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Aggressiveness of Small-Spored *Alternaria* spp. and Their Sensitivity to Succinate Dehydrogenase Inhibitor Fungicides

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Abstract

Brown leaf spot of potato is caused by a number of small-spored *Alternaria* spp. *Alternaria alternata sensu stricto*, *A. arborescens*, and *A. tenuissima* have been reported with increasing frequency in commercial potato fields. Potato cultivars with resistance to small-spored *Alternaria* spp. have yet to be developed; therefore, the application of foliar fungicides is a primary management strategy. Greenhouse inoculation assays demonstrated that isolates of these three small-spored *Alternaria* spp. were pathogenic. Significant differences in aggressiveness were observed across isolates; however, there was no trend in aggressiveness based on species. Significant fungicide by isolate interactions in in vitro fungicide sensitivity and significant differences between baseline and nonbaseline isolates were observed in all three small-spored *Alternaria* spp. The ranges of in vitro sensitivity of *A. alternata* baseline isolates to boscalid (EC_{50} <0.010 to 0.89 µg/ml), fluopyram (<0.010 to 1.14 µg/ml) and solatenol (<0.010 to 1.14 µg/ml) were relatively wide when compared with adepidyn (<0.010 to 0.023 µg/ml). The baseline sensitivities of *A. arborescens* and *A. tenuissima* isolates to all four fungicides were <0.065 µg/ml. Between 10 and 21% of nonbaseline *A. alternata* isolates fell outside the baseline range established for the four succinate dehydrogenase inhibitor (SDHI) fungicides evaluated. In *A. arborescens*, 10 to 80% of nonbaseline isolates had higher sensitivities than the baseline. *A. tenuissima* isolates fell outside the baseline for boscalid (55%), fluopyram (14%), and solatenol (14%), but none fell outside the baseline range for adepidyn. Evaluations of in vivo fungicide efficacy demonstrated that most isolates were equally controlled by the four SDHI fungicides. However, reduced boscalid efficacy was observed for four isolates (two each of *A. arborescens* and *A. tenuissima*) and reduced fluopyram control was observed in one *A. alternata* isolate. Results of these studies demonstrate that isolates of all three species could be contributing to the brown leaf spot pathogen complex and that monitoring both species diversity and fungicide sensitivity could be advantageous for the management of brown leaf spot in potatoes with SDHI fungicides.

Keywords: *Alternaria alternata*, *Alternaria arborescens*, *Alternaria tenuissima*, fungicide resistance, potato, SDHI, *Solanum tuberosum*

Brown leaf spot causes small lesions (pinpoint to 3 mm in diameter) on the foliage of potatoes (*Solanum tuberosum* L.) and has been reported to cause tuber yield losses as high as 18% if conditions are favorable (Droby et al. 1984). Brown leaf spot has been considered a minor disease of potato when compared with early blight, caused by *Alternaria solani* Sorauer; however, symptomatology among these diseases overlap and it is not uncommon for the two diseases to coexist. Because of these factors, brown spot and its causal pathogens have not been studied to the degree that early blight/*A. solani* have been studied. Brown leaf spot caused by small-spored *Alternaria* spp. has been reported with increasing frequency in commercial potato fields (Ding et al. 2019; Fairchild et al. 2013; Tymon et al. 2016b). While historically, *A. alternata sensu stricto* (Fr.) Keissl. had been thought to be the primary causal pathogen of brown spot, *A. alternata sensu lato*, *A. arborescens* E.G. Simmons, *A. arbusti* E.G. Simmons and *A. tenuissima* (Kunze) Wiltshire were associated with potato leaf blight foliage in the Pacific Northwest between 2009 and 2011 (Tymon et al. 2016a, b). These *Alternaria* spp. are nearly indistinguishable morphologically because of overlapping characteristics and plasticity caused by environmental changes but can be differentiated using molecular techniques (Tymon et al. 2016b). Accurate identification of plant pathogens is crucial in the development of accurate disease management strategies including foliar fungicides and the development of resistant cultivars (Tymon et al. 2016b).

In the absence of commercial potato cultivars resistant to early blight or brown leaf spot, the primary management approach is the application of foliar fungicides. Under high disease pressure, applications may occur weekly for as many as 10 weeks from just prior to row closure to senescence. Single-site fungicides such as succinate dehydrogenase inhibitors (SDHIs) are commonly used for early blight management in commercial potato production (Tymon and Johnson 2014). Single-site fungicides are generally considered to be high-risk for resistance development as resistance is based on a single target-site mutation. While *A. alternata* and *A. solani* have been classified as high and medium risk for fungicide resistance, respectively, *A. arborescens* and *A. tenuissima* have not been classified (FRAC 2019). High levels of boscalid resistance were reported in *A. alternata* isolates collected from California pistachios in 2005, just two years after the registration of this fungicide (Avenot and Michailides 2007), and the frequency of resistance increased over time (Avenot and Michailides 2020). Sensitive isolates were most commonly detected from orchards with no history of boscalid use; however, low numbers of resistant isolates were found, indicating that these likely blew in from orchards where this fungicide had been applied. Eleven-point mutations resulting in reduced sensitivity to boscalid were characterized across three subunits in the *Sdh* gene of *A. alternata* isolates collected from pistachio (Avenot et al. 2008, 2009; Avenot and Michailides 2020). In this population, seven mutations were identified in the *AaSdhB* gene, a single mutation was identified in the *AaSdhC* gene and three mutations were identified in *AaSdhD* (Avenot et al. 2008). *A. alternata* isolates collected from peach orchards in South Carolina were determined to be resistant to boscalid, penthiopyrad, fluopyram, and fluxapyroxad (Yang et al. 2015). Resistance to boscalid also has been reported in *A. solani* and *A. alternata* isolates collected from potato in the Columbia Basin (Tymon and Johnson 2014). Fungicide evaluations have been conducted for *A. arborescens* and *A. tenuissima*, but SDHI fungicides have not been included in these evaluations (Everett and Neilson 1996; Hariprasad et al. 2017; Ma et al. 2003; Rani et al. 2018).

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Monitoring a fungal population is important if fungicide resistance management is to be successfully implemented. Considering factors outlined above, monitoring small-spored *Alternaria* spp. for resistance to SDHI fungicides used for the management of early blight and brown leaf spot in potato production particularly important. Based on the lack of SDHI fungicide resistance monitoring for some small-spored *Alternaria* spp., the objectives of this study were to (i) determine the pathogenicity and aggressiveness of three small-spored *Alternaria* spp. (ii) compare the sensitivity of a small-spored *Alternaria* spp. baseline populations with no exposure to SDHI fungicides to nonbaseline isolates, and (iii) determine the brown spot control provided by SDHI fungicides boscalid, fluopyram, solatenol, and adepidyn.

Materials and Methods

Isolate collection, maintenance, and identification. Isolates of small-spored *Alternaria* spp. were recovered from foliage submitted from 12 potato growing states across the United States. For fungicide evaluation purposes, *Alternaria* spp. isolates collected from 1999 to 2004 with no exposure to SDHI fungicides were considered baseline, and isolates with potential SDHI exposure collected from 2011, 2013, 2015, and 2017 were considered nonbaseline. The recovery and isolation of *Alternaria* spp. from foliar tissue into pure culture were similar to that previously described for *A. solani* (Fonseka and Gudmestad 2016; Gudmestad et al. 2013; Pasche et al. 2004, 2005). Foliar sections with lesions characteristic of brown spot were surface sterilized in a 10% sodium hypochlorite solution for 1 min and rinsed in sterile, distilled water. Leaf tissue sections were aseptically excised 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 plate using a sterile glass needle to a clarified V8 (CV-8) medium (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water, 900 ml) amended with 50 mg/ml ampicillin (Sigma-Aldrich; St. Louis, MO). Isolates were incubated under 24-h fluorescent light at room temperature (22 ± 2°C) for 7 days and examined for the presence of *Alternaria* spp. to preserve isolates in long-term cryogenic storage, a 4-mm diameter sterilized cork borer was used to excise sections of media with fungal conidia and mycelia, and placed into 2-ml screw-top centrifuge tubes. The caps were loosely fastened on to 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 also prepared and stored at North Dakota State University for each tissue sample from which isolates of *Alternaria* spp. were obtained. The identity of the small-spored *Alternaria* spp. isolates evaluated in this research was confirmed as *A. alternata*, *A. arborescens*, or *A. tenuissima* via sequencing unique regions in OPA 1-3 (Budde-Rodriguez et al. 2022).

In vivo pathogenicity and aggressiveness of small-spored *Alternaria* spp. Eighteen small-spored *Alternaria* spp. isolates, six of each species including 17 nonbaseline isolates and 1 baseline, were evaluated under greenhouse conditions to determine pathogenicity and estimate aggressiveness following previously described methods for *A. solani* (Pasche et al. 2004, 2005; Pasche and Gudmestad 2008; Table 1). The isolates chosen represent a spatio-temporal distribution from six states (CO, ID, IN, NE, NM, TX). Tomato cultivar Orange Pixie (Tomato Growers Supply Company, Fort Myers, FL) was chosen because of its susceptibility to leaf spot diseases, its compact size compared with potato plants, and the resistance of leaves to dehisce when severely infected. Three tomato seeds were sown in 10 cm^3 plastic pots containing Sunshine Mix LC1 (Sun Gro Horticulture, Bellevue, WA). After emergence, plants were thinned to acquire two uniformly sized plants per pot. A 50-ml suspension of 2.0×10^5 conidia/ml was prepared from 10- to 12-day-old cultures of each small-spored *Alternaria* isolate grown on CV-8 medium under 24-h fluorescent light at 22 ± 2°C (Gudmestad et al. 2013; Pasche et al. 2004, 2005). When the first true leaves were fully expanded, or nearly so, the conidial suspension was applied on plants using a Preval paint-spray gun (Preval Sprayer Division, Prevision Valve Corporation, Yonkers, NY), and inoculated plants were placed in individual humidity chambers (Phytotronic; 1626D) set at >95% RH at 22 ± 2°C. After 24 h, plants were transferred to confinement chambers (plastic chambers with an open ceiling) on greenhouse benches to avoid any cross-contamination. The greenhouse temperature was maintained at 25 ± 2°C, and plants were watered daily. Average percentage brown leaf spot severity was evaluated at 6, 9, and 12 days post-inoculation by estimating the percentage of infected leaf area on the first three true leaves for each isolate using the previously described methods (Gudmestad et al. 2013; Pasche et al. 2004, 2005). The pathogenicity and aggressiveness experiment was performed twice with two samples (two plants per pot) and three replicates (three pots) for each isolate.

In vitro sensitivity of small-spored *Alternaria* spp. to SDHI fungicides. In vitro sensitivity of small-spored *Alternaria* baseline and nonbaseline isolates was determined using a conidial germination

Table 1. In vivo fungicide efficacy of four SDHI fungicides (adepidyn, boscalid, fluopyram, and solatenol) tested on 18 small-spored *Alternaria* spp. isolates based on the area under the dose-response curve (AUDRC)[z]

				Fungicide			
Species	**Isolate**	**Year**	**Location**	**Adepidyn**	**Boscalid**	**Fluopyram**	**Solatenol**
Alternaria alternata	125-1	99	NM	9,978.5 a	9,688.0 ab	9,938.5 a	9,906.4 a
	1714-3	17	TX	9,981.7 a	9,853.0 a	9,987.7 a	9,929.7 a
	1715-7	17	TX	9,887.1 a	9,489.5 abc	9,878.8 a	9,768.4 a
	1716-1	17	TX	9,988.8 a	9,781.2 a	8,680.8 d	9,958.8 a
	Aa3-1	15	IN	9,963.7 a	9,431.2 abc	9,784.4 a	9,967.1 a
	Aa7-1	15	IN	9,982.5 a	9,371.5 abc	9,985.7 a	9,990.0 a
A. arborescens	1294-3	13	NM	9,976.3 a	9,080.9 bcd	9,690.9 ab	9,978.5 a
	1298-2	13	NM	9,975.9 a	9,504.4 abc	9,969.7 a	9,918.1 a
	1713-1	17	CO	9,848.3 a	9,623.0 abc	9,961.2 a	9,973.4 a
	1713-3	17	CO	9,927.0 a	9,799.8 a	9,853.1 a	9,475.2 abc
	1713-6	17	CO	9,986.9 a	8,718.1 d	9,860.4 a	9,982.6 a
	Ar1-1	15	IN	9,968.0 a	9,924.3 a	9,987.0 a	9,975.4 a
A. tenuissima	1317-9	13	NE	9,990.0 a	9,882.7 a	9,976.0 a	9,485.2 abc
	1702-5	17	CO	9,869.8 a	9,835.9 a	9,877.5 a	9,957.8 a
	1714-1	17	TX	9,962.5 a	9,747.1 a	9,959.1 a	9,621.4 abc
	At13-1	15	IN	9,900.6 a	9,972.9 a	9,912.1 a	9,803.7 a
	At8-2	15	IN	9,985.4 a	9,055.1 cd	9,832.6 a	9,916.7 a
	At9-2	15	ID	9,984.2 a	7,009.9 e	9,566.3 abc	9,986.9 a

[z] Across fungicides and isolates, least square means (lsmeans) with the same letters are not significantly different ($\alpha = 0.05$).

assay as previously described (Gudmestad et al. 2013; Pasche et al. 2004, 2005). Twenty *A. alternata*, 7 *A. arborescens*, and 10 *A. tenuissima* baseline isolates collected from 1999 to 2004 with no exposure to SDHI fungicides, and 29 *A. alternata*, 10 *A. arborescens*, and 22 *A. tenuissima* isolates collected from 2011, 2013, 2015, and 2017 were evaluated to determine the in vitro sensitivity to SDHI fungicides. The 98 small-spored *Alternaria* isolates were assayed in 15 trials, with five to eight isolates included in each trial. Trials contained two replications arranged in a split-plot with fungicide as the main effect and isolate as the split plot. Each trial was repeated. Internal control isolates (125-1, an *A. alternata* QoI baseline wild-type isolate, and 1702-5, an *A. tenuissima* QoI reduced-sensitive isolate) were used in each trial to determine assay reproducibility (Wong and Wilcox 2002).

Evaluation of conidial germination and calculation of EC_{50} values was carried out as previously described (Gudmestad et al. 2013; Pasche et al. 2004, 2005). Media containing 2% laboratory-grade agar was amended with technical formulation of 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) dissolved in acetone to reach final concentrations of 0.01, 0.1, 1, 10, and 100 µg/ml. The acetone concentration in all media was 0.1% by volume, including the no-fungicide control. Salicylhydroxamic acid (SHAM) (Sigma Chemical Co., St. Louis, MO) was added at 100 µg/ml to amended and no-fungicide control media to prevent the *Alternaria* spp. from overcoming the activity of the SDHI fungicides through any potential alternative oxidative pathway (Gudmestad et al. 2013; Mallik et al. 2014; Pasche et al. 2004, 2005).

Brown leaf spot control provided by SDHI fungicides. In vivo fungicide efficacy assays were conducted as a 24-h preventative test arranged as split-plot randomized complete block design with small-spored *Alternaria* isolate as the whole plot and fungicide as the split-plot (Gudmestad et al. 2013; Pasche et al. 2004, 2005). The same 18 isolates evaluated for pathogenicity and aggressiveness were included in fungicide efficacy assays (Table 1). When the plants reached a height of 15 to 20 cm and the first three leaves were fully expanded, they were treated with a commercial formulation 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 of active ingredient were applied to the plants to obtain a dose–response curve. The fungicides were applied using a Generation II Research Sprayer (Devries Manufacturing, Hollandale, MN) at approximately 400 kPa. Tomato plants were inoculated individually with 18 small-spored *Alternaria* isolates and average percentage brown spot disease severity was evaluated as described above for pathogenicity and aggressiveness experiments. The fungicide efficacy experiment was performed twice with two samples (two plants per pot) and three replicates (three pots) for each isolate.

Statistical analyses. To estimate aggressiveness of small-spored *Alternaria* isolates in greenhouse experiments, the area under the disease progress curve (AUDPC) was calculated from disease severity ratings taken at 6, 9, and 12 days postinoculation using percentage brown spot severity on tomato plants with no fungicides applied (Shaner and Finney 1977):

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

where W_i represents 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. AUDPC was divided by the total graph area to determine the relative area under the disease progress curve (rAUDPC). An analysis of variance (ANOVA) was conducted using proc glimmix in SAS version 9.4 (SAS Institute, Cary, NC) where isolates were considered as fixed effects and trials and their interaction with isolates were random effects. The comparison of the treatment levels in the aggressiveness test were conducted on least square means (lsmeans) using the Student's t test ($\alpha = 0.05$).

To quantify in vitro fungicide sensitivity, the effective concentration determined to reduce germination by half compared with the 0 µg/ml concentration was deduced from the 50% intercept (EC_{50} value) using SAS (Pasche et al. 2004). EC_{50} values of <0.01 and >100 were analyzed as 0.01 and 100 µg/ml, respectively. 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. Levene's test of homogeneity of variances ($P \leq 0.05$) was conducted to verify that trials could be combined (Milliken and Johnson 1992). ANOVA was conducted with fungicide and isolate as fixed effects within baseline and nonbaseline populations and for each species (total of six groups). Replicates, trials, and their interaction with the treatments were treated as random effects. Mean comparison was conducted on lsmeans using the Student's t test ($\alpha = 0.05$). Pearson correlation test was used to evaluate the association between pairs of fungicides using the EC_{50} values; the analysis was conducted for each species in the baseline and nonbaseline groups. A resistance factor (Rf) was calculated for each fungicide and within species by dividing the mean EC_{50} value of the nonbaseline isolates by the mean EC_{50} value of the baseline isolates.

To determine the efficacy of the four SDHI fungicides, disease severity data from plants treated with each fungicide concentration rated 12 days postinoculation 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, 2005). Area under the dose–response curve (AUDRC) was calculated to determine significant differences in brown spot disease control provided by boscalid, fluopyram, solatenol, and adepidyn:

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

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. AUDRC is calculated across doses of the fungicide evaluated as opposed to AUDPC, which is calculated across time. Interpretations of the AUDRC data are inverse of that for AUDPC. In the traditional use of AUDPC, a high value would indicate that disease development was greater when compared with a lower AUDPC value. In contrast, a high AUDRC value indicates that a fungicide provided a higher degree of control of a fungal pathogen over the tested range of fungicide concentrations compared with a lower AUDRC. For the fungicide efficacy study, isolates and fungicides were treated as fixed effects in the ANOVA while replicates, trials, and their interaction with the treatments were random effects. The comparison of treatment interactions was conducted on lsmeans using Student's t test ($\alpha = 0.05$).

Results

Pathogenicity and aggressiveness of small-spored *Alternaria* spp. All 18 isolates of the three small-spored *Alternaria* spp. were pathogenic on tomato cv. Orange pixie, causing between 11 and 51% disease severity at 12 days postinoculation. Significant differences in aggressiveness, as represented by rAUDPC were observed across isolates of the three species (Fig. 1; $P < 0.0001$). A single *A. alternata* isolate was significantly more aggressive than all other isolates; however, there was no trend in differences in aggressiveness across isolates of all three species. Two general groups, more aggressive (6 isolates) and less aggressive (12 isolates), were observed with isolates from all three species represented in each aggressiveness group.

In vitro SDHI fungicide sensitivity. Independent analysis of variance of in vitro fungicide sensitivity experiments for boscalid, fluopyram, solatenol, and adepidyn determined that variances were homogenous ($P = 0.7409$), and the experiments were combined for further analysis. A significant fungicide × isolate interaction was observed in both baseline and nonbaseline populations ($P < 0.0001$).

EC_{50} values of 20 baseline *A. alternata* isolates for sensitivity to boscalid ranged from 0.01 to 0.89 µg/ml (Fig. 2A; Supplementary Table S1A). Isolates 857-3 (EC_{50} = 0.14 µg/ml), 912-1 (0.51 µg/ml), and 858-1 (0.89 µg/ml) were significantly less sensitive to boscalid than all other *A. alternata* isolates and significant differences in sensitivity were detected among these three isolates. Baseline sensitivity to fluopyram and solatenol both ranged from 0.010 to 1.14 µg/ml. A single isolate, 912-1 (1.14 µg/ml), was less sensitive to both of these chemistries when compared with other isolates. Sensitivity to adepidyn ranged from 0.010 to 0.023 µg/ml and no significant difference in sensitivity was observed among isolates. Significant differences across fungicides were observed with three *A. alternata* isolates (Fig. 2A; Supplementary Table S1A). Isolates 857-3 and 858-1 were less sensitive to boscalid than the other three fungicides. Isolate 912-1 was significantly less sensitive to fluopyram and solatenol when compared with boscalid and adepidyn. Sensitivity to boscalid was significantly lower than that of adepidyn.

The in vitro sensitivity of baseline *A. arborescens* isolates to boscalid ranged from 0.010 to 0.062 µg/ml (Fig. 2B; Supplementary Table S1B). Three isolates, 527-1A (0.033 µg/ml), 342-3 (0.038 µg/ml), and 314 (0.062 µg/ml), were significantly less sensitive than the four other isolates and significant differences were observed among these three isolates. Sensitivity to fluopyram ranged from 0.010 to 0.035 µg/ml, and solatenol sensitivity ranged from 0.010 to 0.062 µg/ml. The same three isolates displaying lower sensitivity to boscalid were also less sensitive to fluopyram (527-1A = 0.016 µg/ml; 314 = 0.027 µg/ml; 342-3 = 0.035 µg/ml), and solatenol (527-1A = 0.050 µg/ml; 314 = 0.031 µg/ml; 342-3 = 0.062 µg/ml). The sensitivity of all the *A. arborescens* isolates to adepidyn was 0.010 µg/ml. Significant differences in sensitivity across fungicides were observed in the same three *A. arborescens* isolates (Fig. 2B; Supplementary Table S1B). Isolate 314 was significantly less sensitive to boscalid, while 342-3 and 527-1A were significantly less sensitive to solatenol when compared with the other three fungicides. All three isolates were significantly more sensitive to adepidyn than the other three fungicides.

The in vitro sensitivity of baseline *A. tenuissima* isolates to boscalid ranged from 0.010 to 0.059 µg/ml (Fig. 2C; Supplementary Table S1C). Three isolates, 128-1 (0.054 µg/ml), 178-2 (0.056 µg/ml), and 154-1 (0.059 µg/ml), were significantly less sensitive to boscalid than the other seven isolates. Sensitivity to fluopyram ranged from 0.010 to 0.052 µg/ml and isolate 122-3 (0.052 µg/ml) was significantly less sensitive to this fungicide than other isolates. Sensitivity to solatenol ranged from 0.010 to 0.026 µg/ml, and isolate 123-6 (0.026 µg/ml) was significantly less sensitive to this fungicide. Sensitivity to adepidyn ranged from 0.010 to 0.025 µg/ml. Significant differences in sensitivity across fungicides were observed with six *A. tenuissima* isolates (Fig. 2C; Supplementary Table S1C). Four isolates, 128-1, 154-1, 178-2, and 302-1, were significantly less sensitive to boscalid compared with other fungicides. Isolate 122-3 was least sensitive to fluopyram and 123-6 was least sensitive to solatenol.

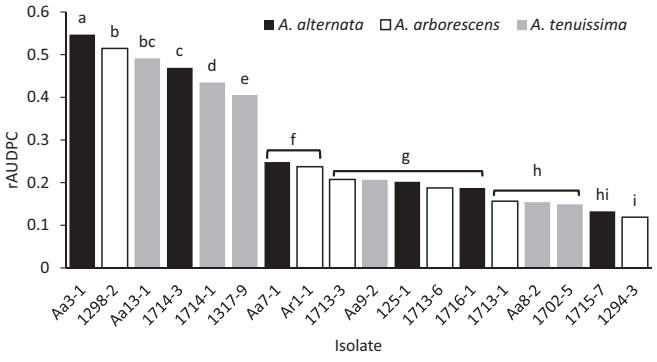

Fig. 1. Aggressiveness of 18 isolates of small-spored *Alternaria* spp. based on relative area under the disease progress curve (rAUDPC) evaluated on tomato plants under greenhouse conditions. Across isolates and species, least square means (lsmeans) with the same letters are not significantly different based on the Student's *t* test (α = 0.05).

The in vitro sensitivity of 29 *A. alternata* nonbaseline isolates to boscalid ranged from 0.01 to 3.85 µg/ml (Fig. 3A; Supplementary Table S1D). The sensitivity of two isolates, 1714-3 (2.20 µg/ml) and 1716-1 (3.85 µg/ml), was significantly lower than all other isolates and sensitivity to boscalid was significantly different between these two isolates. Sensitivity to fluopyram ranged from 0.01 to 3.83 µg/ml. Three *A. alternata* isolates, 1714-3 (2.11 µg/ml), 1708-16 (2.19 µg/ml), and 1716-1 (3.83 µg/ml), were significantly less sensitive to fluopyram than all other isolates. Isolate 1716-1 was significantly less sensitive when compared with the other two isolates. Solatenol sensitivity ranged from 0.01 to 1.87 µg/ml. Ten nonbaseline *A. alternata* isolates were significantly less sensitive (EC_{50} = 0.96 to 1.87 µg/ml) to solatenol when compared with the remaining 19 isolates. Sensitivity to adepidyn ranged from 0.01 to 0.04 µg/ml, and no significant differences were observed across isolates. Significant differences in sensitivity were observed across fungicides in 21 of 29 nonbaseline *A. alternata* isolates (Fig. 3A; Supplementary Table S1D). In nearly half, nine isolates, sensitivity to solatenol was significantly lower when compared with the other three fungicides. Boscalid sensitivity was significantly lower than all other fungicides in two isolates, and lower than solatenol and adepidyn in an additional two isolates. Two isolates were significantly less sensitive to fluopyram when compared with other fungicides. Sensitivity to adepidyn was significantly higher than sensitivity to at least one other fungicide across these 21 nonbaseline *A. alternata* isolates. In vitro sensitivity of nonbaseline *A. alternata* isolates fell outside the baseline range for boscalid (21%) fluopyram (10%), solatenol (24%), and adepidyn (17%) (Figs. 2A and 3A). Sensitivity shifts between the mean EC_{50} values of the baseline and nonbaseline *A. alternata* isolates (Rf) were observed for boscalid (Rf = 5.6), fluopyram (Rf = 6.4), and solatenol (Rf = 8.4). There was no sensitivity shift observed in *A. alternata* isolates to adepidyn (Rf = 0.96).

The in vitro sensitivity of the 10 nonbaseline *A. arborescens* isolates to boscalid ranged from 0.01 to 3.80 µg/ml (Fig. 3B; Supplementary Table S1E). Isolate 1298-2 (3.80 µg/ml) was significantly less sensitive to boscalid than any other isolate. Sensitivity to fluopyram ranged from 0.01 to 2.03 µg/ml. Two *A. arborescens* isolates, 1253-1 (1.76 µg/ml) and 1294-3 (2.03 µg/ml), were significantly less sensitive to fluopyram when compared with the other eight isolates. Sensitivity to solatenol ranged from 0.01 to 2.19 µg/ml. Three isolates, 1298-2 (0.86 µg/ml), 1253-1 (1.82 µg/ml), and 1294-3 (2.19 µg/ml), were significantly less sensitive to solatenol, and sensitivity among these isolates differed. Sensitivity to adepidyn ranged from 0.010 to 0.137 µg/ml with no significant differences in sensitivity observed among the *A. arborescens* nonbaseline isolates evaluated. Sensitivity differed significantly among fungicides in 8 of 10 isolates. Four isolates were less sensitive to fluopyram and solatenol when compared with boscalid and adepidyn. Two isolates, 1298-2 and 1713-7, were significantly less sensitive to boscalid than to the other three fungicides evaluated. Sensitivity to adepidyn was generally higher than to the other fungicides across the 10 nonbaseline *A. arborescens* isolates, significantly so in two isolates. Sensitivity of nonbaseline *A. arborescens* isolates fell outside of the baseline range for boscalid (80%), fluopyram (70%), solatenol (70%), and adepidyn (10%) (Figs. 2B and 3B). Sensitivity shifts were observed for *A. arborescens* isolates for boscalid (Rf = 28.3), fluopyram (Rf = 32.4), solatenol (Rf = 24.9), and adepidyn (Rf = 2.6).

The in vitro sensitivity of 22 nonbaseline *A. tenuissima* isolates to boscalid ranged from 0.010 to 2.66 µg/ml (Fig. 3C; Supplementary Table S1F). Three isolates, 1701-3 (2.66 µg/ml), At8-2 (1.53 µg/ml), and 1701-4 (0.48 µg/ml), were significantly less sensitive to boscalid when compared with the remaining 18, and significant differences were observed among these isolates. Sensitivity to fluopyram ranged from 0.010 to 2.10 µg/ml, and two nonbaseline *A. tenuissima* isolates, 1701-3 (2.10 µg/ml) and 1701-4 (0.60 µg/ml), were significantly less sensitive than all others. Sensitivity to solatenol ranged from 0.010 to 0.027 µg/ml, and sensitivity of all isolates to adepidyn was 0.010 µg/ml. No significant differences in sensitivity to either solatenol or adepidyn were observed among *A. tenuissima* isolates. Sensitivities of 8 of 22 nonbaseline *A. tenuissima* isolates differed across fungicides. Five isolates were significantly less sensitive to boscalid than the other three fungicides, two were significantly less

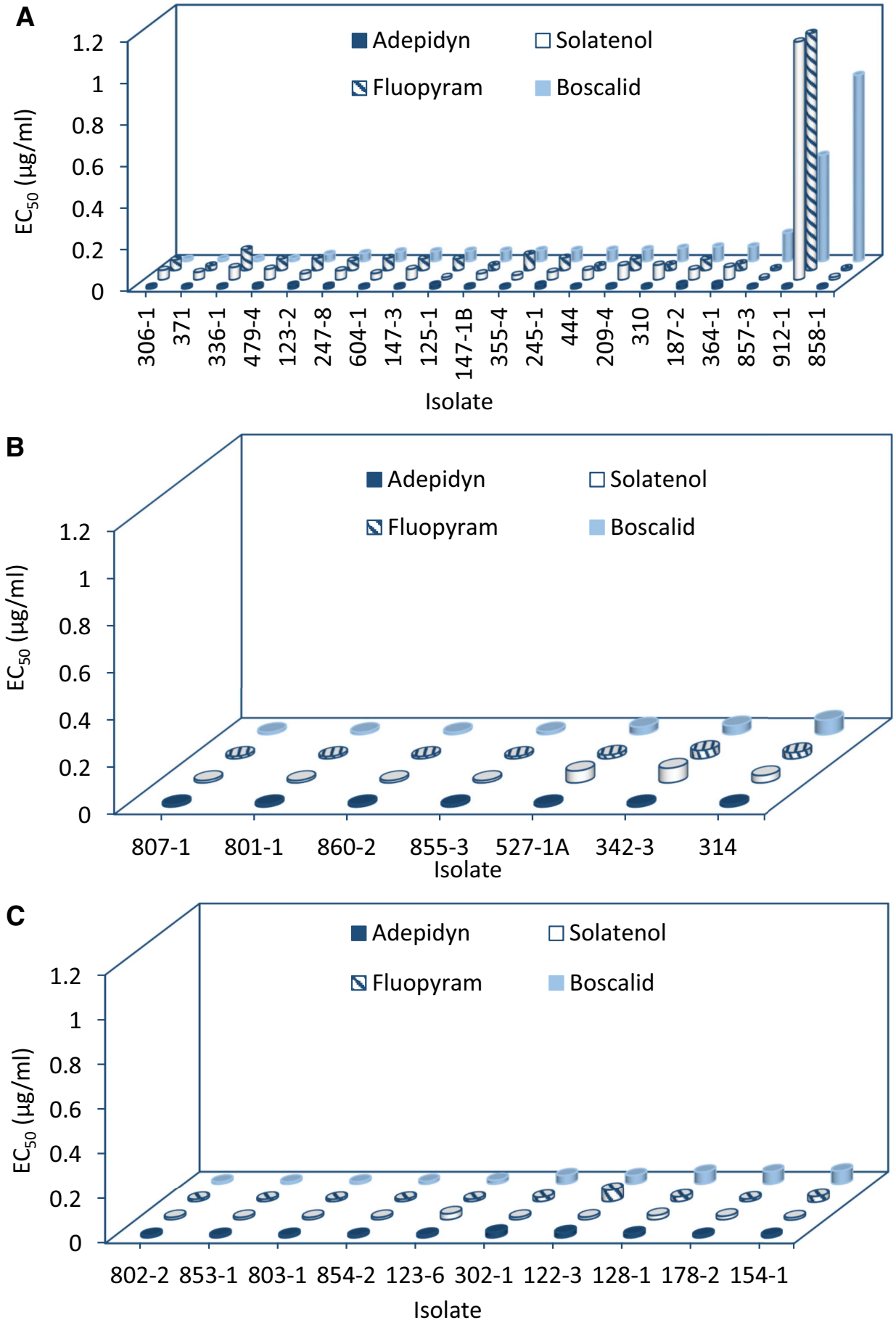

Fig. 2. In vitro fungicide sensitivity of baseline isolates of **A,** *Alternaria alternata* (*n* = 20), **B,** *A. arborescens* (*n* = 7), and **C,** *A. tenuissima* (*n* = 10) to four SDHI fungicides (adepidyn, boscalid, fluopyram, and solatenol) measured as EC_{50} (µg/ml) based on spore germination assays.

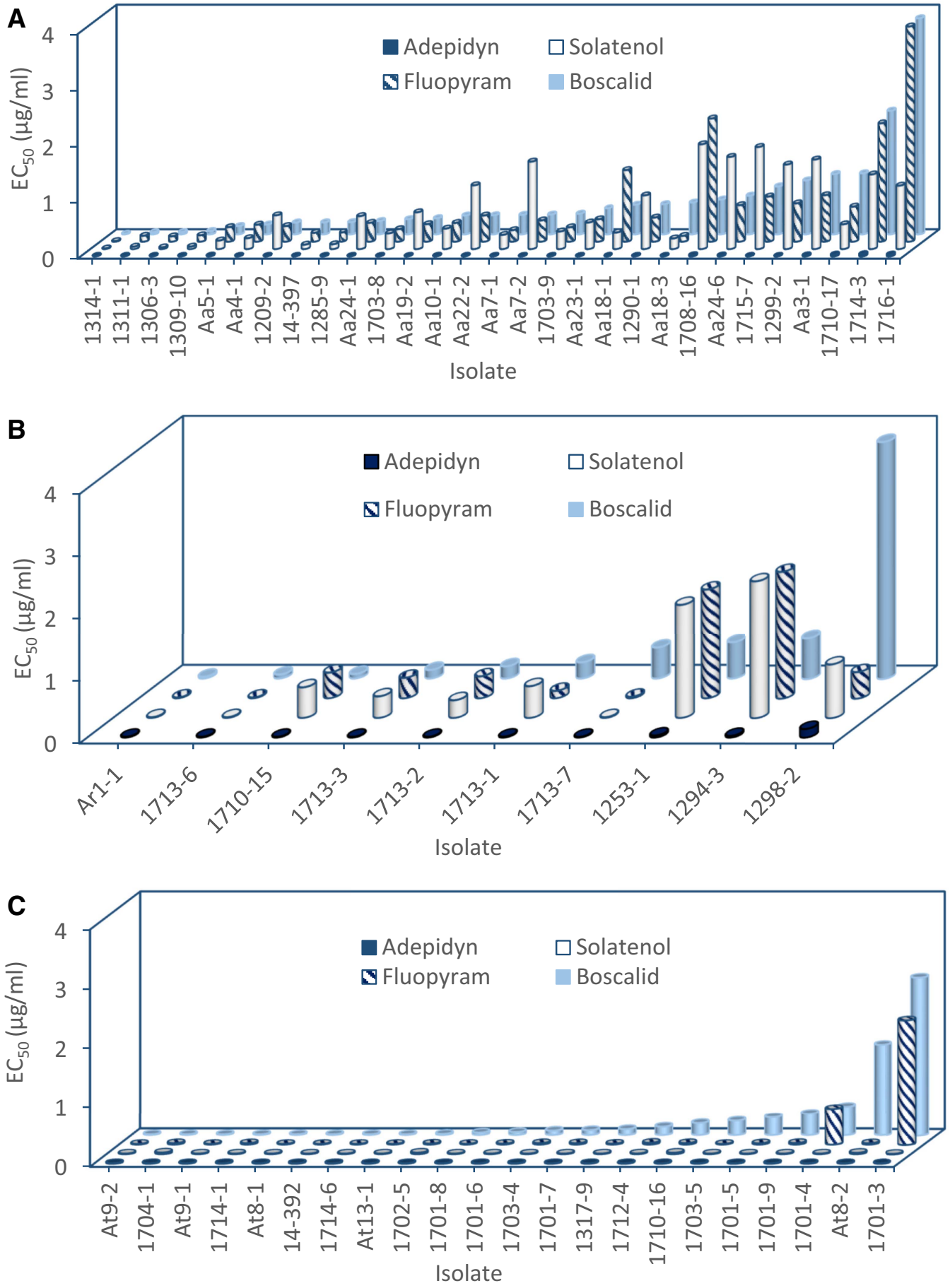

Fig. 3. In vitro fungicide sensitivity of nonbaseline isolates of **A,** *Alternaria alternata* (*n* = 29) **B,** *A. arborescens* (*n* = 10), and **C,** *A. tenuissima* (*n* = 22) to four SDHI fungicides (adepidyn, boscalid, fluopyram, and solatenol) measured as EC_{50} (µg/ml) based on spore germination assays.

sensitive to boscalid than solatenol and adepidyn, and one isolate was significantly less sensitive to fluopyram than the other fungicides. Sensitivity of some nonbaseline *A. tenuissima* isolates fell outside the baseline range for boscalid (55%), fluopyram (14%), and solatenol (14%) (Figs. 2C and 3C). None of the *A. tenuissima* nonbaseline isolates fell outside of the baseline range in sensitivity to adepidyn. Rf for *A. tenuissima* isolates to boscalid and fluopyram were 8.7- and 4.5-fold, respectively. There was no sensitivity shift observed between baseline and nonbaseline isolates for solatenol (Rf = 1.08) or adepidyn (0.74).

No significant correlations were revealed with baseline isolates of all three small-spored *Alternaria* spp. Pearson correlations revealed significant associations between all pairs of tested fungicides for the nonbaseline group of *A. alternata* (Fig. 4) where the strongest correlation was observed between boscalid and fluopyram ($r = 0.79$; $P < 0.0001$; Fig. 4A), adepidyn and boscalid ($r = 0.74$; $P < 0.0001$; Fig. 4B), and adepidyn and fluopyram ($r = 0.70$; $P < 0.0001$; Fig. 4C). Fluopyram ($r = 0.50$; $P < 0.0001$; Fig. 4D) and boscalid ($r = 0.40$; $P < 0.0001$; Fig. 4E) showed moderate to weak associations with solatenol. The weakest, but statistically significant, association was observed between adepidyn and solatenol ($r = 0.33$; $P = 0.0004$; Fig. 4F). In nonbaseline isolates of *A. arborescens*, two strong and statistically significant correlations were observed with boscalid/adepidyn ($r = 0.97$; $P < 0.0001$; Fig. 5A), and solatenol/fluopyram ($r = 0.92$; $P < 0.0001$; Fig. 5B). No additional meaningful and strong correlations were observed between the fungicides for nonbaseline *A. arborescens* and *A. tenuissima* isolates.

In vivo SDHI fungicide efficacy of small-spored *Alternaria* spp. Independent analysis of in vivo disease control experiments for boscalid, fluopyram, solatenol, and adepidyn determined that AUDRC variances were homogenous ($P = 0.25$) and a fungicide × isolate interaction was observed ($P < 0.0001$). Mean separations among the 72 treatments (18 isolates, 6 isolates of each species × 4 fungicides) resulted in seven statistical groupings (Table 1). Disease control among 67 treatments was not significantly different. Four of the remaining five isolates controlled at a significantly lower level were treated with boscalid, including *A. arborescens* isolates 1294-3 (AUDRC = 9080.9) and 1713-6 (8718.1), and *A. tenuissima* isolates At8-2 (9055.1) and At9-2 (7009.9). One *A. alternata* isolate 1716.1 (8680.75) treated with fluopyram was also in this group. *A. tenuissima* isolate At9-2 treated with boscalid was controlled at a significantly lower level than all other isolate/fungicide combinations.

Discussion

The importance of brown leaf spot on potato caused by small-spored *Alternaria* spp. has been increasing in the Pacific Northwest and in some areas of the Midwest (Ding et al. 2019; Tymon et al. 2016a, b). Small-spored *Alternaria* spp. in potato fields have been viewed frequently as secondary colonizers, with *A. solani* being the dominate pathogen (Tymon et al. 2016a). In the Pacific Northwest, *A. arborescens* was the most frequently isolated species from potato, and pathogenicity assays demonstrated it was more aggressive than *A. solani* on wounded potato foliage (Tymon et al. 2016a). The results from the studies reported here represent the first endeavor in monitoring sensitivity levels of *A. alternata*, *A. arborescens*, and *A. tenuissima* populations to SDHI fungicides across multiple years and potato production areas.

Results from greenhouse experiments demonstrated significant differences across isolates within the three species in their aggressiveness on nonwounded tomato foliage. Within each small-spored *Alternaria* spp., from one to three isolates were highly aggressive, indicating that each species may play a role in the brown spot disease complex. However, more isolates of each small-spored *Alternaria* spp. need to be evaluated to provide definitive conclusions about the relative importance of each of these species in the complex. A previous study demonstrated that *A. arbusti* and *A. arborescens* were significantly more aggressive on wounded detached potato leaves than on nonwounded leaves (Tymon et al. 2016a). In that study, only two isolates of each species were evaluated and the results were presented as means of these two isolates. Given our results illustrating significant differences across isolates within a small-spored *Alternaria* spp., subsequent studies should investigate additional isolates to determine whether isolates differ in aggressiveness on potato foliage and whether wounded foliage is important epidemiologically.

The response to SDHI fungicides was variable among isolates within each of the three small-spored *Alternaria* spp. evaluated. This demonstrates, perhaps, a biological significance for understanding the pathogen complex for management of brown leaf spot across potato production areas. Although significant differences in in vitro SDHI fungicide sensitivity were observed in baseline populations of all three *Alternaria* spp., variability in the sensitivity of *A. alternata* isolates was much greater than that observed in *A. arborescens* and *A. tenuissima*. It is important to note that two *A. alternata* baseline isolates with high EC_{50} values resulted in a lower frequency of nonbaseline *A. alternata* isolates falling outside the baseline compared with *A. arborescens* and *A. tenuissima* isolates. Decreased sensitivity to more than one fungicide was observed in some isolates; however, the significant isolate by fungicide interactions observed in both baseline and nonbaseline populations indicate that cross-resistance to the SDHI fungicides evaluated is not universal in isolates of these small-spored *Alternaria* spp. This could be because of the *A. alternata* isolates possessing a mutation in the Sdh complex; however, it is unknown whether *A. arborescens* or *A. tenuissima* develop similar mutations (Avenot et al. 2008). Additionally, the mutations in *A. alternata* (Avenot et al. 2008, 2009) are similar to those present in *A. solani*, and previous research has indicated that these fungal mutations, if present, have a differential impact on conveying resistance to the more recently developed SDHI fungicides (Gudmestad et al. 2013; Mallik et al. 2014). Molecular analyses of the small-spored *Alternaria* spp. evaluated during the current research for the presence of mutations conveying resistance in the *Sdh* gene are in progress. Although it has been suggested previously that accurate identification of *Alternaria* spp. is critical for disease management (Ding et al. 2019; Tymon et al. 2016a), the results reported here suggest that differences in SDHI fungicide sensitivity may depend more on specific isolate exposure to each fungicide rather than which species of small-spored *Alternaria* spp. are prevalent. For example, across nonbaseline populations of *A. tenuissima*, four of five isolates with reduced boscalid sensitivity were recovered from the same grower field in 2017. Previous work in California pistachios indicates that the frequency of resistant *A. alternata* isolates increases with increasing fungicide exposure (Avenot and Michailides 2020). More work is needed to understand any spatio-temporal differences that may exist among potato production areas as they could be an important component in constructing a foliar fungicide program that can effectively manage brown leaf spot given the differences that exist in efficacy of SDHI fungicides across isolates of these species.

The high intrinsic activity detected among the small-spored *Alternaria* spp. to SDHI fungicides may not necessarily translate into observed disease control. Because of the small number of small-spored *Alternaria* isolates evaluated, meaningful correlations between the intrinsic activity and the observed disease control were not reached. Previous studies demonstrated that no loss of early blight disease control occurred with two- and threefold sensitivity shifts in response to famoxadone and fenamidone, respectively, in *A. solani* (Pasche et al. 2005). However, it was unclear how small-spored *Alternaria* spp. would interact with the SDHI fungicides under greenhouse conditions. It is apparent from these results that not only are there significant differences among isolates of these small-spored *Alternaria* spp. in their sensitivity to SDHI fungicides, but there are also differences between these fungicides and the degree of disease control provided. It should not be too surprising that there is a loss of sensitivity among the small-spored *Alternaria* spp. to boscalid given the length of time this fungicide been used in the United States. It also should be of no surprise that the three *Alternaria* spp. evaluated in these studies appear to be more sensitive to adepidyn than they are to SDHI fungicides boscalid and fluopyram. Nonbaseline isolates of the small-spored *Alternaria* spp. used in this study have been exposed to SDHI fungicides boscalid and fluopyram for some time, but they have not

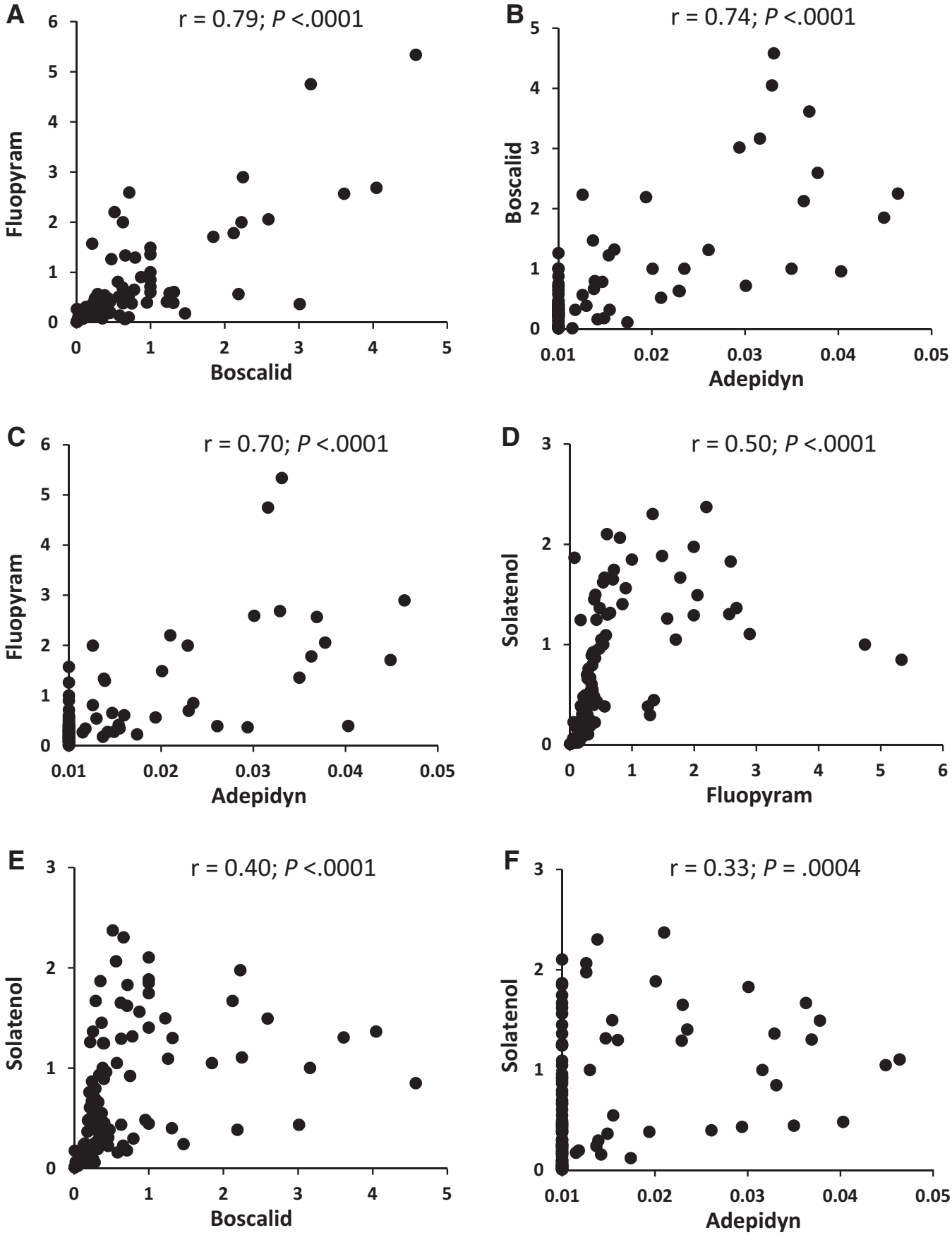

Fig. 4. Significant Pearson correlations between EC_{50} (µg/ml) of all pairs of tested SDHI fungicides (**A,** fluopyram/boscalid; **B,** boscalid/adepidyn; **C,** fluopyram/adepidyn; **D,** solatenol/fluopyram; **E,** solatenol/boscalid; **F,** solatenol/adepidyn) for nonbaseline *Alternaria alternata* isolates (α = 0.05). Please note the differences in the scale used in the *x* and *y* axes among the graphs.

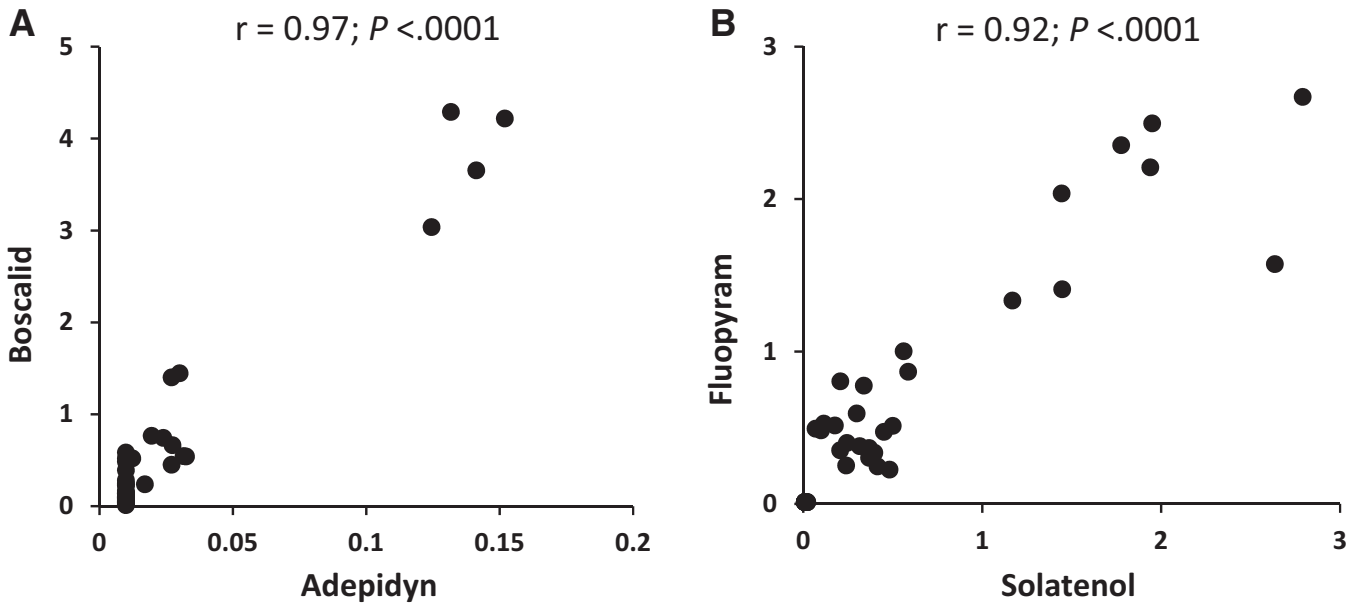

Fig. 5. Significant Pearson correlations between EC_{50} (µg/ml) of **A,** boscalid/adepidyn; **B,** fluopyram/solatenol fungicides for nonbaseline *Alternaria arborescens* isolates (α = 0.05). Please note the differences in the scale used in the *x* and *y* axes between the graphs.

been exposed specifically to adepidyn, which may explain why there was largely no Rf shift detected between the sensitivity of baseline and nonbaseline isolates. Adepidyn (pydiflumetofen) has been shown to possess high intrinsic activity in other plant pathosystems (Breunig and Chilvers 2021; Neves and Bradley 2019, 2021).

It is common among Midwest potato growers to incorporate single-site "specialty" fungicides in rotation with multisite "standard" fungicides for early blight management (Yellareddygari et al. 2016). In a previous study, no significant difference was found in disease severity or yield response between the group 1 (QoIs in rotation with anilinopyrimidines) and group 2 (SDHIs in rotation with QoIs or triazoles) specialty fungicides. Traditionally in the Midwest, small-spored *Alternaria* spp. have been considered a minor pathogen of potato when compared with *A. solani*, although that perspective could be changing considering the increased recovery of small-spored *Alternaria* spp. in Wisconsin (Ding et al. 2019). It may be important to determine which species are important elsewhere and to investigate other single-site fungicides that could be effective in controlling all *Alternaria* spp. detected in Midwestern potato fields.

Given the increased significance of brown leaf spot in many potato production areas of the United States, the development of a more accurate assay to detect and differentiate small-spored *Alternaria* spp. will aid in our understanding of how these pathogens interact with new and existing fungicides. It has been previously demonstrated that it is very difficult to differentiate *A. alternata* from *A. tenuissima* (Tymon et al. 2016b). Furthermore, studies have also demonstrated that there are genotypes of *A. alternata* affecting potato (Ding et al. 2019). In the current study, we demonstrated that aggressiveness did not differ by species, but rather by individual isolates within species. These results contrast previous preliminary pathogenicity studies that suggested *A. arborescens* isolates collected from potato were more aggressive than *A. alternata* (Tymon et al. 2016a). A comprehensive evaluation of the spatio-temporal variation of these pathogens, both as species or genotypes, could be important for the potato industry in choosing the appropriate foliar fungicide regime. It will also be important to have a more complete evaluation of the fungicide chemistries currently available to the potato industry and the activity of other fungicide groups such as demethylation inhibitors, anilinopyrimidines, and phenylpyrroles may have on small-spored *Alternaria* spp. In addition, at the current time, we do not know whether the variation in disease control provided by the SDHI fungicides evaluated is caused by small-spored *Alternaria* isolates possessing an SDHI mutation. Methods to detect SDHI mutations have been developed in *A. alternata*; however, it is unknown whether these primers can accurately detect mutations in the *Sdh* gene among other small-spored *Alternaria* spp. (Avenot et al. 2009), but those molecular studies are warranted.

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