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Sensitivity of *Leptosphaeria maculans* to pyraclostrobin in Alberta, Canada

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Strobilurin fungicides can be used to manage blackleg (*Leptosphaeria maculans*) of canola (*Brassica napus*); however, they have a high risk of selecting for resistance in fungal populations. In 2011, single-spore isolates of *L. maculans* were obtained from infected canola stubble representing six fields in Alberta, Canada. The isolates were grown on fungicide-amended agar plates and percent growth inhibition was calculated based on colony diameter relative to non-amended controls. Using probit regression analysis, the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC₅₀) was determined to be 0.09 mg L⁻¹ based on 13 isolates. To identify highly insensitive isolates, 117 isolates were grown on agar plates amended with a discriminatory dose of 6.25 mg L⁻¹. Isolates in which growth was inhibited <50% were considered insensitive. Growth inhibition values ranged from 66.6% to 100.0% with a mean of 84.3%, indicating that all of the isolates were sensitive to pyraclostrobin. Forty-one of the 117 isolates also were tested via a microtiter plate assay. Based on conidial germination and subsequent growth, the EC₅₀ values obtained for each isolate ranged from 1.0×10^{-4} mg L⁻¹ to 7.4×10^{-3} mg L⁻¹, which were not significantly different from those of the sensitive controls (4.1×10^{-3} mg L⁻¹ and 5.7×10^{-3} mg L⁻¹). While pyraclostrobin insensitivity was not detected in *L. maculans* populations from Alberta, continued monitoring will be necessary to track future changes.

Key words: *Leptosphaeria maculans*, blackleg, *Brassica napus*, canola, pyraclostrobin, fungicide sensitivity

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.) is an ascomycete fungus belonging to the order Pleosporales and causes blackleg disease (synonym: *Phoma* stem canker) in canola rapeseed (*Brassica napus* L.) (Rouxel and Balesdent 2005). Blackleg is one of the most serious diseases of canola in western Canada and caused significant yield losses in the 1970s and 1980s, when canola production expanded (Gugel and Petrie 1992). In recent years, the intensive production of canola and lack of diverse crop rotations has led to an increase in blackleg disease in Alberta (Kutcher et al. 2011).

There are very few fungicidal active ingredients available for the management of blackleg in canola in Alberta (Alberta Government 2015), one of which is the broad spectrum fungicide pyraclostrobin. Pyraclostrobin has been used on various crops since 2003 as the foliar treatment Headline EC (pyraclostrobin, 250 g L⁻¹; BASF Canada Inc., Mississauga, ON). Headline EC was registered on canola in 2010 and first used in 2011, while in 2012 the active ingredient pyraclostrobin was registered as a seed treatment in combination with boscalid (Government of Canada: Health Canada Pest Management Regulatory Agency 2011, 2012). Pyraclostrobin is a strobilurin fungicide, also known as a quinone outside inhibitor (QoI) (Balba 2007). Strobilurin fungicides have single-site activity and inhibit mitochondrial respiration in fungi. Specifically, they act on the Qo site located on the cytochrome bc1 enzyme complex (complex III) of the mitochondria (Gisi et al. 2002). By interrupting the activity in the electron transport chain, QoI fungicides deprive the fungus of energy by preventing the formation of ATP (Gisi et al. 2002) and cause a build-up of electrons in the mitochondria (Inoue et al. 2012). The overabundance of electrons results in the formation of reactive oxygen species (ROS) that damage the mitochondria and contribute to the death of the fungus (Inoue et al. 2012). Strobilurin fungicides, such as pyraclostrobin, have protectant and translaminar activity and are very

effective at inhibiting spore germination, which is an energy demanding stage in the fungal lifecycle (Bartlett et al. 2002).

Strobilurin fungicides are one of the most widely applied fungicide classes worldwide (Balba 2007). However, because of their site-specific activity, they have a high risk of selecting for resistant individuals within a fungal population (Gullino et al. 2000). There are two types of fungicide resistance: polygenic, which results in a gradual shift towards insensitivity due to an interaction of many mutant genes, and major gene resistance, which leads to complete insensitivity as a result of a mutation in one gene (Georgopoulos and Skylakakis 1986). With intensive use and repeated sprays of fungicides from the same class, resistant individuals will be selected and the proportion of resistant individuals will increase. Therefore, fungicide applications will become less effective or eventually ineffective at controlling the disease (Georgopoulos and Skylakakis 1986). Strobilurin resistance is associated with a point mutation in the cytochrome b gene, which leads to an amino acid substitution of glycine with alanine at position 143 (G143A) in the cytochrome b protein (Kim and Hwang 2007). This mutation has been identified in isolates of various fungal species (FRAC 2013; Kim and Hwang 2007). Although less common than the G143A mutation, a second mutation associated with strobilurin resistance is the substitution of phenylalanine with leucine at position 129 (F129L) of the cytochrome b protein, which has been identified in *Pyrenophora teres* Drechs., *Alternaria solani* Kuhn (FRAC 2013), *Pythium aphanidermatum* Edson (Kim and Hwang 2007), and *P. grisea* (Ma and Michailides 2005).

To ensure fungicide resistance management strategies are effective and gain insight into possible resistance issues, it is important to monitor the sensitivity of pathogens to frequently used fungicides (Brent and Hollomon 2007). Molecular methods, such as allele-specific real-

time PCR, are commonly used to detect specific mutations responsible for fungicide resistance (Ma and Michailides 2005). While allele-specific real-time PCR is rapid and highly sensitive, its specificity means it does not detect other mechanisms of resistance. In contrast, phenotypic bioassays can detect the presence of any fungicide resistant genotypes (Vega et al. 2012). Two examples of phenotypic bioassays for fungicide resistance include the conventional mycelial growth plate assay and the microtiter plate assay. When using these methods, the concentration at which fungal growth is effectively inhibited by 50% (EC_{50}) must be determined for a set of isolates, which serves as a sensitivity baseline relative to the pathogen population being assessed. With the growth plate assay, this involves plating the isolates on growth medium amended with different concentrations of the fungicide and measuring inhibition of colony growth. Isolates that have never been exposed to the fungicide are typically used to determine the EC_{50} in order to detect changes in sensitivity within the population being assessed. It is sometimes difficult, however, to obtain such isolates if they were not collected prior to the introduction of the fungicide (Russell 2004). The EC_{50} dose may be used in the future to monitor and detect any shifts in sensitivity of the pathogen population (Russell 2004). The microtiter method involves growing the pathogen in multi-well plates that contain liquid medium amended with various concentrations of the fungicide. A growth indicator dye is added to the wells and spore germination and subsequent growth is measured by monitoring color change with a spectrophotometer (Brent and Hollomon 2007).

Although pyraclostrobin has been registered only recently for the control of blackleg on canola, there are concerns regarding the frequency of resistant isolates initially present in the pathogen population when the fungicide became commercially available. While most canola cultivars grown in Alberta have moderate to high resistance to blackleg, there is always a risk

that genetic resistance will be overcome, especially if the same cultivar is planted repeatedly in short rotations (Marcroft et al. 2012). If genetic disease resistance becomes ineffective, growers may become more dependent on fungicides for blackleg management. Therefore, it is important to provide a current baseline assessment of the sensitivity of *L. maculans* populations to determine the relative merits of and any risks associated with fungicidal products containing this active ingredient. Understanding the current baseline sensitivity, especially for high risk products such as pyraclostrobin, ensures these fungicides remain a sustainable tool for blackleg management in canola, alongside extended crop rotations and strategic deployment of resistance genes.

The objectives of this study were to: 1) determine the EC_{50} value of a set of *L. maculans* isolates to pyraclostrobin fungicide by conducting a radial growth plate assay; and 2) assess the pyraclostrobin sensitivity of a collection of *L. maculans* isolates from canola in Alberta via mycelial growth plate and microtiter plate assays. It was hypothesized that no insensitive isolates would be found, since pyraclostrobin was registered only recently for use on canola to manage blackleg.

MATERIALS AND METHODS

Collection and Preparation of Single-Spore *L. maculans* Isolates

A collection of single-spore *L. maculans* isolates described by Rong et al. (2015) was used in this study. These isolates were obtained in 2011 from infected canola stubble residues from 6 fields located in the Alberta counties of Camrose, Lacombe, Ponoka, Strathcona and Wetaskiwin (Table 1). Within each field, plant residues were collected in a 1 m² area at each of 5 points along a 'W' sampling pattern. Each isolate had been identified as either *L. maculans* or *L.*

biglobosa by means of a PCR assay with β -tubulin based primers (Rong et al. 2015). The isolates were collected after the introduction of QoI fungicides and the registration of pyraclostrobin on canola. However, the history of pyraclostrobin use in these fields at the time of sample collection was unknown. Since the isolates could have been exposed to pyraclostrobin, they may not represent true baseline isolates. The isolates were stored as frozen concentrated stocks of pycnidiospores in 1.5 mL microcentrifuge tubes in sterilized distilled water (SDW) at -20°C until use.

Determining the Mean EC₅₀

To assess the relative sensitivity of the isolates that were collected, it was necessary to first determine the effective concentration of pyraclostrobin required to inhibit the growth of *L. maculans* by 50% (EC₅₀). Determination of the EC₅₀ was performed according to Ahmed et al. (2014) with some modifications. A total of 13 isolates were randomly selected from different locations to serve as 'baseline' sensitivity isolates. To revive the isolates, the pycnidiospores were thawed on ice and 1 μ L of the spore suspension stock of each isolate was added to 500 μ L of SDW. The spore solution was mixed gently and pipetted onto Petri dishes (10-cm diameter) filled with V8 growth medium (composition per L: 850 mL distilled water, 150 mL V8® Original Vegetable Juice, 1.5 g CaCO₃, 15.0 g agar) and spread over the surface of the medium with a sterilized metal plate. After 5 days, 7-mm-plugs of the developing colonies were transferred to fresh V8 medium amended with the pyraclostrobin fungicide. Headline 250 EC (pyraclostrobin, 250 g L⁻¹; BASF Canada Inc., Mississauga, ON) was used as the source of pyraclostrobin. To prepare the fungicide amended plates, a stock solution was first prepared by adding 20 μ L of the formulated product to 3980 μ L of SDW. Appropriate amounts of fungicide were added to cooled autoclaved V8 medium just prior to pouring the plates. The concentrations of active

ingredient in the amended plates were: 0.025 mg L⁻¹, 0.125 mg L⁻¹, 0.250 mg L⁻¹, 0.375 mg L⁻¹, 0.625 mg L⁻¹, and 0.875 mg L⁻¹. The experiments were arranged in a completely randomized design with 10 replicates (Petri dishes) per treatment. Four replicate control dishes that did not contain fungicide were included for each isolate.

The plates were placed on a table under fluorescent lighting for a 24 h period at 20°C ± 2°C. A period of 10 days was sufficient to detect significant growth differences between the fungicide amended and control plates. Using digital calipers, the radial growth of each isolate was determined by measuring the diameter of the colony at its widest point, and by taking a second measurement perpendicular to the first. The two measurements were averaged and then converted to a percentage of radial growth of the un-amended control using the equation: $[(\text{average growth on un-amended} - \text{growth on amended}) / (\text{average growth on un-amended})] \times 100$.

Effect of SHAM and Pyraclostrobin on Mycelial Growth

Some fungal pathogens have been observed to exhibit *in vitro* resistance to QoIs through an alternative respiratory pathway that relies on the alternative oxidase (AOX) enzyme. It is believed that this pathway is responsible for protecting the pathogen against oxidative stress (Inoue et al. 2012). The compound salicylhydroxamic acid (SHAM) blocks alternative respiration; therefore, it is important to include SHAM in sensitivity studies to avoid inaccurate interpretations of resistance (Wise et al. 2008). To determine if *L. maculans* exhibits alternative respiration when exposed to pyraclostrobin *in vitro*, the same 13 baseline isolates that were used to determine the EC₅₀ were tested on V8 growth medium containing salicylhydroxamic acid (SHAM, 99%; Sigma-Aldrich, St. Louis, MO) in addition to pyraclostrobin. The single-spore

isolates were cultured and plated onto medium amended with pyraclostrobin at 0.025 mg L⁻¹, 0.125 mg L⁻¹, 0.250 mg L⁻¹, 0.375 mg L⁻¹, 0.625 mg L⁻¹, or 0.875 mg L⁻¹, as described above, as well as with SHAM at a concentration of 100 µg mL⁻¹ (Wise et al. 2008). To prepare a solution of SHAM, salicylhydroxamic acid was dissolved in methanol. There were a total of 10 replicates (Petri dishes) per treatment for each isolate. There also were 10 replicate control dishes amended with the same concentration of SHAM, but which did not contain any fungicide. After 10 days, the diameters were measured and growth inhibition calculated as indicated previously.

Testing the Sensitivity of *L. maculans* Isolates

Conventional Growth Plate Assay

To detect highly insensitive isolates, a discriminatory dose 69-fold greater than the EC₅₀ was used to screen the full collection of 117 single-spore isolates of *L. maculans*. Isolates were cultured and growth medium was prepared as described previously. The medium contained pyraclostrobin at 6.25 mg L⁻¹ and SHAM at 100 µg mL⁻¹. The treatments were arranged in a completely randomized design, with eight replicates (Petri dishes) per isolate. There were eight control replicate plates per isolate that were amended with SHAM at 100 µg mL⁻¹, but which did not contain any fungicide. The plates were placed on a table under fluorescent lighting at 20°C ± 2°C. The radial growth of each isolate was measured as previously described, and converted to a percentage of radial growth of the un-amended control. Isolates with a growth inhibition <50% were designated as insensitive to pyraclostrobin (Wise et al. 2009).

Microtiter Assay

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A subset of 41 isolates was evaluated for sensitivity to pyraclostrobin via a microtiter plate assay. Briefly, after the isolates were cultured, the spores were collected and immersed in YBA medium (composition per L of deionized water: 20 g yeast extract, 20 g Bacto peptone, 40 g sodium acetate) to increase spore density. The spore concentration was adjusted to 20000 spores mL⁻¹. Since YBA medium was used, the addition of SHAM was not necessary, as this relatively poor nutrient medium prevents the fungus from gaining enough energy to undergo alternative respiration, while providing enough nutrients for sufficient growth (Spiegel and Stammler 2006). Fifty µL of the spore suspension, containing about 1000 spores, was transferred into each well of a 96-well microtiter plate. Formulated Headline 250 EC fungicide was diluted to achieve end concentrations of 0 mg L⁻¹ (control), 0.003 mg L⁻¹, 0.010 mg L⁻¹, 0.030 mg L⁻¹, 0.100 mg L⁻¹, 0.300 mg L⁻¹, 1.000 mg L⁻¹, and 3.000 mg L⁻¹ of the active ingredient. Fifty µL of fungicide solution was added to each well and mixed with the spore suspension. There were 4 replicate wells for each isolate and fungicide concentration. Four replicate wells containing only fungicide and YBA medium also were included for each fungicide concentration and served as blanks. The plates were incubated for 7 days at 18°C in darkness. Pycnidiospore germination and subsequent growth, as indicated by absorbance, was measured with a spectrophotometer at a wavelength of 405 nm. For each isolate and concentration, growth inhibition was calculated using the equation: $[(\text{average absorption with spores and fungicide} - \text{average absorption of blanks}) \times 100] / \text{average absorption of control without fungicide}$. Two isolates of *L. maculans* with known sensitivity to pyraclostrobin were used as sensitive controls. The growth inhibition of the sensitive controls was determined in the same manner as for the other 41 isolates.

Data Analysis

The normality of the growth inhibition residuals of the 13 "baseline" sensitivity isolates in the absence and presence of SHAM were analysed visually and with the Shapiro-Wilk test using the statistical procedure/package `shapiro.test/stats` in R: A Language and Environment for Statistical Computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013). The homogeneity of variance also was analysed visually and tested using the Bartlett test (`bartlett.test/stats`). If residual data visually appeared to be normal and homogeneous, no transformations were applied. Outliers were identified with an outlier test (`outlierTest/car`). If the outlier test reported values with a Bonferroni p-value < 0.05 , the values were removed from the data set. Analysis of variance of the transformed growth inhibition data was carried out using a fit linear mixed-effect model (`lmer/lmerTest`). The model included $\log_{10}$ fungicide concentration, isolate, and $\log_{10}$ fungicide concentration $\times$ isolate interaction. $\log_{10}$ fungicide concentration and isolate were considered fixed, while replication was random. If the effect of isolate was significant on growth inhibition, or if the variance across isolates was not normal, EC_{50} values were calculated separately for each isolate, and averaged to find the baseline EC_{50} .

The EC_{50} value for each of the 13 isolates (in the absence and presence of SHAM) in the growth plate assay and the 41 isolates in the microtiter assay was determined by analysing the growth inhibition data using probit regression analysis. The analysis was done using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0, IBM Corporation, Armonk, NY, USA). A base-10 logarithm ($\log_{10}$) transformation of the fungicide concentrations was used to linearize the growth inhibition data. The resulting linear regression equation was used to estimate the fungicide concentration at which the radial growth was inhibited by 50% (EC_{50}) for each isolate. The EC_{50} values were displayed in a histogram and the normality of the residuals

was analysed visually and tested using the Shapiro-Wilk test in R. The mean EC₅₀ value of the isolates was calculated for each assay.

The mean EC₅₀ values of each of the 13 "baseline" sensitivity isolates in the presence and absence of SHAM were compared using a two-sided paired T-test (t.test/stats). For the microtiter assay, a one-sided t-test (t.test/stats) was used to determine if the EC₅₀ values of the 41 isolates were greater than the two sensitive controls.

The mean growth inhibition values of all of the 117 isolates in the growth plate assay were displayed in a histogram and the normality was analysed visually and tested using the Shapiro-Wilk test (shapiro.test/stats). The relationship of the EC₅₀ values determined from the microtiter assay and the growth inhibition from the mycelial growth plate assay of the 41 isolates was tested using Pearson's product-moment correlation (cor.test/stats).

RESULTS

Determination of EC₅₀ Values of Baseline Isolates

Thirteen isolates of *L. maculans* were randomly selected from different locations in Alberta to serve as baseline isolates. The EC₅₀ values of the 13 individual isolates ranged from 0.01 mg L⁻¹ to 0.28 mg L⁻¹, with a mean ($\pm$ standard deviation) of 0.11 mg L⁻¹ ($\pm$ 0.09 mg L⁻¹) (Fig. 1A; Table 2). The distribution of the residual data was normal ($P = 0.5866$).

Effect of SHAM and Pyraclostrobin on Mycelial Growth

The effect of including SHAM in addition to pyraclostrobin in the growth medium was evaluated on the same 13 baseline isolates. The EC₅₀ values of the individual isolates ranged from 0.02 mg L⁻¹ to 0.24 mg L⁻¹, with a mean of 0.09 mg L⁻¹ ($\pm$ 0.07 mg L⁻¹) (Fig. 1B; Table 2). The

distribution of the residual data was normal ($P = 0.8588$). The result of the two-sided pairwise T-test indicated that there was no significant difference between the EC_{50} values of each isolate tested in the presence or absence of SHAM ($P = 0.3882$).

Sensitivity of a Collection of *L. maculans* Isolates From Alberta

Pyraclostrobin sensitivity in a collection of 117 *L. maculans* isolates from Alberta was assessed in a conventional growth plate assay and a microtiter assay. In the growth plate assay, growth inhibition of the 117 isolates ranged from 66.6% to 100.0% with a mean of 84.3% ($\pm 8.05\%$), relative to a control treatment in which no pyraclostrobin was included. None of the isolates had a growth inhibition of $< 60.0\%$; eight exhibited a growth inhibition of 60.0% to $< 70.0\%$; 24 exhibited a growth inhibition of 70.0% to $< 80.0\%$; 53 exhibited a growth inhibition of 80.0% to $< 90.0\%$; 31 exhibited a growth inhibition of 90.0% to $< 100.0\%$; and a single isolate exhibited a growth inhibition of 100.0% (Fig. 2). The residuals of the growth inhibition data were not normally distributed ($P \leq 0.05$) and were negatively skewed.

In the microtiter assay, the EC_{50} values of the two pyraclostrobin-sensitive controls included in the test were $4.1 \times 10^{-3} \text{ mg L}^{-1}$ and $5.7 \times 10^{-3} \text{ mg L}^{-1}$. The EC_{50} values of the *L. maculans* isolates from Alberta ranged from $1.0 \times 10^{-4} \text{ mg L}^{-1}$ to $7.4 \times 10^{-3} \text{ mg L}^{-1}$, with a mean of $3.6 \times 10^{-3} \text{ mg L}^{-1}$ ($\pm 2.0 \times 10^{-3} \text{ mg L}^{-1}$) (Fig. 3), and were not significantly different from either control ($P = 0.9974$; $P = 1.000$). The distribution of the residual EC_{50} values was not normal ($P < 0.05$) and was positively skewed. No significant correlation ($P = 0.1549$) was found between the EC_{50} values from the microtiter assay and percent growth inhibition from the growth plate assay for the subset of 41 isolates evaluated by both methods.

DISCUSSION

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Many studies have examined the sensitivity of various plant pathogens to pyraclostrobin. For example, pyraclostrobin sensitivity was demonstrated in isolates of *Phytophthora cactorum* Lebert and Cohn from infected strawberries in seven states of the U.S.A. (Rebollar-Alviter et al. 2007), and in isolates of *P. tritici-repentis* from infected wheat in North Dakota, U.S.A (Patel et al. 2012). In contrast, isolates of *Alternaria alternata* Fr. collected from infected pistachio (*Pistacia vera* L.) in California, U.S.A (Avenot et al. 2008), and *Ascochyta rabiei* Pass. from infected chickpea in Alberta (Chang et al. 2007), were found to be insensitive to pyraclostrobin. To our knowledge, however, there have been no reports published on the pyraclostrobin sensitivity of *L. maculans* isolates from Alberta.

The first step in evaluating the sensitivity of *L. maculans* to pyraclostrobin was to determine the EC₅₀ of a set of baseline isolates. Typically, isolates that have never been exposed to a fungicide are used as the baseline in a sensitivity study. For this study, however, there were no sets of isolates available that had been collected prior to the registration and use of pyraclostrobin in Alberta; the isolates analysed were recovered after the registration of this product on canola. Since the sensitivity of the isolates could not be compared with the sensitivity of isolates collected prior to the use of the fungicide, shifts in the sensitivity of the pathogen population could not be determined. Nonetheless, the analysis provided important information on the current status of fungicide sensitivity in *L. maculans* populations from Alberta, which is essential for future monitoring of changes in this sensitivity.

The range of EC₅₀ values obtained in the mycelial growth assays was small (0.01 mg L⁻¹ to 0.28 mg L⁻¹), with a mean of 0.11 mg L⁻¹. In a recent abstract by Liu et al. (2013), the EC₅₀ values of 27 *L. maculans* isolates from 3 fields where pyraclostrobin and azoxystrobin had been sprayed were determined to range from 0.126 mg L⁻¹ to 0.477 mg L⁻¹, with an average of 0.312

mg L⁻¹. While those values are similar to the ones obtained for *L. maculans* in the current study, it is impossible to compare how the sensitivity of the two isolate collections may have shifted from previous years, since the isolates of Liu et al. (2013) had previous exposure to pyraclostrobin and the ones in this report may have been exposed to the fungicide. Differences in fungicide sensitivity also may reflect intrinsic diversity in isolates from different populations of the fungus, and/or varying fungicide spray regimes across western Canada. More frequent or heavier use of fungicides in an area may cause a greater shift in sensitivity relative to areas with less frequent use of the same product (Brent and Hollomon 2007).

There are many sensitivity studies involving pyraclostrobin and other fungal pathogens in the Pleosporales (Ahmed et al. 2014; Avenot et al. 2008; Bowness et al. 2016; Gossen and Anderson 2004; Mondal et al. 2005; Pasche et al. 2004; Patel et al. 2012; Vega and Dewdney 2014; Vega et al. 2012). However, due to natural variation in the fungicide sensitivity of species, different EC₅₀ values are to be expected (Vega and Dewdney 2014). For example, Ahmed et al. (2014) determined the EC₅₀ of *Didymella rabiei* Kovatsch to pyraclostrobin to be 1.0 mg L⁻¹ with a range of 0.3 mg L⁻¹ to 3.8 mg L⁻¹, which is much greater than the values observed for *L. maculans* in both the current study and the abstract of Liu et al. (2013). The EC₅₀ values for the same species in response to different fungicides within the strobilurin group also may be highly variable, given the different chemical properties of each fungicide. Studies involving various strobilurin fungicides have resulted in different EC₅₀ values when tested on the same pathogen species (Patel et al. 2012).

In the current study, a comparison of the EC₅₀ values obtained in the presence or absence of SHAM for the 13 baseline isolates indicated that this compound did not have any effect, strongly suggesting that the baseline isolates did not use alternative respiration to bypass the effects of

pyraclostrobin. To our knowledge, there are no other reports on the effect of pyraclostrobin and SHAM on *L. maculans*. However, there have been many reports indicating the effect of SHAM on other pathogens. Ahmed et al. (2014) reported no effect of SHAM on spore germination in *D. rabiei* in the presence or absence of pyraclostrobin. In contrast, Patel et al. (2012) found that isolates of *P. tritici-repentis* were able to undergo alternative respiration when exposed to pyraclostrobin in the absence of SHAM. Seyran et al. (2010) reported that in the presence of SHAM, but absence of azoxystrobin fungicide, the growth of *Fusicladium effusum* Wint. isolates was reduced, suggesting some toxicity of SHAM towards this fungus. Wise et al. (2008) conducted a study on the baseline sensitivity of *A. rabiei* to various QoI fungicides, including pyraclostrobin. They found that some isolates of the fungus had higher EC₅₀ values when exposed to azoxystrobin in the absence of SHAM, indicating that individual isolates may be capable of alternative respiration. Therefore, despite the absence of an effect of SHAM on any of the 13 baseline isolates tested in the present study, SHAM was added to the medium as a precaution when screening the full collection of *L. maculans* isolates.

The EC₅₀ of the baseline isolates in the presence of SHAM and pyraclostrobin was 0.09 mg L⁻¹. The discriminatory dose used to screen the isolates was 6.25 mg L⁻¹, a concentration 69 times greater than the EC₅₀. Changes in the sensitivity to strobilurin fungicides are qualitative in nature (Gisi et al. 2002), resulting in almost complete insensitivity to treatment with the chemical (Georgopoulos and Skylakakis 1986). When the mechanism of fungicide insensitivity is known and involves a point mutation in a single gene, as is the case with pyraclostrobin insensitivity in *L. maculans*, a high discriminatory dose can be used to clearly detect insensitive isolates (Russell 2004). Indeed, insensitive isolates can tolerate high levels of strobilurin fungicides, often at doses > 10 mg L⁻¹ (Mondal et al. 2005). Wise et al. (2009) reported that two *A. rabiei* isolates

had EC₅₀ values 704 times greater than the mean sensitivity of baseline isolates, indicating insensitivity to pyraclostrobin. In an assessment of the fungicide sensitivity of *A. alternata* isolates, Vega and Dewdney (2014) determined the EC₅₀ of insensitive populations to be 5.507 µg ml⁻¹, which represented a 239-fold decrease in sensitivity relative to the baseline isolates (0.023 µg/ml). The results of the growth plate assay in the current study suggest that there were no isolates insensitive to pyraclostrobin in the collection from Alberta, since no isolates with a < 50% reduction in colony growth were identified. Nevertheless, while the discriminatory dose used in the current study was appropriate for the detection of qualitative changes in the insensitivity to pyraclostrobin, it did not allow for the identification of isolates exhibiting tolerance or intermediate levels of fungicide insensitivity. The distribution of the sensitive isolates was negatively skewed, suggesting that a majority of the isolates had greater sensitivity relative to the entire collection.

Since conidia are more sensitive to QoI fungicides, the EC₅₀ values obtained from microtiter assays generally will be much lower than those from mycelial growth plate assays (Wise et al. 2008). In the current study, the mean EC₅₀ of the baseline isolates in the growth plate assay was almost 18-fold greater than the mean EC₅₀ of the two sensitive control isolates in the microtiter assay. Nevertheless, there were no significant differences between the EC₅₀ values of the sensitive controls and the isolates being screened in the microtiter assay. The results both from the growth plate and microtiter assays support the conclusion that all of the *L. maculans* isolates tested from Alberta are sensitive to pyraclostrobin. However, these isolates represented a relatively small number of fields. Additional evaluation of a larger number of fields and isolates collected over a wider geographic area will be required to confirm this conclusion and for subsequent monitoring of shifts in sensitivity.

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The isolates in this study were collected in the fall after the first season of registered use of pyraclostrobin on canola. Pyraclostrobin (as the commercial formulation Headline EC, BASF) may be applied 1 to 3 times on canola in a single growing season: once at the 2-4 leaf stage for blackleg management, and twice at the early pod stage for the control of *Alternaria brassicae* Berk., *A. alternata*, and *Alternaria raphani* J.W. Groves & Skolko (BASF 2003). While a single year of exposure would not likely result in the development of resistance, there have been cases where resistance has developed within a very short period. For example, Wise et al. (2009) identified fungicide-insensitive isolates of *A. rabiei* in North Dakota after only two years of exposure to pyraclostrobin. In Alberta, pyraclostrobin is also applied to other field crops, including cereals and pulses (BASF 2003). Since *L. maculans* can survive saprophytically on canola residues, it is possible that the fungus could be unintentionally exposed to pyraclostrobin when it is applied to other crops, especially if volunteer canola was present and was infected by *L. maculans*. Nonetheless, the failure to identify any *L. maculans* isolates insensitive to pyraclostrobin suggests that whatever exposure the fungus has had to this fungicide, it has not decreased its fungicide sensitivity substantially. It is possible that continued exposure to pyraclostrobin could change this situation, especially with the repeated applications within and between growing seasons. Pyraclostrobin, along with other strobilurin fungicides, provide plant health benefits independent of disease control such as maintenance of green leaf area, delay of leaf senescence, increase in photosynthetic activity, and increase in yield (Bartlett et al. 2002). These properties may encourage growers to apply pyraclostrobin fungicides regardless of the disease situation. However, the number of applications per season should be limited and fungicide used only when necessary, in order to avoid selection pressure for insensitive isolates (Brent and Hollomon 2007). The judicious use of pyraclostrobin as part of an integrated

blackleg management strategy, along with continued monitoring of *L. maculans* populations for changes in fungicide sensitivity, will be important in ensuring the continued efficacy of this product in the management of blackleg of canola.

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Fig. 1. Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC₅₀) in 13 isolates of *Leptosphaeria maculans* from Alberta, Canada, in the absence (A) or presence (B) of salicylhydroxamic acid (SHAM) at 100 µg mL⁻¹. Individual isolates are grouped in class intervals of 0.05 mg L⁻¹.

Fig. 2. Frequency distribution of the inhibition of mycelial growth in 117 *Leptosphaeria maculans* isolates in response to inclusion of pyraclostrobin (6.25 mg L^{-1}) in V8 growth medium. Inhibition of colony growth is expressed as a percentage relative to a control treatment in which no pyraclostrobin was included. Assays were conducted in the presence of salicylhydroxamic acid (SHAM) at $100 \text{ } \mu\text{g mL}^{-1}$.

Fig. 3. Frequency distribution of the effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC₅₀) in 41 *Leptosphaeria maculans* isolates from Alberta, Canada. The EC₅₀ values were determined for each isolate via a microtiter assay, and were based on the inhibition of pycnidiospore germination and mycelial growth as measured photometrically at 405 nm. Individual isolates are grouped in class intervals of 1×10^{-3} mg L⁻¹.

Table 1. Origin of 117 *Leptosphaeria maculans* isolates

obtained from canola stubble residues collected from 6 fields in Alberta, Canada, and tested for sensitivity to pyraclostrobin fungicide.

Location	Number of isolates tested
Camrose	40
Lacombe	4
Lethbridge	13
Ponoka	37
Strathcona	22
Wetaskiwin	1

Table 2. The effective concentration of pyraclostrobin needed to inhibit mycelial growth by 50% (EC₅₀) in 13 isolates of *Leptosphaeria maculans* in the absence or presence of salicylhydroxamic acid (SHAM). These isolates were used to assess baseline sensitivity to the fungicide.

Isolate	EC ₅₀ (mg L ⁻¹)	
	Without SHAM	With SHAM
33	0.01	0.07
104	0.07	0.04
139	0.03	0.02
144	0.06	0.04
166	0.17	0.13
228	0.10	0.08
269	0.09	0.04
325	0.03	0.04
385	0.09	0.03
394	0.03	0.13
538	0.18	0.17
545	0.28	0.17
626	0.23	0.24
Mean	0.11	0.09

Note: A two-sided paired t-test indicated no significant difference between EC₅₀ values of individual isolates in the absence or presence of SHAM (P = 0.3882).