Blackleg Resistance Stewardship: Improving our management of host resistance

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Executive summary

Leptosphaeria maculans (Desmaz.) Ces. & De Not is a devastating fungal pathogen that cause blackleg of canola (*Brassica napus* L.). This pathogen is prevalent across canola grown regions in Western Canada and is the major limiting factor of canola production. Genetic resistance to blackleg in canola varieties has been widely used in disease control. However, breakdown of host genetic resistance due to population shifts and the emergence of new races of the pathogen is a growing concern in disease control. The gradual increase in both disease incidence and severity of blackleg of canola in Canada in recent years requires updated knowledge in both fungal populations and host resistance. To achieve a better disease management, it's very important to understand the structure of fungal populations in the field and host resistance carried by the canola varieties. The objectives of this study were to identify race structure and frequency of Avirulence genes in the Western Canadian *L. maculans* populations; to characterize resistance genes in Canadian canola varieties/lines; and to understand host defense mechanisms.

For fungal population study, we examined 674 *L. maculans* isolates derived from stubble collected in 2010 and 2011 across the provinces of Alberta, Saskatchewan, Manitoba, and 256 isolates collected from Manitoba in 2012. A total of 930 isolates were examined for 10 avirulence alleles: *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvLlm6*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2*, and *AvrLmS*. In summary results showed that on average the majority of the pathogen population did not carry (therefore virulent on corresponding *Rlm* gene) avirulence genes *AvrLm3, AvrLm9, and AvrLepR2* (<10%), and carried (therefore avirulent) genes *AvrLm 6 and AvrLm7* (>85%). For isolates collected in 2012, the frequency of *Avrm3* was the lowest (1.2%) and it was only detected in three isolates. Among 674 isolates collected in 2010 and 2011, a total of 55 races were detected and the average isolate carried 4.18 avirulence genes.

For characterization of resistance genes, a set of *L. maculans* isolates with known avirulence genotypes were used to characterize the presence of 13 major resistance genes (*Rlm* genes) in 206 Canadian canola varieties/lines, and 104 of these were further evaluated for APR to blackleg under controlled conditions. The results indicated that *Rlm* genes were present in the majority of canola varieties/lines. However, except for the predominant *Rlm3*, the rest of known *Rlm* genes were rarely detected. APR was identified in more than 50% of tested varieties/lines.

The results of this study indicated that the *L. maculans* population in western Canada is diverse and consists of dozens of races, although two are dominant, and the low frequency of a few avirulence alleles including *AvrLm3*. The dominance of *Rlm3* resistance in Canadian canola varieties was observed but this resistance gene was breakdown in Manitoba and many other regions most probably due to the over use of this single resistance gene in disease control.

Introduction

Canola (oilseed rape, *Brassica napus*) is one of the major oil crops grown worldwide. Blackleg, caused by the hemibiotrophic fungal pathogen *Leptosphaeria maculans* is among the major diseases of canola in North America, Australia, Europe and many other regions around the world (Fitt *et al*., 2006). Since the first report of *L. maculans* in Canada (McGee & Petrie 1978), blackleg disease has spread and yield losses peaked at up to 50% in individual fields (Gugel & Petrie, 1992). In recent years, blackleg is ubiquitous across the Prairie Provinces and disease surveys have recorded steadily increasing disease incidence and severity (Canola Council, 2014). The risk posed by blackleg disease lies in the evolutionary potential of the pathogen to rapidly breakdown novel resistance sources through a combination of sexual recombination that increases genetic diversity and the large population's sizes of the asexual pycnidiaspores that can rapidly select for more fit races. The dual sexual and asexual lifecycles in combination with the underlying genetic plasticity of pathogen virulence factors provides the pathogen with an exceptional ability to rapidly overcome resistance sources.

Fungal avirulence genes are commonly studied to better understand the risks posed by different pathogens and to assist in breeding efforts. To date, 16 avirulence genes have been recognized in *L. maculans* based on phenotypic interactions; these are *AvrLm 1, 2, 3, 4, 5 ,6 7, 8, 9, 10, 11, LepR1, LepR2, LepR3, AvrLmS, and AvrLmJ1*. Thus far genetic studies have mapped *AvrLm1, 2, 3, 4, 6, 7, 9, 11, LepR1, and AvrLmJ1*. Most of these are found in two gene clusters, the *AvrLm1*-2-6 cluster (Balesdent et al, 2002) and the *AvrLm 3-4-7-9-AvrLepR1* cluster (Balesdent et al, 2005, Ghanbarnia er al, 2011). In addition to these, six avirulence genes have been cloned; they are *AvrLm1* (Gout et al. 2006), *AvrLm2* (Ghanbarnia et al, unpublished), *AvrLm6* (Fudal et al. 2007), *AvrLm47* (Parlange et al. 2009), *AvrLm11* (Balesdent et al. 2013), and *AvrLmJ1* (Van de Wouw et al. 2013). This rapid adaptation of the pathogen to novel resistance sources indicates the need for extensive knowledge of the pathogen population in

terms of frequency of avirulence genes, race structure, and site to site variation. This knowledge becomes essential in selecting resistance sources that are effective against the majority of the pathogen population, and in developing strategies for maintaining durable resistance.

The disease can be controlled by crop rotation, fungicide application as well as the utilization of resistant varieties (West et al., 2001; Fitt *et al*., 2006). As an environmentally friendly strategy, genetic resistance breeding is generally the most effective disease control methods. Both seedling resistance controlled by major resistance genes (seedling resistance genes; *Rlm* genes) and adult plant resistance mediated by quantitative resistance (minor) genes to *L. maculans* have been identified in canola varieties (Pongam *et al.*, 1998; Balesdent *et al.*, 2001; Pilet *et al.*, 1998, 2001). Major blackleg resistance genes confer race-specific resistance and follow gene-for-gene concept proposed by Flor (1942). To date, at least 18 major resistance genes against *L. maculans* have been identified in *Brassica* species: *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* from *B. napus*, which have been mapped to two *B. napus* linkage groups, N7 and N10 (Ferreria *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* originated from *B. rapa* (Balesdent *et al*., 2002, 2013) ; *Rlm5*, *Rlm6* form *B. juncea* (Chèvre *et al*., 1997; Balesdent *et al*., 2002); *Rlm10* from *B. nigra* (Delourme *et al.*, 2008; Eber *et al*., 2011); *LepR1*, *LepR2*, *LepR3*, *LepR4* in *B. rapa subsp. sylvestris* (Yu *et al.*, 2005, 2007, 2008); and two resistance genes *BLMR1* and *BLMR2* reported by Long *et al.* (2011) and *RlmS* (Van de Wouw et al., 2009). However, only two *Rlm* genes, *Rlm2* and *LepR3* have been cloned by Larkan *et al.* (2013, 2015) so far. In Canada, blackleg resistance breeding programs have been performing successfully in developing resistant varieties for commercial release. However, the basis of blackleg resistance in *B. napus* varieties in Canada is generally unknown (Rimmer, 2006).

Large-scale uses of single resistance source in commercial fields will exert a strong selection pressure on *L. maculans* populations through the co-evolution of host and pathogen. In France, *Rlm1* resistance was overcome within 3 years of commercial release (Rouxel *et al*., 2003). Similarly, the breakdown of 'sylvestris' resistance in Australia was noticed in 2003 (Sprague, *et al.*, 2006; Van de Wouw, *et al.*, 2010). It has been reported that pathogenicity of *L. maculans* populations changed over time in Western Canada. Compared with pathogenicity groups (PGs) of *L. maculans* populations in a collection from 1984 to 2001, Chen and Fernando (2006) found

the presence of more aggressive strains of *L. maculans* in populations collected between 2002 and 2004. Kutcher *et al.* (2010) reported the observation of changes in pathogenicity of populations of *L. maculans* in western Canada, which were believed as a result of the use of specific resistance gene. The association between the specific resistance gene in canola varieties as mentioned by Kutcher *et al.* (2010) and the corresponding avirulence gene in *L. maculans* population can be revealed by investigating *Rlm* genes in canola varieties and avirulence gene frequencies in field fungal populations.

Materials and Methods

Characterization of race structure and frequency of Avirulence genes in the *Leptosphaeria maculans* populations

Canola sample collection. Canola stubble infected with blackleg was collected from unidentified varieties from commercial farmer's fields as part of the provincial disease survey collections. The locations of fields are confidential due to privacy laws. Blackleg infected Canola stubble was also contributed to this study from cooperators and industry disease nurseries. Stubbles collected in 2010 and 2011 were from the provinces of Alberta, Saskatchewan and Manitoba, stubbles collected in 2012 were from Manitoba.

Fungal isolation. Fungal samples were isolates from infected basal stem of Canola plants. The stubble pieces were surface sterilized with 50% bleach for 1 minute and diseased regions from the stem tissue were cut into small pieces and plated onto agar media containing 15g agar, 0.75g calcium carbonate, 800ml distilled water, and 200ml V8 juice (Campbell Soup Company Ltd. Toronto, Ont.). The media was amended with streptomycin sulfate at 0.035 g/liter and plates were grown under light benches in cool white fluorescent light (100-150 E m^{-2} sec^{-1}) at room temperature (22-24℃). Blackleg cultures were selected from other fungal and bacterial growth and re-plated as necessary. Pycnidiaspores were selected from a single pycnidia under a dissecting microscope after 4-6 days of growth. Single pycnidiaspores cultures were left under the light bench for 8-12 days. Both fungal species responsible for blackleg, *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, were recovered from the infected tissue.

Preparation of Fungal inoculum and DNA samples. Pycnidiospores were harvested from agar plates containing single pycnidiaspores using sterile distilled water filtered into 15-ml sterile centrifuge tubes using mira cloth (Fisher Scientific, Pittsburgh, PA). The pyndiaspores were counted using a hemocytometer (Hausser Scientific Company, Horsham, PA) and the spore suspension was diluted to $2x10^{-7}$ spores/ml and stored in sterile microcentrifuge tubes at -20^{o}C until use. The remaining mixture of hyphae, pycnidia, and pycnidiaspores on the agar plates were sampled into sterile 1.5ml microcentrifuge tubes for DNA extraction.

Differential cultivars. A set of *B. napus* and *B. juncea* lines or varieties previously characterized for the resistance genes carried was used in this study as host material for *Leptosphaeria maculans*. The lines used were 'Westar' – no resistance genes (Delourme et al., 2004), 'MT29' – *Rlm1,9* (Delourme et al., 2008), 'Samouraï' – *Rlm2,9* (Rouxel et al., 2003), 'Cooper' – *Rlm1,4* (Balesdent et al., 2002), 'Glacier' – *Rlm2,3* (Balesdent et al., 2002), 'Verona' – *Rlm2,4* (Balesdent et al., 2002), 'Falcon' – *Rlm4*(Rouxel et al., 2003), 'Cutlass' – *Rlm5, Rlm6* (AAFC), '23-2-1' – *Rlm7* (Delourme et al., 2004), 'Darmor' – *Rlm9* (Delourme et al., 2004), 1035 – *LepR1* (AAFC), 1065 – *LepR2* (AAFC), and 'Surpass 400' – *LepR3*, *RlmS* (Van de Wouw et al., 2009; Yu et al., 2008).

L. maculans isolates were inoculated onto this set of differential *Brassica napus* lines to observe the phenotypic reaction and deduce the corresponding avirulence genes carried in the isolates. The presence of multiple resistance genes in the host lines necessitates deduction from multiple phenotypic reactions to determine which resistance gene was responsible to the resistance response. For example, 'Quinta' contains *Rlm3* and *Rlm1* so it cannot be used to determine *Rlm1* if and isolate contains *AvrLm3* or vice versa since the presence of one resistance gene is sufficient to induce resistance and mask the other. Thus isolates which contain multiple avirulence genes are more difficult to deduce and require more differential cultivars; fortunately they are also less frequent. The differential set used does not contain a differential line with *Rlm1* or *Rlm2* only, and thus relies on three lines with two resistance genes each to make a deduction. This limitation results in the differential set of this study being unable to discriminate the presence of absence of *AvrLm1* and *AvrLm2* if an isolate also contains all of *AvrLm3*, *AvrLm4*, and *AvrLm9*. However no isolates were found to contain all three (*AvrLm1*, *AvrLm4*, & *AvrLm9*), and this limitation did not affect the results. 'Cutlass' is the only cultivar with *Rlm5* and *Rlm6*,

thus the two could not be separated and *Rlm5* results are not presented. *Rlm6* was also determined by PCR amplification of the cloned fungal gene using markers, see Table 2. The final limitation of the differential set is in the characterization of *AvrLmS*. The cultivar 'Surpass400' contains both *LepR3* (corresponds to *AvrLm1* in the pathogen) and *RlmS* (corresponds to *AvrLmS*). *LepR3* could not be characterized since there is no other differential cultivar with *RlmS*. *AvrLmS* could be characterized, but only in isolates without *AvrLm1* since *LepR3* interacts with *AvrLm1* (Larkan et al, 2012).

Pathogenicity tests. Canola seeds were seeded into 96 seedling flats using Pro-Mix BX w/ Mycorrhizae (Premier Tech, Rivière-du-Loup, Québec). Flats were watered daily and maintained in growth chambers at 22^{o}C day, 14^{o}C night with 16h light: 8 hour dark photoperiod. After 6 to 7 days of growth, 4 seedlings of each differential line was inoculated with blackleg isolates to be tested. Each lobe of the cotyledons of canola seedlings were wounded using specialized forceps with one arm pointed. A 10 µL droplet of inoculum was pippetted onto each of the two wounds on each cotyledon (four wounds per plant). Flats were left to dry completely before returning to growth chambers. The day following inoculation, the flats were fertilized using 20:20:20 (N:P:K). Emerging true leaves were removed from seedlings to maintain cotyledons. The wounded cotyledons were evaluated for the interaction phenotype (IP) 12-14 days after inoculation on a rating scale of 0 to 9 according to lesion size, chlorosis or necrosis, and presence of pycnidia (Kutcher et al, 2007). The mean score of the 16 lesions between each *L. maculans* isolate- *B. napus* differential line was used to determine if a blackleg isolate was avirulent (IP 0 to 4.9) or virulent (IP 5 to 9) to the differential line. The results were used to determine the avirulence genes carried by each isolate and required minor deduction in lines with more than a single resistance gene.

DNA Extraction, PCR, and sequencing. Extraction of DNA from pure fungal isolates was performed from a mixture of pycnidia, pycnidiaspores, and hyphae harvested from 4-6 day old single pycnidiaspores cultures. DNA was extracted using a modified version of the procedure developed by Lee & Taylor (1990). The samples were mixed with a lysis buffer (containing Tris, EDTA, SDS and NaCl), lysed with mechanical beads at 5000rpm for 30 seconds, incubated at 65°C for 30 minutes, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and

precipitated with 5M NaCl. Following the final centrifugation step, the DNA pellet was dissolved in 100 µL of autoclaved distilled water.

Extracted DNA was identified as either *Leptosphaeria maculans* or *Leptosphaeria biglobosa* using multiplex primers provided by the CFIA (the primer is published and public available) (Liu et al., 2006). *L. maculans* isolates were further tested with primers for detecting the presence/absence of 3 avirulence genes, *AvrLm1, AvrLm6, AvrLm47*. The PCR product for *AvrLm47* was digested with the *HaeIII* enzyme (GG^CC) to detect the SNP mutation of C^{358} to G^{358} that leads to virulence against Rlm4. Virulent isolates produced an additional band after digestion with *HaeIII*. PCR was performed on the Biorad T100^{TM} thermal Cycler with the following cycles: 1 rep of 3 mins at 95℃, 30 reps of 45sec at 95℃, 30 secs at 61℃, 1min at 72℃, 1 rep of 5mins at 72℃.

The PCR products of the three cloned avirulence genes genotyped in this study were sent for sequencing to Macrogen Inc (Seoul, Korea). The PCR products of between 28 to 33 isolates were submitted for single pass sequencing for each of *AvrLm1, AvrLm6,* and *AvrLm47*. The sequences were aligned with the ClustalW (Higgins et al, 1994) algorithm conducted within MEGA version 6 (Tamura, Stecher, Peterson, Filipski, and Kumar 2013). Pairwise alignment in ClustalW was set with a gap penalty of 15 and gap extension penalty of 6.66. After alignment, data was exported to BioEdit software where conserved regions were identified with a minimum 15 residue length, a gap limit of 2 per segment. BioEdit was used to provide nucleotide positional summary and identify SNPs.

Characterization of *Rlm* genes in *Brassica napus* varieties/lines

Characterization of *Rlm* genes. A total of 206 canola varieties/lines were collected for *Rlm* gene characterization, and two trials were performed. Trial I was carried out on 104 Canadian canola varieties or advanced breeding lines which were kindly provided by several companies and research institutions. A set of 22 (D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, D13, D14, S7, ICBN14, PHW1223, R2, AD746, JN2, JN3, J3, J20, Q12; **Table 1**) differential isolates which are able to detect 13 known major blackleg resistance genes (*Rlm* gene) (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, RlmS, LepR1, LepR2*) were used to characterize *Rlm* genes in 104 canola varieties/lines. Three canola varieties/lines (DF78, DF79, DF80) were

resistant to 21 differential isolates and it's very difficult to postulate *Rlm* genes they carried. Thus, twelve more (L-MD7-14, L-PC4-1, L-MP1-8, L-Sb1, L-MP1-6, L-Sb7-6, L-Br17-1, L-Mo5-1, L-Br1-16, RL15, DS103, CV8-7; **Table 1**) *L. maculans* isolates were used to further detect *Rlm* genes in these three canola varieties/lines. Trial II was performed with 102 canola seed samples (considered as canola lines in this paper) collected from different farmers' fields in Manitoba (2012). Each seed sample can be used to predict *Rlm* genes of canola variety grown in that specific field. For this trial, a set of 11 (D3, D4, D5, D7, D10, AD746, JN3, J3, ICBN14, PHW1223, R2; **Table 1**) *L. maculans* isolates were used for characterization of *Rlm* genes.

In both trials, methods for inoculums/plants preparation, inoculation, rating, and so on were the same as described in avirulence gene characterization. However, at least 12 different plants were used for each isolate-variety/line combination. Due to the genetic heterogeneity of seed samples collected from the field and some canola varieties/lines, the percentage of resistance reactions (rating scores 0, 1, 3) was calculated from inoculation sites. When the percentage of resistance reactions was over 50% but less than 100%, genetic heterogeneity was considered as the major cause of the variation and the cultivar was considered resistant. *Rlm* genes were postulated based on gene for gene theory. For example, if a cultivar was resistant to all differential isolates that carrying *AvrLm3* but susceptible to isolates carrying *AvrLm3*, the deduced *Rlm* gene in this cultivar is *Rlm3*.

Adult plant resistance evaluation. Adult plant resistance of 104 Canadian canola varieties/lines provided by different companies and research institutions were preliminary evaluated under controlled conditions, Westar was used as susceptible control. Among all differential *L. maculans* isolates, D3 can infect 101 accessions and damage the whole inoculated cotyledons as early as 12 dpi. Three accessions, DF78, DF79, DF80 were resistant to D3 but susceptible to D13. To reduce the interference of seedling resistance during adult plant resistance evaluation, D13 was used to inoculate DF78, DF79, DF80, and D3 was used to inoculate the rest 101 accessions. For the evaluation, materials were seeded into plastic pots (18 cm in diameter), and inoculated with a single *L. maculans* isolate. The inoculation methods and spore concentration were the same as *Rlm* gene identification. Seedling infection was observed on 14 days after inoculation and plants without success infection were removed. For the experiment design, three replicates were carried out in a completely randomized design, each replicate with 9

plants. Disease severity was scored for basal internal infection with the rating scale of 0-5, adapted from WCC/RRC protocol, where 0 - no noticeable infection, 1 - diseased tissue occupies ≤ 25% of the cross-section, 2 - diseased tissue occupies 25-50% of cross-section, 3 - between 50-75% of the cross-section infected, 4 - more than 75% of the cross-section infected, 5 - 100% of cross-section were diseased, plant dead.

The blackleg resistance category system used is based on relative disease severity: the percentage of the mean disease severity of a tested canola line compared to the susceptible variety Westar. Relative disease severity scores of ≤35% were considered as resistant genotypes (R), scores of =35-50% were assigned as moderately resistant (MR) genotype, scores of =51-65% were considered as moderately susceptible (MS), and relative disease severity of =66-100% were assigned as susceptible (S).

Results

Frequency of *L. maculans* Avirulence genes and race structure in Western Canada. Among 674 isolates collected in 2010 and 2011, the presence or absence of avirulence gene *AvrLmS* was only confirmed in 582 isolates due to the presence of *AvrLm1* in 92 isolates, and the lack of differential lines aside from 'Surpass400' which is resistant to isolates with *AvrLm1*. The other nine avirulence genes were determined for all 674 isolates. The frequency of avirulence genes ranged from as low as 0.15% in *AvrLepR2* to 90.22% in *AvrLm7*. Only a single isolate was detected with *AvrLepR2*. Four other avirulence genes were detected at low frequencies; *AvrLm1* – 13.48%, *AvrLm3* – 8.15%, *AvrLm9* – 1.48%, and *AvrLepR1* – 16%. Along with *AvrLm7*, four other avirulence genes were detected in more than half the population: *AvrLm2* – 80.44%, *AvrLm5* – 71.85%, *AvrLm6* – 89.19%, and *AvrLmS* – 54.87%. **Figure 1** contains the number of virulent and avirulent isolates for the ten examined avirulence genes.

Each combination of avirulence genes is unique race of *L. maculans* following the nomenclature of Balesdent et al (2005). In this study a total of 55 races were found ranging from as high as 22.70% to as low as 0.15%, a single isolate. There were two main races at 22.70% and 22.55% which together account for almost half the population. These two races are followed by a drastic decline to 5.93% for the third most common race. **Figure 2** illustrates the relative frequency of blackleg races in Western Canada. The two main races differ only in the presence

or absence of *AvrLmS* and both contain *AvrLm 2, 4, 6, 7.* The other races are more varied and decline in frequency from 5.93% (40 isolates) to 15 races represented by a single isolate.

When we evaluate the pathogen population in terms of race complexity, number of avirulence genes, we observe that the majority of isolates carry 4 or more avirulence genes with an average of 4.18. There are 299 isolates with 4 avirulence genes followed by 222 isolates with 5 avirulence genes, 102 isolates with 3 avirulence genes, 25 isolates with 6 avirulence genes, 24 isolates with 2 avirulence genes, 1 isolates with 7 avirulence genes, and a single isolate with one avirulence gene. The *L. maculans* population is very genetically diverse as measured using Simpson's index of diversity (Simpson, 1949). The Simpson index of diversity is calculated at 0.89 and weighs the number of races relative to the total number of samples. An index of 1 is a perfectly diverse or random population whereas an index of 0 would represent a single race. The Simpson index of evenness (Simpson, 1949) measures the relative abundance of different races and was calculated at 0.71 for this population indicating a somewhat even population. The two dominant races significantly reduce the evenness score for this population. When we examine provincial differences we can see that Manitoba has both the most diverse and most even population in terms of *L. maculans* races with 0.91 and 0.68 on Simpson's index of diversity and Evenness respectively. Alberta scored 0.87 and 0.61 in terms of diversity and evenness respectively. Saskatchewan had the least diverse and least even pathogen population with 0.77 and 0.39 respectively. In terms of year, the diversity did not change significantly and was calculated at 0.868 in 2010 and at 0.826 in 2011. The Simpsons evenness index dropped from 0.62 in 2010 to 0.50 in 2011. **Table 2** for values of the Simpson's diversity and Even Indices.

The pathogen population is not evenly distributed in western Canada in terms of avirulence genes. On the provincial scale the differences range from as great as a 31.8% decline in *AvrLm2* moving from Saskatchewan to Manitoba, to less than one percent variation in *AvrLepR2* between provinces (**Figure 3**). *AvrLm1, Avlm2, AvrLepR1,* and *AvrLmS* varied by greater than 20% between provinces while *AvrLm3, Avlm7, AvrLm9,* and *AvrLepR2* varied less than 10% between provinces. *AvrLm4* and *AvrLm6* were in between at 16% and 14% provincial variation respectively. On average, avirulence gene frequency varied by 16.1% between provinces.

The frequency of avirulence genes showed significant change in some avirulence gene loci between 2010 and 2011 (**Figure 4**). The frequency shift ranged from a 0.3% increase in *AvrLepR2* to a 12.4% increase in *AvrLepR1*. There were both increases and declines in avirulence genes; *AvrLepR1* had a 12.4% gain while *AvrLm2* declined by 6.7%. Avirulence genes *AvrLm3*, *AvrLm7*, *AvrLm9*, and *AvrLepR2* shifted less than 5% between 2010 and 2011 while avirulence genes *AvrLm6* and *AvrLepR1* shifted greater than 10% within a one year span. *AvrLm2*, *AvrLm4*, and *AvrLmS* ranged in between at a 6.7% decline, 7.4% increase, and a 7.2% increase respectively. On average the frequency of avirulence genes increased by 3.4% from 2010 to 2011. The increase of pathogen complexity and increase in the average avirulence gene frequency from 2010 to 2011 are indicators of less virulence in 2011.

Frequency of *L. maculans* Avirulence genes and race structure in 2012 isolates. The frequency of avirulence alleles corresponding to ten resistance genes, *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm6*, *Rlm7*, *Rlm9*, *RlmS*, *LepR1* and *LepR2* were identified (**Figure 5**). The presence/absence of *AvrLmS* was not determined in 63 isolates due to the presence of *AvLm1* in these isolates. Among all 10 avirulence genes, the frequency of *Avrm3* was the lowest (1.2%) and it was only detected in three isolates. The avirulence genes *AvrLm9* and *AvrLpR2* were detected in 3.9% and 12.1% of tested isolates, respectively. A few avirulence genes were detected in higher frequency, ranging from 25.8% (*AvrLm1*), 35.9% (*AvrLepR1*), 64.1% (*AvrLm2*), to 70.7% (*AvrLm6*). The frequency of *AvrLmS* accounted for 40.4% of 193 isolates. The proportion of avirulence genes *AvrLm4* and *AvrLm7* were ranked highest of all tested isolates, which accounted for 78.5% and 87.9%, respectively.

A total of 75 races were identified, each race represented by 1 to 26 isolates. Most of the isolates belonged to 20 major races (more than 4 isolates in each race), while only 1 to 3 isolates belonged to each race in the remaining 55 races. The top two races were Av2-4-6-7 and Av1-4-6-7-(S), which accounted for 19.2% of all tested isolates. The above results indicated that *L. maculans* populations in the field is highly diversified.

***L. maculans AvrLm1, AvrLm6,* and *AvrLm4-7* genetic variation.** A total of 96 samples were amplified and sequenced at one of three avirulence gene loci: *AvrLm1*, *AvrLm6*, and *AvrLm4-7*. The alignment of the three avirulence genes resulted in significant differences in

terms of the number of SNPs and alleles while the size of the conserved region relative to the PCR product was relatively consistent between the three avirulence genes.

The *AvrLm1* gene of 33 western Canadian *L. maculans* isolates was aligned to the reference French *L. maculans* isolate JN3 and resulted in a single conserved region of 438bp. A total of 58 SNPs was detected among the 34 alignments comprising 13.24% of the conserved region. The 58 SNPs arose from 3 alleles. 31 isolates shared a common allele and 2 isolates from Manitoba 2010 samples had unique haplotypes with many nucleotide substitutions (**Table 3**).

Alignment of the *AvrLm6* gene among 35 Canadian *L. maculans* isolates with the reference genome resulted in a single conserved region of 239bp with a total of 11 SNPs. *AvrLm6* had the least number of SNPs at 4.6% of the conserved region. 11 SNPs resulted in 4 alleles. 32 isolates shared a common allele and 2 isolates from Saskatchewan 2011 exhibited a few unique SNPs. One of the Saskatchewan 2011 isolates shared a SNP with the reference French isolate JN3 that was absent from the other 34 Canadian isolates.

Similar alignment of *AvrLm4-7* from 28 Canadian isolates with the reference genome resulted in 9 conserved regions separated by deletions covering a combined 562bp region. The alignment of *AvrLm4-7* conversely resulted in the greatest number and frequency of SNPs relative to the conserved regions at 21%. 9 alleles resulted in 110 SNPs. 15 isolates shared the most common allele, followed by 5 isolates and 2 isolates for the next two most common alleles. The remaining 6 *AvrLm4-7* alleles were represented by a single isolate each.

Predominance of *Rlm3* in Canadian canola varieties/lines. In experiment I, 85% (88) of total tested accessions showed seedling resistance. A total of eight known *Rlm* genes (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm9, RlmS, LepR1, LepR2*) and unknown *Rlm* gene resistance were identified as present in tested materials. (**Figure 6, Table 4**). However, 16 accessions were susceptible to all *L. maculans* differential isolates and no *Rlm* gene was detected. Unknown *Rlm* gene resistance described in this paper indicated that an accession was resistant to some isolates, but based on the gene for gene theory, none of the 13 *Rlm* genes can be postulated. The presence of unknown *Rlm* gene resistance might be due to the effect of a novel *Rlm* gene, other known *Rlm* genes which were not tested in this study, or a combination of a few *Rlm* genes. Among all detected *Rlm* genes, *Rlm3* was present in 59 accessions, followed by *Rlm1* in 5 accessions and

Rlm2 in 5 accessions. *Rlm9*, *LepR1* were detected in 3 accessions, respectively, *Rlm4*, *RlmS* were present in 2 accessions, respectively, whereas *LepR2* was deduced as present in one accession. In addition, other *Rlm* genes such as *Rlm5*, *Rlm8*, *Rlm10* might be present in some accessions but further confirmation is required.

According to the combination of *Rlm* genes, all tested canola accessions were divided into five groups. A total of 37 accessions carrying single *Rlm* gene resistance (*Rlm1* in one accession, *Rlm3* in 34 accessions, *Rlm4* in one accessions, *LepR2* in one accession) were assigned in group I. Group II included 17 accessions that carried two resistance genes, or a combination of a known resistance gene and unknown resistance (*Rlm3 & LepR2, Rlm3 &* unknown *Rlm* gene resistance). Eight *B. napus* accessions carrying three or more *Rlm* genes were assigned to group III. A total of 26 *B. napus* accessions in group IV carried unknown *Rlm* gene resistance only. Group V contained 16 accessions that were susceptible to all 22 *L. maculans* differential isolates.

In experiment II, seedling resistance was present in 58% of tested seed samples (considered as lines), but only three known *Rlm* genes (*Rlm1, Rlm2, Rlm3*) were detected. Among all 102 tested lines, 50 of them carried *Rlm3*, 3 lines contained *Rlm2*, and 2 lines carried *Rlm1*, Unknown *Rlm* gene resistance was identified as present in 9 lines. Surprisingly, a total of 43 seed samples carried none of the 13 *Rlm* genes that can be detected in this study.

According to the above results, *Rlm3* is the major *Rlm* gene that was widely present in Canadian canola varieties and lines. On the other hand, the absence of the corresponding avirulence gene, *AvrLm3* in field fungal populations in this study indicated the breakdown of *Rlm3* resistance.

Adult plant resistance evaluation under controlled environment. The resistance observed in APR evaluation was assumed to be mediated by adult plant resistance genes only since none of the tested canola varieties/lines materials showed seedling resistance to isolates used. Disease severity of tested materials ranged from 0 to 4.8. Significant difference of relative disease severity among tested canola varieties/lines was observed ($p<0.001$). APR evaluation indicated that 56% of tested accessions showed either resistance or moderate resistance against blackleg, while the remaining 46 accessions were susceptible or moderately susceptible at adult plant stage (**Table 4**). Among 58 accessions that showed APR, 51 of them also had *Rlm* gene

resistance at seedling stage, and seven showed adult plant resistance only. Of 46 susceptible or moderately susceptible accessions, 7 of them were susceptible to all differential isolates at seedling stage. To sum up, a large proportion of canola varieties/lines showed both adult plant resistance and *Rlm* gene resistance (mainly *Rlm3* resistance). Although single source of major gene resistance (*Rlm3*) will exert high selection pressure on fungal population, the presence of adult plant resistance in *Rlm3* canola varieties might partially retard the process of resistance breakdown.

Discussion

The frequency of ten *Avr* genes in 674 *L. maculans* isolates collected from Western Canada (2010 and 2011) indicated a total of 55 races, and among them, two races appeared clearly dominant accounting for almost half of the population and 15 races were represented by only a single isolate each.. A total of 75 races were found in 256 *L. maculans* isolates collected in Manitoba in 2012. An earlier study based on up to 96 isolates found 16 races of *L. maculans* in western Canada (Kutcher *et al*, 2010). This difference suggests that sample size plays a significant role in detecting the presence of low-frequency races of the pathogen.

The frequency of *AvrLm6* was determined primarily based on the presence/absence of the PCR gene product. Although the differential cultivar 'Cutlass' was also used, the presence of both *Rlm5* and *Rlm6* in our seed lot and lack of another host with either *Rlm5* or *Rlm6* alone meant that a resistant phenotype may be due to the presence of either *AvrLm5*, *AvrLm6*, or both in the isolate. Kutcher *et al* (2007) reported the frequency of *AvrLm5* to be relatively low at 10.4% in western Canadian isolates collected from 1997-2005. While only deletions have been observed to lead to virulence at the *AvrLm1* locus, SNP mutations have been observed to cause virulence at the *AvrLm6* locus in several Australian isolates at a frequency of 2.5% (Van de wouw, 2010). Thus the frequency of "true" *AvrLm6* in western Canada may be slightly lower than the reported value if SNP mutations are taken into consideration. *AvrLmS* was determined through phenotypic interaction with 'Surpass400' which contains *RlmS* that interacts with *AvrLmS* and *LepR3* which provides resistance against isolates carrying *AvrLep3* or *AvrLm1* (Larkan *et al*, 2013). Due to the lack of another differential *carrying only RlmS,* a total of 92 *L.*

maculans isolates with *AvrLm1* could not be characterized for *AvrLmS* with the current differential set.

Most *L. maculans Avr* genes reside in two major gene clusters, the first of which is the *AvrLm1*-2-6 cluster (Balesdent *et al*, 2002). This study found that the frequency of *AvrLm1*, *AvrLm2* and *AvrLm6* to be 13.7%, 80.6% and 89.3%, respectively. The decrease in *AvrLm1* has been significant in western Canada, falling from 46% in 1997-2005 samples (Kutcher *et al*, 2010) to 12.6% in 2005-2006 samples (Dilmaghani *et al*, 2009), and to 13.7% in the 2010-2011 isolates of this study. *AvrLm2* declined moderately from 96.6% in 1997-2005 samples (Kutcher *et al*, 2010), to 87.02% in 2005-2006 samples (Dilmaghani *et al*, 2009), and to 80.56% in the 2010-2011 isolates of this study. In 2012 isolates, the frequency of *AvrLm2* was only 64.1%. *AvrLm6* declined only slightly from 100% in 1997-2005 samples (Kutcher *et al*, 2010), to 94.8% in 2005-2006 samples (Dilmaghani *et al*, 2009), to 89.3% in the 2010-2011 isolates of this study, and to 70.7% in 2012 isolates.

The decline in *AvrLm1* in western Canada is most significant in the *AvrLm1-2-6* cluster and the proximity of *AvrLm2* and *AvrLm6* may have led to moderate to slight increases of virulence at those loci. In this study, the alignment of 33 *L. maculans* isolates at the *AvrLm1* locus identified 58 SNPs in the conserved region compared with 11 SNPs in a similar alignment of 28 isolates at the *AvrLm6* locus (**Table 2**). This indicates that *AvrLm1* is under increased pressure from RIP mutations which target repetitive DNA as a defense in ascomycota fungi against transposable elements (Rouxel *et al.*, 2011). The linkage of *AvrLm1-2-6* presents an interesting trend in terms of race structure; 90% of *L. maculans* isolates in Western Canada that carried *AvrLm1* lacked *AvrLm2* and vice versa. Only 3.4% of isolates carried both *Avr* genes, while 6.5% lacked both *AvrLm1* and *AvrLm2*. The low frequency of *AvrLm1* at 13.7% in Western Canada may have contributed to the relatively high frequency of *AvrLm2* (87.0%). This low rate of recombination supports the observation of Ghanbarnia *et al.* (2009) that asexual pycnidiaspores are the primary source of inoculum in Western Canada. *AvrLm4-7* is a single *Avr* gene that interacts with both *Rlm4* and *Rlm7*. Virulence to *Rlm4* is gained through a SNP mutation while virulence to *Rlm7* is gained through a deletion of *AvrLm4-7* (Parlange *et al*, 2009). Our study did not find any isolates that were avirulent on *Rlm4* but virulent on *Rlm7*, while the reverse was relatively common. This supports the position by Parlange *et al*. (2009) that a deletion of

AvrLm4-7 is causes virulence at *Rlm7* but a SNP mutation can cause virulence at *Rlm4*, and this is consistent with this study. The alignment of 28 western Canadian *L. maculans* isolates at the *AvrLm4-7* locus led to 118 SNPs in the conserved region and 8 alleles among the isolates (**Table 3**). Similar alignments lead to 58 SNPs and 3 haplotypes for *AvrLm1* and 11 SNPs and 4 haplotypes for *AvrLm6*. This demonstrates that the *AvrLm4-7* gene is subject the greatest amount of genetic variation among the 3 *Avr* genes sequenced, and yet this cannot be attributed to selection pressure since no *Rlm4* or *Rlm7* cultivars have been identified in Canadian commercial cultivars. *Rlm4* is the only resistance gene overcome primarily through a SNP mutation and the increased frequency of SNPs in the *AvrLm4-7 Avr* gene, in comparison to *AvrLm1* and *AvrLm6,* may explain this observation. The frequency of *AvrLm3* dropped substantially in Western Canada from 52.8% in 2005-2006 samples (Dilmaghani *et al*, 2009) to 13.7% in the 2010-2011 isolates of this study. Selection pressure is likely the most significant factor since the most common resistance gene in Canadian cultivars is *Rlm3.*

Differences in *Avr* gene frequency were observed across the Prairie Provinces. Except for *AvrLm3*, *AvrLm9* and *AvrLepR2,* which were consistently low in each province, most of the other *Avr* genes varied slightly in frequency. *AvrLm2* had the greatest variation in frequency among provinces, at 97% in Saskatchewan and 65% in Manitoba, decreasing by 32% from 84% in Alberta to 65% in Manitoba. The second largest difference was in *AvrLepR1*; at 34% in Alberta but merely 6% in Saskatchewan. The frequency of *AvrLm1* and *AvrLm2* showed the greatest variation from 0 to 63 % and 22% to 100%, respectively. *AvrLm3* was very low or absent at most sites with the exception of Melfort. *AvrLm7*, *AvrLm9* and *AvrLepR2* varied little among the five sites. Greater differences were seen generally between sites in different provinces, although there were exceptions. *AvrLepR1*, for example, was at 58% in Snowflake, Manitoba but only 11% at Roland, Manitoba, which are separated by ~100 km. These differences may be caused by localized selection pressure and it would be interesting to pair pathogen race structure with the resistance genes in canola varieties grown in these fields. The regional and provincial variation demonstrates that a single specific resistance gene would not provide effective control of blackleg at all sites across the Prairie Provinces except *Rlm6* and *Rlm7*, which consistently occurred at high frequencies in each region.

This is the first report on characterization of *Rlm* genes in a collection of Canadian canola cultivars/lines. A total of eight known *Rlm* genes were detected in 104 tested canola varieties/lines and *Rlm3* was the predominent one. Although a lot of Canadian canola varieties/lines carried *Rlm3*, the deficiency of the corresponding *AvrLm3* in *L. maculans* populations indicated the breakdown of *Rlm3* resistance. High frequency of *Rlm3* in Canadian canola varieties/lines might be due to the use of single source of resistance in breeding programs since the first report of this disease in Canada in the 1970s (Gugel, *et al.*, 1992). When pathogenicity groups (PGs) were used to describe *L. maculans* populations, the predominant pathogenicity group in Western Canada was PG2 (Kutcher, *et al*., 2007, 2010; Chen, *et al*., 2006). It can be hypothesized that canola blackleg resistance breeding against PG2 (virulent on *B. napus* cv. Westar, avirulent on Glacier and Quinta) isolates was very popular in Canada for a period of time. Corresponding resistance genes in differential varieties to PG2 isolates, are *Rlm2*, *Rlm3* (Balesdent *et al*., 2002) in Glacier and *Rlm1*, *Rlm3* (Kutcher *et al*., 2010) in Quinta. Due to the presence of *Rlm3* in both Glacier and Quinta, the chance of introducing *Rlm3* into canola varieties is very high. The findings from this study that *Rlm1*, *Rlm2* and *Rlm3* were the top three *Rlm* genes present in canola varieties/lines were in agreement with the hypothesis.

The breakdown of *Rlm3* resistance in Western Canada demonstrated high evolutionary potential of *L. maculans* populations. In Western Canada, *L. maculans* isolates belonging to PG2 were dominant for at least 20 years between 1984 and 2004 (Chen and Fernando, 2006). More specifically, during 1984 and 2001, only PG1 (*Leptosphaeria biglobosa*) and PG2 isolates were observed, but other PG groups (PG3, PG4, PGT) were identified during 2002-2004 (Chen and Fernando, 2006). The frequency of *AvrLm3* in *L. maculans* isolates collected between 1997 and 2005 in Western Canada was 17.7%, much lower than other avirulence alleles (Kutcher *et al*., 2010). In 2010 and 2011, the majority of *L. maculans* isolates collected in Western Canada did not carry avirulence gene *AvrLm3* (less than 10%). In 2012, the frequency of *AvrLm3* in *L. maculans* isolates collected in Manitba was only 1.2% (this study). On the other hand, in 2002, blackleg resistant cultivar Q2 (*Rlm3* resistance) was severely infected in Roland, Manitoba (Chen and Fernando, 2006). In addition, disease incidence and severity of blackleg on canola kept increasing in recent years in Western Canada. In 2012, blackleg was present in almost all 114 farmers' fields sampled in Manitoba, with an average disease severity rating of 2.8 (0-5 scale) based on percentage of disease on cross-section of stubbles. The frequency of *AvrLm3*

declined from 17.7% in more than seven years ago to 8.7% in 2010, 2011, and 1.2% in 2012 indicated the shift of this avirulence gene mainly due to the repeated use of a single *Rlm* gene resistance, *Rlm3* (Kutcher *et al*., 2010; Liban *et al*., 2014, unpublished data). Although the frequency of *AvrLm3* is very low in the field fungal populations, the less use of *Rlm3* resistance varieties may result in the increase of *AvrLm3* frequency in the field.

Marcroft *et al*. (2012b) proved that rotation of resistance genes can minimize disease pressure by manipulating fungal populations. However, rotation of resistance genes in blackleg control in Canada is very challenging at present due to only few limited varieties carried other *Rlm* genes other than *Rlm3*. Although unknown resistance was detected at seedling stage in many canola varieties/lines, further validation is required before these resistance sources can be used in blackleg control. In future breeding programs, using multiple sources of *Rlm* genes with the combination of adult plant resistance will be a better choice. Previous studies showed that, the durability and effectiveness of *Rlm* genes varied in different circumstances. In Australia, research by Marcroft *et al*. (2012a) indicated that *Rlm3* and *Rlm4* were less effective than other seedling resistance genes. In Canada, the durability or effectiveness of *Rlm* genes was difficult to predict due to the lack of knowledge on *Rlm* genes in commercial canola varieties. The durability of several *Rlm* genes under field conditions has been currently carrying out by Dr. Fernando's lab. Unlike the rapid breakdown of 'sylvestris' resistance in Australia and *Rlm1* in Europe (Rouxel *et al*., 2003a; Sprague, *et al.*, 2006; Van de Wouw, *et al.*, 2010) in few years, the breakdown of *Rlm3* resistance in Western Canada was much slower. This is probably due to *Rlm3* resistance was deployed in different canola varieties with different genetic background. In addition, a combination of *Rlm3* and quantitative resistance, a combination of *Rlm3* and other *Rlm* genes in some commercial varieties might have retard the breakdown of *Rlm3* resistance. Although other *Rlm* genes were rarely detected in Canadian canola accessions, they played an important role in blackleg control. They can be used either as donor of a specific *Rlm* gene to other varieties, or as alternative varieties for *Rlm* genes rotation.

The number of *L. maculans* races detected and the geographic variation in *Avr* gene frequency indicated that the *L. maculans* population in Western Canada is quite diverse. Since only *Rlm3* was found commonly in Canadian canola cultivars, the presence of virulent genes in isolates against resistance genes (*Rlm6, Rlm9*) not widely used in commercial cultivars points to

naturally existing virulent races in the absence of selection pressure. Given that *L. maculans* can lose *Avr* genes to gain virulence, often with no fitness cost, sustained use of any single resistance gene will result in loss of effectiveness. This has been observed in Western Canada with the increased of blackleg disease reported from surveys on varieties previously rated as resistant. Resistance genes may be stacked into a single variety to provide protection against multiple races of the pathogen, and this strategy would be most effective if there is a fitness cost associated with the pathogen carrying multiple virulence genes or virulent races are absent at the time of variety deployment. The presence of low frequency races virulent on stacked resistance risks the loss of multiple resistance genes simultaneously when selection pressure degrades resistance. Based on this study, stacking *Rlm6* and *Rlm7* would be effective against all races examined in Western Canada. Similarly, stacking *Rlm2-7-LepR1* would provide resistance against all isolates surveyed. An alternate strategy of rotating resistance genes similar to rotating fungicides or herbicides would more likely increase the longevity of *Rlm1* and *Rlm2.* In Western Canada, most blackleg isolates either carry *AvrLm1* or *AvrLm2,* fewer than 7% of the isolates lack both *AvrLm1* and *AvrLm2.* This may be a case where rotation of the resistance gene *Rlm1* and *Rlm2* would be more effective than stacking them to avoid a scenario found in Chile where both *AvrLm1* and *AvrLm2* were <2% (Dilmaghani *et al*., 2009).The race composition results also support rotating *Rlm3* with *Rlm4* or *Rlm7*. Rotating crops with different blackleg resistance has been shown to be effective in field experiments (Marcroft *et al*, 2012b). Most avirulence genes have no observable correlation in terms of race structure and either stacking or rotation may work equally well. In some cases, a single *L. maculans Avr* gene interacts with multiple resistance genes in *B. napus*, such as *AvrLm1* that can be detected by both *Rlm1* and *LepR3*, and *AvrLm4-7,* which is detected by both *Rlm4* and *Rlm7*. In these cases, stacking *Rlm1* and *LepR3* or *Rlm4* and *Rlm7* would not be advisable since a single gene deletion in the pathogen would render two resistance genes ineffective.

Develop canola varieties with a combination of major gene resistance and adult plant resistance can improve the effectiveness and durability of blackleg resistance (Kiyosawa, 1982; Brun *et al*., 2010). In this study, about half of tested Canadian canola varieties/lines showed both adult plant resistance and *Rlm* resistance. These canola varieties/lines, with the combination of adult plant resistance and major gene resistance, have the potential to increase the durability of major gene resistance (Brun *et al*., 2010; Marcroft *et al*., 2012b). Although adult plant resistance

was evaluated under controlled conditions, it still can be used to preliminarily analyze adult plant resistance in Canadian canola varieties/lines. In addition, quantitative resistance identification under controlled environment can improve the process of blackleg resistance breeding (Huang, *et al*., 2014), and it can be considered as preliminary evaluation and can be further confirmed in the field. Although adult plant resistance can be isolate-specific in some cases as described by Marcroft *et al.* (2012), in most cases, adult resistance identification seems not isolate-specific.

Using genetic resistance is the best strategy to control the disease of blackleg. Characterization of resistance genes in commercial varieties and advanced breeding lines is essential for blackleg resistance breeding (Marcroft *et al.*, 2012a). It has been proposed by Kutcher *et al.* (2010) that changes in pathogenicity populations of *L. maculans* in Western Canada due to the use of a specific resistance gene, which was uncovered in this study to be the predominant *Rlm3* gene. In Western Canada, PG2 was dominant for a long time from 1980s to early 2000s (Chen et al., 2006), and most likely, without the knowledge of *Rlm* genes in breeding lines, resistance against PG2 isolates was incorporated into Canadian canola varieties. This resulted in very limited genetic diversity in *Rlm* genes in Canadian canola varieties, and finally the breakdown of *Rlm3* resistance. Frequent monitoring of avirulence gene frequency in fungal populations and *Rlm* genes in varieties is very important for disease control. In Australia, diversification of blackleg resistance in canola varieties resulted from changes in their breeding programs gives alternative disease control strategies such as rotation of different *Rlm* genes (Marcroft *et al*., 2012b).

L. maculans population in western Canada is diverse. The race combination and changes observed over earlier studies suggest that selection pressure against some resistance genes may alter the frequency of other *Avr* genes and this may be exploited in planning deployment of resistance genes. The potential exists for using field survey data of pathogen race structure to guide breeding efforts and mitigate the risk of widespread resistance erosion. The geographic variation in race structure and some virulence at all avirulence gene loci means that no variety can perform consistently well against blackleg in all regions on the Canadian prairies. We are now learned that although Canadian canola varieties carried resistance genes, most of them carried the same gene, *Rlm3*, and this gene is not effective any more in most canola grown

regions in Western Canada. It's now very important for Canadian canola breeders to develop blackleg resistant varieties with diversified *Rlm* genes and quantitative resistance.

Additional productivity

Conference proceedings/presentations

1. Zhang X, Fernando WGD, Belmonte MF, Beck MG. Transcriptome analysis of defense related mechanisms in *Brassica napus* against the fungal pathogen *Leptosphaeria maculans*. 11th Conference of the European Foundation for Plant Pathology (EFPP), September, 2014, Cracow, Poland.

2. Fernando WGD, Liban SH, Zhang X, Peng G, Lange R. A host-pathogen interaction paradigm: Can a grower change the pattern of rapid adaptation of new races of *Leptosphaeria maculans* to Canadian canola in Western Canada. 11th Conference of the European Foundation for Plant Pathology (EFPP), September, 2014, Cracow, Poland.

3. Zhang X, Belmonte MF, Becker, MG, Fernando WGD. (2014) Transciptome analysis to understand host defense mechanisms in *Brassica napus*. Phytopathology 104 (Suppl. 3):S3.135. http://dx.doi.org/10.1094/PHYTO-104-11-S3.135.

4. Zhang X, Fernando WGD, Peng G, and Kutcher HR. (2013) Characterization of major resistance genes to *Leptosphaeria maculans* in Canadian *Brassica napus* accessions. 10th International Congress of Plant Pathology, Abstracts. ACTA PHYTOPATHOLOGICA SINICA, 43, Supplement: 404.

5. Zhang X., Fernando W.G. Dilantha and Belmonte M. (2013) Roles of lignin metabolism and several disease defense-related genes in *Brassica napus-Leptosphaeria maculans* pathosystem. 10th International Congress of Plant Pathology, Abstracts. ACTA PHYTOPATHOLOGICA SINICA, 43, Supplement: 331.

6. Liban SH, Cross DJ, Fernando WGD, Peng G and Kutcher HR. (2013) Practical genomics: geographic mapping of the race structure in the Canadian *Leptosphaeria maculans* pathogen population. 10th International Congress of Plant Pathology, Beijing China, August 24-30. ACTA PHYTOPATHOLOGICA SINICA, 43, Supplement: 282.

7. W.G. Dilantha Fernando, X. Zhang, S.H. Liban, D.J. Cross, G. Peng, and H.R. Kutcher. (2013) The Canadian response to the rapid face-changing (Bian Lian) of the blackleg pathogen populations in the Prairies. 10th International Congress of Plant Pathology, Beijing China, August 24-30. ACTA PHYTOPATHOLOGICA SINICA, 43, Supplement: 567.

8. Zhang X, Fernando WGD, Kutcher HR, Peng G**.** (2013) Characterization of major resistance genes against blackleg in Canadian canola germplasm. 2012 CPS annual meeting, Abstract, *Can. J. Plant Pathol.*, 35(1): 131.

9. Liban SH, Cross DJ, Peng G, Kutcher HR and Fernando WGD. (2013) Characterization of *Leptosphaeria maculans* race structure in Western Canada. CPS annual meeting, Abstract, *Can. J. Plant Pathol.*, 35(1): 118.

10. Zhang X, Fernando WGD, Belmonte M. Preliminary results of global and site-specific host defense gene expression in *Brassica napus - Leptosphaeria maculans* pathosystem. Canadian Phytopathological Society 2013 Manitoba regional meeting, November, 2013, Carman, MB, Canada (Provincial)

11. Liban SH and Fernando WGD. Genetic Variation between Geographically Isolated Subpopulations of the *Leptosphaeria maculans* Plant Pathogen. Canadian Phytopathological Society 2013 Manitoba regional meeting, November, 2013, Carman, MB, Canada (Provincial)

12. Zhang X, Fernando WGD, Kutcher HR, Peng G. Identifying major resistance genes against blackleg in Canadian canola germplasm. Canadian Phytopathological Society 2012 Manitoba regional meeting, November, 2012, Brandon, MB, Canada (Provincial)

Scientific papers in preparation

Liban SH, Cross DJ, Fernando WGD, Kutcher HR, Peng G. Race structure and frequency of avirulence genes in the Western Canadian *Leptosphaeria maculans* pathogen population. Plant Pathology, submitted.

Zhang X, Liban SH, Peng G, Kutcher HR, Fernando WGD. Breakdown of *Rlm3* resistance in the *Brassica napus - Leptosphaeria maculans* pathosystem. Ready to submit.

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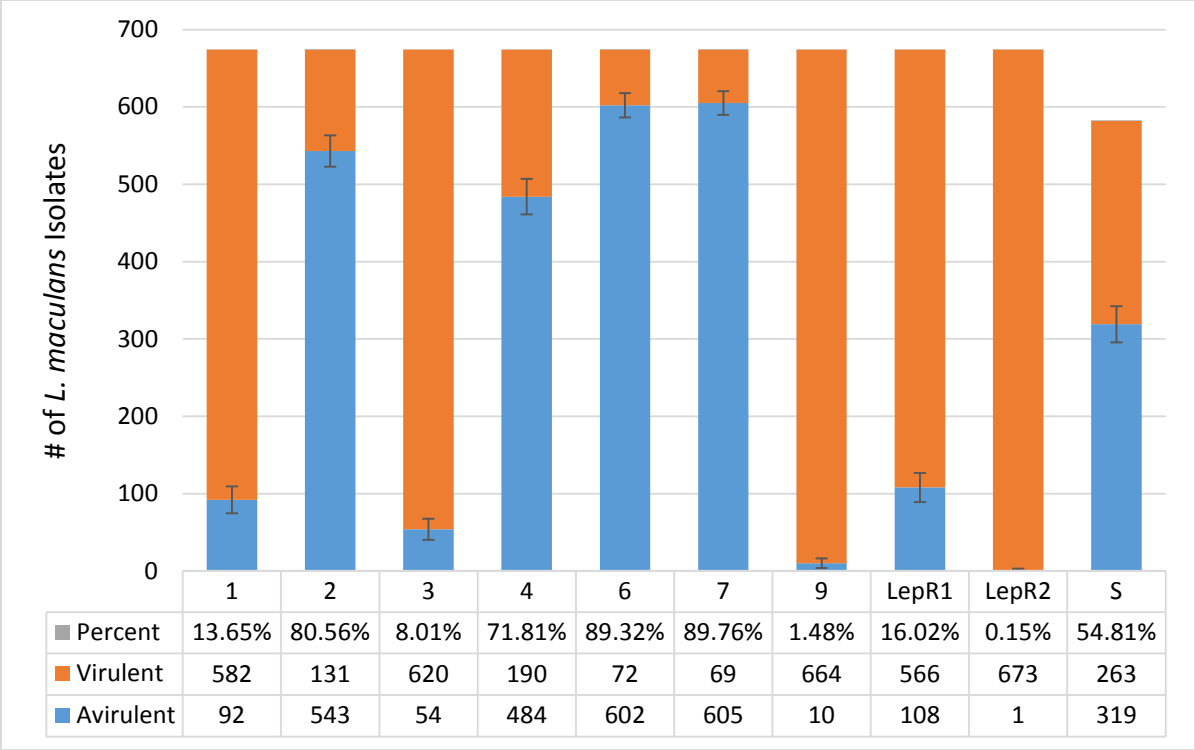


	1	2	3	4	6	7	9	LepR1	LepR2	S
Percent	13.65%	80.56%	8.01%	71.81%	89.32%	89.76%	1.48%	16.02%	0.15%	54.81%
Virulent	582	131	620	190	72	69	664	566	673	263
Avirulent	92	543	54	484	602	605	10	108	1	319

Figure 1 Frequency *Avr* genes in the population of *Leptosphaeria maculans* collected from commercial canola fields in western Canada in 2010 and 2011 based on the analysis using a host differential set and PCR. A total of 674 isolates were examined for potentially carrying 10 known *Avr* genes, with the exception of *AvrLmS* for which 582 isolates were tested

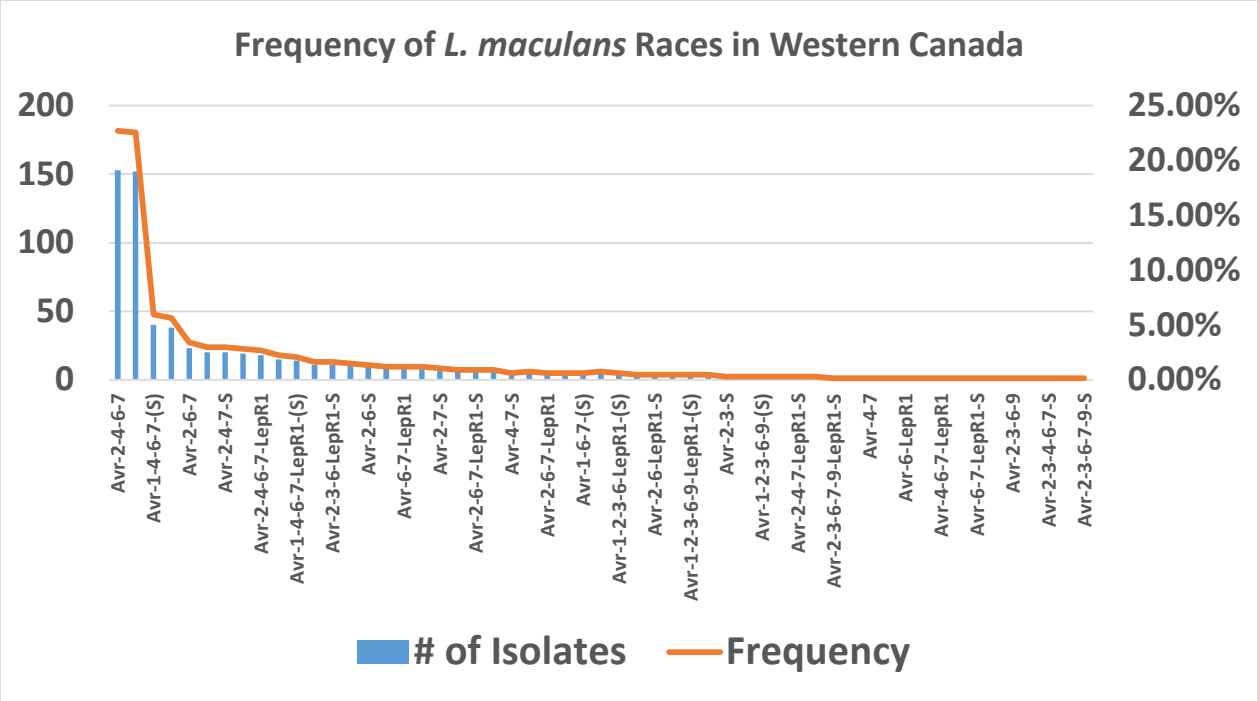
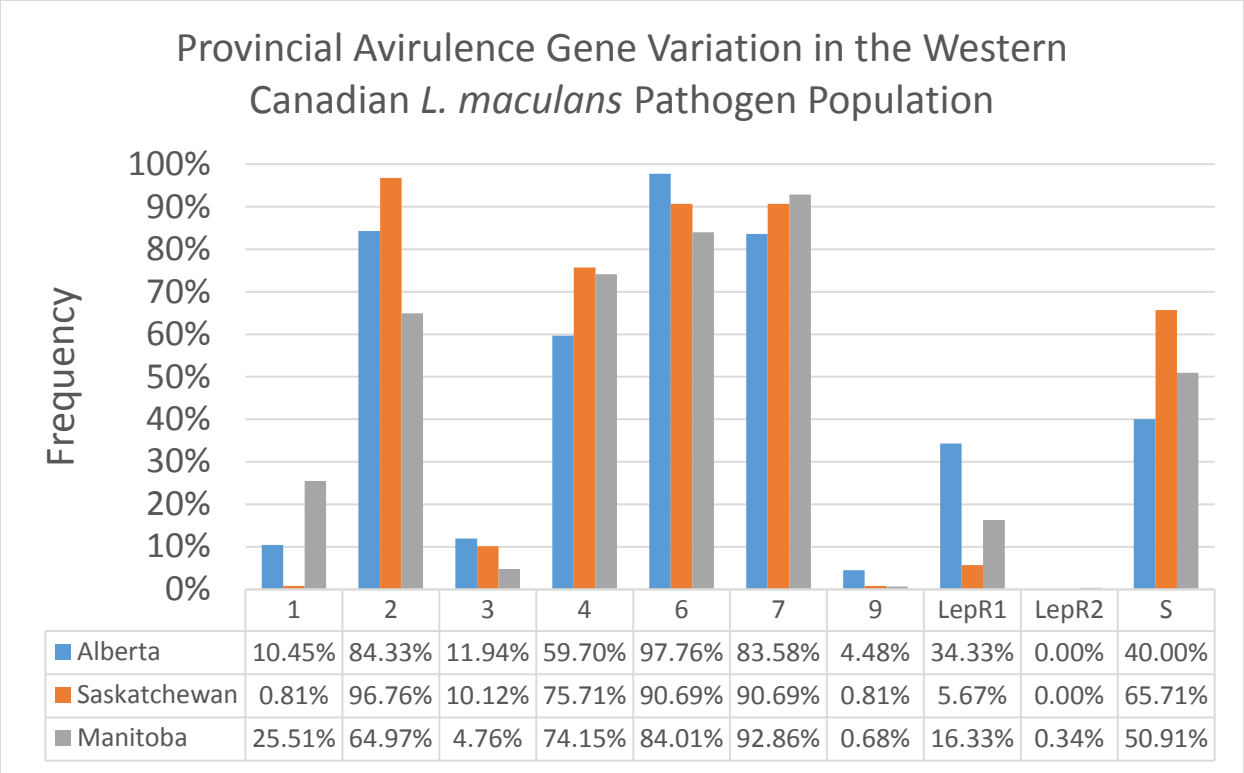
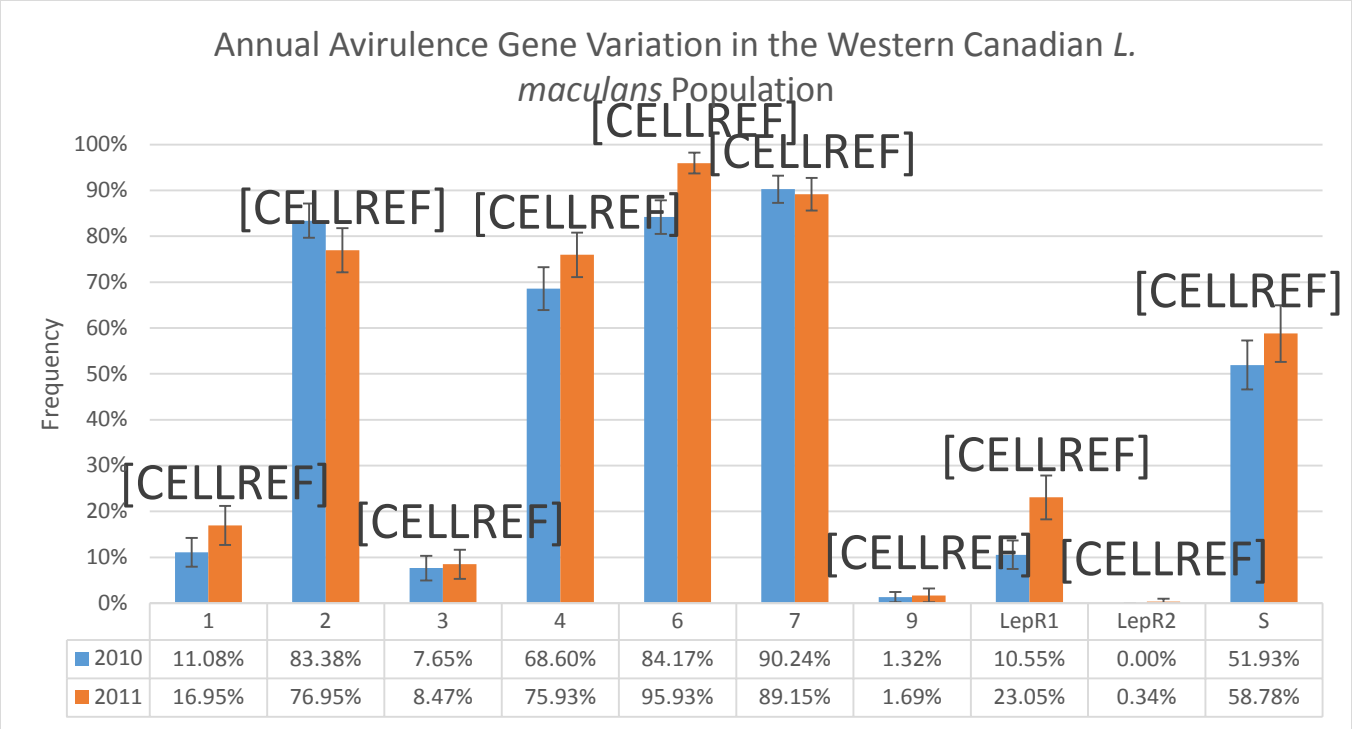


Figure 2 Frequency of 55 *Leptosphaeria maculans* races based on the ten known avirulence genes carried. A total of 674 isolates were examined for all the avirulence genes with the exception of *AvrLmS*/*AvrLepR5* for which 582 isolates were tested.



	1	2	3	4	6	7	9	LepR1	LepR2	S
Alberta	10.45%	84.33%	11.94%	59.70%	97.76%	83.58%	4.48%	34.33%	0.00%	40.00%
Saskatchewan	0.81%	96.76%	10.12%	75.71%	90.69%	90.69%	0.81%	5.67%	0.00%	65.71%
Manitoba	25.51%	64.97%	4.76%	74.15%	84.01%	92.86%	0.68%	16.33%	0.34%	50.91%

Figure 3 Comparison of *Leptosphaeria maculans* avirulence gene frequency between provinces in the Western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS/AvrLepR5* for which 582 isolates were tested.



	1	2	3	4	6	7	9	LepR1	LepR2	S
2010	11.08%	83.38%	7.65%	68.60%	84.17%	90.24%	1.32%	10.55%	0.00%	51.93%
2011	16.95%	76.95%	8.47%	75.93%	95.93%	89.15%	1.69%	23.05%	0.34%	58.78%

Figure 4 Comparison of *Leptosphaeria maculans* avirulence gene frequency in 2010 and 2011 in the Western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS/AvrLepR5* for which 582 isolates were tested.

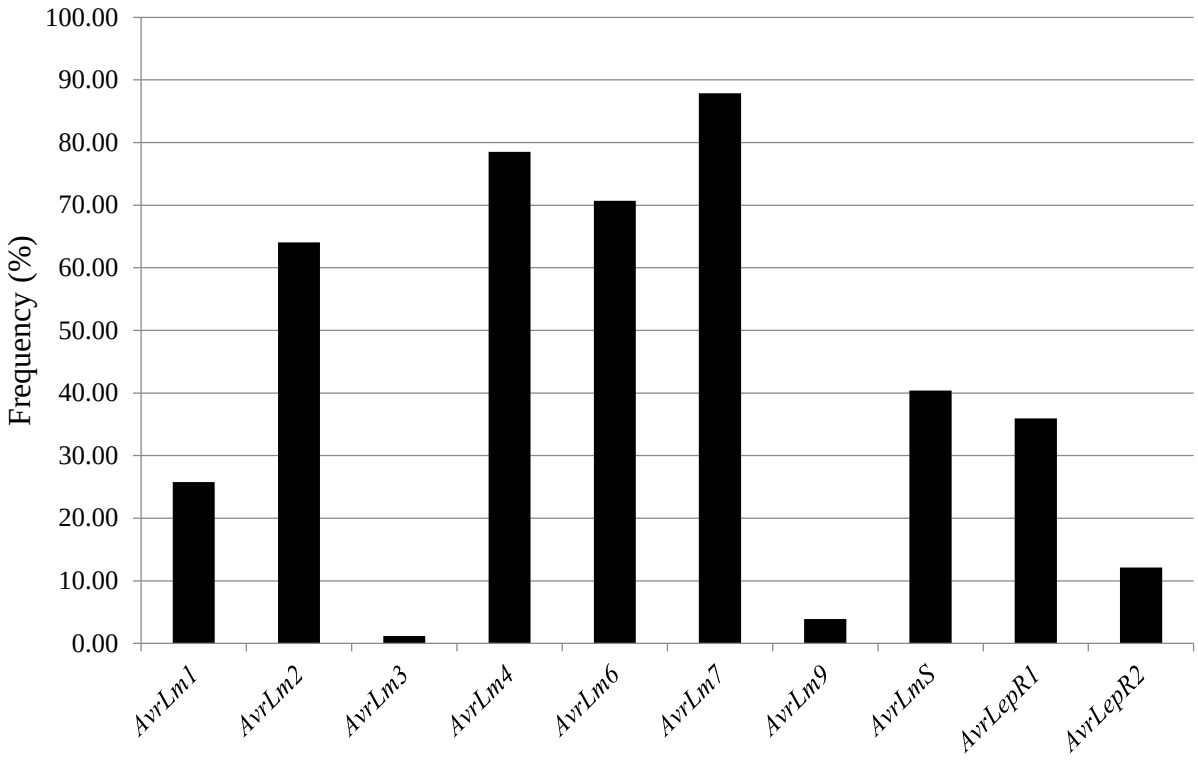


Figure 5 Frequency of avirulence genes in fungal population. *AvrLmS* was assessed in 193 *L. maculans* isolates.

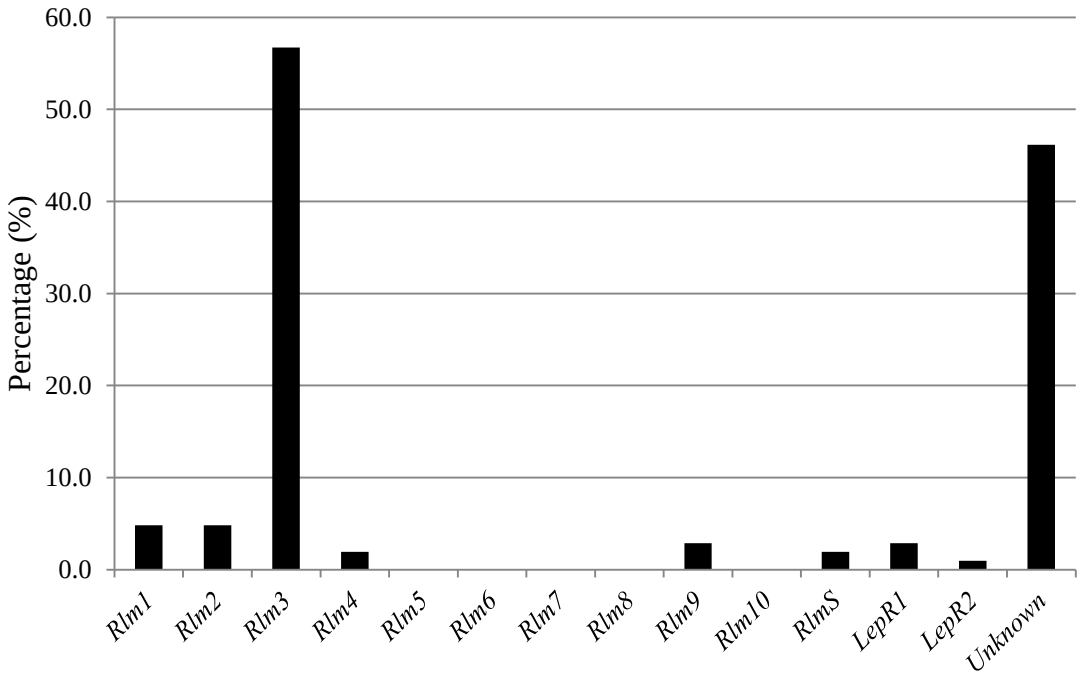


Figure 6 Percentage of *Rlm* genes in 102 canola varieties/lines.

Table 1 Avirulence genotypes of *Leptosphaeria maculans* used as differential isolates.

Isolates	Avirulence genotypes												
	AvrLm1	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm5*	*AvrLm6*	*AvrLm7*	*AvrLm8*	*AvrLm9*	*AvrLm10*	*AvrLmS*	*AvrLepR1*	*AvrLepR2*
D1	-[a]	+[a]	-	-	+	+	-	-	+	nd[b]	+	+	+
D2	-	-	-	-	+	+	-	+	-	nd	+	+	-
D3	-	-	-	-	+	-	-	-	-	nd	-	+	-
D4	-	-	-	+	+	+	+	+	-	nd	-	+	+
D5	+	+	-	+	-	-	+	-	-	nd	+	+	+
D6	+	-	-	-	+	+	-	+	-	nd	+	-	-
D7	+	-	+	-	+	+	-	+	-	nd	nd	+	-
D8	-	-	-	-	+	-	+	nd	-	nd	-	+	-
D9	-	-	-	-	+	+	+	nd	-	nd	-	+	-
D10	-	-	-	-	+	+	-	+	+	nd	+	-	-
D13	-	-	-	+	nd	+	+	nd	-	nd	-	-	-
D14	+	-	-	-	nd	-	+	nd	-	nd	+	+	-
S7	+	-	-	-	+	+	+	nd	-	-	-	+	-
ICBN14	-	-	-	-	+	+	-	-	-	+	-	+	-
PHW1223	-	-	-	-	+	+	-	+	+	-	-	-	-
R2	-	-	-	-	+	-	+	nd	-	+	-	+	-
AD746	-	-	+	-	nd	+	-	nd	-	-	-	+	-
JN2	-	-	-	-	+	+	+	+	-	-	-	+	-
JN3	+	-	-	+	+	+	+	+	-	-	-	-	-
J3	-	+	+	-	nd	+	-	nd	-	nd	+	-	-
J20	-	+	+	-	nd	+	-	nd	-	nd	+	+	-
Q12	-	+	-	+	nd	-	+	nd	-	nd	-	+	-
L-MD7-14	-	-	-	+	nd	+	+	nd	-	nd	-	-	-
L-PC4-1	-	+	-	+	nd	-	-	nd	-	nd	-	-	-
L-MP1-8	-	+	-	+	nd	+	+	nd	-	nd	-	-	-
L-Sb1	-	+	+	-	nd	+	+	nd	-	nd	+	-	-
L-MP1-6	-	-	-	+	nd	+	+	nd	-	nd	-	-	-
L-Sb7-6	-	-	-	+	nd	+	+	nd	-	nd	-	+	-
L-Br17-1	-	-	-	nd	nd	+	+	nd	-	nd	-	+	-
L-Mo5-1	-	+	-	+	nd	+	+	nd	-	nd	-	-	+
L-Br1-16	+	-	-	+	nd	+	+	nd	-	nd	nd	-	-
RL25	-	-	-	-	nd	+	+	nd	-	nd	+	-	-
DS103	-	-	-	-	nd	-	-	nd	+	nd	-	-	-
CV8-7	-	+	-	+	nd	+	+	nd	-	nd	+	-	-

[a] +/- indicates the presence/absence of a specific *AvrLm* gene.

[b] nd indicates not determined.

Table 2 Simpson's Index of Diversity and Evenness for 674 *Leptosphaeria maculans* isolates collected from commercial canola fields in Western Canada in 2010 and 2011. The assessment of evenness and diversity of *L. maculans* races was based on the presence/absence of 10 avirulence genes in the pathogen population

(A) Simpson's Diversity Index*:

	MB	**AB**	**SK**	**Years**
2010	0.922	0.899	0.781	**0.868**
2011	0.891	0.832	0.755	**0.826**
Provinces	**0.906**	**0.865**	**0.768**	**0.885**

* $S = 1 - \sum (n_i^2 - n_i)/(N^2 - N)$

(B) Evenness Index:

	MB	**AB**	**SK**	**Years**
2010	0.700	0.697	0.460	**0.619**
2011	0.667	0.519	0.317	**0.501**
Provinces	**0.684**	**0.608**	**0.389**	**0.712**

* $EH = H/\ln R, \quad \text{with } H = -\sum P_i x \ln P_i$

Table 3 Summary of sequence variation at three *Avr* gene loci among 96 isolates of *Leptosphaeria maculans* from western Canada.

	AvrLm1	*AvrLm6*	*AvrLm4-7*
# of Isolates	33	35	28
PCR Product size (bp)	1123	774	1433
Conserved regions	1	1	9
Conserved regions rize (bp)	438	239	562
Conserved region/PCR product	39%	31%	39%
# of SNPs identified	58	11	118
SNPs / size of conserved regions	13.24%	4.60%	21.00%
% Virulent (Western Canada)	86.52%	10.81%	28.15%
% Avirulent (Western Canada)	13.48%	89.19%	71.85%

Table 4 *Rlm* genes and adult plant resistance (APR) of canola varieties/line.

Accession	*Rlm* gene														Putative *Rlm* genes	APR
	Rlm1	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm5*	*Rlm6*	*Rlm7*	*Rlm8*	*Rlm9*	*Rlm10*	*RlmS*	*LepR1*	*LepR2*	Other		
DF-1	-[a]	-	-	-	-	-	-	-	-	-	-	-	-		None	MS
DF-2	-	-	-	-	-	-	-	-	-	-	-	-	-		None	R
DF-3	-	-	**(+)**[b]	-	-	-	-	-	-	-	-	-	-		*Rlm3* (H)	S
DF-4	-	-	-	-	-	-	-	-	-	-	-	-	-	+[a]	Unknown[d]	S
DF-5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-6	-	-	-	-	-	-	-	-	-	-	-	-	-		None	MS
DF-7	-	-	-	-	-	-	-	-	-	-	-	-	-		None	MR
DF-8	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	Unknown (H)	MR
DF-9	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	Unknown (H)	MS
DF-10	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-11	-	**(+)**	+	-	-	-	-	-	-	**"+"**[c]	-	-	-	(+)	*Rlm2* (H), *Rlm3*, unknown (H)	R
DF-12	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-13	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-14	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-15	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-16	-	-	+	-	-	-	-	-	-	-	-	-	-	(+)	*Rlm3*, unknown	R
DF-17	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-18	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MS
DF-19	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-20	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MS
DF-21	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-22	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MS
DF-23	-	-	**+**	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*	R
DF-24	-	-	**+**	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*	MS
DF-25	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-26	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-27	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-28	-	-	-	-	-	-	-	-	-	-	-	-	-		None	S
DF-29	-	-	-	-	-	-	-	-	-	-	-	-	-		None	S
DF-30	-	-	-	-	-	-	-	-	-	-	-	-	-		None	R
DF-31	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-32	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-33	-	-	-	-	-	-	-	-	-	-	-	-	-		None	S
DF-34	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-35	-	-	-	-	-	-	-	-	-	-	-	-	-		None	S
DF-36	+	-	-	-	-	-	-	-	-	-	-	-	-		*Rlm1*	R
DF-37	+	(+)	+	-	-	-	-	-	-	-	-	-	-		*Rlm1*, *Rlm2* (H), *Rlm3*	R
DF-38	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-39	-	-	**+**	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-40	-	-	**+**	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	S
DF-41	-	-	**+**	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	S

Accession	*Rlm* genes														Putative *Rlm* genes	APR
	Rlm1	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm5*	*Rlm6*	*Rlm7*	*Rlm8*	*Rlm9*	*Rlm10*	*RlmS*	*LepR1*	*LepR2*	Other		
DF-42	+	(+)	+	-	-	-	-	-	-	-	-	-	-		*Rlm1, Rlm2 (H), Rlm3*	R
DF-43	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	MS
DF-44	+	(+)	+	-	-	-	-	-	-	-	-	-	-		*Rlm1, Rlm2* (H), *Rlm3*	R
DF-45	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-46	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	MS
DF-47	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-48	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	MR
DF-49	-	-	**(+)**	-	-	-	-	-	-	-	-	-	-		*Rlm3* (H)	S
DF-50	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-51	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	R
DF-52	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-53	-	-	-	-	-	-	-	-	-	-	-	-	-		None	R
DF-54	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	R
DF-55	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	R
DF-56	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	R
DF-57	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-58	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-59	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MR
DF-60	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-61	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	S
DF-62	-	-	-	-	-	-	-	-	-	-	-	-	-		None	MR
DF-63	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-64	-	-	-	-	-	-	-	-	-	-	-	-	-		None	MS
DF-65	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	R
DF-66	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MS
DF-67	-	-	-	-	-	-	-	-	-	-	-	-	-		None	S
DF-68	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MS
DF-69	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MR
DF-70	+	-	+	-	-	-	-	-	+	"+"	-	-	-		*Rlm1, Rlm3, Rlm9*	R
DF-71	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MR
DF-72	-	-	-	-	-	-	-	-	-	-	-	-	-		None	MR
DF-73	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	S
DF-74	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rl*m3, unknown	MS
DF-75	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MR
DF-76	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MS
DF-77	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	MR
DF-78	-	-	+	-	"+"	-	-	"+"	-	"+"	-	+	-	+	*Rlm3, LepR1*	R
DF-79	-	-	+	-	"+"	-	-	"+"	+	"+"	+	+	"+"	+	*Rlm3, Rlm9, RlmS, LepR1*	R
DF-80	-	-	+	-	"+"	-	-	"+"	+	"+"	+	+	-	+	*Rlm3, Rlm9, RlmS, LepR1*	MR
DF-81	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*Unknown*	R
DF-82	-	-	+	-	-	-	-	-	-	-	-	-	-	(+)	*Rlm3*, unknown	R
DF-83	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-84	-	-	**(+)**	-	-	-	-	-	-	-	-	-	-		*Rlm3*(H)	R

Accession	*Rlm* genes														Putative *Rlm* genes	APR
	Rlm1	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm5*	*Rlm6*	*Rlm7*	*Rlm8*	*Rlm9*	*Rlm10*	*RlmS*	*LepR1*	*LepR2*	Other		
DF-85	-	-	+	-	-	-	-	-	-	-	-	-	-	(+)	*Rlm3*, unknown	R
DF-86	-	-	-	-	-	-	-	-	-	-	-	-	-		None	R
DF-87	-	-	**(+)**	-	-	-	-	-	-	-	-	-	-		*Rlm3*(H)	R
DF-88	-	-	-	-	-	-	-	-	-	-	-	-	-		None	R
DF-89	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	R
DF-90	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*,unknown	MR
DF-91	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	R
DF-92	-	-	+	-	-	-	-	-	-	-	-	-	-	(+)	*Rlm3*, unknown	R
DF-93	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Unknown	R
DF-94	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-95	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	*Unknown (H)*	R
DF-96	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-97	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	R
DF-98	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	R
DF-99	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	Unknown (H)	R
DF-100	-	-	+	-	-	-	-	-	-	-	-	-	-		*Rlm3*	S
DF-101	-	-	-	+	-	-	-	-	-	-	-	-	-		*Rlm4*	MS
DF-102	-	+	+	+	-	-	-	-	-	-	-	-	-		*Rlm2, Rlm3, Rlm4*	MS
DF-103	-	-	+	-	-	-	-	-	-	-	-	-	-	+	*Rlm3*, unknown	S
DF-104	-	-	-	-	-	-	-	-	-	-	-	-	+		*LepR2*	MS

[a]+/- indicates the presence/absence of a specific Rlm gene.

[b](+) refers to accessions with heterogeneous seeds whereby *Rlm* gene was detected in 50-80% of the plants.

[c] "+" indicates possible presence of a specific *Rlm* gene.

[d]Unknown means the resistance genotype can not be determined in this study, accessions showed unknown resistance may due to the presence of a new *Rlm* gene, other known *Rlm* gene or combination of several *Rlm* genes.

APR refers to adult plant resistance, R-resistant, MR-moderately resistant, MS-moderately susceptible, S-susceptible.

Financial Statement (to be added)

Overall, the project stayed on budget.