Decreased Sensitivity of *Leptosphaeria maculans* to Pyraclostrobin in Alberta, Canada

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Abstract

Leptosphaeria maculans, the causal agent of blackleg of canola (*Brassica napus*), can be managed with pyraclostrobin and other strobilurin fungicides. Their frequent application, however, poses a risk for the development of insensitivity in fungal populations. A collection of *L. maculans* single-spore isolates recovered from infected canola stubble in Alberta, Canada, in 2016 was evaluated for its pyraclostrobin sensitivity. In conventional growth plate assays, the concentration of pyraclostrobin required to inhibit fungal growth by 50% (EC₅₀) was determined to be 0.28 mg/liter in a subset of 38 isolates. This EC₅₀ was four times greater than the mean EC₅₀ (0.07 mg/liter) of baseline isolates collected in 2011. Two hundred sixty-three isolates were screened further with two discriminatory doses of 0.28 and 3.5 mg/liter of pyraclostrobin, resulting in growth inhibition

values ranging from 16 to 82% and 41 to 100%, respectively. In microtiter plate assays with the same isolates, the mean EC₅₀ was determined to be 0.0049 mg/liter, almost 3.3 times greater than the mean EC₅₀ (0.0015 mg/liter) of the baseline isolates. The sensitivity of the isolates was also evaluated in microtiter plate assays with discriminatory doses of 0.006 and 0.075 mg/liter of pyraclostrobin, resulting in inhibition values ranging from 20 to 88% and 49 to 100%, respectively. This is the first report of isolates of *L. maculans* with increased insensitivity to pyraclostrobin in Canada, suggesting the need for improved fungicide stewardship.

Keywords: blackleg of canola, fungicide resistance, sensitivity, *Leptosphaeria maculans*, pyraclostrobin

Blackleg (Phoma stem canker) caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not. is the most economically important disease of canola (syn. rapeseed, *Brassica napus* L.) worldwide (Fitt et al. 2006; Howlett 2004; West et al. 2001). The disease can cause significant yield losses and is a major constraint to canola production (Gugel and Petrie 1992; Hwang et al. 2016). Blackleg emerged as a major production challenge for western Canadian canola growers in the 1970s and 1980s, with the first blackleg-resistant cultivars becoming available in the early 1990s (Kutcher et al. 2011). Blackleg-resistant cultivars enabled farmers to manage the disease quite successfully, particularly when these varieties were combined with a 4-year crop rotation (Kutcher et al. 2013). In recent years, however, high economic returns, market opportunities, and cultivar improvements have led to the more intense production of canola and shorter crop rotations in western Canada (Kutcher et al. 2013). From 2010 to 2015, blackleg incidence in this region increased (Zhang and Fernando 2017), resulting in renewed interest in the management of this disease (Kutcher et al. 2011).

On the prairies of western Canada (Alberta, Saskatchewan, and Manitoba), fungicides represent an important tool in blackleg management. There are three registered active ingredients, including propiconazole, azoxystrobin, and pyraclostrobin (Canola Council of Canada 2017). Azoxystrobin and pyraclostrobin are broad-spectrum strobilurin fungicides. The strobilurins are single-site mode-of-action chemicals, also known as quinine outside inhibitors (QoIs) (Bartlett et al. 2002). These fungicides bind to the Qo site of the cytochrome *bc1* enzyme complex (complex III) of the mitochondria, inhibiting mitochondrial respiration (Gisi et al. 2002). This prevents the formation of adenosine triphosphate and results in an energy deficiency in

the fungus (Gisi et al. 2002). Strobilurin fungicides are particularly effective at inhibiting spore germination, which is an energy-demanding stage of fungal development (Bartlett et al. 2002). Inhibition of the respiratory electron transport chain also results in the build-up of reactive oxygen species, which are very damaging to cells and hence contribute to fungal control (Inoue et al. 2012).

Pyraclostrobin has been used widely on various crops in Canada since 2002 (Health Canada 2003; Government of Canada, Health Canada Pest Management Regulatory Agency 2011) and is the active ingredient in the commonly applied fungicide Headline EC (pyraclostrobin, 250 g/liter; BASF 2018). This product was registered on canola in 2010 and was first used in 2011. Because of the site-specific activity of pyraclostrobin, G143A, G137R, and F129L mutations in cytochrome *b* can result in the appearance of fungicide-resistant or -tolerant fungal strains (Vincelli 2002). In the case of *L. maculans*, no pyraclostrobin resistance or specific mutation has been reported to date. The repeated application of pyraclostrobin could, however, select for a fungicide-resistant subpopulation of *L. maculans*, as has been reported for other fungal pathogens (Forcelini et al. 2016; Vincelli 2002).

Fraser et al. (2017) examined the pyraclostrobin sensitivity of a collection of *L. maculans* isolates made from Alberta in 2011, the year following the registration of this fungicide on canola. All of the isolates tested in this earlier study were sensitive to pyraclostrobin, although the potential for future shifts in sensitivity was noted (Fraser et al. 2017). Periodic monitoring is important for proper fungicide stewardship and ensuring continued product efficacy. In this context and in the present study, the fungicide sensitivity of an *L. maculans* collection from Alberta made in 2016, after 5 years of pyraclostrobin application on canola, was investigated in growth plate and microtiter plate assays.

Materials and Methods

Collection and preparation of single-spore isolates. A collection of 251 *L. maculans* single-spore isolates was obtained from infected canola stubble from 18 fields surveyed throughout Alberta in 2016. Stubble was collected from a 1-m² area at each of five points along the arms of 'W' sampling pattern in each field, with the sampling points spaced at least 5 m apart. Single-spore isolations were made following the method of Rong et al. (2015) and stored as pycnidiospores in 1.5-ml microcentrifuge tubes at -80°C in sterilized 20% glycerol.

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The identity of these isolates as *L. maculans* was confirmed by means of a polymerase chain reaction assay with β -tubulin-based primers (Rong et al. 2015). Twelve single-spore isolates obtained from infected canola stubble in Alberta in 2011 (Fraser et al. 2017) were also included in this study and regarded as baseline isolates.

Conventional growth plate assays. *Determining the mean EC_{50} .* A subset of 38 *L. maculans* isolates collected in 2016 was randomly selected from the entire collection and used to determine the effective concentration of pyraclostrobin required to inhibit mycelial growth by 50% (EC_{50}). The EC_{50} of the 12 isolates collected in 2011, previously analyzed by Fraser et al. (2017), also was determined. To revive the isolates, stored pycnidiospores were removed from storage in the microcentrifuge tubes and placed on Petri dishes (10-cm diameter) filled with V8 growth medium (composition per liter: 850 ml of distilled water, 150 ml of V8 Original Vegetable Juice, 1.5 g of $CaCO_3$, and 15.0 g of agar). After 10 days, 6-mm plugs of the developing colonies were transferred to fresh V8 medium amended with different concentrations of pyraclostrobin. Briefly, a fungicide stock solution was prepared by adding 1 ml of Headline 250 EC (pyraclostrobin, 250 g/liter; BASF Canada, Mississauga, ON) to 49 ml of sterilized distilled water, and it was used to obtain final concentrations of active ingredient in the amended plates of 0, 0.0125, 0.025, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, and 0.875 mg/liter by serial dilution. The appropriate amounts of fungicide were added shortly after autoclaving when the medium had cooled to 50 to 60°C. Although Fraser et al. (2017) did not report any significant difference between the EC_{50} values of isolates tested in the presence or absence of salicylhydroxamic acid (SHAM), 0.1 g/liter of this chemical was included in the present assays as a precaution against alternative respiration (Wood and Hollomon 2003). The Petri dishes were placed under fluorescent lighting at $20 \pm 2^\circ C$ for a period of 10 days, at which time two orthogonal measurements of colony diameter (growth) were made using a digital caliper and averaged. The experiment was arranged in a completely randomized design with five replicates (Petri dishes) per treatment and was repeated independently. The percentage of radial growth inhibition relative to the unamended control was determined using the following equation: [(average growth on unamended medium – growth on amended medium)/average growth on unamended medium] $\times$ 100.

*Testing the pyraclostrobin sensitivity of *L. maculans* isolates.* To evaluate the sensitivity of *L. maculans*, two discriminatory doses of pyraclostrobin were used to screen a collection of 251 single-spore isolates from 2016 and 12 single-spore isolates from 2011. The dosages were 4 $\times$ and 50 $\times$ the EC_{50} value of the 12 isolates from 2011. Therefore, V8 growth medium containing pyraclostrobin at 0.28 or 3.5 mg/liter and SHAM at 0.1 g/liter was used to determine the sensitivity of the examined fungal isolates. Inoculation of the cultures and preparation of the medium in Petri dishes were as described above. The Petri dishes were incubated under fluorescent lighting at $20 \pm 2^\circ C$ for 10 days. Two orthogonal measurements of the mycelial growth of each isolate were made and averaged using a digital caliper, with inhibition of radial growth expressed as a percentage of the unamended controls as described above. The treatments included four replicates (Petri dishes) for each isolate plus four control replicates that did not contain any fungicide. Treatments were arranged in a completely randomized design, and the experiment was independently repeated.

*Testing minimum inhibitory dose of *L. maculans* isolates.* An isolate found to have a growth inhibition value of <50% was evaluated further with two sets of discriminatory doses. The first set included doses of 4.9, 7, and 10.5 mg/liter of pyraclostrobin, equivalent to 70 $\times$, 100 $\times$, and 150 $\times$ the mean EC_{50} of the isolates collected in 2011, respectively. The second set of discriminatory doses included 14, 19.6, 28, and 42 mg/liter of pyraclostrobin, equivalent to 50 $\times$, 70 $\times$, 100 $\times$, and 150 $\times$ the EC_{50} of the isolates collected in 2016. Tests were conducted as above in V8 medium containing SHAM at 0.1 g/liter. Similarly, two orthogonal measurements of the mycelial growth of each isolate were made and averaged using a digital caliper, with inhibition of radial growth expressed as a percentage of the unamended controls as described earlier. The treatments included four replicates (Petri dishes) for each isolate plus four control replicates that did not contain any fungicide.

Microtiter plate assays. *Determining the mean EC_{50} .* The same isolates used in the conventional growth plate assays were included in the microtiter plate assays. Fifty isolates including 12 isolates collected in 2011 and 38 isolates collected in 2016 were used to determine the mean EC_{50} . In order to increase spore density, the spores were collected and immersed in YBA medium (composition per liter

Fig. 1. Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 33 isolates of *Leptosphaeria maculans* collected in 2016 and 12 isolates collected in 2011 from Alberta, Canada. Values were determined based on conventional growth plate assays. Individual isolates are grouped in class intervals of 0.05 mg/liter.

of sterilized water: 20 g of yeast extract, 20 g of Bacto peptone, and 40 g of sodium acetate). Streptomycin (100 mg/liter) was added to the medium to avoid bacterial contamination. YBA medium is relatively low in nutrients, so the fungus cannot gain sufficient energy to undergo alternative respiration while still obtaining sufficient nutrients for fungal growth (Spiegel and Stammer 2006). Hence, SHAM was not included in the YBA medium. The fungal spore concentration was adjusted to 2×10^4 spores/ml. Fifty microliters of the spore suspension, containing about 1,000 spores, was transferred into each well of a 96-well microtiter plate, which contained 50 μ l of the fungicide solution of different concentrations prepared in YBA medium. Formulated Headline 250 EC fungicide was diluted to achieve the

Table 1. The effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in a conventional growth plate assay for 12 isolates of *Leptosphaeria maculans* originally collected in 2011 (Fraser et al. 2017)

Isolate	EC_{50} values determined in the current study ^a	EC_{50} values determined by Fraser et al. (2017)
11C78103	0.02	0.07
11C78301	0.05	0.05
11C78384	0.03	0.02
11C78401	0.09	0.04
11P125062	0.07	0.13
11P125232	0.10	0.08
11P125384	0.08	0.04
11S54201	0.05	0.04
11S54041	0.09	0.03
11W189011	0.11	0.17
11L999014	0.05	0.17
11L999241	0.06	0.24
Average	0.07	0.09

^a A paired *t* test indicated that there was no significant difference between the mean EC_{50} values determined by Fraser et al. (2017) and in the current study ($P = 0.29$).

end concentrations, with the EC_{50} determined based on 12 concentrations of pyraclostrobin: 0 (control), 0.0005, 0.001, 0.0015, 0.002, 0.0025, 0.003, 0.005, 0.01, 0.015, 0.05, and 0.15 mg/liter. The combined solution was mixed thoroughly in the microtiter plate. Experiments were arranged in a completely randomized design with four replicate wells assigned for each fungicide concentration/isolate combination. The entire experiment was independently repeated. The plates were incubated for 7 days at 18°C in darkness. The endpoint optical density of pycnidiospore germination was measured with a spectrophotometer (BioTek, Winooski, VT) at a wavelength of 405 nm for the initial and final readings. For each isolate and concentration, final values for absorbance were obtained by subtracting the values of the initial reading from those of the final reading, and growth inhibition was calculated as $1 - (\text{absorption for each treatment/absorption of control})$ and presented as a percentage.

Testing the pyraclostrobin sensitivity of *L. maculans* isolates. Two discriminatory doses of pyraclostrobin were used to screen 251 single-spore isolates from 2016 and 12 single-spore isolates from 2011. To be consistent with the conventional growth plate assays, the discriminatory doses were 4 $\times$ and 50 $\times$ the EC_{50} value obtained for the isolates collected in 2011. Therefore, medium containing concentrations of 0 (control), 0.006, and 0.075 mg/liter of pyraclostrobin were evaluated. Briefly, after an incubation period of 7 days at 18°C in darkness, fungal density was measured at a wavelength of 405 nm. The growth inhibition was calculated as above. The experiment was arranged in a completely randomized design with treatments replicated four times for each isolate, and the entire experiment was repeated.

Testing minimum inhibitory dose of *L. maculans* isolates. An isolate with a growth inhibition value ~50% in the microtiter plate assays was evaluated further with two sets of discriminatory doses (this was the same isolate that had a growth inhibition value <50% in the growth plate assays). The first set included doses of 0.105, 0.15, and 0.225 mg/liter of pyraclostrobin, corresponding to 70 $\times$, 100 $\times$, and 150 $\times$ the EC_{50} of the isolates collected in 2011, respectively. The second set included 0.245, 0.343, 0.49, and

Fig. 2. Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC_{50}) in 38 isolates of *Leptosphaeria maculans* collected in 2016 and 12 isolates collected in 2011 from Alberta, Canada. Values were determined based on microtiter plate assays. Individual isolates are grouped in class intervals of 0.005 mg/liter.

0.735 mg/liter of pyraclostrobin, equivalent to 50 $\times$, 70 $\times$, 100 $\times$, and 150 $\times$ the EC₅₀, respectively, of the isolates collected in 2016. The assays were conducted as described above for the other microtiter plate assays, with fungal density and growth inhibition also determined in the same manner. The treatments included four replicates for each isolate plus four control replicates that did not contain any fungicide.

Data analysis. Data were analyzed with R: A Language and Environment for Statistical Computing (R Core Team 2013). The normality of the EC₅₀ values for 50 *L. maculans* isolates (12 isolates from 2011 and 38 isolates from 2016) was evaluated with the Shapiro-Wilk test using the statistical package shapiro.test. The homogeneity of variance was tested using the Levene test (LeveneTest package). To assess the EC₅₀ concentration, a log₁₀-transformed fungicide concentration was employed to linearize fungal growth inhibition data, and a regression line was calculated. The EC₅₀ values for each isolate were calculated accordingly, and all values were averaged to obtain the mean EC₅₀. The mean EC₅₀ values of the isolates

collected in 2016 and 2011 were compared with a two-sided *t* test. In order to ensure the reproducibility of the data, the mean EC₅₀ values of the isolates from 2011, as determined in the current study, were compared with the corresponding values calculated by Fraser et al. (2017) using a two-sided paired *t* test. The mean growth inhibition values of all 263 isolates included in the discriminatory dose experiments (growth plate and microtiter plate assays) are displayed in histograms. The distribution of growth inhibition of the 263 isolates in the growth plate and microtiter plate assays was compared using the Kolmogorov-Smirnov test.

Results

Determination of EC₅₀ values of baseline isolates. *Conventional growth plate assays.* In the conventional growth plate assays, the EC₅₀ values of the 12 isolates collected in 2011 ranged from 0.02 to 0.11 mg/liter, with a mean of 0.07 mg/liter (Fig. 1; Table 1). A two-sided paired *t* test revealed no significant difference between

Fig. 3. Frequency distribution of the inhibition of mycelial growth in 263 *Leptosphaeria maculans* isolates tested with a discriminatory dose of pyraclostrobin (0.28 mg/liter) representing 4 $\times$ the EC₅₀ for baseline isolates collected in 2011 (**A**), and a discriminatory dose of pyraclostrobin (3.5 mg/liter) representing 50 $\times$ the EC₅₀ for baseline isolates collected in 2011 (**B**), based on conventional growth plate assays. The inhibition of mycelial growth is expressed as a percentage relative to a control treatment with no pyraclostrobin included.

EC₅₀ values calculated in the current study and those calculated by Fraser et al. (2017) for the same set of isolates ($P = 0.29$) (Table 1). The EC₅₀ values of the 38 isolates collected in 2016 ranged from 0.03 to 0.64 mg/liter, with a mean of 0.28 mg/liter (Fig. 1). Five of these isolates had predicted EC₅₀ values greater than the maximum concentration of fungicide employed; therefore, to avoid out-of-range estimates, their estimated EC₅₀ values were excluded from the calculation of the mean EC₅₀ for the 2016 isolates (and only the remaining 33 isolates were used).

Microtiter plate assays. In the microtiter plate assays, the EC₅₀ values of the *L. maculans* isolates collected in 2011 ranged from 0.0001 to 0.0066 mg/liter, with a mean of 0.0015 mg/liter (Fig. 2) and a left-skewed distribution. The EC₅₀ values of the isolates collected in 2016 ranged from 0.0002 to 0.0489 mg/liter, with a mean of 0.0049 mg/liter (Fig. 2).

Pyraclostrobin sensitivity of *L. maculans* isolates. Conventional growth plate assays. Two hundred fifty-one *L. maculans* isolates

collected from Alberta in 2016, along with another 12 isolates collected in 2011, were assessed for pyraclostrobin sensitivity. When tested with the lower (0.28 mg/liter) of the two discriminatory doses, growth inhibition of the 263 isolates ranged from 15.7 to 82%, relative to the control treatment (no pyraclostrobin). Ninety-four (36%) of the isolates had a growth inhibition <50%, 91 (35%) had a growth inhibition of 50 to <60%, 63 (24%) isolates had a growth inhibition of 60 to <70%, 14 (5%) had a growth inhibition of 70 to <80%, and one isolate had a growth inhibition of 80 to <90%. No isolate had a growth inhibition >90% (Fig. 3A). The 12 isolates from 2011 all had a growth inhibition >50%. When tested with the second (higher) discriminatory dose (3.5 mg/liter) of pyraclostrobin, one isolate had a growth inhibition of <50%, seven (3%) had a growth inhibition of 50 to <60%, 19 (7%) had a growth inhibition of 60 to <70%, 80 (30%) had a growth inhibition of 70 to <80%, 98 (37%) had a growth inhibition of 80 to <90%, 55 (21%) had a growth inhibition of 90 to <100%, and three (1%) isolates had a growth inhibition of 100%

Fig. 4. Frequency distribution of the inhibition of mycelial growth in 263 *Leptosphaeria maculans* isolates tested with a discriminatory dose of pyraclostrobin (0.006 mg/liter) representing 4× the EC₅₀ for baseline isolates collected in 2011 (**A**), and a discriminatory dose of pyraclostrobin (0.075 mg/liter) representing 50× the EC₅₀ for baseline isolates collected in 2011 (**B**), based on microtiter plate assays. The inhibition of mycelial growth is expressed as a percentage relative to a control treatment with no pyraclostrobin included.

(Fig. 3B). The growth inhibition of the 263 isolates ranged from 40.9 to 100%, relative to controls that had not been treated with pyraclostrobin. The 12 isolates from the 2011 collection were all in the highly sensitive category (inhibition >90%).

Microtiter plate assays. In the presence of 0.006 mg/liter of pyraclostrobin (4× the mean EC₅₀ of the *L. maculans* isolates collected in 2011), growth inhibition of the 263 isolates ranged from 20 to 87.8%, relative to the control treatment with no pyraclostrobin. Eighty-one (31%) isolates had a growth inhibition of <50%, 94 (36%) had a growth inhibition of 50 to <60%, 61 (23%) had a growth inhibition of 60 to <70%, 18 (7%) had a growth inhibition of 70 to <80%, and nine (3%) isolates had a growth inhibition of 80 to <90%. No isolates had a growth inhibition >90% (Fig. 4A). At the higher discriminatory dose of 0.075 mg/liter of pyraclostrobin (50× the EC₅₀ of the isolates collected in 2011), seven (3%) isolates had a growth inhibition of 50 to <60%, 19 (7%) had a growth inhibition of 60 to <70%, 72 (27%) had a growth inhibition of 70 to <80%, 103 (39%) had a growth inhibition of 80 to <90%, and 62 (24%) isolates had a growth inhibition of 90 to <100% (Fig. 4B). Growth inhibition ranged from 48.8 to 99.5% across the 263 isolates, relative to the control treatment with no pyraclostrobin.

A comparison, using the Kolmogorov-Smirnov test, of the distribution of growth inhibition of the 263 isolates in the conventional growth plate versus microtiter plate assays indicated that there was no significant difference ($P = 0.22$) at the 4× EC₅₀ discriminatory dose, with the data left-skewed in both assays. At the 50× EC₅₀ discriminatory dose, the results from both assays exhibited a right-skewed distribution and also were not significantly different ($P = 0.72$).

Minimum inhibitory concentration of *L. maculans* isolates. The pyraclostrobin sensitivity of isolate 16CW239002, which had growth inhibition values of <50% and ~50%, respectively, in the growth plate and microtiter plate assays, was evaluated further with higher concentrations of the fungicide. In a growth plate assay to evaluate the minimum inhibitory concentration, the percent growth inhibition of this isolate increased to >50% at 4.9 mg/liter of pyraclostrobin (70× the EC₅₀ of the isolates collected in 2011), and it did not grow at 19.6 mg/liter of pyraclostrobin (280× and 70× the EC₅₀ of the isolates from 2011 and 2016, respectively) (Table 2). Similarly, in a microtiter plate assay to evaluate the minimum inhibitory concentration of this isolate, the percent growth inhibition increased to >50% at 0.105 mg/liter of pyraclostrobin (70× the EC₅₀ of the isolates collected in 2011) and to 100% at 0.343 mg/liter of pyraclostrobin (230× and 70× the EC₅₀ of the isolates from 2011 and 2016, respectively) (Table 2).

Discussion

Given the high risk for the development of pyraclostrobin resistance, numerous studies have evaluated the sensitivity of different phytopathogens to this fungicide. Pyraclostrobin-resistant isolates of *Mycosphaerella pinodes* were reported from infected field pea crops in two provinces of Canada and two states of the United States (Bowness et al. 2016). Akhavan et al. (2017) also identified an isolate of *Pyrenophora teres* f. *maculata* (net blotch of barley) from Saskatchewan with decreased sensitivity to pyraclostrobin. In contrast, isolates of *Phytophthora cactorum* (Lebert and Cohn) Schröeter from infected strawberry in the United States (Rebollar-Alviter et al. 2007) and isolates of *Pyrenophora tritici-repentis* (Died.) Drechsler from infected wheat in Canada (MacLean et al. 2017) showed no decrease in sensitivity to this compound. In Canada, pyraclostrobin was first registered on cereals, pulses, and vegetables in 2001 and then on canola in 2010 (Government of Canada, Health Canada Pest Management Regulatory Agency 2011). Limited information is available, however, on the pyraclostrobin sensitivity of *L. maculans* populations in Alberta. Fraser et al. (2017) evaluated the sensitivity in a collection of 117 *L. maculans* isolates from Alberta in 2011 but did not find any evidence of insensitivity to pyraclostrobin at that time.

Isolates with no history of exposure to a fungicide are typically used as a baseline in fungicide sensitivity studies. The isolates characterized by Fraser et al. (2017) were collected in the first year that

pyraclostrobin was registered for blackleg control on canola, and hence there is some possibility that these isolates may have been exposed to the fungicide. Nonetheless, because no *L. maculans* isolates collected prior to the registration of pyraclostrobin in Alberta are available, the same baseline isolates used by Fraser et al. (2017) were included in the current study, along with 38 isolates randomly selected from a collection of *L. maculans* isolates made from Alberta in 2016. A two-sided paired *t* test indicated that the EC₅₀ values obtained by Fraser et al. (2017) were not statistically different from those calculated in this study for the same set of isolates ($P = 0.29$). The mean EC₅₀ for the 2016 isolates was 4× greater than for the 2011 isolates. Moreover, testing with a discriminatory dose of pyraclostrobin 4× greater than the mean EC₅₀ of the 2011 isolates found that approximately a third (80) of the isolates were inhibited <50%; at a discriminatory dose 50× greater than the mean EC₅₀, one isolate was inhibited <50%. These results suggest that the pyraclostrobin sensitivity of *L. maculans* isolates in Alberta decreased from 2011 to 2016. Repeated use of a fungicide with the same mode of action could lead to the development of insensitivity or resistance in fungal populations (Brent and Hollomon 2007). Given that the isolates collected in 2016 may have been exposed to pyraclostrobin several more times than those collected in 2011, it is likely that the selection pressure imposed by the frequent application of this product resulted in an increase in the proportion of the *L. maculans* population exhibiting insensitivity. Differences in fungicide sensitivity also may be influenced by the inherent diversity in isolates from different fungal populations across a region (Kutcher et al. 2007) and/or different fungicide application regimes (Kutcher et al. 2011).

Five of the *L. maculans* isolates used to determine the mean EC₅₀ of the 2016 fungal collection via the growth plate assay had predicted EC₅₀ values greater than the maximum concentration of pyraclostrobin employed and, therefore, were excluded from the calculation of the final mean EC₅₀. This may have led to a slight underestimation of the mean EC₅₀ for the 2016 isolates, further supporting the conclusion that this value had increased for *L. maculans* between 2011 and 2016. The EC₅₀ values obtained with the microtiter plate assay were lower than those calculated based on the conventional growth plate assay. QoIs have a direct effect on spore germination (Olaya et al. 1998), so it is expected that the EC₅₀ values would be much lower in a microtiter plate assay compared with a growth plate assay (Bartlett et al. 2002). Because pyraclostrobin mainly suppresses conidial germination, the microtiter plate assay is likely the preferred and most sensitive method for determining the sensitivity of *L. maculans* isolates to this fungicide (Patel et al. 2012; Wise et al. 2008). Nonetheless, growth inhibition of the isolates at the two discriminatory doses evaluated, in both the conventional growth plate and microtiter plate assays, was not significantly different.

Bowness et al. (2016) observed that isolates of *M. pinodes* on field pea had extreme insensitivity to pyraclostrobin with mean EC₅₀ values that were nearly 1,500× greater than those of sensitive isolates. Based on those observations, they suggested that qualitative insensitivity to pyraclostrobin occurs in *M. pinodes* (Bowness et al. 2016). Akhavan et al. (2017) reported that although some *P. teres* f. *maculata* isolates were insensitive to pyraclostrobin, a dosage 23× the

Table 2. The minimum inhibitory concentration of pyraclostrobin needed to inhibit mycelial growth (%) of *Leptosphaeria maculans* isolate 16CW239002 in conventional growth plate and microtiter plate assays

Conventional growth plate assays		Microtiter plate assays	
Concentration (mg/liter)	Percent growth inhibition	Concentration (mg/liter)	Percent growth inhibition
4.9	71.83%	0.105	75.39%
7	87.55%	0.15	80.4%
10.5	92.21%	0.225	94.32%
14	98.81%	0.245	98.97%
19.6	100.00%	0.343	100.00%
28	100.00%	0.49	100.00%
42	100.00%	0.735	100.00%

average EC₅₀ could still inhibit growth of all isolates by >50%. This led these researchers to conclude that the *P. teres* f. *maculata* isolates had quantitative insensitivity to pyraclostrobin (Akhavan et al. 2017). Based on the current results, only one isolate (16CW239002) appeared to be insensitive to pyraclostrobin. Nonetheless, this isolate failed to grow at a discriminatory dose 70× the EC₅₀ of isolates collected in 2016 (280× and 230× the EC₅₀ of the isolates from 2011 in growth plate and microtiter plate assays, respectively), suggesting this isolate may be tolerant or quantitatively resistant to pyraclostrobin. Three amino acid substitutions, G143A, G137R, and F129L in cytochrome *b* may contribute to resistance or tolerance to QoI fungicides (Grasso et al. 2006; Sierotzki and Frey 2007). Further studies are necessary to determine whether any of the known mutation(s) are associated with this observed insensitivity. Indeed, because the mutations associated with pyraclostrobin insensitivity in *L. maculans* have not been characterized to date, the molecular basis underlying the observed insensitivity should be explored.

To the best of our knowledge, this is the first report of *L. maculans* isolates exhibiting decreased sensitivity to pyraclostrobin. Nonetheless, although insensitivity to pyraclostrobin has not been reported previously in this pathogen, Van de Wouw et al. (2017) found that the sensitivity of Australasian isolates of *L. maculans* to fluquinconazole has also decreased. This suggests the potential for multiple resistance issues in this important fungus and highlights the need for judicious use of fungicides in the management of blackleg of canola. Strategies such as the rotation of fungicides with different modes of action, along with the adoption of cultural and other control methods as part of an integrated disease management plan, will be important for the sustainable and long-term mitigation of blackleg.

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