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Sensitivity of *Alternaria* spp. from potato to pyrimethanil, cyprodinil, and fludioxonil

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ABSTRACT

Early blight, caused by *Alternaria solani,* and brown leaf spot, caused by a number of small-spored *Alternaria* spp. including *Alternaria alternata* sensu stricto*, Alternaria arborescens,* and *Alternaria tenuissima,* are observed annually in all midwestern potato production areas. However, *Alternaria* spp. have developed reduced sensitivity and/or resistance to many single-site mode of action fungicides such as quinone outside inhibitor (QoI; FRAC group 11), succinate dehydrogenase inhibitor (SDHI; FRAC group 7), and anilinopyrimidine (AP; FRAC group 9). Mean in vitro sensitivity EC_{50} values (effective concentration where fungal growth is inhibited by 50%) of *A. alternata* (n = 16)*, A. arborescens* (n = 3)*, A. tenuissima* (n = 5), and *A. solani* (n = 58) in response to the AP fungicides pyrimethanil and cyprodinil and the phenylpyrrole (PP) fungicide fludioxonil were determined via mycelial growth assays. Significant fungicide by isolate interactions were observed for all *Alternaria* spp. evaluated in vitro, indicating reduced-sensitivity of some isolates to individual fungicides. EC_{50} values for three non-baseline *A. solani* isolates collected in 2010, 2011 and 2013 were within the baseline for all three fungicides. A significant correlation was observed between pyrimethanil and cyprodinil EC_{50} values among *A. alternata* isolates, but no relationship was observed with the other fungicides or in *A. solani*. In greenhouse evaluations, a significant loss of disease control was observed for some non-baseline *A. solani* isolates, and this was more pronounced in the AP fungicides, pyrimethanil and cyprodinil. No significant correlation was observed between in vitro EC_{50} value and area under the dose response curve based on greenhouse assays, likely due to the limited number of isolates evaluated. Further research is needed to determine if these reductions affect control of early blight and brown leaf spot in potato under field conditions. Results from this study indicate that fludioxonil and cyprodinil are potentially good additions into fungicide rotation programs or as co-pack chemistries for control of leaf spot diseases and fungicide resistance management.

1. Introduction

Early blight and brown leaf spot are chronic problems in potato production. *Alternaria solani* Sorauer*,* which causes early blight, is the dominant pathogen when compared to small-spored *Alternaria* spp., such as *Alternaria alternata* (Fr.) Keissler, *Alternaria arborescens* E.G. Simmons, and *Alternaria tenuissima* (Kunze) which cause brown leaf spot. Early blight and brown leaf spot can cause potato yield reductions up to 30 and 18%, respectively, if conditions are favorable (Christ and Maczuga, 1989; Droby et al., 1984; Shtienberg et al., 1990).

Specialty fungicides such as quinone outside inhibitors (QoI; FRAC group 11) and succinate dehydrogenase inhibitors (SDHI; FRAC group 7) utilized in rotation with standard protectant fungicides mancozeb or chlorothalonil, can provide early blight disease control and increase potato yield (Yellareddygari et al., 2019). While SDHI and QoI fungicides are useful additions to potato disease management programs, they are regarded as high resistance-risk fungicides due to the single-site modes of action. Specialty fungicides including demethylation inhibitors (DMI; FRAC group 3) and anilinopyrimidines (AP; FRAC group 9) also provide a high level of early blight disease control and are all classified as medium-risk fungicides. The addition of phenylpyrroles (PP; FRAC group 12) also may be useful in the management of early blight and brown leaf spot. PP fungicides are also classified as medium resistance-risk fungicides (FRAC, 2019).

While a low frequency of reduced sensitivity and/or resistance to the AP and PP fungicides has been observed in numerous pathogens, these

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chemistries remain effective for disease management (Avenot and Michailides, 2015; Fairchild et al., 2013; Fonseka and Gudmestad, 2016; Kanetis et al., 2008). A recent study determined that two DMI fungicides, difenoconazole, and metconazole, demonstrated high intrinsic activity against *A. solani* and putative *A. alternata* sensu lato (Fonseka and Gudmestad, 2016). Also in that study, some *A. solani* isolates exhibited reduced sensitivity to the AP fungicide pyrimethanil under in vitro conditions and were controlled to a significantly lesser degree than sensitive isolates in greenhouse evaluations. Pyrimethanil has been utilized in potato grower management programs for over 16 years and reduced sensitivity has only been detected in a small number of *A. solani* isolates in Colorado, Idaho, Minnesota, and Texas (Fairchild et al., 2013; Fonseka and Gudmestad, 2016). Other studies have demonstrated the AP fungicide, cyprodinil, and the PP fungicide, fludioxonil, effectively controlled putative *A. alternata* sensu lato isolates (Avenot and Michailides, 2015). In that study, a few isolates displayed resistance to fludioxonil and/or cyprodinil with no observed fitness penalties.

AP and PP fungicides are frequently used in combination with other fungicide chemistries in pre-packaged mixtures. These mixtures have been highly effective for management of *Botrytis cinerea* (Chapeland et al., 1999; Hilber and Schüepp, 1996), *A. alternata* sensu lato (Avenot and Michailides, 2015), and *Penicillium digitatum* (Kanetis et al., 2008). Fludioxonil is used primarily as a seed treatment fungicide registered on numerous crops, and as a post-harvest fungicide used on several tree fruit crops. Fludioxonil also is used in a pre-packaged mixture with cyprodinil for foliar disease control in pulse crops, and numerous vegetable and fruit crops. In potato, fludioxonil is used as a seed treatment for seed-borne tuber black scurf (*Rhizoctonia solani*) and, more recently has been mixed with other chemistries to manage potato storage diseases such as Fusarium dry rot (*Fusarium* spp.), and silver scurf of potato (*Helimthosporium solani*). The efficacy of cyprodinil and fludioxonil for management of early blight and brown leaf spot has not been studied extensively. Although cyprodinil is not currently registered for foliar use on potato, at the time these studies were initiated it was being considered as a pre-pack partner for recently developed SDHI fungicides. Fludioxonil is used as a pre-packaged mixture partner with the SDHI fungicide adepidyn as a potato foliar fungicide Miravis Prime™ (Syngenta Crop Protection, Greensboro, NC); however, its activity on the *Alternaria* leaf spot pathogens of potato has not been reported.

Previous studies have determined baseline (isolates collected before fungicide was registered) pyrimethanil sensitivity for both *A. solani* and putative *A. alternata* sensu lato (Fonseka and Gudmestad, 2016). A recently published study determined that potato brown spot is caused by four *Alternaria* spp. (*A. alternata, A. arbusti, A. arborescens,* and *A. tenuissima*) (Tymon et al., 2016). In light of this, further examination resulted in reclassification of some isolates of the putative *A. alternata* sensu lato baseline population tested for sensitivity to pyrimethanil (Fonseka and Gudmestad, 2016) as *A. arborescens* and *A. tenuissima* (Budde-Rodriguez, 2020). Given these new findings, reexamination of these isolates is warranted. Additionally, baseline sensitivities of *A. alternata, A. arborescens,* and *A. tenuissima* in response to cyprodinil and fludioxonil have not been established. Analyzing isolate response to new or existing fungicides aids in determining the fungicide risk factors and is the only way to effectively monitor for shifts in sensitivity. *Alternaria solani* and *A. alternata* sensu lato are classified as medium- and high-risk pathogens, respectively (FRAC, 2019). Risk to develop fungicide resistance has not been reported for *A. arborescens* and *A. tenuissima*. Therefore, the objectives of this study were to (i) determine the sensitivity of four *Alternaria* spp. to anilinopyrimidine and phenylpyrrole fungicides, and to (ii) determine the control of *A. solani* provided by pyrimethanil, cyprodinil, and fludioxonil.

2. Materials and methods

2.1. Collection and maintenance of Alternaria spp. isolates

All isolates of *A. solani* and three small-spored *Alternaria* spp. used in this study were recovered from foliage submitted to the laboratory from potato-growing regions across the United States. Recovery of *Alternaria* spp. from potato foliage was performed similarly as previously described (Bauske et al. 2018a, 2018b; Gudmestad et al., 2013; Mallik et al., 2014; Pasche et al. 2004, 2005). Foliar sections with lesions characteristic of early blight and brown spot were surface sterilized in a 10% sodium hypochlorite solution for 1 min and rinsed in sterile, distilled water. Tissue sections were aseptically excised from the foliar surface using a sterile scalpel blade and transferred to 1.5% non-amended agar media (water agar) and incubated at room temperature (22 ± 2 °C) for three to four days until conidia were observed. Purification of the isolates was performed by aseptically transferring a single conidium from the water agar culture to a clarified V8 (CV-8) (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water 900 ml) medium amended with 50 mg/ml ampicillin using a glass needle. Single conidium cultures were incubated under 24 h fluorescent light at room temperature (22 ± 2 °C) for 7 days and examined for growth characteristic of *Alternaria* spp. For long-term cryogenic storage, a 4-mm diameter sterilized cork borer was used to remove plugs of media with fungal conidia and mycelia and placed into 2 ml screw-top centrifuge tubes. The caps were loosely screwed on to the tubes, tubes were labeled and placed in a closed container with silica gel for two to three days to remove excess moisture. After drying, the tubes were capped tightly, sealed with Parafilm, and stored in an −80 °C ultra-freezer. Herbarium specimens were prepared for each tissue sample from which isolates of the four *Alternaria* spp. were obtained.

The identity of the *Alternaria* spp. isolates evaluated in these studies was confirmed as *A. alternata*, *A. arborescens,* or *A. tenuissima* via sequencing. Primer sets OPA-F4 (CGAGCCACATGCTCTGGTTA)/OPA-R4 (AAGTCTAGATCGCTTGCGGG) and OPA-F5 (TTCCACTTTGTCC CCTGCAA)/OPA-R5 (CGTATCTTCTCACGTCGGGC) were designed for this study based on the single-nucleotide polymorphisms (SNPs) using the previously published *OPA 1–3* anonymous locus genomic sequence as a template (Tymon et al., 2016). DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Mallik et al., 2014). Ion express plus fragment library kit (ThermoFisher Scientific, Waltham, MA) was utilized to create the genomic library. Libraries were sequenced using the Ion-Torrent next-generation semiconductor sequencing technology with an Ion Personal Genome Machine System (ThermoFisher Scientific, Waltham, MA). Primers OPA-F4/R4 and OPA-F5/R5 amplified 180 and 219 bp segments, respectively, targeting unique regions in OPA 1–3. Sequences from all isolates were aligned using the Multalin web aligning program (http://multalin.toulouse.inra.fr/multalin/multalin.html hosted by GenoToul bioinformatics platforms) and analyzed using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

To determine sensitivity to pyrimethanil, cyprodinil and fludioxonil, 16 *A. alternata,* three *A. arborescens,* and four *A. tenuissima* baseline isolates collected from 1999 to 2002, one *A. tenuissima* non-baseline collected in 2017, 55 *A. solani* baseline isolates collected from 1998 to 2002, and four non-baseline *A. solani* isolates collected in 2010, 2011 and 2013 were obtained from long-term cryogenic storage (Supp. Table S1). Most of the isolates evaluated during this study were also evaluated in the previous pyrimethanil and DMI fungicide sensitivity study (Fonseka and Gudmestad, 2016). Two *A. alternata,* one *A. arborescens,* one *A. tenuissima,* and six *A. solani* isolates were included in this study in addition to those evaluated previously. The evaluation of pyrimethanil sensitivity was conducted again in these populations to facilitate direct statistical comparisons across fungicides evaluated in this study.

2.2. In vitro sensitivity of Alternaria spp. isolates to pyrimethanil, cyprodinil, and fludioxonil

A study was performed to determine the in vitro sensitivity of isolates from four *Alternaria* spp. to pyrimethanil, cyprodinil, and fludioxonil in a manner similar to those previously described (Bauske et al., 2018b; Fonseka and Gudmestad, 2016; Gudmestad et al., 2013; Pasche et al. 2004, 2005). Twenty-four isolates of three *Alternaria* spp. were assayed in three trials with eight isolates included in each trial. Fifty-eight *A. solani* isolates were assayed in 10 trials with five to seven isolates included in each trial. Internal control isolates for *A. solani* (13-1, a QoI sensitive isolate, and 526-3, a QoI reduced-sensitive isolate) and *Alternaria* spp. (125-1, an *A. alternata* sensu stricto QoI sensitive isolate, and 1702-5, an *A. tenuissima* QoI reduced-sensitive isolate) were used in each trial to determine assay reproducibility (Wong and Wilcox, 2002).

Fungicide sensitivity for pyrimethanil and cyprodinil was determined using a mycelial growth assay on a solid synthetic media containing L-asparagine (asp-agar) (Fonseka and Gudmestad, 2016; Hilber and Schüepp, 1996). The asp-agar procedure was developed for the evaluation of *B. cinerea* sensitivity to AP fungicides. Complex, nutrient-rich media, such as malt-agars, are not appropriate for the in vitro assays as they allow the pathogen to overcome the fungicide activity (Hilber and Schüepp, 1996; Stevenson et al., 2019). Asp-agar media was amended with either technical grade pyrimethanil (95.0% active ingredient; Bayer CropScience, Raleigh, NC), or cyprodinil (98.0% active ingredient; Syngenta Crop Protection, Greensboro, NC) dissolved in acetone to reach final concentrations of 0.1, 1, 10, and 100 μg/ml (Fonseka and Gudmestad, 2016). In vitro evaluation of fludioxonil was also performed using a mycelial growth assay but does not require the synthetic media; therefore, quarter-strength potato dextrose agar (PDA) media was used (5 g Potato Dextrose broth, 15 g agar, 1 L H_2O) (Avenot and Michailides, 2015). Technical grade fludioxonil (98.0% active ingredient; Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to reach final concentrations of 0.1, 1, 10, and 100 μg/ml. A no-fungicide control was included and the acetone concentration in all media was 0.1% by volume. For evaluation of all three fungicides, a 5-mm mycelial plug excised from the edge of a 7-day old *Alternaria* spp. colony was inverted onto the center of the fungicide-amended media so that fungal growth was in contact with the media surface. The plates were incubated at 24 ± 2 °C in the dark for 7 days. After the incubation, mycelial growth diameter of each isolate was measured in two perpendicular directions, with the original 5-mm diameter mycelial plug subtracted from the final measurement.

2.3. In vivo efficacy of pyrimethanil, cyprodinil, and fludioxonil to Alternaria solani

Four *A. solani* baseline isolates expressing the highest measured in vitro EC_{50} values to either cyprodinil and/or fludioxonil and four non-baseline isolates were evaluated under greenhouse conditions (Table 1). Efficacy of AP and PP fungicides against early blight were assayed under greenhouse conditions using a 24 h preventative test similar to methods previously described (Fonseka and Gudmestad, 2016; Gudmestad et al., 2013; Pasche et al. 2004, 2005). The Orange Pixie tomato cultivar (Tomato Growers Supply Company, Fort Myers, FL) was chosen because of its susceptibility to early blight, its compact size compared to potato plants, and the resistance of leaves to dehisce once severely infected. Three tomato seeds were sown in 10 cm^3 plastic pots containing Sunshine Mix LC1 (Sun Gro Horticulture Inc., Bellevue, WA). After emergence, plants were thinned to leave two uniformly-sized plants remaining per pot. When the plants reached a height of 15–20 cm and the first three leaves were fully expanded, the plants were treated with the commercial formulation of pyrimethanil (Scala ® SC, Bayer CropScience LP, St. Louis, MO), cyprodinil (Vangard ® WG, Syngenta Crop Protection, Greensboro, NC), or fludioxonil (Scholar ®, Syngenta Crop Protection, Greensboro, NC). Fungicide concentrations of 0, 0.1, 1, 10, and 100 μg/ml of the active ingredient were applied to the plants using a Generation II Research Sprayer (Devries Manufacturing, Hollandale, MN) at approximately 400 kPa to obtain a dose-response curve.

A suspension containing 2.0×10^5 conidia/ml was prepared from 10 to 12-day old cultures of *A. solani* on CV-8 medium grown under 24 h fluorescent light at 22 ± 2 °C, and 50 ml was applied to the plants using a Preval paint-spray gun (Preval Sprayer Division, Prevision Valve Corporation, Yonkers, NY). Inoculated plants were placed in individual humidity chambers (Phytotronic Inc.; 1626D) set at >95% RH at 22 ± 2 °C for 24 h. Plants were transferred to confinement chambers (plastic chambers with an open ceiling) on the greenhouse benches to reduce the possibility of cross-contamination from other isolates. The greenhouse temperature was maintained at 25 ± 2 °C with daily water applications. Early blight severity was visually rated at 6-, 9- and 12-days post-inoculation by estimating the percentage of infected leaf area on the first three true leaves and recorded as percentage diseased tissue.

Table 1
Alternaria solani isolates selected for in vivo sensitivity to pyrimethanil, cyprodinil, and fludioxonil.

Isolate	State of origin	Collection year
13–1	Nebraska	1998
22–1	Minnesota	1998
38–4	Nebraska	1998
88–1	Wisconsin	1998
1168–3	Idaho	2010
1179–3	North Dakota	2010
1184–14	Colorado	2011
1332–6	Texas	2013

2.4. Statistical analysis

All in vitro experiments were performed twice using a completely random design with two replicates/experiment for each fungicide concentration. The effective concentration where mycelial growth is inhibited by 50% (EC_{50} value) was calculated using the percentage reduction relative to the non-fungicide-amended control plates and regressed against the $\log_{10}$ fungicide concentration (Fonseka and Gudmestad, 2016). For further analysis, EC_{50} values of <0.01 and >100 were considered 0.01 and 100 μg/ml, respectively. Trials were included in the final analysis if the EC_{50} values for the internal control isolates were within the 95% confidence interval (Wong and Wilcox, 2002).

The greenhouse study was performed twice with two samples (two plants per pot) and three replicates (three pots) for each isolate at each fungicide concentration. Greenhouse experiments were arranged as a split-plot randomized complete block design with *A. solani* isolate as the whole plot and fungicide as sub-plots. For every isolate at all fungicide concentrations (0, 0.1, 1, 10, and 100 μg/ml), disease severity data from the 12th collection day was transformed to percentage disease control using the formula: [(1 – (% diseased tissue/% diseased tissue in non-treated plants)) x 100] (Gudmestad et al., 2013; Pasche et al., 2004). Disease control data was utilized for further statistical analyses. Area under the dose-response curve (AUDRC) was calculated to determine significant differences in early blight control provided by pyrimethanil, cyprodinil, and fludioxonil:

$$\mathrm{AUDRC} = \sum_{i=1}^{n} [[(W_{i+1} + W_i) / 2][d_{i+1} - d_i]]$$

W_i is the percentage foliar disease severity at the *i*th observation, d_i dosage at the *i*th observation and *n* the total number of observations. AUDRC is calculated similarly as the area under the disease progress curve (AUDPC) but across all fungicide doses evaluated. Interpretations of the AUDRC data are inverse of that for AUDPC. In the traditional use of AUDPC, a high value would indicate that disease development was greater when compared to a lower AUDPC value. In contrast, a high

AUDRC value indicates that a fungicide provided a higher degree of control of a fungal pathogen over a wide range of fungicide concentrations when compared to a lower AUDRC.

For both in vitro fungicide sensitivity and in vivo fungicide efficacy, Levene's test was used to determine homogeneity of variance between the two independent experiments (Milliken and Johnson, 1992). ANOVA was conducted with fungicide and isolate as fixed effects within each species. Replicates, trials and their interaction with fungicides were treated as random effects. The Student's t-test was used for mean comparisons ($\alpha = 0.05$). Pearson correlation coefficients were calculated to compare in vitro EC_{50} values between pairs of fungicides for *A. alternata* and *A. solani*. Too few isolates of *A. arborescence* and *A. tenuissima* were evaluated to effectively perform the correlation analysis. Pearson correlations were also conducted between in vitro EC_{50} values and AUDRC for each fungicide. All analyses were conducted using the Statistical Analysis System (SAS Institute Inc., Cary, NC).

3. Results

3.1. In vitro sensitivity of baseline Alternaria spp. to pyrimethanil, cyprodinil, and fludioxonil

Levene's test of in vitro fungicide sensitivity experiments for pyrimethanil, cyprodinil, and fludioxonil determined that variances were homogenous across trials and no significant differences were observed between trials for the small-spored *Alternaria* spp. ($P = 0.7258$; $P = 0.8821$), and *A. solani* ($P = 0.0575$; $P = 0.0571$); therefore, the trials were combined for further analysis. A significant fungicide by isolate interaction was observed for *A. alternata* ($P < 0.0001$), *A. arborescens* ($P = 0.0302$), *A. tenuissima* ($P < 0.0001$), and *A. solani* ($P < 0.0001$). Fungicide sensitivity of *A. alternata* baseline isolates to pyrimethanil, cyprodinil, and fludioxonil ranged from <0.10 to 5.85, <0.10 to 1.55, and <0.10–3.36 μg/ml, respectively (Fig. 1a). Three isolates were significantly less sensitive to pyrimethanil compared to any other isolate:fungicide combination. Isolates 444 and 479-4 were significantly less sensitive to fludioxanil than any other isolate. Fungicide sensitivity of *A. arborescens* baseline isolates to pyrimethanil, cyprodinil, and

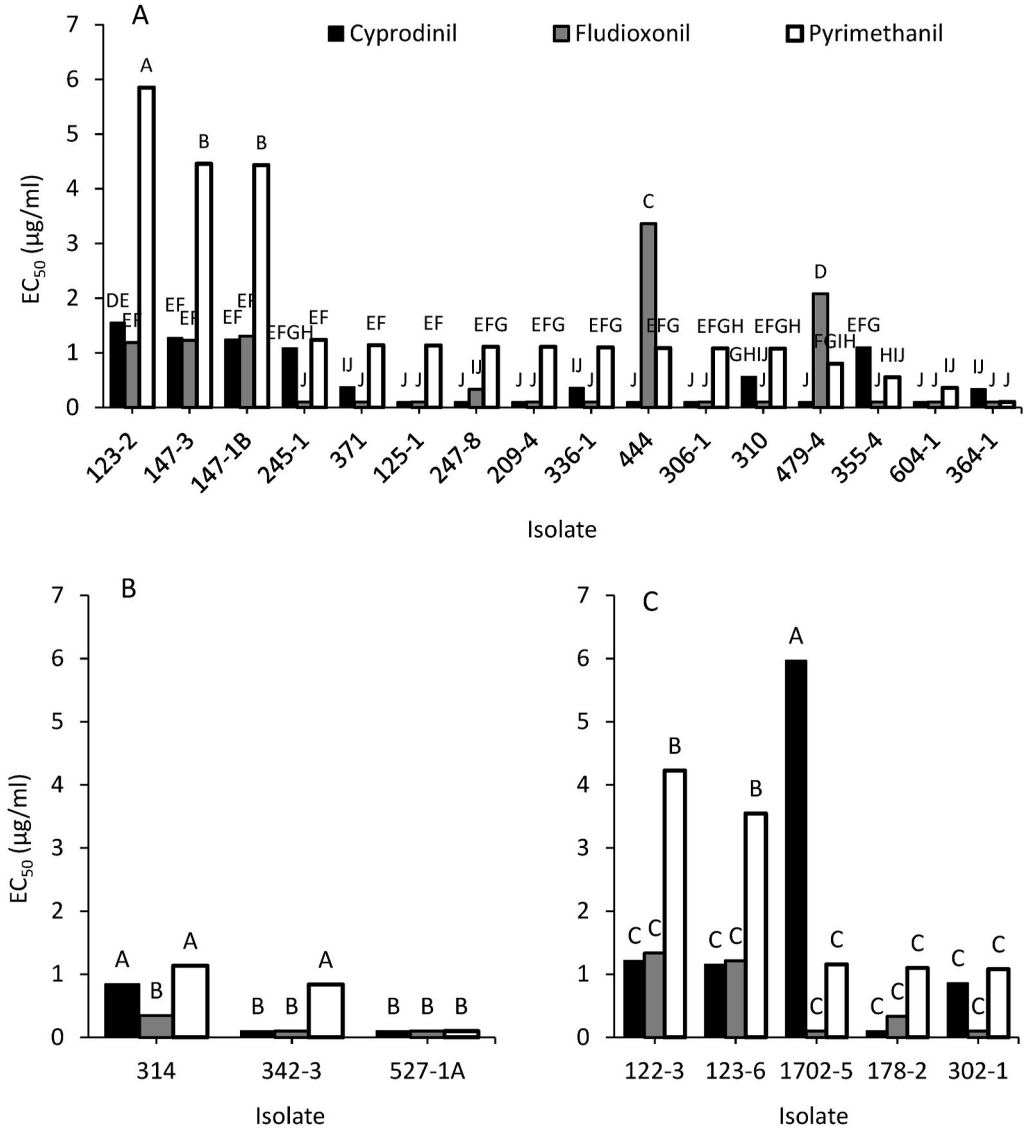

Fig. 1. In vitro fungicide sensitivity of A) *Alternaria alternata* (n = 16) B) *A. arborescens* (n = 3) C) *A. tenuissima* (n = 5) isolates to anilinopyrimidine (pyrimethanil; cyprodinil) and phenylpyrrole (fludioxonil) fungicides measured as EC_{50} (ug/ml) based on mycelial growth assays. Across isolates and fungicides, bars with the same letter are not significantly different based on the Student's t-test ($\alpha = 0.05$).

fludioxonil ranged from <0.10 to 1.14, <0.10 to 0.84, and <0.10–0.35 μg/ml, respectively (Fig. 1b). The nine *A. arborescens* isolate:fungicide combinations were statistically separated into two groups. Isolate 314 was significantly less sensitive to cyprodinil than the other two isolates and isolate 527-1 A was significantly more sensitive to pyrimethanil than 314 and 342-3. Fungicide sensitivities of *A. tenuissima* baseline isolates to pyrimethanil, cyprodinil, and fludioxonil ranged from 1.08 to 4.23, <0.10 to 1.22, and <0.10–1.34 μg/ml, respectively (Fig. 1c). The fifteen *A. tenuissima* isolate:fungicide combinations were statistically separated into three groups. Non-baseline isolate 1702-5 was significantly less sensitive to cyprodinil (EC_{50} = 5.97 μg/ml) than all other isolates were to any fungicide. Baseline isolates 122-3 and 123-6 were significantly less sensitive to pyrimethanil than all other isolates were to any fungicide except 1702-5 to pyrimethanil. Fungicide sensitivities of *A. solani* isolates to pyrimethanil, cyprodinil, and fludioxonil ranged from <0.10 to 1.70, <0.10 to 1.85, and <0.10–1.24 μg/ml, respectively and significant differences were observed across isolate:fungicide combinations (Fig. 2; Supp. Table S2). The frequency distribution was generally bi-modal for all three fungicides, either falling below 0.25 μg/ml or above 1.0 μg/ml. However, more than 60% of isolates displayed sensitivities to cyprodinil and fludioxonil less than 0.25 μg ml and greater than 90% of isolates displayed sensitivities to pyrimethanil greater than 1.0 μg ml. EC_{50} values for three *A solani* isolates collected in 2010, 2011 and 2013 were within the range of the baseline isolates evaluated for all three fungicides.

A strong and significant correlation was observed in the sensitivity of the baseline *A. alternata* isolates to pyrimethanil and cyprodinil (r = 0.77; P = 0.0006) (Fig. 3). No other significant correlations were observed for *A. alternata* and no significant correlations were observed between any of the fungicides among the *A. solani* baseline isolates (data not shown). Due to the low number of *A. arborescens* and *A. tenuissima* isolates correlations could not be conducted.

3.2. Disease control of A. solani isolates to pyrimethanil, cyprodinil, and fludioxonil

In the current research, disease control was established on tomatoes; however, the relationship between in vitro EC_{50} values and control of *A. solani* on tomatoes under greenhouse conditions have been well-corroborated by both molecular and in-field control in numerous previous publications (Bauske and Gudmestad, 2018; Bauske et al., 2018b; Fonseka and Gudmestad, 2016; Gudmestad et al., 2013; Mallik et al., 2014; Pasche and Gudmestad, 2005; Pasche et al. 2004, 2005). Levene's test for greenhouse disease control experiments for pyrimethanil, cyprodinil, and fludioxonil determined that variances were homogenous across trials (P = 0.3801) and trial means were not significantly different (P = 0.9896); therefore, experiments were combined for further analysis. A significant interaction was observed between the whole plot (*A. solani* isolate) and the sub-plot (fungicide) for percentage disease control (P < 0.0001). Pyrimethanil provided significantly less control of four of eight isolates than did the other two fungicides (Fig. 4). Three of these isolates were considered non-baseline, collected from 2010 to 2013. Additionally, pyrimethanil provided significantly lower control than did fludioxonil and similar control as cyprodinil for non-baseline isolate 1332-6. Control provided by cyprodinil was reduced in two non-baseline isolates (1184-14 and 1332-6) when compared to all other isolates. Control provided by cyprodinil of 1184-14 was significantly lower than fludioxonil but higher than pyrimethanil. Disease control of *A. solani* isolate 1332-6 provided by cyprodinil and pyrimethanil were similar, and both provided significantly lower control than did fludioxonil. Significant correlations between in vitro EC_{50} values and AUDRC were not detected (data not shown).

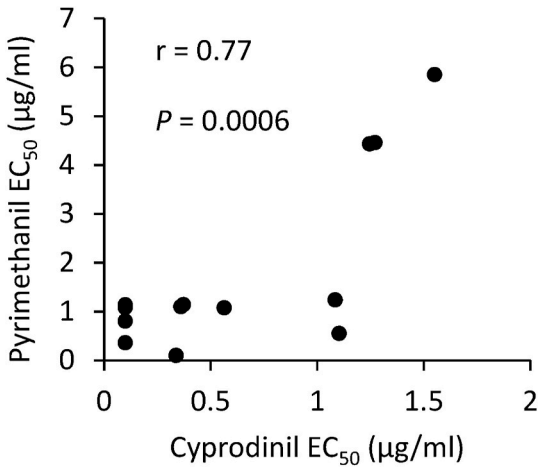

Fig. 3. Pearson correlation comparing in vitro EC_{50} values of *Alternaria alternata* isolates in response to anilinopyrimidine fungicides cyprodinil and pyrimethanil.

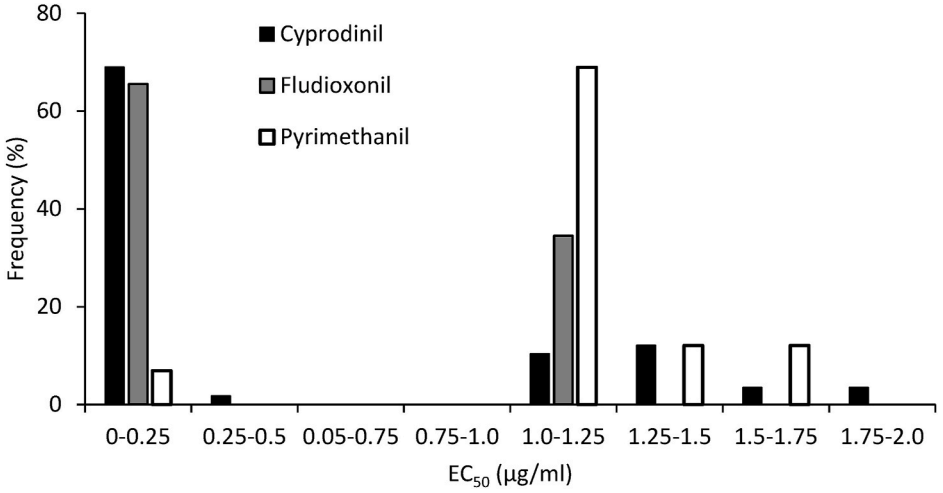

Fig. 2. Frequency (%) distribution of in vitro fungicide sensitivity of *Alternaria solani* (n = 58) isolates to anilinopyrimidine (pyrimethanil; cyprodinil) and phenylpyrrole (fludioxonil) fungicides measured as EC_{50} (ug/ml) based on mycelial growth assays. Across isolates and fungicides, bars with the same letter are not significantly different based on the Student's t-test (α = 0.05).

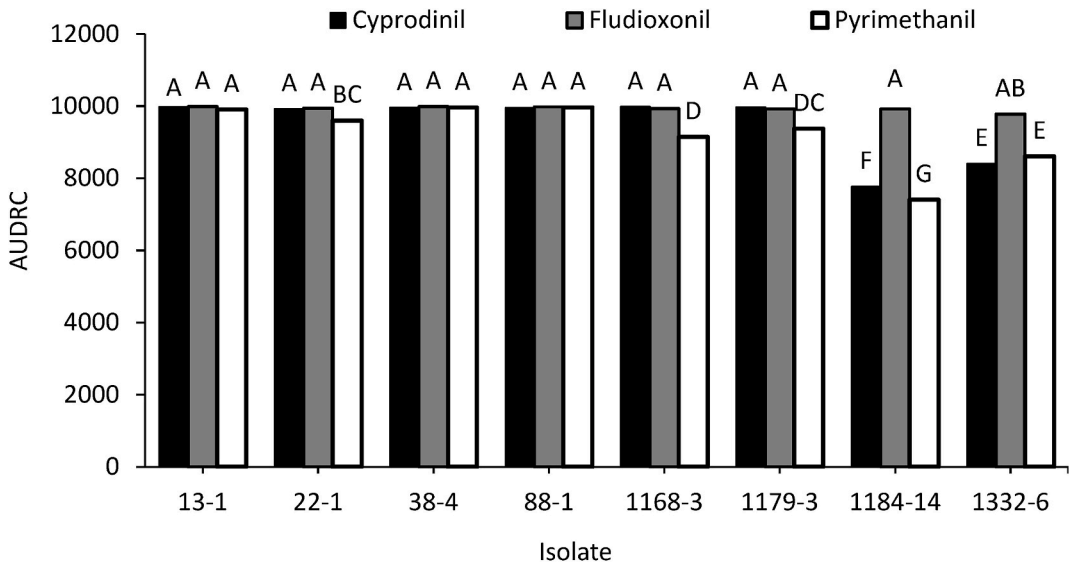

Fig. 4. Area under the dose response curve based on disease severity from greenhouse assays for *Alternaria solani* (n = 8) isolates to anilinopyrimidine (pyrimethanil; cyprodinil) and phenylpyrrole (fludioxonil) fungicides. Across isolates and fungicides, bars with the same letter are not significantly different based on the Student's t-test (α = 0.05).

4. Discussion

Alternaria spp. and *A. solani* have rapidly developed resistance and/or reduced sensitivity to multiple fungicide classes in a relatively short period of time (Avenot and Michailides 2007, 2015; Bauske et al. 2018a, 2018b; Fairchild et al., 2013; Fonseka and Gudmestad 2016; Gudmestad et al., 2013; Malandrakis et al., 2015; Miles et al., 2014; Mallik et al., 2014; Pasche et al., 2004). *A. alternata* sensu lato (Fairchild et al., 2013), *A. solani* (Fonseka and Gudmestad, 2016), *B. cinerea* (Amiri et al., 2013), and *Penicillium expansum* (Xiao et al., 2011) isolates with reduced sensitivity to pyrimethanil have been identified. Resistance to pyrimethanil in *Alternaria* spp. and *A. solani* was first identified in 2010 Idaho field isolates (Fairchild et al., 2013). In that study, one of nine *A. alternata* sensu lato and four of 21 *A. solani* isolates were resistant to pyrimethanil. A later study classified six of 245 *A. solani* isolates to have reduced sensitivity to pyrimethanil, but reduced sensitivity was not observed among the 109 *A. alternata* sensu lato isolates evaluated (Fonseka and Gudmestad, 2016). A loss of sensitivity to DMI chemistries in *A. solani* also has been reported (Fonseka and Gudmestad, 2016). QoI and SDHI resistance has been reported in *A. solani* and other *Alternaria* spp. in Europe (Landschoot et al., 2017; Leiminger et al., 2014; Nottensteiner et al., 2019; Odilbekov et al., 2016). Despite the many reports of fungicide resistance/reduced sensitivity across multiple chemistries in *A. solani* versus other *Alternaria* spp., it is surprising that FRAC continues to consider *A. solani* a medium risk rather than a high risk as is the case with other *Alternaria* spp. (FRAC, 2019). Nonetheless, monitoring the response of *Alternaria* spp. to multiple fungicide classes with different modes of action used in fungicide rotation programs is required to safeguard fungicide options for agricultural producers.

Pyrimethanil was registered for use on potato in the US in 2005 as a stand-alone foliar fungicide and has been utilized as a foliar fungicide in potato for early blight and brown leaf spot management in North Dakota for over a decade (Fonseka and Gudmestad, 2016). Pyrimethanil is also a pre-packaged mixture partner with the single-site SDHI fungicide fluopyram frequently used in rotation with a standard protectant such as mancozeb or chlorothalonil. Baseline sensitivity studies have been established for both *A. solani* and *Alternaria* spp. in response to pyrimethanil, difenoconazole, and metconazole (Fonseka and Gudmestad, 2016). In that study, the 50 *Alternaria* spp. were all classified as *A. alternata* sensu lato. However, it was determined using next-generation sequencing methods that some of these isolates were *A. tenuissima* and *A. arborescens*. Therefore, we felt it was relevant to assess the sensitivity of *Alternaria* spp. to a number of fungicide chemistries. In addition to testing a sub-set of isolates evaluated previously, six *A. solani,* two *A. alternata,* one *A. arborescens*, and one *A. tenuissima* isolates were new to this study (Fonseka and Gudmestad, 2016). In the current study, pyrimethanil sensitivity was conducted to detect differences among the three small-spored *Alternaria* spp. and to determine via direct statistical comparisons if cross-sensitivity exists between the AP and PP fungicides. The *A. solani* baseline isolates from the previously mentioned study were also assayed for sensitivity to AP fungicides, pyrimethanil and cyprodinil in the current study. Additionally, the PP fungicide fludioxonil was evaluated to determine its intrinsic activity on *Alternaria* spp. and potential use as a co-package partner with adepidyn for early blight and brown leaf spot management.

This is the first report establishing baseline sensitivities of *A. solani* and *A. alternata* to cyprodinil, and fludioxonil collected across multiple potato production areas. While a small number of isolates of *A. arborescens,* and *A. tenuissima* were included here, this is the first report of reaction of these *Alternaria* spp. to pyrimethanil, cyprodinil, and fludioxonil collected across multiple potato production areas. More than half of the *A. alternata* sensu stricto isolates evaluated were significantly less sensitive to pyrimethanil than to cyprodinil and fludioxonil. Interestingly, significant and strong cross-sensitivity was detected between pyrimethanil and cyprodinil across *A. alternata* baseline isolates. Strong cross-sensitivity between pyrimethanil and cyprodinil is quite common and it has been detected previously in *B. cinerea* (teleomorph *B. fuckeliana*) (Amiri et al., 2013; Fernández-Ortuño et al., 2013; Hilber and Schüepp, 1996; Myresiotis et al., 2007). Since pyrimethanil and cyprodinil are both AP fungicides, it stands to reason that a cross-sensitivity risk would be higher due to the similar chemical structure. Unfortunately, correlations in pyrimethanil and cyprodinil sensitivity among *A. arborescens* and *A. tenuissima* baseline isolates were not performed because too few isolates were available to be evaluated. Obtaining a larger sample size of all three *Alternaria* spp. that cause brown leaf spot on potato will aid in expanding or establishing baseline sensitivities and increase the understanding of how these pathogens respond across fungicide chemistries.

Fludioxonil was demonstrated to have intrinsic in vitro activity on the majority of *Alternaria* and *A. solani* isolates equal to or higher than cyprodinil or pyrimethanil. While correlations between in vitro and in vivo trials were not significant, likely due to the limited number of

isolates evaluated in the greenhouse, results from greenhouse evaluations generally agreed with in vitro intrinsic activity determined during the current research. In all but one isolate, disease control was significantly greater with fludioxonil and cyprodinil than with pyrimethanil. Fludioxonil consistently provided the best control of all isolates while control of two isolates provided by cyprodinil was significantly lower than fludioxonil. These data demonstrate that fludioxonil will be an excellent addition to the foliar fungicide portfolio for the management of leaf spot diseases of potato and perhaps for resistance management. We hypothesize that lack of effective rotational or co-packaging partners was a contributing factor in the loss of QoI fungicides and several SDHI fungicides due to resistance development in *A. solani.* When azoxystrobin was registered for use on potato in 1999 there were no other fungicides available that possessed similar properties of high intrinsic activity, local and translaminar systemicity, and residual activity on the early blight fungus, which ultimately contributed to the detection of fungicide resistance in 2001 (Pasche et al., 2004). A very similar situation existed when boscalid was registered for use on potato in 2005 and resistance was detected in isolates of *A. solani* collected in 2009 (Gudmestad et al., 2013; Miles et al., 2014). The addition of fludioxonil from the PP class of fungicides, and the only chemistry of this class ever registered as a foliar fungicide on potato in the USA, may assist in reducing the development of resistance in the remaining SDHI and DMI fungicide chemistries.

There are no reports of resistance to AP fungicides being detected in high frequency in fungal plant pathogens. However, AP resistance and cross-resistance within the AP fungicide class has been researched extensively using *B. cinerea* and several studies have proposed models for the type of resistance plant pathogens can develop against these fungicides (Amiri et al., 2013; Fernández-Ortuño et al., 2013; Hilber and Schüepp, 1996; Kanetis et al., 2008). In early studies, three multidrug-resistant (MDR) phenotypes were identified in *B. cinerea* (MDR1, MDR2, and MDR3) (Chapeland et al., 1999; Kretschmer et al., 2009). Isolates of phenotype MDR1 express strong resistance in response to the AP fungicide cyprodinil and the PP fungicide fludioxonil. Isolates of phenotype MDR2 were less sensitive to cyprodinil than the MDR1 isolates. Isolates of phenotype MDR3, which is an MDR1 × MDR2 recombinant, showed the highest level of resistance to both fludioxonil and cyprodinil. Based on the identification of multiple phenotypes with varying resistance responses, these studies suggest that AP resistance in *B. cinerea* may be quantitative as opposed to qualitative. In contrast, some studies have suggested qualitative resistance to be the cause of the loss of efficacy on *B. cinerea* suggesting this type of resistance developed abruptly, should be under single gene(s) control, and should be rapidly detected unless a fitness penalty is present (Amiri et al., 2013; Fernández-Ortuño et al., 2013). In other fungal plant pathogens, including *A. alternata,* qualitative resistance has been observed in isolates that were separated into two main sensitivity groups: fludioxonil-sensitive/cyprodinil-resistant, and fludioxonil-resistant /cyprodinil-sensitive (Avenot and Michailides, 2015). In that study, most of the 126 isolates were sensitive to both or either fungicide, but two isolates with fludioxonil-resistant/cyprodinil-resistant phenotypes were identified. The results of that study suggest that AP and PP resistance in *A. alternata* isolates may be qualitative. It was also concluded that AP and PP fungicides were still effective in controlling *A. alternata* isolates that possessed both a QoI and an SDH mutation (Avenot and Michailides, 2015).

In Midwest potato production areas, brown leaf spot and early blight can be devastating if not treated. While early blight has been regarded as more important than brown leaf spot, isolation frequency of the *Alternaria* spp. has been increasing (Ding et al., 2019). In this study, the high intrinsic activity of all *Alternaria* spp. evaluated, combined with the high early blight disease control exhibited by both cyprodinil and fludioxonil could be useful additions to early blight and brown leaf spot management programs. However, further investigations on the cross-sensitivity and disease control of the AP and PP fungicides should be conducted with a larger spatial and temporal sample size of isolates. Continued monitoring of the current *Alternaria* spp. populations to new and currently utilized chemistries is important in safeguarding these effective fungicide chemistries. A high priority should be given to establishing a more comprehensive baseline for the species and determining the sensitivity of non-baseline isolates of *Alternaria* spp. exposed over several years to AP and PP fungicides in an effort to mitigate the development of resistance to these two fungicide chemistries. Environmental conditions of the Midwest potato production areas are conducive to leaf spot disease development leading to the frequent application of single-site mode of action fungicides to provide control (Yellareddygari et al., 2019). The use of these types of fungicides has contributed to the widespread development of QoI and SDHI resistance in *A. solani* in high frequencies (Bauske et al., 2018a). The potato industry in the Midwest can ill afford to lose additional fungicide chemistries to further development of fungicide resistance.

Author contributions

S. Budde-Rodriguez: Investigation, Data Curation, Formal Analysis, Writing – Original Draft, Writing – Review & Editing, Visualization.

J.S. Pasche: Methodology, Validation, Writing – Review & Editing, Visualization.

I. Mallik: Investigation, Writing – Review & Editing.

N.C. Gudmestad: Conceptualization, Methodology, Validation, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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.2021.105855.

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