Effect of the F129L Mutation in *Alternaria solani* on Fungicides Affecting Mitochondrial Respiration

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ABSTRACT

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Isolates of *Alternaria solani* previously collected from throughout the Midwestern United States and characterized as being azoxystrobin sensitive or reduced sensitive were tested for sensitivity to the Quinone outside inhibitor (Q_oI) fungicides famoxadone and fenamidone and the carboxamide fungicide boscalid. All three fungicides affect mitochondrial respiration: famoxadone and fenamidone at complex III, and boscalid at complex II. *A. solani* isolates possessing reduced-sensitivity to azoxystrobin also were less sensitive in vitro to famoxadone and fenamidone compared with azoxystrobin-sensitive isolates, but the shift in sensitivity was of lower magnitude, approximately 2- to 3-fold versus approximately 12-fold for azoxystrobin. The in vitro EC_{50} values, the concentration that effectively reduces germination by 50% relative to the untreated control, for sensitive *A. solani* isolates were significantly lower for famoxadone and azoxystrobin than for fenamidone and boscalid; whereas, for reduced-sensitive isolates, famoxadone EC_{50} values were significantly lower than all other fungicides. Isolates of *A. solani* with reduced-sensitivity to azoxystrobin were twofold more sensitive in vitro to boscalid than were azoxystrobin-sensitive wild-type isolates, displaying negative cross-sensitivity. All isolates determined to have reduced-sensitivity to azoxystrobin also were determined to possess the amino acid substitution of phenylalanine with leucine at position 129 (F129L mutation) using real-time polymerase chain reaction. In vivo studies were performed to determine the effects of in vitro sensitivity shifts on early blight disease control provided by each fungicide over a range of concentrations. Reduced-sensitivity to azoxystrobin did not significantly affect disease control provided by famoxadone, regardless of the wide range of in vitro famoxadone EC_{50} values. Efficacy of fenamidone was affected by some azoxystrobin reduced-sensitive *A. solani* isolates, but not others. Boscalid controlled azoxystrobin-sensitive and reduced-sensitive isolates with equal effectiveness. These results suggest that the F129L mutation present in *A. solani* does not convey cross-sensitivity in vivo among all Q_oI or related fungicides, and that two- to threefold shifts in in vitro sensitivity among *A. solani* isolates does not appreciably affect disease control.

Additional keywords: *Solanum tuberosum*

Alternaria solani Sorauer (Ellis), a foliar pathogen of potato (*Solanum tuberosum* L.), was considered to be difficult to control, responsible for yield losses of up to 30% (24), until the registration of the first strobilurin fungicide, azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC), in 1999. Strobilurins belong to the group of chemicals classified as Quinone outside inhibitor (Q_oI) fungicides (Fungicide Resistance Action Committee [FRAC] group 11), which inhibit electron transport in mitochondrial respiration at complex III (13). These fungicides have a specific single-site mode of action but, in spite of this, originally were classified as a medium resistance risk in *A. solani* (2). The development of reduced-sensitivity to azoxystrobin was first observed in 2000 in Nebraska by one of the authors (N. C. Gudmestad) and in North Dakota and Minnesota in 2001. Isolates of *A. solani* collected from these fields subsequently were determined to be significantly less sensitive in vitro to azoxystrobin, resulting in decreased disease control in vivo (23). When first registered, azoxystrobin provided significantly improved control of early blight under field conditions compared with protectant fungicides such as chlorothalonil or mancozeb (28). However, more recently, disease control provided by azoxystrobin frequently has been no better than the level of control provided by standard protectant fungicides in the presence of azoxystrobin reduced-sensitive *A. solani* isolates (29).

Resistance to Q_oI fungicides first was detected in *Blumeria graminis* (DC) E. O. Sper f. sp. *tritici* Em. Marchal and was attributed to a mutation in the cytochrome *b* gene causing an amino acid substitution of glycine with alanine at position 143 (G143A) (26). A second amino acid substitution of phenylalanine with leucine at position 129 (F129L) has since been described in one isolate of *Pythium aphanidermatum* (Edson) Fitzp. (1) as well as isolates of *Pyricularia grisea* (Cooke) Sacc., although most isolates of *P. grisea* have undergone the G143A mutation (12,30). Six previously evaluated *A. solani* isolates which had reduced-sensitivity to azoxystrobin were determined to contain the F129L mutation (23). Although the G143A mutation has been shown to convey full resistance in other host–pathogen systems, resulting in a total loss of disease control (8,10,12,14,25,26), the F129L mutation in *A. solani* conveys an approximately 10-fold loss of sensitivity in vitro to fungicides azoxystrobin and pyraclostrobin, resulting in a significant, but not total, loss of disease control under controlled conditions (23). However, reduced-sensitive isolates were only twofold less sensitive to trifloxystrobin and this shift in sensitivity did not appreciably affect disease control in these same studies (23). Similar results were observed with isolates of *P. grisea* in which the F129L mutation was detected (12,30). The G143A mutation has been detected in other *Alternaria* spp., including isolates of *A. alternata* (Fr.:Fr.) Keissl., *A. tenuissima,* (Kunze: Fr.) Wiltshire, and *A. arborescens* Simmons, which failed to be controlled by azoxystrobin (19).

After the registration of azoxystrobin, several other Q_oI fungicides were labeled for use on potato. Related nonstrobilurin Q_oI chemistries famoxadone (Tanos; DuPont Crop Protection, Wilmington, DE) and fenamidone (Reason; Bayer Corporation, Agricultural Division, Kansas City, MO), registered for use on potato in 2003 and 2004, respectively, have modes of action similar to those of azoxystrobin and pyraclostrobin (1,11). Although these chemicals all are classified within the Q_oI group, each is classified by FRAC into different chemical subgroups. Cross-resistance among all fungicides in the Q_oI group, conveyed by the G143A mutation, has been demonstrated in plant-pathogenic fungi such as *Podosphaera xanthii* (Castagne) U. Braun & N. Shishkoff, *Pseudoperonospora cubensis* Berk. & M. A. Curtis) Rostovzev (8,10), *P. grisea* (12,30), *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni in Sacc. (8), *B. graminis* f. sp. *tritici* (8,26), *My-*

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cosphaerella fijiensis Morelet (4,8,25), and *Venturia inaequalis* (Cooke) G. Wint. (14).

Boscalid (Endura; BASF Corporation, Agricultural Products, Research Triangle Park, NC) was registered for use on potato in 2003. Boscalid is a carboxamide fungicide (FRAC group 7) targeting mitochondiral respiration at complex II (succinate-ubiquinone reductase), as opposed to the complex III site targeted by Q_oI fungicides (15). The fully systemic carboxamide fungicides carboxin and oxycarboxin were first reported in 1966 (31). Carboxin was registered in France in 1969 for the control of smut, rust, and bunt diseases of cereal grains (15,16). Natural resistance to this class of fungicide was observed as early as 1981 in *Puccinia horiana* Henn., but was limited to a greenhouse in France (16). In 1985, isolates of *Ustilago nuda* (Jens) Rostr. resistant to carboximide fungicides were found in France (17). Induced resistance to carboxamides in an *U. maydis* (DC) Corda isolate was attributed to substitution of the amino acid histidine with leucine in the iron-sulfur protein subunit (3). Similar results were found with *Mycosphaerella graminicola* (Fuckel) J. Schröt where resistance was conferred by a substitution for histidine of either leucine or tyrosine (27). Resistance to both strobilurin and carboxamide fungicides has been documented in *P. horiana* in England and Europe, respectively (5,6), but resistance to both fungicide groups has not been detected within the same isolate. Carboxamide fungicides have been rated by FRAC as a medium risk for resistance development.

The overall objective of the study was to determine if the previously reported shift in sensitivity of *A. solani* to azoxystrobin and pyraclostrobin also was present in related respiratory-inhibiting fungicides. This objective was accomplished through the establishment of in vitro baseline sensitivity to famoxadone, fenamidone, and boscalid in *A. solani* collected from commercial potato fields in 1998, prior to Q_oI registration and use, compared with *A. solani* collected in 2001, subsequent to registration of Q_oI fungicides. A real-time polymerase chain reaction (PCR) assay for the detection of the F129L mutation was used to verify any shift in sensitivity detected by in vitro spore germination assays. Finally, the significance of any in vitro shift in sensitivity of *A. solani* to famoxadone, fenamidone, and boscalid on early blight disease control was evaluated.

MATERIALS AND METHODS

Collection of *A. solani* isolates. Isolates of *A. solani* were recovered from potato foliar and tuber tissue submitted to our laboratory from various areas throughout the Midwestern United States, mainly Nebraska, North Dakota, and Minnesota (23). *A. solani* isolates tested from 1998 were obtained, prior to the use of azoxystrobin in the United States, via single-spore isolation from hyphal tip cultures (9). Isolates tested from 2001 were obtained via single-spore isolation directly from diseased tissue and transferred to petri plates containing clarified V8 medium (Campbell's V8 juice, 150 ml; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water, 900 ml) amended with ampicillin at 50 mg/ml (CV8+Amp). Isolates were preserved in long-term cryogenic storage (23). Dried specimens also were prepared from each foliar tissue sample from which *A. solani* isolates originated.

In vitro cross-sensitivity assessment. In vitro sensitivities to all fungicides were determined using previously published methods (23). Stock solutions of technical products azoxystrobin (95.3% a.i.), famoxadone (98.7% a.i.), fenamidone (98.0% a.i.), and boscalid (97.4% a.i.) at 100 mg/ml were prepared and diluted serially in acetone. The final concentration of acetone in all media was 0.1% by volume. Q_oI fungicide sensitivity was determined by comparing spore germination on 2% laboratory grade water agar (A360-500; Fisher Scientific, Pittsburgh, PA) amended with azoxystrobin, famoxadone, fenamidone, or boscalid at 0.0, 0.01, 0.1, 1.0, and 10.0 µg/ml fungicide as well as salicylhydroxamic acid (SHAM) in methanol at 100 µg/ml (21). The final concentration of methanol in both the fungicide-amended and media not amended with fungicide was 0.1% by volume. Previous research demonstrated that this concentration of SHAM did not inhibit germination of *A. solani* conidia (23).

Sterile, distilled water was added to *A. solani* cultures and conidia were dislodged using a sterile glass rod. The concentration of the conidial suspension was adjusted to 3×10^5 conidia/ml using a hemacytometer, and 50 µl of the conidial suspension of each isolate was spread across two petri plates (two replications) of water agar amended with each fungicide concentration. The plates were held for 4 h with continuous light at 21°C. After incubation, conidia were examined with a compound microscope at ×100 magnification. A conidium was considered germinated if the germ tube was at least equal in length to the conidium or if there were multiple germ tubes developing normally from a single conidium.

EC_{50} values, the concentration that effectively reduces germination by 50% relative to the untreated control, were determined for each isolate. The mean number of conidia germinated for two replicates was converted into a percent reduction of germination relative to the nonfungicide-amended control. These data were regressed against the logarithm of the fungicide concentration and the EC_{50} value was determined by interpolation of the 50% intercept.

The 51 *A. solani* isolates tested for in vitro cross-sensitivity among the Q_oI fungicides azoxystrobin, famoxadone, fenamidone, and boscalid were chosen with consideration to both geographic location from which the isolate originated and EC_{50} value obtained previously by testing azoxystrobin sensitivity in vitro (23). Of the 25 azoxystrobin-sensitive *A. solani* isolates selected, 24 had been assessed previously in vitro and were determined to have EC_{50} values within the range of the baseline values for isolates sensitive to azoxystrobin (EC_{50} value of 0.01 to 0.07) (23). Of the 26 azoxystrobin reduced-sensitive isolates of *A. solani* also selected, 25 previously had been assessed for sensitivity to azoxystrobin (EC_{50} value of 0.20 to 0.70) (23). Isolates were tested in 14 separate trials, 3 to 24 isolates per trial. In a separate experiment, the same 51 *A. solani* isolates were tested to determine the potential for in vitro cross-sensitivity between azoxystrobin and boscalid. Isolates were tested in 13 separate trials, 2 to 23 isolates per trial. Azoxystrobin was included in each trial. Again, to insure reproducibility of the assays, only those trials were included in which the azoxystrobin EC_{50} value of each isolate remained within either the sensitive or reduced-sensitive group, as in previous experiments (23).

Because famoxadone, fenamidone, and boscalid were not evaluated within the same experiment, a *t* test was performed on azoxystrobin EC_{50} values obtained from the famoxadone-fenamidone experiment and the boscalid experiment using SAS (version 8; SAS Institute, Inc., Cary, NC) to verify that the data could be combined for further analysis. Within each fungicide (azoxystrobin, famoxadone, fenamidone, and boscalid), *t* tests were used to detect differences in the mean EC_{50} values between previously determined azoxystrobin-sensitive and azoxystrobin reduced-sensitive isolate groups. Within the sensitive and reduced-sensitive isolate groups, *t* tests also were used to detect differences in the mean EC_{50} values among all fungicides Correlation analysis was performed on in vitro EC_{50} values for azoxystrobin, famoxadone, fenamidone, and boscalid using Persons correlation coefficients as a measure of the relationships among the fungicides.

Detection of the F129L mutation. *Total genomic DNA extraction.* The mycelium and spore growth of a 12- to 14-day-old culture from a sensitive (#13-1) and a reduced-sensitive (#526-1) isolate previously confirmed to possess the F129L mutation (23) growing on CV8+Amp agar was scraped and ground in liquid nitrogen. Genomic DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI) following manufacturer's instructions. The resulting DNA was rehydrated in 100 µl rehydration solution, contained in the kit, by incubating the solution overnight at 4°C followed by a phenol-chloroform extraction and precipitation. DNA was resus-

pended to 2.5 ng/µl in sterile H_2O based on fluorometric measurements using Hoechst dye 33258 (TD-700 Fluorometer, Turner Designs, Sunnyville, CA).

Amplification, cloning, and sequencing of a fragment of the A. solani *cytochrome* b *gene.* Oligonucleotide primers (As-1F and As-1R; Table 1) were designed based on the alignment of partial *A. solani* cytochrome *b* gene sequences deposited in the GenBank database (accession no. BD260436, AX577579, AX577577, and AX577191). PCR was performed in a total reaction volume of 25 µl containing 1× PCR buffer (Qiagen, Valencia, CA), 1.5 mM $MgCl_2$ (Qiagen), 200 µM each dNTP (Roche, Indianapolis, IN), 0.3 µM each primer, 1 U of Taq DNA polymerase (Qiagen), and 5 ng of genomic DNA. Cycling parameters were 95°C for 10 min; followed by 40 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; with a final extension of 10 min at 72°C and then cooling to 4°C. The amplification product was electrophoresed on a 1% agarose gel with 1× Tris-acetate-EDTA and stained with ethidium bromide.

An 800-bp fragment was excised from the gel, and the PCR product was eluted using the GENECLEAN kit (Bio 101, La Jolla, CA) and ligated directly into the pGEM-T easy vector (Promega Corp.). Plasmids were transformed into *Escherichia coli* JM109 cells (Promega Corp.) according to a standard protocol and plasmid DNA was extracted using the Wizard Plus Minipreps DNA purification system (Promega Corp.). The nucleotide sequence of the plasmid insert was determined using the dideoxynucleotide chain-termination method and T7-1 and SP6 primers at the DNA Sequencing and Synthesis Facility at Iowa State University (Ames). Analysis of the DNA sequence was performed using the bl2seq program provided by the National Center for Biotechnology Information (Bethesda, MD). LightCycler Probe Design Software 2.0 (Roche) was used to determine a primer and probe set that would amplify and differentiate the wild-type *A. solani* isolate from the F129L mutant (Table 1).

Detection of F129L mutation site by real-time PCR. A real-time PCR-hybridization assay was used to detect a single-base-pair mutation changing the amino acid sequence at position 129 (F to L). The method included amplification of a fragment of the cytochrome *b* gene coupled with simultaneous detection of the product by probe hybridization and analysis of the melting point of the DNA fragments. Melting curve analysis has been used previously to detect single-point mutation sites (18,22). A 250-bp fragment of the cytochrome *b* gene of *A. solani* was amplified using sequence-specific primers AS-5F and AS-5R. Two hybridization probes were produced so that the sensor probe, Asol-FL, 3′ labeled with fluorescein, and the anchor probe, Asol-R640, 5′ labeled with LC-Red 640 and 3′ phosphorylated, will hybridize to the amplified product produced by the wild-type isolate. The sensor probe is designed to span the mutation site so that the single nucleotide mismatch position is at least 3 bp away from the sensor probe end. When the sensor probe is melted away from the amplified product, the matching probe-target DNA will separate at a higher melting point temperature (T_m) than probes that are bound to DNA which contain destabilizing nucleotide mismatches. A specific T_m then can be obtained for each genotype.

The PCR and hybridization reactions were carried out using the LightCycler thermocycler (Roche) in glass capillaries in a final volume of 20 µl containing 1× FastStart DNA Master Hybridization Probes (Roche), 2 mM $MgCl_2$ (Roche), 0.5 µM AS-5F forward primer, 2.5 µM AS-5R reverse primer, 0.2 µM sensor (Asol-FL) and anchor (Asol-R640) probes, and 1 ng of DNA. After an initial denaturation step of 95°C for 10 min, PCR was run for 45 cycles using the following conditions: denaturation (95°C, 10 s, ramp rate 20°C/s), annealing (58 to 50°C, 10 s, ramp rate 20°C/s, step size 1°C, acquisition mode: single), and extension (72°C, 8 s, ramp rate 5°C/s). After amplification, melting curves were generated at 95°C (10 s,

Table 1. Oligonucleotides developed for the detection of the F129L mutation in the cytochrome *b* gene of *Alternaria solani* isolates

Oligo name	Sequence (5′ to 3′)
Forward primers	
As-1F	CAGAGCACCTAGAACTCTAGTATGAA
AS-5F	AGAACTCTAGTATGAACTATTGG
Reverse primers	
As-1R	CCTCCTCAAATGAACTCAACAA
AS-5R	ACTTCTTGTAGAATATCCTCTTT
Sensor primer	
Asol-FL	GATGGCTACAGCTTTCCTG-Fluorescein
Anchor primer	
Asol-R640	LC Red 640-TTACCAACATAGCCCAAAATGGTTT-Phosphate

Table 2. Subset designations, origin, in vitro azoxystrobin sensitivity, and real-time polymerase chain reaction melting temperatures for isolates of *Alternaria solani* isolates evaluated in vivo with azoxystrobin, famoxadone, fenamidone, and boscalid

Subset no.	Isolate designation	Geographic origin	Year collected	Sensitivity to azoxystrobin	Fungicide tested in vivo[a]	T_m[b]
1	13-1[c]	O'Neill, NE	1998	Sensitive	azs, fam, fen, bos	55.20
1	528-2	O'Neill, NE	2001	Reduced-sensitive	azs, fam, fen, bos	50.94
1	549-2	Dawson, ND	2001	Sensitive	azs, fam, fen, bos	55.08
1	586-2	Browerville, MN	2001	Reduced-sensitive	azs, fam, fen, bos	51.37
1	588-1	Kearney, NE	2001	Reduced-sensitive	azs, fam, fen, bos	51.49
2	6-1	Park Rapids, MN	1998	Sensitive	azs, fam, fen	ND
2	31-1	O'Neill, NE	1998	Sensitive	azs, fam, fen	55.82
2	40-1	Watertown, SD	1998	Sensitive	azs, fam, fen	56.37
2	535-2	O'Neill, NE	2001	Reduced-sensitive	azs, fam, fen	51.99
2	547-4	Dawson, ND	2001	Reduced-sensitive	azs, fam, fen	50.68
2	577-1	Minden, NE	2001	Reduced-sensitive	azs, fam, fen	51.80
3	22-1	Staples, MN	1998	Sensitive	azs, bos	56.07
3	30-1	Buxton, ND	1998	Sensitive	azs, bos	55.70
3	31-4	O'Neill, NE	1998	Sensitive	azs, bos	ND
3	32-1	Park Rapids, MN	1998	Sensitive	azs, bos	55.57
3	538-2	O'Neill, NE	2001	Reduced-sensitive	azs, bos	50.50
3	547-2	Dawson, ND	2001	Reduced-sensitive	azs, bos	51.06

[a] Azoxystrobin = azs, famoxadone = fam, fenamidone = fen, and boscalid = bos.

[b] Melting temperature (T_m) in degrees Celsius for the sensitive wild-type (55.0 to 58.3°C) and reduced-sensitive mutant (50.5 to 52.5°C) isolates based on real-time polymerase chain reaction; ND = not determined.

[c] Previously shown not to contain the F129L mutation (23).

ramp rate 20°C/s), 45°C (30 s, ramp rate 20°C/s), and 85°C (0 s, ramp rate 0.1°C/s, acquisition mode: continuous). After a final cooling step for 30 s at 40°C, melting curve analysis was performed (18,22). All 25 azoxystrobin reduced-sensitive isolates of *A. solani*, as determined by in vitro evaluations, were tested using real-time PCR to confirm the presence of the F129L mutation. A number of azoxystrobin-sensitive *A. solani* isolates were included as controls. The six *A. solani* isolates previously evaluated for the F129L mutation (23) also were included as internal positive and negative controls for the presence or absence of the F129L mutation.

Effect of sensitivity to fungicides on disease control. All experiments performed to determine the effect of reduced-sensitivity to azoxystrobin on early blight disease control provided by famoxadone, fenamidone, and boscalid were performed as previously described (23). The same five isolates previously evaluated with azoxystrobin, pyraclostrobin, and trifloxystrobin (23) constituted the first subset of isolates of *A. solani* tested to determine disease control provided by azoxystrobin, famoxadone, fenamidone, and boscalid (Table 2). A second subset of six isolates was evaluated for in vivo sensitivity to azoxystrobin, famoxadone, and fenamidone (Table 2). The six isolates from subset 2 were chosen because of low (sensitive) and high (reduced-sensitive) in vitro EC_{50} values for both famoxadone and fenamidone (Fig. 1B and C). Boscalid was evaluated against the first isolate subset in a separate set of experiments. Boscalid was further evaluated against a third, but separate, subset of six isolates (Table 2; Fig. 1D). Again, these isolates were chosen to represent isolates with the lowest and highest EC_{50} values to boscalid, regardless of sensitivity to azoxystrobin.

All isolates were recovered from long-term storage, cultured, and conidia were harvested as described previously for the in vitro assessment and elsewhere (9,23). When dislodging spores, the addition of 5 drops of Tween 20 (Sigma-Aldrich, St. Louis) per 100 ml of sterile distilled water prevented clumping, allowing conidia to be spread more evenly on plant foliage during inoculations.

The in vivo sensitivity assay for *A. solani* isolates was conducted as a 24-h preventative test. Fungicides were applied 24 h prior to inoculation in the greenhouse using tomato plants, cv. Orange Pixie (W. Atlee Burpee and Co., Warminster, PA). Tomato plants were utilized for the in vivo study because of this cultivar's susceptibil-

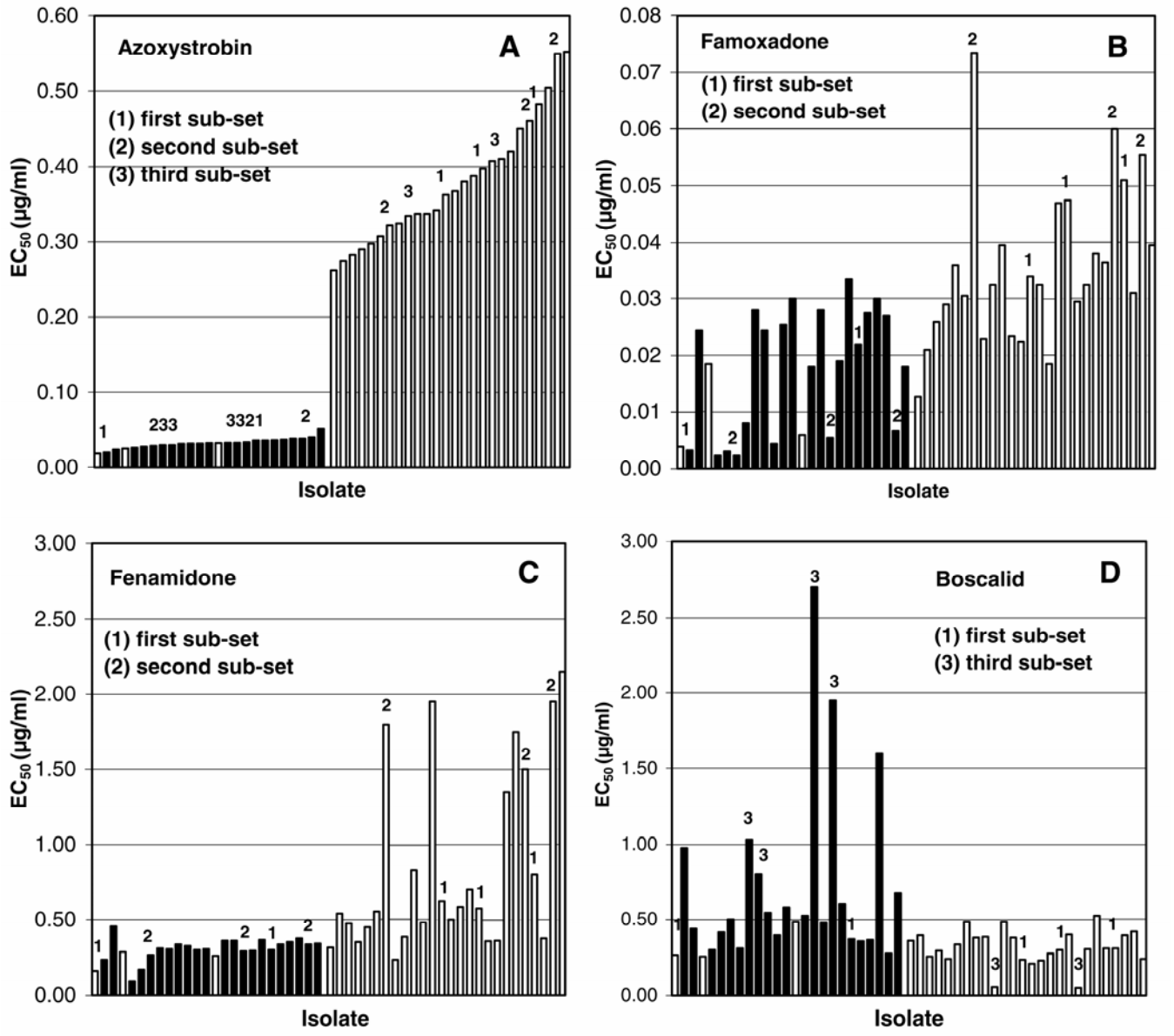

Fig. 1. A, Azoxystrobin, **B,** famoxadone, **C,** fenamidone, and **D,** boscalid EC_{50} values (the concentration that effectively reduces germination by 50% relative to the untreated control) from the in vitro spore germination experiment for *Alternaria solani* isolates collected in 1998 (■) and 2001 (□). Fungicide concentrations are given in micrograms per milliliter. Scales on *y* axis are independent for each fungicide.

ity to early blight. Their compact size compared with potato plants allowed for adequate replication while evaluating multiple fungicides and fungicide concentrations across several *A. solani* isolates. Three tomato seed were sown in each 10-cm^2 pot, in Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA). After emergence, plants were excised to obtain two uniformly sized plants per pot. When three fully expanded leaves were present, and plants had reached a height of 15 to 20 cm, plants were treated with commercial formulations of either azoxystrobin (Quadris), famoxadone + cymoxanil (Tanos), fenamidone (Reason), or boscalid (Endura). Cymoxanil, contained in the Tanos formulation, is active only on oomycetes and has no activity on *A. solani* and, as such, was treated as an inert component of the fungicide (7,33). Final fungicide concentrations of each active ingredient (azoxystrobin, famoxadone, fenamidone, and boscalid) applied to the plants were 0, 0.1, 1.0, 10.0, and 100.0 μg/ml to obtain a dose response curve for each fungicide. Each fungicide was applied to run-off in such a manner as to completely and uniformly wet both the abaxial and adaxial leaf surfaces using a CO_2-powered backpack sprayer at approximately 172 kPa. Twenty-four hours after fungicide application, tomato plants were inoculated with a 3.0×10^5 conidia/ml suspension from 12- to 14-day-old cultures using a Preval paint-spray gun (Preval Sprayer Division, Precision Valve Corp., Yonkers, NY). Tomato plants in each group were inoculated with a single *A. solani* isolate and immediately placed into separate mist chambers (>95% relative humidity, 22 to 24°C, 16-h photoperiod), one isolate per chamber, and held for 24 h, after which plants were transferred to confinement chambers (plastic chambers with open ceilings) on greenhouse benches. Confinement chambers were used to reduce the potential for cross contamination among *A. solani* isolates. The plants were watered once per day, and the temperature in the isolation chambers was maintained at approximately 24 ± 2°C. All experiments were split-plot randomized complete-block designs. Isolates were treated as the whole plot factor. Fungicides at each concentration were treated as the split plots. Early blight disease severity on individual tomato leaves was rated visually by estimating percent infected leaf area of the first three true leaves (three subsamples) and recorded as percent diseased tissue.

Early blight disease severity was rated 6 to 12 days post inoculation for the first subset of isolates and 5 to 8 days post inoculation for the second subset for famoxadone and fenamidone experiments. The in vivo experiment using isolate subset 1 was performed three times and the experiment using isolate subset 2 was performed twice. Disease severity in experiments evaluating disease control provided by azoxystrobin and boscalid was rated as described above 7 days after inoculation for isolate subset 1, and 4 to 8 days after inoculation for isolate subset 3. The experiments with azoxystrobin and boscalid using both *A. solani* isolate subsets 1 and 3 were performed twice.

For all in vivo experiments, three replications (three pots) and two samples (two plants per pot) were tested for each isolate–fungicide concentration. For each isolate, at all fungicide concentrations, disease severity data were transformed to percentage of disease control for further statistical analysis using the formula (1 – [% diseased tissue/% diseased tissue in untreated plants] × 100). Levene's Test (20) was conducted to test for homogeneity of variance among the independent experiments at each fungicide concentration and between the two isolate groups (sensitive and reduced-sensitive) individually for each of the two subsets of isolates. The analysis of variance was performed separately for each fungicide–isolate group combination at each fungicide concentration using SAS (SAS, Inc.). Combined data were analyzed with *t* tests to detect differences at each fungicide concentration.

RESULTS

In vitro cross-sensitivity assessment. For all 51 *A. solani* isolates, azoxystrobin EC_{50} values were not significantly different between the famoxadone-fenamidone and boscalid experiments (*data not shown*); therefore, data from the two experiments were combined for further analysis. Isolates of *A. solani* collected in 1998 all were confirmed to be sensitive to azoxystrobin with EC_{50} values ranging from 0.020 to 0.051 μg/ml (Fig. 1A). The range of EC_{50} values of famoxadone, fenamindone, and boscalid were 0.002 to 0.034, 0.093 to 0.455, and 0.275 to 2.70 μg/ml, respectively (Fig. 1B–D). This represents approximately a 17-, 5-, and 10-fold difference, respectively, in sensitivity to these fungicides within the wild-type *A. solani* group. Isolates of *A. solani* collected in 2001, most of which possessed reduced-sensitivity to azoxystrobin, had in vitro EC_{50} values ranging from 0.019 to 0.553, 0.004 to 0.074, 0.16 to 2.150, and 0.049 to 0.525 μg/ml in response to azosystrobin, famoxadone, fenamidone, and boscalid, respectively (Fig. 1A–D). Among azoxystrobin reduced-sensitive *A. solani* isolates, approximately 19-, 13-, and 11-fold differences in response to famoxadone, fenamidone, and boscalid were observed between the most sensitive and the least sensitive isolates.

Highly significant differences ($P < 0.0001$) were detected between the two isolate groups, sensitive and reduced-sensitive, among the four fungicides, azoxystrobin, famoxadone, fenamidone, and boscalid (Table 3). These shifts represent an 11.7-, 2.1-, and 2.8-fold loss in sensitivity to azoxystrobin, famoxadone, and fenamidone, respectively. A significant isolate group–fungicide interaction resulted because azoxystrobin reduced-sensitive *A. solani* isolates were 2.2-fold more sensitive to boscalid than azoxystrobin-sensitive isolates ($P < 0.01$) (Table 3). It also was determined that, among sensitive *A. solani* isolates, famoxadone and azoxystrobin EC_{50} values were significantly lower than fenamidone and boscalid EC_{50} values. Among reduced-sensitive isolates, famoxadone EC_{50} values were significantly lower than EC_{50} values of the other three fungicides. Correlation of EC_{50} values for isolates exposed to azoxystrobin, famoxadone, and fenamidone in all combinations were weak (Fig. 2A–C). An inverse relationship, negative cross-sensitivity, existed between boscalid and the Q_oI fungicides azoxystrobin, famoxadone, and fenamidone among the isolates tested (Fig. 2D–F).

Detection of F129L mutation site by real-time PCR. T_m has been demonstrated to be very useful and extremely sensitive in detecting single nucleotide base substitutions conferring antibiotic resistance in bacteria and fungicide resistance in *Candida* spp. (18,22), making it an attractive option in the identification of a phenylalanine to leucine shift at position 129 of the mitochondrial genome. The wild-type genotype produced a melting peak range of 55.0 to 58.3°C compared with the F129L mutated genotype, which produced a T_m of 50.5 to 52.5°C. The existence of a nucleotide mismatch between the amplified sequence and the hybridization probe was detected among those 25 *A. solani* isolates determined to have reduced-sensitivity to

Table 3. Mean in vitro concentration that effectively reduces germination by 50% relative to the untreated control (EC_{50} values; μg/ml) of 25 sensitive and 26 reduced-sensitive *Alternaria solani* isolates for four respiratory inhibiting fungicides[a]

	Sensitive		Reduced-sensitive		
Fungicide	**EC_{50}**	**SD**	**EC_{50}**	**SD**	**LSD ($P < 0.0001$)**
Azoxystrobin	0.0324	0.0096	0.3788	0.1458	0.0288
Famoxadone	0.0168	0.0113	0.0355	0.0169	0.0057
Fenamidone	0.3003	0.0856	0.8439	0.6678	0.1889
Boscalid	0.6878	0.6330	0.3175	0.1413	0.1786
LSD ($P < 0.0001$)	0.1051	…	0.1151	…	…

[a] Sensitivity or reduced-sensitivity to azoxystrobin (23); SD = standard deviation; LSD = least significant difference.

azoxystrobin in vitro, confirming the presence of the F129L mutation in all reduced-sensitive isolates tested (Table 2; Fig. 3). The six previously evaluated control isolates (23) reacted as expected, one wild type and five containing the F129L mutation. Real-time PCR also demonstrated that the F129L mutation was not present in wild-type *A. solani* isolates tested and determined to be sensitive to azoxystrobin in vitro (Table 2; Fig. 3).

Effect of isolate sensitivity to fungicides on disease control. *Famoxadone and fenamidone.* Levene's test for homogeneity of variance for in vivo experiments indicated that all and 132 of 144 estimated variances with isolate subsets one and two, respectively, were homogeneous; therefore, data from replicated trials were combined for further analysis.

Data from the first subset of *A. solani* isolates indicated there were significant differences among fungicides only at 100.0 µg/ml and there was a significant fungicide–isolate group interaction at 1.0 and 10.0 µg/ml ($P < 0.05$; Table 4). This interaction occurred because significant differences in disease control between reduced-sensitive and sensitive isolates were observed at 1.0 and 10.0 µg/ml azoxystrobin (Fig. 4A). Significant differences between isolate groups were observed only at a fenamidone concentration of 10.0 µg/ml. No significant differences in disease control between isolate groups were detected at any concentration of famoxadone. A significant difference was detected between reduced-sensitive and sensitive isolates at all concentrations of the three fungicides. This was due primarily to the poor control azoxystrobin provided of reduced-sensitive isolates of *A. solani* relative to famoxadone and fenamidone ($P < 0.01$).

The in vitro shift in azoxystrobin, famoxadone, and fenamidone EC_{50} values between groups in isolate subset 1 was 25-, 3.4-, and 2.9-fold, respectively. Only when EC_{50} values of individual isolates were critically assessed was it determined that additional in vivo studies should be performed. For the second in vivo experiment, a subset of *A. solani* isolates were chosen that were more widely disparate in their sensitivity to famoxadone and fenamidone.

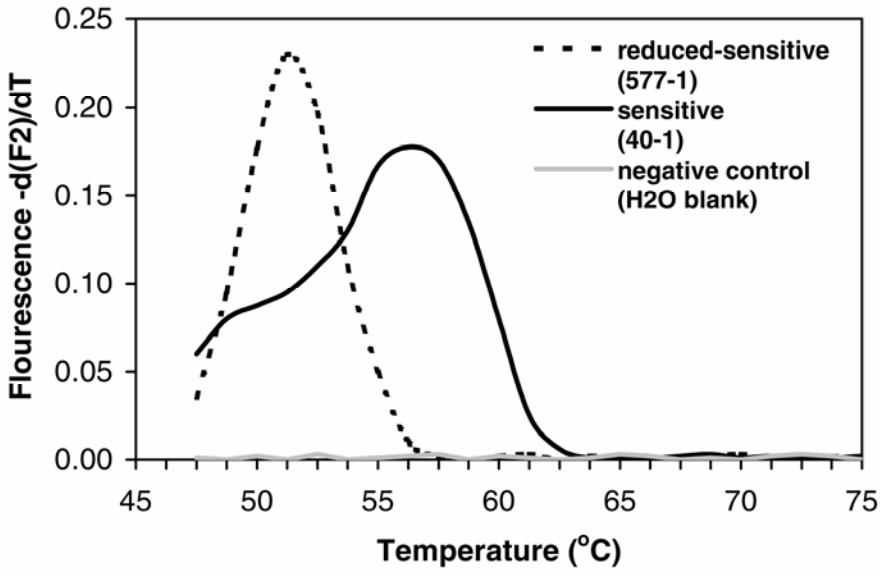

Fig. 3. Representative melting peak analysis of *Alternaria solani* cytochrome *b* genotypes at the F129L mutation position based on real-time polymerase chain reaction. Values on the *y* axis represent the ratio of the first negative derivative of the change in fluorescence (dF) to the variation in temperature (temperature values are given in degrees Celsius).

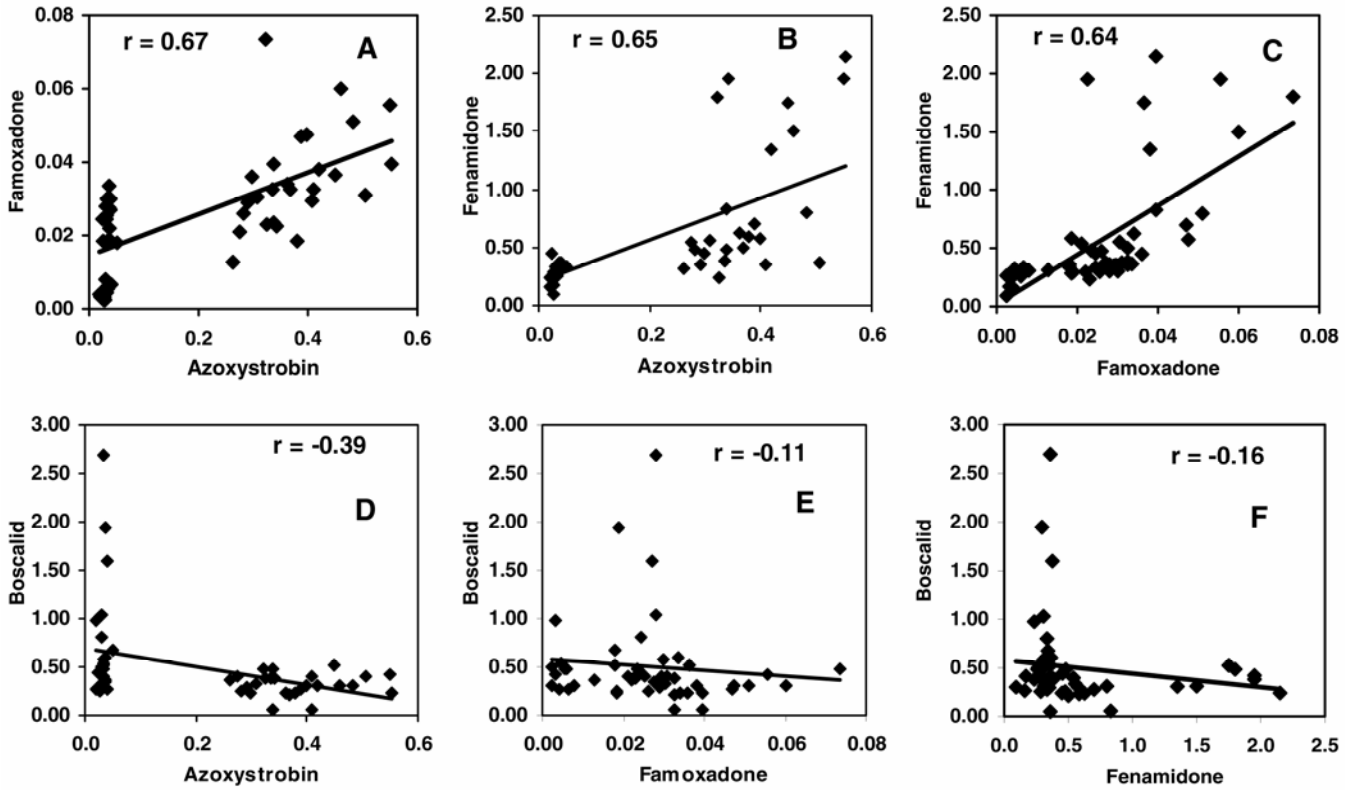

Fig. 2. Correlation of EC_{50} values (the concentration that effectively reduces germination by 50% relative to the untreated control) of azoxystrobin and famoxadone, azoxystrobin and fenamidone, famoxadone and fenamidone, azoxystrobin and boscalid, famoxadone and boscalid, and fenamidone and boscalid of 51 *Alternaria solani* isolates tested by in vitro spore germination assays. Fungicide concentrations are given in micrograms per milliliter.

The resulting in vitro shift in azoxystrobin, famoxadone, and fenamidone EC_{50} values between sensitive and reduced-sensitive *A. solani* groups in isolate subset 2 was 16.5-, 12.6-, and 5.9-fold, respectively.

The evaluation of isolate subset 2 with azoxystrobin, famoxadone, and fenamidone resulted in significant differences between the two isolate groups, sensitive and reduced-sensitive, at all fungicide concentrations ($P < 0.01$), consistent with isolate subset 1 (Fig. 4B). Also consistent with subset 1, all fungicides provided similar control of sensitive *A. solani* isolates, and azoxystrobin provided significantly better disease control of sensitive than reduced-sensitive isolates at all fungicide concentrations ($P < 0.05$; Fig. 4B). Contrary to subset 1, there were significant differences among the three fungicides at all concentrations except 0.1 µg/ml ($P < 0.05$), and no significant fungicide–group interaction was detected at any concentration. Also in contrast to the first isolate subset, fenamidone controlled reduced-sensitive *A. solani* isolates significantly less than sensitive wild-type isolates at both 1.0 and 10.0 µg/ml ($P < 0.05$). Although disease control with famoxadone was numerically less with reduced-sensitive *A. solani* isolates compared with sensitive isolates, these differences were significant only at 100.0 µg/ml ($P < 0.05$; Fig. 4B). Overall, early blight disease control provided by famoxadone was very similar to that observed with the first isolate subset.

Boscalid. In disease control experiments involving azoxystrobin and boscalid on sensitive and reduced-sensitive *A. solani* isolates, Levene's test for variance homogeneity indicated that, for the first and third subset of isolates, 72 of 80 and 90 of 96 variances estimated, respectively, were homogeneous; therefore, for both subsets, the two replicated trials were combined for further analysis.

As with famoxadone and fenamidone, evaluation of an additional subset of isolates was performed. The shift in in vitro azoxystrobin EC_{50} values between sensitive and reduced-sensitive isolate groups in subsets 1 and 3 were similar, 14.7- and 11.4-fold, respectively, whereas the shift in boscalid EC_{50} values was –1.1-fold in subset 1 and –15.6-fold in subset 3 (Fig. 1A and D; Table 2). These differences in in vitro EC_{50} values did not affect disease control of either azoxystrobin or boscalid. For both isolate subsets 1 and 3, a significant fungicide–isolate group interaction ($P < 0.01$) occurred at all fungicide concentrations except 0.1 µg/ml (Table 4) and, similar to previous experiments, azoxystrobin controlled sensitive *A. solani* isolates significantly better than reduced-sensitive isolates at concentrations above 0.1 µg/ml ($P < 0.01$; Fig. 4C and D). No differences in disease control of the two isolate groups were detected at any concentration of boscalid, which controlled both isolate groups as well as azoxystrobin controlled sensitive *A. solani* isolates ($P < 0.01$; Fig. 4C and D). This resulted in a significant fungicide–isolate group interaction ($P < 0.01$) in both isolate subsets (Table 4).

DISCUSSION

In previous studies, we reported a shift in sensitivity of *A. solani* to the Q_oI fungicides azoxystrobin and pyraclostrobin, and that this shift in sensitivity in six isolates was due to the F129L mutation (23). In those studies, isolates of *A. solani* clearly were cross-sensitive in their response to azoxystrobin and pyraclostrobin, but less so in their response to trifloxystrobin, with in vitro shifts in sensitivity to each fungicide approximately 13-, 10-, and 2-fold, respectively. The shift in sensitivity within *A. solani* to these three Q_oI fungicides resulted in a significant loss in the level of disease control provided by azoxystrobin and pyraclostrobin but not with trifloxystrobin in greenhouse efficacy tests (23).

In light of our previous studies with *A. solani*, questions arose whether or not the F129L mutation would convey cross-sensitivity to other fungicides that affect mitochondrial respiration, such as the Q_oI fungicides famoxadone and fenamidone, and the carboxamide fungicide boscalid, all registered on potato. Although a shift in sensitivity to famoxadone and fenamidone has occurred in some isolates, this shift is not universal among all of the azoxystrobin reduced-sensitive isolates we evaluated, indicating that the shift may be quantitative and not due to the F129L mutation, but the action of multiple genes or other rescue mechanisms, including accelerated detoxification (14,32). This may be especially true in the case of famoxadone, where a 17-fold range of in vitro EC_{50} values exists within the baseline sensitive group of isolates and the in vitro EC_{50} values for reduced-sensitive isolates is significantly lower for famoxadone than for the other three fungicides. In addition, the shift from sensitive to reduced-sensitive in vitro does not affect the disease control of famoxadone in greenhouse efficacy tests. The shift in famoxadone and fenamidone in vitro EC_{50} values among all 1998 isolates to 2001 isolates was between two- and threefold, similar to what we previously reported for trifloxystrobin (23). As with earlier greenhouse efficacy tests, demonstrating that a twofold shift in in vitro sensitivity to trifloxystrobin did not result in a significant loss of disease control (23), initial in vivo studies (in vivo isolate subset 1) demonstrated that a shift in sensitivity to famoxadone and fenami-

Table 4. Analysis of variance for the effect of fungicide and fungicide concentration on the control of sensitive and reduced-sensitive isolates of *Alternaria solani* under greenhouse conditions in four separate experiments[a]

		Concentration							
		0.1 µg/ml		1 µg/ml		10 µg/ml		100 µg/ml	
Source of variation	df	*F* value	*P* > *F*	*F* value	*P* > *F*	*F* value	*P* > *F*	*F* value	*P* > *F*
Subset 1 (azs, fam, fen)[b]									
Fungicide	2	0.05	0.9469	1.75	0.1776	3.05	0.0509	5.36	0.0058
Isolate group	1	7.81	0.0060	7.76	0.0061	10.04	0.0019	12.19	0.0007
Fungicide × isolate group	2	2.05	0.1327	4.31	0.0154	3.76	0.0259	1.43	0.2429
Subset 1 (azs, bos)[b]									
Fungicide	1	0.00	0.9453	2.20	0.1437	23.92	<0.0001	39.40	<0.0001
Isolate group	1	2.49	0.1206	14.88	0.0003	16.77	0.0001	8.61	0.0048
Fungicide × isolate group	1	2.32	0.1337	19.22	<0.0001	20.41	<0.0001	23.64	<0.0001
Subset 2 (azs, fam, fen)[b]									
Fungicide	2	0.90	0.4090	5.90	0.0038	3.51	0.0337	4.53	0.0130
Isolate group	1	10.89	0.0013	21.53	<0.0001	27.26	<0.0001	16.57	<0.0001
Fungicide × isolate group	2	0.11	0.8928	2.66	0.0746	1.18	0.3112	0.82	0.4417
Subset 3 (azs, bos)[b]									
Fungicide	1	0.15	0.6966	3.96	0.0507	4.87	0.0306	25.90	<0.0001
Isolate group	1	0.01	0.9233	3.77	0.0563	8.82	0.0041	9.36	0.0032
Fungicide × isolate group	1	0.01	0.9148	17.09	0.0001	9.26	0.0033	11.45	0.0012

[a] Data were transformed from percentage infection to percentage disease control before analysis.
[b] Azoxystrobin = azs, famoxadone = fam, fenamidone = fen, and boscalid = bos.

done also did not significantly affect disease control. In the second subset of *A. solani* isolates chosen for the in vivo studies, disease control was significantly affected by the nearly sixfold shift in in vitro fenamidone sensitivity. These isolates were controlled to a lesser degree by fenamidone compared with the control of azoxystrobin-sensitive isolates at some (but not all) fungicide concentrations. Although reductions in disease control were observed, these reductions were significant only for fenamidone and not famoxadone, despite the fact that reduced-sensitive isolates were nearly 13-fold less sensitive to famoxadone than the sensitive isolates evaluated in the same experiment. These in vivo studies are supported further by preliminary results from field experiments in which famoxadone provided significantly better control of early blight than azoxystrobin in the presence of a reduced-sensitive *A. solani* population (J. S. Pasche and N. C. Gudmestad, *unpublished data*). It is apparent from the studies reported here and elsewhere (23) that, unlike the G143A mutation, the F129L mutation does not convey cross-sensitivity among Q_oI fungicides within *A. solani*.

Similar results were obtained with respect to the gray spot of turf pathogen, *P. grisea*, where the F129L mutation also did not convey a significant loss of disease control provided by trifloxystrobin compared with azoxystrobin, in contrast to the G143A mutation, which conveyed cross-resistance and a total loss of disease control among the Q_oI fungicides investigated (12,30). The G143A mutation has been demonstrated previously to convey cross-resistance among all Q_oI fungicides studied (10,12,14,25,26). The G143A mutation has been shown to convey cross-resistance to famoxadone in *M. fijiensis*; however, because famoxadone was much less active against sensitive isolates of this fungus, resistance factors were much lower than reported for azoxystrobin and trifloxystrobin (25). Köller et al. suggested both qualitative and quantitative shifts in sensitivity of *V. inaequalis* to trifloxystrobin and kresoxim-methyl. The qualitative shifts are attributed to the G143A target-site mutation, whereas the quantitative shifts were

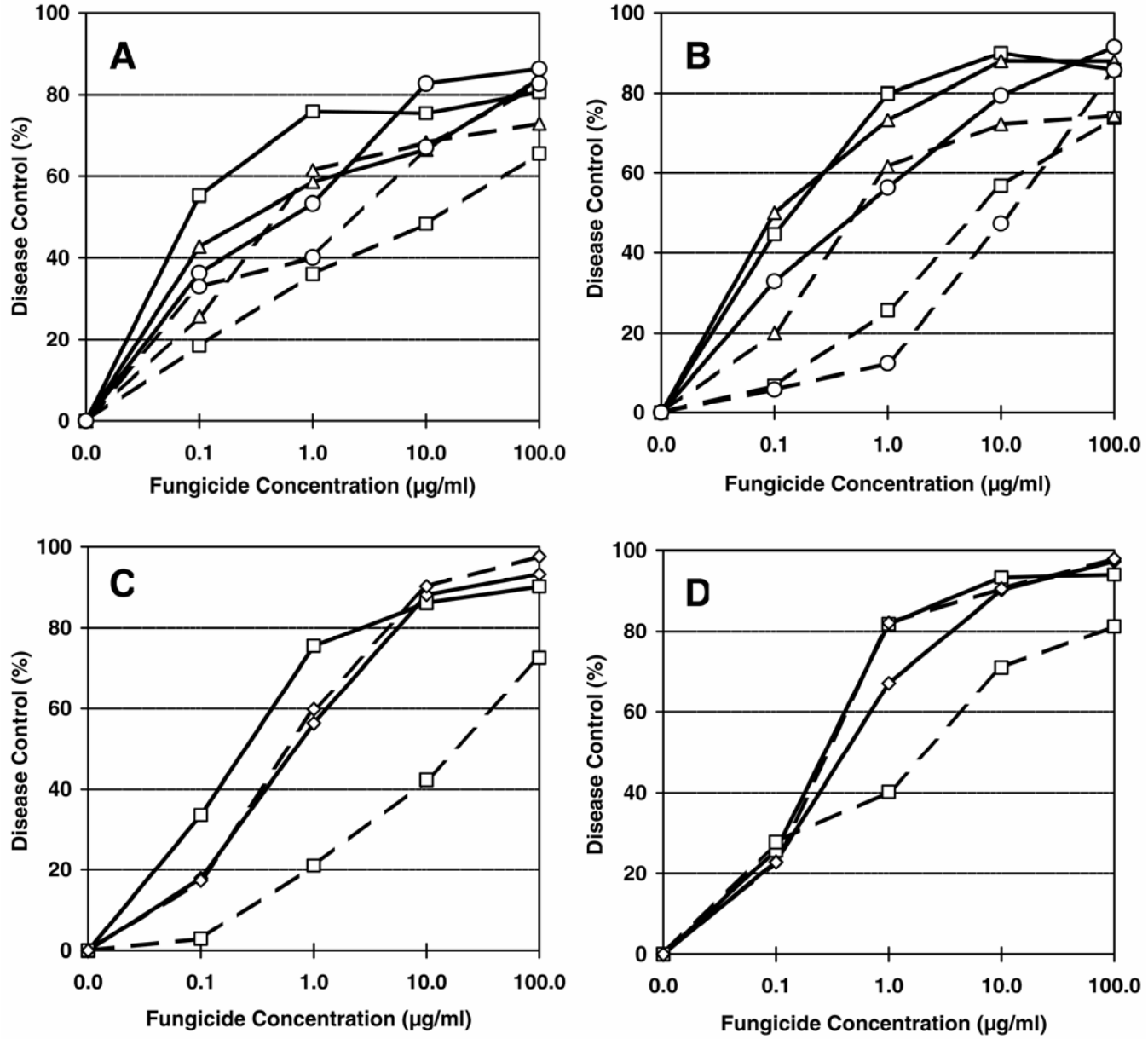

Fig. 4. Mean in vivo percentage disease control for sensitive (—) and reduced-sensitive isolates (---) of *Alternaria solani* to **A** and **B,** azoxystrobin (□), famoxadone (Δ), and fenamidone (○); and **C** and **D,** azoxystrobin (□) and boscalid (◇) at each fungicide concentration. The first subset of *A. solani* isolates tested included two sensitive and three-reduced-sensitive isolates for **A,** axoystrobin, famoxadone, and fenamidone and **C,** axoystrobin and boscalid. The second subset of *A. solani isolates* for azoxystrobin, famoxadone, and fenamidone included **B,** three isolates for both the sensitive and reduced-sensitive groups and **D,** four sensitive and two reduced-sensitive for boscalid.

attributed to the action of multiple genes (14). In addition, because famoxadone is in the oxazolidinedinone subgroup of the Q_oI chemical class of fungicides (1,11), whereas fenamidone is in the imidazolinones subgroup (1), there may be stoichiometric differences that also allow famoxadone to be largely unaffected by the F129L mutation present in the *A. solani* population. However, we cannot discount the fact that, once the *A. solani* population becomes exposed to famoxadone through routine use by the potato industry, this pathogen could be prone to develop resistance to this Q_oI fungicide quite readily, given the short time frame in which it developed reduced sensitivity to azoxystrobin (23). As hypothesized with *V. inaequalis*, these quantitative shifts may be precursors to the qualitative shift (14). Obviously, the early blight fungal population must be monitored carefully for such a shift in sensitivity.

Our previous studies demonstrated that a twofold loss of sensitivity to trifloxystrobin did not affect early blight disease control under greenhouse conditions (23). However, trifloxystrobin never was as effective on a sensitive wild-type *A. solani* population as azoxystrobin or pyraclostrobin (23,28). The presence of the F129L mutation effectively reduced the disease control that azoxystrobin and pyraclostrobin provided of a sensitive population to the level of control provided by trifloxystrobin. This means that, despite the fact that the F129L mutation did not appreciably affect trifloxystrobin disease control, potato growers are unable to take advantage of this because this fungicide provides early blight disease control equal to that of standard protectants (28), except at a higher cost. In studies reported here, we demonstrate a similar situation with famoxadone and fenamidone in that the F129L mutation causes only a two- to threefold loss of sensitivity in vitro and early blight disease control is largely unaffected. Unfortunately, because fenamidone lacks a high intrinsic level of activity on *A. solani*, potato growers are unable once again to take advantage of this through improved early blight disease control. However, the F129L mutation in *A. solani* does not appreciably affect disease control provided by famoxadone on a reduced-sensitive population; therefore, this fungicide chemistry would appear to be an excellent choice, especially under disease pressure conditions of the Midwestern United States, when standard protectants fail to provide adequate control.

The existence of *A. solani* isolates possessing the F129L mutation and reduced-sensitivity to azoxystrobin that are significantly more sensitive to boscalid in vitro than the wild type is not easily explained, especially because azoxystrobin and boscalid inhibit respiration at different sites. However, the negative cross-sensitivity to boscalid among the azoxystrobin reduced-sensitive isolates tested did not appreciably affect disease control in vivo. Similar to the results reported here and elsewhere (23), a twofold difference in in vitro sensitivity does not appear to affect in vivo disease control provided by fungicides that affect fungal respiration. The level of early blight disease control provided by boscalid on a sensitive wild-type population and on an azoxystrobin reduced-sensitive population of *A. solani* is equal to that provided by azoxystrobin on a wild-type population. This is consistent with preliminary field studies, making boscalid an excellent early blight disease control option for potato growers in the Midwest.

Considering the rapidity with which *A. solani* developed reduced-sensitivity to azoxystrobin, we are concerned that, without proper resistance management strategies, this pathogen could also develop reduced-sensitivity to related Q_oI fungicides famoxadone and fenamidone and to the carboxamide fungicide boscalid. The wide range of sensitivity observed among wild-type *A. solani* isolates in baseline testing, an approximately 17-, 5-, and 10-fold in sensitivity to famoxadone, fenamidone, and boscalid, respectively, is concerning. However, because azoxystrobin reduced-sensitive *A. solani* isolates actually are more sensitive to boscalid in vitro then the wild-type, and given the higher degree of efficacy famoxadone has toward *A. solani* compared with fenamidone, we hypothesize that famoxadone:boscalid rotations may be an effective resistance management strategy along with traditional strategies that include tank mixing Q_oI fungicides with other modes of action, alternations with non-Q_oI fungicides, and preventative, not curative applications. This fungicide rotation also could act to effectively delay the development of resistance to boscalid as well. A famoxadone:boscalid rotation, as opposed to a boscalid:famoxadone rotation, takes advantage of the negative cross-sensitivity we report here between Q_oI fungicides and boscalid. Preliminary data from field surveys conducted in 2003 suggest that the use of Q_oI:boscalid rotations also may influence the *A. solani* population in which Q_oI-sensitive isolates increase in their frequency (J. S. Pasche and N. C. Gudmestad, *unpublished data*). Additional studies are underway to investigate more closely this phenomenon.

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