *AvrLm4* (67.6%), *AvrLm7* (77.0%) and *AvrLm5* (91.9%). The frequency of each *Avr* gene in these 74 isolates of *L. maculans* is shown in **Figure 4.2**.

Since 12 *Avr* genes were identified in 74 *L. maculans* isolates, theoretically, there could be 2^{12} or 4096 combinations. Each combination of *Avr* genes is a unique race of *L. maculans* (Balesdent *et al.*, 2005). **Figure 4.3** illustrates all identified *L. maculans* races and their relative frequencies of samples collected in HEAR fields in the 2013 growing season. Thirty-five races

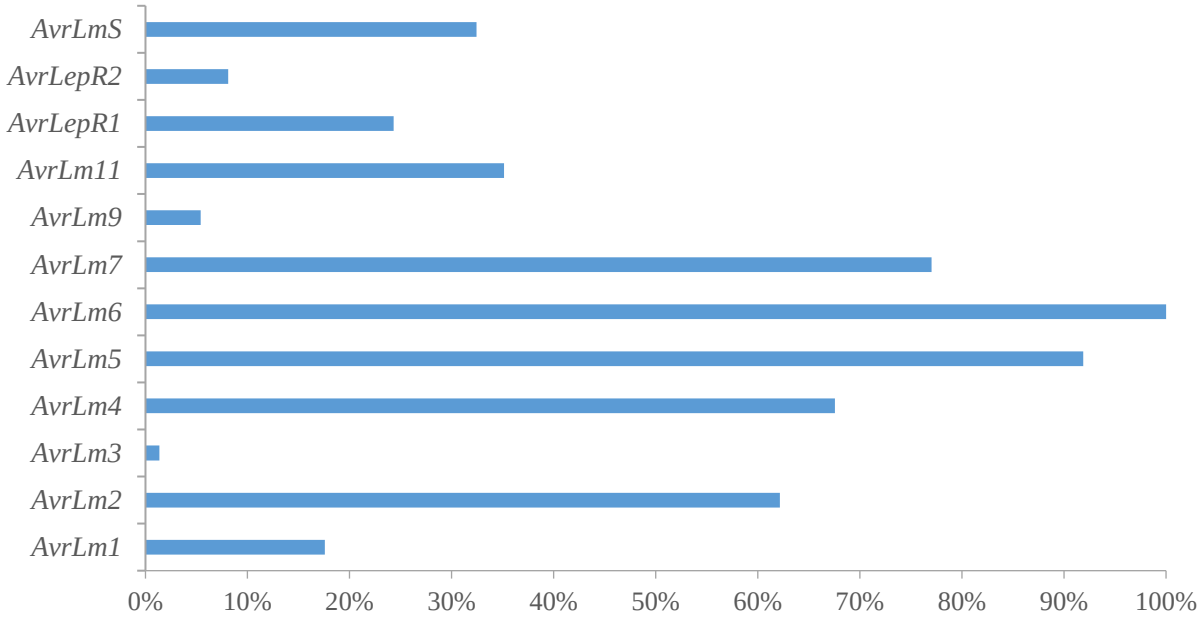

Figure 4.2. The frequency of avirulence alleles in a collection of 74 *Leptosphaeria maculans* isolates collected in HEAR fields in the 2013 growing season. Twelve *Avr* genes were identified. *AvrLmS* was assessed in 67 isolates and could not be confirmed in seven isolates due to the presence of *AvrLm1* in these isolates.

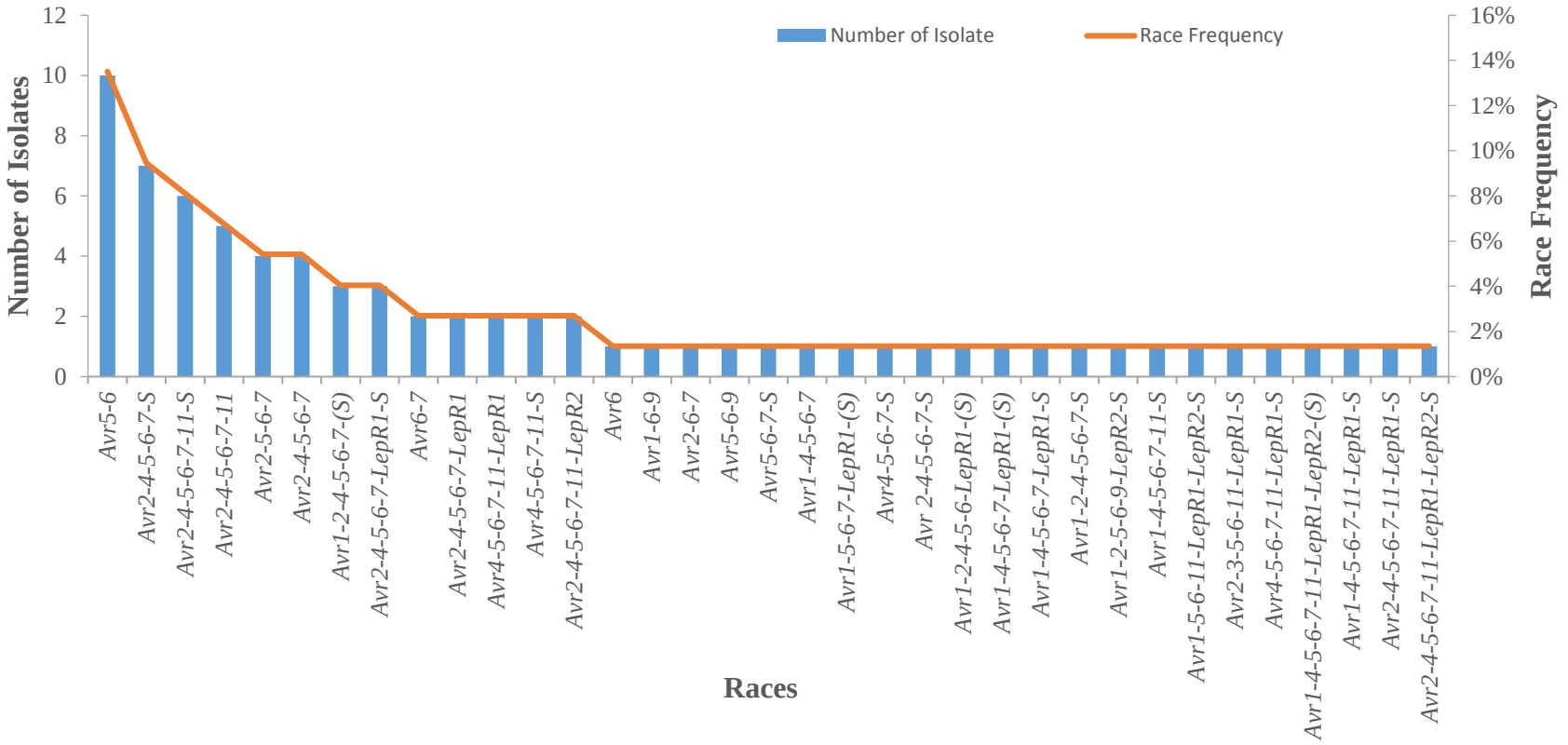

Figure 4.3. Thirty-five races of *Leptosphaeria maculans* and their frequencies identified in 74 isolates collected in HEAR fields in the 2013 growing season. Race structure was identified based on twelve avirulence genes. *(S)* indicates that the presence or absence of *AvrLmS* is unknown and *AvrLmS* was assessed in 67 isolates and could not be confirmed in seven isolates due to the presence of *AvrLm1* in these isolates.

were identified with a percentage as high as 13.5% of *AvrLm5-6* to as low as 1.4% of a single isolate. There were six main races, *AvrLm5-6* (13.5%), *AvrLm2-4-5-6-7-S* (9.5%), *AvrLm2-4-5-6-7-11-S* (8.1%), *AvrLm2-4-5-6-11* (6.8%), *AvrLm2-5-6* (5.4%) and *AvrLm2-4-5-6-7* (5.4%), that contained four or more isolates and these six races accounted for 48.6% of the population. These six main races all carry *AvrLm5* and *AvrLm6*. There were 22 races, each race presented by one single isolate only, which accounted for 62.9% of the total races.

The race structure of the pathogen population was evaluated in terms of complexity (number of *Avr* genes carried by each isolate). Most isolates carry six or seven *AvrLm* genes, with an average of 5.28, accounting for 56.8% of the population. Most (78.4%) of the isolates carry between four to nine *AvrLm* genes. Approximately one-third (33.8%) of isolates carry six *AvrLm* genes, 23.0% of isolates carry seven *Avr* genes, and 9.5% of isolates carry five *AvrLm* genes. *AvrLm2-4-5-6-7-11-LepR1-LepR2-S* carries nine *AvrLm* genes, missing only *AvrLm1*, *AvrLm3* and *AvrLm9*. Another race, composed of one single isolate, carried only *AvrLm6*.

4.3.2 Frequency of Avirulence Genes and Race Structure of *Leptosphaeria maculans* Isolates Collected in Canola Fields in the 2013 growing season

A total of 134 isolates were identified from samples collected from canola fields in the 2013 growing season that included 106 isolates of *L. maculans* and 28 isolates of *L. biglobosa*. All 12 *Avr* genes (*AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2* and *AvrLmS*) were identified from these 106 isolates of *L. maculans*, except for *AvrLmS*, which was assesses in 81 isolates. None of the 12 identified *Avr* genes were absent within the population. As illustrated in **Figure 4.4**, the frequency of *Avr* genes varied from 3.8% in both *AvrLm3* and *AvrLm9* to 100% for *AvrLm6*. Four other *AvrLm* genes were found at low frequencies including *AvrLepR2* (6.6%), *AvrLepR1* (12.3%), *AvrLmS* (19.8%) and

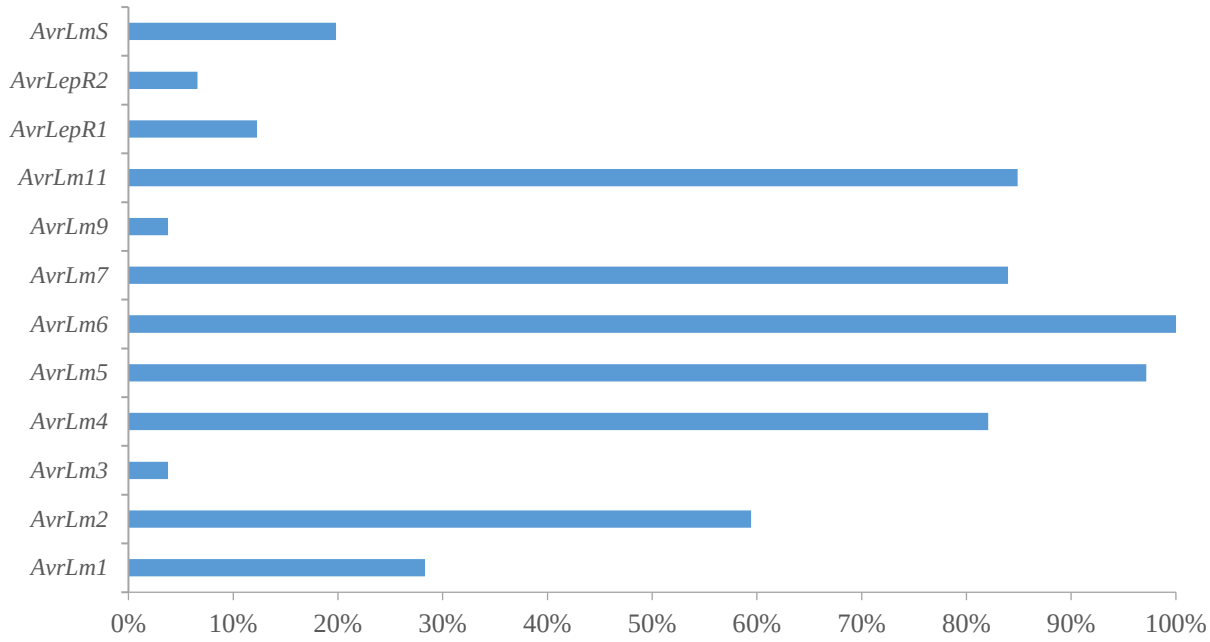

Figure 4.4. The frequency of avirulence alleles in a collection of 106 *Leptosphaeria maculans* isolates collected in canola fields in the 2013 growing season. Twelve *Avr* genes were identified. *AvrLmS* was assessed in 81 isolates and could not be confirmed in 25 isolates due to the presence of *AvrLm1* in these isolates.

AvrLm1 (28.3%). Along with *AvrLm6,* five other *AvrLm* genes were tested at high frequency including *AvrLm2* (59.4%), *AvrLm4* (82.1%), *AvrLm7* (84.0%), *AvrLm11* (84.9%) and *AvrLm5* (97.2%).

Figure 4.5 shows the identified *L. maculans* races and their relative frequencies from samples collected in canola fields in Manitoba in the 2013 growing season. In total, 41 races were identified, of which the six most prevalent races were *AvrLm2-4-5-6-7-11* (20.8%), *AvrLm1-4-5-6-7-11* (9.4%), *AvrLm2-4-5-6-7-11-S* (6.6%), *AvrLm4-5-6-7-11* (5.7%), *AvrLm2-5-6-7-11* (4.7%) and *AvrLm1-2-4-5-6-7-11* (3.8%). These main races contained four or more isolates of each and accounted for 50.9% of the population. There were 21 races, each race represented by only one single isolate, which accounted for 51.2% of the total races and 19.8% of the population.

The race structure of the pathogen population was evaluated in terms of complexity (number of *Avr* genes carried by each isolate). Most isolates carried five or six *Avr* genes, with an average of 5.82, accounting for 61.3% of the population. As many as 95.3% of the isolates carried between four and nine *AvrLm* genes, 35.9% of isolates carried six *AvrLm* genes, 25.5% of isolates carried five *AvrLm* genes, and 17.9% of isolates carried seven *AvrLm* genes. Three races (*AvrLm1-2-3-4-6-7-9-LepR1-LepR2-(S), AvrLm2-4-5-6-7-11-LepR1-LepR2-S* and *AvrLm2-3-4-5-6-9-11-LepR2-S*) contained nine *AvrLm* genes; they all commonly carry four *AvrLm* genes including *AvrLm2*, *AvrLm4, AvrLm6,* and *AvrLepR2*. There was only one single isolate that carried two *AvrLm* genes, *AvrLm5-6*.

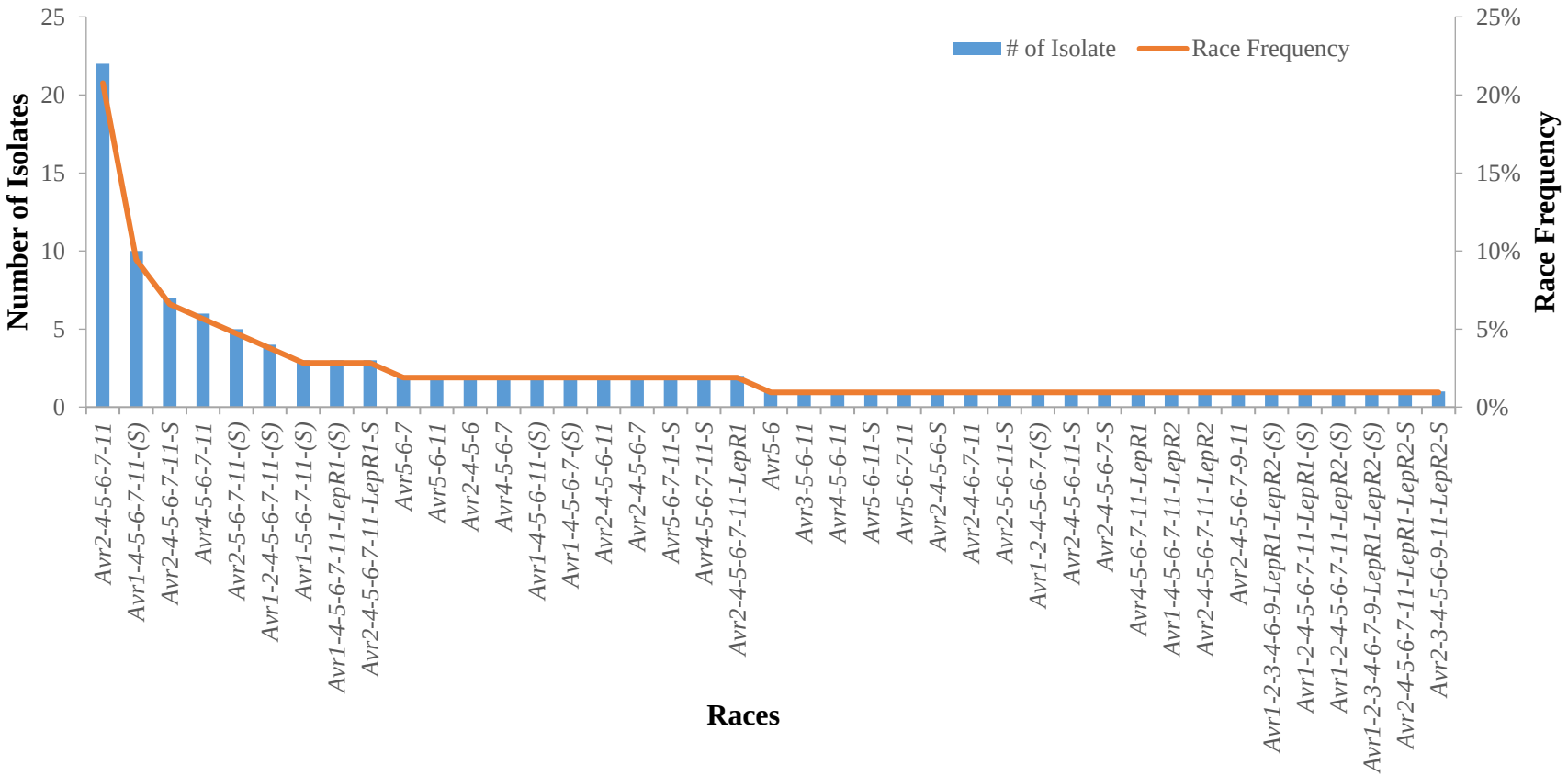

Figure 4.5. Major races of *Leptosphaeria maculans* and their frequencies identified in 106 isolates collected in canola fields in the 2013 growing season. Race structure was identified based on twelve avirulence genes. *(S)* indicates that the presence or absence of *AvrLmS* is unknown and *AvrLmS* was assessed in 81 isolates and could not be confirmed in 25 isolates due to the presence of *AvrLm1* in these isolates.

4.3.3 Frequency of Avirulence Genes and Race Structure of *Leptosphaeria maculans* Isolates Collected in HEAR Fields in the 2014 growing season

A total of 171 blackleg isolates were identified from samples collected from HEAR fields in Manitoba in the 2014 growing season, which included 117 isolates of *L. maculans* and 54 isolates of *L. biglobosa*. Twelve *Avr* genes (*AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2* and *AvrLmS*) were identified from these 117 *L. maculans* isolates, and the frequency is illustrated in **Figure 4.6**. *AvrLmS* was confirmed in 100 isolates only because *AvrLm1* was present in 17 isolates. *AvrLm6* was detected in all of the isolates, but *AvrLm9* was not detected in the population. Five other *AvrLm* genes were found at low frequencies including *AvrLm3* (2.6%), *AvrLepR2* (3.4%), *AvrLepR1* (16.2%), *AvrLm1* (19.7%) and *AvrLmS* (24.8%). Along with *AvrLm6,* five other *AvrLm* genes were tested at high frequency including *AvrLm2* (58.1%), *AvrLm11* (65.0%), *AvrLm4* (68.4%), *AvrLm7* (74.4%) and *AvrLm5* (84.6%).

As illustrated in **Figure 4.7**, 53 races were identified in 117 *L. maculans* isolates, and the relative frequency varied from 14.5% of *AvrLm2-4-5-6-7-11* to 0.9% with a single isolate. There were eight races, *AvrLm2-4-5-6-7-11* (14.5%), *AvrLm5-6* (4.3%), *AvrLm2-4-5-6-11* (4.3%), *AvrLm2-5-6-7-11-S* (4.3%), *AvrLm1-4-5-6-7-11-LepR1-(S)* (4.3%), *AvrLm2-4-5-6-7* (3.4%), *AvrLm*4*-5-6-7-11-S* (3.4%) and *AvrLm2-4-5-6-7-11-S* (3.4%), that contained four or more isolates, and they accounted for 41.9% of the population. These eight main races all carry *AvrLm5* and *AvrLm6,* and seven of them carry *AvrLm7*. There were 29 races, each race represented by only a single isolate, which accounted for 54.7% of the total races and 24.8% of the population.

The race structure of the pathogen population was evaluated in terms of complexity (number of *Avr* genes carried by each isolate). Most isolates carry five or six *AvrLm* genes, with an average of 5.18, accounting for 58.1% of the population. In detail, 83.8% of the isolates carry between four and nine *AvrLm* genes, 34.2% of isolates carry six *AvrLm* genes, 23.9% of isolates carry five *Avr* genes, and 12.0% of isolates carry seven *AvrLm* genes. There was no presence of races carrying nine *AvrLm* genes, while four races, *AvrLm2-4-5-6-7-11-LepR1-LepR2*, *AvrLm1-4-5-6-7-11-LepR1-LepR2*, *AvrLm4-5-6-7-11-LepR1-LepR2-S* and *AvrLm2-4-5-6-7-11-LepR1-S* carry eight *AvrLm* genes with five common genes including *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11* and *AvrLepR2*. Four races, *AvrLm1-6*, *AvrLm5-6*, *AvrLm6-11* and *AvrLm6-7* only carry two *AvrLm* genes.

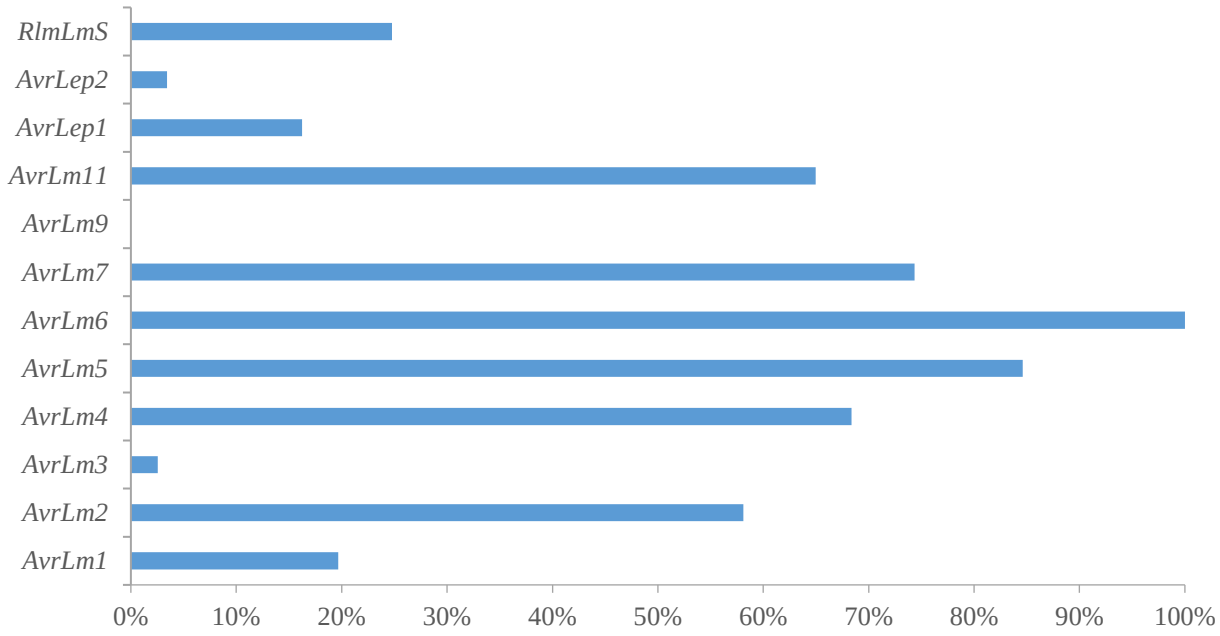

Figure 4.6. The frequency of avirulence alleles in a collection of 117 *Leptosphaeria maculans* isolates collected in HEAR fields in the 2014 growing season. Twelve *Avr* genes were identified. *AvrLmS* was assessed in 100 isolates and could not be confirmed in 17 isolates due to the presence of *AvrLm1* in these isolates.

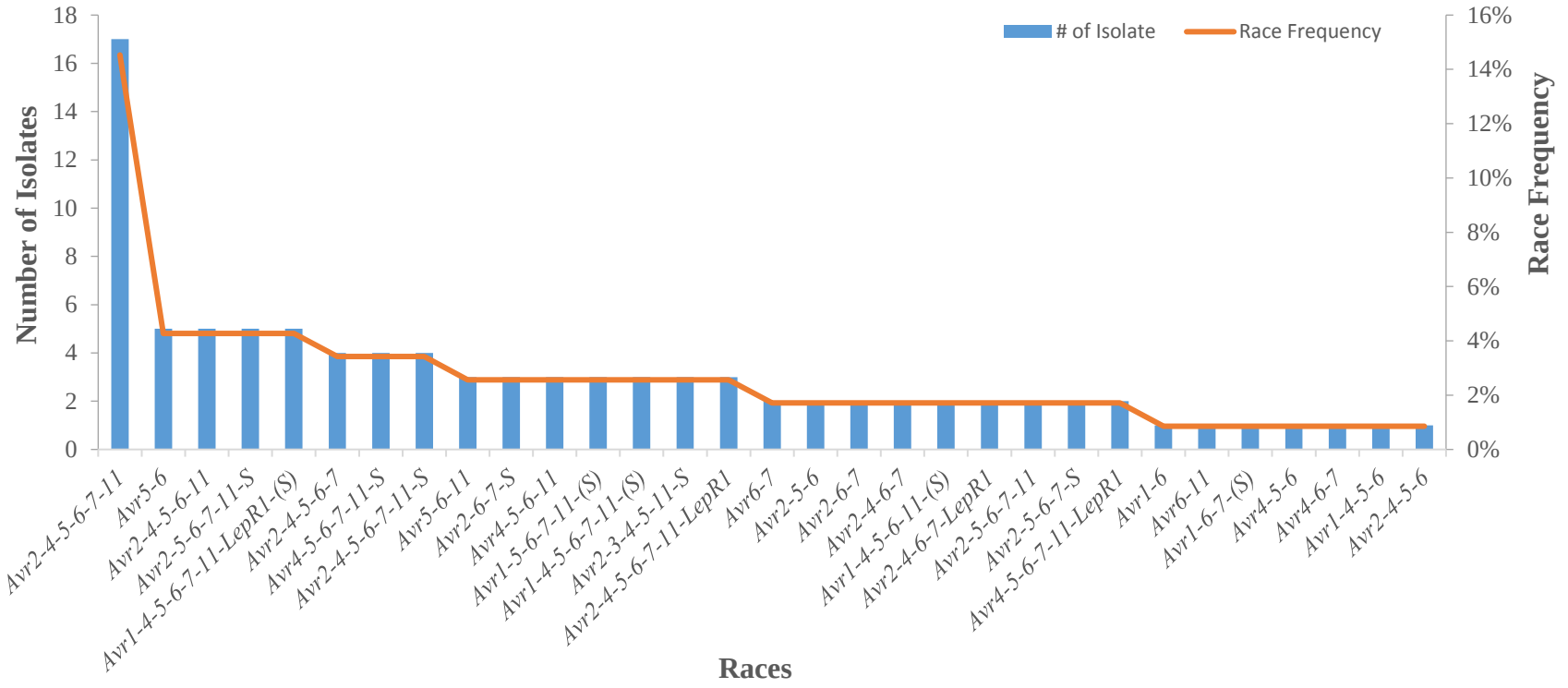

Figure 4.7. Major races of *Leptosphaeria maculans* and their frequencies identified in 117 isolates collected in HEAR fields in the 2014 growing season. Race structure was identified based on twelve avirulence genes. *(S)* indicates that the presence of *AvrLmS* is not known and *AvrLmS* was assessed in 100 isolates and could not be confirmed in 17 isolates due to the presence of *AvrLm1* in these isolates.

4.3.4 Frequency of Avirulence Genes and Race Structure of *Leptosphaeria maculans* in 2013 to 2014

To reflect *L. maculans* race structure in the population in oilseed rape in Manitoba, isolates collected in HEAR and canola fields were pooled together. In total, 386 blackleg isolates were collected in oilseed rape across Manitoba in 2013 to 2014, which included 89 isolates of *L. biglobosa* and 297 isolates of *L. maculans*. Twelve *Avr* genes, *AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm5, AvrLm6, AvrLm7, AvrLm9, AvrLm11, AvrLepR1, AvrLepR2* and *AvrLmS,* were all identified from these 297 *L. maculans* isolates, and the frequency of each *Avr* gene is illustrated in **Figure 4.8**. *AvrLmS* was only confirmed in 248 isolates since *AvrLm1* was present in 49 isolates. The *Avr* gene *AvrLm6* was fixed within the population, while the frequencies among the other *AvrLm* genes were observed to be highly variable. The frequency of the *Avr* gene was as low as 2.4% in *AvrLm9*. Five other *AvrLm* genes were detected at low frequency including *AvrLm3* (2.7%), *AvrLepR2* (5.4%), *AvrLepR1* (16.5%), *AvrLm1* (22.2%) and *AvrLmS* (27.3%). Along with *AvrLm6,* five other *AvrLm* genes were found at high frequencies including *AvrLm2* (59.3%), *AvrLm11* (64.7%), *AvrLm4* (72.7%), *AvrLm7* (78.1%) and *AvrLm5* (90.9%).

In total, 85 races were identified from these 297 isolates collected in 2013 to 2014 in Manitoba, and the relative frequency of each race is illustrated in **Figure 4.9**. There were eight races, *AvrLm2-4-5-6-7-11* (14.8%), *AvrLm2-4-5-6-7-11-S* (5.7%), *AvrLm5-6* (5.4%), *AvrLm1-4-5-6-7-11-(S)* (4.4%), *AvrLm2-4-5-6-7* (3.4%), *AvrLm2-4-5-6-7-S* (3.0%), *AvrLm4-5-6-7-11-S* (2.7%) and *AvrLm1-4-5-6-7-11-LepR1-(S)* (2.7%) that contained 8 to 44 isolates, which accounted for 42.1% of the population. These eight main races all carry *AvrLm5* and *AvrLm6*, and seven of them carry *AvrLm4* and *AvrLm7*. There were 40 races, each race represented by

only one single isolate, which accounted for 47.0% of the total races and 13.5% of the population.

Most isolates carry five or six *AvrLm* genes, with an average of 5.29, accounting for 55.6% of the population. About 86.5% of the isolates carry four to nine *AvrLm* genes, 34.7% of isolates carry six *AvrLm* genes, 20.9% of isolates carry five *AvrLm* genes, and 16.9% of isolates carry seven *AvrLm* genes. There were three races carrying nine *AvrLm* genes, *AvrLm1-2-3-4-6-7-9-LepR1-LepR2-(S)*, *AvrLm2-3-4-5-6-9-11-LepR2-S* and *AvrLm2-4-5-6-7-11-LepR1-LepR2-S*, while only one race, *AvrLm6*, carried one *AvrLm* gene.

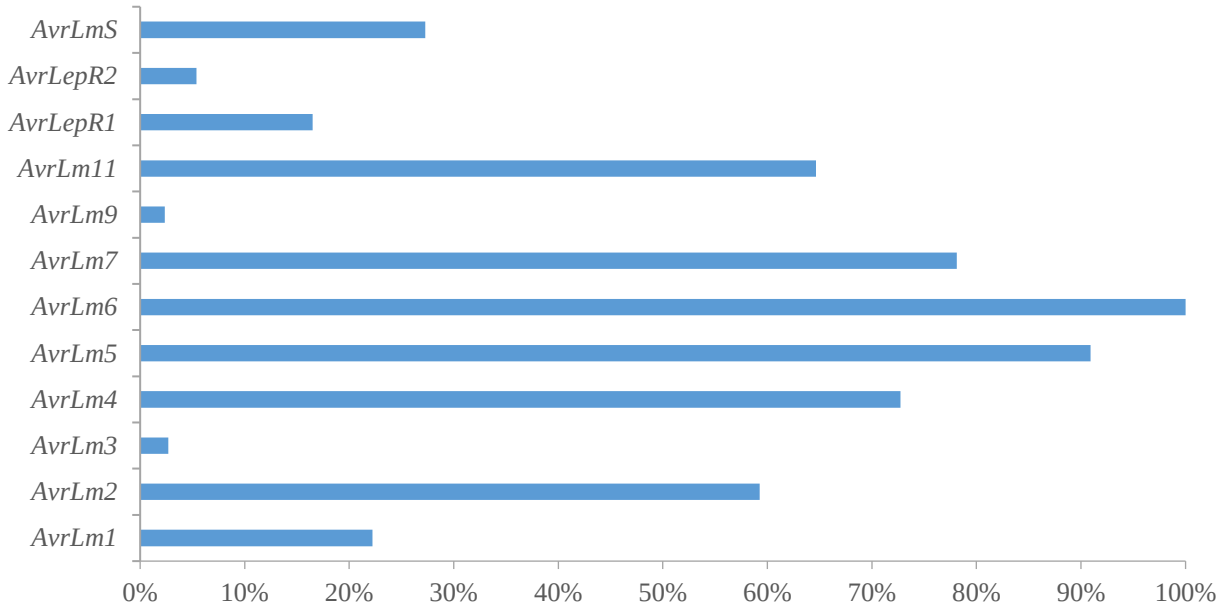

Figure 4.8. The frequency of avirulence alleles in a collection of 297 *Leptosphaeria maculans* isolates collected in 2013 to 2014 in Manitoba. Twelve avirulence genes were identified. *AvrLmS* was assessed in 248 isolates and could not be confirmed in 49 isolates due to the presence of *AvrLm1* in these isolates.

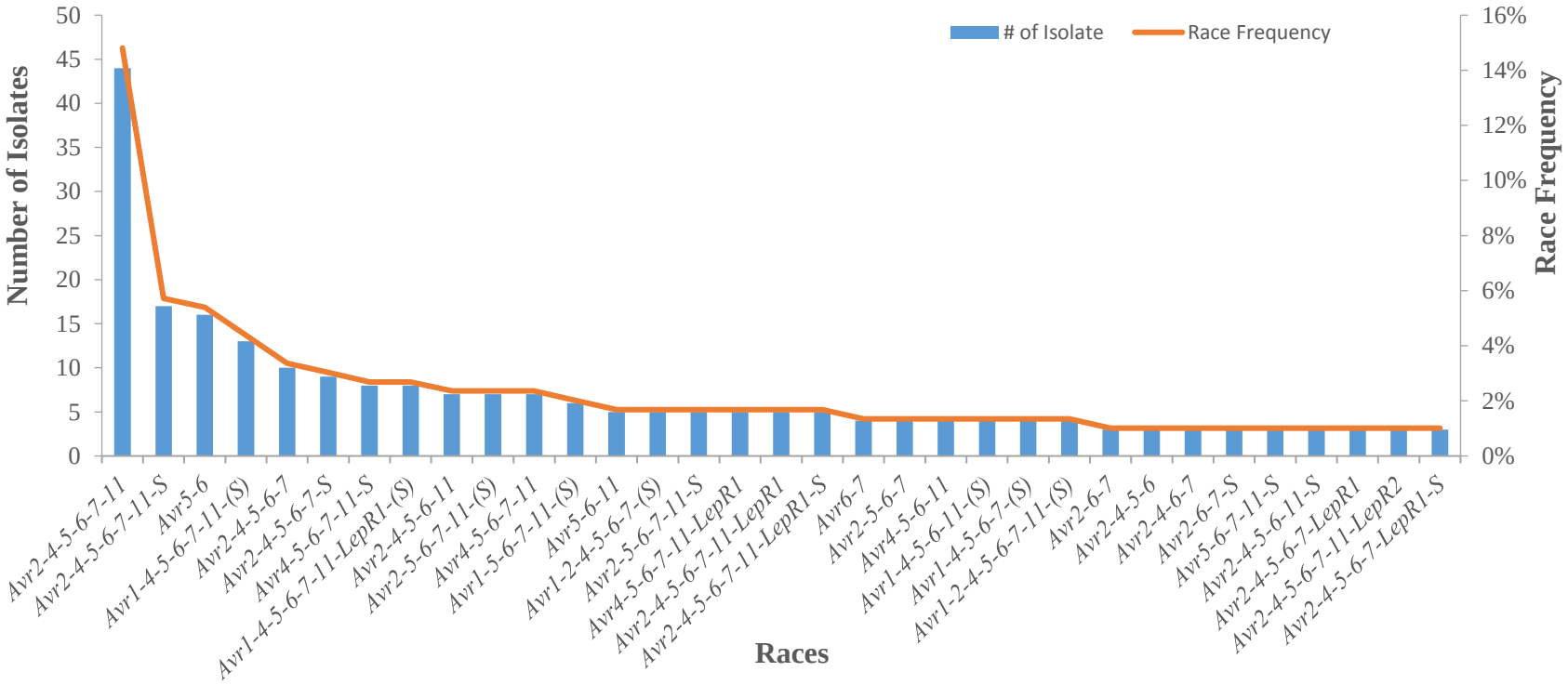

Figure 4.9. Major races of *Leptosphaeria maculans* and their frequencies identified in 297 isolates collected in oilseed rape in Manitoba in 2013 to 2014. Race structure was identified based on twelve avirulence genes. *(S)* indicates that the presence of *AvrLmS* is not known and *AvrLmS* was assessed in 248 isolates and could not be confirmed in 49 isolates due to the presence of *AvrLm1* in these isolates.

4.3.5 Frequency Variation of Avirulence Genes in *Leptosphaeria maculans* Population between HEAR and Canola and Between Seasons

The frequency of twelve *Avr* genes varied in certain *AvrLm* gene loci not only between HEAR and canola but also between the seasons of 2013 and 2014, with both increases and decreases observed (**Figure 4.2, 4.4 and 4.5**). The frequencies of *AvrLm1* (28.3%), *AvrLm4* (82.1%), *AvrLm5* (97.2%), *AvrLm7* (84.0%) and *AvrLm11* (84.9%) in 2013 canola fields were higher than in HEAR fields in both 2013 and 2014, while *AvrLepR1* (12.3%) and *AvrLmS* (19.8%) were lower than in HEAR fields in both 2013 and 2014.

Among isolates sampled from HEAR fields, the frequencies of *AvrLm11* increased markedly by 29.8%, while *AvrLm1*, *AvrLm3* and *AvrLm4* increased only slightly, by less than 2.2%. The frequency of *AvrLmS* decreased from 32.4% in 2013 to 24.8% in 2014. Seven other *Avr* genes (*AvrLm2*, *AvrLm5*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS*) decreased slightly within a range of 1.3% to 7.3%. *AvrLm9* was not detected in samples collected in the 2014 growing season.

4.3.6 Regional Frequency Variation of Avirulence Genes in *Leptosphaeria maculans* Population in Manitoba

To compare the regional variations of *Avr* genes in the *L. maculans* population in Manitoba, the sampled fields were divided into two categories based on their location: southern region and northern region (**Figure 4.1**). As shown in **Figure 4.1**, fields located within the red frames were categorized as southern region, and all other fields were categorized as northern region. The frequency of *Avr* genes from southern region and northern region are listed in **Figure 4.10** by crop and growing season.

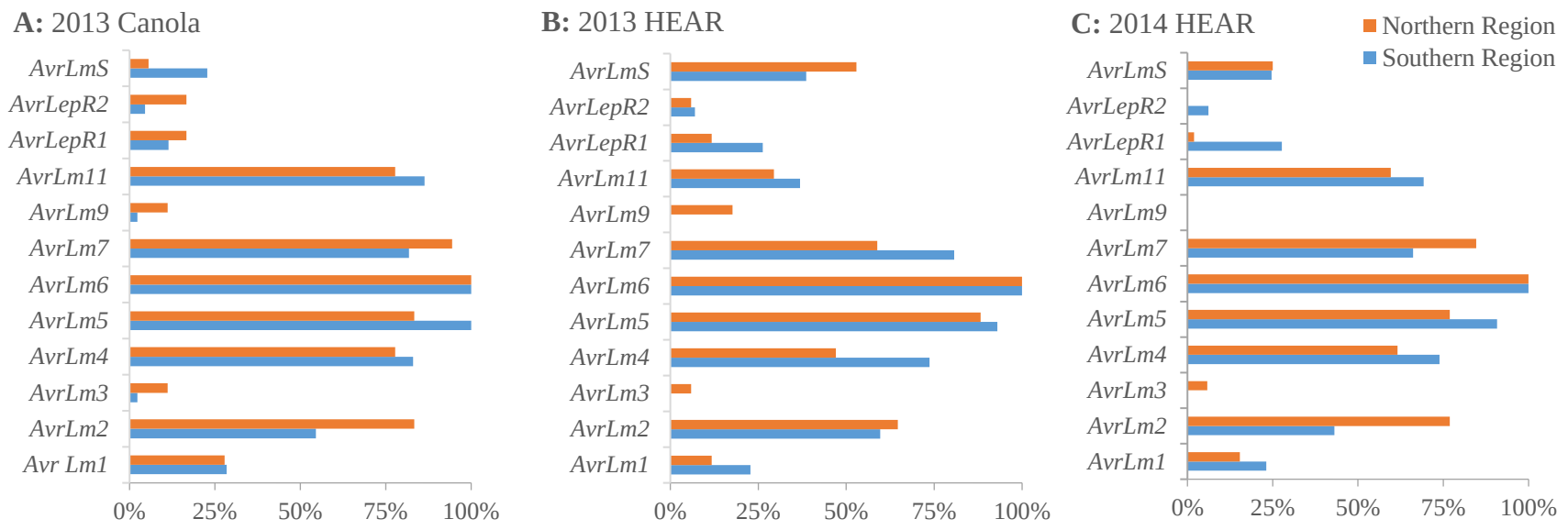

Figure 4.10. The frequency of avirulence alleles in *Leptosphaeria maculans* isolates collected in HEAR and canola fields in Manitoba in 2013 to 2014. A comparison was made between southern region and northern region by crop and growing season. Twelve avirulence genes were characterized. **Panel A**: *L. maculans* isolates were collected from canola fields in the 2013 growing season. Eighty-eight isolates were from southern region, and 18 isolates were from northern region. **Panel B**: *L. maculans* isolates were collected from HEAR fields in the 2013 growing season. Fifty-seven isolates were from the southern region, and 17 isolates were from the northern region. **Panel C**: *L. maculans* isolates were collected in HEAR fields in the 2014 growing season. Sixty-five isolates were from southern region, and 52 isolates were from northern region.

In the *L. maculans* isolates collected in 2013 HEAR fields, 57 isolates were collected from the southern region, with confirmed *AvrLmS* in 50 isolates, and 17 isolates were collected from the northern region, with confirmed *AvrLmS* in nine isolates, based on the twelve identified *Avr* genes. *AvrLm3* and *AvrLm9* were not detected in the southern region, but were detected in the northern region at 5.9% and 17.7%, respectively. Furthermore, the frequencies of *AvrLm2* and *AvrLmS* in the southern region were lower than that in northern region. The remaining seven *AvrLm* genes (*AvrLm1*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm11*, *AvrLepR1* and *AvrLepR2*) were present in the southern region at a higher frequency, ranging from 1.1% to 26.6% compared to the northern region (**Figure 4.10 Panel B**).

In the *L. maculans* isolates collected in 2013 canola fields, 88 isolates were collected from the southern region, with confirmed *AvrLmS* in 69 isolates, and 18 isolates were collected from the northern region, with confirmed *AvrLmS* in nine isolates, based on the twelve identified *Avr* genes. *AvrLm3* and *AvrLm9* were present in both the Southern and northern regions; however, the frequencies in the southern region [*AvrLm3* (2.3%) and *AvrLm9* (2.3%)] were much lower compared to the northern region [*AvrLm3* (11.1%) and *AvrLm9* (11.2%)]. Additionally, *AvrLm2*, *AvrLm7*, *AvrLepR1* and *AvrLepR2* were all present at lower frequency in the southern region than in the northern region, with a difference ranging from 5.1% for *AvrLepR1* to 28.8% for *AvrLm2*. The frequencies of five other *AvrLm* genes (*AvrLm1*, *AvrLm4*, *AvrLm5*, *AvrLm11* and *AvrLmS*) were higher in the southern region compared to the northern region. Both *AvrLm5* and *AvrLm6* were detected in all of the isolates in the southern region, while *AvrLm5* was detected in 83.3% of the isolates in the northern region (**Figure 4.10 Panel A**).

In the *L. maculans* isolates collected in 2014 HEAR fields, 65 isolates were collected from the southern region, with confirmed *AvrLmS* in 53 isolates, and 52 isolates were collected

from the northern region, with confirmed *AvrLmS* in 47 isolates based on the twelve identified *Avr* genes. *AvrLm3* was not detected in the southern region, while there was a frequency of 5.8% in the northern region. *AvrLm9* was not detected in either southern or northern regions. *AvrLepR2* was not detected in the northern region, while there was a frequency of 6.2% in the southern region. *AvrLepR1* was present in 27.7% of isolates in the southern region and only 1.9% of isolates in the northern region. Furthermore, the frequencies of four other *AvrLm* genes (*AvrLm1*, *AvrLm4*, *AvrLm5* and *AvrLm11*) were 7.7% to 13.9% higher in the southern region compared to the northern region. The frequency of *AvrLm2* was 33.8% less in the southern region than in the northern region, and the frequencies of *AvrLm7* and *AvrLmS* in the southern region were 13.9% and 0.4% less, respectively, compared to the northern region (**Figure 4.10 Panel C**).

Since more than half of *L. maculans* isolates were collected in the southern region in both HEAR and canola fields and in both seasons, only isolates collected in the southern region were compared to explore the frequency variation of *AvrLm* genes between crops and seasons. As illustrated in **Figure 4.10 Panel A to C, Southern Region**, 57 isolates were collected from HEAR fields in the 2013 growing season, with confirmed *AvrLmS* in 50 isolates; 88 isolates were collected from canola fields in the 2013 growing season, with confirmed *AvrLmS* in 69 isolates; and 65 isolates were collected from HEAR fields in the 2014 growing season, with confirmed *AvrLmS* in 53 isolates. In both 2013 and 2014, *AvrLm3* and *AvrLm9* were absent from HEAR fields (**Figure 4.10 Panel B and C**), while they were present in 2.3% of the population in 2013 canola fields (**Figure 4.10 Panel A**). *AvrLm1*, *AvrLm4*, *AvrLm5*, *AvrLm7* and *AvrLm11* were present at a higher frequency in canola fields than in HEAR fields.

4.4 Discussion

This study identified 85 races based on 12 *AvrLm* genes in 297 isolates of *L. maculans* collected from HEAR and canola fields in Manitoba in the 2013 and 2014 growing season. Isolates from HEAR and canola fields were tested separately. This study is the first to report the frequency of avirulence alleles and race structure of *L. maculans* in HEAR for two growing seasons and compared race structure of *L. maculans* between HEAR and canola fields.

In earlier research, Kutcher *et al.* (2010b) identified 16 races from 96 isolates of *L. maculan*s based on 10 *AvrLm* genes, and Liban *et al.* (2016) detected 55 races from 674 isolates based on 10 *AvrLm* genes in western Canada. A blackleg disease survey conducted in Manitoba in 2012 identified 150 races from 300 isolates of *L. maculans* based on 12 *AvrLm* (Zhang *et al.*, 2016). Identified races dramatically increased with the increase of identified *Avr* alleles in *L. maculans.* In this study, the main races were *AvrLm2-4-5-6-7-11* (14.8%), *AvrLm2-4-5-6-7-11-S* (5.7%), *AvrLm5-6* (5.4%), *AvrLm1-4-5-6-7-11-S* (4.4%), and *AvrLm2-4-5-6-7* (3.4%). These top five races accounted for 33.7% of the population. The other 80 races contained less than 10 isolates for each race and occurred at a frequency of less than 3.4% based on our sample size of 297 isolates; among these, 40 races were represented by only a single isolate. Between 1997 to 2005, the linkage of the *AvrLm* genes (*AvrLm1*, *AvrLm2*, *AvrLm6* and *AvrLm9*) was the dominant race (*AvrLm1-2-6-(8)-9-10-(LepR3*)) in western Canada (Kutcher *et al.*, 2010b); but this was detected in only three races with one isolate for each in this study (*AvrLm1-2-5-6-9-LepR2-S, AvrLm1-2-3-4-6-9-LepR1-LepR2-(S),* and *AvrLm1-2-3-4-6-7-9-LepR1-LepR2-(S)*). This change was caused by the rapid decrease of *AvrLm9* in the population. In the race structure survey in 2010 to 2011, the two main races were *AvrLm2-4-6-7* and *AvrLm2-4-6-7-S,* which accounted for almost half of the population in western Canada (Liban *et al.*, 2016). In the survey

in 2012, the top three races were *AvrLm2-4-5-6-7-11*, *AvrLm1-2-5-6-7-11-(S)* and *AvrLm2-4-5-6-7-11-LepR1*, which accounted for 17.0% of the population in Manitoba (Zhang *et al.*, 2016). In this study, we also found that *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7* were linked in the main races. However, the top two races in this study accounted for only 20.5% of the population. These differences indicate that the races of *L. maculans* have changed a lot since Kutcher's study (1997 to 2005) in western Canada. Meanwhile, sample size and the number of *AvrLm* genes have been shown to play important roles in detecting the presence of races with lower frequency. Our findings also indicate that the variation of many resistance genes is low, or that many virulence genes may be less fit and, consequently, more difficult to be detected. In this study, 12 *AvrLm* gene loci were identified, but there were at least four other *AvrLm* genes (*AvrLm8*, *AvrLm10*, *AvrLepR3*, and *AvrLepR4*) that were undetectable because neither differential genotypes nor markers for these four *AvrLm* genes were available when this research was started. It is possible that some *AvrLm* genes have not yet been found in *L. maculans*. Our inability to detect all the *AvrLmS* genes in all the isolates may mask the identification of some races. Therefore, the race structure of *L. maculans* is likely more complicated than it was presented by the current research.

In this study, the infected stubble of HEAR and canola was collected directly from farmers' fields. Commercial cultivars of HEAR and canola with different degrees of resistance could change the pathogen population in favour of virulent isolates lacking corresponding *Avr* genes. Frequencies in *Avr* alleles were highly variable among the 12 *Avr* genes characterized in this study, ranging from 2.4% to 100% (**Figure 4.8**).

The avirulence allele *AvrLm6* was carried by all the isolates in the population characterized and tested on genotype Forge (*Rlm6*). Seedlings of Forge showed resistance to all the isolates; however, there were 1 or 2 cotyledons out of 12 that occasionally showed

susceptibility. Forge may carry other resistance genes that we have not yet found; this indicates that the corresponding resistance gene, *Rlm*6, conditioned resistance against all the isolates examined in this study. *AvrLm6* has been at a consistently high frequency in western Canada for many years and was found to be fixed in the European population of *L. maculans* in earlier research (Stachowiak *et al.*, 2006); however, a decline was observed after its repeated use in the same experimental plots in France (Brun *et al.*, 2010). The resistance gene *Rlm6* has been introgressed into *B. napus* from *B. juncea* (Chèvre *et al.*, 1997) and it has been used only in experimental materials and not believed to be present in commercial cultivars of canola. The survey of resistance genes in commercial oilseed rape in western Canada supports this view, which indicates that the major *Rlm* gene prevalent in Canadian cultivars is *Rlm3* (Zhang *et al.*, 2016). Therefore, the fact that all the isolates were avirulent on *Rlm6* is not surprising. *AvrLm6* was present in 100% of isolates of *L. maculans* between 1997 and 2005 based on the seedling tests of FalconMX (*Rlm4,6*) and DarmorMX (*Rlm6,9*) (Kutcher *et al.*, 2010b). The frequency of *Rlm6* was 84.0% in the population of 2010 to 2011 in western Canada (Liban *et al*., 2016) and 66.0% in the population of 2012 in Manitoba (Zhang *et al.*, 2016) based on PCR amplification. Even if Forge (*Rlm6*) might carry some unknown *Rlm* gene(s), *AvrLm6* has a relatively high percentage in the population of *L. maculans* in Manitoba.

AvrLm3 was present in 2.7% of the population, which came from one isolate from 2013 HEAR fields in Dauphin area, four isolates from 2013 canola fields (two from Morden/Morris area and two from Brandon area) and three isolates from 2014 HEAR fields in Dauphin area. In an earlier survey, *AvrLm3* was found in canola fields in Brandon, Morden and Morris (Zhang *et al.*, 2016). In this study, *AvrLm3* was not found in HEAR fields near the Morden/Morris area, but was detected in HEAR fields near the Dauphin area and also in canola fields in the

Morden/Morris area. This is consistent with *AvrLm3* 'erosion', observed in certain locations with a very high percentage relative to other locations (Liban *et al*., 2016; Zhang *et al.,* 2016). The frequency of *AvrLm3* varied between years and locations, as well as among cultivars, as shown by this study related to the cropping practices of a region or individual grower field. From 1985 to 1992, *AvrLm3* was present in 69.2% of isolates in the *L. maculans* population in western Canada, and this decreased dramatically to 17.7% from 1997 to 2005 (Balesdent *et al.,* 2005). Although the frequency of *AvrLm3* was reported as 60% in the *L. maculans* population in samples collected between 2005 and 2006, variation was obvious between locations (Dilmaghani *et al.,* 2009). In samples collected in 2010 and 2011, *AvrLm3* was present in 8.7% of isolates in western Canada, 4.8% specifically in Manitoba (Liban *et al*., 2016); in 2012, *AvrLm3* was present in 2.7% of isolates in Manitoba (Zhang *et al.*, 2016). The frequency of *AvrLm3* decreased dramatically in western Canada in the past three decades due to the overuse of the single resistance gene *Rlm3* in Canadian *B. napus* germplasm for a long period. This resulted in the breakdown of *Rlm3* in western Canada (Zhang *et al.,* 2016). Compared to the rapid breakdown of resistance gene *LepR1* in Australia and *Rlm1* in Europe within three growing seasons (Rouxel *et al.*, 2003a; Sprague *et al.*, 2006; Van de Wouw *et al.*, 2014b), the breakdown of *Rlm3* in Canada was much slower, taking more than two decades since its initial release in the 1990s. There are a few possible reasons for this observation. First, *Rlm3* was introduced into different oilseed rape cultivars with different backgrounds in Canada, in which the quantitative genes may vary and play important roles in disease resistance. Second, the cold weather in Canada can limit the sexual reproduction of *L. maculans* (Liban *et al*., 2016) and thus eliminate the potential of ascospores-meditated infection (Dilmaghani *et al.,* 2009, 2013). Ascospores and pycnidiospores are two important sources of inoculum during disease epidemiology, where ascospores

contribute variation to the population through genetic combination during sexual reproduction (Dilmaghani *et al.*, 2013). In Europe and Australia, the primary source of inoculum is ascospores (Bokor *et al.*, 1975; Gladders and Musa, 1980), whereas pycnidiospores are the primary source of inoculum in western Canada (Ghanbarnia *et al.*, 2011; Guo *et al.*, 2005). Compared to ascospores, pycnidiospores have limited genetic variation and distance spread (Guo *et al.*, 2005). Finally, the blackleg disease was controlled effectively in western Canada by using the recommended four-year rotation along with other management strategies (Kutcher *et al.*, 2013). Agricultural practices, including crop rotation (Kutcher *et al.*, 2013), tillage (Turkington *et al.*, 2000), nitrogen fertilizer application rate (Kutcher *et al.*, 2005) and distribution of resistant cultivars could all affect the pathogen race structure and disease pressure.

Similar to *AvrLm3*, *AvrLm9* has the lowest frequency of 2.4% amongst the 12 *AvrLm* genes. *AvrLm9* was detected in three isolates collected from the Dauphin area in 2013 HEAR fields, and four isolates from 2013 canola fields, of which two were from the Morden/Morris area, and two were from the Brandon area. However, *AvrLm9* was not detected in 2014 HEAR fields. The distribution of *AvrLm9* tends to correlate with *AvrLm3* in Manitoba; it was not detected in HEAR fields in Morden/Morris area but was present in canola fields in the Morden/Morris and Brandon areas. The frequency of *AvrLm9* decreased rapidly in the population in western Canada. It was present in 60.4% of isolates in 1997 to 2005 samples (Kutcher *et al.*, 2010b), 56.5% in 2005 to 2006 samples (Dilmaghani *et al.*, 2009), and fell to 0.7% in 2010 to 2011 samples (Liban *et al*., 2016) and 3.3% in 2012 samples in Manitoba (Zhang *et al.*, 2016). In this study, seven isolates carried *AvrLm9*, six of which also carried *AvrLm3*. In Manitoba, *Rlm9* was detected at a low frequency in commercial oilseed rape cultivars (Zhang *et al.*, 2016). Because of limited knowledge sharing about the resistance genes

carried in commercial oilseed rape cultivars, the progressive decline of *AvrLm9* and *AvrLm3* might have been caused by the wide use of *Rlm3* in western Canada since they are on the same gene cluster. In European winter-type oilseed rape cultivars, *Rlm9* is a commonly used *Rlm* gene, but it was rarely detected in the population (Balesdent *et al.*, 2006; Stachowiak *et al.*, 2006). This indicates that *Rlm9* has been overcome by the selection pressure in Europe (Kutcher, 2010b).

AvrLepR2 was present in only 5.4% of the *L. maculans* population in this study. It was carried by 0.3% of isolates in the population from 2010 to 2011 (Liban *et al*., 2016) and 10.7% in the population from 2012 (Zhang *et al.*, 2016) in Manitoba. In this study, *AvrLepR1* was also observed at a relatively low frequency of 16.5%. The frequency of *AvrLepR1* was 16.3% in the population from 2010 to 2011 (Liban *et al*., 2016) and 39.1% from 2012 (Zhang *et al.*, 2016) in Manitoba. The frequencies of these two genes did not follow any increasing or decreasing trend, as with *AvrLm3* and *AvrLm9*; this is not well-understood, but may have been caused by differences in the size and location of sampling. The *LepR* resistance genes, which correspond to the *AvrLepR* genes in the pathogen, were introduced into *B. napus* genotypes from the interspecies crossing between *B. rapa ssp. sylvestris* × *B. oleracea var. alboglabra* (Crouch *et al.*, 1994). The resistant genotypes '1065' (*LepR1*) and '1135' (*LepR2*) were used in this study to characterize *AvrLepR1* and *AvrLepR2*. The frequencies of *LepR1* and *LepR2* in these two genotypes may be underreported if compared to the newer *LepR1* and *LepR2* introgression genotypes developed by AAFC (Liban *et al*., 2016). *AvrLepR3* could not be determined by using 'Surpass400' (*LepR3* and *RlmS*) because the resistance gene *LepR3* can recognize *AvrLm1* in *L. maculans* and displays resistance to isolates that carry *AvrLm1* or *AvrLepR3* or both (Larkan *et al.*, 2013). *AvrLmS* had a frequency of 27.3% in the population of *L. maculans* from 2013 to 2014 in Manitoba. It was detected in 50.9% of the population from 2010 to 2011 (Liban *et al*.,

2016) and 34.3% of the population from 2012 (Zhang *et al.*, 2016) in Manitoba. In earlier research, *AvrLmS* was detected in 97% of *L. maculans* in the population from 1997 to 2005, which was recorded as *AvrLepR3* by Kutcher *et al.* (2010b) at that time. This was confirmed to be *AvrLepR3* after the resistance gene *LepR3* was cloned and found that it recognizes *AvrLm1* as well (Larkan *et al.*, 2013). *AvrLmS* has decreased significantly in the *L. maculans* population in Manitoba over the past two decades. *RlmS* was detected in commercial oilseed rape in Manitoba at a low frequency and was coupled with other resistance genes (Zhang *et al.*, 2016). It is difficult to explain the decline of *AvrLmS* in the *L. maculans* population in western Canada due to limited knowledge of the presence and history of *RlmS* in commercial cultivars in Canada.

AvrLm5 was detected in 90.9% of the *L. maculans* population in this study based on PCR amplification. It was present at a low frequency of 5.6% in the population from 1997 to 2005 in western Canada (Kutcher *et al.*, 2010b) and 80.7% in the population from 2012 in Manitoba (Zhang *et al.*, 2016). No cultivars/genotypes carrying *Rlm5* resistance was detected in the commercial oilseed rape cultivars in western Canada (Zhang *et al.*, 2016). The dramatic increase of *AvrLm5* frequency in the *L. maculans* population in Manitoba could be explained by the lack of *Rlm5* in commercial cultivars, and thus, no selection pressure was generated in areas of oilseed rape production.

AvrLm2 was present in 59.3% of the *L. maculans* population. *AvrLm2* was present in 83.3% in the population from 1997 to 2005 (Kutcher *et al.*, 2010b), 65.0% from 2010 to 2011 (Liban *et al*., 2016) and 64.3% in 2012 in Manitoba (Zhang *et al.*, 2016). The frequency of *AvrLm2* has decreased gradually in the past three decades. This may have been caused by the presence of resistance gene *Rlm2* in commercial oilseed rape cultivars in western Canada with a low frequency (Zhang *et al.*, 2016). Under selection pressure, the *AvrLm2* eventually switched to

the virulence gene *avrLm2*. In eight most detected races, which accounted for 42.1% of the population, *AvrLm2* was found in four races at a frequency of 26.94. The most frequently detected race was *AvrLm2-4-5-6-7-11*, with a percentage of 14.8%.

AvrLm1 was detected in 22.2% of the *L. maculans* population. It was 33.3% in the population from 1997 to 2005 (Kutcher *et al.*, 2010b), 25.5% in the population from 2010 to 2011 (Liban *et al*., 2016) and 22.0% in the population from 2012 in Manitoba (Zhang *et al.*, 2016). The frequency of *AvrLm1* decreased slowly in western Canada. *Rlm1* was detected in commercial oilseed rape cultivars at a low frequency (Zhang *et al.*, 2016). The *Rlm* genes carried by commercial cultivars are unknown, and their distribution is not publicly disclosed. Thus, more knowledge is required to explain the slow decrease of *AvrLm1* in Manitoba.

In this study, *AvrLm4* and *AvrLm7* were detected in 72.7% and 78.1% of the population respectively. The frequencies of *AvrLm4* and *AvrLm7* were both 33.3% in the population from 1997 to 2005 (Kutcher *et al.*, 2010b), 74.2% and 92.9% in the population from 2010 and 2011, respectively (Liban *et al*., 2016), and 77.1% and 89.2% in the population from 2012 in Manitoba (Zhang *et al.*, 2016). Even if we consider the difference in the sample sizes and locations, the frequencies of *AvrLm4* and *AvrLm7* are increasing gradually in western Canada. *Rlm4* was detected in the commercial oilseed rape cultivars at a very low frequency in western Canada, but no *Rlm7* was detected (Zhang *et al.*, 2016).

In this study, *L. maculans* samples were collected from HEAR and canola fields separately. Race structure and *AvrLm* gene frequencies from different oilseed crops could be compared to observe the effect of different cultivars on the pathogen population. In Manitoba, there is a limited acreage of HEAR and very little information on the presence of resistance genes. There are obvious differences in frequencies among some *AvrLm* genes between HEAR

and canola, namely *AvrLm1*, *AvrLm3*, *AvrLm4*, *AvrLm5*, *AvrLm7*, and *AvrLm11* were present at higher frequencies in canola fields compared to HEAR fields, while *AvrLepR1* and *AvrLmS* were present at lower frequencies in canola fields. Also, the main races varied among the samples collected in HEAR and canola fields. The main races were *AvrLm5-6*, *AvrLm2-4-5-6-7-S* and *AvrLm2-4-5-6-7-11-S* in 2013 HEAR fields, *AvrLm2-4-5-6-7-11*, *AvrLm1-4-5-6-7-11* and *AvrLm2-4-5-6-7-11-S* in 2013 canola fields, and *AvrLm2-4-5-6-7-11*, *AvrLm2-5-6-7-11-S*, *AvrLm5-6* and *AvrLm2-4-5-6-11* in 2014 HEAR fields. Although the sample sizes and locations varied, there were common *AvrLm* genes including *AvrLm2, AvrLm4, AvrLm5, AvrLm6, AvrLm7* and *AvrLm11* that appeared in most races from both HEAR and canola fields. *AvrLm1* had a higher percentage in canola fields and was present in the main races, but it did not appear in samples from HEAR fields. In relation to the sample locations, we found regional variations of frequency among different *AvrLm* genes. *AvrLm3* and *AvrLm9* were only detected in certain regions at a very low frequency. In this study, most samples were collected in the Morden/Morris area. To have a more accurate view of the variation, only isolates from the Morden/Morris area (southern region) were compared (**Figure 4.10 southern region**). The frequency of *AvrLm* genes not only varied between HEAR and canola but also between seasons. *AvrLm2*, *AvrLm5, AvrLm7*, *AvrLepR2* and *AvrLmS* decreased from 2013 to 2014 in HEAR fields, while *AvrLm11* increased significantly and *AvrLm1*, *AvrLm4* and *AvrLepR1* increased slightly. *AvrLm3* and *AvrLm9* were not found in HEAR fields in both 2013 and 2014.

In the early 1980s, the breeding of HEAR mainly focused on increasing erucic acid levels in *B. rapa* and decreasing glucosinolate levels in high erucic acid *B. napus* (Stefansson and Downey, 1995). This breeding goal had been achieved by crossing a high erucic acid Swedish oilseed rape strain with a low glucosinolate progeny from the Bronowski × Target cross

(Stefansson and Downey, 1995). The blackleg resistance from Australian and European cultivars was introduced into Canadian oilseed rape cultivars; other sources of blackleg resistance were introduced as well, which included *B. juncea* and a subspecies of *sylvestris* in *B. rapa* (Love *et al.*, 1990). Resistance genes in commercial HEAR seeds are rarely reported. However, HEAR seeds may carry similar resistance genes to canola since they have a similar genetic background from *B. rapa* and *B. napus* during the breeding process. This could explain the absence of *AvrLm3* and *AvrLm9* in HEAR fields in the Morden/Morris area, as well as the low frequencies in Dauphin area. Additionally, *Rlm3* has already broken down in western Canada (Zhang *et al.*, 2016) and that resulted in a very low frequency of *AvrLm3* in the pathogen population in Manitoba.

This study analyzed the frequency of the *AvrLm* gene among isolates from HEAR and canola fields in Manitoba and race structure was outlined. Our findings are in line with the previous research conducted by Liban *et al.* (2016) and Zhang *et al.* (2016). The isolates in this study consisted of three parts: 2013 HEAR fields, 2013 canola fields and 2014 HEAR fields. Although there were 297 isolates in total, the sample locations were limited and overlapped, especially in the Morden/Morris area. Only 85 races were obtained from this study, which is much less than the 150 races from 300 isolates reported by Zhang *et al.* (2016). For a given country, a sample size of 100 to 200 isolates was suggested to survey *AvrLm* genes frequency and race structure in the population of *L. maculans* (Balesdent *et al.*, 2006). The low frequency of *AvrLm3* and *AvrLm9* in the population of *L. maculans* in this study supports the breakdown of *Rlm3* in western Canada. Meanwhile, *Rlm3* is the major resistance gene carried by oilseed rape cultivars on the market (Zhang *et al.*, 2016), which is creating selection pressure on the pathogen population and *Avr* genes are being replaced by virulence genes. The complexity of *AvrLm* genes

in Manitoba varied from one single *AvrLm* gene to as many as nine *AvrLm* genes. Both extremes had very low frequencies in the population, with five isolates that carried one or nine *AvrLm* genes. Most of the races carried between five and seven *AvrLm* genes, which accounted for 80.8% of the population. The mean complexities varied from 5.28 in 2013 HEAR fields, 5.82 in 2013 canola fields, to 5.18 in 2014 HEAR fields. This indicates that isolates from HEAR fields were more virulent, as there was an increased number of races and decreased average number of *Avr* genes per isolate. The most common *Avr* gene has greater value than that of other less common *Avr* genes in breeding. For example, *Rlm6* had the highest frequency, and it appears in almost all the isolates. This indicates that *Rlm6* could provide resistance to the majority of the pathogen population than the combinations of other less common *AvrLm* genes, such as *AvrLm1*, *AvrLm3*, *AvrLm9, AvrLepR1* and *AvrLepR2*. Based on this study, the resistance genes *Rlm6* and *Rlm5* could be the most effective resistance genes against almost all the *L. maculans* races identified in western Canada. Additionally, *Avr4-7* had high frequencies in the population. These genes could be good candidates in providing effective resistance to oilseed rape. In practice, it is necessary to develop strategies for the deployment of durable resistance genes through breeding and disease management programs (Sprague *et al.*, 2006). The durability of single resistance genes could be increased by single resistance genes rotation (Fitt *et al.*, 2006).

In summary, this reseach showed that the population of *L. maculans* in Manitoba is highly diverse and consists of 85 races with no dominant race. The frequency of *AvrLm* genes and race structure have changed over time since the early studies of the *L. maculans* population in 1997, where *AvrLm* genes may shift to *Avr* genes under selection pressure. An updated knowledge of race structure from field surveys is beneficial to future breeding efforts to mitigate the risk of resistance breakdown. Blackleg disease management is challenging because the

frequency of *AvrLm* genes is dynamic; furthermore, the race structure of *L. maculans* varies across geographic locations, among oilseed rape cultivars and over growing seasons. There is no single *Rlm* gene that can be consistently resistant to the blackleg pathogen in all regions for an extended time. Along with *Avr* genes frequency and race structure surveys, the knowledge of *Rlm* genes in current oilseed rape cultivars, the crop acreage, and the distribution are crucial in developing effective management strategies to control blackleg in Manitoba and western Canada.

Chapter 5

General Discussion and Conclusions

5.1 General Discussion and Conclusions

The fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not. causes severe blackleg disease in canola and other oilseed rape. In Canada, yield loss has been reported to be as high as 50% in individual fields (McLaren *et al.,* 2012). Although blackleg has been managed with several methods, including four-year crop rotation, resistant cultivars and fungicide application, disease incidence and severity have been increasing in recent years (Liban *et al*., 2016; Zhang *et al.*, 2016). The breakdown of the resistance genes *Rlm1, LepR3* and *Rlm3* in canola has been reported in France, Australia and Canada, respectively (Rouxel *et al.,* 2003a; Li *et al.,* 2003; Zhang *et al.*, 2016); this indicates that single dominant gene-mediated resistance is not durable. Disease survey results show that the pathogen race structure in the population changed over time due to increased selection pressure from the wide use of resistant cultivars and the ability of genetic recombination from the dual sexual and asexual lifecycle.

This study included two main areas of work. The first objective focused on the temperature effects on *Rlm* gene-mediated resistance in canola. Seedlings of 14 *Brassica* genotypes carrying known resistance genes were inoculated and treated at four different temperatures. We found that *Rlm1, Rlm2, Rlm3* and *Rlm4* in Topas isogenic lines (ILs) were not temperature sensitive, while *Rlm4* in Falcon became susceptible at 25/20 °C and *Rlm3* in Quantum was susceptible at 25/20 and 30/25 °C. In our findings, some genotypes carrying the same *Rlm* genes responded differently to temperature treatments, indicating that genetic background plays an important role in resistance expression. Resistance genes in genotype Darmor (*Rlm9*), Falcon (*Rlm4*) and Quantum (*Rlm3*) were temperature-sensitive, and resistance could break down at a higher temperatures, while the resistance genes remain effective in Topas ILs. *Rlm6* in Forge is not temperature sensitive, although *Rlm6* in DarmorMX was sensitive to

temperature and could break down at 25 °C in other research (Huang *et al.*, 2006). In other host-pathogen systems, like flax (*Linum usitatissimum* L.)-rust (*Melampsora lini* (Ehrenb) Lev.) and wheat (*Triticum aestivum* L.)-rust (*Puccinia graminis* Pers.f. sp. *ritici* Eriks. & E. Henn), genetic background has been shown to play a key role in determining the degree of dominance of genes conferring resistance.

In Topas ILs, *Rlm1, Rlm2, Rlm3* and *Rlm4* were resistant to *L. maculans* at all of the temperature treatments. However, lesions enlarged with increasing temperature, especially at the higher temperature of 25/20 and 30/25 °C. We found that there was a significant effect of lesion size for each of the isogenic genotypes ($p < 0.05$) and temperature treatment ($p < 0.05$). Topas *Rlm4* developed the largest lesions among the four tested genotypes at 30/25 °C.

Higher and lower temperature affects the growth and sporulation of *L. maculans* and 25 °C was found to be the optimum temperature. Growth and sporulation were restricted by temperatures lower and higher than 25 °C. Temperature and race had significant effects ($p < 0.05$) on sporulation, but the effect of interaction between temperature and race was not significant.

The knowledge of how temperature affects *Rlm* gene-mediated resistance in canola is useful for breeders and regulators. The genetic background should be considered in breeding by introducing *Rlm* genes to specific cultivars. The distribution of different cultivars to different areas should be regulated based on historical peak temperatures in the area during the seedling stage and whether the *Rlm* gene is temperature sensitive.

The second objective of this research sudy examined the race structure of the *L. maculans* population in HEAR and canola fields in Manitoba. In total, 297 isolates of *L. maculans* were

identified. The *AvrLm* genes, *AvrLm6* and *AvrLm5*, were the most prevalent in the pathogen population. *AvrLm7, AvrLm4*, *AvrLm11* and *AvrLm2* were present in more than 50% of the population, while *AvrLm3* and *AvrLm9* were detected at very low frequency. In total, 85 races were identified, and *AvrLm2-4-6-7-11* was the main race, which accounted for 14.8% of the population.

The frequency of *AvrLm* genes varied between high erucic acid rapeseed (HEAR) and canola fields, as well as over the growing seasons. In this study, the frequencies of *AvrLm1, AvrLm4, AvrLm5, AvrLm7* and *AvrLm11* from 2013 canola fields were higher than that in HEAR fields from both 2013 and 2014, while *AvrLepR1* and *AvrLmS* were lower than isolates from HEAR fields. In 2013 canola fields, the frequencies of *AvrLm3, AvrLm9* and *LepR2* were higher in the Dauphin area than in the Morden/Morris area. However, in 2014 HEAR fields, *AvrLm3* was not detected in the Morden/Morris area; *AvrLepR2* was not detected in the Dauphin area, while *AvrLm9* was not detected in either the Morden/Morris or Dauphin areas. In HEAR fields, 191 isolates were tested in 2013 and 2014. *AvrLm3* and *AvrLm9* were absent in southern Manitoba, but in the Dauphin area, was detected in 5.8% and 4.4% of isolates, respectively. *AvrLepR1* frequency was much higher in the south (Morden/Morris) than in the north (Dauphin). Since most samples were collected in the southern region, 220 isolates from canola and HEAR fields from both 2013 and 2014 were tested and identified. The frequency of *AvrLm* genes varied between years and crops. *AvrLm2, AvrLm5, AvrLm7, AvrLepR2* and *AvrLmS* decreased from 2013 to 2014 in HEAR fields, while *AvrLm1, AvrLm4*, and *AvrLepR1* increased slightly.

There is limited information disclosure about the *Rlm* genes carried in HEAR in commercial seeds. However, HEAR seeds may carry similar *Rlm* genes as canola seed, which could explain the low frequency of *AvrLm3* and *AvrLm9*. This work provided knowledge about

AvrLm gene frequency and race structure of *L. maculans* in HEAR and canola in Manitoba and will be useful in HEAR breeding for blackleg disease management. *AvrLm6, AvrLm5*, *AvrLm4* and *AvrLm7* were detected in more than 50% of the isolates, and they could be good candidates for providing effective resistance to *L. maculans* in HEAR and canola cultivars in Canada.

References

Agostini A, Johnson DA, Hulbert S, Demoz B, Fernando WGD, Paulitz T, 2013. First Report of Blackleg Caused by *Leptosphaeria maculans* on Canola in Idaho. *Plant Disease, 97*(6):842-842.

Agrios GN, 2005. Plant Pathology. 5th. *Elsevier Acad. Press*, Burlington, pp 251-257.

Allen EJ, Morgan DG, 1975. A quantitative comparison of the growth, development and yield of different varieties of oilseed rape. *The Journal of Agricultural Science, 85*:159-174.

Ansan-Melayah D, Balesdent MH, Buée M, Rouxel T, 1995. Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. *Phytopathology, 85*:1525-1529.

Ansan-Melayah D, Balesdent MH, Delourme R, Pilet ML, Tanguy X, Renard M, Rouxel T, 1998. Genes for race-specific resistance against blackleg disease in *Brassica napus* L. *Plant Breeding, 117*:373-378.

Aubertot JN, Salam MU, Diggle AJ, Dakowska S, Jedryczka M, 2006. SimMat, a new dynamic module of Blackleg Sporacle for the prediction of pseudothecial maturation of *L. maculans/L. biglobosa* species complex. Parameterisation and evaluation under Polish conditions. *Integrated Control in Oilseed Crops, International Organization for Biological Control Bulletin*, 29:277-285.

Augspurger CK, 2013. Reconstructing patterns of temperature, phenology, and frost damage over 124 years: spring damage risk is increasing. *Ecology, 94*:41-50.

Badawy HMA, Hoppe HH, Koch E, 1991. Differential reactions between the genus Brassica and aggressive single spore isolates of *Leptosphaeria maculans. Journal of Phytopathology, 131*:109-119.

Badawy HMA, Kakau J, Hoppe HH, 1992. Temperature and ageing of host tissue affect the interactions between different oilseed rape cultivars and pathotype groups of *Leptosphaeria maculans. Journal of Phytopathology, 134*:255-263.

Balass M, Cohen Y, Bar-Joseph M, 1993. Temperature-dependent resistance to downy mildew in muskmelon: Structural responses. *Physiological and Molecular Plant Pathology, 43*:11-20.

Balesdent MH, Attard A, Ansan-Melayah D, Delourme R, Renard M, Rouxel T, 2001. Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. *Phytopathology*, *91*:70-76.

Balesdent MH, Attard A, Kühn ML, Rouxel T, 2002. New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology, 92*:1122-1133.

Balesdent MH, Barbetti MJ, Li H, Sivasithamparam K, Gout L, Rouxel T, 2005. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology, 95*:1061-1071.

Balesdent MH, Fudal I, Ollivier B, Bally P, Grandaubert J, Eber F, Rouxel T, 2013. The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytologist*, *198*:887-898.

Balesdent MH, Louvard K, Pinochet X, Rouxel T, 2006. A large-scale survey of races of *Leptosphaeria maculans* occurring on oilseed rape in France. *European Journal of Plant Pathology, 114*:53-65.

Barbetti MJ, Khangura RK, 1999. Managing blackleg in the disease-prone environment of Western Australia. In *Proceedings of the 10th international rapeseed congress*.

Bhardwaj HL, Hamama AA, 2000. Oil, erucic acid, and glucosinolate contents in winter hardy rapeseed germplasms. *Industrial crops and Products*, *12*:33-38.

Biddulph JE, Fitt BD, Leech PK, Welham SJ, Gladders P, 1999. Effects of temperature and wetness duration on infection of oilseed rape leaves by ascospores of *Leptosphaeria maculans* (stem canker). *European Journal of Plant Pathology*, *105*:769-781.

Blenis PV, Chow PS, Stringam GR, 1999. Effects of burial, stem portion and cultivar on the decomposition of canola straw. *Canadian Journal of Plant Science*, *79*:97-100.

Bock A, Sparks TH, Estrella N, Jee N, Casebow A, Schunk C, Menzel A, 2014. Changes in first flowering dates and flowering duration of 232 plant species on the island of Guernsey. *Global Change Biology, 20*:3508-3519.

Bokor A, Barbetti MJ, Brown AGP, MacNish GC, Wood PMcR, 1975. Blackleg of rapeseed. *J. Agric. West, 8*:7-10.

Booth EJ, Gunstone FD, 2004. Rapeseed and rapeseed oil: agronomy, production, and trade. In: *Rapeseed and Canola Oil: Production, Processing, Properties and Uses*. CRC Press LLV, Boca Raton, pp. 1-17.

Borhan MH, 2013. Defining population of the *L. maculans* pathogen in test sites used for canola blackleg resistance trials. Retrieved from http://www.saskcanola.com/quadrant/System/research/reports/Bohran.3.3.3.Short_Report.pdf

Bradley CA, Parks PS, Chen Y, Fernando WGD, 2005. First report of pathogenicity groups 3 and 4 of *Leptosphaeria maculans* in canola in North Dakota. *Plant Disease, 89*:776-776.

Bromfield KR, 1961. The effect of post-inoculation temperature on seedling reaction of selected wheat varieties to stem rust. *Phytopathology, 51:*590-593.

Brown AGP, Barbetti MJ, Wood PM, 1976. Effect of benomyl on 'blackleg' disease of rape in Western Australia. *Australian Journal of Experimental Agriculture, 16*:276-279.

Brun H, Levivier S, Somda I, Ruer D, Renard M, Chèvre AM, 2000. A field method for evaluating the potential durability of new resistance sources: application to the *Leptosphaeria maculans-Brassica napus* pathosystem. *Phytopathology, 90*:961-966.

Canadian Food Inspection Agency, 2017. The Biology of *Brassica napus* L, Canola/Rapeseed. Retrieved from http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/brassica-napus-l-/eng/1330729090093/1330729278970#a14

Canola Council of Canada, 2017a. What is Canola. Retrieved from http://www.canolacouncil.org/oil-and-meal/what-is-canola/the-history-of-canola/

Canola Council of Canada, 2017b. Industry Overview. Retrieved from https://www.canolacouncil.org/markets-stats/industry-overview/

Canola Council of Canada, 2017c. Diseases. Retrieved from https://www.canolacouncil.org/canola-encyclopedia/diseases/

Canola Council of Canada, 2017d. Blackleg. Retrieved from https://www.canolacouncil.org/canola-encyclopedia/diseases/blackleg/

Carlsson AS, Yilmaz JL, Green AG, Stymne S, Hofvander P, 2011. Replacing fossil oil with fresh oil–with what and for what? *European Journal of Lipid Science and Technology, 113*:812-831.

Chen CY, Howlett BJ, 1996. Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves of Indian mustard (*Brassica juncea*) inoculated with avirulent isolates of *Leptosphaeria maculans*. *Physiological and Molecular Plant Pathology, 48*:73-81.

Chen Y, Fernando WGD, 2006. Prevalence of pathogenicity groups of *Leptosphaeria maculans* in western Canada and North Dakota, USA. *Canadian Journal of Plant Pathology*, 28:533-539.

Chèvre AM, Barret P, Eber F, Dupuy P, Brun H, Tanguy X, Renard M, 1997. Selection of stable *Brassica napus-B. juncea* recombinant genotypes resistant to blackleg (*Leptosphaeria maculans*). 1. Identification of molecular markers, chromosomal and genomic origin of the introgression. *Theoretical and Applied Genetics, 95*:1104-1111.

Chèvre AM, Eber F, This P, Barret P, Tanguy X, Brun H, Renard M, 1996. Characterization of *Brassica nigra* chromosomes and of blackleg resistance in *B. napus–B. nigra* addition genotypes. *Plant Breeding, 115*:113-118.

Coonrod D, Brick MA, Byrne PF, DeBonte L, Chen Z, 2008. Inheritance of long chain fatty acid content in rapeseed (*Brassica napus* L.). *Euphytica, 164*:583-592.

Crouch JH, Lewis BG, Mithen RF, 1994. The effect of A genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans*. *Plant Breeding, 112*:265-278.

Cunningham GH, 1927. Dry-rot of swedes and turnips: its cause and control. *New Zealand Department of Agriculture Bulletin*, 133.

Curl EA, 1963. Control of plant diseases by crop rotation. *The Botanical Review, 29*:413-479.

Daun JK, Eskin NAM, Hickling D, 2011. Origin, distribution, and production. In: *Canola: chemistry, production, processing, and utilization.* Elsevier, Amsterdam, The Netherlands, pp. 1-27.

Davies JML, 1986. Disease of oilseed rape. In: *Oilseed Rape.* William Colins Sons & Co Ltd, London, UK. pp. 195-236

de Jong CF, Takken FL, Cai X, de Wit PJ, Joosten MH, 2002. Attenuation of *Cf*-mediated defense responses at elevated temperatures correlates with a decrease in elicitor-binding sites. *Molecular Plant-Microbe Interactions*, *15*:1040-1049.

de Lorgeril M, Salen P, Laporte F, Foulon, T, Payen N, de Leiris J, 2001. Rapeseed oil and rapeseed oil-based margarine for the prevention and treatment of coronary heart disease. *European Journal of Lipid Science and Technology*, *103*:490-495.

Delourme R, Brun H, Ermel M, Lucas MO, Vallee P, Domin C, Barbetti MJ, 2008. Expression of resistance to *Leptosphaeria maculans in Brassica napus* double haploid lines in France and Australia is influenced by location. *Annals of Applied Biology*, *153*:259-269.

Delourme R, Chèvre AM, Brun H, Rouxel T, Balesdent MH, Dias JS, Rimmer SR, 2006. Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *European Journal of Plant Pathology, 114*:41-52.

Delourme R, Pilet-Nayel ML, Archipiano M, Horvais R, Tanguy X, Rouxel T, Balesdent MH, 2004. A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Phytopathology*, *94*:578-583.

Delwiche PA, Williams PH, 1979. Screening for resistance to blackleg of crucifers in the seedling stage. *Cruciferae Newsl*, 4:24.

Delwiche PA, 1980. Genetic aspects of blackleg (*Leptosphaeria maculans*) resistance in rapeseed (*Brassica napus*). Ph. D. Thesis, *University of Wisconsin*, Madison, Wisconsin.

Diederichsen A, McVetty PBE, Daun JK, Eskin NAM, Hickling D, 2011. Botany and plant breeding. In *Canola: chemistry, production, processing, and utilization*. Elsevie, Amsterdam, The Netherlands, pp. 29-56.

Dilmaghani A, Balesdent MH, Didier JP, Wu C, Davey J, Barbetti MJ, Vincenot L, 2009. The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology, 58*:1044-1058.

Dilmaghani A, Balesdent MH, Rouxel T, Moreno-Rico O, 2010. First report of *Leptosphaeria biglobosa* (Blackleg) on *Brassica oleracea* (Cabbage) in Mexico. *Plant Disease*, *94*:791-791.

Dilmaghani A, Gout L, Moreno-Rico O, Dias JS, Coudard L, Castillo-Torres N, Rouxel T, 2013. Clonal populations of *Leptosphaeria maculans* contaminating cabbage in Mexico. *Plant Pathology, 62*:520-532.

Downey RK, 1990. *Brassica* oilseed breeding-achievements and opportunities. In *Plant Breeding Abstracts, 60*:1165-1170.

Downey RK, Röbbelen G, 1989. *Brassica* species. In *Oil Crops of the World, their breeding and utilization*. McGraw-Hill Publishing Co., New York, pp. 339-362.

Downy RK. 1983. The Origin and Description of the *Brassica* Oilseed Crops. In: *High and Low Erucic Acid Rapeseed Oils,* Academic Press Canada, pp. 1-18.

Dyer JM, Stymne S, Green AG, Carlsson AS, 2008. High-value oils from plants. *The Plant Journal*, *54*:640-655.

Eber F, Lourgant K, Brun H, Lode M, Huteau V, Coriton O, Chèvre AM, 2011. Analysis of *Brassica nigra* chromosomes allows identification of a new effective *Leptosphaeria maculans* resistance gene introgressed in *Brassica napus*. In *13th International rapeseed congress,* Prague pp. 5-9.

Fernando WGD, Chen Y, 2003. First report on the presence of *Leptosphaeria maculans* pathogenicity group-3, the causal agent of blackleg of canola in Manitoba. *Plant Disease, 87*:1268-1268.

Ferreira ME, Rimmer SR, Williams PH, Osborn TC, 1995. Mapping loci controlling *Brassica napus* resistance to *Leptosphaeria maculans* under different screening conditions. *Phytopathology, 85*:213-217.

Fitt BD, Brun H, Barbetti MJ, Rimmer SR, 2006. World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). In *Sustainable strategies for managing Brassica napus (oilseed rape) resistance to Leptosphaeria maculans (phoma stem canker).* Springer, Amsterdam, The Netherlands, pp. 3-15.

Fitt BD, Hu BC, Li ZQ, Liu SY, Lange RM, Kharbanda PD, White RP, 2008. Strategies to prevent spread of *Leptosphaeria maculans* (phoma stem canker) onto oilseed rape crops in China; costs and benefits. *Plant Pathology*, *57*:652-664.

Flor HH, 1942. Inheritance of pathogenicity in *Melampsora lini. Phytopathology*, *32*:653-669.

Flor HH, 1971. Status of the gene-for-gene concept. *Annual Review of Phytopathology*, *9*:275-296.

Foglia TA, 2001. Rapeseed oil for oleochemical usage. *Eur. J. Lipid Sci. Technol*, *103*:450-454.

Frey SD, Elliott ET, Paustian K, 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. *Soil Biology and Biochemistry*, *31*:573-585.

Friedt W, Lühs W, 1998. Recent developments and perspectives of industrial rapeseed breeding. *European Journal of Lipid Science and Technology*, *100*:219-226.

Friedt W, Snowdon R, 2009. Oilseed rape. In *Oil crops*, Springer New York. pp. 91-126.

Fudal I, Ross S, Gout L, Blaise F, Kuhn ML, Eckert MR, Rouxel T, 2007. Heterochromatin-like regions as ecological niches for avirulence genes in the *Leptosphaeria maculans* genome: map-based cloning of *AvrLm6*. *Molecular Plant-Microbe Interactions*, *20*:459-470.

Ghanbarnia K, Fernando WGD, Crow G, 2011. Comparison of disease severity and incidence at different growth stages of naturally infected canola plants under field conditions by pycnidiospores of phoma lingam as a main source of inoculum. *Canadian Journal of Plant Pathology*, *33*:355–363.

Ghanbarnia K, Fudal I, Larkan NJ, Links MG, Balesdent MH, Profotova B, Fernando WGD, Rouxel T, Borhan MH, 2015. Rapid identification of the *Leptosphaeria maculans* avirulence gene *AvrLm2*, using an intraspecific comparative genomics approach. *Mol Plant Pathol 6*:1-11

Ghanbarnia K, Lydiate DJ, Rimmer SR, Li G, Kutcher HR, Larkan NJ, Fernando WGD, 2012. Genetic mapping of the *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*. *Theoretical and Applied Genetics*, *124*:505-513.

Gladders P, Musa TM, 1980. Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. *Plant Pathology*, *29*:28-37.

Gout L, Fudal I, Kuhn ML, Blaise F, Eckert M, Cattolico L, Balesdent MH, Rouxel T, 2006. Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the Dothideomycete *Leptosphaeria maculans. Molecular Microbiology*, *60*:67–80

Government Canada, 2017. Historic Data, Retrieved from http://climate.weather.gc.ca/climate_data/daily_data_e.html?StationID=27174

Graboski MS, McCormick RL, 1998. Combustion of fat and vegetable oil derived fuels in diesel engines. *Progress in Energy and Combustion Science*, *24*:125-164.

Gugel RK, Petrie GA, 1992. History, occurrence, impact, and control of blackleg of rapeseed. *Canadian Journal of Plant Pathology*, *14*:36-45.

Guo XW, Fernando WGD, Entz M, 2005. Effects of crop rotation and tillage on blackleg disease of canola. *Canadian Journal of Plant Pathology*, *27*:53-57.

Gupta AK, Saini RG, Gupta S, Malhotra S, 1984. Genetic analysis of two wheat cultivars, '*Sonalika*' and '*WL 711*' for reaction to leaf rust (*Puccinia recondita*). *TAG Theoretical and Applied Genetics*, *67*:215-217.

Gupta SK, Pratap A, 2007. History, origin, and evolution. *Advances in Botanical Research*, *45*:1-20.

Hall R, 1992. Epidemiology of blackleg of oilseed rape. *Canadian Journal of Plant Pathology*, *14*:46-55.

Hall R, Chigogora JL, Phillips LG, 1996. Role of seedborne inoculum of *Leptosphaeria maculans* in development of blackleg on oilseed rape. *Canadian Journal of Plant Pathology*, *18*:35-42.

Hammond KE, 1985. *Systematic infection of Brassica napus* L *spp. oleifera (Metzger) Sink. by Leptosphaeria maculans* (Desm.) Ces. et de Not. Ph.D. Thesis, *University of East Anglia*, Norwich, UK.

Hammond KE, Lewis BG, 1987. The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. *Plant Pathology, 36*:135-147.

Hayward A, Edwards D, Batley J, Parkin I, Kole C, 2011. Introduction-oilseed *Brassicas*. *Genetics, Genomics and Breeding of Oilseed Brassicas*, pp 1-13.

Hayward A, McLanders J, Campbell E, Edwards D, Batley J, 2012. Genomic advances will herald new insights into the *Brassica*: *Leptosphaeria maculans* pathosystem. *Plant Biology, 14*:1-10.

Henderson MP, 1918. The blackleg disease of cabbage caused by *Phoma lingam* (Tode) Desmaz. *Phytopathology, 8*:393–428.

Howlett BJ, 2004. Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology, 26*:245-252.

Howlett BJ, Idnurm A, Pedras MSC, 2001. *Leptosphaeria maculans*, the causal agent of blackleg disease of *Brassicas*. *Fungal Genetics and Biology, 33*:1-14.

Huang YJ, Evans N, Li ZQ, Eckert M, Chèvre AM, Renard M, Fitt BD, 2006. Temperature and leaf wetness duration affect phenotypic expression of *Rlm6*-mediated resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist, 170*:129-141.

Huang YJ, Fitt BD, Jedryczka M, Dakowska S, West JS, Gladders P, Li ZQ, 2005. Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans*) and Poland (*Leptosphaeria biglobosa*). *European Journal of Plant Pathology, 111*:263-277.

Huang YJ, Toscano-Underwood C, Fitt BD, Hu XJ, Hall AM, 2003. Effects of temperature on ascospore germination and penetration of oilseed rape (*Brassica napus*) leaves by A-or B-group *Leptosphaeria maculans* (phoma stem canker). *Plant Pathology, 52*:245-255.

Islam MR, Shepherd KW, Mayo GME, 1989. Effect of genotype and temperature on the expression of *L* genes in flax conferring resistance to rust. *Physiological and Molecular Plant Pathology, 35*:141-150.

Jacobs JL, 2001. A Comparison of Pedigree Selection and Doubled Haploid Line Breeding Methods for Cultivar Development in High Erucic Acid Rapeseed. Ph. D. Thesis, *University of Manitoba*, Winnipeg, Canada.

Jacobsen BJ, Williams PH, 1971. Histology and control of *Brassica oleracea* seed infection by *Phoma lingam*. *Plant Disease Reporter*, *55*:934-938.

Jestin C, Bardol N, Lode M, Duffe P, Domin C, Vallée P, Delourme R, 2015. Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.). *Molecular Breeding*, *35*:167.

Jestin C, Lodé M, Vallée P, Domin C, Falentin C, Horvais R, Delourme R, 2011. Association mapping of quantitative resistance for *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.). *Molecular Breeding*, *27*:271-287.

Johnson T, Newton M, 1941. The effect of high temperature on the stem rust resistance of wheat varieties. *Canadian Journal of Research*, *19*:438-445.

Kaczmarek J, Jedryczka M, 2008. Development of the perfect stage of *Leptosphaeria maculans* and *L. biglobosa* under variable weather conditions of Pomerania in 2004–2008. *Phytopathologia Polonica*, *50*:19-31.

Kaczmarek J, Kedziora A, Brachaczek A, Latunde-Dada AO, Dakowska S, Karg G, Jedryczka M, 2016. Effect of climate change on sporulation of the teleomorphs of *Leptosphaeria* species causing stem canker of *Brassicas*. *Aerobiologia*, *32*:39-51.

Kasprzyk I, Ortyl B, Dulska-Jeż A, 2014. Relationships among weather parameters, airborne pollen and seed crops of Fagus and Quercus in Poland. *Agricultural and Forest Meteorology*, *197*:111-122.

Keri M, Kutcher HR, Rimmer SR, 2001. Virulence of isolates of *Leptosphaeria maculans* from western Canada on *Brassica napus* differentials. *Canadian Journal of Plant Pathology*, *23*:199.

Kharbanda PD, 1993. Blackleg of canola in Alberta: investigations on biology, epidemiology and management. Vegreville, AB: Alberta Environmental Centre, Alberta, Canada. Retrieved from https://archive.org/details/blacklegofcanola00khar

Kharbanda PD, Tewari JP, 1996. Integrated management of canola diseases using cultural methods. *Canadian Journal of Plant Pathology, 18*:168-175.

Kharbanda PD, Yang J, Beatty P, Jensen S, Tewari JP, 1999. Biocontrol of *Leptosphaeria maculans* and other pathogens of canola with Paenibacillus polymyxa PKB1. In *Proceedings of the 10th International Rapeseed Congress.* Canberra, Australia. Retrieved from http://www.regional.org.au/au/gcirc.

Kimber DS, McGregor DI, 1995. The Biology of *Brassica rapa* L. In: *Brassica Oilseeds: Production and Utilization*. CAB International, Wallingford, UK, pp 1–38.

Knott DR, 1981. The effects of genotype and temperature on the resistance to *Puccinia graminis tritici* controlled by the gene *Sr6* in *Triticum aestivum*. *Canadian Journal of Genetics and Cytology*, *23*:183-190.

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

Kuswinanti T, Koopmann B, Hoppe HH, 1999. Virulence pattern of aggressive isolates of *Leptosphaeria maculans* on an extended set of Brassica differentials. *Journal of Plant Diseases and Protection*, 12-20.

Kutcher HR, Balesdent MH, Rimmer SR, Rouxel T, Chèvre AM, Delourme R, Brun H, 2010b. Frequency of avirulence genes in *Leptosphaeria maculans* in western Canada. *Canadian Journal of Plant Pathology, 32*:77-85.

Kutcher HR, Brandt SA, Smith EG, Ulrich D, Malhi SS, Johnston AM, 2013. Blackleg disease of canola mitigated by resistant cultivars and four-year crop rotations in western Canada. *Canadian Journal of Plant Pathology,* 35:209-221.

Kutcher HR, Cross D, Nabetani K, Kirkham CL, 2011. A large-scale survey of races of *L. maculans* occurring on canola in western Canada. Retrieved from *http://www.saskcanola.com/quadrant/System/research/reports/report-Kutcher-maculanssurvey-short.pdf*

Kutcher HR, Keri M, McLaren DL, Rimmer SR, 2007. Pathogenic variability of *Leptosphaeria maculans* in western Canada. *Canadian Journal of Plant Pathology, 29*:388-393.

Kutcher HR, Malhi SS, Gill KS, 2005. Topography and management of nitrogen and fungicide affects diseases and productivity of canola. *Agronomy Journal*, *97*:533-541.

Kutcher HR, Van den Berg CGJ, Rimmer SR, 1993. Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. *Canadian Journal of Plant Pathology*, *15*:253-258.

Kutcher HR, Yu F, and Brun H, 2010a. Improving blackleg disease management of *Brassica napus* from knowledge of genetic interactions *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology*, 32:29-34.

Lamari L. 2008. ASSESS 2.0 Image Analysis Software for Plant Disease Quantification. *American Phytopathological Society*, St. Paul.

Lamey HA, 1995. Survey of blackleg and Sclerotinia stem rot of canola in North Dakota in 1991 and 1993. *Plant Disease*, *79*:322-324.

Larkan NJ, Lydiate DJ, Parkin IAP, Nelson MN, Epp DJ, Cowling WA, Borhan MH, 2013. The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *AvrLm1*. *New Phytologist*, *197*:595-605.

Larkan NJ, Ma L, Borhan MH, 2015. The *Brassica napus* receptor-like protein *RLM2* is encoded by a second allele of the *LepR3/Rlm2* blackleg resistance locus. *Plant Biotechnology Journal*, 13:983-992.

Lazzeri L, Leoni O, Conte LS, Palmieri S, 1994. Some technological characteristics and potential uses of *Crambe abyssinica* products. *Industrial Crops and Products*, *3*:103-112.

Lee SB, Taylor JW, 1990. Isolation of DNA from fungal mycelia and single spores. In: *PCR Protocols, a Guide to Methods and Applications*. Academic Press San Diego, pp. 282–287.

Leonard EC, 1992. High-erucic vegetable oils. *Industrial Crops and Products*, *1*(2):119-123.

LePage R, Penaud A, 1995. Tout se joue avec le premier pic d'ascospores. *Oléoscope*, *28*:23-27.

Li H, Sivasithamparam K, Barbetti MJ, 2003. Breakdown of a *Brassica rapa* subsp. sylvestris pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling Brassicas. *Plant Disease*, *75*:1279–1282.

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

Liban SH, Cross DJ, Kutcher HR, Peng G, Fernando WGD, 2016. Race structure and frequency of avirulence genes in the western Canadian *Leptosphaeria maculans* pathogen population, the causal agent of blackleg in brassica species. *Plant Pathology*, *65*:1161-1169.

Liu C, 2014. Evaluation of Fungicides for Management of Blackleg Disease on Canola and QoI-fungicide Resistance in *Leptosphaeria maculans* in Western Canada, MSc. Thesis, *University of Manitoba, Canada*.

Long Y, Wang Z, Sun Z, Fernando WGD, McVetty PB, Li G, 2011. Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola cultivar 'Surpass400'. *Theoretical and Applied Genetics, 122*:1223-1231.

Love HK, Rakow G, Raney JP, Downey RK, 1990. Development of low glucosinolate mustard. *Canadian Journal of Plant Science*, *70*:419-424.

Lühs W, Friedt W, 1994. Major oil crops.In: *Designer Oil Crops*, pp 5-71.

Luig NH, Rajaram S, 1972. The effect of temperature and genetic background on host gene expression and interaction to *Puccinia graminis tritici*. *Phytopathology*, *62*:1171-1174.

Mahuku GS, Goodwin PH, Hall R, Hsiang T, 1997. Variability in the highly virulent type of *Leptosphaeria maculans* within and between oilseed rape fields. *Canadian Journal of Botany*, *75*:1485-1492.

Manitoba Agriculture, 2017. Canola Production and management. Retrieved from http://www.gov.mb.ca/agriculture/crops/production/canola.html#seeding

Marcroft SJ, Van de Wouw AP, Salisbury PA, Potter TD, Howlett BJ, 2012. Effect of rotation of canola *(Brassica napus*) cultivars with different complements of blackleg resistance genes on disease severity. *Plant Pathology, 61*:934-944.

Mastebroek HD, Lange W, 1997. Progress in a crambe cross breeding program. *Industrial Crops and Products, 6*:221-227.

Mayerhofer R, Good AG, Bansal VK, Thiagarajah MR, Stringam GR, 1997. Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. *Genome, 40*:294-301.

McGee DC, 1977. Blackleg (*Leptosphaeria maculans* (Desm.) Ces. de Not.) of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. *Crop and Pasture Science, 28*:53-62.

McGee DC, Emmett RW, 1977. Black leg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.) of rapeseed in Victoria: crop losses and factors which affect disease severity. *Australian Journal of Agricultural Research, 28*:47-51.

McGee DC, Petrie GA, 1979. Seasonal patterns of ascospore discharge by *Leptosphaeria maculans* in relation to blackleg of oilseed rape. *Phytopathology, 69*:586-589.

McIntosh RA, Dyck PL, 1975. Cytogenetical studies in wheat. VII Gene *Lr23* for reaction to *Puccinia recondita* in Gabo and related cultivars. *Australian Journal of Biological Sciences, 28*:201-212.

McLaren D, Kubinec A, Derksen H, Bisht V, Henderson T, 2012. Manitoba Canola Disease Survey 2012: Results and Impact. Retrieved from https://umanitoba.ca/faculties/afs/agronomists_conf/media/MAC_proceedings_McLaren_28Dec2012.pdf

McVetty PB, Fernando WGD, Li G, Tahir M, Zelmer C, 2009. High-erucic acid, and low-glucosinolate rapeseed (HEAR) cultivar development in Canada. In: *Biocatalysis and Agricultural Biotechnology*. CRC, Boca Raton, FL, USA, pp.43-61.

Mengistu ALEMU, Rimmer SR, Koch E, Williams PH, 1991. Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling *Brassicas. Plant Dis, 75:*1279-1282.

Mikolajczak KL, Miwa TK, Earle FR, Wolff IA, Jones Q, 1961. Search for new industrial oils. V. Oils of Cruciferae. *Journal of the American Oil Chemists Society, 38*:678-681.

Nagaharu U, 1935. Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Jap J Bot, 7*:389-452.

Naseri B, Davidson JA, Scott ES, 2008. Effect of temperature, cultivar and plant tissue on the germination of, and hyphal growth from, ascospores of *Leptosphaeria maculans*. *Australasian Plant Pathology*, *37*:365-372.

Nath UK, 2008. Increasing erucic acid content in the seed oil of rapeseed (*Brassica napus* L.) by combining selection for natural variation and transgenic approaches. Ph. D. Thesis, *Georg-August-Universität Göttingen*, Germany.

Nath UK, Wilmer JA, Wallington EJ, Becker HC, Möllers C, 2009. Increasing erucic acid content through combination of endogenous low polyunsaturated fatty acids alleles with Ld-LPAAT+ Bn-fae1 transgenes in rapeseed (*Brassica napus* L.). *Theoretical and applied genetics*, *118*:765-773.

Nayar SK, Agarwal RK, Nagarajan S, 1987. Sensitivity of leaf rust resistance genes to temperature in wheat. *Cereal Rusts Bull*, 15:1-4.

Oplinger ES, Hardman L, Gritton ET, Doll JD, Kelling KA, 1989. Canola (Rapeseed). *Alternative Field Crops Manual.* Retrieved from http://www.hort.purdue.edu/newcrop/afcm/canola.html

Parlange F, Daverdin G, Fudal I, Kuhn ML, Balesdent MH, Blaise F, Rouxel T, 2009. *Leptosphaeria maculans* avirulence gene *AvrLm4-7* confers a dual recognition specificity by the *Rlm4* and *Rlm7* resistance genes of oilseed rape, and circumvents *Rlm4*-mediated recognition through a single amino acid change. *Molecular Microbiology*, *71*:851-863.

Paul VH, Rawlinson CJ, 1992. *Diseases and pests of oilseed rape*. *Gelsenkirchen-Buer*: Verlag Theodore Mann, Germany, pp. 52-83

Pérès A, Poisson B, Le Sourne V, Maisonneuve C, 1999. *Leptosphaeria maculans*: effect of temperature, rainfall and humidity on the formation of pseudothecia. In *Proceedings of the 10th international rapeseed congress*, 26-9.

Petrie GA, 1978. Occurrence of a highly virulent strain of blackleg (*Leptosphaeria maculans*) on rape in Saskatchewan. *Canadian Inventaire Plant des maladies Disease des Plantes Survey*, 21.

Petrie GA, Vanterpool TC, 1965. Diseases of rape and cruciferous weeds in Saskatchewan in 1965. *Can. Plant Dis. Surv, 45*:111-112.

Petrie GA, Vanterpool TC, 1974. Infestation of crucifer seed in western Canada by the blackleg fungus *Leptosphaeria maculans*. *Canadian plant disease survey*.

Pilet ML, Delourme R, Foisset N, Renard M, 1998. Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in winter rapeseed (*Brassica napus* L.). *TAG Theoretical and Applied Genetics, 96*:23-30.

Pilet ML, Duplan G, Archipiano M, Barret P, Baron C, Horvais R, Delourme R, 2001. Stability of QTL for field resistance to blackleg across two genetic backgrounds in oilseed rape. *Crop Science, 41*:197-205.

Platford G, 1985. Manitoba canola survey. Report to the Western Committee on Plant Disease Control. In *10th Annual Meeting, Winnipeg*, 66:51-53.

Plissonneau C, Daverdin G, Ollivier B, Blaise F, Degrave A, Fudal I, Balesdent MH, 2016. A game of hide and seek between avirulence genes *AvrLm4-7* and A*vrLm3* in *Leptosphaeria maculans*. *New Phytologist, 209*:1613-1624.

Pongam P, Osborn TC, Williams PH, 1998. Genetic analysis and identification of amplified fragment length polymorphism markers linked to the alm1 avirulence gene of *Leptosphaeria maculans*. *Phytopathology, 88*:1068-1072.

Puc M, Kasprzyk I, 2013. The patterns of Corylus and Alnus pollen seasons and pollination periods in two Polish cities located in different climatic regions. *Aerobiologia, 29*:495-511.

Raman H, Raman R, Larkan N, 2013. Genetic dissection of blackleg resistance loci in rapeseed (*Brassica napus* L.). *Plant Breeding from Laboratories to Fields*, *4*:86-120.

Ramarathnam R, Dilantha FWG, 2006. Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans*, the blackleg pathogen of canola. *Biocontrol Science and Technology, 16*:567-582.

Ratnayake WMN, Daun JK, 2004. Chemical composition of canola and rapeseed oils. In: *Rapeseed and Canola Oil. Production, processing, properties and uses.* CRC Press LLV, Boca Raton, pp 37-73.

Rimmer SR, 2006. Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. *Canadian Journal of Plant Pathology*, *28*: S288-S297.

Rimmer SR, 2007. Chasing genes for resistance to blackleg and Sclerotinia in *Brassica napus*. In *Proceedings 12th International Rapeseed Congress*, Wuhan, China.

Rimmer SR, Van den Berg CGJ, 1992. Resistance of oilseed Brassica spp. to blackleg caused by *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology*, *14*:56-66.

Roelfs AP, McVey DV, 1979. Low infection types produced by *Puccinia graminis* f. sp. *tritici* and wheat lines with designated genes for resistance. *Phytopathology*, *69*:722-730.

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

Rouxel T, Willner E, Coudard L, Balesdent MH, 2003b. Screening and identification of resistance to *Leptosphaeria maculans* (stem canker) in *Brassica napus* accessions. *Euphytica*, *133*:219-231.

Roy N, Reeves J, 1975. Breeding better rape and linseed for Western Australia. *Journal of Agriculture. Western Australia*.

Roy NN, 1984. Interspecific transfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus*. *Euphytica*, *33*:295-303.

Samborski DJ, Dyck PL, 1968. Inheritance of virulence in wheat leaf rust on the standard differential wheat varieties. *Canadian Journal of Genetics and Cytology*, *10*:24-32.

Sanyal A, Pinochet X, Merrien A, Laustriat M, Decocq G, Fine F, 2015. Erucic acid rapeseed: 1. Prospects of Improvements. *OCL, 22*:303.

Sawatsky WM, 1989. Evaluation of screening techniques for resistance to *Leptosphaeria maculans* and genetic studies of resistance to the disease in *Brassica napus*. M.Sc. Thesis, *University of Manitoba*, Winnipeg, Canada.

Scarth R, McVetty PBE, Rimmer SR, Stefansson BR, 1991. Hero summer rape. *Canadian Journal of Plant Science, 71*:865-866.

Shoemaker RA, Brun H, 2001. The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Canadian Journal of Botany, 79*:412-419.

Sivasithamparam K, Barbetti MJ, Li H, 2005. Recurring challenges from a necrotrophic fungal plant pathogen: a case study with *Leptosphaeria maculans* (causal agent of blackleg disease in Brassicas) in Western Australia. *Annals of Botany, 96*:363-377.

Snowdon R, Luhs W, Friedt W, 2007. Oilseed Rape. In: *Genome Mapping and Molecular Breeding in Plants*. Springer, Berlin, Germany, pp. 55-114.

Sonntag NOV, 1995. Industrial utilization of long-chain fatty acids and their derivatives. In: *Brassica Oilseeds. CAB International*, Oxon, UK, pp. 339-352.

Sprague SJ, Balesdent MH, Brun H, Hayden HL, Marcroft SJ, Pinochet X, Howlett BJ, 2006. Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *European Journal of Plant Pathology, 114*:33-40.

Sprague SJ, Marcroft SJ, Lindbeck KD, Ware AH, Khangura RK, Van de Wounw AP, 2017. Detection, prevalence and severity of upper canopy infection on mature *Brassica napus* plants caused by *Leptosphaeria maculans* in Australia. *Crop and Pasture Science* (online)

Stachowiak A, Olechnowicz J, Jedryczka M, Rouxel T, Balesdent MH, Happstadius I, Evans N, 2006. Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *European Journal of Plant Pathology*, *114*:67-75.

Statistics Canada, 2017. Field Crops in Canada. Retrieved from http://www.statcan.gc.ca/pub/11-627-m/11-627-m2017012-eng.htm

Stefansson BR, 1983. The development of improved rapeseed cultivars. In: *High and Low Erucic Acid Rapeseed Oils,* Academic Press Canada, pp. 143-159.

Stefansson BR, Downey RK, 1995. Rapeseed. In: *Harvest of Gold*. University Extension Press, Saskatoon, pp. 140–152

Stefansson BR, Hougen FW, 1964. Selection of rape plants (*Brassica napus*) with seed oil practically free from erucic acid. *Canadian Journal of Plant Science, 44*:359-364.

Stefansson BR, Kondra ZP, 1975. Tower summer rape. *Canadian Journal of Plant Science 55*:343-344.

Tewari JP, Shinners TC, Briggs KG, 1997. Production of calcium oxalate crystals by two species of *Cyathus* in culture and infested plant debris. *Zeitschrift für Naturforschung C, 52*:421-425.

Tewari JP, Strelkov SE, Orchard D, Hartman M, Lange RM, Turkington TK, 2005. Identification of clubroot of crucifers on canola (*Brassica napus*) in Alberta. *Canadian Journal of Plant Pathology*, *27*:143-144.

The Science of Climate Change, 2015. Environment and Climate Change Canada, Retrieved from http://publications.gc.ca/site/eng/9.836086/publication.html

Thürwächter F, Garbe V, Hoppe HH, 1999. Ascospore discharge, leaf infestation and variations in pathogenicity as criteria to predict impact of *Leptosphaeria maculans* on oilseed rape. *Journal of Phytopathology, 147*:215-222.

Toscano-Underwood C, Huang YJ, Fitt BD, Hall AM, 2003. Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathology, 52*:726-736.

Trautwein EA, 1997. Food quality of rapeseed oil–aspects from a nutritional point of view. *GCIRC Bull, 14*:123-128.

Turkington TK, Clayton GW, Klein-Gebbinck H, Woods DL, 2000. Residue decomposition and blackleg of canola: influence of tillage practices. *Canadian Journal of Plant Pathology, 22*:150-154.

US Department of Agriculture, 2017. Canola. Retrieved from http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx

USDA Foreign Agricultural Service, 2017. Oilseeds: World Markets and Trade. Retrieved from http://usda.mannlib.cornell.edu/usda/current/oilseed-trade/oilseed-trade-09-12-2017.pdf

Uysal A, Kurt Ş, 2017. Influence of inoculum density, temperature, wetness duration, and leaf age on infection and development of spinach anthracnose caused by the fungal pathogen *Colletotrichum spinaciae*. *European Journal of Plant Pathology*, 149:1041-1052.

Van de Wouw AP, Lowe RG, Elliott CE, Dubois DJ, Howlett BJ, 2014a. An avirulence gene, *AvrLmJ1*, from the blackleg fungus, *Leptosphaeria maculans*, confers avirulence to *Brassica juncea* cultivars. *Molecular Plant Pathology, 15*:523-530.

Van de Wouw AP, Marcroft SJ, Barbetti MJ, Hua L, Salisbury PA, Gout L, Balesdent MH, 2009. Dual control of avirulence in *Leptosphaeria maculans* towards a *Brassica napus* cultivar with 'sylvestris-derived' resistance suggests involvement of two resistance genes. *Plant Pathology, 58*:305-313.

Van de Wouw AP, Marcroft SJ, Ware A, Lindbeck K, Khangura R, Howlett BJ, 2014b. Breakdown of resistance to the fungal disease, blackleg, is averted in commercial canola (*Brassica napus*) crops in Australia. *Field Crops Research, 166*:144-151.

Vanniasingham VM, Gilligan CA, 1989. Effects of host, pathogen and environmental factors on latent period and production of pycnidia of *Leptosphaeria maculans* on oilseed rape leaves in controlled environments. *Mycological Research, 93*:167-174.

Vanterpool TC, 1961. Rape diseases in Saskatchewan in 1961. *Can. Plant Dis. Surv, 41*:372-373.

Wang YP, Sonntag K, Rudloff E, 2003. Development of rapeseed with high erucic acid content by asymmetric somatic hybridization between *Brassica napus* and *Crambe abyssinica*. *Theoretical and Applied Genetics*, *106*:1147-1155.

Webb KM, Ona I, Bai J, Garrett KA, Mew T, Cruz V, Leach JE, 2010. A benefit of high temperature: increased effectiveness of a rice bacterial blight disease resistance gene. *New Phytologist, 185*:568-576.

West JS, Balesdent MH, Rouxel T, Narcy JP, Huang YJ, Roux J, Schmit J, 2002. Colonization of winter oilseed rape tissues by A/Tox+ and B/Tox0 *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology*, *51*:311-321.

West JS, Biddulph JE, Fitt BD, Gladders P, 1999. Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. *Annals of Applied Biology*, *135*:535-546.

West JS, Evans N, Leech PK, Fitt BDL, Welham SJ, Jedryczka M, Penaud A, 2000. Predicting leaf infection by *Leptosphaeria maculans* on winter oilseed rape. *IOBC WPRS BULLETIN*, *23*:23-28.

West JS, Fitt BD, 2005. Population dynamics and dispersal of *Leptosphaeria maculans* (blackleg of canola). *Australasian Plant Pathology*, 34:457-461.

West JS, Kharbanda PD, Barbetti MJ, Fitt BD, 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology*, *50*:10-27.

Williams PH, 1992. Biology of *Leptosphaeria maculans*.*Canadian Journal of Plant Pathology, 14*:30-35.

Williams PH, Delwiche PA, 1979. Screening for resistance to blackleg of crucifers in the seedling stage. In: *Proceucarpia conference on the breeding of cruciferous crops*, Wageningen, The Netherlands, pp.164–170.

Wood P, Barbetti MJ, 1977. The role of seed infection in the spread of blackleg of rape in Western Australia. *Animal Production Science, 17*:1040-1044.

Wratten N, Mailer RJ, 1989. *Brassica napus* L. var. napus (rapeseed) cv. Eureka. *Animal Production Science, 29*:602-602.

Wright KM, Duncan GH, Pradel KS, Carr F, Wood S, Oparka KJ, Santa Cruz S, 2000. Analysis of the *N* gene hypersensitive response induced by a fluorescently tagged tobacco mosaic virus. *Plant Physiology, 123*:1375-1386.

Yu F, Gugel RK, Kutcher HR, Peng G, Rimmer SR, 2013. Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus*× *B. rapa* subsp. sylvestris. *Theoretical and applied genetics, 126*:307-315.

Yu F, Lydiate DJ, Hahn K, Kuzmicz S, Hammond C, Rimmer SR, 2007b. Identification and mapping of a novel blackleg resistance locus *LepR4* in the progenies from *Brassica napus* x *B. rapa* subsp. sylvestris in *12th International Rapeseed Congress.Wuhan, China*

Yu F, Lydiate DJ, Rimmer SR, 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theoretical and Applied Genetics, 110*:969-979.

Yu F, Lydiate DJ, Rimmer SR, 2007a. Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa* subsp. sylvestris. *Genome, 51*:64-72.

Zhang S, Klessig DF, 1998. Resistance gene *N*-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. *Proceedings of the National Academy of Sciences, 95*(13):7433-7438.

Zhang X, Peng G, Kutcher HR, Balesdent MH, Delourme R, Fernando WGD, 2016. Breakdown of *Rlm3* resistance in the *Brassica napus–Leptosphaeria maculans* pathosystem in western Canada. *European Journal of Plant Pathology, 145*:659-674.

Zhang, X, Fernando, WGD, 2017. Insights into fighting against blackleg disease of *Brassica napus* in Canada. *Crop and Pasture Science*. Canada, online (https://doi.org/10.1071/CP16401).

Zhu B, Rimmer SR, 2003. Inheritance of resistance to *Leptosphaeria maculans* in two accessions of *Brassica napus*. *Canadian Journal of Plant Pathology, 25*:98-103.

Appendices

Appendix I. Lesion development on the cotyledons of Topas *Rlm1* infected with *Leptosphaeria maculans* isolate R2-7 under different temperatures.

DPI	15–10 °C				20–15 °C				25–20 °C				30–25 °C			
	Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2			
2	0.470[1]	±	0.185[2]	a[3]	0.458	±	0.110	a	0.850	±	0.143	a	0.825	±	0.153	a
3	0.637	±	0.188	a	0.713	±	0.116	ab	0.965	±	0.144	a	0.823	±	0.149	a
4	0.557	±	0.152	a	0.752	±	0.205	ab	1.107	±	0.154	a	0.908	±	0.183	a
5	0.624	±	0.219	a	0.867	±	0.165	ab	1.112	±	0.103	a	0.989	±	0.190	a
6	0.677	±	0.266	a	1.019	±	0.255	abc	1.421	±	0.277	ab	2.244	±	1.162	ab
7	0.709	±	0.224	a	1.120	±	0.244	bc	1.743	±	0.425	bc	2.856	±	1.221	ab
8	1.154	±	0.141	b	1.264	±	0.325	bc	2.047	±	1.032	cd	4.722	±	1.398	bc
9	1.165	±	0.195	b	1.562	±	0.568	cd	2.055	±	0.614	cd	5.987	±	3.483	c
10	1.176	±	0.431	b	2.079	±	0.807	de	2.518	±	1.246	de	5.811	±	3.474	c
11	1.204	±	0.459	b	2.117	±	0.963	e	2.591	±	1.081	de	6.691	±	5.299	cd
12	1.265	±	0.244	b	2.251	±	0.860	e	3.033	±	1.274	ef	8.362	±	5.652	de
13	1.270	±	0.740	b	2.280	±	1.749	e	3.103	±	1.222	ef	9.401	±	6.102	e
14	1.311	±	0.459	b	2.472	±	1.773	e	3.613	±	1.815	f	9.648	±	5.324	e

[1]Refers to the average lesion size of ten seedlings under the same treatment.
[2]Refers to the standard error.
[3]The letter in the same column refers to the significance of lesion size under the same temperature treatment.

Appendix II. Lesion development on the cotyledons of Topas *Rlm2* infected with *Leptosphaeria maculans* isolate Sb1 under different temperatures.

DPI	15–10 °C				20–15 °C				25–20 °C				30–25 °C			
	Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2			
2	0.361[1]	±	0.072[2]	a[3]	0.409	±	0.078	a	0.713	±	0.132	a	0.795	±	0.191	a
3	0.441	±	0.129	ab	0.629	±	0.209	ab	0.947	±	0.189	a	0.808	±	0.195	a
4	0.597	±	0.145	bc	0.700	±	0.149	b	1.059	±	0.199	ab	1.095	±	0.147	ab
5	0.714	±	0.175	c	0.837	±	0.184	bc	1.076	±	0.216	ab	1.100	±	0.177	ab
6	0.723	±	0.214	c	1.032	±	0.119	cd	1.462	±	0.441	abc	2.105	±	0.756	abc
7	0.725	±	0.161	c	1.071	±	0.201	cd	1.400	±	0.292	abc	2.573	±	1.275	bc
8	0.974	±	0.248	d	1.151	±	0.247	d	1.808	±	0.923	bcd	3.008	±	1.323	cd
9	0.988	±	0.238	d	1.168	±	0.321	de	1.981	±	1.137	cde	3.094	±	1.516	cde
10	1.162	±	0.264	de	1.419	±	0.456	ef	2.634	±	1.560	de	4.282	±	2.614	def
11	1.248	±	0.304	ef	1.567	±	0.399	f	2.360	±	0.900	ef	4.678	±	3.312	ef
12	1.404	±	0.296	fg	1.618	±	0.530	f	3.143	±	1.190	f	5.201	±	3.529	f
13	1.443	±	0.470	g	1.910	±	0.901	g	4.323	±	1.950	g	7.651	±	3.528	g
14	1.555	±	0.637	g	1.945	±	0.644	g	4.317	±	2.695	g	9.096	±	5.328	g

[1]Refers to the average lesion size of ten seedlings under the same treatment.
[2]Refers to the standard error.
[3]The letter in the same column refers to the significance of lesion size under the same temperature treatment.

Appendix III. Lesion development on the cotyledons of Topas *Rlm3* infected with *Leptosphaeria maculans* isolate Sb1 under different temperatures.

DPI	15–10 °C				20–15 °C				25–20 °C				30–25 °C			
	Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2			
2	0.458[1]	±	0.138[2]	a[3]	0.581	±	0.184	a	0.860	±	0.255	a	0.722	±	0.123	a
3	0.676	±	0.140	b	0.781	±	0.198	ab	1.060	±	0.147	ab	0.882	±	0.217	ab
4	0.727	±	0.148	b	0.856	±	0.257	bc	1.095	±	0.155	ab	0.918	±	0.151	ab
5	0.836	±	0.138	bc	0.852	±	0.385	bc	1.089	±	0.129	ab	0.935	±	0.102	ab
6	0.995	±	0.154	cd	0.857	±	0.162	bc	1.356	±	0.250	ab	1.292	±	0.209	ab
7	1.023	±	0.285	cd	0.944	±	0.207	bc	1.587	±	0.313	ab	1.339	±	0.378	ab
8	1.034	±	0.284	cd	0.968	±	0.343	bc	1.969	±	0.908	abc	2.843	±	2.639	bc
9	1.095	±	0.347	de	1.083	±	0.372	cd	2.027	±	0.536	abc	3.698	±	3.226	cd
10	1.105	±	0.164	de	1.082	±	0.253	cd	2.186	±	1.020	bc	4.719	±	4.430	cde
11	1.199	±	0.297	def	1.256	±	0.272	de	2.900	±	0.995	cd	5.383	±	4.708	de
12	1.280	±	0.606	ef	1.282	±	0.532	de	3.678	±	2.534	de	6.194	±	3.758	ef
13	1.288	±	0.480	ef	1.392	±	0.410	e	4.228	±	2.656	ef	6.281	±	3.911	ef
14	1.389	±	0.648	f	1.660	±	0.418	f	5.143	±	4.111	f	7.563	±	4.254	f

[1]Refers to the average lesion size of ten seedlings under the same treatment.
[2]Refers to the standard error.
[3]The letter in the same column refers to the significance of lesion size under the same temperature treatment.

Appendix IV. Lesion development on the cotyledons of Topas *Rlm4* infected with *Leptosphaeria maculans* isolate Sb2 under different temperatures.

DPI	15–10 °C				20–15 °C				25–20 °C				30–25 °C			
	Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2				Lesion Size/mm^2			
2	0.471[1]	±	0.150[2]	a[3]	0.642	±	0.195	a	0.681	±	0.151	a	0.568	±	0.095	a
3	0.739	±	0.184	a	0.846	±	0.176	a	0.753	±	0.187	a	0.769	±	0.188	a
4	0.944	±	0.225	ab	1.443	±	0.489	ab	1.050	±	0.210	a	1.058	±	0.224	a
5	1.104	±	0.266	ab	2.139	±	0.948	bc	1.077	±	0.248	a	1.328	±	0.935	a
6	1.745	±	0.490	c	2.223	±	1.272	bc	1.640	±	0.381	b	3.904	±	1.975	b
7	3.229	±	1.653	d	2.833	±	1.058	cd	1.715	±	0.748	bc	4.621	±	4.785	bc
8	3.324	±	1.414	d	3.020	±	1.164	cd	1.783	±	0.651	bc	5.276	±	2.526	bc
9	3.678	±	1.378	de	3.115	±	1.219	cd	2.233	±	0.776	cd	5.632	±	3.466	bc
10	3.816	±	1.635	de	3.351	±	1.477	d	2.227	±	0.946	cd	6.793	±	3.213	c
11	3.914	±	1.577	de	3.457	±	1.413	d	2.482	±	1.048	d	7.386	±	3.762	cd
12	3.918	±	1.248	de	3.545	±	1.351	d	2.587	±	1.273	d	9.546	±	6.262	de
13	4.153	±	1.558	de	3.772	±	1.415	d	3.308	±	1.329	e	9.888	±	7.638	de
14	4.375	±	2.587	e	5.535	±	3.328	e	3.320	±	1.449	e	10.222	±	6.767	e

[1]Refers to the average lesion size of ten seedlings under the same treatment.
[2]Refers to the standard error.
[3]The letter in the same column refers to the significance of lesion size under the same temperature treatment.

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INTRODUCTION

Appendix VI. Lists of abbreviations

Avr…….Avirulence

avr……..Virulence

DPI…….Days post inoculation

IL……Isogenic line

HEAR…….High erucic acid rapeseed

PCR……Polymerase chain reaction

R…….Resistant

S…….Susceptible