CHARACTERIZING *AVR* GENES OF *LEPTOSPHAERIA MACULANS* AND RESISTANCE RESPONSES AMONG CANADIAN CANOLA CULTIVARS (CCCS) IN WESTERN CANADA

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Abstract

Blackleg of canola, caused by *Leptosphaeria maculans* (Desmaz.) Ces. & de Not, is a serious concern in western Canada. The disease had been managed successfully since 1990s with use of resistant cultivars and extended crop rotations until recent years when both blackleg incidence and severity increased noticeably. This may be attributed to changes in the pathogen population that erodes the resistance of canola cultivars. The resistance associated with Canadian canola (*Brassica napus* L.) cultivars (CCCs) in Canada is not clearly understood. The current study was conducted to investigate the race structure of *L. maculans* in commercial canola fields and determine its role in blackleg incidence and severity. In addition, resistance (*R*) genes in representative CCCs were characterized to understand their role in blackleg control against the current population of *L. maculans*.

A total of 372 *L. maculans* isolates collected from 16 canola fields with different levels of blackleg severity in 2012 and 2013 were analysed for the presence or absence of particular avirulence (*Avr*) alleles by inoculating 12 lines of a host differential set with known *R* genes. The results indicated that the alleles *AvrLm1*, *AvrLm3*, *AvrLm9* and *AvrLep2* were at very low or undetectable levels in these fields, while *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7* were generally common. Since only the *R* genes *Rlm1* and *Rlm3* are found commonly in CCCs, this result indicates that most of our cultivars are no longer effective against the current pathogen population on the prairies.Variation in *Avr* gene frequency was observed, depending on the cultivar, field or region studied, but these differences alone appeared insufficient to explain the variability in blackleg severity in these fields, and the erosion of *Rlm1* and *Rlm3* would unlikely be the primary cause of isolated blackleg outbreaks for most of the fields investigated.

Nonspecific resistance may exist in many CCCs. To characterize it, eight common CCCs were selected and assessed using a cotyledon inoculation assay for specific *R* genes

carried using a set of *L. maculans* isolates carrying known *Avr* genes. Three of the CCCs were assessed further for non-race-specific resistance using both the cotyledon inoculation method and a petiole inoculation method with virulent *L. maculans* isolates carrying neither *AvrLm1* nor *AvrLm3*, as well as with droplet digital PCR (ddPCR) and fluorescent microscopy. The colonization of leaf tissue and progression of the pathogen into the petiole and stem was more limited in CCCs than in Westar, based on the hyphal spread of a GFP (green fluorescence protein)-labelled *L. maculans* isolate and on the amount of *L. maculans* DNA found in the petioles and stems of CCCs relative to those of Westar. Additionally, inoculated cotyledons showed substantially lower disease severity on CCCs (scores of 5–6) than on Westar (scores 8–9) at 14 dpi. It is therefore possible that many Canadian CCCs carry nonspecific blackleg resistance in their genetic background, which hinders the spread of pathogen hyphae from the cotyledon to the petiole and stem, as well as pathogen development in the stem.

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Chapter 1. Background

1.1. The importance of Canola to the Canadian economy

Canola occupies a distinct position in oilseed crops among many countries of the world. A study conducted by LMC International for the Canola Council of Canada revealed that canola adds $19.3 billion to the Canadian economy every year, and the industry supports more than 249,000 jobs and $12.5 billion in wages (Canola Council of Canada 2013).

In Canada, over 90% of canola is produced on the prairies; Saskatchewan is the biggest producer, with an output of 8.9 million tonnes of canola seed annually, which is almost 50% of the national production. Alberta has slightly higher production than Manitoba; together they make up about another 50% of the national production. There has been a continuous increase in seeded canola acreage since its development as a crop in western Canada (Statistics Canada 2016). More than 75% of canola produced in Canada is exported.

1.2. Blackleg of canola

The fungus *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.), which causes ‘stem canker’ or ‘blackleg’ disease, is a pervasive pathogen of oilseed rape and canola (Rouxel and Balesdent 2005). The disease has caused significant yield and quality losses of canola in Europe, Australia and Canada (West et al. 2001; Kutcher et al. 2010), and is an on-going threat to canola production in western Canada (Liban et al. 2016; Zhang et al. 2015). Since this pathogen species has not been reported in China, it is on the quarantine list of crop pests. Therefore blackleg is a threat to Canadian canola exports to China, a market worth $4 billion annually (Canola Council of Canada 2016).

Blackleg of canola can be caused by a complex of two species: *L. maculans* and *L. biglobosa* (Shoemaker and Brun 2001), with *L. maculans* being more virulent and reported from almost all canola/oilseed rape growing regions of the world except China (West et al. 2001; Fitt et al. 2006). These species had previously been referred to as highly and weakly virulent forms of *L. maculans* until the latter was designated as *L. biglobosa* (Cunningham 1927; Petrie 1978; Mendes-Pereira et al. 2003)

Severe blackleg symptoms were first reported on canola in western Canada in the 1970's, and the disease spread rapidly after the initial report in Saskatchewan (Petrie 1995b). The disease was present on oilseed rape crops in the province earlier, but was not considered as a significant issue with respect to the canola production (Vanterpool 1961). Blackleg was the most damaging disease on canola in Saskatchewan between the early 1980's and the 1990's, with yield losses up to 50% reported in individual fields (Gugel and Petrie 1992). The first disease epidemic occurred in 1982, resulting in an estimated 6% yield loss in western Canada and in some fields the loss was a much as 56% (Petrie1985b). Control recommendations at that time included using clean seed, fungicide seed treatment, burial of infested canola residues destruction of volunteers and susceptible weeds, adhering to crop rotations of four years, and avoiding seeding canola adjacent to previously diseased fields (Martens et al. 1988).

With the introduction of resistant cultivars and use of extended crop rotations since the early 1990s, blackleg was controlled quiet successfully in western Canada for many years, although genetic changes were observed in the pathogen population (Chen and Fernando 2006; Keri et al. 2001; Kutcher et al. 2007). By 2009, blackleg was reported from an increasing number of canola fields. Based on annual provincial disease surveys, blackleg is increasingly observed in many areas in western Canada, with substantial damage observed on even resistant-rated (*R*-rated) cultivars.

1.3. Brief background related to proposed research

The fungus which causes blackleg of canola has a strong ability to evolve and adapt; it mutates frequently and consequently rapidly erodes host resistance (Kutcher et al. 2007). Although blackleg was managed successfully for many years in western Canada by use of resistant cultivars and extended crop rotations, the disease appears to be increasingly problematic, especially in southern Manitoba and east-central Alberta. In Saskatchewan, higher levels of blackleg have been reported more often from the north-western and south-eastern regions (Miller et al. 2012; McLaren DL et al. 2012; Miller et al. 2013; McLaren DL et al. 2013,). This rising trend may be caused by a shift in the pathogen population that erodes the resistance genes carried by current cultivars, due to shortened crop rotations or a combination of both. Race-specific resistance targeting certain avirulence (*Avr*) alleles in the pathogen population tends to be highly effective at the seedling stage, as well as adult-plant stages, but this type of resistance can often break down in short period of time, with changes in the pathogen race structure. It was not clear if the severe blackleg disease observed on some of the *R*-rated cultivars would be due to the loss of certain *Avr* alleles in the pathogen corresponding to the *R* genes in these cultivars. About 70% of canola cultivars or breeding lines in Canada carry the *R* genes *Rlm1* or *Rlm3* or both, whereas other *R* genes are found relatively uncommon (Zhang et al. 2015).

The main purpose of this study was to determine if the variable levels of blackleg severity observed on *R*-rated canola cultivars would be related to the race structure of the pathogen. Additionally, non-race-specific resistance was investigated and characterized using representative CCCs, and its role in managing blackleg in western Canada assessed. These data, when combined with the crop management and cultivar information from the commercial fields studied, may provide insights into the cause of the recent increase in blackleg.

1.3. Hypotheses

1. The severe cases of blackleg observed on *R*-rated canola cultivars might be due an adaptation of the pathogen to specific *R* genes carried by CCCs.
2. Both qualitative and quantitative resistance may exist in many Canadian CCCs, which reduces the risk of widespread failure of CCCs with *Avr*-gene fluctuation in the pathogen population.
3. Quantitative resistance may reduce the impact of blackleg on canola by reducing the pathogen spread from infected cotyledons to the stem and limiting infection development in basal stem tissues.

1.4. Objectives

The objectives of this research were to: 1) characterize the *Avr*-gene profile of *L. maculans* in commercial canola fields with different levels of blackleg damage to decipher if lack of certain *Avr* genes in the pathogen population would be the cause of the severe blackleg observed; and 2) assess the interactions of *R*-rated canola cultivars with *L. maculans* carrying and not carrying corresponding *A*vr genes, as well as the non-race-specific resistance based on pathological, microscopic and molecular characterization of the infection process. This information may help producers, as well as industry to better understand the risk of blackleg associated with pathogen-cultivar dynamics, as well as other factors for optimal management of the disease.

Chapter 2. Literature review

2.1. Canola

Canola has become one of the world's most important oilseed crops in a relatively short period of time (Lin et al. 2013) and a moneymaking commodity for producers in Canada. The word canola was coined in 1979, representing “Canadian oil” (Statistics Canada 2009). To produce canola, the products must meet internationally recognized standards principally, low levels of erucic acid and glucosinolate (Mag 1983; Lin et al. 2013). Canola is the third most common cooking oil by volume after palm and soybean (Lin et al. 2013). The global production of canola in 2010 and 2011 was about 38 million metric tonnes (MMt). Canada became the biggest producer that year, with 15.4 MMt, followed by China at 14.0 MMt. of the five top producing countries in 2012 (Figure 2.1.), Canadian canola accounted for approximately 31% of world production. World production soared to 46.5 MMt in 2012 (Morrison et al. 2016).

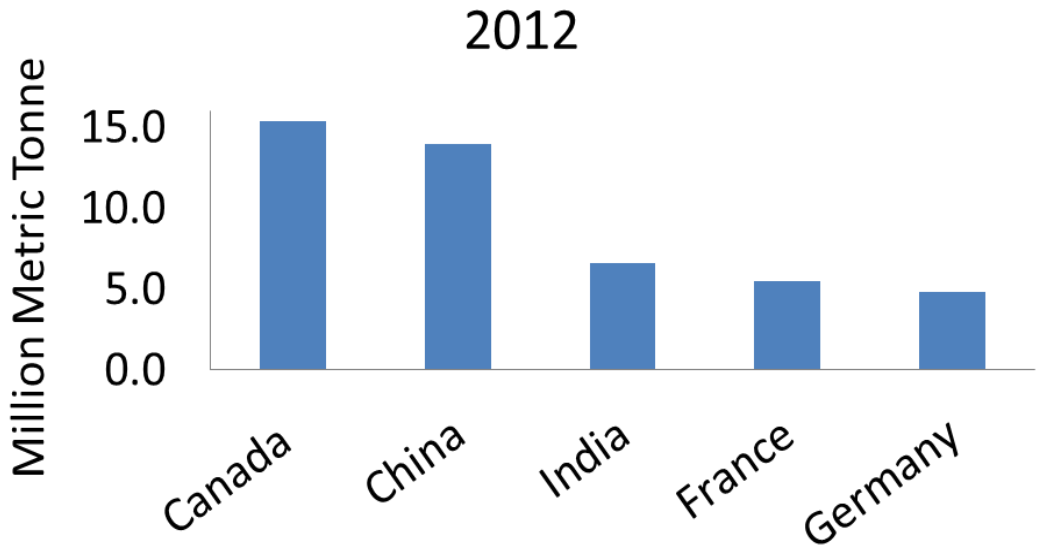


Figure 2.1. The global production of canola (million metric tonnes) in 2012. The largest producer was Canada, with 15.4 million tonnes (FAOSTAT 2012).

Canola contributes approximately 19.3 billion dollars to the Canadian economy each year and is the second largest crop seeded in Canada next to wheat. The production of canola has increased continuously for many years, with a yield increase from 1,330 kg ha^{-1} to 2,025 kg ha^{-1} between 2000 and 2013 (Morrison et al. 2016). The industry provides work for about 261,000 people, by creating 194,000 paid jobs. The sector provides approximately $12.5 billion in wages (Canola Council of Canada 2013). In addition, about 65,000 farm families (excluding growers) who are involved in canola production are supported by this sector. Saskatchewan accounts for almost 50% of national canola production, contributing $5 billion in the provincial economy (Statistics Canada 2016).

Oilseed rape was grown as early as 2000 BC; mostly in Asia and the Mediterranean region during ancient civilisations (Colton and Potter 1999). Europeans started growing this crop as a source of lamp oil in the beginning of the 13th century (Colton and Potter 1999). In Canada, commercial cultivation of oilseed rape began in 1942 during World War II for use as a lubricant on war ships (Statistics Canada 2009).

Canola belongs to the plant family Brassicaceae (previously Cruciferae) comprised of about 375 genera and 3,200 species. Brassica is one of the genera and *B. napus* L. subsp. *oleifera*, originating from a cross of *B. oleracea* and *B. rapa* (Nagaharu 1935), is a crop species commonly known as oilseed rape, rapeseed or canola (Jessop et al. 1986).

In Canada, edible oil was extracted from oilseed rape for the first time in 1956 (Colton and Potter 1999). Canola, developed by breeders in Saskatchewan and Manitoba from rapeseed plants through conventional breeding during the decade of 1960-70, was the first rapeseed crop with low erucic acid. The cultivar LEAR, an abbreviation of Low Erucic Acid Rape, was released in 1968 (*B. napus* cv. oro). This was followed by the release of the first double low (low erucic acid and low glucosinolate) variety in 1974 (Potter et al. 1995). Since then, Canada has become the world leader in the production of low erucic acid rapeseed or canola. Canola is grown primarily for seed, which contains up to 45% pure oil. Canola oil is mainly used for cooking, although use as margarine is common. Canola meal, a by-product after oil extraction, is a good source of high protein animal feed.

Based on the study of chloroplast nuclear and mitochondrial DNA, Song and Osborn (1992) suggested that *B. montana* is closely related to a prototype of *B. napus* that appears to share the cytoplasm of both *B. oleracea* and *B. rapa*. Most cultivated *B. napus*, however, carry a cytoplasm different from either *B. oleracea* or *B. rapa*. It has also been suggested that *B. napus* has multiple origins, but the current species resulted from crosses between *B. oleracea* and *B. rapa*. Canola growth and development can be divided into several stages. The longevity of each stage is affected by factors including day length, temperature, nutrients, moisture (humidity) and cultivar. Temperature is often the most important environmental factor that regulates the growth and development of canola in Western Canada (Canola Council of Canada 2016a).

Canola (*B. napus*) is a cool-season crop (Johnston et al. 2002) that cannot tolerate drought. The crop has acclimatised to many regions of the world, and does well over a wide range of soil conditions if fertility and moisture are sufficient (Nielsen 1997, 1998). The ideal temperature for maxim growth is above 20ºC (ranging between 12ºC and 30ºC). Diseases, insects and weeds are limiting factors for canola production and extended crop rotations are generally good practices for alleviating the negative impacts of these pest problems by reducing the buildup of the pest population (Canola Council of Canada 2016b; Kutcher et al. 2011a).

Except for a few hardy winter type canola cultivars seeded in the fall in southern Ontario, most of the canola cultivars grown in Canada are annual spring types. Though *B. napus* is self-pollinated, up to 20-30% outcrossing has been reported (Rakow and Woods 1987). The transmission of pollen is mostly through insects, but it can also be done by physical contact of racemes of flowers. There are no reports of vegetative propagation, and consequently seed is the only source for successive generations (Rakow and Woods 1987). Reproduction through parthenogenesis has been reported in *B. napus* (Eenink 1974) when in some conditions pollen from different species of plants lands on the stigma (Rieger et al. 2002).

2.2. Blackleg of Canola

Oilseed rape or canola can be affected by many diseases, but stem canker, also known as blackleg, caused by *Leptosphaeria maculans*, can be extremely damaging. The fungal pathogen was first identified as *Sphaeria lingam* on cabbage in 1791 (Henderson 1918), and blackleg disease of cruciferous vegetable crops has been known for over 100 years. The name, *Leptosphaeria maculans* (Desm.) Ces. & de Not, was suggested by Tulasne in 1863 (Balesdent et al. 2005), and its anamorph is *Phoma lingam* Tode ex Fr. Blackleg can be caused by a complex of two species: *L. maculans and L. biglobosa* (Mendes-Pereira et al.

2003), but *L. maculans* is the most prominent and damaging species in most canola/oilseed rape production regions, including Australia, Canada and Europe. The species *L. biglobosa* 'Canadensis' is abundant on oilseed rape in Canada since it was first reported in 1957, but is considered a weak pathogen of canola (Williams and Fitt 1999; Fitt et al. 2006). It normally appears late in a growing season, causing superficial wounds and posing little economic damage to the crop (Canola Council of Canada 2016c). Worldwide, this species is considered less aggressive than *L. maculans* (Fitt et al. 2006). In China, canola imports carrying *L. maculans* from Australia and Canada are considered a major risk factor for introduction of the pathogen. In Canada, *L. maculans* was found initially in Saskatchewan in 1975 and the pathogen spread subsequently to Alberta by 1983 and Manitoba by 1984 (Gugel and Petrie 1992).

The prevalence and severity of blackleg varies among growing seasons (Howlett 2004), cropping system and canola varieties (Aubertot et al. 2004, 2006). It may also vary in different agro-ecological settings (Sosnowski et al. 2004). *Leptosphaeria maculans* is believed to have been present for over 60 years in Europe (Aubertot et al. 2004), 80 years in Australia (Sivasithamparam et al. 2005), and 40 years in Canada (Gugel and Petrie 1992; McGee and Petrie 1978). Diversity has been reported in this pathogen, and characterized on the basis of race structure (Petrie 1988), physiology (McGee and Petrie 1978), biochemical properties (Pedras and Biesenthal 2000), genetic variation (Gall et al. 1995), and molecular markers (Goodwin and Annis 1991). Evolution in virulence has been observed in western Canada (Chen and Fernando 2006; Kutcher et al. 2007), which was linked to the adaptation of the pathogen to canola cultivars with genetic resistance (Kutcher et al. 2007). The same happened in France (Rouxel et al. 2003a) and Australia (Li et al. 2003; Sprague et al. 2006), where major-gene resistance was overcome when *L. maculans* adapted to the *R* genes *Rlm1* in France and *LepR3* in Australia (Rouxel et al. 2003; Sprague et al. 2006).

2.3. Pathogen biology

Leptosphaeria maculans is an ascomycete fungus, and has historically been classified as a Loculoascomycete, a class comprising of over 6000 species (Silva-Hanlin and Hanlin 1999). The fungus affects mostly Brassica crops, especially *B. napus* and *B. rapa* oilseed rape or canola (Balesdent 2005). It is now in the class Dothideomycetes (syn. Loculoascomycetes). Frequent revisions of taxonomic subdivisions, which include the genus *Leptosphaeria*, have been described by Liew et al. (2000). At present, *L. maculans* belongs to the largest order, Pleosporales, which encompasses several important plant pathogens including *Phaeosphaeria nodorum* (formerly *L. nodorum*) and the genera *Alternaria, Cochliobolus*, *Pleospora*, *Pyrenophora* and *Venturia* (Berbee 2001).

Several species of *Leptosphaeria* have described on crucifers, either as saprophytes or pathogens (Petrie 1969; Rouxel et al. 2004). Over time, most of these species were believed to be a single species, i.e. *L. maculans*, initially separated into 'highly virulent' (A or Tox^{+}) and 'weakly virulent' (B or Tox^{0}) groups producing destructive stem canker and less damaging stem lesions, respectively (Rouxel et al. 2004; Williams and Fitt 1999). Molecular and biochemical differentiation of *L. maculans* and *L. biglobosa* isolates resulted in up to seven subspecies, of which some were represented by only a few isolates and were hypothesized to have a very narrow host range (Mendes-Pereira et al. 2003). The absence of *L. maculans* in Canada until the 1970s and in Poland until the early 1990s suggests that this is a 'younger' species, which continues to spread and is slowly replacing the less virulent *L. biglobosa* as is observed in western Canada and Poland (Rouxel et al. 2004; West et al. 2002, 2004). The continued spread of *L. maculans* is certainly a concern to countries such as China, where only *L. biglobosa* has been reported (Fitt et al. 2008).

Leptosphaeria maculans is a single-cross hybrid ascomycete with a genome size of about 34 Mb (Cozijnsen et al. 2000) carrying about 10,000-12,000 genes on 15 to 16

chromosomes, which are easily detected through pulsed field gel electrophoresis (Howlett et al. 2001). The genome of *L. maculans* has a unique structure; it is divided into separate irregular portions. These blocks are either gene rich or gene poor; those gene-poor regions hold some active genes, which are the main players during host infection as effector-avirulence genes, when no corresponding *R* genes exist in canola (Howlett et al. 2001).

2.4. Disease cycle

The life cycle of *L. maculans* can be complex. The existence of this pathogen is based on several modes but colonization in seed and crop stubble is the primary mode of survival and dispersal (Williams and Fitt 1999). Both pseudothecia and pycnidia contain viable spores for many years and under favourable conditions, high RH and temperature, pseudothecia will release ascospores and pycnidia will ooze a pink-colored mass of pycnidiospores (Ash 2000). Germ tubes of conidia and ascospores find their way into host leaf tissues via stomata or wounds, where fungal hyphae grow inter-cellularly after initial infection (Hammond and Lewis 1987). This phase of infection is symptomless initially but can lead to a necrotrophic phase as the infection progresses. By colonizing canola residues, *L. maculans* continues its saprotrophic mode (Punithalingam and Holliday 1972). Sexual production occurs when the two mating types are present under particular temperature, radiation, and suitable organic substances (Williams 1992; Petrie and Lewis 1985a).

2.5. Disease epidemiology and impact

In a way, the infection by blackleg is similar to that of mono-cyclic diseases; the epidemics are commonly initiated by airborne ascospores and/or pycnidiospores on cotyledons or lower leaves (Hall 1992; Mahuku et al. 1997). The disease incidence is often affected by the amount of initial inoculum, mostly from infested crop residues in the field (Hall 1992; Thurwachter et al. 1999) and, to a much less extent, from infected seed (Jacobsen and Williams 1971; Wood and Barbetti 1977a). The inoculum of *L. maculans* may also be blown

from other host cruciferous species (Hall 1992). High moisture, in the form of rain (Peres and Poisson 1997) or dew (McGee 1977), facilitates release of ascospores from pseudothecia on infested woody residues (Hall 1992; Mahuku et al. 1997), and this activity can last for an extended period of time but often coincides with the emergence of canola when plants are susceptible.

The release of ascospores often occurs in late fall or early winter in Australia, eastern Canada or western Europe (Rempel and Hall 1993; Gladders and Musa 1980), which coincides with the infection of "winter canola" seedlings. The situation in western Canada or Eastern Europe, however, is different; ascospores often are released in much greater amounts in spring when spring-seeded canola is in the seedling stage (McGee and Petrie 1979; Jedryczka et al. 1999).

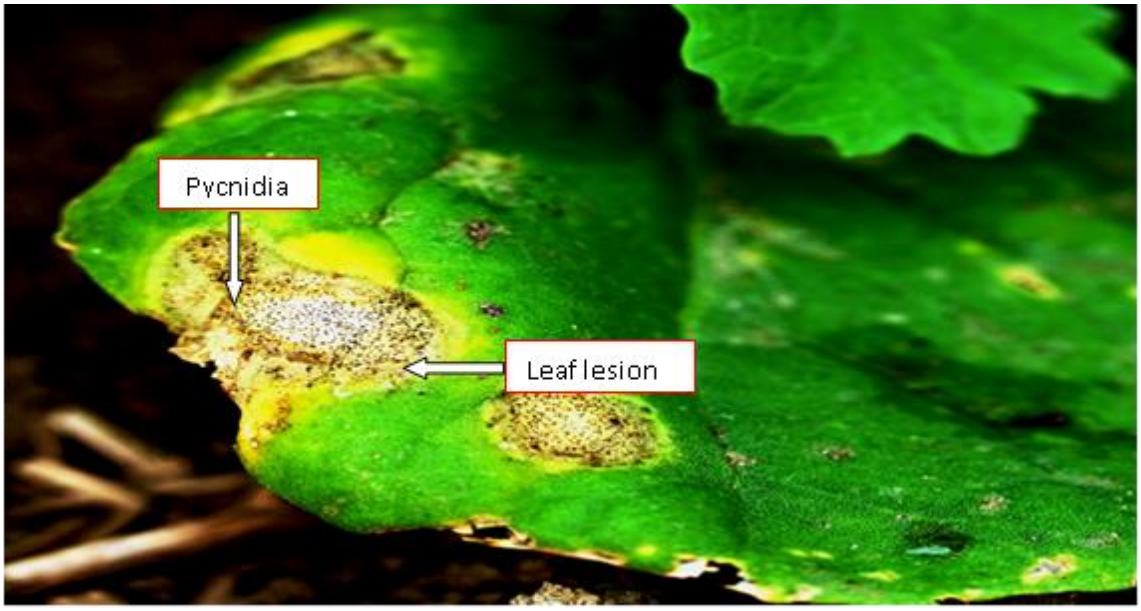


Figure 2.2. Lesions on young leaves of canola resulting from *L. maculans* infection under field conditions.

Wind is the key factor for inoculum dispersal (Shoemaker and Brun 2001; West et al. 2001). The *L. maculans* ascospores (~50×7 μm), generally come from the stubble of previous crops, penetrates cotyledons or young leaves through stomata or wounds, and proceeds further to the stem via petioles (West et al. 2001). The infection of cotyledons or young leaves and stem tissues may also be inferred as primary and secondary infections. On infected cotyledons or leaves, the tissues usually display greyish-green lesions (**Figure 2.2**) with invisible boundaries and quite often pycnidia (Brun et al. 1997; Ansan-Melayah et al. 1997). After the pathogen grows inside the stem, external symptoms are rarely observed until the plant reaches maturity, when basal stem cankers may be observed. The weakening of the lower stem by the disease can cause pre-mature ripening or lodging (Rouxel and Balesdent 2005).

Major epidemics were reported on oilseed rape in France in 1950 (Li et al. 2003; Rouxel et al. 2003). Australia encountered severe epidemics of blackleg on oilseed rape in the early 1970's, which devastated its thriving oilseed industry (McGee 1977). Similar epidemics imperiled the production of canola/oilseed rape in Canada and Europe in the 1970s and 1980s (Gugel and Petrie 1992). In contrast, blackleg is quite rare in Scotland or in Asia where large areas of oilseed rape are grown annually.

Yield loss caused by blackleg has been recognized for almost 100 years on many horticultural crucifer crops (Henderson 1918) but the information is sporadic on canola. In general, total crop destruction due to seedling death is rare and yield loss of approximately 10% may occur when disease incidence is high. However, 30 - 50% yield loss can happen under extreme disease situations (Hall et al. 1993; Zhou et al. 1999; Barbetti and Khangura 1999). In western Canada, shortly after the identification of *L. maculans* in 1975, the average incidence of blackleg increased tenfold between 1978 and 1981. By 1982 the disease was estimated to cause overall yield loss of 6% in Saskatchewan (Petrie 1985b), but in some

fields, the loss was as much as 56%. Field survey results in Saskatchewan indicated that 65% of *L. maculans* isolates were virulent on canola in 1986 (Jesperson 1986). In the 1980s, the disease became epidemic in Alberta, Manitoba and Ontario, and by 1988 several regions in Alberta reported yield losses of approximately 10% (Juska et al. 1997). By 1987 blackleg had been found in 83% of canola fields surveyed in Manitoba (Platford 1988), with approximately 8% yield loss. In Ontario, disease incidence increased from 32% in 1986 to 69% in 1987, resulting in approximately 8% yield loss. Both incidence and severity of blackleg continued to increase until the early 1990s (Gugel and Petrie 1992).

Weather conditions affect the persistence of pseudothecia on crop residues due to effects on the rate of decomposition, which is influenced by soil moisture and temperature. Under hot and dry conditions in Western Australia, canola residues may remain as an inoculum source for up to 4 years (Barbetti and Khangura1997). In western Canada, pseudothecia may also last for several years due to cold winters and relatively dry summers (Petrie 1986). In contrast, the mild wet climate in the UK encourages rapid decomposition of debris often within 2 years (West et al. 1999).

2.6. Blackleg resistance

Rouxel and Balesdent (2005) referred to Muller (1953) and Muller et al. (1957) and reported that *L. maculans* also infects non-cruciferous plants, including *Artemisia campestris* (Asteraceae), *Humulus* sp. (Moraceae), *Phaseolus* sp. (Fabaceae), *Swertia perennis* (Gentianaceae) and *Teucrium* sp. (Labiatae). Later studies have generally indicated that *L. maculans* is strictly a pathogen of crucifers and affects mainly Brassica crops (Petrie 1969). Molecular approaches have determined that many isolates from cruciferous weeds belong to several subspecies of *L. biglobosa* (Mendes-Pereira et al. 2003). Recent work by Li et al. (2005) showed that *L. maculans* would be able to infect several non-brassica species in the Brassicaceae family under field conditions, including *Raphanus sativus* (radish), *R.*

raphanistrum (wild radish), *Sinapis alba* (white mustard) and *Eruca vesicaria* ssp. sativa (rocket salad). The model plant *Arabidopsis thaliana* has also been suggested to be a potential host for *L. maculans*, at least under laboratory conditions (Bonham et al. 2004).

Two types of blackleg resistance exist in canola; quantitative and qualitative (Rimmer and Van den Berg 1992). Quantitative resistance is non-race specific and may be controlled polygenically. Often the resistance is not conspicuous until the plant attains the adult stage. Race-specific or qualitative resistance is controlled by specific *R* genes that interact with the pathogen in a "gene-for-gene" fashion (Ansan-Melayah et al. 1998). This type of resistance is usually effective at an early growth stage. It is generally believed that all canola cultivars in Canada carry a certain level of resistance to *L. maculans* although the source and nature of the resistance is generally unknown (Rimmer 2006).

2.7. Host-pathogen Interactions

Like other living organisms, crop plants tend to guard themselves by resisting against attacks from pathogens, invertebrates or even parasitic plants (Runyon et al. 2010). Due to lack of a circulatory system to activate immunity in response to pathogen attack, each single cell of a plant may be equipped with a built-in mechanism of defence inducible upon infection. This singularity separates the defence mechanism of plants from the immune system of vertebrates (Walbot 1985). Plant breeders generally acknowledge that most of the time disease resistance is inherited as a single dominant or semi dominant gene (Keen 1990). With genomic studies on both host plants and pathogens, and the knowledge of avirulence alleles in the pathogen population, our understanding of interactions between *L. maculans* and Brassica spp. has increased greatly in recent years (Balesdent et al. 2005; Stachowiak et al. 2006).

To understand these interactions, it is important to differentiate between two types of resistance carried by the host: 1) qualitative resistance expressed at seedling and adult-plant stages, and 2) quantitative resistance which is more pronounced at the adult stage with

reduced incidence and severity of basal stem canker or blackleg. Adult plant resistance (APR) in the field may be conferred by race-specific major genes or race non-specific polygenes (Delourme et al. 2006). The presence or absence of specific *R* genes in *B. napus* or *Avr* genes in *L. maculans* can only be determined by genotyping the host or pathogen population. The *R* genes that confer resistance at cotyledon and adult plant stages are expected to differ from each other (Ballinger and Salisbury 1996), and in most cases they may be linked (Li and Cowling 2003; Zhu and Rimmer 2003). The mechanism of quantitative resistance may change with the host/infection stage or resistance source used (Delourme et al. 2006). Hence, further characterization of the interaction in relation to APR and race-specific resistance at different plant/infection stages may help better decipher the modes of action involved in blackleg resistance. Often host resistance responses include the hypersensitive reaction surrounding the infection site that may quickly arrest pathogen hyphae. Additionally, the production of callose, phytoalexin and lignin, accumulation of pectin in the lumen of xylem vessels, and induction of pathogenesis-related (PR) proteins may all contribute to the resistance responses of plant hosts (Hammond et al. 1985; Chen et al. 1996).

2.8. Strategies of host-plant resistance to disease

Many plants are bestowed with a well-developed and well-organized defence mechanism, through which they can resist attacks by microbial pathogens (Dangl and Jones 2001). Upon infection, the plant discerns the pathogen through pathogen associated molecular patterns (PAMPs), which involves the recognition of common features of microbes (Zipfel and Rathjen 2008). Pathogens may also have well-developed mechanisms to escape recognition through the production of "pathogenicity effectors" (Chisholm et al. 2006). The battle does not stop here and the process of guard and decoy between the pathogen and host is dynamic (Chisholm et al. 2006; Jones and Dangl 2006). In plants, Dangl and Jones (2001) proposed the guard theory, which suggests the indirect recognition of a virulence factor or pathogen. It

suggests that *R* proteins act by watching (guarding) for the effectors, targeting proteins encoded by resistance genes are sometimes called (r proteins) in plants and any change in them results in the activation of *R* proteins which triggers disease resistance in the host (Dangl and Jones 2001; Jones and Dangl 2006; Van Der Biezen and Jones 1998). This recognition is triggered by avirulence (*Avr*) genes in the pathogen that correspond to *R* genes in the host following the gene-for-gene theory (Flor 1942). This facilitates the defense mechanism of a plant to respond to a particular tactic of pathogenesis at a faster rate than that of the plant can adapt to. Where, one NBS-LRR protein is able to recognize the effects of more than one virulence factor or effector (Dodds and Rathjen 2010)

Effectors secreted by the pathogens during early infection are small molecules that facilitate the infection by the pathogen (de Jonge et al. 2011). Effectors, which are *Avr* proteins co-evolved with host *R* proteins encoded by major *R* genes. This relationship abides the gene-for-gene principle between an *Avr* gene and its corresponding *R* gene that enables the plant to neutralize the effects of *Avr* proteins. This phenomenon is called effector-triggered immunity (ETI) by Plissonneau et al. (2016).

2.9. Additional host resistance strategies

The interaction between Brassica spp. and *L. maculans* can be complicated and may not always follow the gene-for-gene model, as shown in recent studies when *AvrLm3* and *AvrLm4-7* alleles were involved (Plissonneau et al. 2016). The highly expressed allele of *AvrLm3* seems not to have identifiable homologues in other fungal species, not even those closely related to *L. maculans*, including *L. biglobosa* and *L. maculans* f. sp. *lepidii* (Grandaubert et al. 2014). Although *AvrLm3* possesses all the features commonly found in *Avr* proteins of *L. maculans*, the presence of *AvrLm4-7* would deter its recognition by the corresponding *R* gene, *Rlm3*. Conversely, if *AvrLm4-7* is absent, *AvrLm3* functions as expected. This may be due to interactions between *AvrLm3* and *AvrLm4-7*, where *Rlm3*-

trigered resistance is ‘concealed’ by the functional alleles of *AvrLm4-7* (Houterman et al. 2008). It appears that co-existence of *AvrLm3* and *AvrLm4-7* in *L. maculans* would subdue pathogen recognition by *Rlm3* (Plissonneau et al. 2016).

A similar situation occurred in the interactions between the *Avr* genes *AvrLm1* and *AvrLepR3* with the *R* genes *Rlm1* and *LepR3*; cloning of *LepR3* confirmed that *Rlm1* and *LepR3* are separate *R* genes that both interact with the corresponding avirulence genes *AvrLm1* and *AvrLep3* (Larkan et al. 2013). Therefore, when the cv. “Surpass 400”, which was initially believed to carry only *LepR3*, was used to identify *Avr* genes in the pathogen, the reactions were complicated because the cultivar would also react to the isolates that carried *AvrLm1* (Van de Wouw et al. 2009). As *LepR3* interacts with *AvrLm1,* and *Rlm1* with *AvrLepR3*, additional tests were required to identify *AvrLm1* and *AvrLep3* in the pathogen population.

2.10. Mechanisms of blackleg resistance

Both quantitative or APR, and qualitative, or race-specific resistance have been reported in canola against *L. maculans* (Delourme et al. 2006). In some cultivars both types of resistance may exist. For example, Zhang et al. (2015) reported that more than 50% of CCCs smight carry a level of APR against blackleg. Race-specific resistance is conferred by a known *R* gene and expressed strongly at seedling stages. It is generally believed that the recognition between a specific *R* gene and it’s corresponding *Avr* gene in the pathogen results in a cascade of host-defense responses (Ansan-Melayah et al. 1998).

To date, at least 18 specific *R* genes have been reported in Brassica species against *L. maculans* (Zhang et al. 2015); *Rlm1, Rlm2, Rlm3, Rlm4, Rlm7* and *Rlm9* were originally from *B. rapa* or *B. oleracea*, but have been identified in *B. napus*. These *R* genes have been mapped to the linkage groups N7 and N10 in *B. napus* (Ferreira et al. 1995; Mayerhofer et al. 1997; Ansan-Melayah et al. 1998; Zhu and Rimmer 2003; Rimmer 2006; Delourme et al.

2006). *Rlm8* and *Rlm11* were identified also in *B. rapa* (Balesdent et al. 2002, 2013), *Rlm5* and *Rlm6* were in *B. juncea* (Chevre et al. 1997; Balesdent et al. 2002), and *Rlm10* in *B. nigra* (Chevre et al. 1996; Eber et al. 2011). The genes *LepR1*, *LepR2*, *LepR3*, *LepR4* and *RlmS* were identified from *B. rapa* subsp. *sylvestris* (Yu et al. 2005, 2007; Van de Wouw et al. 2009), and *BLMR1* and *BLMR2* from *B. napus* cv. Surpass 400 (Long et al. 2011). Of these only the *R* genes *LepR3* (also interacts with *AvrLm1*) and *Rlm2* have been cloned so far (Larkan et al. 2013, Larkan and Borhan 2015). At least seven of the *Avr* genes in *L. maculans* have been cloned, which include *AvrLm1* (Gout et al. 2006), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLm3* (Plissonneau et al. 2016), *AvrLm5*/*AvrLmJ1* (Van de Wouw et al. 2014), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007) and *AvrLm11* (Balesdent et al. 2013).

Plants, with quantitative resistance deters infection and colonization by pathogen, hence the disease development on the host (Poland et al. 2009; Young 1996). It is often characterized as partial, polygenic, horizontal, non-race specific or field resistance. Against blackleg, this type of resistance is more manifested in the adult-plant stage than in seedling stage.

2.11. *Avr* genes in the *L. maculans* population

With sexual recombination, *L. maculans* has a great ability to adapt to, and overcome a specific *R* gene, especially when the pathogen population is large the host plants are abundant (Rouxel and Balesdent 2005; West and Fitt 2005). Race-specific *R* genes exert strong selection pressure on the pathogen population, allowing virulent mutants to adapt to the host rapidly (McDonald and Linde 2002). There have been examples of *L. maculans* adapting rapidly to new *R* sources in oilseed rape on which artificial inoculum was applied (Brun et al. 2000) or in commercial fields where canola/rapeseed cultivars carrying new *R* genes were introduced (Li et al. 2003; Rouxel et al. 2003; Sprague et al. 2006). In each case, the

pathogen population likely changed and became virulent only three to four years after the introduction of the new cultivars. To deploy effective *R* genes and restore cultivar resistance to blackleg, it is useful to understand the *Avr* gene structure in the pathogen population (Dilmaghani et al. 2009).

A total of 16 *Avr* genes have been identified in *L. maculans*, and a differential set of hosts with known specific *R* genes can be used to study the *Avr* gene structure in a pathogen population (Kutcher et al. 2007). Differences among *L. maculans* populations in terms of race structure, diversity and complexity have been shown globally; 18 races have been found in Australia carrying an average of 5.11 virulence alleles per isolate but only 8 races identified in Europe carrying 4.33 virulence alleles per race (Balesdent et al. 2005).

Studies of the *L. maculans Avr*-allele structure were conducted in France (Balesdent et al. 2006) and northern Europe (Stachowiak et al. 2006), with variations noticed in pathogen populations in different regions. Balesdent et al. (2006) suggested that even with the high frequency of certain *Avr* alleles, the virulence alleles already present in the pathogen population would eventually be capable of overcoming each of the nine *R* genes (*Rlm1* - *Rlm9*) identified. For example, *AvrLm6* and *AvrLm7* were detected in greater than 99% of the 1,787 isolates characterized, but *AvrLm2* and *AvrLm9* were absent (Balesdent et al. 2006). Using *Rlm6* or *Rlm7* may rapidly select for the corresponding virulent alleles in the pathogen population. Similarly, in a pan-European study involving isolates from the UK, Germany, Sweden and Poland, Stachowiak et al. (2006) did not detect the alleles *AvrLm2*, *AvrLm3* or *AvrLm9* among the 603 isolates examined. The alleles *AvrLm1* and *AvrLm4* were only at low levels (< 10%), while the frequency of alleles *AvrLm5* (86%), *AvrLm6* (100%) and *AvrLm7* (> 99%) was high.

Balesdent et al. (2005) characterized race structure of 63 isolates from a global collection by classifying them into pathogenicity groups (PGs). The analysis of those isolates

revealed geographical differences in the proportion of PGs. Of 26 races, 18 were identified from Australia, which was higher than Europe with eight races. The average number of virulence alleles in isolates collected from Australian was also higher (5.11) than in Europe (4.33) or Canada (3.46).

In Germany, 644 isolates of *Leptosphaeria maculans* were collected from four areas in northern Germany in autumn 2011 and 2012. A total of 13 races were determined by inoculating the canola cultivars carrying six specific resistance genes: *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9*, where race *Av5-6-7-*(*8*), virulent to (*Rlm1-4 and Rlm9*) was detected in 85% of the isolates, race *Av1-5-6-7-*(*8*) (virulent to *Rlm2–4* and *Rlm9*) detected in 10% of the isolates, and only six of the 644 isolates exhibited (0.9 %) virulence to *Rlm7* (Winter and Koopmann 2016).

In western Canada, signs of virulence evolution in the pathogen population were observed in late 1990s (Chen and Fernando 2005; 2006; Keri et al. 2001). A pathogenesis-grouping (PG) system was used initially to detect changes in the pathogen population (Kutcher et al. 2007). Further genetic studies identified a large number of *R*-genes in Brassica spp.; therefore the PG classification system, which was based on three resistance genes, explained limited variability of the pathogen population on the prairies. Thus, races of *L. maculans* were described on the basis of avirulence genes carried by each isolate (Kutcher 2010). More recently, following the same approach, the analysis of *L. maculans* isolates collected from the prairies showed that about 98% of the isolates carried 3 to 6 *Avr* genes (Kutcher et al. 2010). In general the frequency of *AvrLm1*, *AvrLm3, AvrLm9* and *AvrLep2* were very low in many regions of the prairies, indicating that canola cultivars carrying the corresponding *R* genes *Rlm1, Rlm3, Rlm9* or *LepR2* would no longer be effective.

2.12. Management of blackleg in canola

Blackleg of canola was managed successfully in western Canada for almost 20 years with a combination of resistant cultivars and diverse crop rotations (Kutcher et al. 2011a). In the early 1990's resistant cultivars of canola were introduced and extended crop rotation adopted by many growers. Both race-specific (monogenic) and non-race-specific (quantitative) resistance may have been present in these cultivars (Rimmer 2006), and some of the race-specific *R* genes may have similar molecular mechanisms against the races of the pathogen (Larkan et al. 2013).

These measures reduced the impact of blackleg in canola, but did not eliminate the pathogen, which changed in virulence since late 1990's (Keri et al. 2001; Chen and Fernando 2005; 2006). Only a limited number of known *R* genes have been found in CCCs, namely *Rlm1* (10%) and *Rlm3* (70%) (Zhang et al. 2015). Non-race-specific (quantitative) resistance or APR is not well understood in Canadian cultivars.

There are examples of the defeat of race-specific resistance due to changes in the pathogen *Avr* gene profile in response to the *R* gene used (Balesdent et al. 2006). Therefore, understanding the genetic basis for blackleg resistance is important to deploying or rotating *R* genes for effective control of blackleg (Kutcher et al. 2010). For sustainable management of blackleg, knowledge of the dynamics between *R* genes and the pathogen population is important; the durability of race-specific resistance depends on the amount of pathogen inoculum and the *L. maculans* race composition, which is often correlated with infested stubble from preceding crops (Kutcher et al. 2010).

Combining race-specific with race non-specific (horizontal) resistance could be the best technique for managing blackleg of canola. Kutcher et al. (2010) further suggested that for effective use of specific *R* genes, the pathogen population should be monitored at regular intervals to track the changes in race structure. A management plan involving selection of

resistant varieties based on the pathogen population or rotation of *R* genes over time, in combination with quantitative resistance and diverse crop rotation should safeguard canola production. This strategy was adopted in France and Australia where growers and the industry are provided with the timely information on the regional pathogen race composition and cultivars carrying *R* genes effective on a regional basis (Peres and Poisson 1997; Marcroft et al. 2004).

The epidemics of blackleg in canola are closely related to the abundance of infested stubble of the prior crop in which the pathogen survives as long as the residue persists in the field. This period may range from one to four years depending on climatic conditions (West et al. 2001). Because infested crop residue is the most important source of *L. maculans* inoculum, canola should not be seeded into canola stubble or close to fields that have had diseased crops within the past three years (Kutcher et al. 2011a). A diverse crop rotation can complement resistant cultivars by alleviating the pressure of pathogen inoculum, hence reduction the risk and speed of resistance breakdown. An early season fungicide treatment can reduce disease severity, but the yield benefit is often insignificant if the cultivar is even moderately resistant (Peng et al. 2014).

2.13. New techniques potentially useful to studying plant-pathogen interactions

2.13.1. Droplet-digital PCR

Many molecular tools have been developed in recent years that are useful to study host-pathogen interactions, including droplet-digital PCR (ddPCR) and green florescent protein (GFP) labelling.

The quantification of pathogen DNA in diseased plants using the latest molecular methods is becoming increasingly popular due to the sensitivity and accuracy of these technologies. These methods include real-time and end-point PCR, which yields delicate and highly specific DNA detection from fungi, viruses, bacteria and other microbial organisms in

a very short time. Conventionally, the detection and quantification of plant pathogens are performed by isolation using selective media or by conducting biochemical, chemical or immunological analyses (Mihail et al. 1992). These methods often can identify and confirm the presence of a target pathogen, but are generally labour intensive and require skilled taxonomical expertise (Lievens et al. 2005). By contrast, molecular-based techniques such as polymerase chain reaction (PCR) assays have proved to be rapid, highly specific and sensitive, overcoming some of the shortcomings with conventional bioassays (Sankaran et al. 2010; Schena et al. 2004). PCR identification may also be applied to non-culturable microorganisms (Lievens et al. 2005).

To assess host resistance, it is often relevant to measure pathogen movement or proliferation in plant tissues (Bonants et al. 2004), and PCR techniques, such as real-time PCR (RT-PCR), can provide quite reliable estimates. Many plant pathogens can be detected or quantified using PCR-based methods (Schaad et al. 2003), and new technologies have been adopted continuously. For example, the ddPCR system is a platform that allows a substantial gain in dynamic range while bringing the cost of analysis down relative to other PCR procedures. Therefore, it has been popular for many applications (Baker 2012; Pinheiro et al. 2012). Unlike regular quantitative PCR (qPCR), which uses a standard curve, ddPCR offers an accurate and absolute measurement allowing precise calculation of copy numbers of target DNA without the need for a standard (Bhat et al. 2009; Corbisier et al. 2010; Hindson et al. 2011). The detection and quantification of viral and bacterial pathogens have also been carried out using ddPCR (Roberts et al. 2013). One of the advantages of ddPCR is its low sensitivity to inhibitors (Maunula et al. 2009). Therefore, it is a desired platform for detection and quantification of pathogens in plant tissues.

2.13.2. Fluorescence microscopy

Microscopic studies in the field of plant pathology have continued to be important since the invention of the microscope in late 17th century (Puglia and Vannacci 2012). In the interaction between a fungus and a plant, it is important to investigate disease progression based on the amount of pathogen bio-mass in infected plant tissues. Therefore, markers that can be easily measured and visualized are required to determine pathogen growth and development. Fluorescence microscopy can be used for observing biological samples *in vivo* and this need has prompted rapid development of many highly specific techniques and applications (Ishikawa-Ankerhold et al. 2012; Shaw and Ehrhardt 2013).

The Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* Victoria (Cody et al. 1993) can be used as a universal reporter for a broad range of heterologous living cells and organisms (Chiu et al. 1996). Improvements have been made to render GFP a versatile and sensitive reporter in a variety of living plant cells and in transgenic plants (Maor et al. 1998). One of the greatest advantages of using GFP as a marker is that fluorescence intensity is directly proportional to the amount of protein (Maor et al. 1998). As such, measuring the amount of GFP will provide a means of quantifying the organism if the GFP gene is controlled by a constitutive promoter (Littlejohn et al. 2015). Additionally, as GFP is species-independent it can be studied in living tissue without the disintegration of cells or demolition of tissue (Chiu et al. 1996). The fluorescence of GFP is very stable under a range of conditions, including temperatures up to 65ºC and a pH range of 3-12. (Yang et al. 1996). GFP has been used in a large variety of organisms, including animals, plants, fungi and bacteria (Chalfie 1994; Casper and Holt 1996; Spellig et al. 1996). A modified version of the original GFP gene, *sgfp*, has been expressed at high levels in a number of filamentous fungi (Maor et al. 1998; Sexton and Howlett 2001).

The original GFP displayed a unique spectrum; it was excited by peak wavelengths of 395 or 475 nm, emitting a green fluorescence with a peak at 509 nm that can be directly visualized or captured in organisms by imaging detection instruments (Chalfie 1994; Heim et al. 1994). Recently, through codon mutagenesis within the chromophore region by dozens of laboratories, numerous GFP variants displaying distinct excitation and emission maxima have been developed (Cormack et al. 1996; Cubitt et al. 1995), allowing multiple GFP variants to be simultaneously expressed and tracked in the same cells or tissues. GFP markers have inherent fluorescence that allows for non-invasive detection without the introduction of cofactors or destruction of the biological sample (Chalfie 1994).

GFP has been gaining the popularity as a potent bioluminescent marker in the study of physiological, molecular, genetic and biochemical events in plants and other organisms (Baulcombe et al. 1995; Haseloff et al. 1997; 1998; Sheen et al. 1995). This approach has enabled the investigation of the location of subcellular proteins (Schneider 2000), compartments in mitochondria, plastids or the cytosol (Englert et al. 2007; Galvez et al. 1998; Hedtke et al. 1999; Noji et al. 1998; Zhu et al. 1997), in groups of cell motors (Hasezawa et al. 2000), and repetitious domains in chromosomes of a genome (Lindhout et al. 2007). GFP has also been utilized to trace intracellular or intercellular protein movement and trafficking in response to changes in environmental signals (Kircher et al. 1999; Yamaguchi et al. 1999) and in developmental stages (Imlau et al. 1999; Itaya et al. 1998), as well as during pathogen infection (Carette et al. 2000; Peleg et al. 2007). Furthermore, adopting GFP as a visible marker also facilitated the study of fundamental biological processes such as microbe-plant symbiosis (Gage et al. 1996), insect pest and plant host interactions (Urwin et al. 1997), plant development (Ottenschlager et al. 1999), host stress responses (Manak et al. 2002) and anti-virus or gene-silencing mechanisms (Baulcombe et al. 1995; Dalmay et al. 2000; Voinnet et al. 1998). The GFP marker also offers several advantages over other reporters such as b-

glucuronidase (GUS) or luciferase (LUC), whose detection would require either destructive, X-glcA, {The most commonly used chemical compound, with the molecular formula $C_{14}H_{13}BrClNO_{7,}$ in experiments of molecular biology as a marker for GUS histochemical analysis (Nguyen 2002)}, staining followed by ethanol (Jefferson 1987) or luciferin treatment, which would kill the tissue or affect tissue permeability of mature plants (Millar et al. 1992).

2.14. Proposed research

The literature highlighted above testifies to the fact that *L. maculans* has a great ability to evolve and adapt. Although blackleg of canola was managed successfully for many years in western Canada between the early 1990s and 2009 by the use of resistant cultivars and extended crop rotations, the disease has increased noticeably in the past few years. This may be caused by a shift in the pathogen race structure, which makes some of the current resistant cultivars, susceptible.

Race-specific resistance targeting certain *Avr* genes in the pathogen population can be highly effective but this type of resistance can often be defeated rapidly with changes in the pathogen. It was not clear if losses of certain *Avr* alleles in the pathogen population are the primary cause of severe blackleg damage on certain *R*-rated canola cultivars. Race-nonspecific resistance may exist in many widely distributed canola cultivars, but there is little information on how it works. This type of resistance will not stop foliar infection at the seedling stage, but may limit the spread of pathogen into the stem, as well as disease development in the stem. The mechanism of this type disease resistance is still not clear, but it may be useful to blackleg management in western Canada where the growing season is much shorter for blackleg development than in other parts of the world.

The overall goal of this proposed research was to understand the potential uniqueness of the pathogen race structure in commercial canola fields with high levels of blackleg,

relative to those observed in regional monitoring. Knowledge of the pathogen race structure, together with management and cultivar information, may provide a clue to the cause of relatively isolated cases of severe blackleg damage in some fields. Additionally, *R* genes in common CCCs were determined and the main resistance mechanisms of the cultivars determined to understand how the current cultivars resist blackleg disease. This information will help to identify the primary mechanisms of blackleg resistance associated with current canola cultivars and develop optimal host resistance strategies for blackleg management.

Chapter 3. Analysis of the *Avr*-gene profile of *Leptosphaeria maculans* in Canadian canola fields

3.1. Abstract

Blackleg of canola, caused by the fungal pathogen *Leptosphaeria maculans*, had been managed successfully in western Canada for many years with use of resistant cultivars and extended crop rotations until recent years when both disease incidence and severity increased noticeably. This may be attributed to changes in the pathogen population that erodes the resistance of canola cultivars. Additionally, the resistance associated with Canadian canola cultivars (CCCs) in Canada was not well understood. The current study was conducted to investigate the race structure of *L. maculans* in selected commercial canola fields and its impact on blackleg incidence and severity observed in these fields. A total of 372 *L. maculans* isolates collected from 17 canola fields with different levels of blackleg severity in 2012 and 2013 were analysed for the presence or absence of certain avirulence (*Avr*) alleles using a set of 12 host differentials carrying known *R* genes. The results showed great diversity in the pathogen population, with a total of 90 races identified in these fields. The races *AvrLm2-4-6-7* and *AvrLm2-4-6-7-S* are most prevalent, accounting for 11.6% and 10.8% of the population, respectively. The alleles *AvrLm1*, *AvrLm3, AvrLm9* and *AvrLep2* were at very low or undetectable levels in these fields, while *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7* were generally common. Some variations in *Avr* profile were observed between fields or cultivars, but since only the *R* genes *Rlm1* and *Rlm3* may be present commonly in CCCs, these results indicate that most of our cultivars do not carry the specific *R* genes corresponding to the prevalent *Avr* genes in the current pathogen population on the prairies.

3.2. Introduction

The fungus *L maculans,* causing blackleg disease in canola, has a strong ability to evolve and adapt. Although the disease was managed successfully in western Canada between the 1990s and 2000s by use of resistant cultivars and extended crop rotation, recent field surveys since 2010 have shown noticeable increases in blackleg incidence as well as severity, especially in southern Manitoba and east-central Alberta. This trend might be a result of a shift in pathogen race structure that had made some of the current cultivars susceptible or more intensive canola production. Race-specific resistance targeting corresponding avirulence (*Avr*) genes in the pathogen population has been a key strategy against blackleg (Ansan-Melayah et al. 1997; Rouxel et al. 2003). This type of resistance can be expressed at both seedling and adult-plant stages, but may also be ineffective rapidly with changes in the pathogen race structure. It was not clear if the severe blackleg disease observed on some of the resistant cultivars was due to the loss of certain *Avr* alleles in the pathogen population or to other reasons. Approximately 60% of current canola cultivars or breeding lines carry the resistance (*R*) gene *Rlm3* and 10% carry *Rlm1*, but other specific *R* genes seem to be relatively uncommon (Zhang et al. 2015).

Blackleg pathogen races on the Canadian prairies have been studied since the early 1990s, first using a pathogenesis-group (PG) system which identified the evolution of the pathogen population from predominantly PG2 to a mixture of PG2, PG3, PGT and PG4 (Chen and Fernando 2006, Kutcher et al. 2007). More recently, the pathogen population was characterized on the frequency of *Avr* genes using a set of Brassica hosts carrying specific *R* genes (Kutcher et al. 2007). Using this approach and *L. maculans* isolates from "Westar" (susceptible) trap plots across the prairies in 2007, Kutcher et al. (2011) was able to present a regional picture on the *Avr* gene profile and showed that *AvrLm1* and *AvrLep2* were generally at very low levels in the pathogen population. This means that the *R* genes *Rlm1* and *LepR2* are no longer effective in many areas of the prairies. Regional differences were also revealed

by this study; *AvrLm2* and *AvrLm3* were scarce in southern Manitoba but relatively common in Saskatchewan and parts of Alberta. This regional *Avr* profile, when taken together with the information on *R* genes in commercial cultivars, can provide a basis for selecting canola cultivars for blackleg management on a regional basis.

The development of new pathogen races, coupled with increasingly more intensive canola production on the prairies, poses a risk to the genetic resistance carried by many canola cultivars (Fleury 2013). Provincial canola disease surveys in recent years have found fields of *R*-rated cultivars with high levels of blackleg both incidence and severity, especially in southern Manitoba and east-central Alberta (Fleury 2013). In Saskatchewan, blackleg has been found more frequently in the northwestern and southeastern regions (Cross et al. 2012; Liban et al. 2013). There were an increased number of reports on blackleg in 2012, compared with previous years, and some of the fields in southern Manitoba were severely damaged (Fleury 2013). It was possible that the increase in disease damage was caused by a shift in pathogen races in these fields. If this is the case, changing the canola cultivar to one carrying *R* genes effective against the current pathogen population would lower the risk of blackleg damage.

The objectives of this study were to: 1) conduct annual field surveys in a number of regions on the prairies to identify canola fields with different level of blackleg incidence and severity, 2) characterize the pathogen *Avr*-gene profile to understand the pathogen race composition in each individual field, and 3) analyze the role of *Avr* change in causing blackleg disease based on the information from *Avr* structure, canola cultivar, crop rotation, fungicide application as well as other management practices.

This research was aimed to assess whether the “resistance breakdown” observed in isolated commercial fields was caused by a shift in the pathogen *Avr*-gene structure. The information will be useful for assessing the resistance mechanisms and effectiveness of

current canola cultivars against the current pathogen population in western Canada, allowing producers and the industry to fine tune cultivar resistance strategies by rotating cultivars or crops based on the knowledge of pathogen *Avr* profile and host resistance characteristics.

3.3. Materials and Methods

3.3.1. Field surveys and pathogen isolation

To collect diseased canola stubble, field surveys were conducted between 2012 and 2014 in consultation with regional crop specialists/agronomists. Most of the fields reported with blackleg were in central Alberta, northwestern Saskatchewan, and southern Manitoba. In a region, multiple fields within about a 10-km radius and 10 km from another canola crop were selected, wherever possible, to represent the region. In a region, wherever possible, multiple cultivars and break intervals from canola were also selected, for additional comparisons. A questionnaire (Appendix 1) was used to collect the information. In each of these fields, >100 plants at growth stage 5.2 (Harper and Berkenhamp 1975) or within a week after swathing were pulled at five random locations along a "W" path across the field. These were examined for incidence of basal stem canker and then cut at the crown area to assess blackleg symptoms on the cross section using a 0-5 scale (Appendix 2) for disease severity. Diseased stem pieces from each field were kept for isolation of *L. maculans*.

3.3.2. Production of *L. maculans* inoculum for host inoculation

Diseased stubble collected during the 2012 and 2013 cropping seasons were surface sterilized in 70% ethanol for 5 sec, then in 10% Clorox® Bleach (0.6% sodium hypochlorite) for 60 sec, and rinsed in tap water twice before plating on V8-juice (Campbell Soup Ltd, Toronto, ON) agar amended with 100-ppm streptomycin sulphate. The plates were placed at 20ºC with 12 h cool white fluorescent lighting for 5-10 d to allow *L. maculans* to produce pycnidia. A small amount of ooze was transferred from a single pycnidium to fresh V8-juice agar plates as to

get a genetically uniform isolate, and incubated for about 7 d under the same conditions as above. Only one *L. maculans* isolate from a diseased stubble piece was kept.

Each plate was then flooded with 7 mL of sterile distilled water and gently scrapped with a bent stain-less steel rod to dislodge conidia from pycnidia. A Falcon™ cell strainer (70 μm pore size, Fisher Scientific Canada, Edmonton, AB), designed to fit in a 50-ml Falcon™ centrifuge tube, was used to filter mycelia or other debris from the conidial suspension. The concentration of spore suspension was estimated using a haemocytometer and adjusted to about 1×10^7 spore mL^{-1} with sterile water for plant inoculation (Kutcher et al. 2011b).

3.3.3. Host differentials for detection of *Avr* genes in the pathogen population

A set of 12 Brassica cultivars/lines (Table 3.1) carrying at least 11 specific *R* genes (*Rlm1-4*, *Rlm6-7, Rlm9, LepR1-3* and *RlmS*) against blackleg were used to determine the presence or absence of *Avr* genes in the pathogen population. The canola cultivar Westar, which carries no *R* gene, was used as a susceptible control throughout the study.

Table 3.1. Differential set of Brassica cultivars/lines carrying specific *R* genes for detection of *Avr* genes in *L. maculans*.

Cultivar/lines	***R* gene carried**	***Avr* to be detected**	**Source of *R* genes**
Westar	none	none	N/A
Quinta	*Rlm1, Rlm3*	*AvrLm1,3; AvrLep3*	*B. napus*
Cooper	*Rlm1, Rlm4*	*AvrLm1,4*	*B. napus*
MT29	*Rlm1, Rlm9*	*AvrLm1,9*	*B. napus*
Topas-*Rlm2*	*Rlm2*	*AvrLm2*	*B. napus*
Falcon	*Rlm4*	*AvrLm4*	*B. napus*
Forge	*Rlm6(8)*	*AvrLm6(8)*	*B. juncea*
01-23-02	*Rlm7*	*AvrLm7*	*B. napus,*
Darmor	*Rlm9*	*AvrLm9*	*B. napus*
1065	*LepR1*	*AvrLep1*	*B. rapa*
1135	*LepR2*	*AvrLep2*	*B. rapa*
Surpass 400	*LepR3, RlmS*	*AvrLm1,S; AvrLep3*	*B. rapa*

The differential lines were randomly seeded in the Sunshine #3 soil-less planting mix (Sun Gro Horticulture Canada Ltd., Vancouver, BC) amended with 12.5 g L^{-1} Osmocote Plus 16-9-12 (N-P-K; Scotts Miracle-Gro Canada, Mississauga, ON) in flats (65×40×5 cm) with each cultivar/line in two blocks. A plastic panel was used as a seeding guide to maintain the distance between cultivars/lines. Plants were watered from the bottom of the flats (trays) as described by Kutcher et al. (2011). Seeded trays were placed in a growth cabinet at 22/16°C (day/night) with a 16 h photoperiod provided with cool florescent tubes (512 μmol m^{-2} s^{-1}). After emergence, the plants were thinned to three per row, thus there were six plants of each genotype in two blocks (Appendix 3).

3.3.4. Inoculation of host differentials and assessment of disease severity

Cotyledons of each differential line were inoculated with the spore suspension of a *L. maculans* isolate at 7 d after seeding. Before inoculation both lobes of a cotyledon were wounded (0.5-mm hole) with a pair of modified tweezers, and a 10-μl droplet of spore suspension was placed over the wound. Inoculated plants were kept in the growth cabinet,

with emerging true leaves removed regularly to delay the senescence of cotyledons. At 12-14 d after inoculation, these plants were assessed for blackleg severity using a 0-9 scale (Delwiche 1980), which takes into the consideration both lesion size and pycnidium formation on cotyledons (Appendix 4). The highest score on the four inoculated lobes of two cotyledons of each differential line (cultivar) was recorded, whereas the average score over the 6 plants in two separate blocks was used to determine the nature of each isolate-host interaction following Kutcher et al. (2011), where the average score ≤ 4.9, that is below 5 where the lesion size is small and pycnidia is absent (Appendix 3), was considered a resistant reaction and ≥5.0 as susceptible.

3.3.5. Data analysis

Up to 25 *L. maculans* isolates might be used to represent the pathogen population in each field, but in most cases, only 17-25 isolates were tested for *Avr* profile. Infection scores on cotyledons were averaged over the six plants (three in each block) of each differential line in two blocks randomly positioned; a resistant reaction (≤ 4.9), where the lesion size is small and pycnidia absent (Appendix 3), was interpreted as the presence of an *Avr* gene(s) corresponding to at least one of the *R* genes in the host. Sometimes, several *Avr* genes could be identified in a single pathogen isolate based on deduction using multiple differentials. The *Avr* profile was expressed as a frequency (%) for each *Avr* gene identified in a pathogen population.

3.4. Results

3.4.1. *Avr* allele profile in targeted commercial canola fields in 2012

A total of 179 *L. maculans* isolates were collected from eight (CCCs) carrying *Rlm1* or *Rlm2* (Zhang et al. 2015) in two Alberta fields, three Saskatchewan fields and three Manitoba fields in 2012 (Table 2). These were tested on the host differentials for the frequency of *Avr* alleles in the pathogen population.

Table 3.2. Isolates of *L. maculans* collected from eight commercial fields in western Canada in 2012 and tested for the frequency of *Avr* alleles.

Province	**Location (Nearest town)**	**Number of *L. maculans* isolates tested**
Alberta	Olds	17
	Trochu	17
Saskatchewan	Speers	30
	Bigger	22
	Watrous	25
Manitoba	Cartwright	22
	Holland	23
	Winkler (blackleg nursery)	23
	Total	179

3.4.2. *Avr* allele frequencies in Alberta (2012)

In Alberta, samples were obtained from two sites near Olds and Trochu. The Trochu site had two adjacent fields seeded with cv. Invigor-5440 (Bayer CropScience) and 45S52 (Pioneer-DuPont). The former was listed as blackleg resistant in the 2012 Alberta Seed Guide (http://seed.ab.ca/-Spring2012AlbertaSeedGuide), while 45S52 was moderately resistant. The prior crops were canola (2010) and cereal (2011). Disease incidence was approximately 80% on Invigor-5440 with a yield of 1.84 t ha-1 and 10% on 45S52 with a yield between 2.25 and 2.37 t ha-1. The isolates of *L. maculans* were mostly obtained from the Invigor-5440 stubble. The field near Olds was seeded with cv. VT500 RR, which was also listed as resistant in the Seed Guide. The disease incidence was 50% and the yield was 1.66 t ha-1.

Profiling of *AvrLm* genes in the *L. maculans* population indicated variation among locations; *AvrLm6 and AvrLm7* were generally common in all fields and were detected in >90% of pathogen isolates (**Figure 3.1**). The *AvrLm1*and *AvrLm9* alleles were detected in few *L. maculans* isolates (<15%) in the Trochu fields, and were absent in the Olds field. The frequencies of *AvrLm2* and *AvrLmS* (Van de Wouw et al. 2009) were significantly higher (70-80%) in the Trochu fields, relative to those found in the Olds field (<30%). The most striking difference was with *AvrLm4*; it was present in nearly 60% of *L. maculans* isolates from Trochu but absent in the Olds population (**Figure 3.1**).

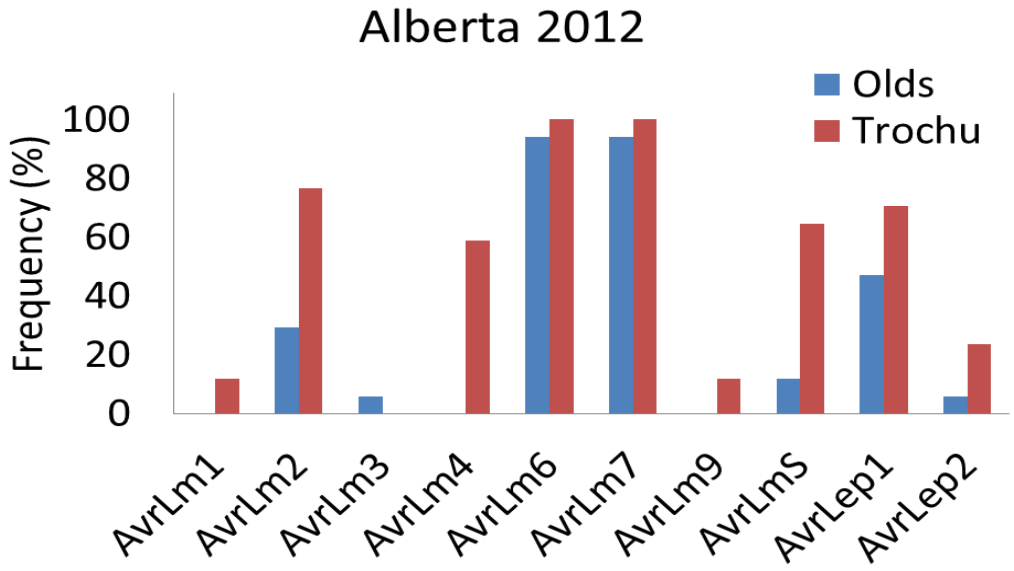


Figure 3.1. The frequency of *Avr* allele in *L. maculans* population detected in isolates collected from two severely diseased fields near Olds and Trochu, at Alberta.

3.4.3. *Avr* allele frequencies in Saskatchewan (2012)

Stubble samples were collected from three sites in the province where the blackleg incidence ranged from 70% to 80% at early crop maturity stage (GS 5.2). The cultivar in the field near Speers was 45S52, which was rated resistant in the 2012 Saskatchewan Seed Guide. The disease incidence in this field was 80%, but the cultivars in other two fields were unknown

nor was information on crop rotations and yields. The *Avr*-gene profile was similar among these fields (**Figure 3.2**) with the frequencies for *AvrLm2*, *AvrLm*6 and *AvrLm*7 generally >80%. The genes *AvrLm6* and *AvrLm7* were often found surpassing 90% in these fields, whereas the *AvrLm4* fluctuated between 60 and 80%. Similar to the picture in Alberta fields, the *AvrLm3*, *AvrLm9* and *AvrLep2* genes were low or absent in the pathogen population, while *AvrLm1*, *AvrLmS* (Van de Wouw et al. 2009) and *AvrLep1* were at moderate or very low frequencies (10-70%). *AvrLm1* was more common in the Saskatchewan fields than in the Alberta fields, especially in the field near Watrous where it was detected in approximately 70% of the *L. maculans* isolates.

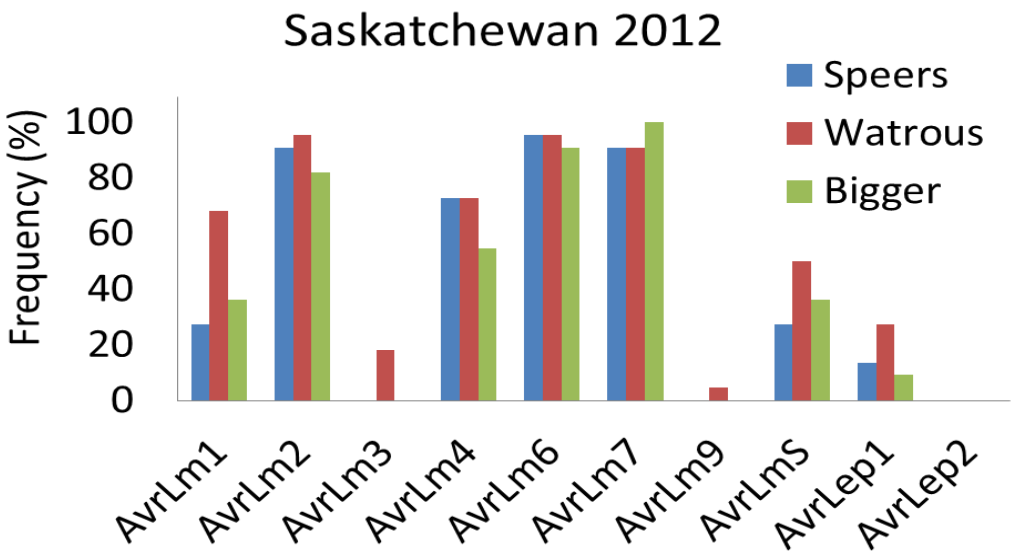


Figure 3.2. The frequency of *Avr* allele in *L. maculans* population detected in isolates collected from three severely diseased fields near Speers, Watrous and Bigger, at Saskatchewan.

3.4.4. *Avr* allele frequencies in Manitoba (2012)

Manitoba experienced dry and hot weather during July and August, 2012, and the average canola yield ranged between 1.48 to 1.59 t ha-1 (Honey 2013). Stubble samples were collected from three sites near Holland, Cartwright and Winkler (canola disease nursery). The cv. VT500 RR was seeded in the field near Holland, while cv. Nexera RR 1012 was grown in the field near Cartwright. Both cultivars were registered as resistant in the Saskatchewan Seed Guide 2012. The cultivar sampled from the field near Winkler was Canterra 1950 RR (>90% disease incidence), which was listed as moderately resistant. The disease incidence was >70% and >90%, respectively, in the fields near Holland and Cartwright. The crop rotation was back to back canola in the Holland field with reported yield at about 1.13 t ha-1, but rotation information was not available for the other two fields.

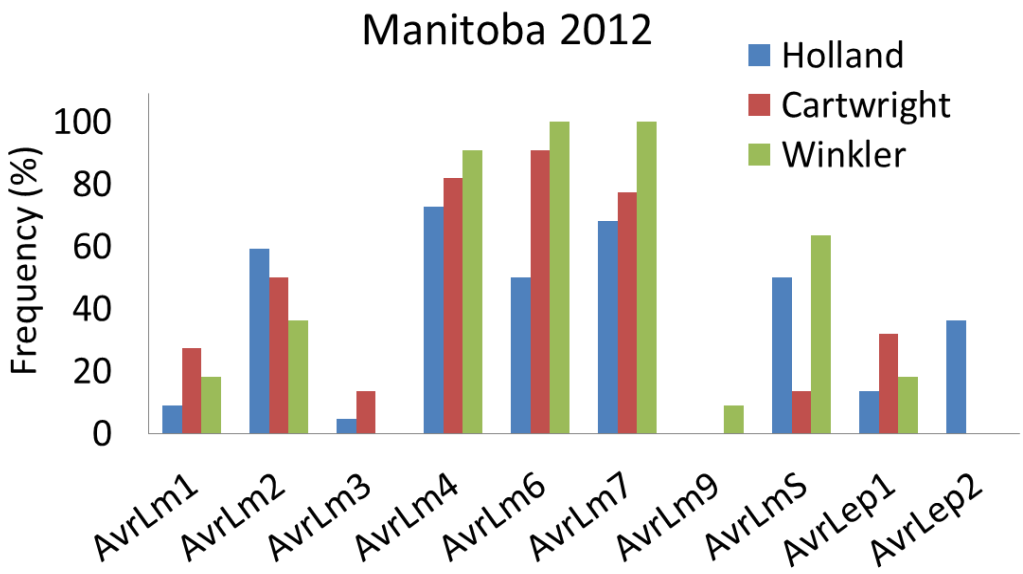


Figure 3.3. The frequency of *Avr* allele in *L. maculans* population detected in isolates collected from three severely diseased fields near Holland, Cartwright and Winkler, at Manitoba.

The *Avr*-gene profile was similar among the fields in Manitoba with only minor variation; the frequencies of *AvrLm4, AvrLm6* and *AvrLm7* were generally high (>70%), except in the Holland field where *AvrLm6* was only 50% (**Figure 3.3**). Genes *AvrLm1, AvrLm3* and *AvrLm9* were found at low (<30%) to undetectable levels, and this pattern was similar to those in Alberta and Saskatchewan. The gene *AvrLep2* was present in almost 40% of *L. maculans* isolates in the Holland field but was absent in the other two fields.

3.4.5. Race identification in the 2012 *L. maculans* population

A total of Sixty three (63) races, with different combinations of *Avr* genes, were identified from the 2012 population of *L. maculans* collected from eight diseased fields in western Canada (Table 3.3.). Of these races, 16 (with 2 to 6 *Avr* genes) were identified in >2% of the *L. maculans* isolates, but only five were detected in all three provinces (**Figure 3.4**). The race present at the highest frequency (11.2%) carried *Av2-4-6-7-(8),* and was the most common race in all three provinces. The races *Av2-6-7-(8)- Av6-7-(8)* and *Av4-6-7-S -(8)* were also

common and accounted for approximately 8%, 6% and 5%, respectively, of the races in the pathogen population (**Figure 3.4**). The races *Av2-6-7-(8)* and *Av6-7-(8),* though common in each province, varied in frequency depending on the location. In contrast, the race *Av4-6-7-S-(8)* was uncommon in Alberta.

Table 3.3. The races of *L. maculans* identified among 179 isolates collected from eight severely diseased canola fields in western Canada in 2012.

***Avr* alleles**	**No. of *Avr* alleles**	**Frequency (%)**	**No. of isolates for the race**
Av2-4-6-7-(8)	4	11.2	20
Av2-6-7-(8)	3	7.3	13
Av6-7-(8)	2	5.6	10
Av4-6-7-S-(8)	4	5.0	9
Av2-4-6-7-S-(8)	5	4.5	8
Av1-2-4-6-S-(8), AvLep1	5	4.5	8
Av2-6-7-S-(8)	4	3.9	7
Av1-2-4-6-(8)	4	3.4	6
Av1-2-4-6-S-(8)	5	2.8	5
Av1-4-6-7-S-(8)	4	2.8	5
Av2-4-6-7-S-(8)-AvLep1	6	2.8	5
Av6-7-(8)-*AvrLep1*	3	2.8	5
Av1-2-4-6-7-(8)	5	2.2	4
Av2-4-6-(8)	3	2.2	4
Av4-6-7-(8)	3	2.2	4
Av4-6-7-(8)-*AvrLep1*	4	2.2	4
Av4	1	1.7	3
Av2-4-6-7-(8)-*AvrLep1*	5	1.7	3
Av2-4-7-*AvrLep2*	4	1.7	3
Av6-7-S-(8)-*AvrLep1*	5	1.7	3
Av1-2-3-4-6-7-(8)	6	1.1	2
Av1-2-3-4-6-7-S-(8)	7	1.1	2
Av1-2-6-7-(8)	4	1.1	2
Av1-2-6-7-S-(8)-*AvrLep1*	6	1.1	2
Av1-4-6-7-(8)	5	1.1	2
Av2-6-7-S-(8)-*AvrLep1*	5	1.1	2
Av4-6-(8)	2	1.1	2
Av4-6-7-S(8)-*AvrLep1*	5	1.1	2
Av7	1	0.6	1
Av1-2-3-4-6-7-9-(8)	7	0.6	1
Av1-2-4	3	0.6	1
Av1-2-4-6-7-(8)-*AvrLep1*	6	0.6	1
Av1-2-4-7	4	0.6	1
Av1-2-6-7-S-(8)	5	0.6	1
Av1-3-4	3	0.6	1
Av1-3-9-S-*AvrLep2*	4	0.6	1
Av1-4-6-7-S-(8)-*AvrLep1*	6	0.6	1
Av1-6-7-(8)	3	0.6	1
Av1-6-7-S-(8)-*AvrLep1*	5	0.6	1
Av1-2-4-6-7-9-S-(8)-,*AvrLep1-AvrLep2*	10	0.6	1
Av1-2-4-6-7-S-(8)-*AvrLep1*	8	0.6	1
Av2-3-4-6-7-S-(8)-*AvrLep1*, *AvrLp2*	9	0.6	1
Av2-3-6-(8)	3	0.6	1
Av2-3-6-7-(8)	5	0.6	1
Av2-4-6-(8)-*AvrLep1*	4	0.6	1
Av2-4-6-7-9-S-(8)-*AvrLep1-AvrLep2*	9	0.6	1
Av2-4-6-7-S-(8)-*AvrLpe1-AvrLep2*	8	0.6	1

Av2-4-7	3	0.6	1
Av2-4-7-S	4	0.6	1
Av2-4-7-S-*AvrLep1-AvrLep2*	6	0.6	1
Av2-4-7-S-*AvrLep2*	5	0.6	1
Av2-4-*AvrLep2*	3	0.6	1
Av2-6-S-(8)	3	0.6	1
Av2-6-7-9-(8)	4	0.6	1
Av2-6-7-9-(8)-*AvrLep1*	6	0.6	1
Av2-6-7-(8)-*AvrLep1*	5	0.6	1
Av2-7-*AvrLep1*	3	0.6	1
Av2-7-S	3	0.6	1
Av2-*AvrLep2*	2	0.6	1
Av4-6-7-9-S-(8)	5	0.6	1
Av4-6-7-9-S-(8)-*AvrLep1*	6	0.6	1
Av6-(8)-*AvrLep1*	3	0.6	1
Av7-*AvrLep2*	2	0.6	1

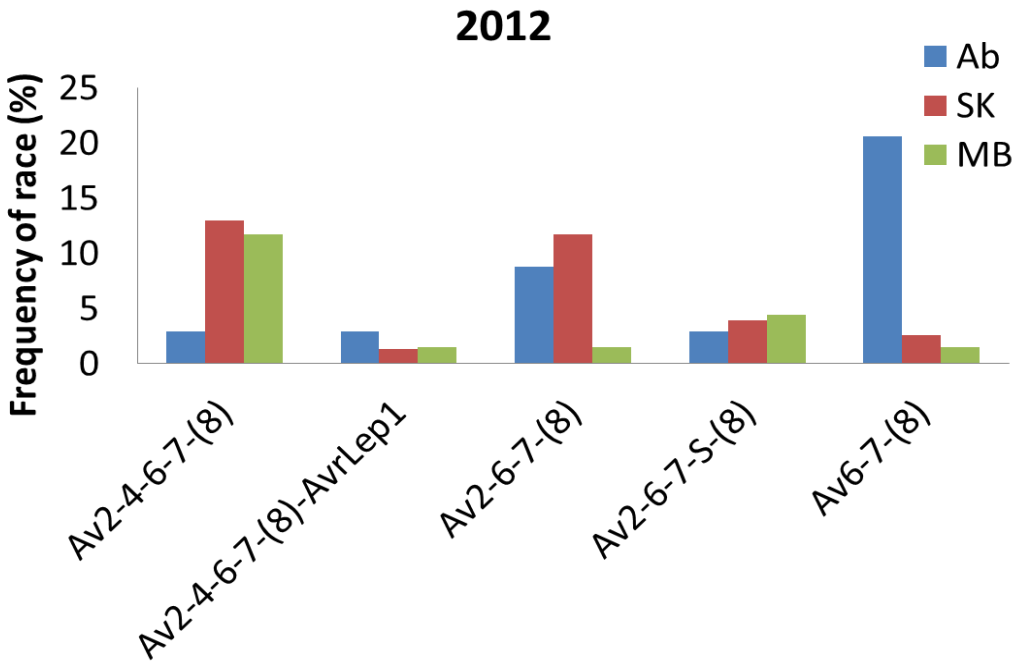


Figure 3.4. The most common races of *L. maculans* identified in canola fields in western Canada (2012).

3.4.6. *Avr* allele profile in targeted commercial canola fields in 2013

In 2013, blackleg of canola was less severe in many regions of western Canada relative to 2012, especially in Alberta. Therefore, diseased stubble was collected only from Saskatchewan and Manitoba. A total of 193 *L. maculans* isolates from eight commercial fields were tested for *Avr* alleles. The disease incidence varied substantially among these fields; most fields in Saskatchewan had <10% blackleg incidence.

Table 3.4. Isolates of *L. maculans* collected from eight commercial fields in western Canada in 2013 and tested for the frequency of *Avr* alleles.

Province	Location (nearest town)	# *L. maculans* tested
Saskatchewan	Meota	25
	Goodeve (1)	23
	Goodeve (2)	23
	Langenburg	31
Manitoba	Killarney	25
	Lowe Farm (1)	23
	Lowe Farm (2)	24
	Winkler (blackleg nursery)	19
	Total	193

3.4.7. *Avr* allele frequencies in Saskatchewan (2013)

Diseased stubble from four sites, namely Meota, Goodeve 1, Goodeve 2 and Langenburg (Table 3.4). Samples from two adjacent fields near the Meota and Langenburg sites were combined and considered as a single site at each location. Samples collected from two adjacent fields near Meota, was seeded with Invigor 5440 and canola had been grown there continuously from 2009 to 2012 with a cultivar rotation of 5440, 71-45, L150 and 73-45 in the previous years. All cultivars were rated resistant to blackleg in the Saskatchewan Seed Guide (2012), except 71-45, which was rated moderately resistant. Blackleg incidence was 50-78% in these two fields. For both fields in Goodeve, the cultivar was L150 (resistant); canola had been grown continuously in one of the fields for three years and in the other field a canola-lentil-canola rotation was grown the prior three years. In Langenburg, cv. Nexera 1012 and L150 were grown in the two fields where the crop rotation was wheat-canola-canola-wheat prior to the current canola crop. The disease incidence was generally <10% in these fields. Yield data for all the fields was not known.

The *Avr* profiling showed a similar pattern among fields in Saskatchewan, regardless of the disease level, cultivar or crop rotation practice. Genes *AvrLm2*, *AvrLm4*, *AvrLm*6 and *AvrLm*7 were detected in >90% of the isolates (**Figure 3.5**), whereas *AvrLm1, AvrLm3, AvrLm9 and AvrLep2* were very low or undetectable in all the fields. The frequency of

AvrLm3 and *AvrLep1* were approximately 30% and 80% respectively, in the fields near Meota; these two *Avr* genes were rarely found in other fields in western Canada during the study.

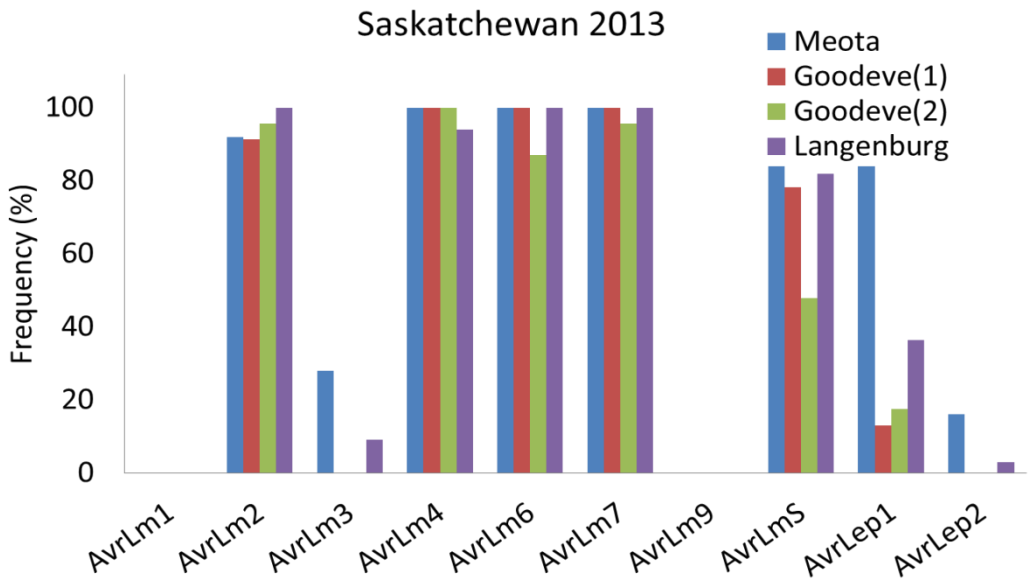


Figure 3.5. The frequency of *Avr* allele in *L. maculans* population detected in isolates collected from four commercial fields with variable disease severity, near Meota, Goodeve 1, Goodeve 2 and Langenburg, at Saskatchewan.

3.4.8. *Avr* allele frequencies in Manitoba (2013)

Stubble samples collected from four diseased sites in southern Manitoba near Killarney, Lowe Farm 1, Lowe Farm 2 and Winker had disease incidences of >75%, 10%, 20% and ~50%, respectively. Near Winkler samples were taken from blackleg nursery with continuous canola for three years, prior (cv. Canterra 1950 RR). The fields near Killarney and Lowe Farm (2) were seeded with cultivars: L130 LL, L156H and 9560 CL, respectively, and the crop rotation were canola-wheat in each of these fields. Unfortunately, the yield data was not available from these fields. Incidence of blackleg was high at Killarney between 2009 and 2011 (Gerald Martin, BASF, personal communication).

Only slight variation in *Avr* profile was observed among fields; *AvrLm4*, *AvrLm*6 and *AvrLm*7 were detected in >90% of pathogen isolates, while *AvrLm2* and *AvrLmS* (Van de Wouw et al. 2009) were generally >60% (**Figure 3.6**). Isolates carrying *AvrLm3*, *AvrLm*9 or *AvrLep2* were rarely detected in these fields, or were at very low levels. In Lowe Farm Field 1, the frequency of *AvrLm2* was lower than those in other fields surveyed in 2013. *AvrLm1* was often >20% in Manitoba fields, but was not detected in the Saskatchewan fields (**Figure 3.6**).

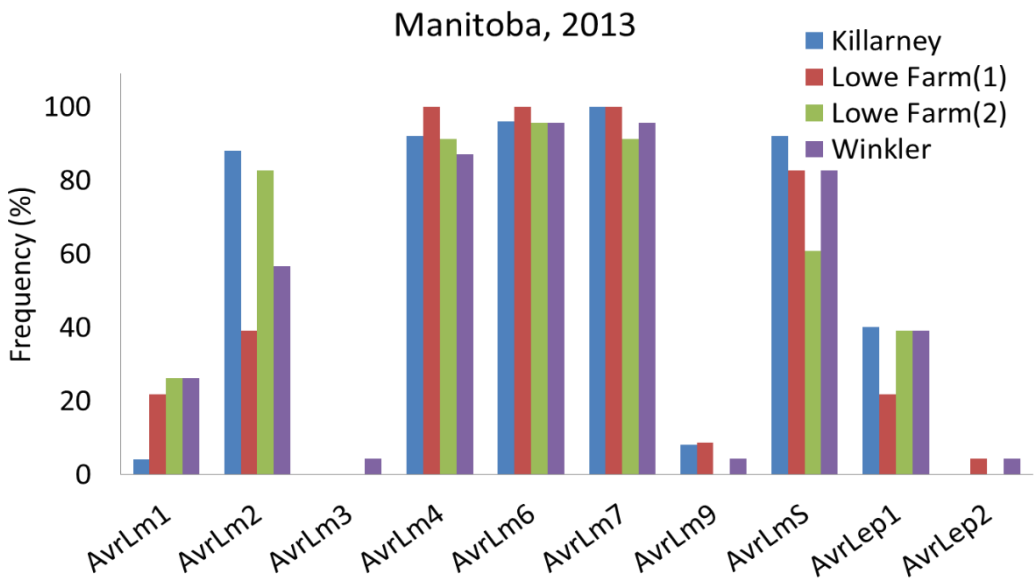


Figure 3.6. The frequency of *Avr* allele in *L. maculans* population detected in isolates collected from four commercial fields with variable disease severity, near Killarney, Lowe Farm 1, Lowe Farm 2 and Winkler, at Manitoba.

3.2.9. Race identification in the 2013 *L. maculans* population

A total of 46 races were identified from the 2013 population of *L. maculans* collected from eight targeted canola fields in Saskatchewan and Manitoba in 2013 (Table 3.5), and 15 were present in both provinces (**Figure 3.7**). Race *AvrLm2-4-6-7-S-(8)* had the highest frequency (20%), and was common in both provinces. Other common races included: *Av2-4-6-7-(8)*

(11.9%), *Av2-4-6-7-S-(8)-AvrLep1* (9.8%) and *Av2-3-4-6-7-S-(8)* (5.7%) (**Figure 3.7**). Similar to the 2012 picture, avirulence genes: *AvrLm2, AvrLm6,(8)* and *AvrLm*7 were common in most of the fields. A total of 26 and 35 races were identified in Saskatchewan and Manitoba, respectively in 2013, but only 9 and 11 of them were >2% in respective fields.

Table 3.5. Races of *L. maculans* identified among 193 isolates from eight canola fields in Saskatchewan and Manitoba during 2013.

***Avr* allele carried**	**No. of avirulence alleles**	**Frequency (%) at the site**	**No. of isolates in each race**
Av2-4-6-7-S-(8)	5	20.7	40
Av2-4-6-7-(8)	4	11.9	23
Av2-4-6-7-S-(8)-*AvrLep1*	6	9.8	19
Av2-3-4-6-7-S-(8)	6	5.7	11
Av2-3-4-6-7-S-(8)-*AvrLep1*	7	5.7	11
Av1-4-6-7-S-(8)-*AvrLep1*	6	3.6	7
Av2-4-6-7-(8)-*AvrLep1*	5	3.1	6
Av4-6-7-S-(8)	4	3.1	6
Av1-2-3-4-6-7-S-(8)-*AvrLem1*	8	2.6	5
Av1-2-4-6-7-S-(8)-*AvrLep1*	7	2.6	5
Av1-2-4-6-7-S-(8)-*AvrLep1*	7	2.6	5
Av2-3-4-6-7-(8)	5	2.6	5
Av4-6-7-S-(8)-*AvrLep1*	5	2.6	5
Av4-6-7-(8)	3	2.1	4
Av2-3-4-6-7-(8)-*AvrLep1*	6	1.6	3
Av2-4-7	3	1.6	3
Av1-2-3-4-6-7-S-(8)	7	1.0	2
Av1-2-4-6-7-S-(8)	6	1.0	2
Av1-3-4-6-7-S-(8)	6	1.0	2
Av2-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	1.0	2
Av2-6-7-S-(8)	4	1.0	2
Av4	1	0.5	1
Av1-2-3-4-6-7-9-S(8)*AvrLem1*	9	0.5	1
Av1-2-3-4-6-7-(8)-*AvrLep2*	7	0.5	1
Av1-2-4-6-7-9-S(8)	7	0.5	1
Av1-2-4-6-7-9-S-(8)-*AvrLep1*	8	0.5	1
Av1-2-6-7-S-(8)	5	0.5	1
Av1-3-4-6-7-9-S-(8)-*AvrLep1-AvrLep2*	9	0.5	1
Av1-4-6-7-(8)	4	0.5	1
Av1-4-6-7-S-(8)	5	0.5	1
Av1-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	0.5	1
Av1-4-7-S	4	0.5	1
Av1-6-7-S-(8)	4	0.5	1
Av2-3-4-6-7-(8)-*AvrLep1-AvrLep2*	7	0.5	1
Av2-3-6-7-S-(8)	5	0.5	1
Av2-4-6-7-(8)-*AvrLp2*	5	0.5	1
Av2-4-7-S	4	0.5	1
Av2-7-S	3	0.5	1
Av3-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	0.5	1
Av3-6-9-S-(8)-*AvrLep1*	5	0.5	1
Av4-6-7-(8)-*AvrLep1*	4	0.5	1
Av4-6-7-9-S-(8)	5	0.5	1
Av4-6-7-(8)-*AvrLep1*	4	0.5	1
Av4-7	2	0.5	1
Av4-7-*AvrLep1*	3	0.5	1
Av6-7-(8)	2	0.5	1

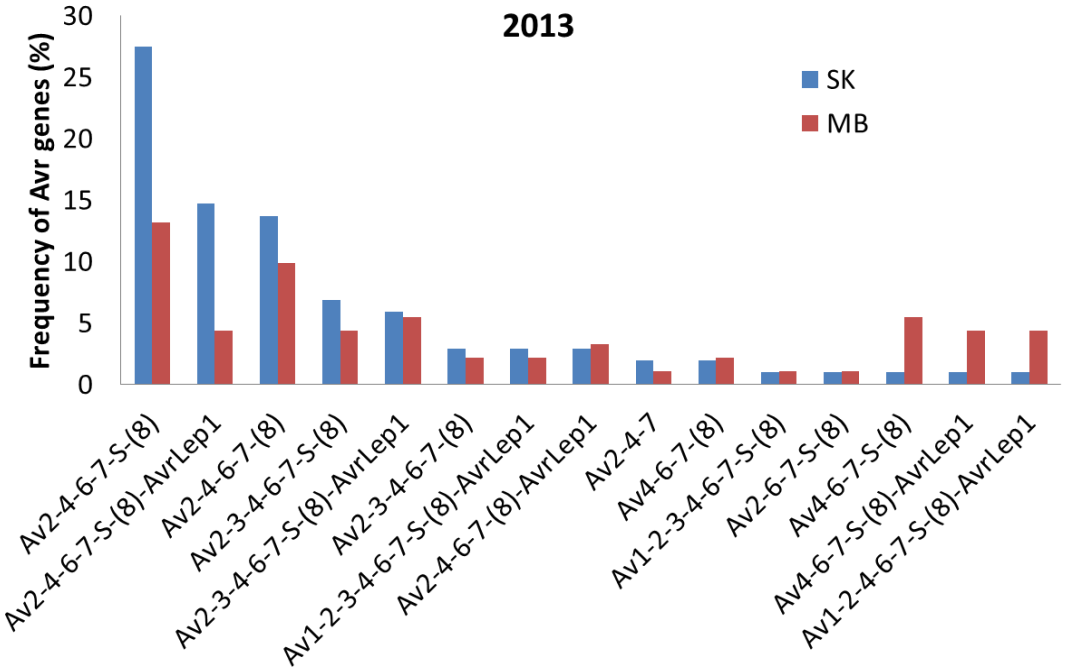


Figure 3.7. The most common races of *L. maculans* identified in canola fields in Saskatchewan and Manitoba (2013).

3.2.10. Race composition of *L. maculans* isolates collected in 2012 and 2013

A total of 90 races of *L. maculans* were identified when the data from 2012 and 2013 (372 isolates) were combined (Table 3.6), with 19 races common between provinces and years (**Figure 3.8**). Race *Av2-4-6-7-(8)* had the highest overall frequency in the pathogen population (11.6%), and was detected in both years in almost all fields. Other races that were observed frequently included *Av2-4-6-7-S-(8)* at 10.8% and *Av2-4-6-7-S-(8)-AvrLep1* at 6.5% (Table 3.6). However, for these races there were slight variations in prevalence between years; *Av2-4-6-7-(8)* was (11.2%) the most prevalent race in 2012 (Table 3.3), however, it was *Av2-4-6-7-S-(8)* (20.7%) in 2013 (Table 3.5).

Table 3.6. Races of *L. maculans* identified among 372 isolates from sixteen canola fields in western Canada in 2012 and 2013.

***Avr* alleles carried**	**No. of avirulence alleles**	**Frequency (%)**	**No. of isolates of each race**
Av2-4-6-7-(8)	4	11.6	43
Av2-4-6-7-S-(8)	5	10.8	40
Av2-4-6-7-S-(8)-*AvrLep1*	6	6.5	24
Av4-6-7-S-(8)	4	4.0	15
Av1-2-4-6-S-(8)-*AvrLep1*	6	3.8	14
Av2-6-7-(8)	3	3.5	13
Av6-7-(8)	2	3.0	11
Av2-3-4-6-7-S-(8)-*AvrLep1*	7	3.0	11
Av2-3-4-6-7-S-(8)	6	3.0	11
Av1-2-4-6-7-S-(8)-*AvrLep1*	7	2.7	10
Av2-6-7-S-(8)	4	2.4	9
Av4-6-7-(8)	3	2.2	8
Av2-4-6-7-S-(8)	5	2.2	8
Av4-6-7-S-(8)-*AvrLep1*	5	1.9	7
Av2-4-6-7-(8)-*AvrLep1*	5	1.6	6
Av1-4-6-7-S-(8)	4	1.6	6
Av1-2-4-6-(8)	4	1.6	6
Av6-7-(8)-*AvrLp1*	3	1.3	5
Av2-3-4-6-7-(8)	5	1.3	5
Av1-2-4-6-S-(8)	5	1.3	5
Av1-2-3-4-6-7-S-(8)-*AvrLem1*	8	1.3	5
Av4-6-7-S-(8)-*AvrLep1*	4	1.1	4
Av2-4-7	5	1.1	4
Av2-4-6-(8)	3	1.1	4
Av1-2-4-6-7-(8)	5	1.1	4
Av1-2-3-4-6-7-S-(8)	7	1.1	4
Av4	1	1.1	4
Av6-7-S-(8)-*AvrLep1*	5	0.8	3
Av2-4-7-*AvrLep2*	4	0.8	3
Av2-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	0.8	3
Av2-4-6-7-(8)-*AvrLep1*	5	0.8	3
Av2-3-4-6-7-(8)-*AvrLep1*	6	0.8	3
Av1-4-6-7-(8)	5	0.8	3
Av4-6-7-9-S-(8)	5	0.5	2
Av4-6-(8)	2	0.5	2
Av2-6-7-S-(8)-*AvrLep1*	5	0.5	2
Av2-4-7-S	4	0.5	2
Av1-3-4-6-7-S-(8)	6	0.5	2
Av1-2-6-7-S-(8)-*AvrLep1*	6	0.5	2
Av1-2-6-7-S-(8)	5	0.5	2
Av1-2-6-7-(8)	4	0.5	2
Av1-2-4-6-7-S-(8)	6	0.5	2
Av1-2-3-4-6-7-(8)	6	0.5	2
Av7-*AvrLep2*	2	0.3	1
Av6-(8)-*AvrLep1*	3	0.3	1
Av4-7	2	0.3	1
Av4-7-*AvrLep1*	3	0.3	1

Av4-6-7-9-S-(8)-*AvrLep1*	6	0.3	1
Av4-6-7-(8)-*AvrLep1*	4	0.3	1
Av3-6-9-S-(8)-*AvrLep1*	5	0.3	1
Av3-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	0.3	1
Av2-*AvrLep2*	2	0.3	1
Av2-7-S	3	0.3	1
Av2-7-S	3	0.3	1
Av2-7-*AvrLep1*	3	0.3	1
Av2-6-7-(8)-*AvrLep1*	5	0.3	1
Av2-6-7-9-(8)-*AvrLep1*	6	0.3	1
Av2-6-7-9-(8)	4	0.3	1
Av2-6-S-(8)	3	0.3	1
Av2-4-*AvrLep2*	3	0.3	1
Av2-4-7-S-*AvrLep2*	5	0.3	1
Av2-4-7-S-*AvrLep1-AvrLep2*	6	0.3	1
Av2-4-6-7-(8)-*AvrLep2*	5	0.3	1
Av2-4-6-7-9-S-(8)-*AvrLep1-AvrLep2*	9	0.3	1
Av2-4-6-(8)-*AvrLep1*	4	0.3	1
Av2-3-6-7-S-(8)	5	0.3	1
Av2-3-6-7-(8)	5	0.3	1
Av2-3-6-(8)	3	0.3	1
Av2-3-4-6-7-S-(8)-*AvrLep1-AvrLep2*	9	0.3	1
Av2-3-4-6-7-(8)-*AvrLep1-AvrLep2*	7	0.3	1
Av1-6-7-S-(8)-*AvrLep1*	5	0.3	1
Av1-6-7-S-(8)	4	0.3	1
Av1-6-7-(8)	3	0.3	1
Av1-4-7-S	4	0.3	1
Av1-4-6-7-S-(8)-*AvrLep1-AvrLep2*	7	0.3	1
Av1-4-6-7-S-(8)-*AvrLep1*	6	0.3	1
Av1-3-9-S-*AvrLep2*	4	0.3	1
Av1-3-4-6-7-9-S-(8)-*AvrLep1-AvrLep2*	9	0.3	1
Av1-3-4	3	0.3	1
Av1-2-4-7	4	0.3	1
Av1-2-4-6-7-S-(8)-*AvrLep1*	8	0.3	1
Av1-2-4-6-7-(8)-*AvrLp1*	6	0.3	1

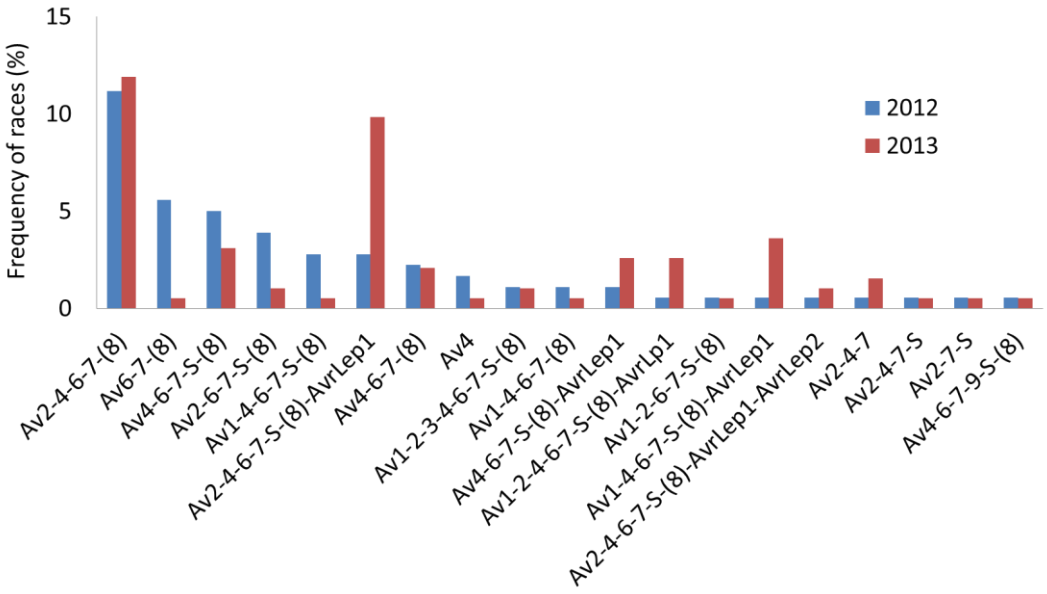


Figure 3.8. The most common races of *L. maculans* found in canola fields in Saskatchewan and Manitoba (2012 and 2013).

3.5. Discussion

The relationship between the *Avr* gene profile in specific canola fields and blackleg incidence or severity was more complex than originally hypothesized, due partially to the lack of information on *R* genes carried by CCCs, and to the possibility there is more than one type of resistance present in many of the current cultivars. The initial hypothesis stated that the increased level of blackleg on some of the *R*-rated canola cultivars might be the result of selection pressure exerted by specific *R* genes that compelled the pathogen to adapt once a mutation or recombination occurs. Thus, the virulence frequency increased on these alleles in the pathogen population, consequently rendering the corresponding *R* gene(s) in the cultivar ineffective. For example, a cultivar carrying *Rlm3* as the sole source of resistance may be defeated once a massive shift occurs in the pathogen population from *AvrLm3* (avirulent) to *avrLm3* (virulent). This change will allow the pathogen to adapt to the cultivar and the virulent population to rapidly increase. To understand this dynamic, information on the presence/absence of *Avr* genes in the pathogen population and *R* genes in canola cultivars is

required. In realty however, knowledge of *R* genes carried by specific canola cultivars is, for the most part, still unclear in Canada. A recent study found that *Rlm1* and *Rlm3* accounted for about 10% and 60% of CCCs/breeding lines, respectively (Zhang et al. 2015). This information is not specific to any canola cultivar. Furthermore, the nature and mechanisms of blackleg resistance are also unknown for most canola cultivars. The current study analyzed the *Avr* profile in selected canola fields with the blackleg incidence ranging between <10% and >75%, and generally detected low to undetectable levels of *AvrLm1* and *AvrLm3*. If *Rlm1* and *Rlm3* are the only *R* genes carried in CCCs (Zhang et al. 2015), then the variable blackleg incidence among these fields may be due to factors beyond the mere lack of certain *Avr* genes in the pathogen population since the corresponding *AvrLm1* and *AvrLm3* were low in all these fields.

The study provides insight into the pathogen race structure in response to *R* genes in cultivars by analyzing field-specific data in connection with blackleg disease levels. The genes *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7* were common in most fields, regardless of blackleg incidence. This is possibly because none of the corresponding *R* genes had been used widely in CCCs (Zhang et al. 2015). By the same token, the general lack of *AvrLm1* and *AvrLm3* in these fields is likely related to the long-term use of *Rlm1* and *Rlm3* in CCCs. When compared with an earlier report (Kutcher et al. 2011b), there was a decrease in *AvrLm3* accompanied by a sharp increase in *AvrLm7*. This opposite trend (or correlation?) between these two *Avr* genes in the pathogen population was suggested in a field survey in France; *AvrLm7* was at >99%, whereas *AvrLm3* was <1%. Only one of 1,797 *L. maculans* isolates was virulent to *Rlm7* and this isolate was avirulent to *Rlm3* (Balesdent et al. 2006). Plissonneau et al. (2016) observed an interaction between *AvrLm3* and *AvrLm4-7*; the co-existence of these two *Avr* genes subdues recognition of *AvrLm3* by *Rlm3*, consequently

failing to activate host defense responses. Therefore, it is possible that *AvrLm3* might have been underestimated in western Canada due to the presence of *AvrLm7*.

The results of this study were similar to studies of broader-range collections in western Canada during 2010 and 2011 (Cross et al. 2014; Liban et al. 2016). This may indicate that the pathogen population in these fields reflected that which occurs over large regions of western Canada and varies little over short distances. Greater changes were observed when the *Avr* profile was compared with those identified between 2005 and 2007 (Dilmaghani et al. 2009; Kutcher et al. 2011b); *AvrLm3* and *AvrLm9* had declined from ~60% to very low levels, while *AvrLm7* increased from ~50% to >95%.

The population of *L. maculans* in western Canada was considered genetically diverse (Dilmaghani et al. 2009) and maintained certain *Avr* alleles that are not always present in other parts of the world (Balesdent et al. 2006). The diversity is reflected by the number of pathogen races identified; Kutcher et al. (2010) reported 16 races based on 96 isolates of *L. maculans* from western Canada, but Liban et al. (2016) identified 55 races based on 674 isolates studied, with the races *AvrLm2-4-6-7* (22.7%) and *AvrLm2-4-6-7-S* (22.5%) being most prevalent. In contrast, Balesdent et al. (2006) identified only 11 races in France based on a population of 1,797 *L. maculans* isolates. The current study of 372 isolates characterized to 90 races indicated greater diversity of the pathogen population than that in the previous reports by Kutcher et al. (2010) and Liban et al. (2016) (Table 3.6). Interestingly, the most prevalent races were exactly the same as those identified earlier by Liban et al. (2016) though with noticeably lower frequencies (11.6% and 10.8%, respectively). More than 85% of the isolates carried 3 to 6 *Avr* genes, but >10% of the isolates carried 7-10 *Avr* genes. The dominance of races *AvrLm2-4-6-7* and *AvrLm2-4-6-7-S* reflected an uneven pathogen population in western Canada, but also provided important clues for selecting effective *R*

genes, including *Rlm2*, *Rlm4*, *Rlm6* and *Rlm7*, for blackleg management. The high pathogen race diversity also indicated that caution is required in deploying these *R* genes because each can be defeated by at least one of the races in the pathogen population in western Canada. An integrated approach combining race-specific *R* genes with race-nonspecific resistance (Delourme et al. 2006; Brun et al. 2010) and extended crop rotation (Kutcher et al. 2011a) may be more sustainable for blackleg management.

In this study, variations in *Avr* gene frequencies were observed among fields, especially in Alberta. For example, *AvrLm4* was found in 60% of the isolates in the Trochu field in 2012, but this *Avr* gene was absent among isolates in the Olds field. The absence of *AvrLm4* appears unusual because the corresponding *R* gene was not found in any of the CCCs or even breeding lines (Zhang et al. 2015); therefore, absence of *AvrLm4* could not have been caused by selection pressure from the *R* gene. After cloning *AvrLm7*, Parlange et al. (2009) suggested that *AvrLm4* and *AvrLm7* are two distinct alleles of a single gene. In the current study, pathogen isolates from most fields *AvrLm4* and *AvrLm7* had similar gene frequencies, with only a few cases where *AvrLm4* was slightly lower than *AvrLm7*. Although the absence of *AvrLm4* from the Olds field was in contrast with results in other fields, its implication is not yet clear since it appears not to be related to the disease levels observed in this field. None of the cultivars in western Canada carries the corresponding *Rlm4* (Zhang et al. 2015). The field-dependent variation was less noticeable in Saskatchewan and Manitoba, with only a slight exception for *AvrLm3* in Saskatchewan, which was undetectable at most locations but 20% in Watrous (2012) and 30% in Meota (2013) fields. This may be due to specific cultivars (carrying no *Rlm3*) used in the two latter fields, but this can only be verified when the specific *R* genes of these cultivars are known.

Between the years, the most noticeable change in the *Avr* gene profile was the dramatic decrease of *AvrLm1* in Saskatchewan; >20% of the isolates collected in 2012 carried this *Avr*

gene, with frequency of approximately 70% in the Watrous field, but it was completely absent in all Saskatchewan fields sampled in 2013. This change could be due to the cultivars grown, but may also indicate that *Rlm1* is used in many new hybrids. Another detectable change between years was the increase of *AvrLm4* in Saskatchewan and *AvrLm6* and *AvrLm7* in Manitoba, although the causes are not understood (Figures; 3.5; 3.6). With the increased frequency of *AvrLm7* in Manitoba, *AvrLm3* declined (Figure 3.6) and this follows the pattern of interaction between these two *Avr* genes reported previously (Plissonneau et al. 2016). The largest between province differences in *Avr* gene profile were the lower presence of *AvrLm1* and *AvrLm4* in the Alberta fields in 2012, relative to those in Saskatchewan and Manitoba. In 2013, *AvrLm1* and *AvrLm9* were not found in Saskatchewan (Figure 3.5), but were detectable in most Manitoba fields (Figure 3.6). In contrast, *AvrLm2* was found in >90% of *L. maculans* isolates in Saskatchewan, but was <60% in two of the four Manitoba fields. Due to the lack of information on specific *R* genes in most of these cultivars, the real cause of these variations was unclear (Figures: 3.5; 3.6).

The *AvrLm1* and *AvrLm3* genes were very low or absent in almost all the commercial fields examined; this would indicate that the *R* genes *Rlm1* and *Rlm3* carried by many canola cultivars are no longer effective against blackleg in these fields. Though the initial hypothesis was largely correct that the pathogen population had shifted and was capable of overcoming the *R* genes in canola cultivars, there was insufficient evidence to attribute the high blackleg incidence in any of these fields to the low frequencies of *AvrLm1* or *AvrLm3* because these *Avr* gene frequencies were not much higher in low incidence fields, especially in 2013. It is unlikely that the low frequency or absence of *AvrLm1* and *AvrLm3* genes in the pathogen population was the primary cause of high blackleg incidence in some of these fields. It is more likely that the intensive production of canola in almost all these fields, either a 1-year break from canola or even continuous canola for several years contributed to the buildup of

pathogen inoculum (Guo and Fernando 2005; Guo et al. 2008, Kutcher et al 2011). Blackleg incidence and severity can also be affected by weather conditions (Guo and Fernando 2005), and a combination of high pathogen inoculum coupled with conducive local weather events, including localized rain showers during early infection or hail damage during the growing season might have influenced blackleg development on the same cultivar at different locations. Field-specific pathogen inoculum and weather data would be useful to assess these factors.

Prior studies have documented the race structure of *L. maculans* in western Canada to provide an overall picture of the evolution of the pathogen and the effectiveness of specific *R* genes in canola cultivars (Kutcher et al. 2010; 2011; Liban et al. 2016). The current study focused on individual fields that varied in blackleg incidence and severity to determine whether the severe-disease cases were caused by unique and virulent pathogen races. The results indicated that isolates making up the pathogen population in each of these fields were virulent on multiple *R* genes; however, for some specific *R* gene in the host, the expected correlation with the corresponding *Avr* genes in the *L. maculans* population was not detected. In reality, however, severe blackleg disease was observed in only a few of the fields, and even resistant cultivars from the same seed company (L130 LL, L156H) varied substantially in blackleg incidence between two fields, despite the similarity of the *Avr* gene profile (Figure 3.6). It is possible that additional factors also played a role. Zhang et al. (2015) reported adult plant resistance (APR) in many CCCs (CCCs), which is not believed to be controlled by major *R* genes (Lindhout 2002; Stuthman et al. 2007). Therefore, it is possible that many CCCs in western Canada carry a level of background blackleg resistance that may be deemed insufficient at the seedling stage. However, there is very little characterization of this type of resistance in CCCs, especially of the resistance mechanism. This information will

be useful to assess the value of APR to be used in blackleg management in western Canada, the research subject presented in the following chapter.

3.6. Conclusion

Increased prevalence and incidence of blackleg disease was reported in recent years. This study was initiated to determine whether the fields with high disease incidence were the result of changes in *Avr* profiles resulting from pathogen adaptation to the canola cultivars used. A total of 17 fields were investigated in 2012 and 2013, with disease incidence ranging from <10% to >75%. Great diversity was detected in the pathogen population, with 90 races identified. The races *Av2-4-6-7* and *Av2-4-6-7-S* were most prevalent, accounting for 11.6% and 10.8% of the population, respectively. More than 85% of the isolates carried 3 to 6 *Avr* genes. *AvrLm2, AvrLm4, AvrLm6* and *AvrLm7* were common, but *AvrLm1, AvrLm3, AvrLm9* and *AvrLep2* were rarely detected in these fields. Since the majority of CCCs carry only the *R* genes *Rlm1* and/or *Rlm3*, the pathogen is considered virulent to all of the current cultivars. Therefore, the variation in blackleg incidence among these fields was unlikely to have resulted from only the breakdown of *Rlm1* and *Rlm3*. Additional factors may play a role, including resistance not controlled by a specific *Avr-R* gene interaction. This type of resistance, however, has not been characterized for CCC used in western Canada. The genetic diversity of the pathogen population observed in this study also highlights the need for caution when we consider new *R* genes for blackleg management. Each gene currently available can be defeated by at least one of the races identified in the current pathogen population. Race-specific and non-specific resistance, in combination with extended crop rotations, may be warranted for sustainable blackleg management in western Canada.

Chapter 4. Characterization of blackleg resistance associated with common canola cultivars used in western Canada

4.1. Abstract

Many of the canola (*Brassica napus* L.) cultivars grown in western Canada claimed to be resistant (*R*), to blackleg disease [*Leptosphaeria maculans* (Desmaz.) Ces. & de Not.] carry *Rlm1* and *Rlm3*. The absence of the corresponding avirulence (*AVR*) genes (*AvrLm1* and *AvrLm3*) in the pathogen population prevailing in western Canada, indicates that these *R* genes are no longer effective. However, the relatively few cases of blackleg damaged crops that are produced from cultivars that carry *Rlm 1* and *Rlm3*, suggest that additional resistance may be present in these CCCs. Three CCCs carrying *Rlm1*, *Rlm3* or both were assessed against virulent *L. maculans* isolates. The infection of cotyledons and spread of the pathogen into the stem via the petiole were evaluated using a visual scale and fluorescence microscopy. The DNA of the pathogen in the petiole and stem tissues was quantified using droplet digital PCR (ddPCR). At 14 days post-inoculation (dpi), all inoculated cotyledons exhibited infection symptoms, but the mean severity was lower for CCCs then on the susceptible check. Limited hyphal spread of the pathogen was observed in CCC cotyledons compared with the susceptible check ten days after inoculation with a virulent isolate of *L. maculans*, with green fluorescent protein (GFP). The amount of *L. maculans* DNA detected by ddPCR was substantially lower in petioles and stems of inoculated CCCs relative to the susceptible check. The results indicate that race non-specific resistance associated with the CCCs plays a role in resistance to blackleg by delaying or reducing the spread of fungal hyphae from infected cotyledons into stems, which is where the most severe symptoms occur on the plant.

4.2. Introduction

An understanding of the host-pathogen interaction is essential to use cultivar resistance for effective management of blackleg in canola. To date a total of 18 race-specific major *R* genes have been identified (Zhang et al. 2015), often unintentionally, when high levels of blackleg resistance under field conditions were recognized (Ansan-Melayah et al. 1997; Rouxel et al. 2003). These *R* genes are identified in the A, B and C genomes of *Brassica* spp. (Fredua-Agyeman et al. 2014). The genetic resistance conferred by major *R* genes, however, can no longer be effective once the *L. maculans* population shifts and the frequency of the corresponding *Avr* genes diminish in the pathogen population. The high frequency of sexual recombination in *L. maculans* and mass distribution of airborne ascospores each year provides the pathogen with great potential to bring about changes in their genetic makeup (West et al. 2001) and adapt to canola cultivars carrying specific *R* genes (McDonald and Linde 2002). Experiences in western Canada have indicated that the pathogen can change rapidly after the introduction of resistant canola cultivars (Kutcher et al. 2011b).

Two types of resistance to blackleg, namely race-specific (major *R*-gene) and non-specific (quantitative) resistance, have been reported in *B. napus* (Delourme et al. 2006). The former can be highly effective when the corresponding *Avr* genes are present in the pathogen population. This type of resistance may not be durable because it exerts strong selection pressure for virulence genes in the pathogen population, especially when the same *R* genes are used continuously in short crop rotations (Rouxel et al. 2003; Li et al. 2003; Gladders et al. 2006; Sprague et al. 2006). The data presented in the Chapter 3 of this thesis showed that the current pathogen population in western Canada generally lacks *AvrLm1* and *AvrLm3*. The frequency these *Avr* genes in the western Canadian pathogen population has declined over time (Liban et al. 2016, Dilmaghani et al. 2009; Kutcher et al. 2010; 2011). This is likely due to the long-term use of the *R* genes *Rlm1* and *Rlm3* in CCCs (Zhang et al. 2015).

Quantitative resistance (*QR*) to blackleg is not well characterized but generally believed to be polygenic (Pilet et al. 1998). Often resistance expression is on a continuous scale (Geiger and Heun 1989). It may not be recognized at the seedling stage, but becomes noticeable in adult plants by lower disease incidence and severity under field conditions (Ansan-Melayah et al. 1998). Prior to the early 1980s, well before the introduction of first resistant cultivar "Quantum" carried *Rlm3,* quantitative resistance might have been the most common type of blackleg resistance in *B. napus* because most cultivars seemed to respond similarly to different isolates of *L. maculans* (Thurling and Venn 1977; Cargeeg and Thurling 1980; Newman 1984).

Blackleg disease was successfully controlled in western Canada between the late 1980s and 2009 through the use of resistant canola cultivars and 4-year crop rotations (Kutcher et al. 2013). The source of resistance in these cultivars was unknown, so was the role of different types of resistance played in blackleg management (Rimmer 2006). In Australia, cultivars carrying different complements of *R* genes are recommended to reduce disease severity and plant mortality (Marcroft et al. 2012). This strategy is believed to reduce selection pressure towards particular *R* genes, minimizing the frequency of virulent isolates in the pathogen population. This approach has not yet been adopted in western Canada. There is evidence that using both types of resistance is of benefit for blackleg management (Brun et al. 2010), but little information is available for assessing *QR.* Despite the ineffectiveness of *Rlm1*and *Rlm3* against the current pathogen population, most Canadian cultivar was rated blackleg resistant at the time of cultivar registration (Saskatchewan Seed Growers Association 2012)

It is possible that the resistance in most of these cultivars is conferred by genetics beyond *Rlm1* or *Rlm3*; likely *QR* also plays a role. In 2012 and 2013, some of the *R*-rated canola cultivars had high levels of blackleg (Peng et al. 2014) that could not be attributed

solely to the presence or absence of any *Avr* genes in the pathogen population (refer to the Chapter 3). This was because most of the cultivars in western Canada carry similar major *R* genes and the pathogen race composition is often similar in fields showing different levels of blackleg (Zhang et al. 2015).

The mechanism of *QR* against blackleg of canola is not well understood; it does not result in a hypersensitive reaction in the host and cannot completely prevent host colonization (Huang et al. 2014), rather show more severe symptoms, but development of the disease is often slower relative to that on susceptible cultivars. As, the initiation of blackleg disease mostly coincide with the emergence of young canola plants, as such cotyledons and young leaves are the main avenue for the pathogen to infect the stem (West et al. 2001), *QR* may limit the spread of the pathogen from cotyledons to stems before cotyledon senescence, consequently reducing the incidence and severity of blackleg. This information will help to understand the key modes of action associated with CCC for blackleg resistance against the current pathogen population, facilitating disease management. It was hypothesized that most CCCs carry a level of *QR* that limits the spread of the pathogen from cotyledons into stems, thus reducing the severity of stem infection. It may also reduce disease development in the stem, alleviating the severity of blackleg at crop maturity. The main purpose of this research was to assess *QR* or non-race-specific resistance in common CCCs and understand it in relation to the typical infection avenue leading to stem canker. The objectives were to: 1) characterize the development of blackleg on CCCs originating from cotyledon infection to identify clues for resistant host responses, and 2) verify the observed resistance responses based on the spread of pathogen within the host in critical stages of infection using sensitive and accurate molecular and fluorescence microscopic approaches.

4.3. Materials and Methods

Three experiments were conducted under controlled-environment conditions (growth cabinet and greenhouse) to characterize the resistance response of canola cultivars representative of those used in western Canada. Eight CCCs were provided by Bayer CropScience, Monsanto Canada, Pioneer Hybrid Canada, Crop Production Services, and Agrium Inc. Although most of the CCCs were not identified, these seed companies supply hybrid seeds for the majority of the canola grown in western Canada. The cv. Westar was used as a susceptible control throughout this study.

4.3.1. Identification of *R* genes carried in CCCs

A differential set of ten *L. maculans* isolates carrying known *Avr* genes (Table 4.1.) was used to inoculate cotyledons of CCC to determine the presence of specific *R* genes in each cultivar. Seven of the CCCs were rated resistant (*R*-rated) and one moderately resistant (*MR*) to blackleg based on public and private multi-site coop trials across the prairies. The cotyledon-inoculation method described in Chapter 3 was used to perform this test. The purpose was to determine *R* genes, if any, carried by the CCCs and to assess potential differences in the infection severity on cotyledons. The test was carried out twice.

Table 4.1. The *Avr* gene composition of *Leptosphaeria* maculans isolates used to determine the presence of specific resistance genes in CCCs.

Isolate	*Avr* genes involved	Expected reaction on canola varieties or lines carrying the Rgenes											
		Rlm1	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm5*	*Rlm6*	*Rlm7*	*Rlm8*	*Rlm9*	*Rlm10*	*LepR1*	*LepR3*
S7‡	1, 5, 6, 7, (8)*	***Avr*** [1]	*avr* [1]	*avr*	*avr*	***Avr***	***Avr***	***Avr***	-	*avr*	*avr*	*avr*	*avr*
P27D	1, 5, 6, 7, (8)*, 10	***Avr***	*avr*	*avr*	*avr*	***Avr***	***Avr***	***Avr***	-	*avr*	***Avr***	*avr*	*avr*
V45-30	2, 7, (10)*	*avr*	***Avr***	*avr*	*avr*	*avr*	*avr*	***Avr***	*avr*	*avr*	-	*avr*	*avr*
19.4.24	3, 5, 6, 8, (10)*	*avr*	*avr*	***Avr***	*avr*	***Avr***	***Avr***	*avr*	***Avr***	*avr*	-	*avr*	*avr*
V23-2.1	4, 5, 6, 7, 8, (10)*	*avr*	*avr*	*avr*	***Avr***	***Avr***	***Avr***	***Avr***	***Avr***	*avr*	-	*avr*	*avr*
IBCN 14	5, 6	*avr*	*avr*	*avr*	*avr*	***Avr***	***Avr***	*avr*	*avr*	*avr*	***Avr***	*avr*	*avr*
290 Cdn	5, 6, 7, 8, 10, Lep3	*avr*	*avr*	*avr*	*avr*	***Avr***	***Avr***	***Avr***	***Avr***	*avr*	***Avr***	*avr*	***Avr***
NZ-T4	5, 6, 8, (10)*	*avr*	*avr*	*avr*	*avr*	***Avr***	***Avr***	*avr*	***Avr***	*avr*	-	*avr*	*avr*
PHW1223	5, 6, 8, 9	*avr*	*avr*	*avr*	*avr*	***Avr***	***Avr***	*avr*	***Avr***	***Avr***	*avr*	*avr*	*avr*
R2†	5, 7, (8)*, 10	*avr*	*avr*	*avr*	*avr*	***Avr***	*avr*	***Avr***	-	*avr*	***Avr***	*avr*	*avr*
CR07-96	(5)*,6,7, Lep1	*avr*	*avr*	*avr*	*avr*	***Avr***	***Avr***	***Avr***	*avr*	*avr*	*avr*	***Avr***	*avr*

[1] ***Avr*** and *avr*: Representing incompatible and compatible host reactions, respectively.
* *Avr* genes may be carried by these isolates

4.3.2. Pathogen isolates and inoculum

Virulent isolates of *L. maculans* were selected for this study; based on the *R* genes carried by the CCCs, none of the pathogen isolates carried any corresponding *Avr* genes, and thus a resistant response was not the result of a specific *R-Avr* gene interaction. Three *L. maculans* isolates: 12CC09 [*Av6,(8)*], 13CCMB02-19 [*Av4-6-7-(8)*] and 13CCMB04-06 [*Av2-4-6-7-S-(8)*], were selected. These isolates did not carry *AvrLm1* or *AvrLm3* alleles, corresponding to the *Rlm1* and *Rlm3*, carried by CCCs. Only isolate 12CC09 was used throughout the study; the other two isolates were used only in limited cotyledon-inoculation experiments for validation the results obtained in experiment 1. The production of *L. maculans* followed the same protocol described in Chapter 3. Briefly, the fungal culture was transferred to V8-juice agar and incubated at room temperature for about 10 d before harvest by flooding with sterile distilled water. The concentration of pycnidiospore suspension was adjusted to about 1×10^7 spore mL^{-1} for inoculation (Kutcher et al. 2011b).

4.3.3. Plants

Each CCC was seeded in 10-cm square pots using Sunshine #3 soil-less planting mix amended with 16-9-12 (N-P-K) at 12.5 g L^{-1} as described earlier. Fertilization was sufficient to support canola plants to maturity in the greenhouse. Canola plants were grown in small pots (3-3/8" x 4") that were placed in flats and kept initially in a growth chamber at 22/16°C (day/night) with a 16 h photoperiod (512 μmol m^{-2} s^{-1}). Plants were watered from the bottom of the tray with about 2.3 L of tap water per tray (Kutcher et al. 2011b). After germination, plants were thinned to 2 per pot and each CCC had about 15 pots per run. Each experiment had three runs. Cotyledons of each plant were inoculated at 7 after seeding (DAS), or on petioles at 14 DAS, depending on the experiment. Seven days post inoculation (dpi) the plants were transplanted to 13-cm plastic pots (one plant per pot) and placed randomly on a greenhouse bench until early maturity stage of 5.2 (Harper and Berkenhamp, 1975) to assess

blackleg severity originating from cotyledon or petiole inoculation. The temperature in the greenhouse was 22/16°C with 16 h natural daylight supplemented by 430 watt high-pressure sodium lamps (230 μmol m^{-2} s^{-1}). All plants were checked daily and watered as needed.

4.3.4. Plant inoculation and infection/disease assessment

To simulate infection development from infected cotyledons or petioles into the stem, wounds were made on cotyledons with tweezers as described above or on petioles with a needle. A 10-μl droplet of *L. maculans* spore suspension was applied to each wound. The inoculation was carried out at 7 or 14 DAS depending on the experiment, and unlike the earlier study, true leaves were not removed. Inoculated plants were placed in a dew chamber (Percival Scientific, Perry, IA) at 20°C in the dark for 24 h to facilitate infection, and then transferred back to the growth chamber until the assessment of disease severity on the cotyledons. Additionally, some of these plants were assessed for pathogen spread from the inoculation site (IS) into the stem using droplet digital PCR (ddPCR) and fluorescence microscopy (with green fluorescent protein -GFP) as described in the following sections. Additional inoculated plants were kept in the greenhouse until maturity and monitored weekly for plant mortality commencing at 14 dpi. At each assessment point, dead plants were recorded and removed. A plant showing permanent wilting for longer than a week was considered "dead" and assessed at severity level 5 at the final disease severity rating. At early maturity, growth stage (GS) 5.2 (Harper and Berkenhamp, 1975), all plants were cut at the cotyledon abscission point on the stem, and the internal blackening of stem was assessed using the 0-5 scale based on the percentage of diseased area of the stem cross section (**Appendix 3**).

4.3.5. Fluorescent microscopy for observation of *L. maculans* in cotyledon/petiole tissues

The spread of *L. maculans* in inoculated canola cotyledons was assessed using a pathogen isolate carrying only *AvrLm6,(8)* that had been transformed with a green-florescent-protein

(GFP) gene which expressed strongly in the cell-wall membrane, as well as in the cytoplasm of the fungal hyphae. The untransformed wild type was the *L. maculan*s isolate 12CC09, and therefore the GFP-transformed isolate was given the name 12CC09-GFP. Fluorescent microscopy was performed on inoculated cotyledons at 10 dpi using a Zeiss Stereo-Lumar fluorescent microscope outfitted with 0.8× NeoLumar S lens. Leaf samples were placed on a microscope slide, submerged in a drop of water, and covered with a glass slip. The GFP imaging was done with a microscope with an HBO100 mercury lamp and GFP filter. A bright field image was obtained using a KL-2500 LCD white-light source, no filter, and a 1.5 s exposure time. The brightness and gamma were adjusted to optimize the visibility of the green-fluorescent fungal hyphae in overlaid images

4.3.6. Quantifying *L. maculans* in petiole and stem tissues using ddPCR

At 14 dpi, the petioles of inoculated cotyledons and stem pieces cut between 1 cm above and 1 cm below the petiole were sampled separately, and freeze dried for DNA extraction using the BioSprint robot and kit (Qiagen, Toronto, ON). The quality of DNA was confirmed on a Nanodrop (Agilent Technol. Mississauga, ON), and all DNA samples were kept at 4°C until use. Primers were designed using the *L. maculans* sequence provided by Dr. H. Borhan, AAFC Saskatoon Research and Development Centre, and validated using genomic DNA of *L. maculans*. The actin genes of *B. napus* were used as housekeeping genes (required for the upkeep of basic cellular activities of an organism) and the primers validated using the DNA extracted from the double haploid *B. napus* line DH12075 (AAFC). The primer and probe information is provided in **Table 4.2**. For validation, the mixture of forward and reverse primers, DNA samples, water, dNTP's, 10× PCR buffer with Mg and Taq for each primer pair along with markers were loaded on the standard 1% agarose gel (100 ml TAE buffer, 1 g Agarose and 2 µl Enviro Safe dye), and electrophoresed at 90V for 30 min. The effectiveness of the primers/probes was confirmed.

Table 4.2. Primer and probe DNA sequences used in the quantification of *L. maculans* and *Brassica napus* DNA in the ddPCR assay.

Primer & probe for L. maculans	
Lm HyPro F1	GCGCGAATCACCAGATACA
Lm HyPro Probe	ACGAAGTGTGAGGTCGTCTGTGAGA
Lm HyPro R1	CTCCTCTAGGGAAGGACATACA
Primer & probe for B. napus	
Bn. Actin F	CAGTGGTCGTACTACTGGTATTG
Bn. Probe	TGCTGGATTCTGGTGATGGTGTGT
Bn. Actin R	GATGGCGTGTGAAAGAGAGA

Before testing, DNA samples extracted from petiole or stem tissues were digested with the enzyme HindIII (a restriction endonuclease that recognizes the sequence and cuts best) by adding 1µl of DNA to 9 µl of the enzyme cocktail in each well. After mixing, samples were placed in an oven at 37°C for 1 h. The PCR cocktail was prepared in bulk by vortexing 12.5 µl Super mix for Probes (Bio-Rad Canada, Mississauga, ON), 1 µl Lm HyPro F1 and R1 primers (22.5 µM), 1 µl Lm HyPro probe (6.2 µM, FAM dye), 1 µl Bn. actin F and R primers (22.5 µM), 1 µl Bn. Probe (6.25 µM, HEX dye) and 2.5 µl sterile deionized water. Four µl of digested template DNA were mixed with the PCR cocktail to make a 25 µl reaction.

A disposable cartridge was placed in the cartridge holder cassette to generate droplets. A multi-channel pipet was used to add 20 µl of PCR reaction solution to the centre row of the droplet generator cartridge. Care was taken during this step to avoid bubbles as they would disturb droplet formation. Droplet generation oil was added (70 µl) to the bottom-row wells of the cartridge using the multi-channel pipet. The gasket was fitted over the top of the cassette before it was placed in the droplet generator. After analysis, the cassette was

removed from the generator and 40 µl of droplet suspension was pipetted from the top row very slowly, and the droplet suspension was then pipetted into a sealable PCR plate, very slowly to avoid bubbles.

After transferring the droplet suspension to a Twin-tech sealable PCR plate, a single foil seal was placed on the top of the PCR plate to secure the content in the wells and the plate was placed in a sealer programed at 180ºC for 10 s. The PCR plate was then removed and placed in the BIO-RAD C1000 thermocycler programmed as follows: at 95ºC for 10 s, 94ºC for 30 s, 58.3ºC for 1 min with a ramp of 2ºC/s (annealing temperature), 50 cycles of 30 s at 94ºC, 98ºC for 10 s and 12ºC hold. The program QuantaSoft™ was used to read and analyze the PCR reaction in a large number of droplets. Before reading, a sample template was entered and when the PCR was finished the plate was taken to the BIO-RAD Q100 reader. Each well took approximately 1.5 minutes to read. The quantity of *L. maculans* DNA was expressed as a proportion of the total *B. napus* DNA from the petiole or stem sample.

4.3.7. Experiment 1: Resistance to blackleg and/or plant mortality resulting from the cotyledons inoculated with *L. maculans*

Three CCCs that varied slightly in terms of cotyledon resistance in pre-trials were selected for this experiment. All carried the *R* genes *Rlm1* and/or *Rlm3*, but none was considered highly resistant to *L. maculans* Isolate 12CC09, carrying *AvrLm6,(8)* (**Appendix** 2). The cv. Westar was used as the susceptible control. As described earlier, test cultivars were seeded initially in small 10-cm pots and at 7 DAS, both lobes of each cotyledon were wounded and inoculated with Isolate 12CC09 carrying no corresponding *Avr* genes to *Rlm1* or *Rlm3* found in the CCCs. At 14 dpi, the disease severity on cotyledons was assessed. Fifteen to 32 inoculated plants, depending on the cultivar or trial repetition, were then transplanted individually into 13-cm pots and kept in the greenhouse until the end of the experiment. These plants were examined weekly for basal canker or mortality. Up to 10 non-

inoculated Westar plants were included as an additional check for disease symptoms and plant mortality. All plants were cut at the cotyledon abscission point on the stem and assessed for blackleg severity using a 0-5 scale (**Appendix 3**). The experiment was carried out three times; each time the pathogen inoculum and plant materials were prepared independently. A disease severity index (DSI) was calculated for each CCC and for Westar for each repetition using the following formula (Grau et al. 1982):

$$\text{DSI (\%)} = \frac{\sum [(\text{rating class})\ (\#\ \text{plants in the class})]}{(\text{Total \# of plants})\ (5)} \times 100$$

For fluorescent microscopy to trace the pathogen hyphal movement after inoculation, cotyledon samples were taken from 5 to 10 random plants from each repetition of each trial at 10 dpi. The GFP transformed *L. maculans* isolate (12CC09-GFP) was used for inoculation. Samples were examined immediately under a fluorescent microscope for the spread of pathogen from inoculation sites. The assessment of CCCs for hyphal spread was compared to that in Westar.

For the ddPCR assay, petiole and stem samples were taken from three to eight random plants at 14 dai of cotyledon inoculation experiments in each trial. The petiole and stem samples were separated for each CCC and the control. The quantity of *L. maculans* DNA based on ddPCR represented the relative amount of pathogen hypha that had reached the petiole or stem tissue. Three tests were conducted for both GFP and ddPCR assays.

4.3.8. Experiment 2: Resistance to blackleg and/or plant mortality resulting in the inoculation of petioles with *L. maculans*

To assess the potential resistance of CCCs after pathogen establishment in the stem, same three CCCs were selected, and the petioles of the cotyledons were inoculated at 14 DAS with

L. maculans Isolate 12CC09 at a location adjacent to the stem axil. This method of inoculation increased the probability of successful stem infection due to the short distance from the site of inoculation, and generally 100% stem infection was achieved in preliminary trials. About 15 plants were inoculated for each CCC in each trial. These plants were then transplanted individually into 13-cm pots at approximately 7 dpi, kept in the greenhouse, and examined weekly for basal canker or mortality. All plants were cut at the cotyledon abscission point on the stem at early maturity and assessed for blackleg severity using the 0-5 scale. The experiment was conducted three times, with plant materials and pathogen inoculum prepared independently.

4.3.9. Experiment 3: Test of CCC resistance with additional *L. maculans* isolates

To validate the results of experiment 1, same CCCs were also inoculated with two additional *L. maculans* isolates (Isolates13CCMB02-19 [*Av4-6-7-(8)*] and 13CCMB04-06 [*Av-2-4-6-7-S-(8)*]) These isolates carry different *Avr* genes from those of Isolate 12CC09, but none correspond to any of the *R* genes (*Rlm1* and/or *Rlm3*) in the CCCs. The experimental protocol and inoculation and assessment methods were similar to Experiment 1 (4.3.7) and the same three CCCs were used. The experiment was carried out twice at different times, with ten plants for each CCC as well as the Westar control.

4.3.10. Data analysis

The determination of *R* genes carried by CCC was similar to that described for *Avr* gene detection in Chapter 3. Six plants were arranged in two blocks for each repetition, and disease severity scores on cotyledons were averaged over a total 6 plants (2 blocks) of each CCC or Westar. A resistant reaction (≤ 4.9), where the lesion size is small and pycnidia absent (Appendix 3), was interpreted as the potential presence of *R* genes corresponding to at least one of the *Avr* genes in the *L. maculans* isolate. All other experiments used a completely randomized design, with experiments carried out three times, unless otherwise indicated.

The same disease rating scale as in Experiment 1 was used to evaluate disease severity on cotyledons. Resistance was accepted if disease severity scores were ≤ 5 Plant mortality and disease severity index (DSI) values from different repetitions were used in statistical analysis using the R Statistical Software (V3.1.2, R Foundation for Statistical Computing, Vienna, Austria). The DSI (%) data was log transformed and confirmed for normality based on the Shapiro-Wilk Test ($P > 0.05$) prior to the analysis of variance (ANOVA) using R Software. Treatments were separated with Tukey's Test ($P = 0.05$). The same statistical analysis was applied to the ddPCR data.

4.4. Results

4.4.1. *R* genes carried by CCCs

Most of the CCCs were resistant to infection by *L. maculans* isolates carrying *AvrLm1* or *AvrLm3*, based on the disease severity of inoculated cotyledons. One of the CCCs (CCC7) appeared moderately resistant to infection by Isolate 290 Cdn, which carries neither *AvrLm1*, nor *AvrLm3*. It was therefore deduced that all of the CCCs carried the specific *R* genes *Rlm1*, *Rlm3* or both and CCC7 might also carry additional *R* genes.

Table 4.3. Reaction of CCCs on cotyledons to inoculation using *L. maculans* isolates carrying various *Avr* genes[1].

Isolate	*AvrLm*	Westar	CCC1	CCC2	CCC3	CCC4	CCC5	CCC6	CCC7	CCC8
S7	*1,5,6,7(8)*	5.7	1.0	4.0	4.7	5.0	4.0	5.3	5.0	4.7
P27D	*1,5,6,7(8),10*	6.7	1.0	3.3	4.0	3.3	3.7	3.3	3.3	5.3
V45-30	*2,7,(10)*	7.9	5.9	6.2	7.0	6.8	6.8	5.0	7.0	7.0
19.4.24	*3,5,6,8,(10)*	7.3	1.3	1.0	3.3	1.0	1.3	6.3	4.3	1.3
V23-2-1	*4,5,6,7,8,(10)*	8.1	6.1	7.0	7.0	5.0	7.9	5.0	7.4	7.4
IBCN	*5,6*	7.4	7.0	7.0	6.6	5.2	5.0	6.6	7.0	7.0
290CDN	*5,6,7,8,10,Lep3*	7.3	5.0	5.0	6.0	4.7	5.0	5.7	6.0	5.3
NZT-4	*5,6,8,(10)*	9.0	5.9	5.7	7.9	6.3	7.9	5.7	7.9	7.0
PHW1223	*5,6,8,9*	7.4	5.4	7.0	7.0	6.1	7.0	5.4	7.0	7.0
R2	*5,7,(8),10*	7.4	5.4	7.0	7.0	6.1	7.0	5.4	7.0	7.0

[1] Averaged over 6 plants.

4.4.2. Experiment 1: Resistance of CCCs to blackleg and/or plant mortality base on cotyledon assessment

The average disease severity on inoculated cotyledons was 5.3 to 6.7 for the CCCs, compared to 7.7 on Westar at 14 dpi. The infection generally continued to spread into the stem, causing variable levels of blackleg and plant mortality on CCCs. Often dark lesions were visible on the stem by the juncture of the stem and petiole of the inoculated cotyledon at about 28 dpi and plant wilting and death sometimes occurred. Non-inoculated Westar plants showed no such symptoms. There were noticeable differences in the trend of plant mortality on different cultivars; CCC1 had the lowest mortality and none of the plants died until 49 dpi (**Figure 4.1**). Plant mortality of CCC2 and CCC3 began slightly earlier (42 dpi), and had substantially higher final mortalities relative to CCC1. Plant mortality of CCC2 and CCC3 was delayed compared with CCC1 and the final mortality was lower than the susceptible control Westar. Cumulative

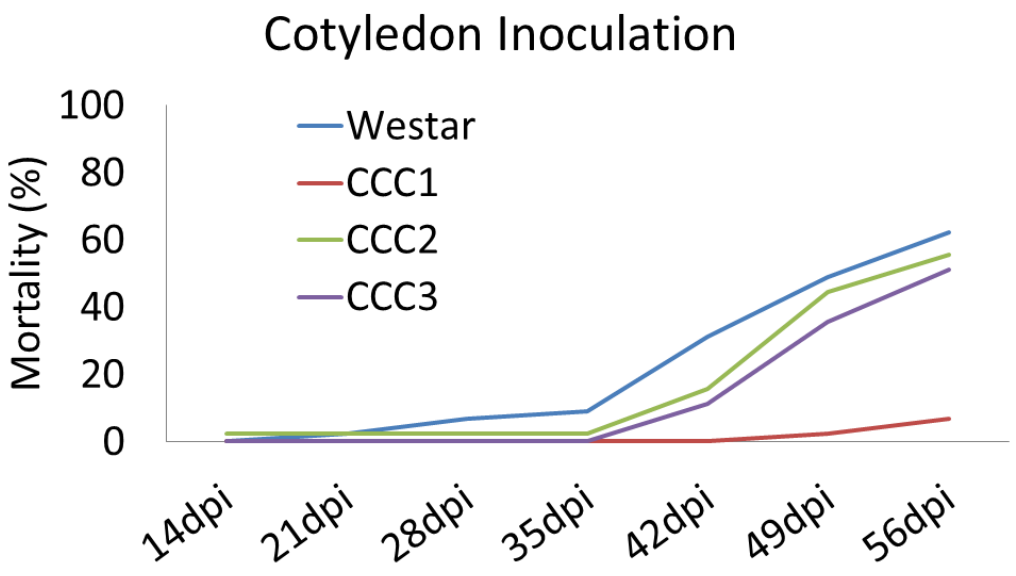


Figure 4.1. Plant mortality of selected CCCs originating from the inoculation of cotyledons with the *L. maculans* isolate 12CC09. Westar was a susceptible control.

At early maturity, the mean DSI was approximately 80% on Westar, with plant mortality of >90% (**Figure 4.2**). Both DSI and plant mortality varied substantially among CCCs; CCC1 had the lowest DSI and final plant mortality of all the cultivars tested, with about 50% reduction in both categories when compared with Westar. The CCC2 and CCC3 appeared less resistant than CCC1, but still had substantially less plant mortality compared with Westar (Tukey's Test, **Appendix 5**). Although the DSIs of CCC2 and CCC3 were not different from each other (Tukey's Test, P >0.05), but were significantly different from that of the control (Westar).

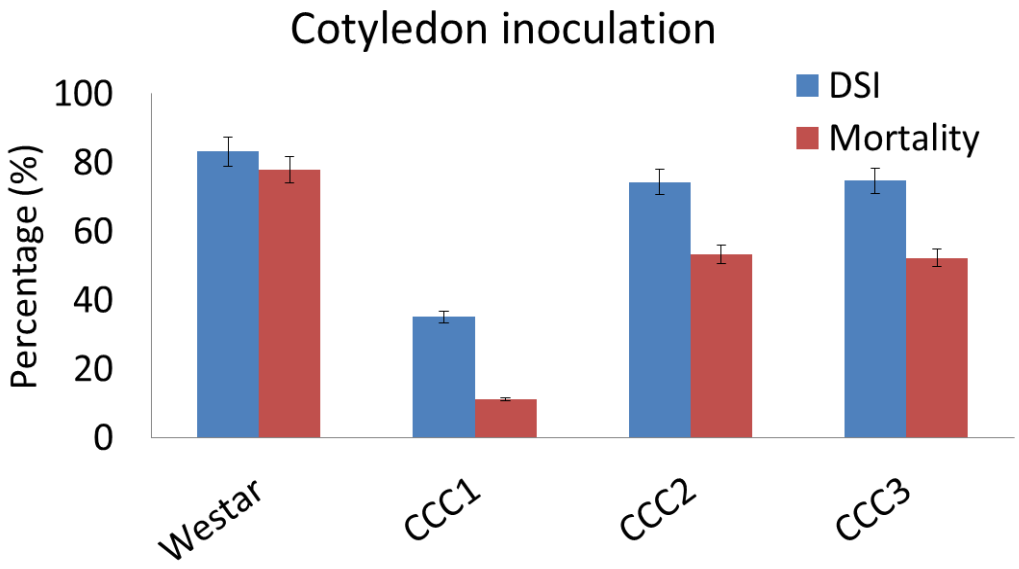


Figure 4.2. Disease severity index (DSI) and final plant mortality at the early maturity stage of canola after cotyledon inoculation using *L. maculans* isolate 12CC09 in greenhouse trials.

The amount of *L. maculans* DNA was calculated against the amount of *B. napus* DNA in the sample; this quantified the pathogen relative to the host tissue. The amount of pathogen DNA detected in petioles of inoculated cotyledons was substantially less in the CCCs than for Westar at 14 dpi (**Figure 4.3**). In stem tissues, adjacent to the petiole, the amount was also significantly less (<90%) in the CCCs than in Westar (**Figure 4.3**). In general, the amount of pathogen DNA was lower in stems than petioles at 14 dpi, regardless of cultivar.

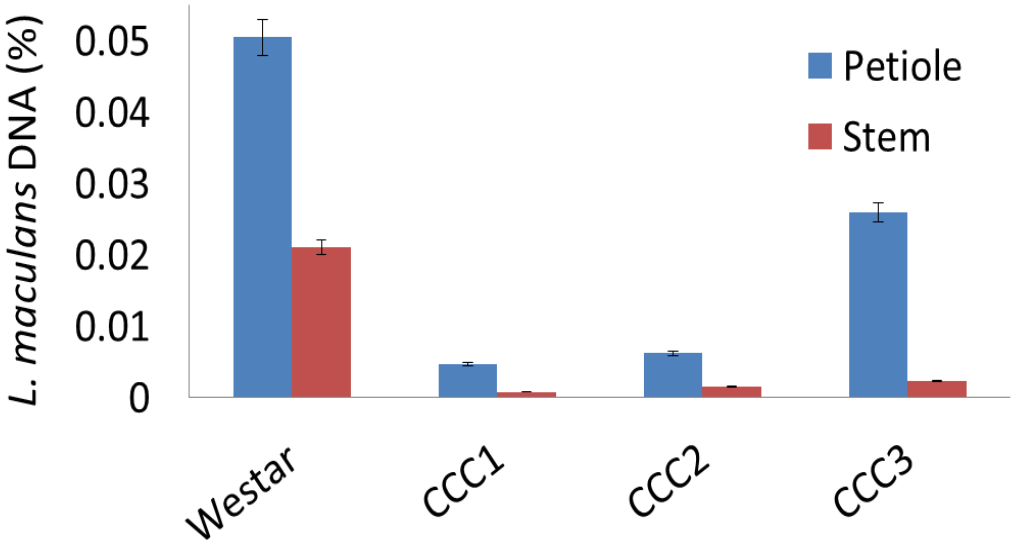


Figure 4.3. The amount of *L. maculans* DNA in the petiole of Westar (control) was approximately 0.05% and in stem tissues adjacent to the petiole was nearly 0.02%, which was higher relative to the CCCs at 14 dpi.

Florescent microscopy clearly illustrated the movement of GFP-labelled *L. maculans* hyphae from the inoculation site (IS) on the Westar cotyledon into the petiole at 10 dpi (**Figure 4.4**). Movement was limited on CCCs, especially CCC1 and CCC2 (**Figure 4.5** and **4.6**). At this stage, there was limited necrosis around the IS on all cultivars, but lesions could be clearly defined. The colonization of the cotyledon by *L. maculans* appeared to occur earlier than visible disease symptoms.

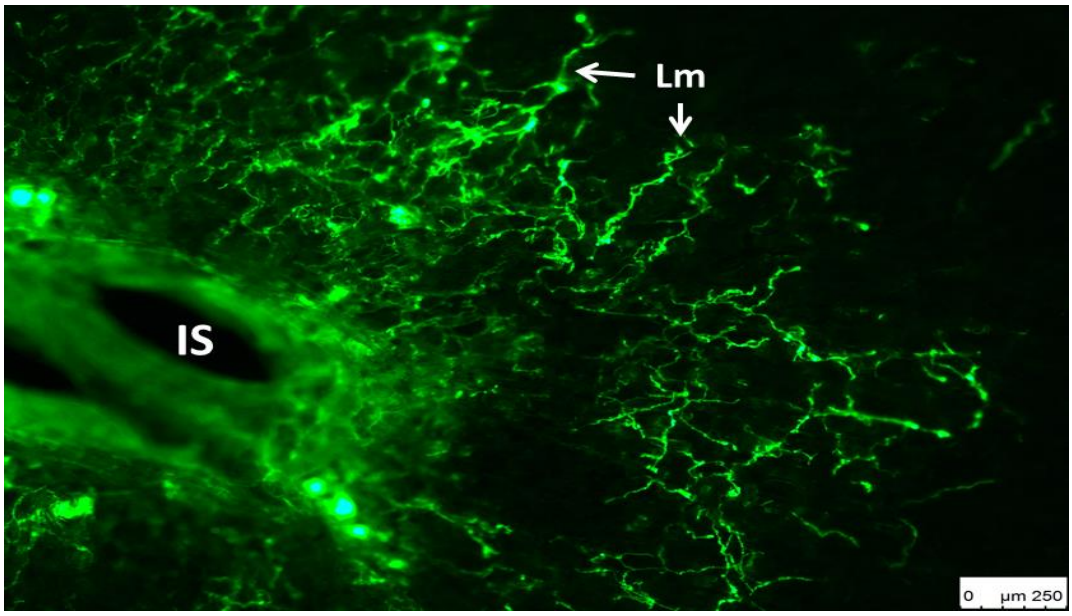


Figure 4.4. The colonization of cotyledon tissue by *L. maculans* isolate 12CC09-GFP from the inoculation site (IS) on Westar; Lm indicates pathogen hyphae (arrow pointed) under the florescent microscope.

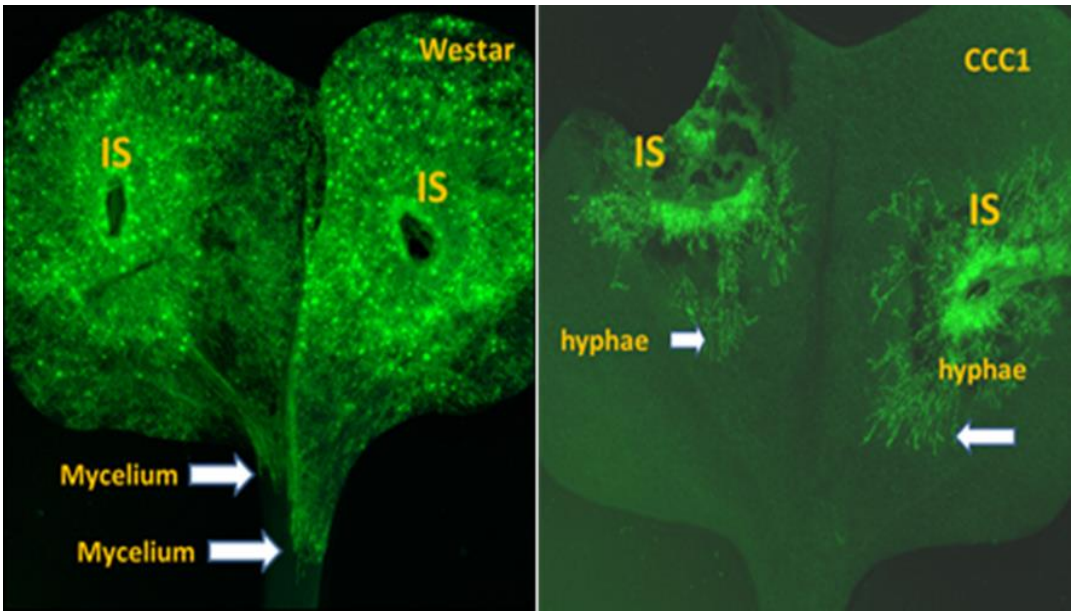


Figure 4.5. The spread of GFP-labelled *L. maculans* hyphae (white arrows) in cotyledon and petiole tissues from the inoculation site (IS) on Westar and CCC1 at 10 dpi.

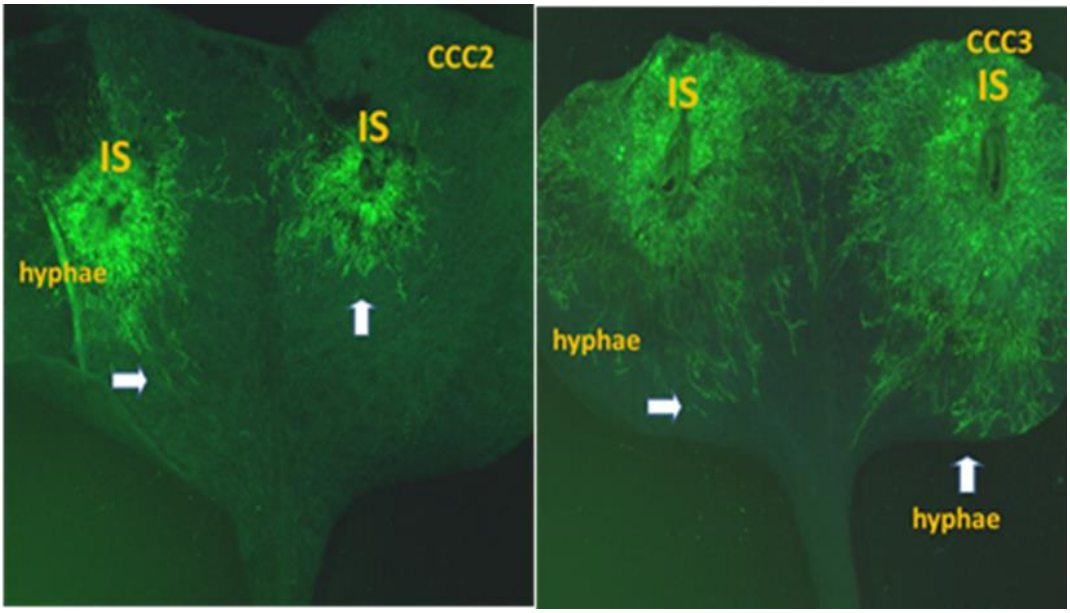


Figure 4.6. The spread of GFP-labelled *L. maculans* hyphae (white arrows) in the cotyledon tissue from the inoculation site (IS) on CCC2 and CCC3 at 10 dpi.

4.3.3. Experiment 2: Resistance of CCCs to blackleg and/or plant mortality after petiole inoculation

It took less time for stem infection and plant mortality with petiole inoculation as opposed to cotyledon inoculation; plant mortality was observed on Westar and each of the CCCs by 21 dpi (**Figure 4.5**), as opposed to 35 dpi on CCCs after cotyledon inoculation. Mortality increased only marginally on CCC1 after the initial observation and was <20% at the end of the experiment (56 dpi). The CCC2 and CCC3 had more rapid increase in mortality and close to 80% of these plants died by 56 dpi, although the mortality was still slightly lower than that of Westar at each time point of assessment after 21 dpi (**Figure 4.6**).

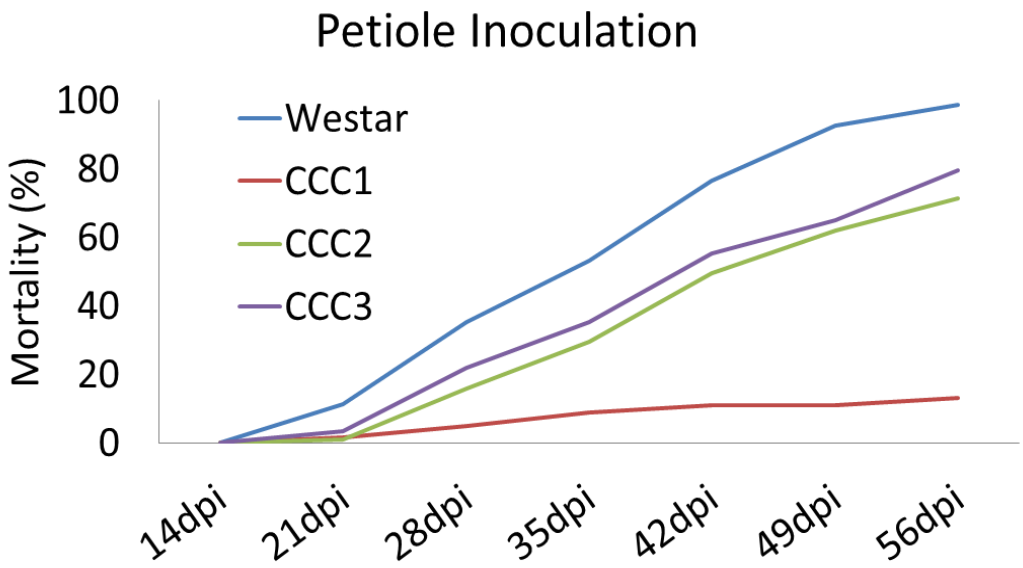


Figure 4.7. Plant mortality of selected CCCs originating from the inoculation of petioles with the *L. maculans* isolate 12CC09. Westar was a susceptible control.

The disease severity at early maturity after petiole inoculation was also higher than that after cotyledon inoculation; the average DSI on Westar was almost 100% and none of the plants survived to that stage (**Figure 4.8**). The plant mortality of CCC2 and CCC3 was reduced slightly compared with Westar, but it was CCC1 that had the most substantially reduced DSI (<40%) and plant mortality. More than 80% of CCC1 plants survived to early maturity, as opposed to ~20% for CCC2 or CCC3. The latter two cultivars had approximately 80% DSI, which was not significantly different from that of Westar.

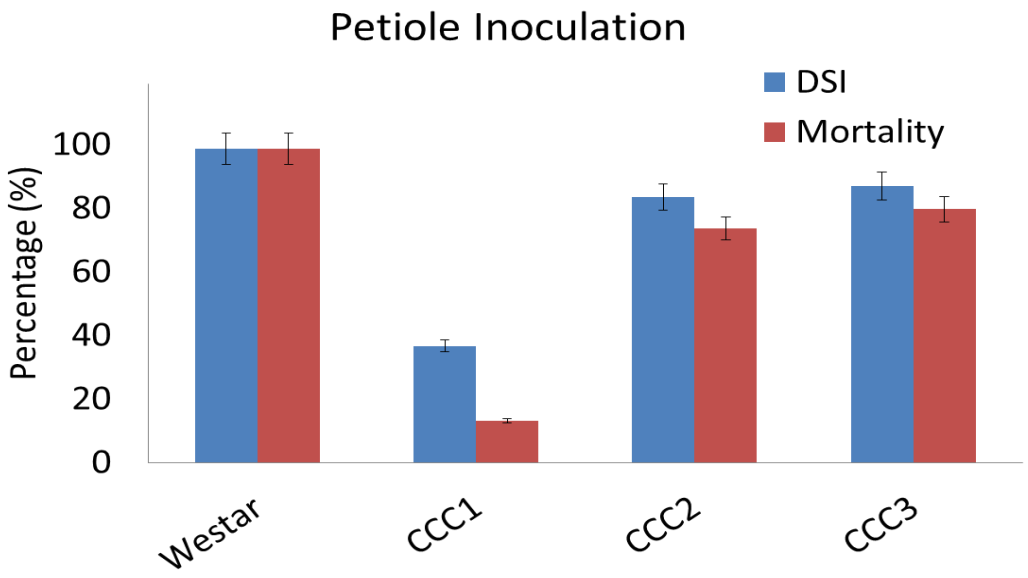


Figure 4.8. Disease severity index (DSI) and final plant mortality at the early maturity stage of canola after petiole inoculation using *L. maculans* isolate 12CC09 in greenhouse trials.

4.3.4. Experiment 3: Resistance of CCC to infection caused by additional *L. maculans* isolates

Two additional *L. maculans* isolates with different *Avr* composition from that of Isolate 12CC09 were used to compare the CCCs for blackleg resistance after cotyledon inoculation. These two isolates, 13CCMB02-19 and 13CCMB04-06, appeared less aggressive than Isolate 12CC09 and resulted in lower DSI (<50%) and plant mortality (<30%) on Westar at early maturity (**Figure 4.9**). In contrast, cotyledon inoculation with Isolate 12CC09 generally resulted in both a DSI and plant mortality of >80%, at a similar growth stage. Nevertheless, these CCCs reduced DSI and plant mortality significantly when compared with Westar; CCC1 was the most resistant, whereas the other two CCCs did not differ from each other.

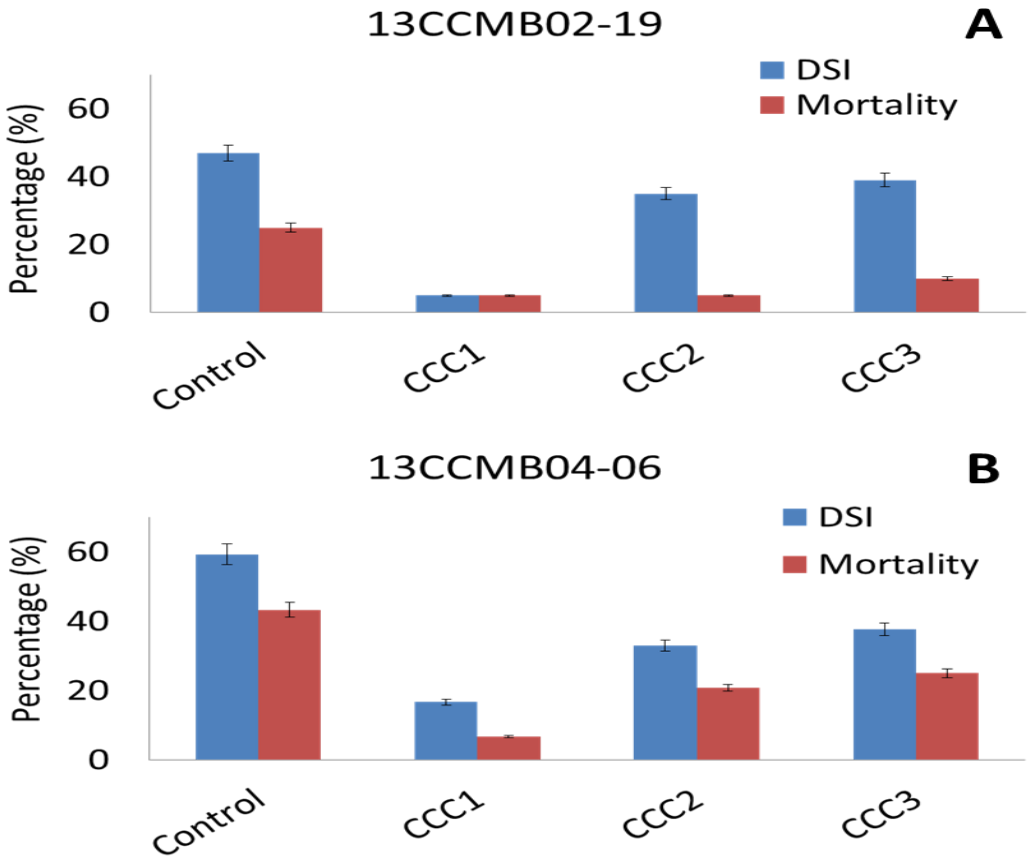


Figure 4.9. Disease severity index (DSI) and final plant mortality at the early maturity stage of canola after cotyledon inoculation using two *L. maculans* isolates: 13CCMB02-19 [*Avr4-6-7-(8)*] (A) and 13CCMB04-06 [*Avr2-4-6-7-S-(8)*] (B).

4.3. Discussion

Most CCCs are believed to carry some level of resistance to blackleg, although the source or sources of that resistance is largely unknown (Rimmer 2006). The eight CCCs provided by several seed companies that supply the majority of the canola seed in western Canada all carry the *R* genes *Rlm1* and/or *Rlm3.* CCC4 may also carry *LepR3*, but this was not validated by testing with a *L. maculans* isolate carrying *AvrLep3* without *AvrLm1*. Additionally, severe symptoms on cotyledons were not observed; average disease severity was 4.7. The result is generally consistent with that reported by Zhang et al. (2015) who investigated a broad range of canola cultivars and breeding lines in Canada. Most of the canola cultivars tested in our study carried *R* genes that are not effective against the current pathogen population, in which *AvrLm1*, *AvrLm3* and even *AvrLep3* are at extremely low levels (Liban et al. 2016; Chapter 3). This is likely the response of the pathogen to the cvs. Quantum (*Rlm3*) and Q2 (*Rlm1, Rlm3*) introduced in the early 1990s' (Kutcher et al. 2009), as well as to many later cultivars carrying *Rlm1* and/or *Rlm3* as revealed in this study. *Rlm1* also conditions resistance to *L. maculans* carrying *AvrLep3* (Larkan et al. 2013), resulting in the low frequency of this *Avr* gene in the pathogen population. Despite the fact that only *Rlm1* and/or *Rlm3* were found in these CCCs, all are rated blackleg resistant in western Canada based on their field performance. This suggests that for many canola cultivars additional factors beyond *Rlm1* or *Rlm3* are involved in blackleg resistance; race-nonspecific or *QR*, which is more evident when assessed in adult-plant stages (Zhang et al. 2015), may play an important role.

The genetics of, and modes of action for *QR* are not well understood; it is usually not expressed strongly at seedling stages but often results in reduced disease damage under field conditions (Brun et al. 2010). Its identification, however, can be complicated because the process depends heavily on field experimentation (Pilet et al. 1998; Fitt et al. 2006) and the results can be influenced by many environmental and management factors. Huang et al.

(2009) observed *QR* in the cv. Darmor (*B. napus*) in field trials, and in controlled environment they observed that the pathogen colonized the stem more slowly in Darmor than on a susceptible cultivar, although the pathogen spread from leaves to stems rapidly in both cultivars. Later they also observed slower spread of *L. maculans* in leaves and petioles of another *QR* line. Delourme et al. (2006) suggested that the modes of action for *QR* may vary with the host, infection stage or resistant genotype involved. In the current study, selected CCCs were verified not to be carrying *R* genes corresponding to any of the *Avr* genes in the *L. maculans* isolates used. Therefore, any resistant response of the CCCs would likely have resulted from *QR* mechanisms. ddPCR and GFP-fluorescent microscopy (**Figure 4.3-4.6**) provided strong evidence of restricted pathogen spread from inoculated cotyledons via the petioles into the stems of CCCs, and this mechanism is likely important because infected cotyledons and petioles often separated from the stem shortly after 14 dpi (data not shown). This prevented the pathogen from entering the stem if it had not already done so by this time. The reduced DSI and plant mortality of CCCs, especially CCC1 (**Figure 4.1** and **4.2**) relative to Wester after cotyledon infection, also indicated restricted pathogen spread, and therefore blackleg resistance. In petiole inoculation, differences in DSI and plant mortality between the CCCs and Westar highlighted the stem resistance within the CCCs; this inoculation method caused almost 100% stem infection of all cultivars (data not shown), however DSI was much lower on the CCCs, especially on CCC1 (**Figure 4.7** and **4.8**).

Increased lignification of pith cells may strengthen stem resistance to blackleg (Hammond and Lewis 1987), but experimental data are generally lacking for molecular mechanisms of *QR*. In the current study, the *QR* identified in connection with infection of cotyledon, petiole and stem tissue of CCCs, suggested their relevance to blackleg resistance and that they may also play an important role in current disease management strategies in western Canada. The characterization of cotyledon and stem infection confirmed differences

between CCCs and the susceptible control, which may pave the way for further studies on molecular mechanisms of *QR* resistance by selecting stages of infection to identify the specific genes or pathways involved. Monitoring plant mortality after inoculation provided an additional measurement on stem resistance to blackleg; CCCs delayed the onset of plant mortality by one and three weeks, respectively, in petiole and cotyledon inoculations relative to that of Westar. This was possibly due to slower infection of CCC stem tissues. CCC1 stood out in this aspect, not only did it have the latest onset of mortality after inoculation, but also infection progressed more slowly than in other CCCs. This resulted in much reduced final plant mortality at early maturity relative to other cultivars, which would likely reduce the impact of blackleg.

This study was not intended to compare or rank the CCCs for blackleg resistance due to the limited environmental conditions used for testing, but rather to determine *QR* of these common CCCs in western Canada. Nevertheless, variation was observed among the CCCs evaluated; CCC1 appeared to be more resistant than other CCCs in almost each aspect of assessment, including DSI and plant mortality after cotyledon or petiole inoculation.

Pathogen spread was determined by ddPCR, GFP-labelled fluorescent microscopy, and to multiple isolates of *L. maculans*. The highly repeatable results indicated the potential for screening and preliminary selection of more robust *QR* under controlled-environment conditions. Conventional strategies for *QR* identification against blackleg rely heavily on field trials (Ansan-Melayah et al. 1997; Rouxel et al. 2003; Fitt et al. 2006); this approach is laborious, inefficient and sometimes produces unreliable results due to environmental variability (Fitt et al. 2011; McDonald 2010). Large-scale, multi-year field experiments are often carried out for *QR* assessment, which makes the process costly and time-consuming.

Huang et al. (2014) explored qPCR plus GFP fluorescent microscopy to assess *QR* under controlled conditions and identified relative resistance to stem colonization by *L.*

maculans in a *B. napus* line. The current study provides further evidence that supports initial *QR* assessment based on pathogen spread from cotyledons to stems and the progress of disease development in stem tissues under a controlled environment. As shown with the three CCCs, the assessment using ddPCR plus GFP-labelled fluorescent microscopy produced highly repeatable results and the process can be streamlined to efficiently screen a large amount of germplasm. Promising candidates can be evaluated further under field conditions for validation. In western Canada, it is of great benefit to include strong *QR* to blackleg in CCCs because it may complement the specific *R* genes that target the avirulent fractions of pathogen population, which alleviates selection pressure on virulent isolates, thus enhancing the durability of specific *R* genes (Brun et al. 2010). It also helps maintain a high level of blackleg protection when major *R* genes are overcome by the pathogen. Studies of Zhang et al. (2015) and Liban et al. (2016) reported that *Rlm1* and *Rlm3* are no longer effective; however, blackleg is still relatively well managed in western Canada.

The generally higher DSI and plant mortality after petiole inoculation relative to cotyledon inoculation are likely due to the shorter distance that the pathogen has to progress to reach the stem. With almost 100% success in reaching and establishing in stem tissues, the petiole inoculation method appears to be a reasonable protocol for the assessment of stem resistance to blackleg. It can also differentiate canola genotypes by the extent of pathogen spread in cotyledon and petiole tissues. So far, these two aspects of blackleg resistance appear to be the key mechanisms associated with most of canola cultivars in western Canada, where specific *R* genes corresponding to the prevalent *Avr* genes in the current pathogen population (Liban et al. 2016) are generally not found (Zhang et al. 2015). This two-stage assessment may be used for screening *QR* against blackleg in controlled conditions, with the aid of proper enabling techniques such as ddPCR and GFP-labelled fluorescent microscopy. The results of the current study also highlight the potential value of these resistance mechanisms

of *QR* in blackleg management in western Canada; restricting pathogen spread from cotyledon to stem and limiting disease development in stem tissues reduces blackleg incidence and disease severity of canola. These resistance mechanisms of *QR* may be of greater benefit in western Canada where the growing season for canola (90-105 days) is much shorter than in many other parts of the world, including >180 days in Australia (Walton et al. 1999) and >300 days for winter rapeseed in Europe (Walton et al. 1999). Both the resistance mechanisms: hypersensitive response by qualitative resistance and restricting pathogen spread from cotyledon to stem and limiting disease development in stem tissues by quantitative resistance may likely reduce the incidence and severity of blackleg more substantially on shorter-season canola crops.

Use of ddPCR appears to be of great benefit to assess the spread of *L. maculans* inside leaf and petiole tissues before blackleg symptoms became visible. It provides sensitive quantification (Bhat et al. 2009) of blackleg, based on the absolute amount of DNA (Corbisier et al. 2010), which is calibrated against the amount of host DNA. Therefore, it is a robust method for quantitative analysis during *QR* identification. Usually *L. maculans* progresses into the canola stem via the petiole of infected cotyledons (West et al. 2001); therefore it is critical for the pathogen to grow into the petiole rapidly to maximum the chance to reach stem tissues before the cotyledon abscises. The ddPCR data showed that the amount of pathogen DNA was consistently higher in the petioles of Westar cotyledons than in the CCCs; this likely means that the pathogen had moved into the petiole and colonized it more extensively in the susceptible control, than in the CCCs. Consequently, this resulted in higher incidence of stem infection and blackleg severity. On the CCCs, the pathogen took longer to move through the cotyledon and petiole, increasing the risk of failure to reach the stem before cotyledon abscission. The ddPCR technology also indicated substantially smaller amounts of *L. maculans* DNA in stem tissues of CCCs adjacently to the petiole of inoculated

cotyledons at 14 dpi than in the susceptible cultivar. This further suggests a lower rate of successful establishment and/or colonization of the pathogen in stem tissues. Therefore ddPCR should be further explored to its efficiency to screening canola germplasm for *QR*.

4.4. Conclusion

This study characterized *QR* associated with CCCs in two distinct stages of infection process: 1) the spread of the pathogen from an infected cotyledon via the petiole into the stem, and 2) disease development in stem tissues. The ddPCR and GFP-fluorescent microscopy techniques proved to be useful to assess pathogen spread and identify QR. The study suggests that canola germplasm can be screened for *QR* under controlled-environment conditions by evaluating the efficiency of the process and consistency of resistance responses. Many CCCs do not carry *R* genes corresponding to the prevalent *Avr* alleles in the pathogen population in western Canada, but most of these cultivars remain resistant to blackleg due to race-nonspecific resistance. This resistance appears to restrict pathogen spread in cotyledons and petioles, consequently limiting the opportunity for the pathogen to reach the stem before abscission. As a result, the incidence of stem infection may be reduced. Additionally, the CCCs seem to resist infection and colonization of stem tissues by the pathogen, further alleviating the severity of blackleg.

Chapter 5. General discussion and conclusion

5.1. General discussion

The recent increase in the incidence and severity of blackleg of canola in many regions on the Canadian prairies was suggested to be due to shifts in pathogen race structure, as well as shortened crop rotations (Kutcher et al. 2013). In a recent study, Liban et al. (2016) reported a generally low frequency of *Avr* genes *AvrLm1*and *AvrLm3* in the pathogen population on the prairies, and another study, Zhang et al. (2015) reported that most CCCs in Canada carry only the *R* genes *Rlm1* and/or *Rlm3*. It is therefore evident that most CCCs grown in western Canada do not carry specific *R* genes corresponding to the prevalent *Avr* genes in the pathogen population. The current study of the *Avr* profile of *L. maculans* in commercial canola fields also detected low frequency of *AvrLm1* and *AvrLm3* (Soomro et al. 2015), and together these results indicate that the *R* genes *Rlm1* and *Rlm3* are no longer effective against the current pathogen population on the prairies. Despite these finding, disease surveys in all provinces detected only a small percentage of canola fields with serious blackleg damage (Miller et al 2014, Canadian Plant Disease Survey 2012; 2013. Additionally, the characterization of the *L. maculans* race structure in this study did not explain the substantial differences in blackleg incidence among commercial canola fields because the majority of CCCs in Canada carry similar *R* genes, i.e. *Rlm1* and/or *Rlm3* (Zhang et al. 2015). Therefore, the relationship between cultivar resistance and pathogen *Avr*-gene profile appears more complex, and factors beyond the major genes *Rlm1* and *Rlm3* may play a role in the variation in disease incidence and severity observed.

The current study also provides further insights into the pathogen race structure in these targeted commercial fields. Although the overall *Avr*-gene picture is similar to that

described in an earlier study (Liban et al. 2016), with low frequencies of *AvrLm1*, *AvrLm3*, *AvrLm9, AvrLep2* and *AvrLep3*, and high frequencies of *AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7*. Some interesting phenomena were observed in these fields; for example, *AvrLm2* and *AvrLm4* were noticeably lower in Alberta fields than in Saskatchewan or Manitoba fields. These results also indicate that several *R* genes might be used to improve blackleg control on the prairies, including *Rlm2, Rlm4, Rlm6* and *Rlm7*. When compared with the earlier data (Kutcher et al. 2011b), there was a clear decrease in *AvrLm3* in almost all the fields, accompanied by a high frequency of *AvrLm7*, similar to what was observed in France previously (Balesdent et al. 2006), likely due to a masking effect of *AvrLm4-7* over *AvrLm3* (Plissonneau et al. 2016). *AvrLm3* may be recognized more readily by *Rlm3* once *AvrLm7* begins to decrease in the pathogen population were *Rlm7* to be used in Canadian CCCs. Therefore, it is important to continue *Avr* monitoring to deploy effective *R* genes in canola cultivars and provide early warning that a specific *R* gene may be under selection pressure from the pathogen population. If the data can be generated over all regions, it may help guide the regional deployment or rotation of *R* genes corresponding to the prevalent *Avr* genes, as practiced in Australia (Marcroft et al. 2012).

The population of *L. maculans* in western Canada is considered genetically diverse (Dilmaghani et al. 2009) and includes certain *Avr* alleles rarely found in other parts of the world. The diversity is also reflected by the number of pathogen races identified by Liban et al. (2016) who reported 55 races, with races *AvrLm2-4-6-7* (22.7%) and *AvrLm2-4-6-7-S* (22.5%) the most prevalent on the prairies. In contrast, Balesdent et al. (2006), identified only 11 races in France based on characterization of 1,797 *L. maculans* isolates. The current study demonstrated even greater diversity in the pathogen population, with a total of 90 races. Interestingly, the most prevalent races were exactly the same as those identified earlier by Liban et al. (2016), although the frequencies were lower (11.6% and 10.8%, respectively).

The dominance of certain races reflects an uneven pathogen population in western Canada, but also provides important information for the selection of effective *R* genes for blackleg resistance breeding. The high pathogen race diversity also indicates the need for caution when deploying new *R* genes because all known *R* genes can be defeated by at least one of the races identified in the current study. To improve resistance durability, race-specific and race-nonspecific resistance may be combined for optimal blackleg management (Delourme et al. 2006; Brun et al. 2010).

In Canada, most CCCs may carry a level of resistance to blackleg, although the source and nature of resistance is largely unknown (Rimmer 2006). This was consistent with the assessment of eight CCCs from major seed companies; each of the CCCCs carries *Rlm1* and/or *Rlm3*. This result is also generally consistent with that reported by Zhang et al (2015). These results highlight that most Canadian CCCs carry no *R* genes effective against the current pathogen population in which the corresponding *AvrLm1* and *AvrLm3* are at extremely low levels (Liban et al. 2016; Chapter 3). All CCCs used in this study were considered resistant to blackleg based on their field performances, despite the fact they do not carry effective *R* genes to the pathogen population in western Canada. This is likely due to factors beyond *Rlm1* or *Rlm3*, including race-nonspecific or *QR*. The genetics of *QR* are not well understood (Brun et al. 2010) and its identification has traditionally depended on extensive field experimentation (Pilet et al. 1998; Rouxel et al. 2003; Fitt et al. 2006). The current study used *L. maculans* isolates carrying no *Avr* genes corresponding to any of the *R* genes in the CCCs to characterize race-nonspecific resistance. Droplet digital PCR and GFP-fluorescent microscopy provided strong evidence that pathogen spread from infected cotyledons into the stem was restricted in CCCs, and this mechanism appears highly relevant to blackleg resistance because it can potentially reduce stem infection if the cotyledon abscises before the pathogen passes through the petiole and reaches the stem. Droplet digital

PCR may be developed further for robust assessment of *L. maculans* spread inside the canola leaf, petiole and stem for sensitive quantification of infection (Bhat et al. 2009; Corbisier et al. 2010). It may be used to screen for and identify *QR* related to the mechanism of reduced stem infection via infected cotyledons.

Since the modes of action of *QR* may vary depending on the host or infection stage (Delourme et al. 2006), resistance to stem infection should also be assessed to complement screening for pathogen spread in cotyledon and petiole tissues, as described above. The greater blackleg incidence and severity associated with petiole inoculation, relative to that with cotyledon inoculation, demonstrated that petiole inoculation results in high incidence of stem infection and this protocol may be adopted for screening resistance against stem infection and colonization by the pathogen. Petiole inoculation can be easily accomplished in controlled environments and used with quantitative pathogen measurements in stem tissues (Huang et al. 2014) for *QR* identification. Monitoring of plant mortality after petiole inoculation provides an additional measurement for stem resistance based on the onset of plant mortality and final plant survival relatively to the susceptible control. Each CCC delayed the onset of plant mortality and improved plant survival at maturity. These effects will likely reduce the impact of blackleg. Screening for *QR* based on cotyledon and stem infection differentiates the growth stages related to blackleg resistance; resistance may be expressed at one or both stages of infection (Huang et al 2009). Quantitative resistance limits pathogen spread from infected cotyledons into the stem, as well as reduces the infection in stem tissues, which would certainly be ideal since the resistance mechanism is activate throughout the whole infection process. Promising candidates can be evaluated further under field conditions for validation. In western Canada, it is useful to include a strong *QR* background for blackleg resistance because it complements specific *R* gene resistance, which targets avirulent fractions of the pathogen population by alleviating the selection pressure on

virulent isolates, enhancing the durability of specific *R* genes (Brun et al. 2010). It also helps maintain a level of protection when major *R* genes are overcome by the pathogen,

For most of the Canadian CCCs, it appears that the key mechanism of blackleg resistance is through limiting pathogen spread in cotyledon and petiole tissues before the leaf abscises. This mechanism can reduce the incidence of stem infection. Another mechanism is to reduce disease development in stem tissues. These two aspects of resistance may alleviate the impact of blackleg in western Canada where specific *R* genes corresponding to the prevalent *Avr* genes in the current pathogen population (Liban et al. 2016) are generally not carried by current CCCCs (Zhang et al. 2015). The results of the current study highlight the value of these resistance mechanisms for blackleg management in western Canada where the growing season is substantially shorter than in most other parts of the world (Walton et al. 1999).

5.2. General conclusions

This study was initiated to assess whether the high levels of blackleg may be caused by unique *Avr* gene profiles in the pathogen population due to adaptation to resistant canola cultivars. A total of 17 fields were investigated, with disease incidence ranging from <10% to >75% and a total of 90 races were identified. The races *AvrLm2-4-6-7* and *AvrLm2-4-6-7-S* were the most prevalent, accounting for 11.6% and 10.8% of the population. Since most CCCs in western Canada carry only *Rlm1* and/or *Rlm3*, the pathogen is considered virulent because the corresponding *AvrLm*1 and *AvrLm3* were at low levels in most of these fields. Variation in blackleg incidence may be caused by factors beyond the breakdown of *Rlm1* or *Rlm3* in these commercial fields. Many CCCs carry a level of blackleg resistance not controlled by a specific *R* gene. The genetic diversity of the pathogen population found in this study highlights the need for caution when new *R* genes are considered because each known *R* gene can be defeated by at least one of the races identified in the study. Race-specific and

race-nonspecific resistance, in combination with extended crop rotations, may be warranted for sustainable blackleg management in western Canada.

This study characterized the *QR* associated with CCCs in two distinct stages of the infection process: the spread of the pathogen from infected cotyledons into the stem, and disease development in stem tissues. Droplet digital PCR and GFP-fluorescent microscopy proved to be effective in aiding the assessment of pathogen spread and these technologies may be adopted for efficient *QR* screening under controlled-environment conditions. Many Canadian CCCs, although they carry no *R* genes corresponding to the prevalent *Avr* alleles in the pathogen population in western Canada, remain resistant to blackleg in the field due to race-nonspecific resistance mechanisms. This is due to the restriction of pathogen spread in infected cotyledons and petioles, reducing the chance for pathogen to reach the stem before the leaf abscises. The CCCs also suppress disease development of stem tissues after infection, alleviating the severity of blackleg.

5.3. Suggested Future Research

As the pathogen *L. maculans* has great ability to evolve and the current pathogen population has adapted to the *R* genes *Rlm1* and *Rlm3*, the success of controlling blackleg disease in commercial fields of canola lies in continuing regional monitoring of *Avr* alleles in the pathogen population for optimal deployment or rotation of specific *R* genes in CCCs. Since the nonspecific resistance seems to play an important role in many current CCCs, as shown in the current study and severe blackleg sometimes also occurs on these CCCs, it is important to assess environmental impact such as, high temperature, drought and plant injury caused by mechanical or insect damage, on the performance of *QR* in CCCs. This information will help determine additional management practices, including well-timed fungicide or insecticide application to alleviate the additional pressure favoring infection or disease development. .

The current study has highlighted the effectiveness of ddPCR in quantifying the spread of *L.*

maculans infection in canola cotyledons, petioles and stems, which appears correlated with *QR* expression of the CCCs. It may be feasible to develop a ddPCR-based protocol for efficient screening of *QR* in commercial breeding lines to continue improving nonspecific blackleg resistance in CCCs. Since *QR* plays a major role in blackleg resistance under western Canadian conditions, it will be useful to also investigate molecular mechanisms associated with *QR* during cotyledon and stem infection to understand and utilize different modes of action for sustainable blackleg management.

APPENDICES

Appendix 1. 2014 Blackleg Infected Canola Reporting Form

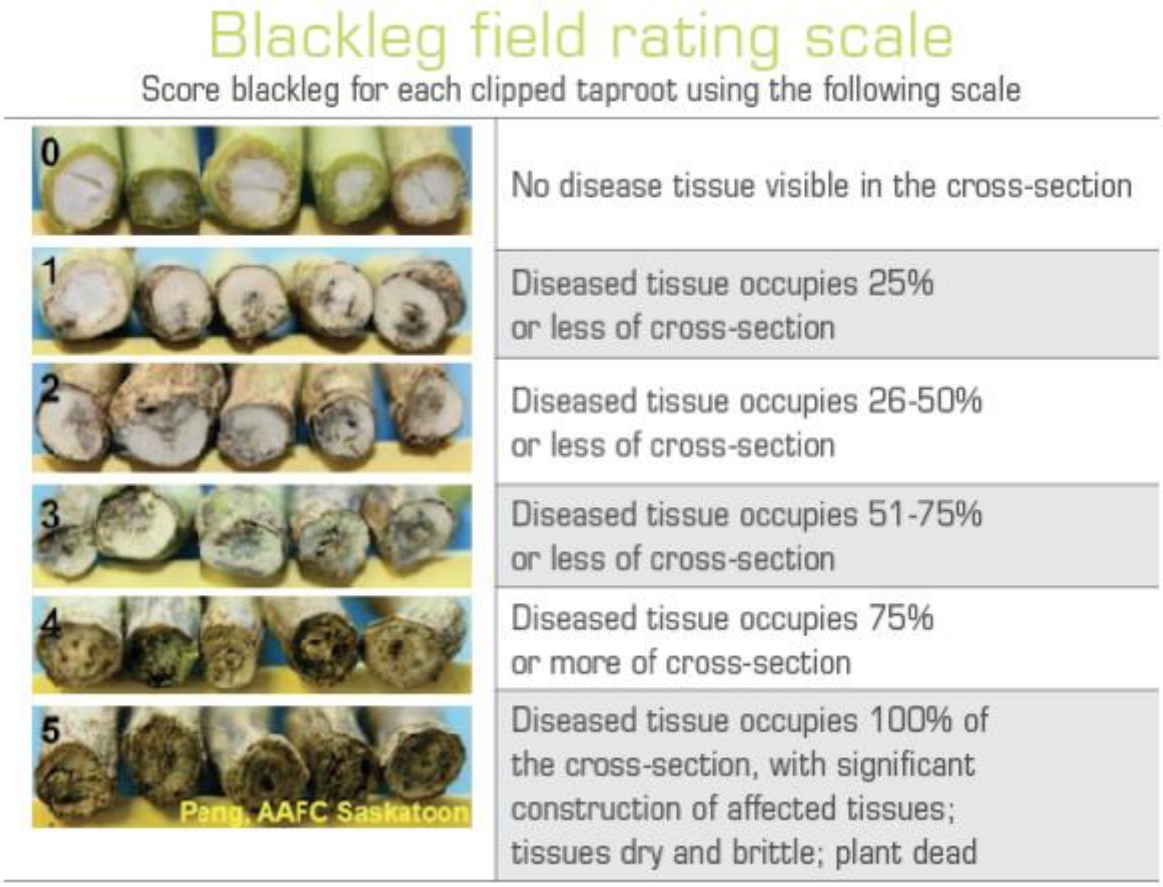
Date:
Evaluator(s) :
Field Location (GPS coordinates and nearest town name): Field Size:
Variety (s):
Crop Stage (before or days after swathing): Hail damage (Y/N)
Evidence of blackleg in 2013 Description of disease (symptoms, field patterns, etc.): Estimated incidence (%): Estimated severity (0-5): Evidence of blackleg included (Y/N)? – i.e. photos of canker or inside blackening of basal stems:

Field history :

Year	Crop	Variety (s)	Blackleg (Y/N)	Tillage type	Residue Management
2012					
2011					
2010					
2009					

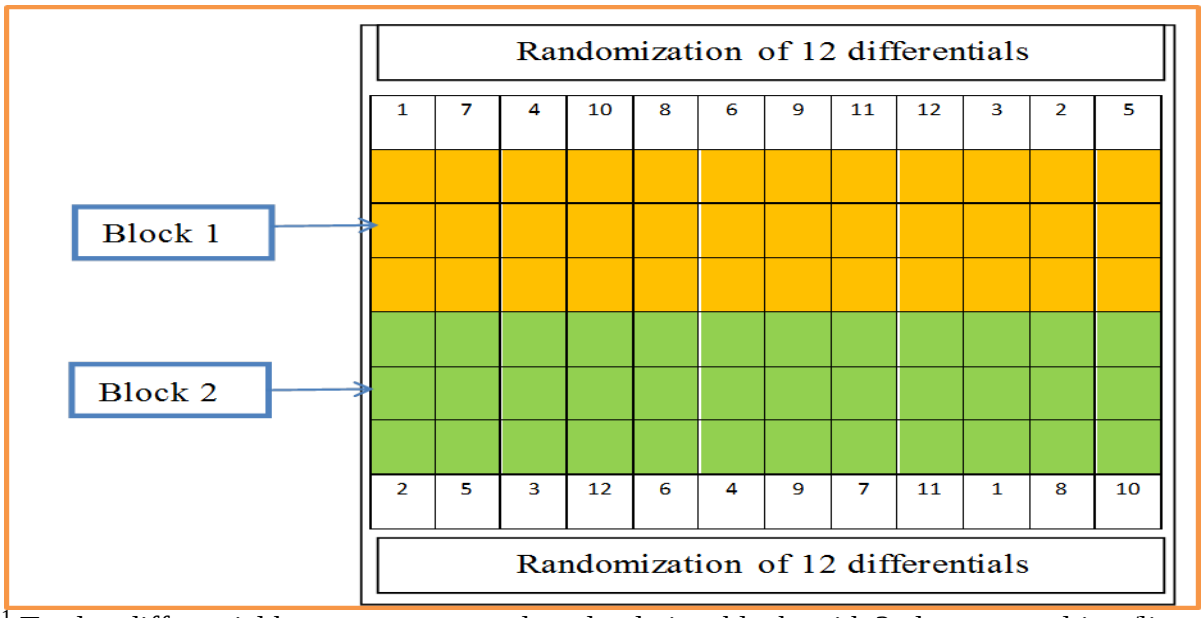
Please forward completed forms and stubble samples to Gary Peng (**gary.peng@agr.gc.ca**) at:
AAFC Saskatoon Research Centre
107 Science Place
Saskatoon, SK S7N 0X2.

Appendix 1. Blackleg rating scale 5.1 and 5.5 (WCC/RRC 2009)



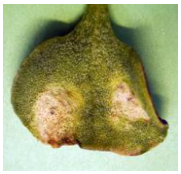
Blackleg lesions that occurred on the upper portions of the stem were assessed separately from basal stem cankers. Stem lesions were recorded as present or absent. Basal stem cankers were scored using a disease severity scale based on area of diseased tissue in the cross-section of the stem where 0 = no diseased tissue visible in the cross section and 5 = diseased tissue occupied 100% of cross section with plant dead (WCC/RRC, 2009).

Appendix 2. The layout of the differential set during planting for *Avr* identification in isolates of *Leptosphaeria maculans*[1]

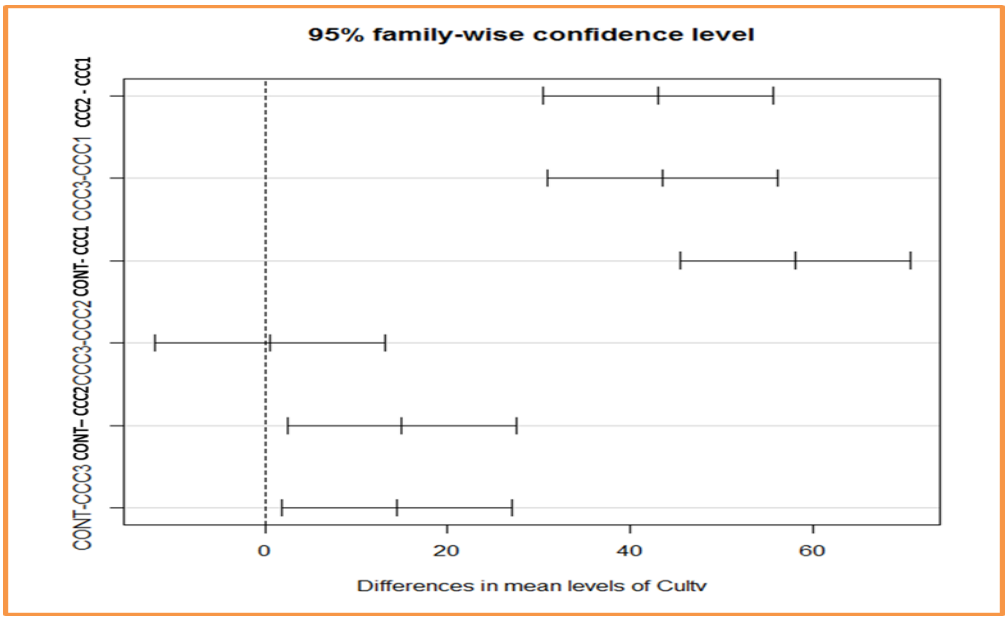


[1] Twelve differential hosts were arranged randomly in a block, with 3 plants per cultivar/line in a tray, and replicated similarly in the Block 2.

Appendix 3. Blackleg Rating Scale (Dolweche 1981)

Disease rating	Disease description	
0	No darkening of tissue around the wound. Typical response of non-inoculated cotyledon.	
1	Limited blackening around wound; lesion diameter 0.5-1.5 mm. Faint chlorotic halo may be present.	
3	Dark necrotic lesion, 1.5-3.0 mm. Chlorotic halos may be present. Sporulation absent.	
5	3.0-6.0 mm lesion may be delimited by darkened necrotic tissue or may show greyish-green tissue collapse. Sporulation absent.	
7	Greyish-green tissue collapse. Lesion 3.0-6.0 mm, with sharply delimited, non-darkened margins, limited number of pycnidia may be present.	
9	Rapid tissue collapse at about 10 days accompanied by presence of many pycnidia in large lesions (>than 5 mm) with diffuse, non-darkened margins.	

Appendix 4. Tukey HSD post hoc test



Tukey's HSD (post hoc) test for multiple comparisons following an ANOVA was performed to determine the significance of variation showed consistency by cultivars in the results of the experiments conducted in greenhouse, by ddPCR and by microscopy. The above chart clearly shows that Westar showed high level of susceptibility when inoculated with the isolate of *Leptosphaeria maculans* that carried *AvrLm6*,(8), whereas CCC1 suffered little blackleg disease despite lacking the corresponding resistance gene. The other two CCCs also lacking corresponding resistance genes and did not show high levels of resistance but performed well in comparison to the Westar. Cultivars CCC2 and CCC3 carried same level of resistance and were different from CCC1, but not from each other.

This test validated the results and indicated that the difference among CCCs was statistically significant. It also indicated that all CCCs were less severely diseased than Westar. This further supported our findings that the growth of the pathogen inside the cotyledon and petiole of these cultivars was slower than in Westar.

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