A Six-Year Investigation of the Dynamics of Avirulence Allele Profiles, Blackleg Incidence, and Mating Type Alleles of *Leptosphaeria maculans* Populations Associated with Canola Crops in Manitoba, Canada

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Abstract

Blackleg, caused by the fungal pathogen *Leptosphaeria maculans*, is one of the most economically important diseases of canola (*Brassica napus*, oilseed rape) worldwide. This study assessed incidence of blackleg, the avirulence allele, and mating type distributions of *L. maculans* isolates collected in commercial canola fields in Manitoba, Canada, from 2010 to 2015. A total of 956 *L. maculans* isolates were collected from 2010 to 2015 to determine the presence of 12 avirulence alleles using differential canola cultivars and/or PCR assays specific for each avirulence allele. *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLmS*

were detected at frequencies ranging from 97 to 33%, where the *AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLepR1*, and *AvrLepR2* alleles were the least abundant. When the race structure was examined, a total of 170 races were identified among the 956 isolates, with three major races, *AvrLm-2-4-5-6-7-11*, *AvrLm-2-4-5-6-7-11-S*, and *Avr-1-4-5-6-7-11-(S)* accounting for 15, 10, and 6% of the total fungal population, respectively. The distribution of the mating type alleles (*MAT1-1* and *MAT1-2*) indicated that sexual reproduction was not inhibited in any of the nine Manitoba regions in any of the years *L. maculans* isolates were collected.

Blackleg, caused by the fungal pathogen *Leptosphaeria maculans*, has been identified as one of the major diseases affecting production in many canola (oilseed rape, *Brassica napus*) growing regions worldwide (Fitt et al. 2006). This disease can be controlled by the utilization of resistant varieties, crop rotations, and fungicide applications (Kutcher et al. 2013). Both qualitative (*R*-gene resistance) resistance and quantitative (adult plant resistance, APR) have been identified in *Brassica* species (Balesdent et al. 2001; Delourme et al. 2006; Jestin et al. 2011; Rimmer 2006). Prior genetic studies indicated a gene for gene relationship between an *R* gene and the corresponding avirulence (*Avr*) gene for this pathogen (Ansan-Melayah et al. 1998; Balesdent et al. 2001). Using *R* gene-mediated resistance is often a very effective strategy for disease control when the fungal populations harbor a high frequency of the corresponding avirulence alleles (Daverdin et al. 2012). However, intensive use of single *R* genes will exert selection pressure on the fungal populations, and can result in pathogen adaptation to *R* genes in the host plants. Previous studies indicated that the pathogen can adapt to the selection pressure within a few years under both experimental conditions and commercial agricultural systems (Zhang et al. 2016). In experimental conditions, *Rlm6* resistance was not effective after three growing seasons (Brun et al. 2010). In France, *Rlm1* resistance was overcome by the fungal populations within three growing seasons (Rouxel et al. 2003). Similarly, *LepR3* resistance in Australia lost effect a few years after the release of a commercial *LepR3*-carrying variety (Sprague et al. 2006; Van de Wouw et al. 2010, 2014). In western Canada, outbreaks of blackleg in crops of commercial canola varieties, carrying blackleg resistance genes, has been recorded

frequently since 2007 (Chen and Fernando 2006; W. G. D. Fernando, unpublished data). More recently, the breakdown of *Rlm3* resistance was reported in western Canada due to intensive use of *Rlm3*-carrying canola varieties (Zhang et al. 2016).

Avirulence genes of *L. maculans* are usually located in AT-rich, gene-poor regions of the genome (Rouxel et al. 2011). These regions comprise repetitive DNA derived from transposable elements (TEs) and, therefore, provide an unstable genomic environment that promotes rapid gain and loss of avirulence genes (Soyer et al. 2014). For example, deletions, amino acid substitutions, and repeat-induced point (RIP) mutations of *AvrLm1* were observed in *L. maculans* isolates collected before and after the breakdown of 'sylvestris' resistance in Australia (Van de Wouw et al. 2010). To date, at least 14 *Avr* genes have been identified in *L. maculans*, and seven of them have been cloned, including *AvrLm1* (Gout et al. 2006b), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLm3* (Plissonneau et al. 2016), *AvrLm5/AvrLmJ1* (Van de Wouw et al. 2014; Balesdent and Howlett, unpublished data), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013). More than 18 *R* genes in *Brassica* species have been identified to date: *Rlm1* to *Rlm11*, *LepR1* to *LepR4*, *RlmS*, *BLMR1*, and *BLMR2* (Balesdent et al. 2013; Eber et al. 2011; Long et al. 2011; Rimmer 2006; Van de Wouw et al. 2009). Only two *R* genes, *LepR3* (which interacts with *AvrLm1*) and *Rlm2* (which interacts with *AvrLm2*) have been cloned (Larkan et al. 2013, 2015).

Frequent monitoring of fungal populations can provide relevant data that can be used as part of an effective blackleg control strategy. Reports on *L. maculans* populations in France and North America have provided useful knowledge for manipulating the planting of different resistant varieties with different *R*-genes for control of blackleg (Balesdent et al. 2006; Dilmaghani et al. 2009; Liban et al. 2016; Rouxel et al. 2003). However, there is an increasing demand to monitor long-term dynamics of populations of *L. maculans* due to the high evolutionary potential of the pathogen. Identification of avirulence alleles in *L. maculans* populations can be achieved via pathogenicity tests using a set of differential varieties with known *R* genes and/or polymerase chain reaction (PCR) assays specific for cloned avirulence genes. Although pathogenicity tests are labor-intensive and time-consuming, the results are generally very reliable. PCR assays are a rapid and high-throughput alternative for determining the presence of avirulence alleles. However, PCR markers for avirulence alleles need

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to be validated to confirm that the phenotypic results match genotypic data to account for misleading genetic effects such as partial gene deletions, mutations, allelic variants, and gene interactions. The need for marker validation was shown by the gene masking or 'camouflage' effect that *AvrLm4-7* has on the *AvrLm3* allele, where the *AvrLm3* gene is not recognized by host *Rlm3* when *AvrLm4-7* is also present and a virulent phenotype occurs (Plissonneau et al. 2016).

The life cycle of *L. maculans* typically involves annual sexual reproduction by production of ascospores, and asexual reproduction by production of pycnidiospores (Gout et al. 2006a). Sexual reproduction is extremely important for producing primary inoculum via ascospores, and generating genetic variations in fungal populations. *L. maculans* has two idiomorphs of a single mating type (*MAT*) locus, *MAT1-1* and *MAT1-2* (Cozijnsen and Howlett 2003). Earlier studies inferred a lack of sexual reproduction in *L. maculans* populations in western Canada since pycnidiospores were observed as the primary inoculum source (Dilmaghani et al. 2009; Ghanbarnia et al. 2011).

Annual disease surveys conducted in Manitoba, a province in the Canadian prairies, have reported the incidence of blackleg each year. However, these surveys have not provided information on the *Avr* alleles and pathogen strains/populations in different regions of Manitoba. This type of data would inform local growers on which varieties are most resistant to pathogen strains in various regions of the province. The objectives of this study were to evaluate *Avr* allele profiles in Manitoba from 2010 to 2015, blackleg disease incidence, mating type alleles, and changes in avirulence alleles of these *L. maculans* isolates by region and across all areas of the province surveyed. Knowledge of *Avr* allele frequencies will provide guidance for blackleg resistance breeding and facilitate optimal use of available *R* genes for growers.

Materials and Methods

Sample collection. Blackleg infected canola stems were collected from commercial canola fields across Manitoba from 2010 to 2015 as part of the Canadian Plant Disease Survey program (<http://phytopath.ca/publication/cpds/>). All samples were collected in August when symptoms of blackleg were visible on stems, including the stem base (basal canker). The number of canola fields from which canola basal stem samples were collected were 103 in 2010, 81 in 2011, 108 in 2012, 106 in 2013, 127 in 2014, and 112 in 2015. Fields were distributed across nine regions defined by township as shown in Figure 1. The nine regions were depicted with the following letters and the number of fields within each of the regions indicated in brackets: A, Steinbach area (17 fields); B, Selkirk area (20 fields); C, Swan Valley (18 fields); D, Dauphin (39 fields); E, Central Plains (33 fields); F, Pembina Valley (101 fields); G, Roblin, Russell, Rosburn area (49 fields); H, Southwest area (40 fields); I, and Brandon area (42 fields). A total of 956 *L. maculans* isolates were obtained from blackleg infected stems: 139 isolates from stems collected in 68 fields in 2010, 61 isolates from 55 fields in 2011, 112 isolates from 54 fields in 2012, 143 isolates from 74 fields in 2013, 207 isolates from 97 fields in 2014, and 302 isolates from 71 fields in 2015.

Determination of blackleg incidence and severity. The incidence of blackleg (disease incidence) in each field was evaluated based on the percentage of blackleg infected stems (stems with basal canker) in 100 plants sampled in each field per year. The percent range of blackleg incidence was tabulated for each region/year. Mean value and standard deviation were not determined because samples collected within each of the nine regions were not collected from the same field (i.e., single replicate) each year due to crop rotation of canola by farmers. Disease severity of each plant (total of 100) was rated on a 0 to 5 rating scale as described by Zhang et al. (2016). Briefly, disease severity of basal stems was scored on a 0 to 5 rating scale: 0 = no noticeable infection; 1 = diseased tissue occupies $\leq 25\%$ of the cross-section; 2 = diseased tissue occupies 25 to 50% of cross-section; 3 = between 50 and 75% of the cross-section infected; 4 = more than 75% of the cross-section infected; and 5 = 100% of cross-section diseased, plant dead.

Fungal isolation and inoculum preparation. Fungal isolation, purification, inoculum preparation, and maintenance were performed as described by Liban et al. (2016) with some modifications. All

isolates were collected from the stem or stem base of infected canola stems. Briefly, infected stems were surface-sterilized in 50% (v/v) bleach for 1 min, rinsed with sterilized water, air dried, and cut into small pieces. These stem pieces were placed on nonclarified V8 agar juice medium (200 ml of V8 juice, 800 ml water, 15 g agar, 0.75 g CaCO_3) amended with 0.0035% (w/v) streptomycin sulfate and incubated under continuous fluorescent light at room temperature (25°C) for 7 days. Each pure culture of *L. maculans*, obtained from a single spore, was transferred onto a new Petri plate containing V8 agar, as described in previous studies (Liban et al. 2016). Subsequently, each Petri plate was flooded with 3 ml of sterile distilled water and *L. maculans* spores were harvested for pathogenicity testing under greenhouse conditions.

DNA extraction and PCR assays. Genomic DNA was extracted from pycnidiospores suspensions of purified *L. maculans* isolates using the method described by Liban et al. (2016). The presence or absence of avirulence (*Avr*) alleles in *L. maculans* isolates collected from growers' field (2010 to 2015) was determined using PCR with primers that were specific to the six cloned *Avr* genes, derived from the original reports on cloning of the respective genes: *AvrLm1* (Gout et al. 2006a), *AvrLm2* (Ghanbarnia et al. 2015), *AvrLmJ1/AvrLm5* (Van de Wouw et al. 2014; Balesdent and Howlett, unpublished data), *AvrLm4-7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013). The presence of a PCR product indicated the presence of the avirulent allele, while the absence of the PCR product indicated that the avirulent allele was not present (Rong et al. 2015; Van de Wouw et al. 2010). For *AvrLm4-7*, two allelic variance of this avirulence gene interact with host *Rlm4* and *Rlm7* resistance genes where the presence and absence genotype indicates the presence and absence of *AvrLm7*; however, a single nucleotide change renders the gene virulent toward *Rlm4*. In the case of *AvrLm4*, an SNP marker would be needed to accurately determine the phenotype and for the purposes of this study, the pathogenicity assay was used to determine the frequency of the *AvrLm4* allele. The distribution of two mating type alleles, *MAT1-1* and *MAT1-2*, in *L. maculans* populations was also determined by using the multiplex PCR assay developed by Cozijnsen and Howlett (2003). In some cases, the PCR assay was repeated to verify results.

Pathogenicity tests. Avirulence profiles of *L. maculans* isolates were determined by inoculating the isolates onto a set of *B. napus* differentials listed in Table 1 (Zhang et al. 2016). Four cotyledon lobes of 7-day-old *B. napus* seedlings were wounded with modified tissue forceps to generate the route of infection. Each wounded site was

Fig. 1. Manitoba map of the nine regions in which blackleg infected canola stems were sampled. Sites are represented by letters and are as follows: A, Steinbach region; B, Selkirk area; C, Swan Valley; D, Dauphin area; E, Central Plains; F, Pembina Valley; G, Roblin, Russell, Rosburn, and Roblin; H, Southwest area; I, Brandon area. Black pins in each site represent the area where fields were sampled.

inoculated with a 10- μ l droplet of the appropriate pycnidiospore suspension (2×10^7 spores/ml). Disease symptoms on inoculated cotyledons were evaluated 14 days post inoculation (dpi) using a rating scale of 0 to 9 as described by Williams and Delwiche (1979). At least six plants of each differential variety were used for the pathogenicity test of each isolate. A total of 24 disease scores (four cotyledon lobes $\times$ six plants) were obtained for each isolate-*B. napus* interaction, and the average score was used to determine the interaction phenotype (IP), with avirulent IP scores ranging from 0 to 4.9 and virulent IP scores ranging from 5 to 9 (Liban et al. 2016). *B. napus* differentials used in this study (Table 1) enabled the identification of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2*, and *AvrLmS*. Surpass 400 (*LepR3*, *RlmS*) was used to identify *AvrLmS* in the population of fungal isolates collected. Since *AvrLm1* interacts with *LepR3* (Larkan et al. 2013), the presence of the *AvrLmS* allele could not be identified in isolates carrying *AvrLm1*. The race structure of each isolate was determined according to Balesdent et al. (2005). The distribution and frequency of *AvrLm2*, *AvrLm3*, *AvrLm9*, *AvrLepR1*, *AvrLepR2*, and *AvrLmS* alleles within the fungal population were assessed using differential cultivars. The avirulence frequency of *AvrLm5*, *AvrLm6*, and *AvrLm11* was assessed with PCR. The avirulence frequency of *AvrLm1*, *AvrLm4*, and *AvrLm7* were assessed with both PCR and differential cultivars.

Data analysis. *L. maculans* isolates collected from all regions in a given year were evaluated as a population, and isolates collected from a given site in a given year was evaluated as a subpopulation. The isolates in each population and subpopulation were analyzed for the frequency of *Avr* alleles. Genetic diversity of these *L. maculans* populations and subpopulations was studied individually. Simpson's index of diversity was calculated to estimate genetic diversity of the population (Balesdent et al. 2006). The Margalef index, which measures the richness in species and/or races of a population, was also calculated (Balesdent et al. 2006). The distribution of two mating alleles, *MAT1-1* and *MAT1-2*, were determined in the *L. maculans* populations collected each year and in the subpopulations of isolates collected within each of the nine Manitoban regions (i.e., isolates from each region collected from 2010 to 2015 pooled together). The ratios between the two alleles were analyzed with a χ^2 test (Ugoni and Walker 1995) to determine if the ratios varied from 1:1. A χ^2 homogeneity test was also performed to determine if the mating type allele distribution in the collected isolates was similar among all years and regions. Because the significance level of $\alpha = 0.05$ was used for testing the allele ratios, χ^2 95% (with degrees of freedom [df] corresponding to the number of samples - 1) from the χ^2 table was used to perform the χ^2 homogeneity test.

Results

Disease incidence and severity of blackleg. The six-year survey of canola crops in Manitoba, Canada, from 2010 to 2015 has shown a general trend in blackleg incidence across nine regions, where the average disease incidence increases from 0 to 98% (Table 2). The Steinbach (A), Central Plains (E), Pembina Valley (F), and Roblin/Russell/

Rosburn (G) regions shared a similar pattern in terms of blackleg incidence where there was a continuous increase observed in each of these regions ranging from 0 to 98%, 0 to 92%, 6 to 84%, and 5 to 65% (A, E, F, and G, respectively) from 2010 to 2015 (Table 2). The Swan Valley region (C) had the lowest range of blackleg incidence over the six years, ranging from 0 to 51%. The Dauphin (D) region exhibited a range of 1 to 4% blackleg incidence in 2010, and in 2013 the range of disease incidence increased up to 30%, where it remained constant to 2015 (Table 2). The Southwest area (H) exhibited a range of blackleg incidence of 1 to 17% in 2010, increasing to 50% in 2011, and decreasing to 10 and 4% in 2012 and 2013, respectively. The Selkirk area (B) exhibited the exact pattern in terms of increase and decrease of blackleg incidence. The Brandon (I) area also exhibited a similar pattern, where the blackleg incidence in 2010 was relatively low ranging from 1 to 20%, and in 2012 increased to a range up to 50%, decreasing in 2013 and then increasing once again to a range of 13 to 55% blackleg incidence in 2014. However, unlike regions H and B, the range of blackleg incidence increased up to 90% in 2015 (Table 2). Disease severity, rated on a 0 to 5 scale, ranged from 1.4 to 1.6 from 2010 to 2015, indicating very little change in severity from year to year within the nine regions of Manitoba sampled (data not shown).

Avirulent alleles in fungal populations. The presence or absence of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4-7*, *AvrLm5*, *AvrLm6*, *AvrLm9*, *AvrLm11*, *AvrLepR1*, *AvrLepR2*, and *AvrLmS* alleles was determined using *B. napus* differentials, alone or coupled with PCR assays, for each *L. maculans* collected in this six-year survey of canola in Manitoba. The frequency and distribution of the *Avr* alleles carried by the fungal isolates were first analyzed by year of sampling, with years representing populations of the pathogen (Fig. 2). From the total 956 *L. maculans* isolates collected in 2010 to 2015, seven *Avr* alleles, *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLmS*, were the most abundantly identified and were present in 50 to 100% of the collected isolates over the six-year period (Fig. 2A). When each year was examined more closely, isolates collected in 2013 appeared to harbor lower frequency of *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLmS* alleles (63, 76, 66, 65, 77, and 12%, respectively; Fig. 2A). However, in 2015, the frequency of these alleles, with the exception of *AvrLm5*, was greater than in the other years of sampling (77, 99, 99, 96, and 92%, respectively; Fig. 2A). The *AvrLm2* allele frequency, on the other hand, appeared to remain relatively similar in isolates collected in 2012 to 2015 (67, 63, 64, and 62%, respectively; Fig. 2A).

Table 2. % Range of blackleg incidence observed in each region per year of surveillance

Region ^a	% Range of blackleg incidence ^b					
	2010	2011	2012	2013	2014	2015
A	0–10	5–45	1–30	9–84	7–98	11–50
B	0–5	8–50	1–11	0–7	11–16	4–50
C	0	2	ND ^c	1–6	51	1–8
D	1–4	1–11	2–19	1–30	8–30	3–30
E	0–10	3–15	3–50	11–40	10–92	24–76
F	6–40	0–8	5–21	3–50	15–84	21–60
G	5–14	1–8	1–30	5–33	2–65	1–13
H	1–17	21–50	0–10	2–4	5–30	4–90
I	1–20	1–15	4–50	1–15	13–55	0–6

^a *Leptosphaeria maculans* isolates were collected from canola crops obtained in nine Manitoba regions (as shown in Fig. 1): Areas are defined by letters and are as follows: A, Steinbach area; B, Selkirk area; C, Swan Valley; D, Dauphin; E, Central Plains; F, Pembina Valley; G, Roblin, Russell, Rosburn area; H, Southwest area; I, Brandon area.

^b Incidence of blackleg evaluated based on the percentage of blackleg infected stems (stems with basal canker) in 100 collected crop samples/year/field within each region. Blackleg incidence (%) reported as the range of incidence for each region. Mean value and standard deviation was not calculated because crop samples collected for each year within each of the nine regions were not from the same fields (i.e., single replicate) due to crop rotation of canola by producers.

^c Not determined; no isolates obtained in this region.

Table 1. *Brassica napus* varieties/lines used as differentials to identify avirulence genotypes of *Leptosphaeria maculans* isolates

Cultivar/line	R-genes	Reference
MT29	<i>Rlm1,9</i>	Delourme et al. 2008
01-23-2-1	<i>Rlm7</i>	Dilmaghani et al. 2009
Quinta	<i>Rlm1,3</i>	Kutcher et al. 2010
Surpass 400	<i>LepR3, RlmS</i>	Larkan et al. 2013
1065	<i>LepR1</i>	Kutcher et al. unpublished
1135	<i>LepR2</i>	Kutcher et al. unpublished
Glacier	<i>Rlm2,3</i>	Balesdent et al. 2001
Jet Neuf	<i>Rlm4</i>	Gout et al. 2006b
Goéland	<i>Rlm9</i>	Balesdent et al. 2006
Bristol	<i>Rlm2, Rlm9</i>	Balesdent et al. 2005
02-22-2-1	<i>Rlm3</i>	Gout et al. 2006b
Westar	<i>No R gene</i>	Balesdent et al. 2001

As shown in Figure 2B, the alleles that were detected the least abundantly in the 956 isolates collected from 2010 to 2015 were *AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLepR1*, and *AvrLepR2*. The *AvrLm1* allele was detected in isolates collected in all six years and was relatively stable from 2010 to 2014 (15.2% in 2010, 16.4% in 2011, 12.5% in 2012 and 2013, and 11.1% in 2014; Fig. 2B). In 2015, the *AvrLm1* allele was detected at the highest observed frequency of 28%. Interestingly, the *AvrLm3* allele illustrated a pattern of decreasing frequency in the collected isolates from 2010 to 2014 (Fig. 2B), where the allele frequency decreased from 6.52% in 2010 to 4.92% in 2011, to 1.79% in 2012, to 0.7% in 2013, and to 0% in 2014. However, in 2015, this allele was detected only in 1.3% of the 302 *L. maculans* isolates collected (Fig. 2B). The *AvrLm9* allele shared a similar decreasing pattern as was observed for *AvrLm3*, but the *AvrLm9* allele was not detected in any isolates in 2010. From 2011, when the allele was detected, to 2013, the observed *AvrLm9* allelic frequency in isolates collected those years decreased from 3.2% in 2011 to 0.8% in 2012, 0.7% in 2013, and 0% in 2014 (Fig. 2B). An intermittent frequency pattern from *L. maculans* isolates collected in 2010 to 2015 was observed in the *AvrLepR2* allele, where it was not detected in isolates collected in 2010, 2012, or 2013, but was detected in isolates collected in 2011 (1.6%), 2014 (6.7%), and 2015 (0.3%) (Fig. 2B). The *AvrLepR1* allele frequency

observed from the collected isolates, on the other hand, appeared to fluctuate the most from year to year (4% of the 139 isolates collected in 2010, 49% of the 61 isolates collected in 2011, 38% of the 105 isolates collected in 2012, 20% of the 143 isolates collected in 2013, 7% of the 207 isolates collected in 2014, and 26% of the 302 isolates collected in 2015; Fig. 2B).

When the *L. maculans* isolates were analyzed by region of Manitoba in which canola crops were sampled, the detected frequency of *AvrLm5*, *AvrLm6*, *AvrLm7*, and *AvrLm11* alleles were great in each region surveyed (Fig. 3A) with an average frequency of 90.2% ($\pm 3\%$), 85% ($\pm 8\%$), 86% ($\pm 7\%$), and 81% ($\pm 6\%$), respectively. Conversely, larger fluctuations of the *AvrLm1*, *AvrLm4*, and *AvrLepR1* allele frequencies were observed among the nine Manitoban regions (Fig. 3A and B). The average frequency of *AvrLm1* was 15.4% ($\pm 10\%$) and ranged from 3.8% in the Central Plains to 33.0% in Pembina Valley (Fig. 3B), while the average frequency of *AvrLm4* was 70% ($\pm 13\%$), ranging from 43.9% in the Southwest area to 88.4% in the Steinbach area (Fig. 3A). The average *AvrLepR1* allele frequency within the nine regions was 16.8% ($\pm 6\%$), ranging from 6% in the Swan Valley region to 24% in the Russell/Roblin/Rossburn area (Fig. 3B). The less frequently detected *AvrLm3*, *AvrLm9*, and *AvrLepR2* alleles ranged from 0 to 7% across the nine regions (Fig. 3B).

Fig. 2. Frequency of *Avr* alleles in *Leptosphaeria maculans* populations (2010 to 2015). **A**, The frequency of the most prevalent alleles (*AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLmS*) within the *L. maculans* population. **B**, Frequency of the least prevalent alleles (*AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLepR1*, and *AvrLepR2*). The frequency of the avirulence allele in each year was calculated based on the percentage of isolates carrying the avirulence allele among all isolates tested in that year. The presence of *AvrLm1*, *AvrLm2*, *AvrLm4*, and *AvrLm7* was assessed using a combination of PCR and pathogenicity assays, while the presence of *AvrLm5*, *AvrLm6*, and *AvrLm11* alleles were determined using PCR only. Presence of the other five *Avr* alleles was determined with pathogenicity assays only.

Fig. 3. Frequency of avirulence alleles in *Leptosphaeria maculans* populations from nine Manitoba regions (see below). **A**, The frequency of the most prevalent alleles (*AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm11*, and *AvrLmS*). **B**, Frequency of the least prevalent alleles (*AvrLm1*, *AvrLm3*, *AvrLm9*, *AvrLepR1*, and *AvrLepR2*) within the *L. maculans* population. The presence of *AvrLm1*, *AvrLm2*, *AvrLm4*, and *AvrLm7* was assessed using a combination of PCR and pathogenicity assays, while the presence of *AvrLm5*, *AvrLm6*, and *AvrLm11* were determined using PCR only. Presence of the other five *Avr* alleles was determined with pathogenicity assays only. Nine regions (Fig. 1) are as follows: A, Steinbach region; B, Selkirk area; C, Swan Valley; D, Dauphin area; E, Central Plains; F, Pembina Valley; G, Russell, Rossburn, and Roblin; H, Southwest area; I, Brandon area.

Genetic diversity and race structure analysis in *L. maculans* populations. A total of 12 avirulence alleles were detected and analyzed for the *L. maculans* isolates collected from 2010 to 2015. A total of 27 race structures was identified for isolates of *L. maculans* collected and analyzed in 2010, 23 races from isolates collected in 2011, 53 from 2012 isolates, 62 from 2013 isolates, 59 from 2014 isolates, and 51 from 2015 isolates (Table 2). In total, 170 races of *L. maculans* were identified from 956 isolates collected from 2010 to 2015. The three most prevalent races were: *Avr-2-4-5-6-7-11* detected in 143 isolates, *Avr-2-4-5-6-7-11-(S)* in 93 isolates, and *Avr-1-4-5-6-7-11-(S)* in 54 isolates (Fig. 4). Seven isolates were identified, with race structure of *Avr* null (no detectable avirulent alleles), *Avr-1*, *Avr-4*, *Avr-5*, *Avr-7*, and *Avr-11*. The race structure also appeared to be relatively stable from year to year in that a majority of the isolates collected each year had a race structure similar to *Avr-2-4-5-6-11-(S)* (Fig. 4), with some minor changes. For example, in 2011, *AvrLepR1* was prominent within the race structure while in 2014, *AvrLm1* was identified more frequently within the race structure. Over the six years of the survey in Manitoba, there was a high

level of race diversity detected in the *L. maculans* isolates. With exception of 2010, for which the isolates collected had a Simpson index of 0.77, Simpson index in isolates collected in the other five years was more than 0.90, indicating high genetic diversity of populations each year (Table 3). The Margalef index values also indicated high race diversity in these *L. maculans* populations in Manitoba, ranging from 5.27 in 2010 to 5.35 in 2011, 11.02 in 2012, 12.29 in 2013, 10.88 in 2014, and 8.76 in 2015.

Genetic diversity and race structure analysis in *L. maculans* subpopulations. The *L. maculans* isolates race structure was also analyzed based on regions surveyed in Manitoba (Table 4). As shown in Table 4, the number of races within each region varied depending on the number of isolates collected within each region, with the greatest number of races detected in the Brandon and Pembina Valley regions (118 and 117 races, respectively). As shown in Table 5, the two most frequently observed races, in seven of the nine regions, were *Avr-2-4-5-6-7-11* and *Avr-2-4-5-6-7-11-(S)*. The Selkirk, Southwest, and Pembina Valley areas, however, differed from other regions sampled. The top two races detected in Selkirk were *Avr-2-4-5-7-11*

Fig. 4. Frequency of 29 major *Leptosphaeria maculans* races (each race with five or more isolates) based on their avirulence alleles. A total of 964 *L. maculans* isolates were analyzed. It should be noted that the race “*Avr*” represents *Avr*-null.

Table 3. *Avr* allele diversity (i.e., races) of the collected *Leptosphaeria maculans* populations from 2010 to 2015 and the distribution of mating type alleles year to year

Year	No. of isolates	No. of races	Margalef index ^a	Simpson index ^b	Number of fields	<i>MATI-1</i> ^c	<i>MATI-2</i> ^c	χ^2	<i>P</i> value
2010	138	27	5.27	0.77	68	54	84	6.52	0.01
2011	61	23	5.35	0.93	55	27	34	0.80	0.44
2012	105	53	11.02	0.97	54	53	52	0.009	1.00
2013	143	62	12.29	0.95	74	78	65	1.18	0.32
2014	207	59	10.88	0.95	97	115	92	2.56	0.13
2015	302	51	8.76	0.92	71	122	180	11.13	0.001
χ^2 for homogeneity ^d								22.21	0.002

^a Margalef index was used to determine the race richness within each *L. maculans* population from year to year. The higher the value, the “richer” the race of a given population.

^b Simpson’s index was used to determine the allele race diversity within each *L. maculans* population from year to year. Values close to 1 indicate a high race diversity.

^c Number of isolates carrying the *MATI-1* and *MATI-2* alleles as determined via PCR. Ratio between both mating types determined as illustrated by χ^2 (χ^2 where *P* values less than 0.05 are significantly different than a 1:1 ratio).

^d χ^2 for homogeneity was determined to see whether the isolates collected from each year were behaving similarly in terms of mating type allele distribution (i.e., a 1:1 ratio between *MATI-1* and *MATI-2* alleles in all years). A χ^2 homogeneity value of 22.21 is >11.07 (1–0.05; df = 5), which indicates that the mating type allele distribution among the *L. maculans* populations from year to year are different than a 1:1 ratio.

and *Avr-1-4-5-7-11-LepRI-(S)* (9 and 6% of the 34 isolates collected, respectively) while the top two races detected in the Southwest area were *Avr-2-5-6-7-11-(S)* and *Avr-2-4-5-6-7-11-(S)* (15 and 7% of the 73 isolates collected, respectively). Isolates collected from the Pembina Valley area showed a top two race structure of *Avr-2-5-6-7-11* and *Avr-1-4-5-6-7-11-(S)* (14 and 11% of the 250 isolates collected, respectively; Table 5).

Within the nine Manitoban regions sampled, there was a high level of race diversity detected in the collected *L. maculans* isolates. With the exception of the Steinbach, Selkirk, and Swan Valley, for which the isolates collected had Simpson indices of 0.75, 0.88, and 0.84, respectively, the Simpson index in isolates collected in the other regions was ≥ 0.90 , indicating high genetic diversity within those regions (Table 4). The Margalef index values also indicated high race diversity in the *L. maculans* subpopulations in Manitoba, ranging from 6.41 to 21.50 (Table 4). Furthermore, the assortment of races found within each of the nine regions also illustrated high levels of diversity, where none of the regions sampled shared the exact same race structures.

Identification of mating type alleles in *L. maculans* populations and subpopulations. To better understand the long-term change of the pathogen populations, PCR products corresponding to one of the two mating type alleles were amplified from 956 *L. maculans* isolates tested. Both mating type alleles were present in the populations within all nine Manitoban regions examined (Table 4), indicating that isolates with the two mating alleles were well adapted to field conditions in Manitoba, Canada, and that mating type did not restrict sexual recombination. Overall, 449 *MAT1-1* alleles and 507 *MAT1-2* alleles were identified from 897 isolates. The ratio of the two mating type alleles did not appear to deviate significantly from a 1:1 ratio in isolates collected from 2011 to 2014 (Table 3). However, in *L. maculans* isolates collected in 2010 and 2015, χ^2 values of 6.652 ($P = 0.013$) and 11.13 ($P = 0.001$) were calculated, respectively, indicating that isolates collected within those years did not carry the *MAT1-1* and *MAT1-2* alleles in a 1:1 ratio. In fact, in both years the *MAT1-2* allele was more frequently detected in the collected isolates. χ^2 for homogeneity was also determined to see whether the isolates collected from each year were behaving similarly throughout the six

Table 4. Margalef index, Simpson index, and mating type alleles distribution in *Leptosphaeria maculans* subpopulations

Region ^a	No. of isolates	No. of races	No. of fields	Margalef index ^b	Simpson index ^c	<i>MAT1-1</i> ^d	<i>MAT1-2</i> ^d	χ^2	<i>P</i> value
A	43	29	17	7.44	0.75	27	16	2.81	0.13
B	34	25	20	7.37	0.88	15	19	0.47	0.61
C	31	23	18	6.41	0.84	11	20	2.61	0.15
D	88	47	39	10.27	0.97	36	52	2.91	0.11
E	94	48	33	10.34	0.95	46	48	0.04	0.92
F	250	117	101	21.01	0.97	124	123	0.004	1.00
G	113	65	49	13.54	0.96	43	70	6.45	0.01
H	73	50	40	11.42	0.90	34	39	0.34	0.64
I	230	118	102	21.50	0.96	113	117	0.07	0.84
						χ^2 for homogeneity ^e		15.71	0.003

^a *L. maculans* isolates were collected from canola crops obtained in nine Manitoba regions (as shown in Fig. 1): Areas are defined by letters and are as follows: A, Steinbach area; B, Selkirk area; C, Swan Valley; D, Dauphin; E, Central Plains; F, Pembina Valley; G, Roblin, Russell, Rossburn area; H, Southwest area; I, Brandon area.

^b Margalef index was used to determine the race richness within each *L. maculans* population from year to year. The higher the value, the “richer” the race of a given population.

^c Simpson’s index was used to determine the allele race diversity within each *L. maculans* population from year to year. Values close to 1 indicate a high race diversity.

^d Number of isolates carrying the *MAT1-1* and *MAT1-2* alleles as determined via PCR. Ratio between both mating types determined as illustrated by χ^2 (χ^2) where *P* values less than 0.05 are significantly different than a 1:1 ratio.

^e χ^2 for homogeneity was determined to see whether the isolates collected from each year were behaving similarly in terms of mating type allele distribution (i.e., a 1:1 ratio between *MAT1-1* and *MAT1-2* alleles). A χ^2 homogeneity value of 15.71 is >15.51 (1–0.05; df = 8), which indicates that the mating type allele distribution among the *L. maculans* populations from year to year are different from one another.

Table 5. The top five races identified in the collected *L. maculans* isolates from each of the nine Manitoban regions

No. of isolates ^a	Region A ^b	No. of isolates	Region B	No. of isolates	Region C
8 (18%)	<i>Avr-2-4-5-6-7-11-(S)</i>	3 (9%)	<i>Avr-2-4-5-7-11</i>	8 (26%)	<i>Avr-2-4-5-6-7-11</i>
7 (16%)	<i>Avr-2-4-5-6-7-11</i>	2 (6%)	<i>Avr-1-4-5-7-11-LepRI-(S)</i>	5 (6%)	<i>Avr-2-4-5-6-7-11-(S)</i>
6 (14%)	<i>Avr-1-4-5-6-7-11-(S)</i>	2 (6%)	<i>Avr-2-4</i>	1 (3%)	<i>Avr-null</i>
3 (7%)	<i>Avr-2-4-5-6-7-11-LepRI-(S)</i>	2 (6%)	<i>Avr-2-4-5-6-7-11</i>	1 (3%)	<i>Avr-1</i>
2 (5%)	<i>Avr-4-5-6-7-11</i>	2 (6%)	<i>Avr-2-4-5-6-7-11-LepRI-(S)</i>	1 (3%)	<i>Avr-1-2-4-7-9-LepRI-(S)</i>
No. of isolates	Region D	No. of isolates	Region E	No. of isolates	Region F
23 (26%)	<i>Avr-2-4-5-6-7-11-(S)</i>	25 (27%)	<i>Avr-2-4-5-6-7-11</i>	35 (14%)	<i>Avr-2-4-5-6-7-11</i>
22 (25%)	<i>Avr-2-4-5-6-7-11</i>	8 (9%)	<i>Avr-2-4-5-6-7-11-(S)</i>	27 (11%)	<i>Avr-1-4-5-6-7-11-(S)</i>
5 (6%)	<i>Avr-2-5-6-7-11</i>	8 (9%)	<i>Avr-2-5-6-11</i>	13 (5%)	<i>Avr-2-4-5-6-7-11-LepRI</i>
3 (3%)	<i>Avr-1-4-5-6-7-11-(S)</i>	3 (3%)	<i>Avr-1-4-5-6-7-11-(S)</i>	12 (5%)	<i>Avr-2-4-5-6-7-11-(S)</i>
3 (3%)	<i>Avr-2-5-6-7-11-(S)</i>	3 (3%)	<i>Avr-2-4-5-6-7</i>	11 (4%)	<i>Avr-4-5-6-7-11</i>
No. of isolates	Region G	No. of isolates	Region H	No. of isolates	Region I
15 (13%)	<i>Avr-2-4-5-6-7-11</i>	11 (15%)	<i>Avr-2-5-6-7-11-(S)</i>	27 (12%)	<i>Avr-2-4-5-6-7-11-(S)</i>
13 (12%)	<i>Avr-2-4-5-6-7-11-(S)</i>	5 (7%)	<i>Avr-2-4-5-6-7-11-(S)</i>	18 (8%)	<i>Avr-2-4-5-6-7-11</i>
9 (8%)	<i>Avr-2-5-6-7-11-(S)</i>	5 (7%)	<i>Avr-2-4-5-7-11</i>	17 (7%)	<i>Avr-2-5-6-7-11</i>
5 (4%)	<i>Avr-1-4-5-6-7-11-(S)</i>	4 (5%)	<i>Avr-2-5-6-7-11-LepRI-(S)</i>	17 (7%)	<i>Avr-2-5-6-7-11-(S)</i>
5 (4%)	<i>Avr-2-4-5-6-7-11-LepRI-(S)</i>	3 (4%)	<i>Avr-2-4-5-6-7-11-LepRI</i>	10 (4%)	<i>Avr-2-4-5-6-7-11-LepRI-(S)</i>

^a Number of each isolate collected within the region that harbors the race structure with percentage of the total collected isolates from each region represented in brackets.

^b The following letters represent the following Manitoba regions with total isolates collected in each of those regions in brackets: A, Steinbach area (43); B, Selkirk area (34); C, Swan Valley (31); D, Dauphin (88); E, Central Plains (94); F, Pembina Valley (250); G, Roblin, Russell, Rossburn area (113); H, Southwest area (73); I, Brandon area (230).

years in terms of mating type allele distribution (i.e., a 1:1 ratio between *MAT1-1* and *MAT1-2* alleles in all years). As shown in Table 3, the χ^2 homogeneity value calculated was 22.21, which is >11.07 (1–0.05; df = 5). This indicates that the mating type allele distribution of the *L. maculans* populations collected in 2010 to 2015 are not behaving similarly from year to year.

When the mating type distribution in the *L. maculans* subpopulation (i.e., by region) was analyzed by χ^2 test, the mating type ratio in isolates collected from eight of the nine regions did not appear to deviate from a 1:1 ratio (Table 4). However, in isolates collected from the Roblin, Russell, Rosburn area, a χ^2 value of 6.45 ($P = 0.01$) was calculated for that particular region, indicating that the isolates collected from this region appear to deviate from carrying the mating type alleles in a 1:1 ratio. In addition, the *MAT1-2* allele appears to be harbored more frequently in isolates collected from this region. χ^2 for homogeneity was determined to see whether the isolates collected from each region were behaving similarly from region to region in terms of mating type allele distribution (i.e., a 1:1 ratio between *MAT1-1* and *MAT1-2* alleles). As shown in Table 4, a χ^2 homogeneity value of 15.71 was calculated, which is >15.51 (1–0.05; df = 8). This indicates that the mating type allele distribution of the *L. maculans* subpopulation from region to region are behaving differently.

Discussion

This study reports on avirulence (*Avr*) and mating type allele frequency in *L. maculans* isolates collected from canola fields across nine regions in Manitoba, Canada, over a six-year period from 2010 to 2015. The presence of the *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm5*, *AvrLm6*, *AvrLm9*, *AvrLm11*, *AvrLepR1*, *AvrLepR2*, and the *AvrLmS* alleles was determined in 956 *L. maculans* isolates collected from 2010 to 2015 through pathogenicity testing, PCR, or a combination thereof. The PCR markers used for the three avirulence alleles without corresponding pathogenicity testing in this study (*AvrLm5*, *AvrLm6*, and *AvrLm11*) were shown to correspond to the phenotypic response as described in the cloning of the respective genes *AvrLmJ1/AvrLm5* (Van de Wouw et al. 2014; Balesdent and Howlett, unpublished data), *AvrLm6* (Fudal et al. 2007), and *AvrLm11* (Balesdent et al. 2013). Van de Wouw et al. (2010) also illustrated that a positive PCR product was correlated with the presence of *AvrLm1* and *AvrLm6* avirulence alleles and that large deletions in these genes, rendering them virulent, could be distinguished (Van de Wouw et al. 2010).

The results obtained from these assays indicated that the most predominant alleles identified in the collected *L. maculans* isolates each year were *AvrLm2*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7*, *AvrLm11*, and *AvrLmS* alleles. It has been reported that *L. maculans* isolates collected from 2010 and 2011 in western Canada (Alberta, Saskatchewan, and Manitoba) shared a similar genotype, where more than 85% of the isolate population carried both *AvrLm6* and *AvrLm7* (Liban et al. 2016). *AvrLm1* was also detected at low frequencies in the study by Liban et al. (2016), which is similar to the findings in this study. Here, the *AvrLm1* allele was identified in less than 28% of the isolates collected, and the frequency of this allele was highly variable among the nine Manitoban regions ranging from 3.8% in the Dauphin area to 33% in the Pembina Valley. There are likely influencing factors that attribute to the variability observed in the *AvrLm1* allele frequency in the *L. maculans* isolates collected from region to region. Agronomic practices, crop rotation, and selection pressure of *Rlm* genes carried in the host likely play an influential role in shaping the *Avr* allele profiles and contributing to the diversity of *Avr* alleles in the *L. maculans* isolates from region to region. It should also be noted that samples for this study were not obtained from the same field each year; blackleg infected canola crop stems from different fields within the same nine regions were collected and used to isolate *L. maculans*, which would also add variation to the frequency of *Avr* alleles.

From 2010 to 2015, the *L. maculans* isolates collected within each of the nine regions of Manitoba harbored the *AvrLm9*, *AvrLepR2*, and *AvrLm3* alleles less abundantly. When examining each of the nine

regions, the Dauphin area appeared to harbor isolates carrying *AvrLm9* and *AvrLepR2* alleles in less than 2% of the collected fungal population, while *AvrLm3* was not detected in any of the isolates tested within this region. The *AvrLm3* allele was not detected in isolates collected from crops in the Steinbach or Swan Valley regions, and identified in less than 2% of the population in isolates collected from the Central Plains, Pembina Valley, and Brandon areas. These findings are supported by the study conducted by Liban et al. (2016), where *L. maculans* isolates collected from Alberta, Saskatchewan, and Manitoba carried *AvrLm3*, *AvrLm9*, and *AvrLepR2* in less than 10% of the fungal population. A previous study conducted by Zhang et al. (2016) suggested that *Rlm3* was one of the most frequently used *R* genes in Canadian *B. napus* canola crop varieties. Consequently, the decrease in the frequency of the *AvrLm3* allele from 2010 to 2014 observed in this study may be explained by the strong selection pressure exerted by the presence of the *Rlm3* gene in Canadian canola varieties. In a study conducted by Liban et al. (2016), all collected *L. maculans* isolates (in western Canada) that carried the *AvrLm3* allele also carried *AvrLm9*. The authors suggested that the decline in *AvrLm9* frequency observed in this study may be due to negative selection against the *AvrLm3* allele and a potential linkage between *AvrLm3* and *AvrLm9*. Although it was not verified in this study if all collected isolates carrying *AvrLm3* also carried *AvrLm9*, a linkage between the alleles may explain why both alleles are found in such low frequencies.

A hide and seek relationship between *AvrLm3* and *AvrLm4-7* was reported by Plissonneau et al. (2016), where *L. maculans* isolates carrying *AvrLm4-7* also carried *AvrLm3* (but the function of *AvrLm3* was blocked by *AvrLm4-7*). A very recent paper by Plissonneau et al. (2017) demonstrated the “camouflaged model,” where the loss of *Rlm3*-mediated resistance response in isolates virulent toward *Rlm3* occurred by two methods. The first method was through deletion or inactivation mutations of *AvrLm4-7*, which resulted in virulent isoforms of the *AvrLm3* protein. The second method was through point mutations in *AvrLm4-7*, which allowed for suppression of the *AvrLm3* phenotype to be maintained. The identification of three race structures of *Avr-2-3-5-6-7-11-LepR1-(S)* and *Avr-2-3-5-6-7-9-11-LepR1-S* within the *L. maculans* population in this study suggests that other factors are influencing this interaction. The *AvrLm3* gene was recently cloned (Plissonneau et al. 2017), and collected *L. maculans* isolates from 2015 were subjected to PCR assays using *AvrLm3* specific primers. Of the 302 isolates collected in 2015, 288 isolates carried the *AvrLm3* allele despite the low frequency of the *AvrLm3* phenotype reported in this study. This supports the “camouflage” model (Plissonneau et al. 2017) since the majority of these isolates also carried *AvrLm4-7*, which was reported to mask the *AvrLm3* recognition by host *Rlm3*.

The race structure of the 956 isolates collected from 2010 to 2015 were evaluated, and three races, *Avr-2-4-5-6-7-11*, *Avr-2-4-5-6-7-11-(S)*, and *Avr-1-4-5-6-7-11-(S)*, were highly dominant. These findings indicate an uneven pathogen population, while the 170 races detected emphasizes the diversity. The stability of two major races *Avr-2-4-5-6-7-11* and *Avr-2-4-5-6-7-11-(S)* within seven of the nine regions in the *L. maculans* population over the six-year study period points toward lower rates of sexual recombination. Early studies in western Canada indicated that pycnidiospores were the major source of inoculum for *L. maculans* infection and reported a lack of sexual reproduction in the *L. maculans* populations (Dilmaghani et al. 2009; Ghanbarnia et al. 2011; Guo et al. 2005). However, there have been no large-scale analysis of mating type alleles in Canadian *L. maculans* populations. The current data in this study illustrates that *MAT1-1* and *MAT1-2* alleles in two of the six years as well as two of the nine regions (in all years) were significantly different from a 1:1 ratio, indicating that sexual reproduction may be restricted in some regions. Interestingly, the dominant stable race in the collected *L. maculans* isolates also suggests that the fungal population primarily undergoes asexual reproduction within Manitoba. However, there were some regions that did exhibit an equal distribution of both mating type alleles, illustrating that sexual reproduction may not be limited to these regions in Manitoba but that other environmental factors may attribute to *L. maculans* reproductive lifecycle.

The genetic diversity and complexity of the *L. maculans* population in western Canada has changed over time. Chen and Fernando (2006) reported the prevalence of PG2 isolates (carrying *AvrLm2* and/or *AvrLm3*; carrying *AvrLm1* and/or *AvrLm4*) in central Canada. Dilmaghani et al. (2009) reported an *AvrLm3* and *AvrLm9* allele frequency of 56.4% in collected fungal isolates in western Canada. However, the frequency in which the *AvrLm3* and *AvrLm9* allele was detected in *L. maculans* isolates appeared to decrease to 1.5% in recent years as reported by Liban et al. (2016). In our study, a total of 170 races were identified from 956 *L. maculans* isolates. Interestingly, the considerably diverse number of races found within each region in *L. maculans* suggests the potential for blackleg to affect canola production in Manitoba because each resistance gene examined in the study can be overcome by at least one of the pathogen races detected. In addition, isolates with race structure *Avr* null, *Avr-1*, *Avr-4*, *Avr-5*, *Avr-7*, and *Avr-11* were also detected in this study. Isolates with these race structures would contribute greatly to the disease progression as these isolates would likely be virulent toward most of the commercially available cultivars grown by local farmers. Simpson's evenness index, which measures relative frequency of races, were all relatively close to 1 throughout the years, suggesting a high risk of resistance breakdown within these regions of Manitoba. In addition, the pattern of increased disease incidence across the nine regions over the six-year period also suggested a potential breakdown in resistance; however, further studies are required to determine if there is an actual correlation between disease incidence and *Avr* allele frequency and if other factors such as weather conditions or changes in cropping rotations are contributing factors. The results reported here describe the avirulence allele profile of *L. maculans* populations in Manitoba, Canada. The regional *Avr* allele distribution observed in the *L. maculans* isolates collected in this study validates the notion that a single specific resistance gene may not provide effective control of blackleg across Manitoba. Therefore, it is necessary to understand the *Avr* genotype in the pathogen and the relationship between races and different resistance genes under selection pressure, as these are primarily the most important factors to consider when breeding for blackleg resistance in Canada.

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