Sensitivity of *Mycosphaerella pinodes* to Pyraclostrobin Fungicide

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Abstract

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Mycosphaerella blight, caused by *Mycosphaerella pinodes*, is a destructive disease of field pea that is managed using foliar fungicides. Strobilurin fungicides have been used in western Canada for disease management since 2003. To assess the baseline sensitivities of *M. pinodes* isolates to the strobilurin fungicide pyraclostrobin, the effective concentration to reduce mycelial growth by 50% (EC_{50}) was determined for 70 isolates collected prior to 2003 from Alberta, Saskatchewan, North Dakota, and Washington State. Each of these isolates was sensitive to pyraclostrobin, with EC_{50} values ranging from 0.03 to 0.29 mg liter⁻¹. The pyraclostrobin concentrations required to reduce conidia

germination by 50% was lower, ranging from 0.008 to 0.041 mg liter⁻¹. In all, 324 isolates collected in 2010 and 2011 were tested for high levels of insensitivity by examining mycelial growth using a discriminatory dose of 5 mg liter⁻¹. Nineteen isolates were highly insensitive to pyraclostrobin, with EC_{50} values of 80 to 216 mg liter⁻¹. Conidia of these isolates germinated when exposed to a discriminatory dose of 0.1 mg liter⁻¹. Insensitive isolates infected field pea plants treated with pyraclostrobin but sensitive isolates did not. The identification of insensitive isolates indicates that insensitivity may be emerging in the pathogen population.

Mycosphaerella blight, caused by *Mycosphaerella pinodes*, is a destructive foliar disease of field pea in western Canada and around the world (Banniza and Vandenberg 2003; Beasse et al. 1999; Bretag et al. 2006; Davidson and Ramsey 2000). Yield losses vary considerably but, in Canada, losses were reported to be as high as 50% (Conner et al. 2007). *M. pinodes* is part of the Ascochyta blight complex, which comprises three fungal pathogens: *Ascochyta pisi* Lib., (Chilvers et al. 2009), *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vesterg. (anamorph *A. pinodes* L. K. Jones), and *Phoma pinodella* (L. K. Jones) Morgan-Jones & K. B. Burch. Of the three, *M. pinodes* is by far the most abundant in Canada, causing 90% of reported Ascochyta blight infections (Ali et al. 1982).

M. pinodes is best managed by reducing the amount of available inoculum and suppressing the subsequent epidemic. The most commonly utilized strategy for management of *Mycosphaerella* blight is the application of fungicide sprays in a preventative and systematic schedule (Beasse et al. 2000). Multiple sprays, initiated at early to midflowering, provide effective disease control and improve yield (Warkentin et al. 2000). The use of fungicides for disease management on field crops has increased rapidly in recent years (Gossen et al. 2014). However, the selection pressure imposed by repeated use of fungicides is the leading contributor to fungicide insensitivity in pathogen populations (Gisi et al. 1997). This risk must be taken into consideration when applying a single fungicide repeatedly, or those with the same mode of action (Brent and Holloman 2007b).

The strobilurin group of fungicides have been widely used since 1996. They are single-site mode-of-action fungicides classified as

quinine outside inhibitors (QoI), because they bind the Qo site of cytochrome b, inhibiting mitochondrial respiration (Bartlett et al. 2002). This causes an energy deficiency due to lack of single ATP (Bartlett et al. 2002). The Fungicide Resistance Action Committee (FRAC 2015) has identified strobilurins as a high risk for insensitivity due to their site-specific mode of action, especially with repeated use.

Pyraclostrobin is a strobilurin fungicide that has been widely used in Canada since 2002, and is the active ingredient in the fungicide Headline 250 EC (BASF Canada, Mississauga, ON, Canada). It provides broad-spectrum control of many plant pathogens, including the *Ascochyta* and *Mycosphaerella* spp. on pulse crops (BASF 2011). In Canada, the research into pyraclostrobin insensitivity in field crop pathogens has focused on *A. rabiei* on chickpea, which has developed qualitative insensitivity to this product (Chang et al. 2007; Gossen and Anderson 2004; Thaher 2011; Wise et al. 2008, 2009). Similarities in the risk characteristics between *A. rabiei* on chickpea and *M. pinodes* on field pea indicate that there is a possibility of fungicide insensitivity developing in *M. pinodes*. Also, three species in the *Mycosphaerella* genus are known to be insensitive to strobilurins: *M. fijiensis*, *M. citri*, and *M. graminicola* (Gisi et al. 1997; Grasso et al. 2006; Keinath 2009; Miguez et al. 2003; Mondal et al. 2005).

There are two types of fungicide insensitivity: quantitative and qualitative. Quantitative insensitivity results in the pathogen becoming less sensitive to the fungicide, although higher rates or more fungicide applications are still effective. Qualitative insensitivity causes the pathogen to become completely insensitive to the active ingredient and control is no longer possible at field rates. Insensitivity to the strobilurin fungicides is generally qualitative (Ypema and Gold 1999).

In vitro research on fungal respiration in the presence of respiration-inhibiting fungicides such as the strobilurins has shown that certain microorganisms can use an alternative respiration pathway, involving the production of a cyanide-insensitive alternative oxidase (AOX) (Miguez et al. 2003). This enables the bc complex in the mitochondrial respiration chain to be bypassed and allows mycelial growth and spore germination even in the presence of

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the fungicide (Olaya and Koller 1999; Vincelli and Dixon 2002; Ziogas et al. 1997). Although alternative respiration has not been directly identified in QoI-insensitive pathogens (Avila-Adame et al. 2003), the induction of this alternative pathway of respiration can serve as an effective rescue mechanism when pathogen sensitivities are tested in the absence of a host. Salicylhydroxamic acid (SHAM) is used to inhibit AOX. This chemical is routinely included in QoI in vitro fungicide sensitivity assessments, because alternative respiration may affect the results of these assays, leading to incorrect assessments of fungicide sensitivity (Miguez et al. 2003; Wise et al. 2009).

Establishing the baseline sensitivity of the pathogen prior to exposure of the population to the active ingredient in the fungicide is very useful for subsequent monitoring to detect the development of fungicide insensitivity (Avenot and Michailides 2007; Brent and Holloman 2007a). Studies on strobilurin insensitivity in *M. pinodes* have not been conducted. Considering the widespread use of the site-specific mode of action of pyraclostrobin fungicide and the identification of strobilurin insensitivity in closely related species, it was suspected that similar insensitivity issues may have already developed.

The objectives of this study were to (i) quantify the baseline sensitivity of *M. pinodes* isolates from the Canadian prairies and northern Great Plains of the United States to pyraclostrobin fungicide using radial growth assessments, (ii) determine whether either inhibiting AOX with SHAM or using the formulated product (Headline) instead of the technical-grade product affected the baseline sensitivity assessments, (iii) determine whether baseline assessments using conidial germination produced the same pattern of response as radial growth assessments, and (iv) compare the reaction of *M. pinodes* isolates collected in 2010 and 2011 with that of baseline isolates to determine whether sensitivity to pyraclostrobin is changing in western Canada and the Northern Great Plains of the United States.

Materials and Methods

Baseline sensitivity. *Preparation of isolates.* In all, 40 *M. pinodes* isolates were obtained from the Saskatoon Research Centre, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada and 30 isolates were obtained from the *M. pinodes* collection at the United States Department of Agriculture in Pullman, WA. These cultures were collected prior to 2003, before the registration of QoI fungicides in those geographical areas, and, therefore, represent a true baseline collection of isolates that have not been exposed to QoI fungicides. These isolates were confirmed to be *M. pinodes* based on colony and spore morphology. They were mass transferred onto water agar (WA; Difco Laboratories), allowed to grow for 5 days, and purified by isolation from hyphal tips. The cultures were then transferred onto commercial potato dextrose agar (PDA; Difco Laboratories) and incubated at $20 \pm 2^\circ\text{C}$ for 14 days under white fluorescent light with a 16-h photoperiod, and their identity was again confirmed by colony and spore morphology, as before.

Radial growth assessments. Seventy baseline isolates of *M. pinodes* were inoculated onto PDA and incubated for 1 week. Then, 5-mm-diameter agar plugs taken from the edge of each actively growing culture were transferred, mycelium side down, onto the center of a fresh petri dish of PDA measuring 15 by 100 mm. To determine whether formulation affected the sensitivity of *M. pinodes* to pyraclostrobin, the response to a formulated commercial product (Headline) was compared with the technical-grade product (89% active ingredient [a.i.]; BASF) in a radial growth study. Technical-grade pyraclostrobin was prepared by dissolving the powdered product into acetone. The solution was filter sterilized before adding to the cooled, autoclaved medium. The final concentration of acetone in the medium was 0.01% by volume. For the formulated product, Headline fungicide was serially diluted using sterilized deionized water. The Headline solutions were added to autoclaved PDA after it had cooled to 55°C . The treatments were concentrations of fungicide in amended PDA of pyraclostrobin at 0 (control), 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, or 50 mg liter⁻¹. After inoculation, the dishes were incubated for 7 days. The colony diameter (radial growth) was determined by

measuring each culture at its widest point and then at a right angle to the first measurement. The two measurements were averaged and the 5-mm core subtracted. The colonies were incubated for another 7 days and measured again. The measurements taken 14 days after inoculation are presented because they showed more pronounced and consistent differences among the isolates than the assessments at 7 days. The experiment was arranged in a completely randomized design with one dish per replicate and four replicates per treatment. The experiment was repeated once. This design was used in all subsequent in vitro studies.

To assess the effect of SHAM (Sigma-Aldrich) on the response of *M. pinodes* to pyraclostrobin, a radial growth study was conducted using formulated product media amended with and without SHAM. SHAM was prepared by dissolving 100 mg of SHAM into 1 ml of methanol and adding the mixture to the PDA medium at a concentration of 0.01% by volume (100 $\mu\text{g ml}^{-1}$), as described by Wise et al. (2008). SHAM was filter sterilized and added to the autoclaved medium after cooling to 55°C . The study was conducted using the methods and pyraclostrobin treatment concentrations described previously.

Conidial germination assessments. Of the 70 baseline isolates, 50 were inoculated onto PDA and incubated as described previously. After 7 days, conidia were collected from each culture by adding 2 ml of sterilized deionized water containing 0.05% (vol/vol) Tween 20 to each petri dish and then gently dislodging the conidia with a glass rod or a transfer loop. The resulting conidial suspension was collected and adjusted with a hemocytometer to 2×10^5 spores ml⁻¹, and 100 μl of suspension was pipetted onto each of four replicate dishes of Headline-amended PDA plus SHAM at 100 $\mu\text{g ml}^{-1}$. Based on the results of the radial growth trials, only formulated product was used. The treatments consisted of pyraclostrobin at 0 (control), 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10 mg liter⁻¹. Following 24 h of incubation at $20 \pm 2^\circ\text{C}$, the germination of 100 conidia per replicate petri dish was assessed under a compound microscope with a $\times 200$ objective lens. A conidium was considered germinated if the germ tube was at least as long as the conidium itself (Pasche et al. 2004).

Sensitivity of *M. pinodes* isolates collected in 2010 and 2011.

Preparation of isolates. In total, 324 isolates of *M. pinodes* were obtained from infected plant samples collected from commercial fields in Alberta, Saskatchewan, North Dakota, and Washington State in 2010 and 2011. These isolates represented populations that may have been exposed to pyraclostrobin fungicides for up to 8 years. Sections of the leaf containing disease lesions were removed and surface sterilized in 5% NaOCl for 25 s, rinsed three times with deionized water, and allowed to dry for 15 min in a laminar flow hood. The leaf sections were transferred onto PDA and incubated for 7 days at $20 \pm 2^\circ\text{C}$. Putative cultures of *M. pinodes* were then transferred onto WA plates, incubated for 5 days, and purified by transferring hyphal tips onto PDA. The pure cultures were incubated for 14 days, their identity was confirmed based on culture and spore morphology, and they were transferred onto PDA slants and stored at 4°C .

Testing for sensitivity. Each of the 324 isolates collected in 2010 and 2011 was tested using the radial growth procedure described previously at a discriminatory dose of 5.0 mg liter⁻¹ using formulated product (Headline) and 0.1% SHAM. This concentration was selected to identify highly insensitive isolates based on the response of the baseline isolates, where 5.0 mg liter⁻¹ consistently reduced colony growth by more than 70% but some growth still occurred. Isolates were categorized as sensitive if growth was reduced by more than 70%, intermediate if growth was reduced between 30 and 70%, and insensitive if growth was reduced by less than 30% by Headline (adapted from Thaher 2011). The response of the exposed isolates (from 2010 and 2011) was compared with the response of the baseline isolates to see if the sensitivity level of the populations had changed.

Each isolate that was designated as insensitive in the radial growth study was also tested for insensitivity at a discriminatory dose of 0.1 mg liter⁻¹ using the conidial germination procedure described

Table 1. Analysis of variance for radial growth of the baseline isolates of *Mycosphaerella pinodes* from Saskatchewan and Washington to assess their response to formulated product versus technical-grade formulation of pyraclostrobin fungicide

Isolate	<i>Pr</i> > <i>F</i>	<i>F</i> value	<i>R</i> ²	Coefficient of variation	Log ₁₀ mean growth ^a
Saskatchewan					
Sep0002	0.280	1.28	0.95	9.70	1.47
Sep0004	0.715	0.14	0.95	9.86	1.46
Sep0008	0.085	3.52	0.91	20.61	1.34
Mar0405	0.283	1.25	0.72	25.65	1.40
Mar0412	0.162	2.22	0.97	11.92	1.33
Mar0413	0.659	0.21	0.95	12.74	1.44
Mar0419	0.423	0.69	0.92	19.71	1.35
Mar0425	0.372	0.87	0.95	14.24	1.39
Mar0426	0.180	2.02	0.96	9.72	1.42
Mar0427	0.297	1.19	0.95	14.91	1.31
Mar0435	0.035	5.68	0.97	10.43	1.34
Mar0448	0.562	0.36	0.96	8.25	1.49
Mar0452	0.791	0.07	0.95	10.55	1.50
Mar0458	0.476	0.54	0.95	11.67	1.47
Mar0463	0.973	0.00	0.95	8.85	1.48
Mar0464	0.274	1.29	0.65	46.31	1.26
Apr0404	0.126	2.70	0.94	11.98	1.45
Apr0405	0.373	0.86	0.94	12.45	1.47
Apr0409	0.994	0.00	0.95	11.98	1.41
Aug0002	0.056	4.55	0.97	11.85	1.25
Aug0004	0.964	0.00	0.80	53.14	1.12
Aug0006	0.043	5.11	0.94	22.64	1.18
Nov0456	0.373	0.86	0.89	17.43	1.48
Washington					
MP01	0.146	2.42	0.93	19.21	1.34
MP03	0.340	0.99	0.95	20.50	1.25
MP04	0.641	0.23	0.96	11.30	1.42
MP05	0.022	7.09	0.98	7.82	1.44
MP06	0.265	1.37	0.95	13.38	1.37
MP07	0.246	1.50	0.95	11.19	1.48
MP10	0.250	1.47	0.92	19.82	1.39
MP11	0.555	0.37	0.89	24.50	1.32
MP12	0.343	0.98	0.92	18.63	1.39
MP14	0.870	0.03	0.94	17.99	1.33
MP15	0.084	3.56	0.96	11.83	1.35
MP17	0.225	1.65	0.92	13.41	1.52
MP18	0.203	1.83	0.95	12.43	1.44
MP22	0.966	0.00	0.93	18.47	1.33
MP24	0.656	0.21	0.85	35.71	1.33
MP26A	0.089	3.48	0.94	21.37	1.26
MP26B	0.115	2.89	0.96	10.40	1.40
MP27	0.779	0.08	0.95	20.10	1.24
MP28	0.816	0.06	0.93	14.76	1.36
MP30	0.035	5.68	0.95	13.83	1.40
AP10	0.093	3.34	0.97	11.01	1.35

^a Dependent variable was log₁₀ transformation of percent growth response with 1 degree of freedom and *P* ≤ 0.05.

Fig. 1. Radial growth of a representative sensitive isolate of *Mycosphaerella pinodes* (R0101R) in response to media amended with a range of concentrations of pyraclostrobin (formulated product, no salicylhydroxamic acid).

previously. An additional 25 isolates designated as sensitive in the radial growth study were also assessed. The lower discriminatory dose was chosen based on the previous conidial germination study, which indicated that a lower dose is required to inhibit conidial germination than is necessary to inhibit radial growth, and was selected to identify highly insensitive isolates. The isolates were classified as sensitive, intermediate, or insensitive, as described previously. The trial was repeated once.

Effective concentration to reduce mycelial growth of insensitive isolates. To assess the degree of insensitivity of the insensitive isolates, a radial growth study was conducted using Headline-amended PDA and 0.01% SHAM, as described previously. The treatments consisted of pyraclostrobin at 0, 5, 10, 20, 40, 80, 160, or 320 mg liter⁻¹. Colony growth was measured after 14 days and effective concentration to reduce mycelial growth by 50% (EC₅₀) values were calculated. The trial was repeated once.

Effect of fungicide application on disease development. A study was conducted to compare the efficacy of pyraclostrobin against sensitive and insensitive isolates inoculated onto field pea plants under controlled conditions, adapted from Thaher (2011). The study was set up in a randomized complete block design with four replicates and 10 pots per experimental unit. Six treatments were applied: (i) nontreated control (no fungicide, no inoculation); (ii) pyraclostrobin application only (fungicide control); (iii) inoculation with sensitive isolates only (inoculated control); (iv) inoculation with insensitive isolates only (inoculated control); (v) pyraclostrobin application, then inoculation with sensitive isolates; and (vi) pyraclostrobin application, then inoculation with insensitive isolates.

Ten plants of field pea 'CDC Meadow' were grown in each 15-cm-diameter pot of soil-less mix (ProMix BX; Premier Horticulture) in a growth chamber set with a 16-h photoperiod at 20°C (day) and 15°C (night). The spore suspensions were produced using the procedure from the conidial germination study, with conidia of five randomly selected insensitive isolates combined into one spore suspension, and a similar spore suspension prepared using five sensitive isolates. The plants were sprayed to saturation with the recommended rate of pyraclostrobin (0.04 ml per 10 ml of water) at 21 days after planting and inoculated 1 day later by spraying plants with a spore suspension (2 × 10⁵ spores ml⁻¹). A handheld spray bottle was used for both fungicide application and inoculation. After inoculation, the pots were incubated in a transparent-plastic moist chamber at high humidity for 48 h with a 16-h photoperiod at 18°C (day) and 15°C (night). The plants were then returned to the growth chamber, where the humidity was kept as high as possible with the use of a humidifier. Each plant was rated for *Mycosphaerella* blight lesion severity at 7 days after inoculation using the 0-to-9 scale of Xu et al. (1996), where 0 = no disease symptoms and 9 = the plant is completely covered with lesions.

Data analysis. For each isolate in the radial growth studies, mean growth per treatment was converted to a percentage of the growth of an unamended control, as follows: [1 – (growth on amended medium/growth on unamended medium) × 100]. In the conidial germination study, counts were converted to percent germination relative to the unamended control, as follows: [1 – (germination on amended medium/germination on unamended medium) × 100] (Thaher 2011). In the study to assess the effect of SHAM, the two response curves for each isolate with and without SHAM were graphed and tested against each other to identify differences.

All statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC) and differences were considered significant at $P \leq 0.05$. The EC₅₀ value represents the effective concentration of the amount of fungicide active ingredient required to inhibit growth of the pathogen by 50%. The EC₅₀ values for each isolate for both radial growth and conidial germination were determined by fitting the data to a nonlinear equation and using nonlinear regression (PROC NLIN). The residual data were tested for normality using PROC UNIVARIATE (Shapiro-Wilk test; Wise et al. 2008), and did not follow a normal curve because of the nonlinear dose response of the pathogen. To normalize the residuals, the data were transformed

using a square root transformation. To compute variance and eliminate outliers, mean EC₅₀ values that were three, two, and then one standard deviations from the mean were removed from the data set (R. C. Yang, University of Alberta, personal communication).

Analysis of variance (ANOVA) was conducted for each isolate within a data set. The ANOVA tables used to obtain the EC₅₀ values were then compared against each other. *F* tests were conducted on the values obtained from the ANOVA tables to ensure homogeneity of the data (Wise et al. 2009). Once the data were homogeneous, the value was recorded and a discriminatory dose was chosen from the homogeneous data set (R. C. Yang, University of Alberta, personal communication).

Table 2. Effective concentration to inhibit 50% growth (EC₅₀) of baseline isolates of *Mycosphaerella pinodes*, based on radial growth of media amended with formulated product (FP) and technical-grade (TG) pyraclostrobin with and without salicylhydroxamic acid (SHAM)

Isolate	EC ₅₀ (µg ml ⁻¹) ^a		
	No SHAM FP	With SHAM FP	With SHAM TG
R010R	0.104	0.074	N/A
Sep0002	0.072	0.187	0.116
Sep0004	0.076	0.165	0.136
Sep0008	0.077	0.114	0.059
Mar0405	0.216	0.152	0.059
Mar0412	0.075	0.090	0.118
Mar0413	0.131	0.137	0.142
Mar0419	0.121	0.091	0.169
Mar0425	0.199	0.107	0.092
Mar0426	0.110	0.101	0.099
Mar0427	0.121	0.090	0.084
Mar0435	0.071	0.065	0.050
Mar0448	0.122	0.173	0.137
Mar0452	0.128	0.179	0.147
Mar0458	0.185	0.167	0.125
Mar0460	0.151	0.166	N/A
Mar0463	0.052	0.149	0.100
Mar0464	0.139	0.084	0.102
Apr0404	0.078	0.169	0.063
Apr0405	0.133	0.149	0.142
Apr0409	0.100	0.089	0.095
Aug0002	0.065	0.079	0.034
Aug0004	0.031	0.092	0.037
Aug0006	0.091	0.082	0.036
Nov0456	0.295	0.128	0.124
MP01	0.209	0.203	0.117
MP03	0.091	0.094	0.066
MP04	0.121	0.216	0.146
MP05	0.093	0.089	0.077
MP06	0.117	0.120	0.124
MP07	0.185	0.148	0.150
MP08	0.177	0.206	N/A
MP09	0.150	0.142	N/A
MP10	0.100	0.180	0.118
MP11	0.138	0.153	0.110
MP12	0.158	0.129	0.142
MP14	0.148	0.156	0.188
MP15	0.090	0.104	0.093
MP17	N/A	0.164	0.241
MP18	N/A	0.189	0.123
MP22	0.081	0.128	0.176
MP23	0.145	0.130	N/A
MP24	0.248	0.185	0.240
MP26A	0.057	0.108	0.159
MP26B	0.106	0.121	0.126
MP27	0.087	0.104	0.150
MP28	0.145	0.104	0.119
MP30	0.173	0.137	0.148
AP10	N/A	0.067	0.125

^a N/A – not available.

To assess the effect of the pyraclostrobin source on the expression of sensitivity in the baseline isolates, the two response curves (formulated versus technical-grade active ingredient) for each isolate were tested against each other independently to compare the EC₅₀ values and graphed. The data were tested to ensure that the assumptions of ANOVA were met and means were separated using Fisher's protected least significant difference at $P \leq 0.05$. The data were then log-transformed to linearize the logarithmic curve and compared using the general linear model (PROC GLM). Although the isolates would be considered a random effect, because each isolate was tested individually, isolates were not included in the model statement. The same log transformation and statistical analysis was used to assess the effect of SHAM (with and without SHAM added; R. C. Yang, University of Alberta, personal communication).

The EC₅₀ values for the insensitive isolates were determined using nonlinear regression (PROC NLIN). Treatment means between the insensitive and sensitive isolates from all geographical areas were compared using the general linear model (PROC GLM) with

Tukey's multiple range test and orthogonal single-degree-of-freedom contrasts.

Results

Baseline sensitivity. Radial growth assessments. In the initial radial growth study of the baseline isolates, there was no main effect of isolate or source of pyraclostrobin (technical-grade versus formulated product) (Table 1). Data indicated that the range of EC₅₀ values was between 0.05 and 0.1 mg liter⁻¹ a.i. (Fig. 1). The EC₅₀ values of individual isolate for pyraclostrobin were 0.03 to 0.29 mg liter⁻¹, with a mean of 0.12 mg liter⁻¹ (Table 2). In companion trials, there was no significant effect of adding SHAM to the media (Table 3) and there were no differences in the EC₅₀ values (Table 2). When graphed and compared, the response curves of the two data sets for each isolate were similar (Fig. 2; Supplementary Fig. S1). All isolates were sensitive to pyraclostrobin.

Conidial germination assessment. Similarly, conidial germination of the baseline isolates in response to pyraclostrobin varied slightly

Table 3. Analysis of variance for radial growth of the baseline isolates of *Mycosphaerella pinodes* isolates in response to the formulated product of pyraclostrobin with and without salicylhydroxamic acid

Isolate	<i>Pr</i> > <i>F</i>	<i>F</i> value	<i>R</i> ²	Coefficient of variation	Log ₁₀ growth ^a
Saskatchewan					
R010R	0.176	2.04	0.91	24.79	1.25
Sep0002	0.138	2.44	0.90	45.75	1.02
Sep0004	0.009	9.05	0.94	26.89	1.11
Sep0008	0.042	5.03	0.97	12.45	1.27
Mar0405	0.166	2.13	0.94	17.04	1.33
Mar0412	0.186	1.94	0.90	34.24	1.14
Mar0413	0.998	0.00	0.95	16.04	1.27
Mar0419	0.616	0.26	0.97	12.26	1.26
Mar0425	0.298	1.17	0.92	34.43	1.10
Mar0426	0.522	0.43	0.96	11.81	1.35
Mar0427	0.285	1.24	0.95	19.87	1.17
Mar0435	0.028	6.01	0.92	29.51	1.13
Mar0448	0.101	3.08	0.95	15.65	1.29
Mar0458	0.081	3.55	0.91	26.61	1.21
Mar0460	0.930	0.01	0.96	13.74	1.28
Mar0464	0.117	2.79	0.92	31.21	1.15
Apr0404	0.052	4.50	0.96	15.58	1.25
Apr0405	0.246	1.47	0.96	14.98	1.28
Apr0409	0.123	2.69	0.92	34.90	1.09
Aug0002	0.129	2.62	0.86	42.92	1.11
Aug0004	0.040	5.14	0.85	52.00	1.07
Aug0006	0.413	0.71	0.81	60.64	1.09
Nov0456	0.178	2.01	0.93	16.38	1.38
Washington					
MP01	0.159	2.21	0.96	12.25	1.33
MP03	0.493	0.50	0.95	18.01	1.23
MP04	0.802	0.07	0.95	13.72	1.33
MP05	0.067	3.87	0.77	27.19	1.34
MP06	0.007	10.0	0.96	17.13	1.20
MP07	0.660	0.20	0.95	10.31	1.45
MP08	0.212	1.72	0.92	12.27	1.51
MP09	0.027	6.07	0.96	15.26	1.24
MP10	0.261	1.38	0.92	17.76	1.39
MP11	0.742	0.11	0.90	24.32	1.30
MP12	0.777	0.08	0.92	18.89	1.31
MP14	0.230	1.59	0.94	14.66	1.38
MP15	0.146	2.37	0.95	15.01	1.25
MP22	0.020	6.92	0.97	14.64	1.20
MP23	0.033	5.53	0.72	45.47	1.23
MP24	0.156	2.27	0.87	24.07	1.41
MP26A	0.637	4.05	0.97	21.16	1.00
MP26B	0.524	0.43	0.98	8.15	1.35
MP27	0.120	2.75	0.96	19.91	1.10
MP28	0.582	0.40	0.94	16.62	1.28
MP30	0.555	0.37	0.96	8.63	1.45

^a Dependent variable was log₁₀ transformation of percent growth response with 1 degree of freedom and $P \leq 0.05$.

but did not differ significantly among isolates or source of pyraclostrobin. Data indicated that the EC_{50} value was approximately $0.01 \text{ mg liter}^{-1}$ a.i. (Supplementary Fig. S2). The EC_{50} values for pyraclostrobin were 0.008 to $0.041 \text{ mg liter}^{-1}$, with a mean of $0.015 \text{ mg liter}^{-1}$ (Supplementary Table S1). Although the EC_{50} values were lower than for the radial growth procedure, based on conidial germination, the classification of the isolates were the same and they were sensitive to pyraclostrobin.

Sensitivity of *M. pinodes* isolates collected in 2010 and 2011.

Testing for sensitivity. The sensitivity to pyraclostrobin of 324 isolates from four geographical areas (Saskatchewan, Alberta, Washington, and North Dakota) was assessed at a discriminatory dose of $5.0 \text{ mg liter}^{-1}$ using the radial growth procedure. Of those isolates, 19 were insensitive, 304 were sensitive, and 1 was intermediate (Table 4; Supplementary Table S2; Supplementary Fig. S3) based on the classification system outlined above. The 19 isolates that were identified as insensitive were assessed using the conidial germination procedure at a discriminatory dose of $0.1 \text{ mg liter}^{-1}$. The isolates followed the same response pattern and were classified as insensitive (Table 4). Of the 19 insensitive isolates, 9 were collected from central Alberta, 5 from north-central Alberta, and 5 from across central and southern Saskatchewan (Table 4).

EC_{50} of insensitive isolates. The radial growth response of the insensitive isolates to the high concentrations of the pyraclostrobin fungicide varied. Data indicated EC_{50} values as high as $260 \text{ mg liter}^{-1}$ and as low as $120 \text{ mg liter}^{-1}$. Upon analysis, the EC_{50} values for the 19 insensitive isolates indicated pyraclostrobin at 80 to $261 \text{ mg liter}^{-1}$, with a mean of $180 \text{ mg liter}^{-1}$ (Table 4). The dose response curve and EC_{50} values for the insensitive isolates differed significantly from that of the sensitive isolates (Fig. 3). The mean EC_{50} value of the insensitive isolates was nearly 1,500 times higher than the mean for the sensitive isolates.

Effect of fungicide application on disease development. Application of pyraclostrobin had no effect on *Mycosphaerella* blight severity resulting from inoculation with the insensitive isolates but substantially reduced severity on the plants inoculated with the sensitive isolates. The mean *Mycosphaerella* blight rating for the sensitive isolates sprayed with the fungicide was 0.2 (of 9), whereas the mean rating of the insensitive isolates was 6.4 .

Discussion

Repeated application of strobilurin fungicides during the growing season is the most effective option for management of *M. pinodes* on field pea (Bretag 1985; Warkentin et al. 2000). However, strobilurins

have a very site-specific mode of action; therefore, many pathogen populations have a high risk of development of insensitivity within the pathogen population. Repeated application increases the selection pressure for fungicide insensitivity (Gisi et al. 1997; Ma and Michailides 2005). Insensitivity to the strobilurins has already been reported in populations of several pathogens in the genus *Mycosphaerella* and has also developed in populations of *A. rabiei* on chickpea across the Northern Great Plains (Gossen et al. 2008; Thayer 2011; Wise et al. 2008, 2009). Therefore, a study to develop a baseline for *M. pinodes* for sensitivity to strobilurin fungicides and to determine the occurrence in the shift in pyraclostrobin sensitivity was timely. This is the first report of isolates of *M. pinodes* that are insensitive to pyraclostrobin.

The baseline sensitivity of *M. pinodes* to pyraclostrobin was established based on the EC_{50} value response of 70 isolates collected from western Canada, the northern Great Plains, and the northwestern United States. Insensitivity to a fungicidal mode of action may occur in a pathogen population that has not been previously exposed to that fungicide as a consequence of naturally occurring mutations (Brent and Holloman 2007b) or even insensitivity of the entire species. In the current study, there was concern that such isolates could be represented in the sample and could affect the baseline measurement. However, each of the isolates in the baseline sensitivity assessment had a substantial level of inhibition by the fungicide and we concluded that each of the isolates in the baseline collection was sensitive to pyraclostrobin.

There are two main methodologies used to test the sensitivity of a pathogen to a fungicide in vitro: radial growth and conidial germination. Several studies have reported that conidial germination was more effective than radial growth for assessment of pathogen insensitivity (Demirci et al. 2003; Vincelli and Dixon 2002). In the current study, the EC_{50} values of the baseline isolates were much lower in the conidial germination assay (0.008 to $0.041 \text{ mg liter}^{-1}$) than in the radial growth assay (0.031 to $0.294 \text{ mg liter}^{-1}$) but there were no differences in the classification of isolates using either method.

Previous research involving in vitro testing indicated that SHAM is necessary to inhibit alternative respiration in some pathogens to minimize the incidence of false negatives for insensitivity. For example, there were differences in the EC_{50} values with and without SHAM for *A. rabiei* (Wise et al. 2008, 2009). However, another study reported that differences associated with SHAM in *A. rabiei* were much smaller than those associated with the qualitative resistance; therefore, SHAM was not essential for differentiating sensitive

Fig. 2. Dose response curves for radial growth of a representative sensitive isolate of *Mycosphaerella pinodes* (Apr0404) to the formulated product of pyraclostrobin, with and without salicylhydroxamic acid (SHAM), relative to a nontreated control.

Fig. 3. Dose response curve of the mean of all *Mycosphaerella pinodes* isolates that were insensitive and sensitive to the formulated product of pyraclostrobin fungicide collected from Alberta (AB) and Saskatchewan (SK) in 2010 and 2011.

and insensitive isolates for routine assessments (Thaier 2011). In the current study, there were no significant differences in the EC₅₀ values or response curves with or without SHAM. However, even though alternative respiration was not detected, SHAM was included in the insensitivity testing of isolates collected in 2010 and 2011 as a precaution to ensure that an accurate response to the fungicide was obtained.

Both formulated product and technical-grade active ingredients of pyraclostrobin have been used in testing for fungicide insensitivity in previous in vitro studies. Formulated product is more readily available than technical-grade active ingredient but the components of the formulation may occasionally affect the results. In the current study, however, there was no difference between formulated product and the technical-grade active ingredient.

The discriminatory dose used to test for insensitivity needs to be appropriate to the pathogen, the combination with the fungicide, and the research being conducted. Concentrations range from 4 to 200× the baseline EC₅₀ (Avila-Adame et al. 2003; Mondal et al. 2005; Rebollar-Alviter et al. 2007; Wise et al. 2009). In all research conducted, the dose is chosen based on previous growth observations. A discriminatory dose of 5 mg liter⁻¹ was selected to assess the isolates collected in 2010 and 2011, which was about 50× the baseline EC₅₀ and 2.5× the recommended application rate. This level was chosen to identify only highly insensitive isolates. At this high concentration of pyraclostrobin, sensitive isolates were only capable of slow growth, whereas the growth of highly insensitive isolates was only slightly reduced.

Only 19 of the 324 isolates of *M. pinodes* collected from the four geographical areas in 2011 and 2012 were insensitive to pyraclostrobin and 1 isolate had intermediate sensitivity. Although sensitivity to only one strobilurin fungicide, pyraclostrobin, was examined, cross-resistance among the actives in the strobilurin group is the norm due to their highly specific mode of action (Avila-Adame et al. 2003; Bartlett et al. 2002).

There are two mutations in the mitochondrial cytochrome b gene known to confer insensitivity to strobilurins: G143A and F129 L (Gisi et al. 2000; Torriani et al. 2009). The G143A mutation is a single nucleotide change on amino acid 143 from glycine to alanine and results in qualitative insensitivity. The F129 L mutation is a change on amino acid 129 from phenylalanine to leucine, which produces only moderate insensitivity; therefore, effective disease reduction with QoI fungicides is still possible (Gisi et al. 2000). The high level

of insensitivity detected in the 19 pyraclostrobin-insensitive isolates of *M. pinodes* indicates that it is the result of the G143 mutation. Therefore, increasing the amount of fungicide used or the frequency of fungicide application will not result in disease suppression. Indeed, in the current study, the average EC₅₀ value of the insensitive isolates was determined to be 180 mg liter⁻¹, which corresponds to about 65× the label rate.

Of the 19 pyraclostrobin-insensitive isolates, 9 isolates were collected from Central Alberta, where field pea is grown intensively and fungicides are applied almost every year as a preventative measure. Five of the insensitive isolates were collected from more northern areas of Alberta, where field pea cultivation is also widespread and pyraclostrobin is frequently used. The remaining five isolates were collected from across central and southern Saskatchewan, where field pea is frequently encountered but not a normal component of the cropping rotation in every field. None of the isolates collected from North Dakota or Washington State were classified as insensitive. This may reflect less frequent exposure of the pathogen population to strobilurin fungicides in those areas. The acreage of field pea in Canada is about seven times higher than in the United States, and that disparity is increasing (USDA 2010). More intensive production of field pea has almost certainly resulted in a more frequent application of pyraclostrobin for *Mycosphaerella* blight management, with a corresponding increase in the intensity of selection pressure for insensitive strains. More frequent cultivation of a susceptible host, a large pathogen population, and frequent application of fungicide all favor the occurrence of insensitive individuals in the population. Nevertheless, the prevalence of insensitive strains may eventually increase even in areas where selection pressure is not high, because of the airborne nature of the sexual spores of *M. pinodes*. Fungicidal insensitivity can be spread over long distances through the dissemination of airborne ascospores (Torriani et al. 2009).

When insensitive isolates were inoculated onto pea plants and sprayed with pyraclostrobin to observe the response in planta, the insensitive isolates caused severe foliar symptoms, similar to that in the nonsprayed but inoculated control, but almost no disease developed following inoculation of fungicide-treated plants with sensitive isolates. These results indicate that the aggressiveness of the insensitive isolates is similar to that of sensitive isolates. Under field conditions, insensitive isolates generally become more common because of the selection pressure imposed by continued fungicide application (Ma

Table 4. Isolates of *Mycosphaerella pinodes* collected in 2010 and 2011 from Saskatchewan and Alberta with reduced sensitivity to pyraclostrobin fungicide (formulated product), based on reduction in radial growth and conidial germination

Year	Culture	Growth reduction (%)	Germination reduction (%)	EC ₅₀ (µg ml ⁻¹)	Classification
Alberta					
2010	Barrhead 8	28.57	6.33	144.4	Insensitive
	Barrhead 7	25.33	9.76	260.7	Insensitive
	Barrhead 6	16.89	9.12	186.0	Insensitive
	Mannville F11A-2	16.86	1.68	211.5	Insensitive
	Mannville F11A-4	15.18	11.33	255.2	Insensitive
	Kelsey 5	30.74	4.18	176.0	Insensitive
2011	Hwy 611 11	25.31	2.71	226.8	Insensitive
	Red Deer F1-3	20.95	16.60	149.0	Insensitive
	Lacombe F2-1	15.54	5.19	148.3	Insensitive
	Red Deer F2-3	15.14	19.63	161.3	Insensitive
	New Norway 1	10.41	2.51	216.5	Insensitive
	Hwy 611 1	-5.29	1.00	185.8	Insensitive
	Hwy 611 5	-10.61	7.88	230.5	Insensitive
	Hwy 611 8	-35.29	6.86	136.4	Insensitive
Saskatchewan					
2010	Swift Current 3	29.30	13.98	80.3	Insensitive
	Wingard Ferry 4	21.06	6.62	179.9	Insensitive
	Swift Current 6	16.22	17.72	189.2	Insensitive
	Wolsley 3	12.57	3.05	97.6	Insensitive
	Saskatoon 1	-23.95	-0.40	176.8	Insensitive
2011	Purdue 17	50.19	N/A	5.0	Intermediate

and Michailides 2005). Thus, as long as the isolates remain pathogenic, and given the sexual reproduction of *M. pinodes*, the frequency of fungicide-insensitive isolates will likely continue to increase, and application of the fungicide will no longer be sufficient to control the disease. This will mean that strobilurins will not be effective in controlling *M. pinodes* in field pea and other fungicides will have to be used.

In chickpea, the increase in insensitive isolates and associated breakdown of effective *Ascochyta* blight reduction using strobilurin fungicides occurred over a period of only 2 to 3 years (Gossen et al. 2008; Taher 2011; Wise et al. 2008, 2009), forcing producers to utilize a fungicide with an alternative mode of action. This type of abrupt change in the sensitivity to strobilurin fungicides has not yet been observed with *M. pinodes*, likely because of lower selection pressure. Only 6% of the 324 isolates of *M. pinodes* from across the northern Great Plains region were insensitive to the pyraclostrobin, while the overwhelming majority were sensitive. Pyraclostrobin has not been applied as intensively onto field pea as it has been on chickpea because many producers do not consider it economically feasible to make repeated applications of fungicide to field pea crops. However, the identification of insensitive isolates is a cause for concern. It is critically important to continue to monitor *M. pinodes* populations for increased frequency of strobilurin insensitivity. The agricultural industry must ensure the prudent use of this fungicide chemistry in the future and continue to work diligently to develop other management strategies, such as cultivar resistance for the control of *Mycosphaerella* blight in field pea.

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