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Baseline sensitivity of *Fusarium graminearum* from wheat, corn, dry bean and soybean to pydiflumetofen in Michigan, USA

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ABSTRACT

Fusarium graminearum is perhaps best known for causing head blight in wheat and ear and stalk rot in corn. However, *F. graminearum* can also cause seedling diseases and root rot on many field crops including wheat, corn, soybean, and dry bean. In 2019, a new Succinate Dehydrogenase Inhibitor (SDHI) fungicide, pydiflumetofen (Syngenta, Switzerland), was registered for management of Fusarium Head Blight of wheat and corn ear rot. Here, we determined the sensitivity of isolates of *F. graminearum* from wheat, corn, soybean and dry bean from Michigan to this new active ingredient. *In vitro* sensitivity of 94 isolates was assessed with a poison plate mycelial growth assay, and a subset with 21 isolates was also tested with a spore germination assay. The effective concentration that reduced growth or germination by 50% (EC_{50}) was determined for each isolate. Mycelial growth EC_{50} values ranged from 0.008 to 0.263 μg/mL, with a mean of 0.060 μg/mL. EC_{50} values for spore germination assays ranged from 0.167 to 0.538 μg/mL, with a mean of 0.321 μg/mL. This study demonstrates both mycelial growth and spore germination are inhibited by this new chemistry *in vitro*. This data also establishes baseline sensitivity values of Michigan populations of *F. graminearum* before widespread application of this chemistry on commercial fields.

1. Introduction

Fusarium graminearum is a major pathogen worldwide including the United States, causing economic losses due to Fusarium Head Blight (FHB) in wheat and ear mold in corn (Nganje et al., 2004; Windels, 2000). Not only does this pathogen cause yield losses, but also contamination of wheat and corn grain with mycotoxins, mainly deoxynivalenol (DON). These mycotoxins can be hazardous for both human and animal consumption (Pestka, 2010). The reduction in grain quality by infection and mycotoxins leaves a grower unable to sell the grain or result in grain unsuitable for feed. *F. graminearum* can also infect roots, including corn, wheat, dry bean and soybean roots, where it can contribute to stand and yield losses (Bilgi et al., 2011; Broders et al., 2007; Xue et al., 2007). While these are diverse crops and tissues, there is currently no evidence to suggest genetic or phenotypic differentiation between the populations of *F. graminearum* isolated from these different hosts (Burlakoti et al., 2008; Kuhnem et al., 2015a, 2015b).

In management of FHB in wheat and ear mold of corn, fungicides remain a very important tool for growers (McMullen et al., 2012; Wegulo et al., 2015), as complete genetic resistance to *F. graminearum* has not been fully elucidated nor integrated into all commercial varieties. Historically, demethylation inhibitor (DMI) fungicides (FRAC Code 3) have been the only fungicide class available for management of head scab to wheat growers in the United States, and have been in widespread use since 1998 when Folicur (tebuconazole) was first marketed for FHB and DON reduction in some states (McMullen et al., 2012). By 2008, four DMI fungicides had full federal registration for FHB on wheat: tebuconazole, prothioconazole, metconazole, and propiconazole. In Michigan, fungicides are frequently used on soft white wheat (SWW) to manage FHB, with past survey results indicating at least 40% of large scale growers of SWW use a fungicide every year (Black and Nagelkirk, 2014). Similarly, in corn, a DMI product, Proline 480 SC (prothioconazole), is the only product labelled to suppress *Fusarium* ear molds and reduce mycotoxin contamination. However, in 2019, a new active ingredient with a different mode of action, pydiflumetofen (Syngenta, Switzerland), was registered for head scab management in wheat and ear rot in corn. The trademark name for the active ingredient is Adepidyn™ and is marketed in wheat as Miravis™ Ace, and Miravis™ Neo in corn as a pre-mix with additional chemistries. Pydiflumetofen inhibits respiration and is in the succinate dehydrogenase inhibitor

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(SDHI) group of fungicides (FRAC code 7). It is a N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide with the chemical formula $C_{16}H_{16}Cl_3F_2N_3O_2$.

Currently, there is no documented resistance to pydiflumetofen, but the fungicide resistance action committee (FRAC) classifies this fungicide as medium to high risk for resistance development (FRAC code list 2019, www.frac.info/publications). Resistance to SDHI fungicides has been demonstrated in numerous pathosystems (Avenot et al., 2012, 2008; Miyamoto et al., 2010; Popko, Jr. et al., 2018; Rehfus et al., 2016; Veloukas et al., 2011) resulting in failure of disease management at the field level. The SDH enzyme has four subunits, three of which are involved in fungicide binding and subsequently where mutations conferring resistance are found (subunits B, C and D).

While baseline *in vitro* sensitivity to pydiflumetofen in a few other pathosystems has been published (Ayer et al., 2019; Miller et al., 2020), including a different FHB pathogen *F. asiaticum* (Hou et al., 2017), there is not yet any population level sensitivity data published for *F. graminearum*. Here, *in vitro* sensitivity of *F. graminearum* to pydiflumetofen was determined by both mycelial growth and spore germination assays, in order to confirm effectiveness and determine a baseline for future sensitivity monitoring in Michigan before widespread use of the chemistry occurs.

2. Materials and methods

2.1. Isolate collection

A subset of 94 isolates was chosen from a *F. graminearum* collection originating from Michigan wheat, corn, dry bean, and soybean surveys spanning 2011–2017, with a majority collected in 2016 and 2017. Isolates were chosen to reflect geographic diversity across Michigan when possible (Fig. 1.). Samples originated from 65 unique fields, with one to three isolates per field, and no more than one isolate was used from a single plant. The majority of isolates originated from wheat (n = 57) and corn (n = 22) hosts where pydiflumetofen is currently registered for *Fusarium* suppression. In addition, isolates from soybean (n = 5) or drybean (n = 10) roots were included to increase diversity and time frame of collection. Meta-data for each isolate can be found in supplementary table 1. All were single spored and identity confirmed by PCR amplification and sanger sequencing of the translation elongation factor 1-α (EF-1α) according to (O'Donnell et al., 1998). Isolates were stored in 35% glycerol at −80 °C or on #1 Whatman filter paper in 4 °C. Isolates were removed from storage immediately prior to assays.

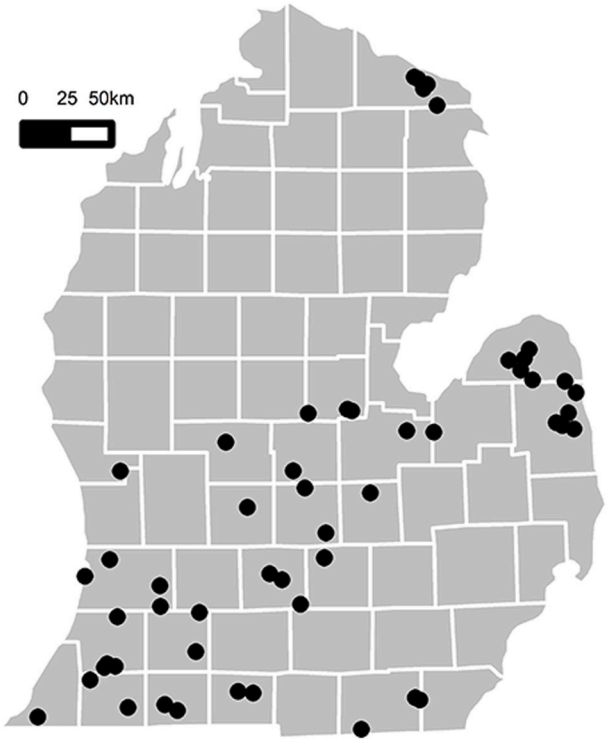

Fig. 1. Map of the state of Michigan, USA with counties shown. Dots indicating approximate locations of commercial fields where isolates of *F. graminearum* in this study were collected.

2.2. Evaluation of mycelial growth sensitivity

Baseline sensitivity of the 94 isolates was determined by mycelial growth assays on fungicide amended medium to determine EC_{50} values (effective concentration to reduce growth by 50%). Isolates were grown on potato dextrose agar (PDA; Acumedia, Lansing, MI) for 5d at 24 °C. A 5 mm cork borer was used to produce equally sized agar plugs from the leading edge of the colony. A single agar plug was placed mycelial side down directly in the center of the assay plate (100 mm Petri plate). Assay plates were made with PDA, autoclaved, and cooled to approximately 52 °C and amended with pydiflumetofen stock solutions. Stock solutions were prepared by dissolving technical grade pydiflumetofen (Syngenta Crop Protection) in methanol at five concentrations (5000, 1000, 250, 50, and 10 μg/mL), and then 1 mL of stock solution was used in 1L of the final medium to produce 0, 0.01, 0.05, 0.25, 1.00, or 5.00 μg/mL final concentration in assay plates. The non-treated control was amended with methanol only. These fungicide concentrations and PDA growth medium were chosen as they have been previously used (Hou et al., 2017) and appeared to best fit a dose response curve in preliminary studies compared to other more minimal medias.

Inoculated assay plates were incubated in the dark at 24 °C for 4 days. Then radial growth was measured in two perpendicular directions with a digital caliper (Absolute Digimatic Caliper, model CD-6" AX, Mitutoyo Corp., Sakado 1-Chome, Japan). Relative growth at each concentration was determined by dividing the average of the two measurements by the average radial growth of the same isolate on the non-treated control plate. Each experiment had two Petri plates replicating each concentration and isolate combination, and two experimental runs for each isolate were performed. Four total experiments were run, with sets of 45–50 isolates run in a single experiment.

2.3. Determination of spore germination sensitivity

Since SDHIs have demonstrated ability to inhibit spore germination, a subset of isolates was also evaluated for their sensitivity in spore germination assays. Twenty-one isolates were chosen to represent a range of EC_{50} values from the mycelial growth assays as well as crop and location diversity. Water agar was prepared with 20 g/L of agar (Sigma, St. Louis, MO) and amended with pydiflumetofen in the same manner as mycelial growth assays. Spore suspensions were generated by growing isolates in liquid carboxymethyl cellulose broth and shaken at room temperature for 4–5 days, which produces ample macroconidia (Cappellini and Peterson, 1965). The day of inoculation, spore suspensions were prepared by filtering the carboxymethyl cellulose broth through three layers of miracloth (EMD Millipore, Billerica, MA), centrifuged to pellet spores (5000 rpm for 5 min), and broth was decanted off. Spores were then resuspended in sterile water, quantified in a hemocytometer, and diluted to 2×10^5 spores/mL. Approximately 100 μl of the spore suspension was pipetted onto the agar surface and spread evenly across the plate with a glass rod. Plates were incubated in darkness at 24 °C for 20 h, then assessed visually under a microscope. Each experiment had two Petri plates replicating each concentration and isolate combination, and two experimental runs were performed. At least 50 spores were evaluated on each Petri plate. Spores were considered germinated if germ tubes were longer than the length of the spore. Relative percent germination was determined by dividing the percentage of spores

germinated on the plate by the average percent germination of the two control plates for the same isolate.

2.4. Data analysis

All data analysis was conducted in R (R Core Team, 2018). Model selection and EC_{50} determination were generated in R package 'drc' (Ritz et al., 2015). Since EC_{50} can be significantly affected by model choice (Noel et al., 2018), two common models, the 3- parameter log-logistic model, and the 4-parameter log logistic model, were evaluated by Akaike information criterion (AIC). The 3-parameter model was chosen, as it best fit the greatest proportion of isolates for both assays. Absolute EC_{50} values were estimated separately for each run, and mean of the two runs presented. Correlation between assays was assessed with Spearman correlation, and differences between mean EC_{50} values from the two assays were evaluated using a paired *t*-test. Shapiro-Wilk test of normality was performed with the shapiro.test from base R 'stats' (R Core Team, 2018). All code for data processing and analysis is publicly available on https://github.com/mikbreunig/Pydiflumetofen.

3. Results

3.1. Sensitivity of mycelial growth of F. graminearum to pydiflumetofen

EC_{50} values for mycelial growth assays for each of the 94 isolates are listed in Supplementary table 1. The mean EC_{50} was 0.060 μg/mL, median of 0.050 μg/mL, and values ranged from 0.008 to 0.263 μg/mL (Fig. 2). Values were not normally distributed ($P < 0.0001$), and skewed right. There appeared to be no trends in EC_{50} values related to year, host, or geographic location.

3.2. Sensitivity of spore germination of F. graminearum to pydiflumetofen

A subset of 21 *F. graminearum* isolates was used for both mycelial growth and spore germination assays (Table 1). EC_{50} values for spore germination had a mean of 0.321 μg/mL, median of 0.300 μg/mL, and range of 0.167–0.538 μg/mL (Fig. 3). Values were normally distributed ($P = 0.215$).

3.3. Comparison of assay type

The 21 isolates that were tested in both assays were analyzed to determine the relationship between spore germination and mycelial growth sensitivity. EC_{50} values from spore germination assays (mean = 0.320 μg/mL) were significantly greater ($P < 0.0001$) than the EC_{50} values for mycelial growth assays (mean = 0.073 μg/mL) (Table 1), with a mean difference of 0.246 μg/mL. There does not appear to be a significant correlation (r = 0.335, $P = 0.137$) between the EC_{50} values determined by mycelial growth and the EC_{50} values determined by spore germination.

4. Discussion

Fungicides remain a key tool in management of *Fusarium* and reduction of the resulting mycotoxins including deoxynivalenol in wheat and corn. The results of this study provide us with phenotypic data to evaluate the current sensitivity of Michigan populations of *Fusarium graminearum* to pydiflumetofen, a recently registered chemistry in the new SDHI chemical group N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides. *F. graminearum* appears sensitive to pydiflumetofen *in vitro* for both mycelial growth and spore germination, with EC_{50} values at 0.538 μg/mL and below for spore germination, and 0.263 μg/mL and below for mycelial growth. All isolates in this study were collected from commercial fields before this fungicide was registered, so this data can be used as a baseline sensitivity measurement for future efficacy monitoring in Michigan. While this active ingredient is a component of formulations in numerous other field and vegetable crops, these did not receive EPA registration until May 2018, so isolates of *F. graminearum* would not have been exposed to pydiflumetofen prior to collection. Other SDHI products could have been used in these systems previously, but none would be in the same chemical group as pydiflumetofen.

EC_{50} values were significantly greater for spore germination than mycelial growth, but the growth response curves do not necessarily indicate that spore germination is less sensitive to this chemistry. At the highest doses (1 μg/ml, 5 μg/ml), spore germination was completely inhibited, while mycelial growth was only inhibited 80–90%. Conversely, mycelial growth began to be inhibited at the lowest dose of 0.01 μg/mL, while spore germination was mostly unaffected. Selected growth response curves from each assay from a single isolate demonstrate this pattern (Fig. 4). It is not yet determined if these responses could simply be a function of assay methods or true biological differences in response at these different life stages. Evaluation of a closely related head blight pathogen, *Fusarium asiaticum* by Hou et al., in 2017 revealed similar results with isolates sensitive to pydiflumetofen and conidial germination EC_{50} values slightly greater than mycelial growth

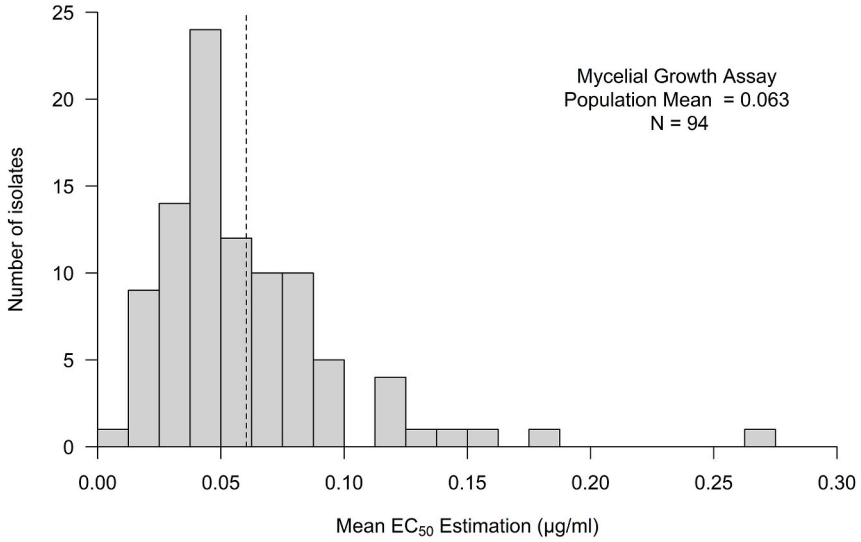

Fig. 2. Distribution of mean EC_{50} values for 94 isolates of *F. graminearum* as determined by mycelial growth assay, with dashed line indicating the mean.

Table 1
EC_{50} of 21 isolates determined by spore germination experiments and mycelial growth experiments, and mean difference between the two estimates.

Isolate name	EC_{50} Estimation				Mean Difference
	Spore Germination		Mycelial Growth		
	Mean (μg/ml)	95% CI	Mean (μg/ml)	95% CI	
119C	0.421	0.269–0.574	0.022	0.004–0.039	−0.399
53F	0.445	−0.646–1.536	0.082	0.056–0.109	−0.363
73S	0.186	−0.339–0.712	0.058	−0.011–0.127	−0.128
C6B	0.512	−1.755–2.78	0.082	0.062–0.101	−0.430
LEN9-3_S2_15F	0.167	−0.097–0.432	0.066	−0.207–0.339	−0.101
SAG_13-3-S1_150	0.327	−0.810–1.465	0.034	−0.058–0.126	−0.293
121D	0.304	0.118–0.491	0.042	0.019–0.065	−0.262
57G	0.300	−0.427–1.026	0.099	0.009–0.190	−0.201
66B	0.335	−0.283–0.953	0.055	−0.220–0.330	−0.280
76O	0.344	0.037–0.65	0.139	−0.140–0.419	−0.205
82B	0.538	−1.607–2.683	0.120	0.014–0.225	−0.418
C5-2D	0.287	0.136–0.438	0.029	0.016–0.041	−0.258
MI-CF-4-5	0.478	0.202–0.754	0.154	0.143–0.165	−0.324
104B	0.223	−0.502–0.949	0.018	−0.050–0.086	−0.205
89C	0.352	0.194–0.509	0.042	−0.066–0.149	−0.310
90A	0.274	−0.625–1.173	0.046	−0.043–0.134	−0.228
C5-3C	0.260	−0.931–1.451	0.079	−0.161–0.318	−0.181
F_15_115	0.246	−0.055–0.548	0.057	−0.073–0.186	−0.189
Ingham_P104	0.189	−0.495–0.872	0.038	0.011–0.065	−0.151
21A	0.286	0.140–0.432	0.264	−1.641–2.168	−0.022
94B	0.256	0.085–0.428	0.025	−0.040–0.090	−0.231
Mean	0.320		0.073		−0.246

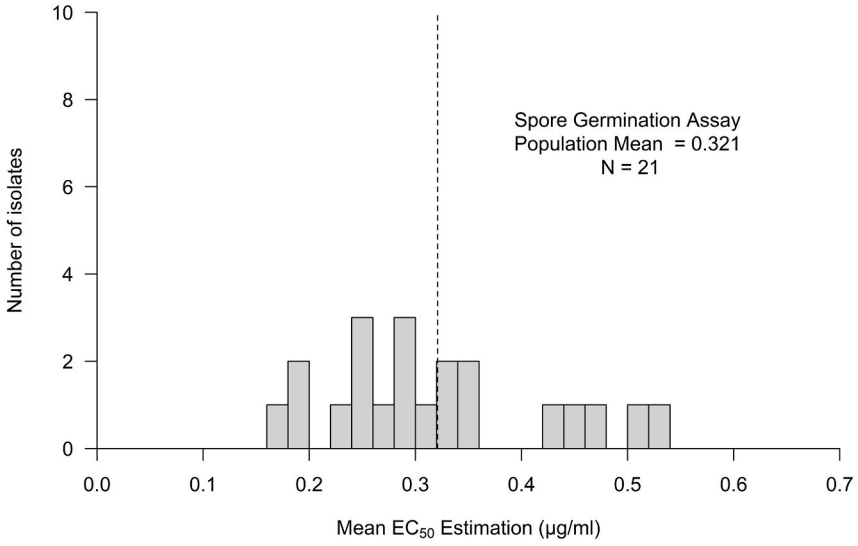

Fig. 3. Distribution of mean EC_{50} values for 21 isolates of *F. graminearum* as determined by spore germination assays, with dashed line indicating the mean.

EC_{50} values.

Differences between the mycelial growth and spore germination assays were also explored by analyzing the correlation between the EC_{50} values from each assay. We did not find a correlation in this study, but that could be due to the low level of variation in EC_{50} values. If isolates with larger EC_{50} values were found, this relationship could be more thoroughly investigated. Because of this unclear relationship, future studies investigating pydiflumetofen should consider using both assays for a portion of isolates.

While we did not detect any resistance in our study, it is possible that we failed to detect resistance present at low levels due to the sample size of isolates tested (n = 94). This was explored using power analysis, with a power calculation for one sample proportion using pwr.p.test function in pwr package in R (Champely, 2018) with $\alpha = 0.05$. Given 94 isolates, the power for detecting a resistance proportion of 5% is 99%. However, if resistance is present at only 1%, the statistical power is reduced to 61%. Nonetheless, the results of this study are a good indication that resistance is not currently present at significant levels.

Besides testing additional isolates and geographies phenotypically, future studies could monitor the population molecularly for mutations in sdh subunits. One published study has investigated resistance risk of *F. graminearum* to pydiflumetofen, not by testing natural populations but by generating mutants (Sun et al., 2020). In the resistant mutants, four different mutations in the succinate dehydrogenase subunit C gene were discovered; these and other mutations could be assayed for in future studies of field populations.

Future work could also investigate the relationship between *in vitro* EC_{50} sensitivity values and *in vivo* efficacy for this chemistry. Such studies would be important to put *in vitro* data into context. However, to date, field testing from Michigan and across the United States demonstrates pydiflumetofen applications alone or as a premix perform similar to or better than other fungicide options for FHB suppression on wheat

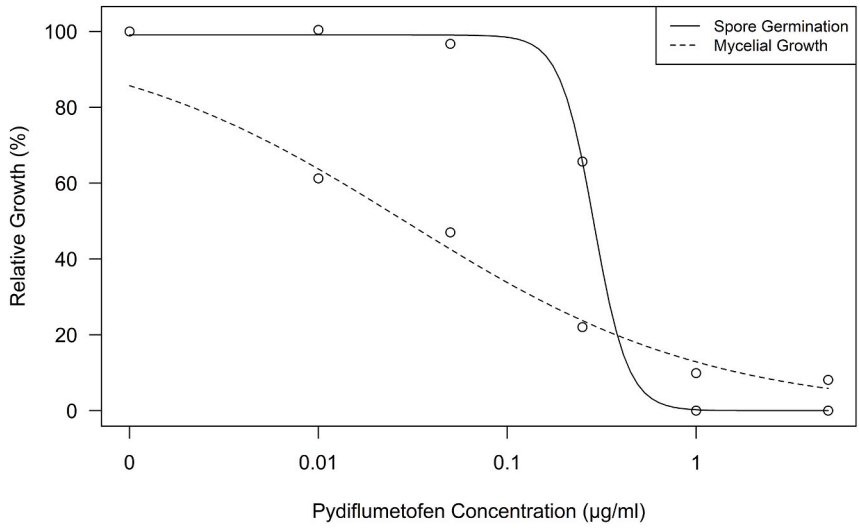

Fig. 4. Dose response curves from a representative isolate (C5-2D). Open circles represent average of relative growth or relative percent germination at each concentration, and lines the dose response model for each assay, solid line representing the spore germination assay and dashed mycelial growth assay.

(Breunig et al., 2017; Paul et al., 2019), suggesting *in vivo* efficacy at these EC_{50} levels. Likewise, studies with pydiflumetofen in other *Fusarium* species confirmed field efficacy at similar mycelial growth EC_{50} values. In the closely related *Fusarium asiaticum,* EC_{50} values ranged from 0.0900 to 0.2084 µg/ml and authors found 80% control of FHB in field studies (Hou et al., 2017). In the more distantly related *Fusarium oxysporum* f. sp. *niveum, sensitivity* ranged from 0.34 to 1.88 µg/ml, and showed similar control to prothioconazole in the field for watermelon wilt (Miller et al., 2020).

Prior to pydiflumetofen, no other SDHI chemistries were shown to be effective for *F. graminearum* or closely related species, and only one other SDHI (fluopyram) effective on a limited number of *Fusarium* species. Fluopyram is in the pyridinyl-ethylbenzamides chemical group and registered for Fusarium root rot diseases, mainly caused by members of the *Fusarium solani* species complex.

The introduction of pydiflumetofen is significant, as it introduces another mode of action for management of FHB in the United States beside DMI chemistries. Unpublished data determining sensitivity to DMIs for a subset of these isolates revealed isolates less sensitive to tebuconazole (EC_{50} values ranging from 1 to 4 µg/ml) were still quite sensitive to pydiflumetofen (<0.1 µg/ml) (Breunig and Chilvers, unpublished). If triazole resistance were to develop, this could mean pydiflumetofen would be a valuable rotation or mixing partner for reducing resistance risk and managing Fusarium head blight in the field. Ultimately the result of this baseline sensitivity measurement demonstrates populations of *F. graminearum* in Michigan are sensitive to pydiflumetofen *in vitro* for both mycelial growth and spore germination, and Adepidyn products could be valuable in management of diseases caused by *Fusarium graminearum.*

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2020.105419.

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