# Spatial and Temporal Sensitivity of *Alternaria* Species Associated With Potato Foliar Diseases to Demethylation Inhibiting and Anilino-Pyrimidine Fungicides

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## Abstract

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Early blight and brown spot, caused by *Alternaria solani* and *Alternaria alternata*, respectively, are important foliar diseases of potato, affecting both tuber yield and quality. Most of the commercial cultivars lack resistance; therefore, the application of foliar fungicides remains a primary disease management strategy. Baseline sensitivities of *A. solani* to difenoconazole and metconazole (demethylation inhibitors) using mycelial growth assay exhibited similar intrinsic activity against the pathogen with mean $EC_{50}$ (the effective concentration at which the fungal growth is inhibited by 50%) values of 0.09 μg/ml. However, the sensitivity of individual baseline *A. solani* isolates to each fungicide varied substantially, resulting in very low and nonsignificant correlation coefficients among fungicides. Mean $EC_{50}$ values for baseline *A. alternata* isolates in response to difenoconazole and metconazole were 0.14 and 0.26 μg/ml, respectively. The sensitivity of the majority of *A. solani* and *A. alternata* isolates collected from 2010 to 2014 from various potato production states was consistent with baseline isolates, therefore, these potato pathogens remain sensitive to the two demethylation inhibitor chemistries used to manage it. Baseline sensitivity assays of pyrimethanil (anilino-pyrimidine) also indicated great intrinsic activity against both foliar pathogens with mean $EC_{50}$ values of 0.44 and 0.35 μg/ml for *A. solani* and *A. alternata*, respectively. Although *A. alternata* remains largely sensitive to pyrimethanil, 6 out of 245 *A. solani* isolates collected from 2010 to 2014 exhibited reduced-sensitivity to the fungicide in in vitro assays. Reduced-sensitive isolates were not controlled at most pyrimethanil doses except at 100 μg/ml in greenhouse in vivo efficacy tests. These chemistries remain valuable options for fungicide rotation programs in areas of high disease pressure.

*Alternaria solani* Sorauer and *A. alternata* (Fries.) Keissler cause the potato (*Solanum tuberosum* L.) foliar diseases early blight and brown spot, respectively. *A. solani* creates characteristic dark brown to black lesions with concentric rings on older, senescing leaves, which produce a 'target spot' effect, a diagnostic symptom of early blight (Franc and Christ 2001). *A. alternata* produces similar symptoms and is frequently isolated with *A. solani* in leaf spot–diseased tissue. These ubiquitous diseases are potential threats when potatoes are grown under irrigation and during periods of heavy dew (Rotem 1994). Primary damage is attributed to premature defoliation of the potato plants, resulting in tuber yield reduction. The pathogens may also cause a type of dry rot of tubers, further reducing both the quantity and quality of marketable tubers (Nnodu et al. 1982). The majority of commercially acceptable potato cultivars are susceptible to early blight and brown spot (Franc and Christ 2001) and cultural practices alone are insufficient to reduce the inoculum, thus frequent applications of foliar fungicides are necessary for disease management.

Frequent application of protectant fungicides from early in the growing season until vine-kill are essential under growing conditions prevalent in the midwestern United States, but these are insufficient at high inoculum pressure and conducive environmental conditions (Pasche and Gudmestad 2008). Therefore, the application of locally systemic and translaminar fungicides such as quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), demethylation inhibitors (DMIs), and anilino-pyrimidines (APs) is needed to slow the development of an epidemic. The QoI fungicides were first introduced in 1999 on potato and provided excellent disease control (Pasche and Gudmestad 2008; Stevenson and James 1999). Reduced efficacy to QoI fungicides were reported within two years, due to increased selection pressure caused by extensive usage in intensive production systems (Pasche et al. 2004). SDHI fungicides boscalid and fluopyram were registered for the use on potato in 2005 and 2012, respectively, and became reliable alternatives to QoI fungicides (Gudmestad et al. 2013; Pasche and Gudmestad 2008; Pasche et al. 2005).

The target site of SDHI fungicides is mitochondrial complex II at either the succinate ubiquinone reductase or succinate dehydrogenase complex, which is a functional part of tricarboxylic acid cycle and the mitochondrial electron transport chain (Yin et al. 2011). The frequent use of SDHI fungicides caused resistance to boscalid in various pathosystems, such as *A. alternata* of peach, pistachio, and potato (Avenot and Michailides 2007; Fairchild et al. 2013; Tymon and Johnson 2014; Yang et al. 2015), *Botrytis cinerea* of apple (Yin et al. 2011), *Didymella bryoniae* of watermelon (Thomas et al. 2012), and *A. solani* of potato (Gudmestad et al. 2013; Tymon and Johnson 2014; Wharton et al. 2012). Current studies demonstrate that *A. solani* isolates resistant to boscalid are not resistant to fluopyram (Fairchild et al. 2013; Gudmestad et al. 2013). However, with other SDHI fungicides registered on potato, such as penthiopyrad and fluxapyroxad, there is a potential for additional cross-resistance among fungicides of the SDHI class (Gudmestad et al. 2013). Therefore, it is likely that the increased use of fluopyram will place significant selection pressure on the early blight pathogen, thus placing additional pressure on other systemic and highly efficacious fungicide chemistries.

First introduced in the 1970s, DMIs are one of four classes of sterol biosynthesis inhibitors having a broad spectrum of activity on a number of fungal pathogens from the Ascomycetes and Basidiomycetes to Fungi imperfecti (Thomas et al. 2012). Difenoconazole is a translaminar fungicide with durable preventive activity during penetration and haustoria formation of phytopathogenic fungi causing various leaf spot diseases, powdery mildews, rust, and scab of annual and perennial crops (Bouwman et al. 2011). Metconazole, another DMI fungicide, has excellent activity on various smut and rust diseases, root rots, and powdery mildews (Friskop et al. 2015). Their mode of action is the inhibition of fungal cell membrane development by preventing ergosterol biosynthesis, thereby causing disruption of membrane function, leakage of cytoplasmic contents, and hyphal inhibition (Bouwman et al. 2011; Brent and Holloman 2007a). Difenoconazole and metconazole were first registered for use on potato in

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2011 and have the potential to be key rotational fungicides in the growers' spray program to control pervasive potato foliar diseases. Currently, little is known about DMI resistance in *A. solani* and *A. alternata* pathogen populations.

Pyrimethanil, an anilino-pyrimidine fungicide targeting methionine biosynthesis, was registered on potato for early blight disease control in 2005. Pyrimethanil and other AP fungicides have largely been used to control several pathogens, including *B. cinerea* (Zhao et al. 2010) and *Venturia inaequalis* (Köller et al. 2005). The AP fungicides have been classified by the Fungicide Resistance Action Committee (FRAC) as fungicides possessing medium risk of resistance development. However, resistance to AP fungicides has been reported in field isolates of *B. cinerea* of many crops (Amiri et al. 2013; Leroux et al. 1999; Myresiotis et al. 2007), *Pecillium* spp. of apple and citrus (Kanetis et al. 2008; Xiao et al. 2011), and *V. inaequalis* of apple (FRAC 2015). Pyrimethanil resistance was first detected in 2010 in Idaho with 19 and 11% of *A. solani* and *A. alternata* isolates, respectively, demonstrating insensitivity to the fungicide (Fairchild et al. 2013). Despite this report, prevalence and impact of pyrimethanil resistance in a diverse pathogen population has yet to be determined.

The incidence of fungicide resistance has grown substantially, due to the introduction of fungicides with a single mode of action (Skylakakis 1982). Due to the countless examples of plant pathogens with reduced sensitivity or resistance to agrochemicals, the plant protection community has taken steps to avoid or delay resistance development in newly developed chemistries, including the formation of FRAC. One of the key elements in resistance management is the establishment of baseline sensitivity and the monitoring of pathogen populations over time (Jutsum et al. 1998; Russell 2005). Further monitoring of a fungal plant pathogen can detect shifts in pathogen sensitivity, predict efficacy of fungicide regimes, and provide information necessary to recommend resistance management tactics (Thomas et al. 2012). The specific objectives of the current study were to determine the difenoconazole, metconazole, and pyrimethanil sensitivity of *Alternaria* spp. isolates from a baseline population, with isolates collected from a wide potato production area in the midwestern United States, and to determine the effect of any in vitro reduced sensitivity of *Alternaria* spp. that might be detected on disease control.

## Materials and Methods

***A. solani* and *A. alternata* isolate collection and maintenance.** *A. solani* and *A. alternata* isolates that were collected before 2012 were obtained from long-term cryogenic storage (Gudmestad et al. 2013; Pasche et al. 2004). Isolates collected from 2012 to 2014 were obtained from leaf and tuber samples submitted to our laboratory from potato production areas across the nation, including North Dakota, Minnesota, Texas, Nebraska, Michigan, New Mexico, Wisconsin, Colorado, Illinois, Washington, and Idaho. Early blight and brown spot lesions from leaf samples were transferred to 1.5% agar media and were incubated at room temperature (22 ± 2°C) for 3 to 4 days, until conidia were produced (Holm et al. 2003). A single conidium was transferred to a petri plate containing clarified V8 medium (CV-8) (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water, 900 ml) amended with 50 mg of ampicillin per milliliter. Isolates were incubated under 24 h of fluorescent light at 22 ± 2°C for a week and were examined for the presence of *A. solani* or *A. alternata* conidia (Pasche et al. 2004). For long-term cryogenic storage, 4-mm diameter plugs of media with fungal mycelia and conidia were cut using a sterilized cork borer, and the plugs were placed in screw-top centrifuge tubes. The loosely capped tubes were placed in a closed container with silica gel for 2 to 3 days to remove moisture from the media and then, were capped, sealed with Parafilm, and preserved at −80°C in an ultrafreezer.

**In vitro sensitivity to DMI fungicides.** DMI sensitivity was determined via mycelial growth assays conducted as described previously in evaluations of *D. bryoniae* sensitivity to DMI fungicides (Keinath and Hansen 2013). Working cultures were transferred onto CV-8 medium and were incubated under 24 h of fluorescent light at 22 ± 2°C. After 4 days, 5-mm agar plugs were excised from the leading edge of growth and were inverted onto 60 mm petri plates containing 2% laboratory-grade agar (A360-500; Fisher Scientific, Pittsburgh, PA) amended with technical grade formulations of difenoconazole (95% active ingredient [a.i.]) (Syngenta Crop Protection, Greensboro, NC) and metconazole (99% a.i.) (Valent U.S.A. Corporation, Walnut Creek, CA) dissolved in acetone to reach final concentrations of 0, 0.01, 0.1, 1, and 10 mg/ml. The final concentration of acetone in all media was 0.1% by volume. Two perpendicular measurements of mycelial growth for each isolate was measured, with the original plug diameter (5 mm) subtracted, after incubation at 25 ± 2°C in darkness for 7 days for all the in vitro fungicide sensitivity assays.

Fifty-seven baseline *A. solani* and 50 baseline *A. alternata* isolates were evaluated for in vitro sensitivity to DMI fungicides (Table 1). These isolates were collected from 1998 to 2002 and have had no exposure to DMI fungicides. *A. solani* isolates collected in 2010 (55 isolates), 2011 (109 isolates), 2012 (eight isolates), 2013 (58 isolates), and 2014 (15 isolates) were also tested for shift in sensitivity to DMI fungicides (Table 1). Additionally, *A. alternata* isolates collected in 2011 (19 isolates), 2013 (75 isolates), and 2014 (15 isolates) were also tested for sensitivity to DMI fungicides (Table 1).

**In vitro sensitivity to pyrimethanil.** Fungicide sensitivity was determined via mycelial growth assay on a synthetic medium containing L-asparagine (asp-agar), as described previously for evaluating *B. cinerea* sensitivity to group 9 fungicides (Hilber and Schüepp 1996). Media containing asp-agar were amended with technical grade pyrimethanil (95% a.i.) (Bayer CropScience, Raleigh, NC) dissolved in acetone to reach final concentrations of 0, 0.1, 1, 10, and 100 μg/ml. The highest concentration was not required for baseline evaluations. Five stock solutions were prepared for pyrimethanil sensitivity testing. Asp-agar consisting of $K_2HPO_4 \cdot 3H_2O$ (1.31 g) and $MgSO_4 \cdot 7H_2O$ (1 g), each dissolved in 30 ml of distilled water, were stocks I and II, respectively. Stock III contained KCl (0.5 g) and $FeSO_4 \cdot 7H_2O$ (0.01 g) dissolved 30 ml of distilled water. Stock IV was produced by dissolving L-asparagine (2 g) and agar (15 g) dissolved in 400 ml of distilled water. Stock V contains $C_6H_{12}O_6 \cdot 1H_2O$ (22 g) dissolved in 490 ml of distilled water. The precipitate that forms when stocks I and II are combined was dissolved by adding 12 M hydrochloric acid dropwise. A precipitate again was observed with the addition of stock III but dissolved with the addition of stock IV. Stock solutions I to IV and stock V were combined after autoclaving, and pyrimethanil was added after cooling.

All *Alternaria* isolates tested for DMI sensitivity were evaluated also for pyrimethanil sensitivity (Table 1).

**Effect of reduced sensitivity of *A. solani* to pyrimethanil on disease control.** The significance of an in vitro shift in sensitivity of *A. solani* to pyrimethanil on disease control was determined under greenhouse conditions, as previously described (Gudmestad et al. 2013; Pasche et al. 2004, 2005). A subset of six isolates of *A. solani* was tested for in vivo studies based on in vitro pyrimethanil sensitivity. Two sensitive and four pyrimethanil reduced-sensitive isolates were used. All isolates were recovered from long-term storage and were maintained, and conidia were harvested.

The in vivo sensitivity assay was conducted as a 24-h preventive test. Pyrimethanil was applied 24 h prior to inoculation in the greenhouse using tomato plants, cv. Orange Pixie VFT hybrid (Tomato Growers Supply Company, Fort Myers, FL). This cultivar was used because of its susceptibility to early blight and compact size compared with potato plants. This allowed for adequate replication for evaluating multiple fungicide concentrations across several *A. solani* isolates. Three tomato seeds were sown in each 10-$cm^3$ plastic pot containing Sunshine Mix LC1 (Sun Gro Horticulture Inc., Bellevue, WA), and after emergence, plants were thinned to obtain two uniformly sized plants per pot. When the first three leaves were fully expanded and plants had reached a height of 15 to 20 cm, plants were treated with a commercial formulation of pyrimethanil (37.4% a.i.) (Scala 400 SC; Bayer). Ten-fold fungicide concentrations of the active ingredient were applied to the plants (0, 0.1, 1, 10, and 100 μg/ml) to obtain a dose response curve. Fungicide was applied using a Generation II research sprayer (Devries Manufacturing, Hollandale, MN) at approximately 400 kPa. At 24 h after fungicide application, plants were inoculated, using 50 ml of a $2.0 \times 10^5$ conidia per milliliter suspension produced from 10- to 12-day-old-cultures of *A. solani* maintained on CV-8 medium for 7 to 14 days under 24 h

fluorescent light at 22 ± 2°C. A Preval paint-spray gun (Preval Sprayer Division; Precision Valve Corporation, Yonkers, NY) was used for inoculation, and inoculated plants were kept in individual humidity chambers (Phytotronic Inc., Earth City, MO) (1626D) for 24 h at >95% relative humidity and 22 ± 2°C. Plants were transferred to confinement chambers (plastic chambers with open ceilings) on greenhouse benches, to avoid cross contamination among *A. solani* isolates, and were maintained at 25 ± 2°C with daily application of water. Early blight disease severity was rated visually at 6, 9, and 12 days postinoculation by estimating the percentage of infected leaf area of the first three true leaves (three subsamples), recorded as percentage of diseased tissue. Two samples (two plants per pot) and three replications (three pots) were tested for each isolate × fungicide concentration. The in vivo experiment was performed three times.

**Table 1.** Origin of *Alternaria* spp. isolates used in in vitro fungicide sensitivity assays from 1998 to 2014

| Year | State of origin | No. of *Alternaria solani* isolates | No. of *Alternaria alternata* isolates |
|---|---|---|---|
| 1998[z] | Idaho | 2 | – |
| | Minnesota | 5 | – |
| | Nebraska | 13 | – |
| | North Dakota | 1 | – |
| | South Dakota | 1 | – |
| | Texas | 1 | – |
| | Wisconsin | 1 | – |
| 1999[z] | Colorado | – | 1 |
| | Idaho | – | 1 |
| | Minnesota | – | 3 |
| | Nebraska | – | 1 |
| | New Mexico | – | 6 |
| | North Dakota | – | 1 |
| | Texas | – | 11 |
| 2000[z] | Idaho | – | 3 |
| | Minnesota | – | 1 |
| | North Dakota | – | 8 |
| | Texas | – | 1 |
| | Wisconsin | – | 1 |
| 2001[z] | Minnesota | 5 | – |
| | Nebraska | 17 | – |
| | North Dakota | 11 | 3 |
| 2002[z] | Minnesota | – | 1 |
| | Nebraska | – | 1 |
| | North Dakota | – | 6 |
| | Texas | – | 1 |
| 2010 | Idaho | 27 | – |
| | Nebraska | 12 | – |
| | North Dakota | 16 | – |
| 2011 | Colorado | 4 | – |
| | Florida | 2 | – |
| | Idaho | 15 | 3 |
| | Minnesota | 29 | 2 |
| | Nebraska | 31 | 1 |
| | North Dakota | 10 | 13 |
| | Texas | 15 | – |
| | Wisconsin | 3 | – |
| 2012 | Colorado | 1 | – |
| | Idaho | 1 | – |
| | Nebraska | 3 | – |
| | Texas | 2 | – |
| | Wisconsin | 1 | – |
| 2013 | California | – | 6 |
| | Colorado | 9 | 7 |
| | Idaho | 1 | 5 |
| | Illinois | 2 | 21 |
| | New Mexico | 4 | 8 |
| | Michigan | 2 | – |
| | Minnesota | 8 | 1 |
| | Nebraska | 5 | 9 |
| | North Dakota | 11 | 5 |
| | Texas | 10 | 11 |
| | Washington | 1 | 1 |
| | Wisconsin | 5 | 1 |
| 2014 | Idaho | 1 | 4 |
| | Michigan | 1 | – |
| | Nebraska | 3 | – |
| | North Dakota | 2 | – |
| | Texas | 8 | 11 |

[z] *Alternaria solani* and *Alternaria alternata* isolates collected from 1998 to 2002 had no exposure to demethylation inhibitor (DMI) and anilino-pyrimidine (AP) fungicides and, hence, are categorized as baseline isolates.

**Statistical analyses.** All in vitro experiments were performed twice in completely random design with two replicates for each fungicide concentration. To determine the $EC_{50}$ value (effective concentration at which the fungal growth is inhibited by 50%) for each isolate, the percent reduction in mycelial growth relative to the nontreated control was calculated (Pasche et al. 2004). These data were regressed against the $\log_{10}$ fungicide concentration, and the $EC_{50}$ value was determined by interpolation of 50% intercept, using the Statistical Analysis System (SAS Institute Inc., Cary, NC). The experiments were analyzed separately, and the F test was used to test for homogeneity of variance among experiments. In all in vitro studies involving *A. alternata*, the coefficient of variability (standard error/mean) of log-10 transformed $EC_{50}$ values among all experimental repeats was calculated as a measure of assay reproducibility (Thomas et al. 2012). In all in vitro studies involving *A. solani*, control isolates 13-1, a wild-type *A. solani* isolate, and 526-3, a QoI resistant isolate, were used in each trial as internal controls to determine reproducibility of the assay. Assay reproducibility calculations were applied to the internal controls (Wong and Wilcox 2002). The assay reproducibility calculations generated approximate limits for 95% confidence interval for the two internal controls. Trials in which the $EC_{50}$ values of the internal controls are within the 95% confidence interval were included in further statistical analyses. Correlation analysis ($\alpha = 0.05$) was performed using Pearson correlation coefficients to compare in vitro fungicide $EC_{50}$ values for both baseline and 2010 to 2014 *A. solani* and *A. alternata* isolates. A resistance factor (Rf) was calculated for each fungicide by dividing the $EC_{50}$ value of an individual isolate by the mean baseline $EC_{50}$ value for the *Alternaria* species being evaluated.

All in vivo experiments were split-plot randomized complete block designs with *A. solani* isolates as the main plot and fungicide concentrations as split-plots. For each isolate, at all fungicide concentrations, disease severity data were transformed to percent disease control, using the formula 1 − (% diseased tissue/% diseased tissue in nontreated plants) × 100. Levene's test was conducted to test for homogeneity of variance among three independent experiments (Milliken and Johnson 1992). The analysis of variance (ANOVA) was performed separately for isolate × fungicide group combination at each fungicide concentration using SAS, and *t* tests were used on the combined data to detect differences at each fungicide concentration. Area under the disease progress curve for dose-response curves were calculated to determine if there is a significant difference in disease control provided by pyrimethanil in controlling sensitive and reduced-sensitive isolates (Shaner and Finney 1977).

## Results

**Determination of baseline in vitro sensitivity of *A. solani* and *A. alternata* to difenoconazole, metconazole, and pyrimethanil.** Experimental variances were homogenous ($P = 0.05$) for $EC_{50}$ values calculated from in vitro fungicide sensitivity experiments. $EC_{50}$ values of the *A. solani* isolate sensitivity to difenoconazole and metconazole ranged from 0.02 to 0.30 and 0.04 to 0.18 μg/ml, respectively, with a mean $EC_{50}$ value of 0.09 μg/ml to each fungicide (Fig. 1A). $EC_{50}$ values of the *A. alternata* isolate sensitivity to difenoconazole and metconazole ranged from 0.03 to 0.33 and 0.04 to 0.48 μg/ml with mean $EC_{50}$ values of 0.14 and 0.26, respectively (Fig. 1B). The correlation coefficient comparing $EC_{50}$ values for difenoconazole and metconazole baseline sensitivities of individual *A. solani* isolates was very low ($r = 0.0205$), indicating that the association between these two fungicides was very weak and not significant

($P = 0.8797$) (Fig. 2A). In contrast, the correlation analysis disclosed a positive and statistically significant association between $EC_{50}$ values for difenoconazole and metconazole baseline sensitivities among individual *A. alternata* isolates ($r = 0.7141$, $P < 0.0001$) (Fig. 2B).

$EC_{50}$ values of the *A. solani* isolate sensitivity for pyrimethanil ranged from 0.35 to 0.58 μg/ml with a mean $EC_{50}$ value of 0.44 (Fig. 1C). $EC_{50}$ values for baseline sensitivity of *A. alternata* isolates to pyrimethanil ranged from 0.15 to 0.42 μg/ml, with mean $EC_{50}$ value of 0.35 (Fig. 1C).

**Determination of in vitro sensitivity of *A. solani* isolates collected from 2010 to 2014.** Independent ANOVA of in vitro fungicide sensitivity experiments for difenoconazole, metconazole, and pyrimethanil $EC_{50}$ values determined that error variances were homogenous ($P = 0.05$); thus, experiments were combined by individual fungicides. $EC_{50}$ values of *A. solani* isolate sensitivity to difenoconazole and metconazole ranged from 0.05 to 0.35 and 0.04 to 0.42 μg/ml, respectively. The overall mean fungicide sensitivities of the fifty-five 2010 *A. solani* isolates to difenoconazole and metconazole were 0.12 and 0.18 μg/ml, respectively (Fig. 3A). Mean $EC_{50}$ values for the 109 isolates from 2011 were 0.13 and 0.19 μg/ml for difenoconazole and metconazole, respectively. Eight isolates from 2012 had mean $EC_{50}$ values of 0.14 and 0.25 μg/ml for difenoconazole and metconazole, respectively (Fig. 3A). Mean $EC_{50}$ values for the 58 isolates from 2013 were 0.07 and 0.16 μg/ml for difenoconazole and metconazole, respectively, while 15 isolates from 2014 had mean $EC_{50}$ values of 0.06 and 0.10 μg/ml for difenoconazole and metconazole (Fig. 3A).

Individual analysis of *A. solani* isolate $EC_{50}$ values for each fungicide revealed a significant difference between the mean $EC_{50}$ value

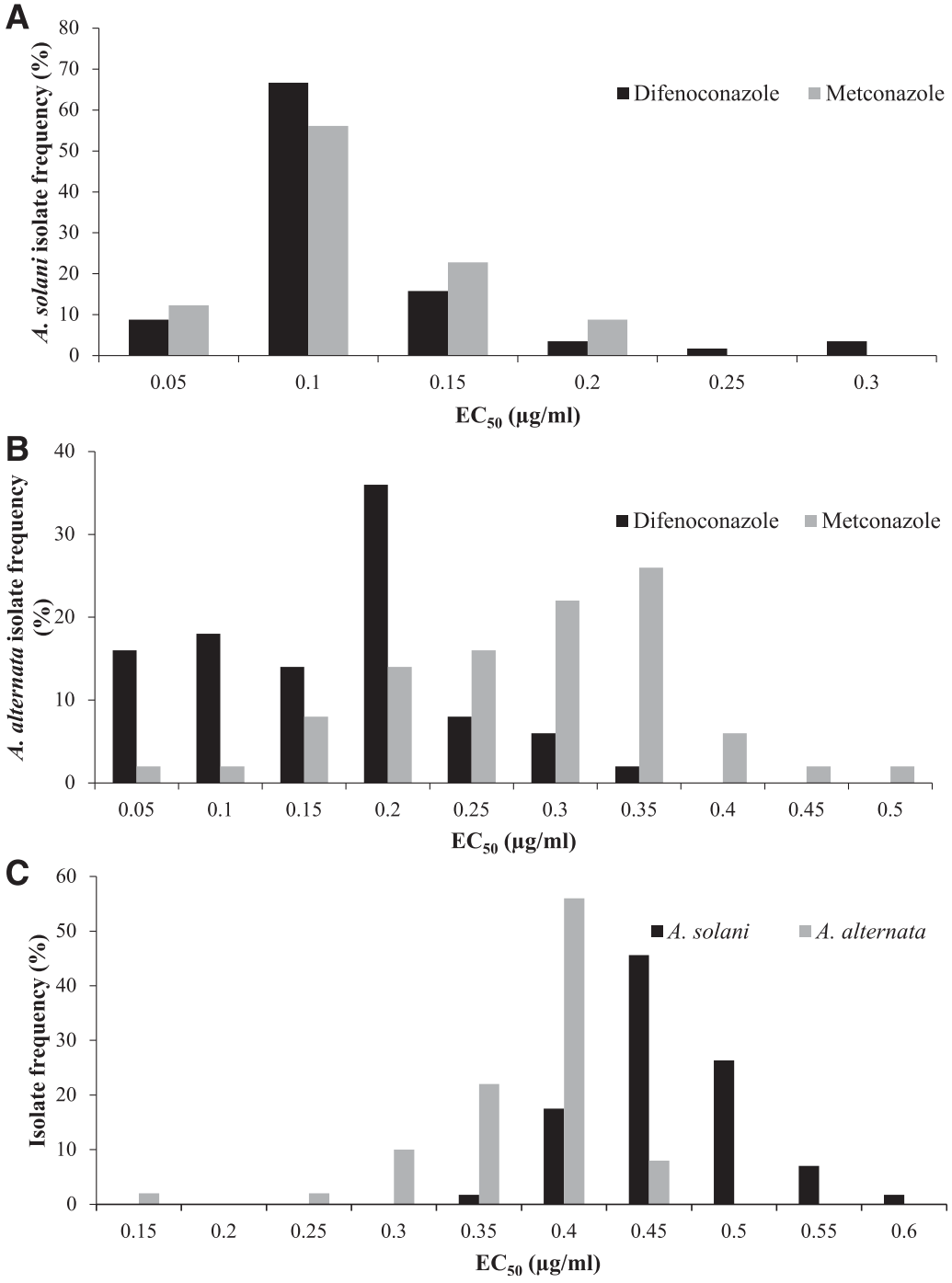


**Fig. 1.** Frequency distribution of sensitivity of **A,** 57 baseline *Alternaria solani* isolates to demethylation inhibitor (DMI) fungicides, **B,** 50 baseline *Alternaria alternata* isolates to DMI fungicides, and **C,** *Alternaria* spp. isolates to pyrimethanil, based on in vitro methods to determine the effective concentration that inhibits mycelial growth by 50% compared with the nontreated control ($EC_{50}$ μg/ml). These isolates were collected from 1998 to 2002 from several potato production areas in the United States.

of baseline and fungicide-exposed isolates for both difenoconazole and metconazole ($P$ = 0.0228 and $P < 0.0001$), respectively (Fig. 3A). In this study, the Rf values for *A. solani* sensitivity testing were 1.2 and 2.0, respectively, for difenoconazole and metconazole. Isolates collected in 2012 had significantly higher $EC_{50}$ values for metconazole than each of the preceding years, while isolates collected in 2012 had the highest $EC_{50}$ value for difenoconazole, and isolates collected in 2014 had the lowest $EC_{50}$ value for difenoconazole sensitivity, relative to isolates collected in other years (Fig. 3A). In contrast to the baseline *A. solani* isolates, correlation analysis disclosed a significant association between $EC_{50}$ values for difenoconazole and metconazole sensitivities of individual *A. solani* isolates collected from 2010 to 2014 ($r$ = 0.4962, $P < 0.0001$) (Fig. 4A). However, the difference may be due to the number of isolates used in the baseline ($n$ = 57) and nonbaseline ($n$ = 245) sensitivity testing.

$EC_{50}$ values of the *A. solani* isolate sensitivity for pyrimethanil ranged from 0.36 to 28.26 μg/ml. The overall mean pyrimethanil sensitivity for the 55 *A. solani* isolates collected in 2010 was 0.74 μg/ml (Fig. 5). The mean $EC_{50}$ value for the 109 isolates from 2011 was 0.95 μg/ml, while eight isolates from 2012 had a mean $EC_{50}$ value of 0.50 μg/ml (Fig. 5). The mean $EC_{50}$ value for the 58 isolates from 2013 was 0.54 μg/ml, while 15 isolates from 2014 had a mean $EC_{50}$ value of 0.57 μg/ml (Fig. 5). Although there was no significant ($P$ = 0.4453) difference between mean $EC_{50}$ value of baseline and fungicide exposed isolates (Fig. 5), six *A. solani* isolates had $EC_{50}$ values greater than mean baseline value by fourfold (Table 2).

**Determination of in vitro sensitivity of *A. alternata* isolates collected from 2011 to 2014.** Independent ANOVA of in vitro fungicide sensitivity experiments for difenoconazole, metconazole, and pyrimethanil $EC_{50}$ values determined that error variances were homogenous ($P$ = 0.05); thus, experiments were combined by individual fungicides. $EC_{50}$ values of the *A. alternata* isolate sensitivity to difenoconazole and metconazole ranged from 0.03 to 0.28 and 0.05 to 0.46 μg/ml, respectively. The overall mean fungicide sensitivities of the nineteen 2011 *A. alternata* isolates to difenoconazole and metconazole were 0.12 and 0.20 μg/ml, respectively (Fig. 3B). Mean $EC_{50}$ values for the 75 isolates from 2013 were 0.12 and 0.18 μg/ml for difenoconazole and metconazole, respectively, while 15 isolates from 2014 had mean $EC_{50}$ values of 0.07 and 0.12 μg/ml for difenoconazole and metconazole (Fig. 3B). Individual analysis of *A. alternata* isolate $EC_{50}$ values for each fungicide revealed a significant difference between mean $EC_{50}$ value of baseline and fungicide-exposed isolates for both difenoconazole and metconazole ($P$ = 0.0020 and $P < 0.0001$), respectively (Fig. 3B). In this study, the Rf values for *A. alternata* sensitivity testing were 0.8 and 0.7, respectively, for difenoconazole and metconazole. Similar to baseline *A. alternata* isolates, correlation analysis disclosed a significant association between $EC_{50}$ values for difenoconazole and metconazole sensitivities among individual *A. alternata* isolates collected from 2011 to 2014 ($r$ = 0.8673, $P < 0.0001$) (Fig. 4B).

*A. alternata* isolates collected from 2011 to 2014 were tested for pyrimethanil sensitivity with significant differences observed between mean $EC_{50}$ values of baseline and nonbaseline (fungicide-exposed) isolates ($P < 0.0001$) (Fig. 5). $EC_{50}$ values of the *A. alternata* isolate sensitivity for pyrimethanil ranged from 0.31 to 1.27 μg/ml. The overall mean pyrimethanil sensitivity for 19 *A. alternata* isolates collected in 2011 was 0.52 μg/ml (Fig. 5). The mean $EC_{50}$ value for the 75 isolates from 2013 was 0.47 μg/ml, while 15 isolates from 2014 had a mean $EC_{50}$ value of 0.61 μg/ml (Fig. 5). In this study, the Rf value for *A. alternata* sensitivity testing was 1.4 for pyrimethanil.

**Effect of reduced-sensitivity of *A. solani* to pyrimethanil on disease control.** Independent analysis of in vivo disease control experiments for pyrimethanil determined that variances were homogenous ($P$ = 0.05); thus, experiments were combined for further analysis. A significant interaction between the main plot (isolate) and subplot factor (fungicide concentrations) ($P < 0.0001$) was observed for percentage disease control of pyrimethanil on *A. solani*–infected greenhouse-grown tomato plants. Significant effects ($P < 0.0001$) were also

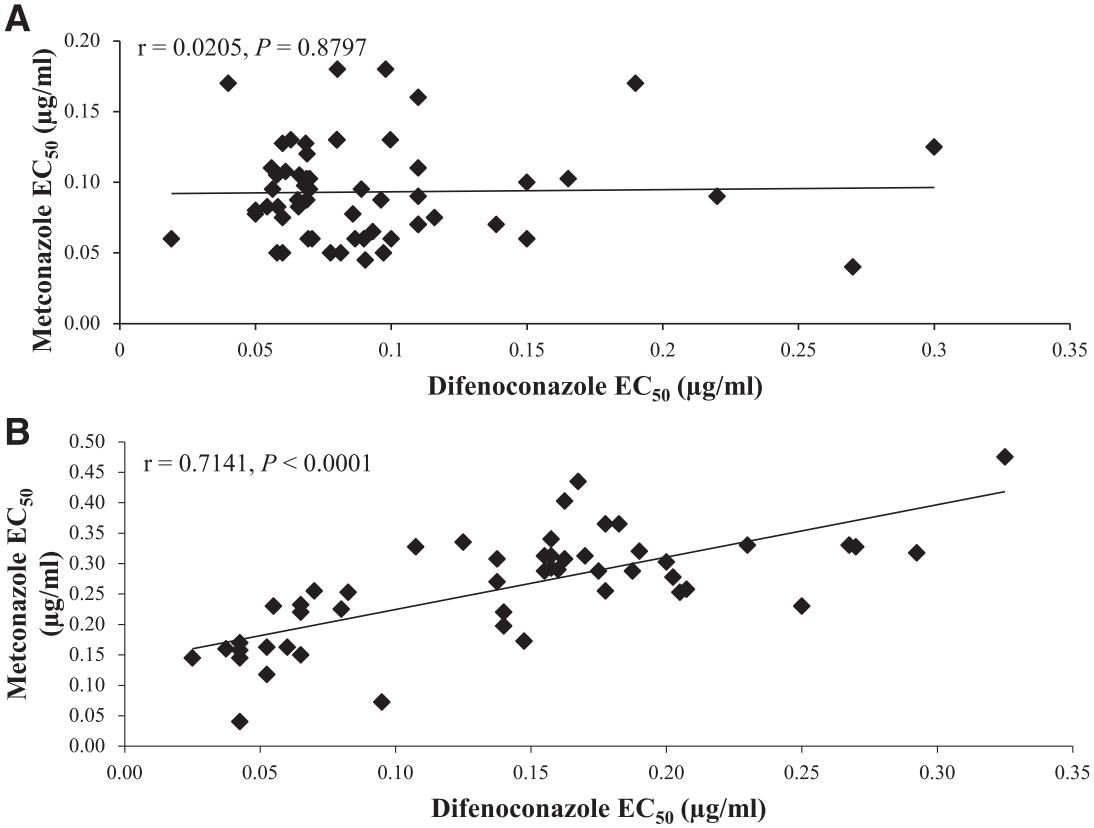


**Fig. 2.** Linear correlation between **A,** in vitro difenoconazole and metconazole sensitivity of 57 baseline *Alternaria solani* isolates and **B,** in vitro difenoconazole and metconazole sensitivity of 50 baseline *Alternaria alternata* isolates.

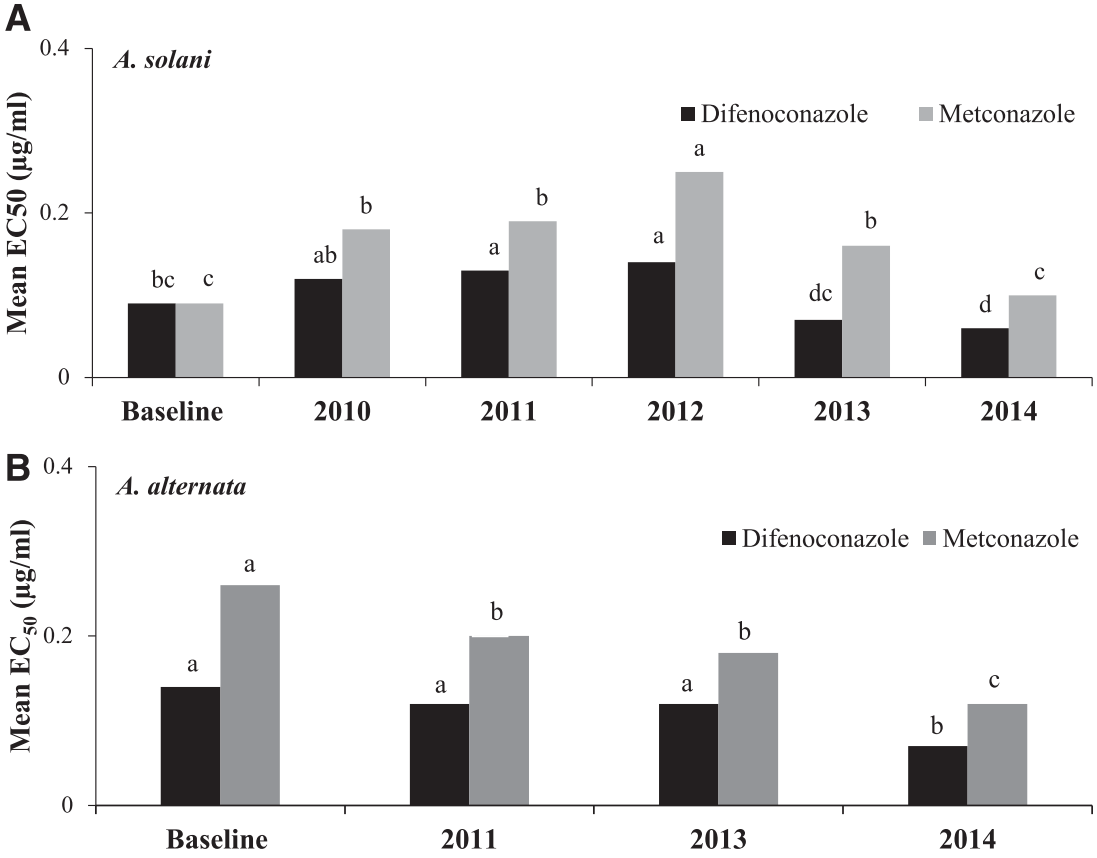


**Fig. 3.** Mean $EC_{50}$ (the effective concentration at which the fungal growth is inhibited by 50%) values for in vitro isolate sensitivity of **A,** *Alternaria solani* and **B,** *Alternaria alternata* to demethylation inhibitor fungicides across years. Within fungicides, columns with the same letter are not significantly different, based on Fisher's protected least significant difference at the $P = 0.05$ level. In vitro $EC_{50}$ values were not determined for *A. alternata* isolates for 2010 and 2012 due to lack of brown spot–infected potato samples sent to the laboratory.

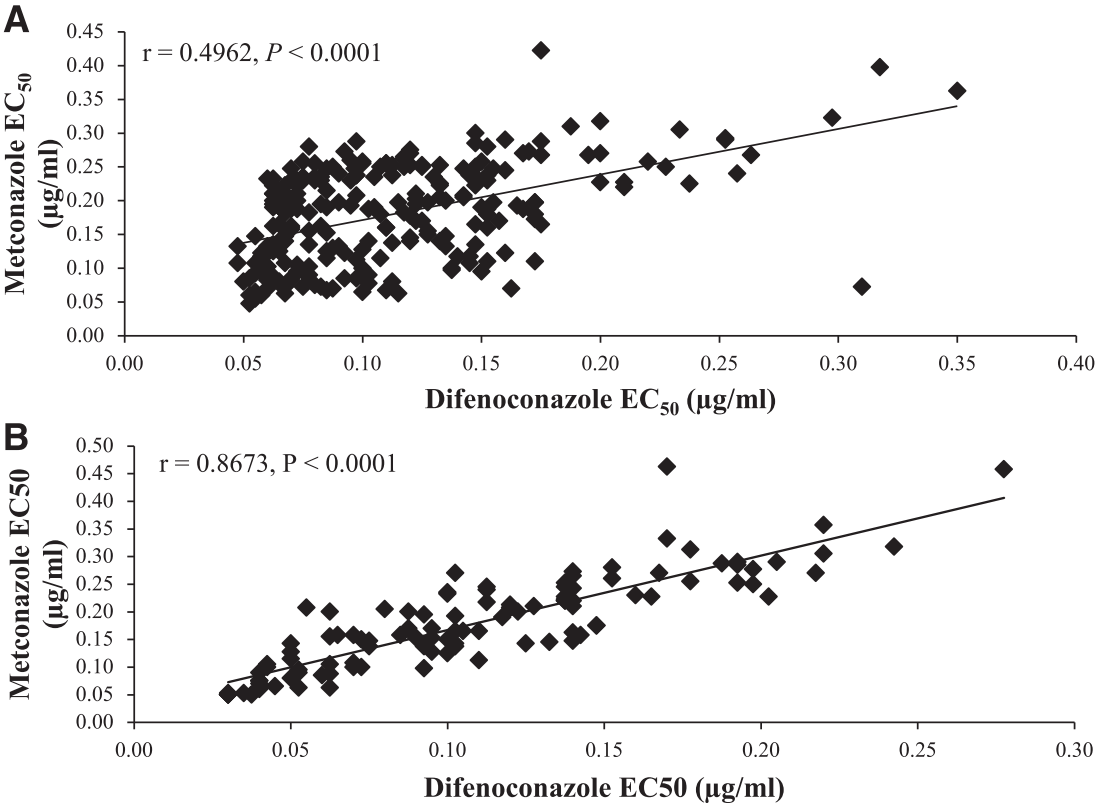


**Fig. 4.** Linear correlation between **A,** in vitro difenoconazole and metconazole sensitivity of 245 *Alternaria solani* isolates collected from 2010 to 2014 and **B,** in vitro difenoconazole and metconazole sensitivity of 109 *Alternaria alternata* isolates collected from 2011 to 2014.

observed for isolate and fungicide concentration for percent disease control. Dose response curves indicate that sensitive *A. solani* isolates (13-1 and 1179-3) were controlled similarly at all fungicide concentrations except 1 μg/ml (Table 3; Fig. 6). Significant differences were observed in the disease control of reduced-sensitive isolates (1168-3, 1184-14, 1191-13, and 1332-6) at concentrations of 0.1, 1, and 10 μg/ml (Table 3). For all concentrations, disease control of reduced-sensitive isolates of *A. solani* was significantly less than the disease control provided by pyrimethanil on sensitive isolates (Table 3).

## Discussion

This is the first report of monitoring sensitivity levels of *A. solani* and *A. alternata* populations to difenoconazole, metconazole, and pyrimethanil across multiple years and production areas. The two DMI fungicides were registered in 2011 for use on potato, but pyrimethanil has been used by growers for the management of early blight and brown spot for almost 10 years. The in vitro sensitivities of the fungicides described in this study are based on $EC_{50}$ values derived from the inhibition of mycelial growth. Assays developed in other pathogen systems have successfully used this method (Bolton et al. 2012; Hilber and Schüepp 1996). DMI fungicides and pyrimethanil inhibit mycelial growth of fungal pathogens, the development stage related to progression and proliferation of subcuticular stromata but cause no inhibition of spore germination (Daniels and Lucas 1995; Smith et al. 1991). It has been determined previously that complex media such as malt agar was not suitable for in vitro assays of AP fungicides if mycelial plugs are used as inoculum from agar plates that have been incubated for more than 23 h (Hilber and Schüepp 1996). In that study, the incubation period of the inoculum did not affect the activity of anilino-pyrimidines, when asp-agar was used. Therefore, the synthetic medium containing asp-agar was used for in vitro pyrimethanil testing in this study.

Baseline isolates of *A. solani* and *A. alternata* used in this study had relatively high levels of variability in response to difenoconazole, with the difference between the most- and least-sensitive isolates being 15- and 11-fold, respectively. Similarly, wider distribution (45-fold) was reported in the sensitive *V. inaequalis* isolates (Villani et al. 2015). However, the distribution ranges of difenoconazole sensitivity reported in *Cercospora beticola* (Bolton et al. 2012), *Colletotrichum coccodes* (Olaya et al. 2010), and *D. bryoniae* (Thomas et al. 2012) were narrow, displaying limited variation within baseline isolates. A few reports assessing in vitro fungicide sensitivity of metconazole on other fungal pathogens are available. The range of $EC_{50}$ values for metconazole was narrow for baseline *A. solani* isolates (4.5-fold) and was similar to that reported in *Galactomyces geotrichum* (McKay et al. 2012). The range of sensitivity of *Fusarium graminearum* isolates in response to metconazole was higher (17.2-fold) than what this study reports for *A. solani*; however, since those *Fusarium* isolates had previous exposure to tebuconazole, this does not constitute a valid baseline group for *Fusarium graminearum* and a wider range of values could be expected from such a population (Spolti et al. 2014). In the baseline sensitivity established in the current study for *A. alternata*, there is a 12-fold difference in sensitivity from the most metconazole-sensitive isolate to the least sensitive. The distribution of pyrimethanil sensitivity of baseline *A. solani* and *A. alternata* isolates in this study was narrow and is comparable with ranges of $EC_{50}$ values reported in *B. cinerea* (Sun et al. 2010) and *V. inaequalis* (Köller et al. 2005) but is unlike the wider distribution reported in a *Penicillium* sp. (Sholberg et al. 2005).

The two DMI fungicides, difenoconazole and metconazole, appear to exhibit great intrinsic activity against both *A. solani* and *A. alternata*, perhaps due to the postinfection activity reported for these fungicides (Wong and Midland 2007). The sensitivity of the majority of *A. solani* and *A. alternata* isolates collected from 2010 to 2014 was consistent with baseline isolates; therefore, these isolates remain sensitive to the two DMI chemistries. Some baseline isolates of *A. solani* and *A. alternata* had high DMI $EC_{50}$ values, which may be due to some level of reduced sensitivity already present in the population but not present in the isolates we have available to us. The high $EC_{50}$ values might also be due to the great amount of variation in morphology, physiology, genetic makeup, and pathogenicity among *Alternaria* spp. isolates, despite having only an asexual life cycle (Aradhya et al. 2001; van der Waals et al. 2004; Woudenberg et al. 2015). In vivo trials were not conducted assessing disease control provided by these fungicides, as previous studies have demonstrated that two- to fourfold changes in Rf values in *A. solani* do not affect early blight disease control under greenhouse conditions (Gudmestad et al. 2013; Pasche et al. 2004, 2005). It should be noted, however, that DMIs are not the primary 'specialty' fungicides used to manage early blight. Instead, tank mixtures of QoIs and SDHIs with

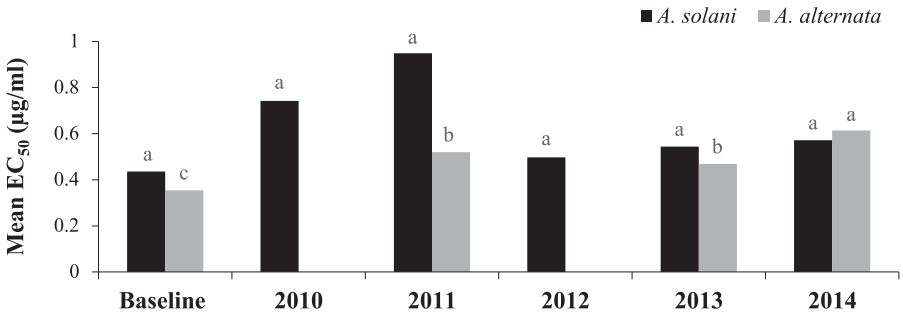


**Fig. 5.** Mean $EC_{50}$ (the effective concentration at which the fungal growth is inhibited by 50%) values for in vitro isolate sensitivity of *Alternaria solani* and *Alternaria alternata* to pyrimethanil across years. Within species, columns with the same letter are not significantly different, based on Fisher's protected least significant difference at the $P = 0.05$ level.

**Table 2.** Origin of *Alternaria solani* isolates determined to be reduced-sensitive to pyrimethanil in the in vitro assessment

| Isolate | Origin | Cultivar | Collection year | $EC_{50}$[z] (μg/ml) |
|---|---|---|---|---|
| 1169-4 | Acequia, ID | Unknown | 2010 | 2.06 |
| 1172-8 | Acequia, ID | Unknown | 2010 | 1.77 |
| 1184-14 | Wray, CO | FL 1867 | 2011 | 3.70 |
| 1191-13 | Wadena, MN | Unknown | 2011 | 28.26 |
| 1226-12 | Dalhart, TX | FL 1867 | 2011 | 4.10 |
| 1332-6 | Dalhart, TX | Russet Norkotah | 2013 | 2.42 |

[z] $EC_{50}$ = the effective concentration at which the fungal growth is inhibited by 50%.

protectant fungicides such as chlorothalonil and mancozeb are used in fungicide rotation programs (Yellareddygari et al. 2016). Regardless, these results do not necessarily indicate that the pathogen populations may not shift toward DMI resistance in the future.

FRAC states that it is wise to accept that cross-resistance is present among DMI fungicides active against the same fungus. Examples of cross-resistance among the same chemical class have been reported in *Alternaria* spp. and other closely related fungi, so there is a high risk for cross-resistance between these two DMI fungicides in *A. solani* and *A. alternata* (Gudmestad et al. 2013; Pasche et al. 2004, 2005). However, given the fact that *A. solani* developed resistance against fungicides with single modes of action quite rapidly, it is highly likely that sequential use of these two DMIs in the same fungicide regime may increase the selection pressure on the pathogen population (Thomas et al. 2012). In this study, the correlation coefficient comparing $EC_{50}$ values for difenoconazole and metconazole baseline sensitivities of *A. solani* isolates was very low. The difference in $EC_{50}$ values of the two fungicides may be due to either the intrinsic activity of each fungicide, the genetic control of the variation in sensitivity to the two DMI fungicides, or both, despite having the same mode of action (Gubler et al. 1996). Currently, DMI fungicides are applied as mixtures to reduce the shift toward fungicide insensitivity. Difenoconazole is registered for use on potato in combination with mandipropamid as Revus Top (Syngenta Crop Protection) and metconazole as Quash (Valent U.S. A. Corporation). Difenoconazole has a limitation of no more than two consecutive applications before rotating to an alternate mode of action and metconazole has a restriction of two applications per season (Friskop et al. 2015). Increased risk of reduced-sensitivity toward difenoconazole, has been reported in *V. inaequalis* populations in Uruguay (Mondino et al. 2015). In that study, Rf values of 6.6 and 11.7 were reported in apple orchards with moderate (up to four applications per season) and intensive use (more than five applications per season) of the DMI chemistry, respectively.

Reduced sensitivity of *A. solani* and *A. alternata* to pyrimethanil was first detected in field isolates collected in 2010 from Idaho (Fairchild et al. 2013). In that study, four of 21 *A. solani* and one of nine *A. alternata* isolates were reported as resistant, although $EC_{50}$ values were reported only for two isolates. The spiral gradient dilution method, an alternative to the classical 'poison agar' plating technique, was utilized to determine in vitro fungicide sensitivity of those isolates from Idaho. Despite the use of an alternative fungicide sensitivity screening method, potato dextrose agar, a complex media was used for the in vitro assays. The use of complex media instead of synthetic media might have had inhibitory effects on pyrimethanil, as a previous study has demonstrated that the activity of AP fungicides in fungicide sensitivity assays is low when complex media are used (Hilber and Schüepp 1996). The use of complex media, along with small sample size, may have contributed to the detection of relatively high frequency of resistance in the Idaho pathogen population. In the study reported here, 109 *A. alternata* isolates recovered from across all locations and years that were tested for sensitivity to pyrimethanil were found to still be sensitive to the fungicide. Additionally, only six of 245 *A. solani* isolates exhibited reduced sensitivity to the AP fungicide, a frequency of only 2.4% and much lower than previously reported by Fairchild et al. (2013). These isolates demonstrated approximately four- to 64-fold loss in sensitivity compared with the baseline population.

As expected, the level of disease control loss was consistent with the $EC_{50}$ values obtained from in vitro sensitivity assays. Pyrimethanil provided similar levels of control of early blight disease caused by the two sensitive *A. solani* isolates, which was significantly superior to the control provided on reduced-sensitive isolates. Reduced-sensitive isolates were not controlled by pyrimethanil except at the highest concentration. In this study, the term 'reduced-sensitivity' was used, instead of resistance, to describe the shift in sensitivity. Resistance to a specific fungicide should equate to a 100% loss of disease control at every concentration tested, demonstrating the fungicide would be of no value to the potato grower (Pasche et al. 2004). The

**Table 3.** Mean in vivo percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays

| | | | Pyrimethanil concentration (µg/ml) | | | | |
|---|---|---|---|---|---|---|---|
| **Isolate** | **$EC_{50}$[w] (µg/ml)** | **Sensitive/reduced-sensitive[x]** | **0.1** | **1** | **10** | **100** | **AUDRC[y]** |
| 13-1 | 0.52 | S | 7.4 a | 37.0 a | 88.9 a | 94.8 a | 8,854.5 a |
| 1179-3 | 0.75 | S | 5.5 ab | 32.3 b | 89.4 a | 96.4 a | 8,927.4 a |
| 1168-3 | 1.57 | RS | 5.0 b | 28.2 c | 64.7 c | 90.7 b | 7,427.1 c |
| 1184-14 | 3.70 | RS | 2.2 c | 22.2 d | 63.6 c | 91.0 b | 7,355.6 c |
| 1191-13 | 28.26 | RS | 3.7 bc | 11.5 f | 60.9 c | 91.1 b | 7,171.5 d |
| 1332-6 | 2.42 | RS | 2.4 c | 18.0 e | 69.5 b | 91.1 b | 7,932.3 b |
| $LSD_{P=0.05}$[z] | | | 2.1 | 3.1 | 4.1 | 1.8 | 171.7 |

[w] $EC_{50}$ (the effective concentration at which the fungal growth is inhibited by 50%) values were obtained for pyrimethanil from the in vitro assessment.
[x] Isolates were characterized as sensitive (S) or reduced-sensitive (RS) based on resistance factor values (S = ≤4, RS = >4).
[y] AUDRC= Area under dose response curve.
[z] Least significant difference at the $P = 0.05$ level.

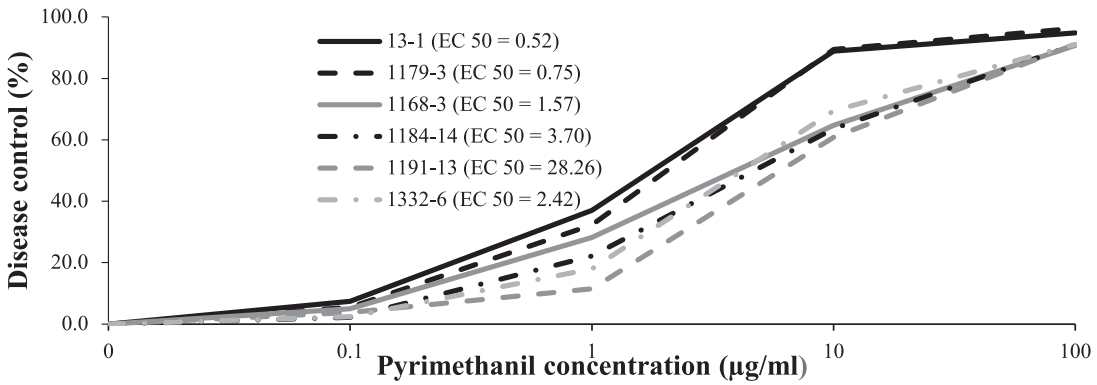


**Fig. 6.** Mean in vivo percentage disease control of *Alternaria solani* isolates by pyrimethanil as determined in greenhouse assays.

in vivo data of this study do not portray a total loss of disease control; hence, the use of the term reduced-sensitivity to reflect the shift in sensitivity of the *A. solani* population in response to pyrimethanil.

The single-site mode of action of AP fungicides has been suggested to result in the inhibition of the secretion of fungal hydrolytic enzymes essential during infection as well as interfere with biosynthesis of methionine (Heye et al. 1994; Masner et al. 1994). Pyrimethanil as well as other AP fungicides cyprodinil and mepanipyrim are considered to be medium risk chemistries in the development of fungicide resistance in fungal pathogens (FRAC 2015). However, resistance to AP fungicides has been reported in *B. cinerea* (Amiri et al. 2013; Chapeland et al. 1999; Fernández-Ortuño et al. 2013; Hilber and Hilber-Bodmer 1998; Latorre et al. 2002; Leroux et al. 1999; Moyano et al. 2004; Myresiotis et al. 2007; Sun et al. 2010), *Oculimacula* spp. (Leroux et al. 2013), *Penicillium digitatum* and *Penicillium expansum* of apple and citrus, respectively, (Kanetis et al. 2008; Xiao et al. 2011), and *V. inaequalis* of apple (FRAC 2015).

*B. cinerea*, one of the most important phytopathogenic fungi, is considered to be a high-risk pathogen for fungicide resistance development and resistance to AP fungicides is common in some field crops and greenhouses (Brent and Holloman 2007b). Different phenotypes of AP resistance have been detected, particularly in *B. cinerea*, with resistance levels varying from low to very high (Leroux et al. 1999; Myresiotis et al. 2007). Cross-resistance in *B. cinerea* has also been reported among the three fungicides within the AP class (Hilber and Schüepp 1996; Latorre et al. 2002; Myresiotis et al. 2007). *B. cinerea* has a high risk of AP resistance development due to resistant isolates demonstrating to be fit as sensitive isolates, based on parameters of lesion growth and sporulation (Fernández-Ortuño et al. 2013; Moyano et al. 2004). Genetic information is a crucial element in determining resistance risk to AP fungicides in *B. cinerea* populations. Genetic analysis of *Botryotinia fuckeliana*, the teleomorph of *B. cinerea*, indicated that resistance to AP fungicides segregated in a 1:1 ratio, and therefore, the gray mold pathogen displays a high inherent resistance risk to this specific chemistry, due to its monogenic resistance (Chapeland et al. 1999; Hilber and Hilber-Bodmer 1998). It is believed that the single-gene mode of resistance resulted in approximately 50% resistant isolates in some research studies focused on AP resistance in *B. cinerea* (Amiri et al. 2013; Fernández-Ortuño et al. 2013; Myresiotis et al. 2007). This major gene resistance reported in *B. cinerea* suggests that change in sensitivity should be rapid once resistant strains were detected. In contrast, field isolate *Oculimacula* spp. (formerly *Tapesia* spp.) displayed a gradual shift in sensitivity, suggesting polygenic control of resistance (Babij et al. 2000). This implies that there might be more than one mechanism of AP resistance in *Alternaria* spp.

The research reported here lays the groundwork for monitoring the shift in sensitivity in *A. solani* and *A. alternata* to difenoconazole, metconazole, and pyrimethanil, thereby evaluating the efficacy of resistance management programs for these pathogens in intensive production systems. It is important to collect additional pathogen isolates from potato production regions to monitor sensitivity to those fungicides. Monitoring studies will be valuable for continuous use of those chemistries in disease management programs. The two DMI fungicides and pyrimethanil should be used in rotation with other systemic and protectant fungicides, to safeguard their efficacy. The risk of resistance to pyrimethanil developing in *A. solani* may be increased because of pre-existing resistance to QoIs and boscalid. The *A. solani* isolates that demonstrated reduced sensitivity to pyrimethanil in this study also contain mutations that confer resistance to strobilurins and boscalid (Mallik et al. 2014). The qualitative nature of resistance may be the reason for reduced sensitivity toward pyrimethanil. Similar changes in population sensitivity have been observed for QoIs and SDHIs (Brent and Holloman 2007b). Qualitative resistance is defined as a sudden and marked loss of efficacy and the presence of a definite sensitive and resistant target population with extensively differing responses (Brent and Holloman 2007a). The primary mode of action is yet undiscovered for AP chemistries; therefore, the resistance mechanism is currently unknown for this established fungicide group.

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