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Impact of SDH Mutations in *Alternaria solani* on Recently Developed SDHI Fungicides Adepidyn and Solatenol

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Abstract

Early blight, caused by *Alternaria solani*, is observed annually in all midwestern potato production areas. The use of foliar fungicides remains a primary management strategy. However, *A. solani* has developed reduced sensitivity or resistance to many single-site fungicides such as quinone outside inhibitor (QoI, FRAC group 11), succinate dehydrogenase inhibitor (SDHI, FRAC group 7), demethylation inhibitor (DMI, FRAC group 3), and anilinopyrimidine (AP, FRAC group 9) fungicides. Boscalid, fluopyram, solatenol, and adepidyn are EPA-registered SDHI fungicides used commercially on a variety of crops, including potato. Five SDH mutations have been characterized previously in *A. solani* that affect the efficacy of boscalid while only one of these mutations has been demonstrated to negatively affect fluopyram efficacy. Conidial germination assays were used to determine if a shift in sensitivity has occurred in these SDHI fungicides. *A. solani* isolates collected prior to the commercial application of SDHI fungicides (baseline) were compared with recently collected isolates (nonbaseline). Greenhouse evaluations were conducted also to evaluate the efficacy of boscalid, fluopyram, solatenol, and adepidyn on *A. solani* isolates possessing individual SDH mutations. Additionally, field trials were conducted to determine the effects of application of these SDHI fungicides on the frequency of SDH mutations. Fluopyram, solatenol, and adepidyn had high intrinsic activity against *A. solani* when compared with boscalid, based on in vitro assays. The application of adepidyn and solatenol resulted in greater early blight control than the application of boscalid and fluopyram in greenhouse experiments. Molecular characterization of *A. solani* isolates collected from the field trials determined that the frequency of the H134R-mutation can increase in response to more recently developed SDHI fungicides. In contrast, the H278R/Y- and H133R-mutations decreased to the point of being nearly absent in these field experiments.

Keywords: *Alternaria solani*, SDHI fungicides, SDH mutations, fungicide resistance

Early blight, caused by *Alternaria solani*, causes economic losses annually in potatoes across all United States growing regions. Potato yield losses as high as 20 to 30% have been recorded in the U.S. during severe early blight epidemics (Christ and Maczuga 1989; Shtienberg et al. 1990), with the greatest yield losses occurring during early bulking (growth stages III to IV, weeks 7 to 9) (Yellareddygari et al. 2018). The primary method of early blight management is through the application of foliar fungicides. Single-site mode of action fungicides such as the succinate dehydrogenase inhibitors (SDHI, FRAC group 7) are highly efficacious for managing early blight (Pasche and Gudmestad 2008). Therefore, the application of single-site mode of action fungicides during early tuber bulking is important to limit yield losses from early blight (Yellareddygari et al. 2016, 2018).

EPA-registered next generation SDHI fungicides boscalid, fluopyram, solatenol, and adepidyn have been used commercially on a variety of crops, including potato. Boscalid was registered for early blight disease control on potato in 2005 and resistance was detected in *A. solani* in 2009 and 2010 (Fairchild et al. 2013; Gudmestad et al. 2013). Fluopyram was registered on potato for early blight disease control in 2012 and resistance was reported in 2014, with evidence of reduced disease control in the greenhouse (Mallik et al. 2014) and field settings (Bauske et al. 2018b). Reduced-sensitivity and/or resistance to boscalid and fluopyram also has been identified in a variety of pathogens including *Alternaria alternata* (Avenot et al. 2014), *Botrytis cinerea* (Fernández-Ortuño et al. 2013), *Blumeriella jaapii* (Outwater et al. 2019), various *Colletotrichum* spp. (Ishii et al. 2016), and *Didymella bryoniae* (Thomas et al. 2012). Solatenol and adepidyn were labeled for use on potato in 2015 and 2018, respectively. Baseline studies have been established for solatenol in *Bipolaris maydis* (Hou et al. 2018), *Colletotrichum* spp. (Ishii et al. 2016), and *Venturia inaequalis* (Villani et al. 2016). Adepidyn baseline studies have been established for *Cercospora zeae-maydis* (Neves and Bradley 2019), *Fusarium asiaticum* (Hou et al. 2017), and *Sclerotinia sclerotiorum* (Duan et al. 2019). Currently, there have been no reports of reduced sensitivity or resistance to either solatenol or adepidyn; however, it is possible that cross resistance to other SDHI fungicides exists in some pathosystems. Baseline sensitivity in *A. solani* to adepidyn and solatenol fungicides have not been determined, but these have been established for boscalid, penthiopyrad, and fluopyram (Bauske et al. 2018b; Gudmestad et al. 2013; Mallik et al. 2014). Without an established baseline sensitivity, it would be difficult to determine if reduced sensitivity or resistance has developed in a fungal population.

The five single-point mutations conveying reduced sensitivity to boscalid in *A. solani* have been found on three *Sdh* genes (Mallik et al. 2014). Two mutations have been identified on the *AsSdhB* gene (H278R and H278Y), one on the *AsSdhC* gene (H134R), and two on the *AsSdhD* gene (D123E and H133R). In a 2011–2012 *A. solani* survey, *AsSdhB* H278Y- and H278R-mutants were recovered at the highest frequencies across all sampled U.S. potato producing regions (Mallik et al. 2014). In contrast, *AsSdhC* and *AsSdhD* H133R-, H134R-, and D123E-mutants were recovered at lower frequencies and were more regionally specific. Results from a 2013–2015 survey indicated that the H134R-mutant was predominate (50, 36, and 27%, respectively), while the presence of the H278Y-mutant increased over these three years (18, 38, and 40%, respectively) and the H278R-mutant decreased over the same three-year period (Bauske et al. 2018a). Over the three-year period, the H133R-mutant increased slightly from 14 to 16% and the D123E-mutant increased from 4 to 12%. No predicted or realized fitness penalties were detected among *A. solani* isolates carrying any SDH mutation (Bauske and Gudmestad 2018).

The overall objective of this study was to establish a more comprehensive understanding of *A. solani* and recently registered SDHI fungicides. The baseline sensitivity of target plant pathogenic fungi is an important component in potentially managing fungicide resistance.

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Additionally, because resistance to SDHI fungicides already exists in *A. solani*, it is important to know the impact, if any, existing SDHI mutations may have on the efficacy of adepidyn and solatenol. The primary objective was accomplished by determining (i) the in vitro sensitivity of a baseline population and nonbaseline isolates collected from 2010 to 2015 to adepidyn and solatenol, (ii) the effect of SDH mutations on the efficacy of boscalid, fluopyram, solatenol, and adepidyn, and (iii) the impact of recently registered SDHI fungicides on the frequency of SDH mutations in *A. solani* isolates under field conditions.

Materials and Methods

***A. solani* isolate collection and maintenance.** *A. solani* isolates were recovered from foliage submitted to the laboratory from potato growing regions across the United States. Fifty-seven *A. solani* isolates with no exposure to SDHI fungicides (baseline) collected from 1998 to 2001 were obtained from long-term cryogenic storage (Supplementary Table S1). One hundred and ten *A. solani* isolates with exposure to SDHI fungicides (nonbaseline) were isolated from early blight infected potato foliage submitted in 2010, 2013, and 2015. The nonbaseline isolates used in this study were collected from long-term storage and subsequently assayed for presence of SDH mutations. These 110 nonbaseline isolates were composed of 21 to 22 *A. solani* isolates possessing each of the five known SDH mutations (H278R, H278Y, H134R, H133R, and D123E). Two isolates with no SDH mutations (SDH-wild-type) were also included in this nonbaseline population.

To determine the SDHI mutations present in each fungicide treatment, *A. solani* isolates were recovered from potato leaves collected in the field experiments described below. Foliar sections with lesions characteristic of early blight 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 edge of the foliar lesion using a scalpel blade and transferred to a 1.5% nonamended agar media (water agar) and incubated at room temperature (22 ± 2°C) for 3 to 4 days until conidia were produced. Purification of the isolates was performed by transferring a single conidium from the water agar to solid clarified V8 (CV8) medium (100 ml Campbell's V8 juice, 1.5 g $CaCO_3$, 15 g agar, and 900 ml distilled water) amended with 50 mg/ml of ampicillin using a sterile glass needle. Cultures were incubated under 24 h fluorescent light at room temperature (22 ± 2°C) for 7 days (Bauske and Gudmestad 2018; Bauske et al. 2018a, b; Gudmestad et al. 2013; Pasche et al. 2004, 2005). To preserve isolates in long-term cryogenic storage, a 4-mm diameter sterilized cork-borer was used to remove circular sections of media with *A. solani* conidia and mycelia and placed into 2 ml screw-top centrifuge tubes. The caps were loosely screwed onto the tubes, tubes were labeled and placed in a closed container with silica gel for 2 to 3 days to remove excess moisture. After drying, the tubes were capped tightly, sealed with Parafilm, and stored in a –80°C ultra-freezer. Herbarium specimens were made for each tissue sample from which *A. solani* isolates were obtained.

Characterization of SDH mutations. To identify mutations present in *A. solani* isolates selected for nonbaseline in vitro and in vivo assays, and isolates collected from field trials, DNA was extracted using the Omega Mag-Bind Plant DNA Plus Kit (Omega Bio-tek Inc., Norcross, GA) with the KingFisher Flex benchtop automated extraction instrument (Thermo Fisher Scientific Inc., Waltham, MA). Using a sterile toothpick, spores were scraped from the 7-day-old pure *A. solani* cultures into a 2-ml screw-top tube containing one ceramic bead and 500 ml of CSPL buffer. The tube was placed in the FastPrep instrument (MP Biomedicals, Santa Ana, CA) and agitated at a speed of 6.0 m/s for 40 s to homogenize the spores and buffer. The tubes were incubated at 56°C for 30 min while the additional buffer plates were prepared.

A total of six plates were used for the KingFisher automated extraction instrument. The first of the five buffer plates (plate 2) contained 500 µl of CSPW1; the second buffer plate (plate 3) contained 500 µl of CSPW2 buffer. Plates 4 and 5 each contained 500 µl of SPM wash buffer and the final buffer plate (plate 6) contained 200 µl of elution buffer. After the 30 min incubation, the tubes were centrifuged at 14,000 × *g* for 10 min, 400 µl of supernatant was transferred to a clean 96-well plate (plate 1), and 5 µl of RNaseA was added to each well. Following incubation at room temperature for 10 min, 400 µl of isopropanol and 15 µl of Mag-Bind particles were added to each well of plate 1. The plates were placed in the KingFisher instrument and the DNA extraction program Omega Plus 1 was activated. Samples were incubated at room temperature for 5 min, mixed using a vortex for 90 s, and rested for 90 s. Next, the Mag-Bind particles were collected in five rounds at 5 s intervals and moved into plate 2. The beads were released into the buffer and samples were vortexed for 60 s, allowed to settle for 30 s, and vortexed again for 30 s before beads were collected as previously described (five rounds at 5 s intervals) and moved to plate 3. The samples and beads went through two separate rounds of SPM wash buffer (plates 4 and 5), collected, and set at room temperature for 10 min to dry. The dry beads were released into plate 6 at 65°C and gently mixed with a vortex for 30 s twice and allowed to rest for 5 min in between mixing events. The beads were collected from the plate and discarded. DNA was transferred to 0.5 ml labeled snap-cap tubes and stored in the –20°C freezer.

The H134R and H133R mutations in the *AsSdhC* and *AsSdhD* genes, respectively, were detected using previously described PCR methods (Mallik et al. 2014). Multiplex PCR assays were performed using 25 µl volume consisting of 20 ng of DNA, 2 mM $MgCl_2$, 0.2 mM dNTP, 5 µM SdhBSen-F, 5 µM SdhBSen-R, 3 µM SdhC-F, 3 µM SdhC-R1, 5 µM SdhD-F, and 5 µM SdhD-R1 primers and 1 U of Go Taq polymerase (Promega Corp., Madison, WI). The multiplex was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA) with an initial preheat of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. A final extension at 72°C for 7 min was executed at the end of the program. A 475-bp product or a 72-bp product was amplified when a mutation was detected in *AsSdhC* or *AsSdhD* genes, respectively. Amplification of a 235-bp product indicated that no mutation was detected in the *AsSdhB* gene.

If no product was amplified in the multiplex PCR, further evaluation was conducted with a MAMA-PCR to determine if a mutation was detected in the *AsSdhB* gene (H278R or H278Y) (Mallik et al. 2014). The H278R mutation was detected with MAMAB1-F and MAMABM-R primers and the H278Y mutation was detected with MAMAB1-F and MAMABR-R primers. MAMA-PCR assays were performed using 25 µl volume consisting of 20 ng of DNA, 1.5 mM MgCl2, 0.2 mM of each primer, and 1 U of Go Taq polymerase. The MAMA-PCR program for amplifying *AsSdhB* mutations was the same as the multiplex program previously described, except an annealing temperature of 60°C was used. An additional PCR assay was performed if no amplifications were expressed in the multiplex or MAMA-PCR assays to detect the presence of the D123E mutation. This additional assay was completed using a 25 µl volume consisting of 20 ng of DNA, 1.5 mM $MgCl_2$, 0.5 mM dNTP, 5 µM of each primer, and 1 U of Go Taq polymerase. The thermal cycler PCR program was as described for the MAMA-PCR program. A 127-bp amplified product signifies the presence of the D123E mutation (Bauske et al. 2018a). All amplified products were separated by gel electrophoresis at 100 volts for 30 min in a 1.2% agarose gel.

In vitro sensitivity of *A. solani* isolates to SDHI fungicides. A study was performed to determine the in vitro sensitivity of 57 *A. solani* baseline isolates and the 110 nonbaseline isolates to boscalid, fluopyram, solatenol, and adepidyn using a conidial germination inhibition assay. Given the number of baseline and nonbaseline *A. solani* isolates to be tested, it would be impossible to perform this study in a single experiment. Therefore, the 167 *A. solani* isolates were assayed in 18 individual trials, with eight to 10 isolates included in each trial. Individual trials were combined by utilizing internal control isolates (13-1, an *A. solani* wild-type isolate, and 526-3, an *A. solani* QoI reduced-sensitive isolate containing the F129L mutation; neither isolate had any SDH mutation) in each trial to determine assay reproducibility (Wong and Wilcox 2002). This statistic is used to determine if individual trials can be combined into a single experiment. A trial in which the internal controls differed by more than 5% of the known EC_{50} value was omitted and not used in further analysis.

The in vitro sensitivity assays were conducted to directly compare intrinsic activity of adepidyn and solatenol fungicides to boscalid and fluopyram, the latter two fungicides which had been previously used in characterizing *A. solani* isolates for sensitivity to SDHI

fungicides (Bauske et al. 2018b; Gudmestad et al. 2013; Mallik et al. 2014). Nonbaseline isolates in this study have all been exposed to boscalid, and isolates recovered in 2013 and 2015 also had exposure to fluopyram. None of the nonbaseline isolates have had exposure to adepidyn and solatenol fungicides.

All isolates were grown on CV-8 agar for 7 to 9 days at 22 ± 2°C under 24 h fluorescent light (Pasche et al. 2004). A sterile glass rod was used to scrape conidia from the agar surface using distilled H_2O. The conidia concentration was adjusted to a 2×10^5 conidia/ml using a hemocytometer and 150 µl was added to the surface of each fungicide amended media plate and spread using a sterile glass rod. Media containing 2% laboratory-grade agar (A360-500 Fischer Scientific, Pittsburgh, PA) was amended with technical formulation of either boscalid (99% active ingredient; BASF Corporation, Research Triangle Park, NC), fluopyram (97.78% active ingredient; Bayer CropScience, Raleigh, NC), solatenol (97% active ingredient; Syngenta Crop Protection, Greensboro, NC), or adepidyn (98.3% active ingredient; Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone, to reach final concentrations of 0.01, 0.1, 1, 10, and 100 µg/ml. A no-fungicide control was included and the acetone concentrations in all media were 0.1% by volume. Salicylhydroxamic acid (SHAM) was added at 100 µg/ml to the media to prevent *A. solani* from overcoming the activity of the SDHI fungicides through an alternative oxidative pathway (Bauske and Gudmestad 2018; Bauske et al. 2018a, b; Gudmestad et al. 2013; Mallik et al. 2014; Pasche et al. 2005). Previous studies have demonstrated that SHAM does not inhibit spore germination among *A. solani* isolates (Pasche et al. 2004, 2005); media was incubated at 21 ± 2°C in the light for 4 h. Percentage spore germination (50 conidia for each treatment) was estimated using a compound microscope at 100× magnification. A conidium was classified as germinated if one germ tube was at least the length of the conidium, or if multiple germ tubes developed from a single conidium. This study was performed twice with two replicates per trial.

In vivo efficacy of boscalid, fluopyram, solatenol, and adepidyn under greenhouse conditions. *A. solani* isolates with low and high solatenol and adepidyn EC_{50} values were selected for in vivo sensitivity assays. Isolates were also selected based on the presence of specific SDH mutations. Nonbaseline isolates possessing neither the F129L or any SDH mutation (three *A. solani* isolates each with either H278R, H278Y, H134R, H133R, or D123E mutation) and sensitive to SDHI fungicides (SDH-wild-type) were also used in these in vivo studies.

SDHI fungicides boscalid, fluopyram, solatenol, and adepidyn were assayed for efficacy against early blight was assayed under greenhouse conditions using a 24 h preventative test (Bauske et al. 2018b; 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 with potato plants, and the resistance of leaves to dehisce when severely infected. Three tomato seeds were sown in a single 10 cm^3 plastic pot containing Sunshine Mix LC1 (Sun Gro Horticulture Inc., Bellevue, WA). After emergence, plants were thinned to acquire two uniformly sized plants per pot. When the plants reached a height of 15 to 20 cm and the first three leaves were fully expanded, they were treated with commercial formulations of boscalid (Endura, BASF Corporation, Research Triangle Park, NC), fluopyram (Luna Privilege, Bayer CropScience, Raleigh, NC), solatenol (Aprovia, Syngenta Crop Protection, Greensboro, NC), or adepidyn (Miravis, Syngenta Crop Protection, Greensboro, NC). Fungicide concentrations of 0, 0.1, 1, 10, and 100 µg/ml active ingredient were applied to the plants to obtain a dose-response curve using a Generation II Research Sprayer (Devries Manufacturing, Hollandale, MN) at approximately 400 kPa.

A 50 ml suspension of 2.0×10^5 conidia/ml was prepared from 10- to 12-day old cultures of *A. solani* grown under 24 h fluorescent light at 22 ± 2°C on CV-8 medium and applied to plants using a Preval paint-spray gun (Preval Sprayer Division, Prevision Valve Corporation, Yonkers, NY). Inoculated plants were placed in humidity chambers (Phytotronic Inc.; 1626D) at >95% RH at 22 ± 2°C for 24 h. The plants were transferred to confinement chambers (plastic cages with an open ceiling) on the greenhouse benches to avoid cross-contamination from other isolates.

The greenhouse temperature was maintained at 25 ± 2°C and plants were watered daily. Early blight severity was visually rated at 6-, 9-, and 12-days postinoculation by estimating percentage infected leaf area on the first three true leaves and recorded as percentage diseased tissue. This in vivo experiment was performed twice with two samples (two plants per pot) and three replicates (three pots) and per isolate at each fungicide concentration.

Effect of SDHI fungicides on early blight severity and frequency of SDH mutations. Two field trials were conducted in 2018 to determine the impact of fungicide selection on mutation frequency following previously described methodology (Bauske et al. 2018b; Pasche and Gudmestad 2008). The trials were performed under irrigated conditions near Inkster and Larimore, ND, using the early blight and brown spot susceptible cv. Ranger Russet.

The experiment consisted of 10 treatments with four replicates in a randomized complete block design. Plants were grown in four row plots, approximately 3.6 m wide and 9 m long (Table 1). Two treatments consisted of the standard protectants chlorothalonil and mancozeb applied at 7-day intervals throughout the growing season. Two treatments were developed to be similar to foliar fungicide programs followed by commercial potato grower in North Dakota. These consisted of single-site foliar fungicides tank mixed with standard protectant fungicides, either fluopyram/pyrimethanil or adepidyn/fludioxonil (Pasche and Gudmestad 2008). Solatenol was applied in-furrow alone, or mixed with two rates of the biological control *Bacillus subtilis* (Serenade Soil, Bayer CropScience). Fluopyram was also applied in-furrow alone, or with the high rate of *B. subtilis*. *B. subtilis* has been demonstrated previously to reduce the incidence of the D123E mutation in *A. solani* when applied in furrow at planting (Bauske et al. 2018b). This trait may be due to the ability of *B. subtilis* to enhance disease resistance in plants associated with the accumulation of salicylic acid and pathogenesis-related proteins (Métrauxs 2001; van Loon and van Strien 1999). In-furrow applications were directed at the seed-piece using a planter-mounted CO_2 sprayer with a single nozzle with a 6501 tip. Foliar fungicides were applied with a water volume of 560 liters/ha and a pressure of 375 kPa. Foliar disease percentage was recorded in the center two rows at approximately 7-day intervals, beginning from 60 to 70 days after planting. These disease severity evaluations were recorded on a 0 to 100% diseased leaf tissue scale weekly for 11 weeks, terminating one week following the final foliar fungicide application (Pasche and Gudmestad 2008).

All treatments were inoculated using conidial suspensions of four *A. solani* isolates collected in 2011 and 2012, two isolates each containing the H278Y mutation, and two isolates classified as wild-type (no F129L or SDH mutations). Isolates were grown in CV8 media under constant fluorescent light for 2 weeks at room temperature (22 ± 2°C). Distilled water was added to the cultures, and conidia were dislodged with a glass rod and diluted into a 0.25% gelatin solution to a concentration of 6.7×10^3 conidia/ml. This suspension was applied twice using customized ATV application equipment to the outside two rows of each four-row treatment at a rate of 104 ml/row in mid-July and early-August (approximately 2 weeks apart). Immediately following the final foliar disease severity rating, approximately 10 compound leaves exhibiting symptoms typical of early blight were sampled arbitrarily from all four replicates of each treatment, including the nontreated control. The leaves were placed in unsealed plastic bags inside a cooler for transport to the laboratory. The infected leaf tissue was transferred to 1.5% nonamended agar media and isolations were made as described above (Holm et al. 2003). Thirty-five to 41 *A. solani* isolates were collected from each treatment and DNA was extracted and evaluated for the presence of SDH mutations as previously described. A total of 477 *A. solani* isolates were characterized for SDH mutations from the Larimore location and 357 from the Inkster location for a total of 834 isolates evaluated in field experiments.

Statistical analysis. To quantify in vitro fungicide sensitivity, the effective concentration where fungal germination is inhibited by 50% (EC_{50} value) was deduced from the 50% intercept (EC_{50} value) using SAS version 9.4 (SAS Institute Inc., Cary, NC), (Pasche et al. 2004). Isolates with EC_{50} values of <0.01 and >100 were analyzed

as 0.01 and 100 µg/ml, respectively. An F-test was used to determine homogeneity of variance across experiments. Assay reproducibility was determined using the approximate limits for a 95% confidence interval for two internal controls included in every trial (Wong and Wilcox 2002). Trials were included in the final analysis if the internal control EC_{50} values were within the 95% confidence interval. Mean separation was determined using Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Pearson correlation coefficients were calculated to compare the in vitro fungicide EC_{50} values for SDHI baseline and nonbaseline *A. solani* isolates within each fungicide. A resistance factor (Rf) was calculated for each fungicide by dividing the mean EC_{50} value of the nonbaseline isolates by the mean EC_{50} value of the baseline isolates.

Greenhouse experiments were conducted as split-plot arrangement in a randomized complete block design with *A. solani* isolate as the whole plot and fungicide as the split-plots. For every isolate at all fungicide concentrations, disease severity data were transformed to percentage disease control using the formula: [1 – (% diseased tissue/ % diseased tissue in nontreated plants) × 100] (Gudmestad et al. 2013; Pasche et al. 2004). Disease control data were utilized for further statistical analyses and the Levene's test was used to determine homogeneity of variance between two independent experiments (Milliken and Johnson 1992). A two-way ANOVA was also conducted to evaluate the main effects of (and interactions among) trial, isolate, and fungicide to determine if the trials could be combined for further analyses using proc GLM SAS. Area under the dose-response curve (AUDRC) was calculated to determine if there were significant differences in early blight control provided by boscalid, fluopyram, solatenol, and adepidyn across fungicide concentrations:

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

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.

For field trials, the area under the disease progress curve (AUDPC) was calculated using percentage early blight severity, recorded as previously described above (Shaner and Finney 1977):

$$\text{AUDPC} = \sum_{i=1}^{n} [[(W_{i+1} + W_i)/2][t_{i+1} - t_i]]$$

W_i is the percentage foliar disease severity at the *i*th observation, t_i the time in days at the *i*th observation, and *n* the total number of observations. The relative area under the disease progress curve (RAUDPC) was calculated for each treatment of the replicated trials from each location-year by dividing AUDPC values by the total area of the graph and analyzing using ANOVA (Proc GLM SAS version 9.4, Cary, NC). Fisher's protected LSD ($\alpha = 0.05$) was used to differentiate mean RAUDPC values. SDH-mutant frequency field data were unbalanced and therefore required $\log_{10}$ transformation prior to analysis. Back-transformed data are presented.

Results

In vitro sensitivity of *A. solani* to SDHI fungicides. Independent analysis of variance across in vitro fungicide sensitivity experiments for boscalid, fluopyram, solatenol, and adepidyn determined that variances were homogenous ($P = 0.7506$) and the experiments were combined for further analysis. In vitro fungicide sensitivity of *A. solani* baseline isolates to boscalid, fluopyram, solatenol, and adepidyn ranged from <0.01 to 0.97, <0.01 to 1.31, <0.01 to 0.23, and <0.01 to 0.08 µg/ml, respectively (Fig. 1). Baseline isolates of *A. solani* were significantly less sensitive to boscalid (0.20 µg/ml) than to all other SDHI fungicides evaluated and these isolates were less sensitive to fluopyram (0.14 µg/ml) compared with solatenol (0.03 µg/ml) and adepidyn (0.01 µg/ml) (Table 2). The mean sensitivity of *A. solani* isolates to fluopyram was significantly lower when compared with solatenol and adepidyn. *A. solani* nonbaseline isolate sensitivity to boscalid, fluopyram, solatenol, and adepidyn ranged from <0.01 to 65.03, <0.01 to 8.64, <0.01 to 3.43, and <0.01 to 0.69 µg/ml, respectively (Fig. 1). As observed with the baseline isolates, nonbaseline *A. solani* isolates were significantly less sensitive to boscalid (4.56 µg/ml) than to fluopyram (0.74 µg/ml), solatenol (0.20 µg/ml), and adepidyn (0.05 µg/

Table 1. Fungicide treatment, use rates, and application schedule of treatments evaluated in field trials conducted in 2018 in Inkster and Larimore, ND

Treatment[x]	Active ingredient(s)	Rate	Schedule[y]	Interval[z]
Nontreated	Nontreated	-	-	-
Chlorothalonil	Chlorothalonil	2.5 liters/ha	Full season	7-day
Mancozeb	Mancozeb	2.25 kg/ha	Full season	7-day
Grower standard/adepidyn + fludioxonil	Azoxystrobin +	0.5 liters/ha	1, 3	7-day
	Chlorothalonil +	0.1% v/v +		
	Mancozeb	2.25 kg/ha		
	Chlorothalonil	1.5 liters/ha	2	
	Chlorothalonil	2.5 liters/ha	4, 6, 8–10	
	Adepidyn/fludioxonil	0.7 liters/ha	5	
	Mancozeb	2.25 kg/ha		
	difenoconazole +	0.5 liters/ha	7	
	Mancozeb	2.25 kg/ha		
Solatenol-in-furrow	Solatenol	1.4 liters/ha	In-furrow	At planting
Solatenol + *B. subtilis* (low rate) in-furrow	Solatenol +	1.4 liters/ha	In-furrow	At planting
	Bacillus subtilis	4.7 liters/ha		
Solatenol + *B. subtilis* (high rate) in-furrow	Solatenol +	1.4 liters/ha	In-furrow	At planting
	Bacillus subtilis	9.4 liters/ha		
Fluopyram in-furrow	Fluopyram	0.6 liters/ha	In-furrow	At planting
Fluopyram + *B. subtilis* (high rate) in-furrow	Fluopyram +	0.6 liters/ha	In-furrow	At planting
	Bacillus subtilis	9.4 liters/ha		
Grower standard/fluopyram+ pyrimethanil	Fenamidone +	0.5 liters/ha	1, 3	7-day
	Chlorothalonil +	0.1% v/v +		
	Mancozeb	2.25 kg/ha		
	Chlorothalonil	1.5 liters/ha	2	
	Chlorothalonil	2.5 liters/ha	4, 6, 8–10	
	Fluopyram/pyrimethanil	11 oz/ha	5	
	Mancozeb	2.25 kg/ha		
	Pyrimethanil +	0.5 liters/ha	7	
	Mancozeb	2.25 kg/ha		

[x] '+' indicates a tank mixture, and '/' refers to a formulated chemical mixture.
[y] Week(s) fungicide was applied.
[z] Days between subsequent applications of treatments.

ml) (Table 2). However, there were no significant differences in sensitivity among *A. solani* nonbaseline isolates to fluopyram, solatenol, and adepidyn. Baseline *A. solani* isolates were more sensitive to all four fungicides than were nonbaseline isolates. The boscalid, fluopyram, solatenol, and adepidyn sensitivities of 52%, 24%, 22%, and 15% of the nonbaseline *A. solani* isolates fell outside the baseline range.

Resistance factors calculated from in vitro fungicide sensitivity assays varied among the SDHI fungicides after comparing the mean sensitivities of baseline and nonbaseline isolates. The Rf for *A. solani* nonbaseline isolates compared with the baseline isolates in response to boscalid, fluopyram, solatenol, and adepidyn were 23.3-, 5.4-, 5.7-, and 3.8-fold, respectively. No meaningful correlations were observed between fungicides evaluated in either baseline or nonbaseline *A. solani* populations.

When evaluated within SDH mutation, all nonbaseline *A. solani* isolates with a mutation were significantly less sensitive to boscalid compared with the other fungicides evaluated and no difference in sensitivity was observed among those three fungicides (Table 3). Nonbaseline, SDH wild-type isolates (no mutation in the SDH genes) were less sensitive to boscalid and fluopyram compared with solatenol and adepidyn. Nonbaseline *A. solani* isolates with no SDH mutations were significantly more sensitive to all SDHI fungicides compared with isolates with a mutation except isolates with the H278R mutation to fluopyram and solatenol. While significant differences were observed across *A. solani* isolates with SDH mutations for each of the four SDHI fungicides evaluated, no consistent patterns were observed (Table 3). Generally, the H278R, H133R, and D123E reduced sensitivities to the greatest degree to two or three fungicides.

Greenhouse evaluation of the impact SDH mutations in *A. solani* on SDHI fungicide efficacy. Independent analysis of greenhouse disease control experiments for boscalid, fluopyram, solatenol, and adepidyn determined that variances were homogenous (P = 0.8158), and experiments were combined for further analysis. A significant interaction was observed between the whole plot (isolate) and subplot (fungicide) for percentage disease control, as represented by AUDRC ($P < 0.0001$). Across all SDH mutation types, including the SDH-wild-type, disease control was significantly different among all fungicides with adepidyn providing the best control, followed by solatenol, fluopyram, and boscalid (Table 4). Comparisons within SDH mutation type indicate that disease control was significantly lower with boscalid, as compared with all other fungicides, for *A. solani* isolates with the H133R, D123E, and SDH-wild-type isolates. Control provided by boscalid and fluopyram did not differ for isolates with the H278R, H278Y, or H134R mutations but these two fungicides provided less control of isolates with these mutations, and SDH-wild-type isolates, when compared with solatenol. Boscalid and fluopyram provided less control of isolates possessing any mutation than adepidyn. Disease control provided by the application of fluopyram was

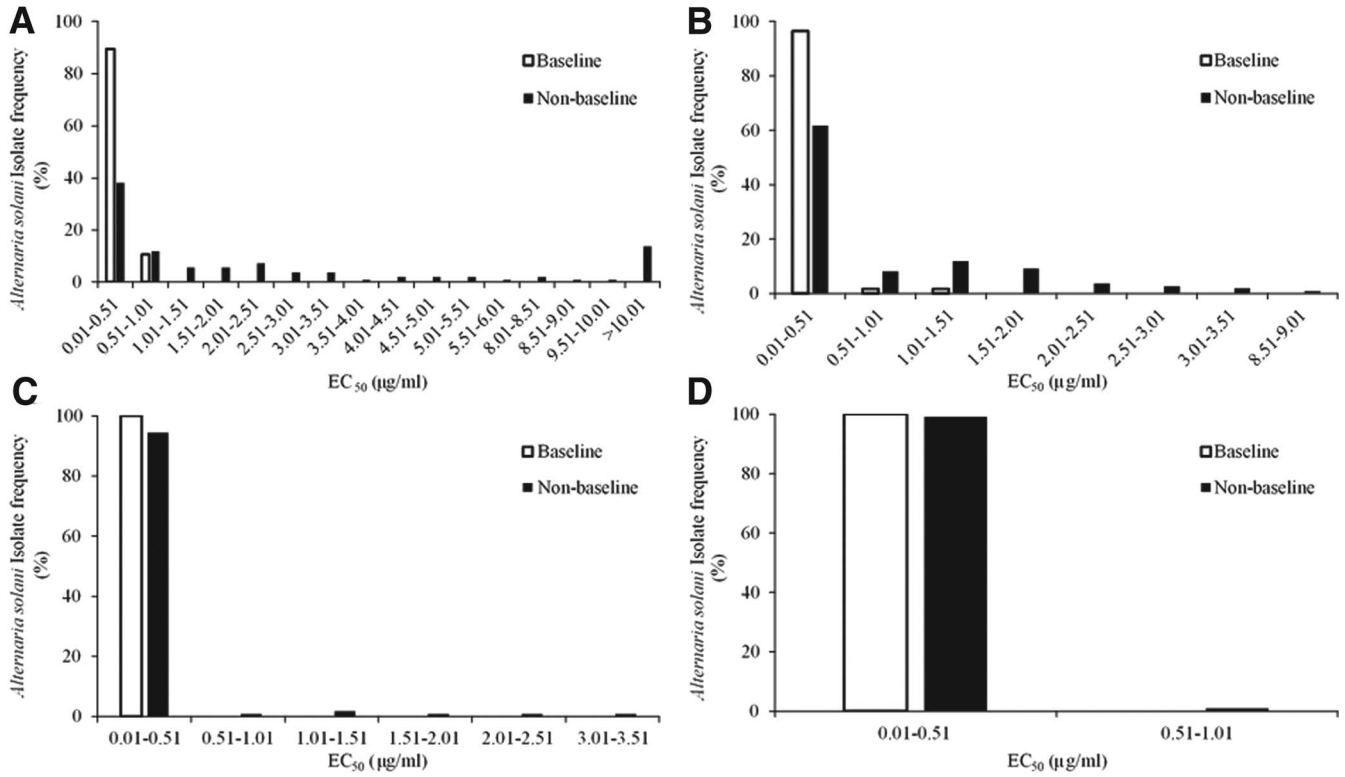

Fig. 1. Frequency distribution of sensitivity of 57 baseline and 110 nonbaseline *Alternaria solani* isolates to the SDHI fungicides **A,** boscalid, **B,** fluopyram, **C,** solatenol, and **D,** adepidyn. The sensitivity is determined based on the effective concentration that inhibits the spore germination growth by 50% compared with the nontreated control (EC_{50} µg/ml).

Table 2. In vitro fungicide sensitivity (mean EC_{50} value) of baseline and nonbaseline *Alternaria solani* isolates to boscalid, fluopyram, solatenol, and adepidyn

	Mean fungicide EC_{50} values (µg/ml)				
***A. solani* population**	**Boscalid**	**Fluopyram**	**Solatenol**	**Adepidyn**	**$LSD_{P = 0.05}$[z]**
Baseline (n = 57)	0.12 b A	0.14 b B	0.03 b C	0.01 b C	0.030
Nonbaseline (n = 110)	4.56 a A	0.74 a B	0.19 a B	0.05 a B	0.719
$LSD_{P = 0.05}$[z]	0.87	0.08	0.03	0.005	

[z] Fisher's protected least significant difference at $\alpha = 0.05$; rows containing the same uppercase letters represent no significant difference between the SDHI fungicides within the baseline/nonbaseline. Columns containing the same lowercase letters represent no significant difference between the baseline and nonbaseline means within the SDHI fungicide.

significantly lower than provided by solatenol for isolates with all SDH mutation types with the exception of the H133R and the D123E. Disease control provided by solatenol was lower than that provided by adepidyn only for isolates with the H278R and H278Y mutations (Table 4).

Significant differences in disease control were also observed within fungicides across SDH mutation type. SDH-wild-type isolates were controlled better than isolates with SDH mutations with all fungicides except adepidyn, but these differences were not always significant (Table 4). All SDHI fungicides provided similar control of isolates with the H278Y mutation and SDH-wild-type isolates. No significant difference in disease control by boscalid and adepidyn was observed across the other SDH mutation types. Only the H134R and the H278R mutations resulted in a significant loss of disease control by fluopyram when compared with SDH-wild-type isolates. The application of solatenol provided better disease control of *A. solani* isolates with the H134R mutation compared with isolates with the H278R, H133R, and D123E mutations. Isolates with the H278R mutation were controlled least by solatenol.

Early blight severity and SDH mutation frequency under field conditions. Results from field trials conducted at Larimore and Inkster, North Dakota, in 2018 demonstrated there were significant differences among fungicide treatments in the control of early blight. At both locations, all fungicide treatments provided significantly better control of early blight compared with the nontreated control plots and similar trends were observed across all treatments (Fig. 2A and B). At Larimore, the grower standard with adepidyn and fludioxonil provided significantly better control of early blight compared with the solatenol and the fluopyram in-furrow-based treatments (Fig. 2A). Disease control provided by chlorothalonil, mancozeb, and the grower standard with fluopyram and pyrimethanil was not significantly different than the grower standard with adepidyn and fludioxonil. At Inkster, both grower standard treatments provided significantly better control of early blight compared with the other treatments with the exception of chlorothalonil and mancozeb full-season (Fig. 2B). The level of disease control provided by solatenol applied in furrow at planting alone, and with the high rate of *B. subtilis*, was lower than the control provided by fluopyram applied in furrow at Larimore and all treatments that included solatenol in-furrow provided significantly lower control than fluopyram in-furrow at Inkster.

Significant differences were observed in total yield at both field trial locations in 2018. As was the case with early blight disease severity, similar trends were observed across fungicide treatment regimes. At Larimore, the grower standard with adepidyn and fludioxonil was the only treatment that resulted in significantly higher yield than the nontreated control, but yield in this treatment was not significantly different from chlorothalonil, mancozeb, solatenol in-furrow with the low rate of *B. subtilis*, or the grower standard with fluopyram and pyrimethanil (Fig. 3A). At Inkster, the application of mancozeb full-season was the only treatment resulting in significantly higher yield than the nontreated control (Fig. 3B). However, the yield resulting from the application of mancozeb full-season was not significantly higher than the grower standard with adepidyn and fludioxonil, solatenol in-furrow with the low rate of *B. subtilis*, fluopyram in-furrow with the high rate of *B. subtilis*, and the grower standard with fluopyram and pyrimethanil.

All known *A. solani* SDH mutations were detected among isolates collected from these trials. At both field locations, the prevalence of collected H278Y- and H278R-mutants was low (<14%), despite inoculating the plots with two SDHI-wild-type and two H278Y-mutant isolates (Table 5). No significant difference was observed in the frequency of isolates with the H278R mutation at either site. Isolates with this mutation were detected in the nontreated and solatenol in-furrow treatments at both locations and in three and one additional treatments at Larimore and Inkster, respectively. A significant difference was observed in the frequency of H278Y-mutant detection only at Inkster. The application of solatenol in-furrow resulted in the highest frequency of detection of H278Y, but this was only significantly higher than

Table 3. In vitro fungicide sensitivity (mean EC_{50} values) of the baseline *Alternaria solani* isolates and the succinate dehydrogenase (SDH) mutations within nonbaseline *A. solani* isolates to boscalid, fluopyram, solatenol, and adepidyn

SDH mutations (number of isolates)	Mean fungicide EC_{50} values (µg/ml)				$LSD_{P = 0.05}$[z]
	Boscalid	Fluopyram	Solatenol	Adepidyn	
Baseline (n = 57)	0.20 A	0.14 B	0.03 C	0.01 C	0.030
Nonbaseline (n = 110)					
H278R (n = 22)	4.28 b A	0.27 c B	0.02 c B	0.03 d B	2.244
H278Y (n = 21)	3.29 b A	0.90 a B	0.36 a B	0.07 a B	0.951
H134R (n = 22)	2.71 b A	0.67 b B	0.15 b B	0.05 bc B	0.732
H133R (n = 21)	6.54 a A	1.00 a B	0.13 b B	0.06 b B	1.304
D123E (n = 22)	6.31 a A	0.90 a B	0.31 a B	0.04 cd B	2.245
SDH-Wild-type (n = 2)	0.13 c A	0.09 c A	0.03 c B	0.01 e B	0.046
$LSD_{P = 0.05}$[z]	1.947	0.186	0.061	0.012	

[z] Fisher's protected least significant difference at the $\alpha = 0.05$; rows containing the same uppercase letters represent no significant difference between the fungicides within the SDHI-mutation and columns containing the same lowercase letters represent no significant difference detected between SDH mutations within the SDHI fungicide.

Table 4. Mean area under the dose response curve (AUDRC) for *Alternaria solani* isolates possessing an SDH mutation (H278R, H278Y, H134R, H133R, D123E, or SDH-wild-type) among the SDHI fungicides boscalid, fluopyram, solatenol, and adepidyn

SDH mutations	Mean AUDRC				$LSD_{P = 0.05}$[z]
	Boscalid	Fluopyram	Solatenol	Adepidyn	
Combined *A. solani* isolates (n = 18)	7,483.2 D	8,049.2 C	9,366.4 B	9,700.8 A	313.6
H278R (n = 3)	6,825.8 c C	7,178.1 b C	8,655.7 d B	9,587.9 b A	863.5
H278Y (n = 3)	8,222.6 ab C	8,402.4 a C	9,729.7 ab B	9,949.5 a A	639.0
H134R (n = 3)	6,589.6 c B	6,077.9 c B	9,550.7 b A	9,496.4 b A	983.7
H133R (n = 3)	7,052.7 c C	8,791.6 a B	9,205.5 c AB	9,587.9 b A	439.6
D123E (n = 3)	7,353.0 bc C	8,627.6 a B	9,235.8 c AB	9,670.7 b A	721.2
SDH-Wild-type (n = 3)	8,855.2 a C	9,217.5 a B	9,820.7 a A	9,938.4 a A	528.4
$LSD_{P = 0.05}$[z]	1,147.9	834.2	263.6	186.6	

[z] Fisher's protected least significant difference at the $\alpha = 0.05$; rows containing the same uppercase letter indicate no significant differences existed between the fungicides. Columns containing the same lowercase letter indicate no significant differences exist between the SDH mutations within the fungicide.

treatments in which the mutation was not detected (0%). The frequency of detection of isolates with the H134R mutation was generally higher than any other mutation, ranging from 4.2 to 85.2%. Differences in the detection of this mutation were only significant at Larimore. Here, only the application of chlorothalonil full-season resulted in a higher frequency of detection of the H134R mutation compared with the nontreated control. A single isolate possessing the H133R mutation was detected in the low rate of *B. subtilis* treatment/solatenol in-furrow treatment at Inkster. The detection frequency of the D123E mutation was significantly different at both trial locations. At Larimore, the application of solatenol in-furrow resulted in the highest detection of the D123E mutation, significantly higher than the nontreated control, but was not significantly higher than mancozeb full-season or either grower standard. At Inkster, the D123E mutation was observed most frequently when mancozeb was applied full-season and was similar when chlorothalonil was applied full-season and when the grower standard of fluopyram and pyrimethanil was used. At Inkster, *A. solani* isolates possessing the D123E mutation were detected in all treatments except the two treatments that included applications of fluopyram in-furrow. Between zero and 21% of isolates were determined to be SDH-wild-type across both locations and differences were only significant at Larimore. The application of fluopyram and the high rate of *B. subtilis* in-furrow resulted in the highest frequency of SDH-wild-type isolates, but this was not significantly different than the nontreated control. Mutation status could not be determined in 7.7 to 82.5% of isolates and the inability to determine mutations in *A. solani* isolates using currently available methods was highest when solatenol was applied in-furrow with the low rate of *B. subtilis* at both trial locations.

Discussion

A substantial amount of previous research on SDHI fungicide resistance in *A. solani* dealt primarily with the characterizing of point mutations conveying resistance in this fungus and the impact those mutations had on boscalid (Fairchild et al. 2013; Gudmestad et al. 2013; Mallik et al. 2014). Further studies investigated how SDH mutations in *A. solani* impact more recently developed SDHI fungicides such as penthiopyrad and fluopyram (Bauske and Gudmestad 2018; Bauske et al. 2018a, b; Mallik et al. 2014). One of the goals of the current study was to determine the sensitivity of the *A. solani* baseline and nonbaseline isolates to four SDHI fungicides, two of which were very recently introduced into the U.S. potato market. The results reported here, and elsewhere, clearly indicate that there has been a shift in sensitivity of *A. solani* to boscalid. While a proportion of nonbaseline *A. solani* isolates fell outside the baseline sensitivity range for each fungicide, we have demonstrated that SDH-mutant *A. solani* isolates were significantly more sensitive to fluopyram, solatenol, and adepidyn than they were to boscalid, regardless of the mutation.

An additional goal of this study was to determine the impact SDH mutations may have on the efficacy of more recently EPA-registered SDHI fungicides solatenol and adepidyn compared with older SDHI fungicides using greenhouse efficacy evaluations. Results from greenhouse evaluations indicate that adepidyn provided the highest level of disease control of *A. solani* isolates possessing the H278R and H278Y mutations among the SDHI fungicides currently registered on potato. For all other isolates of *A. solani* evaluated, disease control provided by adepidyn was statistically similar to solatenol. Furthermore,

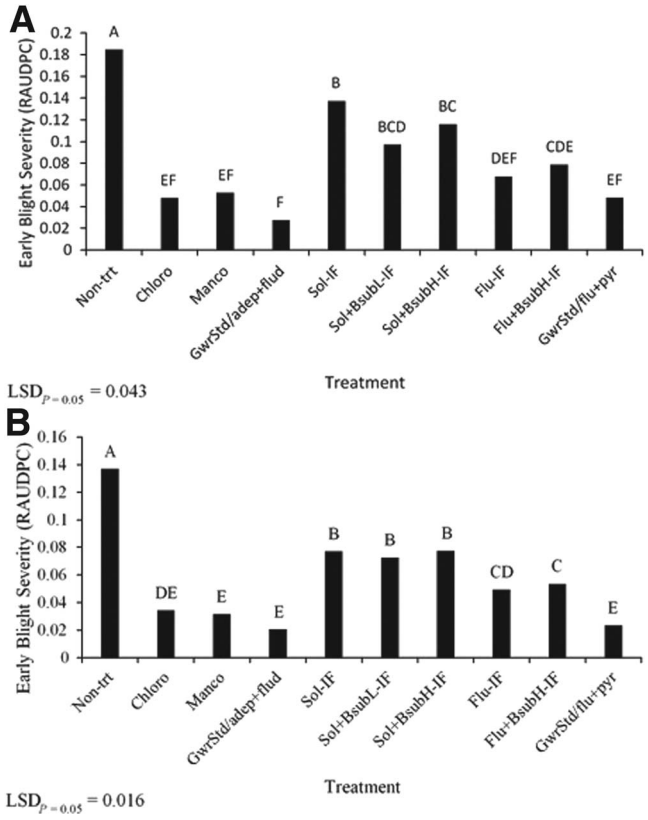

Fig. 2. Early blight severity, expressed as relative area under the disease progress curve (RAUDPC) from 2018 field trials conducted in **A,** Larimore and **B,** Inkster, ND. Treatments included a nontreated control (nontrt); chlorothalonil (Chloro); mancozeb (Manco); separate applications of QoI, a new foliar SDHI and PP, and DMI fungicide each mixed with standard protectants (GwrStd/adep+flud); solatenol in-furrow (Sol-IF); solatenol in furrow with 4.7 liters/ha and 9.4 liters/ha of *Bacillus subtilis* (Sol+BsubL-IF and Sol+BsubH-IF, respectively); fluopyram in-furrow (Flu-IF); fluopyram in-furrow with 9.4 liters/ha of *B. subtilis* (Flu+BsubH-IF); and separate applications of QoI, SDHI-fluopyram mixed with and anilinopyrimidine-pyrimethanil, each mixed with standard protectants (GwrStd/Flu+Pyr). Columns with the same letter are not significantly different according to Fisher's protected least significant difference test (α < 0.05).

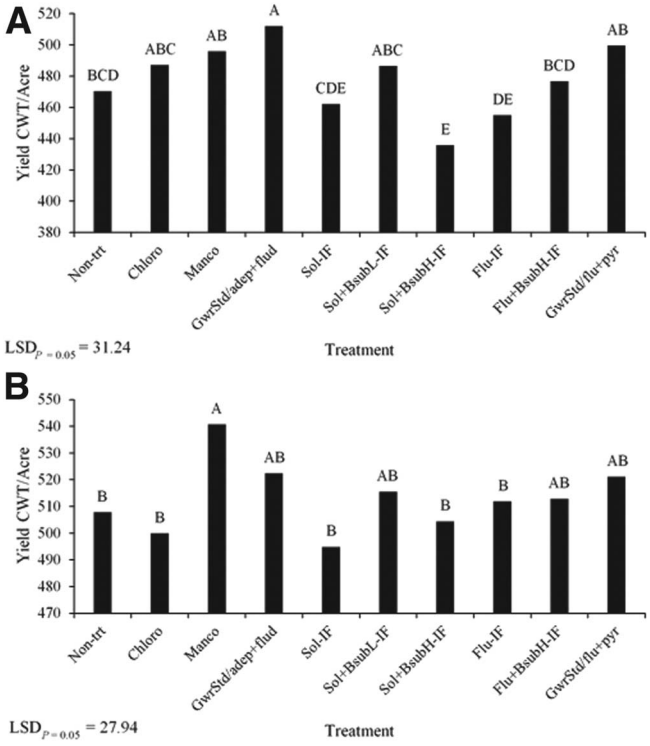

Fig. 3. Yield data expressed in hundredweight (CWT) per acre from 2018 field trials conducted in **A,** Larimore and **B,** Inkster, ND. Treatments included a nontreated control (nontrt); chlorothalonil (Chloro); mancozeb (Manco); separate applications of QoI, a new foliar SDHI and PP, and DMI fungicide each mixed with standard protectants (GwrStd/adep+flud); solatenol in-furrow (Sol-IF); solatenol in furrow with 4.7 liters/ha and 9.4 liters/ha of *Bacillus subtilis* (Sol+BsubL-IF and Sol+BsubH-IF, respectively); fluopyram in-furrow (Flu-IF); fluopyram in-furrow with 9.4 liters/ha of *B. subtilis* (Flu+BsubH-IF); and separate applications of QoI, SDHI-fluopyram mixed with and anilinopyrimidine-pyrimethanil, each mixed with standard protectants (GwrStd/Flu+Pyr). Columns with the same letter are not significantly different according to Fisher's protected least significant difference test (α < 0.05).

solatenol provided significantly higher disease control of isolates possessing the *AsSdhC* and *AsSdhB* mutations compared with fluopyram and boscalid. Previous studies suggest that solatenol may bind to the SdhD protein in the SDH complex (Ishii et al. 2016). However, *A. solani* isolates possessing the H278R mutation (*AsSdhB* gene) were controlled less by solatenol compared with isolates possessing the other SDH mutations and SDH-wild-type isolates. Interestingly, fluopyram was the only SDHI fungicide evaluated that controlled isolates possessing the D123E-mutation as well as the SDH-wild-type isolates. Previous aggressiveness studies determined that *A. solani* isolates possessing the D123E mutation are more aggressive under greenhouse conditions compared with the SDH-wild-type isolates (Bauske and Gudmestad 2018). These differences could be explained by the differing number of isolates used in these two greenhouse studies and the high levels of variability within *A. solani* (Bauske and Gudmestad 2018; van der Waals et al. 2004; Woudenberg et al. 2015). In previous studies, a larger subset of *A. solani* isolates were evaluated in the greenhouse compared with the greenhouse isolates analyzed in this study (Bauske and Gudmestad 2018). Utilizing a larger number of *A. solani* isolates would provide a more comprehensive look at the aggressiveness and response of the SDH-mutants to the SDHI fungicides, and these studies are warranted.

The final goal of this study was to determine the impact of newly registered SDHI fungicides on the frequency of the SDH mutants under field conditions. Initial studies on the distribution of SDH mutations in *A. solani* in the United States demonstrated that mutations in the *SdhB* gene, such as H278R and H278Y, were the most prevalent (Mallik et al. 2014). More recent studies have demonstrated that isolates with a mutation in the *SdhB* gene have diminished substantially in frequency (Bauske et al. 2018a) and without any apparent parasitic fitness penalty that would explain the lower frequency (Bauske and Gudmestad 2018). In the studies reported here, *A. solani* isolates possessing the H278Y mutation were controlled as effectively as the SDHI-wild-type isolates across all SDHI fungicide treatments, possibly explaining why the H278Y mutation is less commonly detected in the field. Under greenhouse conditions, *A. solani* isolates possessing the H134R mutation were not controlled as effectively with any SDHI fungicide evaluated as were the SDH-wild-type isolates. This is not surprising; a previous survey determined that the H134R-mutants are becoming predominate in the field after fluopyram was introduced into early blight foliar fungicide programs (Bauske et al. 2018a). Fluopyram has been shown to bind to a different region within the SDH complex compared with boscalid (Avenot et al. 2014). This suggests that the *A. solani* H134R-mutants may have a substantial negative impact on the efficacy of fluopyram in the field. Nonetheless, it continues to remain clear from the studies reported here and elsewhere (Bauske et al. 2018a, b; Gudmestad et al. 2013; Mallik et al. 2014) that the complex of SDH mutations in *A. solani* have a differential effect on the intrinsic activity of these fungicides and the disease control efficacy they provide. Interestingly, QoI resistance in *A. solani* conveyed by the F129L mutation had a similar effect on in vitro intrinsic activity and fungicide efficacy on that class of fungicides (Pasche et al. 2004, 2005).

The frequency of the D123E mutation has been reportedly increasing in potato field production areas (Bauske et al. 2018a). The D123E mutation was first identified in a Nebraska potato field in 2011 and was determined to possess very high resistance to boscalid and penthiopyrad, while remaining sensitive to fluopyram (Mallik et al. 2014). In that study, approximately 1.5% of collected isolates possessed the D123E mutation (Mallik et al. 2014). A recent study determined that *A. solani* isolates with the D123E mutation possessed a high level of resistance to boscalid and possessed a low level of reduced-sensitivity to fluopyram (Bauske et al. 2018b). In that study, it was determined that in response to fluopyram, percentage disease control was significantly lower in D123E-mutants compared with other SDH mutations such as H278R, H133R, and H134R. A survey of *A. solani* isolates collected across the U.S. potato production areas conducted in 2013–2015 determined the frequency of the D123E mutation in *A. solani* has increased while the frequency of the *AsSdhB* mutations (H278R and H278Y) decreased, perhaps due to the use of fluopyram (Bauske et al. 2018a). It will be interesting to note what, if any, impact more recently introduced SDHI fungicides such as solatenol and adepidyn will have on the frequency of SDH mutations in *A. solani*.

Table 5. Frequency of *Alternaria solani* SDH mutations recovered from each treatment in 2018 from two locations[x]

	SDH mutations						
Location, treatments[y]	**H278R[z]**	**H278Y**	**H134R**	**H133R**	**D123E**	**SDH-Wild-type**	**Unknown[x]**
Larimore, ND							
Nontreated	5.8 a	5.3 a	46.9 bc	0.0 a	5.8 c	8.6 ab	27.7 bcd
Chlorothalonil	0.0 a	0.0 a	85.2 a	0.0 a	5.2 c	1.9 b	7.7 d
Mancozeb	0.0 a	0.0 a	6.3 e	0.0 a	17.6 abc	9.6 ab	63.5 abc
GrwStd/adep+flud	1.7 a	3.8 a	5.8 e	0.0 a	20.2 abc	0.0 b	68.5 ab
Sol-IF	2.1 a	1.9 a	33.7 bcd	0.0 a	34.7 a	2.1 b	27.8 bcd
Sol + BsubL-IF	1.7 a	2.1 a	4.2 e	0.0 a	7.5 c	2.1 b	82.5 a
Sol + BsubH-IF	3.6 a	4.5 a	19.6 de	0.0 a	12.3 c	7.1 ab	57.4 abc
Flu-IF	0.0 a	1.8 a	35.2 bcd	0.0 a	6.9 c	0.0 b	56.1 abc
Flu + BsubH-IF	0.0 a	0.0 a	53.3 b	0.0 a	12.4 bc	21.2 a	13.1 d
GrwStd/flu+pyr	6.0 a	0.0 a	24.2 cde	0.0 a	33.6 ab	13.9 ab	22.4 cd
LSD $_{P = 0.05}$	6.0	5.3	24.4	0.0	21.3	14.5	41.3
Inkster, ND							
Nontreated	1.8 a	9.8 ab	27.7 a	0.0 a	15.7 bc	3.6 a	41.4 ab
Chlorothalonil	1.6 a	1.6 ab	8.7 a	0.0 a	49.2 ab	21.4 a	17.5 b
Mancozeb	0.0 a	0.0 b	10.4 a	0.0 a	56.3 a	16.7 a	16.7 b
GrwStd/adep+flud	0.0 a	11.1 ab	38.3 a	0.0 a	8.3 c	6.1 a	36.1 ab
Sol-IF	4.2 a	13.2 a	44.6 a	0.0 a	10.4 bc	15.8 a	18.1 b
Sol + BsubL-IF	0.0 a	0.0 b	18.5 a	2.3 a	7.8 c	1.9 a	69.5 a
Sol + BsubH-IF	0.0 a	5.0 ab	21.8 a	0.0 a	12.5 bc	20.0 a	40.7 ab
Flu-IF	0.0 a	0.0 b	24.2 a	0.0 a	0.0 c	14.3 a	61.5 ab
Flu + BsubH-IF	0.0 a	0.0 b	36.7 a	0.0 a	0.0 c	8.7 a	54.6 ab
GrwStd/flu+pyr	0.0 a	0.0 b	30.8 a	0.0 a	36.5 abc	0.0 a	32.7 ab
LSD $_{P = 0.05}$	4.2	13.1	38.8	2.3	38.9	28.6	48.6

[x] Isolates could not be confidently characterized and are currently undergoing further examination. SDH-mutant frequency field data were unbalanced and therefore required $\log_{10}$ transformation prior to analysis. Back-transformed data are presented.

[y] Treatments included a nontreated control; chlorothalonil; mancozeb; separate applications of QoI, a new foliar SDHI and *PP* mix, and DMI fungicide each mixed with standard protectants (GrwStd/adep+flud); solatenol in-furrow (Sol-IF); solatenol in furrow with 4.7 liters/ha and 9.4 liters/ha of *Bacillus subtilis* (Sol+BsubL-IF and Sol+BsubH-IF, respectively); fluopyram in-furrow (Flu-IF); fluopyram in-furrow with 9.4 liters/ha of *B. subtilis* (Flu+BsubH-IF); and separate applications of QoI, SDHI-fluopyram mixed with and anilinopyrimidine-pyrimethanil, each mixed with standard protectants (GrwStd/flu+pyr).

[z] Numbers followed by different lowercase letters within columns are significantly different according to Fisher's protected least significant difference test at $\alpha = 0.05$.

Previous studies have suggested that the use of fluopyram in potato, both as an in-furrow, at planting application and as a foliar fungicide, resulted in the increased frequency of the D123E mutation in *A. solani* (Bauske et al. 2018b) In field experiments reported here, *A. solani* isolates with the D123E mutation were detected in all treatments at both locations except in the two fluopyram in-furrow treatments at the Inkster location, which is in contrast to previous studies (Bauske et al. 2018b). In the previous study, the frequency of the D123E-mutant detection was significantly higher in treatments where fluopyram was applied in-furrow at planting, suggesting this fungicide was driving the increase in frequency of this mutation in *A. solani* (Bauske et al. 2018b). In the current studies, the D123E mutation was detected in *A. solani* isolates in much higher frequencies than in the previous study regardless of the fungicide chemistry and frequently in the absence of an SDHI fungicide. For example, at the Inkster location, the highest detection of the D123E mutation was in isolates recovered from treatments with chlorothalonil or mancozeb applied full-season without any other fungicide chemistry. Furthermore, a previous study demonstrated that in-furrow, at planting applications of *B. subtilis* with fluopyram significantly reduced the frequency of the D123E mutation in *A. solani* compared with fluopyram applied alone (Bauske et al. 2018b). In contrast in the current study, when *B. subtilis* was applied in-furrow with either solatenol or fluopyram, no consistent reduction in the frequency of the D123E mutation was observed. The application of *B. subtilis* with solatenol did significantly reduce the frequency of the D123E mutation in *A. solani* isolates recovered compared with solatenol applied alone, but only at the Larimore location. No such reduction in the D123E mutation occurred at the Inkster location. *B. subtilis* applied with fluopyram had no effect on the frequency of the D123E mutation at either location. These results are perhaps reflective of the inconsistencies of biological control products in disease and resistance management or could be attributable to the increasing spatiotemporal variability in the *A. solani* population (Bauske et al. 2018a; Fairchild et al. 2013; Mallik et al. 2014). The variability in the early blight pathogen is clearly illustrated in the studies presented here by the relatively high frequency of isolates of the fungus that could not be characterized using the PCR methods employed here. These isolates did not have any known SDH mutation but were also not wild-types. Pathogen variability is also highlighted by the differences that existed in fungicide efficacy and frequency of mutations in the two field trials conducted in the same year and in locations only about 40 km apart. It is also interesting to note that some of the highest frequencies of "unknown" isolates with putative SDHI mutations were found in treatments in which *B. subtilis* was applied in furrow at planting. Nonetheless, results of the current study do not support the data generated previously that suggested fluopyram played a substantial role in the increased frequency of the D123E mutation and that *B. subtilis* can be used to effectively manage the frequency of this mutation in *A. solani* (Bauske et al. 2018b).

Perhaps the most interesting observation made during the field studies was that the H134R-mutation was detected at a higher frequency than the other SDH mutations at both locations across most fungicide treatments. Interestingly, the H134R-mutants were detected at a higher frequency in fluopyram in-furrow treatments while previous research determined that the application of in-furrow fluopyram increased the frequency of *A. solani* isolates possessing the D123E mutation (Bauske et al. 2018b). However, as previously discussed, in the current study it was observed that fluopyram applied in-furrow had little or no impact on the frequency of *A. solani* D123E-mutants recovered from those treatments. Since solatenol and adepidyn fungicides were included in the current studies but not in the previous studies, it begs the question whether or not these two relatively new fungicides may exert additional pressures on the *A. solani* population. We hypothesize that solatenol and adepidyn may cause additional shifts in the frequency of SDH mutations. To support this hypothesis, it should be noted that a substantial proportion of the *A. solani* isolates recovered from all fungicide treatments in the field studies could not be characterized using the PCR methods employed here. A large number of these isolates have been sequenced and preliminary evidence suggests that there may be additional mutations present in *A. solani* SDH genes that have not been previously characterized. Results from the studies reported here continue to support the need for *A. solani* population monitoring as it appears to still be very fluid and continue to develop across the United States as SDHI chemistries continue to be developed and introduced into the potato industry.

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