Shift in Sensitivity of *Alternaria solani* in Response to Q_oI Fungicides

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ABSTRACT

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Isolates of *Alternaria solani*, cause of potato early blight, collected in 1998 through 2001 from various potato growing areas across the midwestern United States, were tested for sensitivity to azoxystrobin. Isolates collected in 1998, prior to the introduction of azoxystrobin, were tested to establish the baseline sensitivity of the fungus to this fungicide. Isolates collected in subsequent years, not necessarily from the same sites as baseline isolates, were tested to determine if populations of *A. solani* had become less sensitive to azoxystrobin. Azoxystrobin sensitivity was determined utilizing an in vitro spore germination assay. The effective fungicide concentration that inhibited spore germination by 50% (EC_{50}) was determined for each isolate. There was no significant difference in mean EC_{50} values between baseline isolates and all other isolates collected through 1999. Mean azoxystrobin EC_{50} values of *A. solani* isolates collected in 2000 and 2001 were significantly higher compared with means from previous years, and mean azoxystrobin EC_{50} values from 2001 were significantly higher than means from isolates collected in 2000. A subset of 54 *A. solani* isolates was evaluated in vitro for cross-sensitivity to pyraclostrobin and trifloxystrobin. A highly significant and strong correlation among the isolates tested for fungicide cross-sensitivity was detected between azoxystrobin and pyraclostrobin; however, the correlation between azoxystrobin and trifloxystrobin, and between trifloxystrobin and pyraclostrobin, was significant but weak. A second subset of five isolates was chosen for in vivo assessment of azoxystrobin, pyraclostrobin, and trifloxystrobin sensitivity. Disease severity on plants treated with azoxystrobin and pyraclostrobin was significantly greater with reduced-sensitive *A. solani* isolates compared with sensitive isolates. Disease severity was not statistically different between azoxystrobin reduced-sensitive and sensitive *A. solani* isolates on plants treated with trifloxystrobin. This is the first report of a shift in sensitivity to Q_oI fungicides in a fungus possessing only an anamorphic stage.

Additional keywords: reduced sensitivity, *Solanum tuberosum*

Potato early blight occurs worldwide and is prevalent wherever potatoes are grown. *Alternaria solani* Sorauer has long been recognized as a foliar pathogen of potato (*Solanum tuberosum* L.) and is considered to be a difficult pathogen to control (3), largely because few cultivars possess resistance (2). The most effective early blight control measure is frequent application of protectant fungicides from early in the growing season until vine desiccation (12).

Late in the 1998 growing season, North Dakota, Minnesota, Nebraska, and Wisconsin were granted emergency use labels for azoxystrobin (Quadris, Syngenta Crop Protection, Greensboro, NC) by the Environmental Protection Agency (EPA); full registration was granted in 1999. Initially, excellent early blight control was provided by this new chemistry; however, reduced disease control was first observed in isolated commercial potato fields in 2000. Quinol-oxidizing inhibitor (Q_oI) fungicides, such as azoxystrobin, have a single site mode of action, interfering with the electron transport of the cytochrome bc_1 complex (4). Related Q_oI chemistries trifloxystrobin (Gem, Bayer Corporation, Agricultural Division, Kansas City, MO) and pyraclostrobin (Headline, BASF Corporation, Agricultural Products, Research Triangle Park, NC), registered for use on potato in 2001 and 2002, respectively, have the same mode of action as azoxystrobin.

Previous research indicated that a shift in sensitivity to azoxystrobin in field isolates of *A. solani* might have been detected as early as 1999 (5). Based on these preliminary results and field observations of inadequate early blight control by azoxystrobin, a study was conducted to determine if a shift in the sensitivity of *A. solani* to azoxystrobin has occurred. The specific objectives of the study were: the establishment of an in vitro baseline sensitivity distribution of *A. solani* isolates collected from commercial potato fields in 1998, prior to azoxystrobin registration and use; determination of in vitro azoxystrobin sensitivity of *A. solani* isolates collected in 1998, 1999, 2000, and 2001, subsequent to azoxystrobin registration; establishment of in vitro baseline sensitivity to pyraclostrobin and trifloxystrobin; and determination of the significance of the in vitro shift in sensitivity of *A. solani* to azoxystrobin, pyraclostrobin, and trifloxystrobin on disease control.

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MATERIALS AND METHODS

Collection, isolation, and maintenance of *A. solani.* Isolates of *A. solani* were recovered from potato foliar and tuber tissue submitted to our laboratory from various areas throughout the midwestern United States. *A. solani* isolates tested from 1998 and 1999 were obtained via single spore isolation from hyphal tip cultures (5). Emergency use labels for azoxystrobin were granted and first applications were made after 30 July 1998 in Minnesota, North Dakota, Nebraska, and Wisconsin, and full label registration was not available in other states until 1999. Therefore, only isolates collected prior to these dates in each respective state were included in the baseline assessment. A total of 21 isolates, collected in 1998 after the emergency registration of azoxystrobin in Minnesota, North Dakota, Nebraska, and Wisconsin, were placed into a "1998 nonbaseline group." These isolates are included in a separate nonbaseline group because they were collected after azoxystrobin had been used in commercial potato fields during the 1998 growing season and we cannot discount the possibility that they were exposed to this fungicide. Isolates tested from 2000 and 2001 were obtained via single spore isolation directly from diseased tissue and transferred to petri plates containing clarified V8 medium (CV-8) (Campbell's V8 juice, 100 ml; $CaCO_3$, 1.5 g; agar, 15 g; and distilled water, 900 ml). Isolates collected during each of the 4 years were not necessarily collected from the same sites within each state.

Isolates were preserved in long-term cryogenic storage. Each isolate was grown on CV-8 for 7 to 10 days under 24-h light at room temperature (25 ± 2°C) until the agar surface was covered with profuse mycelium and spores. Agar plugs with fungal mycelia and conidia were excised using a sterilized 4-mm cork borer. Plugs were placed in small screw-top centrifuge tubes and placed with loosened lids in the laminar flow hood for 24 to 48 h to remove moisture from the medium. The tubes were sealed with Parafilm and stored at –80°C.

Herbarium specimens were also prepared from each tissue sample from which *A. solani* isolates originated.

In vitro assessment of Q_oI sensitivity. An experiment was conducted to determine the effect of salicylhydroxamic acid at 100 µg/ml (SHAM) (Sigma Chemical Co., St. Louis, MO) on spore germination of *A. solani* (10). SHAM is frequently added to media to prevent the fungus from overcoming the toxicity of a Q_oI fungicide through an alternative oxidative pathway (7,10,15). A stock solution of 10,000 µg/ml SHAM in methanol was prepared by adding 100 mg of SHAM to every 1 ml of methanol and warming in a 37°C water bath for 1 to 2 min to completely dissolve the SHAM. The final concentration of methanol in both amended and unamended media was 0.1% by volume. Thirty-one *A. solani* isolates, representing 1998 and 2001 isolate collections, were randomly selected, and conidia from these isolates were plated onto water agar with and without SHAM.

Technical formulations of azoxystrobin (95.3% a.i.), pyraclostrobin (93.5% a.i.), and trifloxystrobin (99.4% a.i.) were dissolved in acetone (10.5, 10.7, and 10.1 mg of fungicide per ml, respectively) to obtain stock solutions of 10,000 µg/ml. Azoxystrobin sensitivity was determined by comparing spore germination on 1.5% water agar amended with 0.0, 0.01, 0.1, 1.0, and 10.0 µg/ml azoxystrobin and 100 µg/ml SHAM (10). For cross-sensitivity assessments, azoxystrobin, pyraclostrobin, and trifloxystrobin sensitivities were determined by comparing spore germination on 2% water agar amended with 0, 0.001, 0.01, 0.1, 1.0, and 10.0 µg/ml technical formulations of each fungicide and 100 µg/ml SHAM. The final concentration of acetone in the media was 0.1% by volume.

Sterile distilled water containing 5 drops of Tween 20 (Sigma) per 100 ml was added to *A. solani* cultures, and conidia were dislodged using a sterile glass rod. The concentration of the conidial suspension was adjusted to 1×10^5 conidia per ml using a hemacytometer, and 500 µl of the conidial suspension of each isolate was added to two replicate plates of each fungicide dilution. The suspension was spread across each plate using a sterile glass rod. The plates were allowed to partially dry in a laminar flow hood for approximately 20 min. Each plate was sealed with Parafilm and held in a Precision incubator (GCA Corporation, Chicago, IL) with continuous light at 21°C for 4 h. For the Q_oI cross-sensitivity testing, a few changes were implemented into the technique in order to increase efficiency. These changes included the use of 2% laboratory grade agar (Fisher Scientific A360-500) and the omission of Tween 20 in the water used to prepare the spore suspension. A further modification to the spore germination assay was that petri plates were not dried or sealed with Parafilm prior to incubation. Plates not sealed with Parafilm dried sufficiently for clear examination of the spores during the 4-h incubation. 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.

The concentration that effectively reduces germination by 50% relative to the untreated control (EC_{50}) was determined for each isolate tested in the in vitro azoxystrobin sensitivity study. The mean number of conidia germinated for two replicates was converted into a percent reduction of germination relative to the untreated 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.

A total of 180 *A. solani* isolates was evaluated within the azoxystrobin sensitivity assessment in 12 separate trials, 7 to 24 isolates per trial. Internal controls (isolates 13-1, sensitive to azoxystrobin, and 526-3, which is azoxystrobin reduced-sensitive) were included in each of the 12 trials to determine the reproducibility of the germinated spore test. The assay reproducibility calculations used by Wong and Wilcox (17) were applied to the internal control isolates. The assay reproducibility calculations generated the approximate limits for a 95% confidence interval for the two internal controls. These limits were approximated because the Land's Coefficients had to be estimated. Only those experiments in which the EC_{50} values of the internal controls were within the 95% confidence interval were included in further statistical analyses. Each isolate tested in an experiment was set in a completely randomized design with two replications per fungicide concentration. Fifty conidia were examined per replication. All experiments were performed twice. Based on EC_{50} values from the in vitro azoxystrobin sensitivity assessment, isolates were placed into two groups, azoxystrobin sensitive and azoxystrobin reduced-sensitive.

Trials were analyzed separately and an *F* test was performed to test for homogeneity of variance among the experiments. The analysis was performed using the general linear models procedure on the mean EC_{50} values using the Statistical Analysis System (SAS Institute, Cary, NC). Further analyses were conducted using the combined EC_{50} values for the 32 baseline isolates compared with those with exposure to azoxystrobin, including the 21 isolates from 1998 not included in the baseline, the 11 isolates from 1999, the 13 isolates from 2000, and the 103 isolates from 2001. Mean EC_{50} values for each isolate were separated using the least significant difference test (LSD, $P = 0.05$). It is important to note that results for 2001 are biased as a consequence of isolates being collected primarily from fields in which azoxystrobin applications had yielded insufficient early blight disease control.

A subset of 54 *A. solani* isolates previously tested in vitro for sensitivity to azoxystrobin were tested in vitro for cross-sensitivity among the Q_oI fungicides azoxystrobin, pyraclostrobin, and trifloxystrobin. Isolates were chosen both by geographic location from which they originated and EC_{50} values obtained in the in vitro azoxystrobin tests. Twenty-six azoxystrobin sensitive *A. solani* isolates from 1998 were selected for the in vitro cross-sensitivity assessment, 25 of which had been tested previously in the in vitro azoxystrobin sensitivity experiments and were determined to have EC_{50} values within the range of the baseline isolates. Twenty-seven azoxystrobin reduced-sensitive isolates of *A. solani* from 2001 were selected, 22 of which had been determined previously to have EC_{50} values above the range established for the baseline isolates. An additional *A. solani* isolate was included that had an azoxystrobin EC_{50} value between the baseline and reduced-sensitive ranges.

Isolates were tested in 10 separate trials, 4 to 16 isolates per experiment. Control isolates were included in the first eight trials to ensure the results obtained from the new methods correlated with the previously established results. For each fungicide, *t* tests were used to detect differences in the mean EC_{50} values between the azoxystrobin sensitive and azoxystrobin reduced-sensitive groups of isolates using the Statistical Analysis System. Correlation analysis was also performed on the EC_{50} values from azoxystrobin, pyraclostrobin, and trifloxystrobin in vitro cross-sensitivity assessment using the Persons correlation coefficients as a measure of the relationships among the three fungicides.

Effect of isolate sensitivity to Q_oI fungicides on disease control. A subset of five isolates of *A. solani* previously tested in vitro for sensitivity to azoxystrobin, pyraclostrobin, and trifloxystrobin were selected for an in vivo assessment to determine the effect of azoxystrobin reduced-sensitivity on disease control provided by these three Q_oI fungicides. The isolates were selected based on the EC_{50} values obtained in the in vitro cross-sensitivity test and on the geographic location where the isolates were obtained. The isolates were recovered from long-term storage, maintained, and conidia were harvested as described previously for the in vitro Q_oI assessment.

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. Bonny Best (To-

tally Tomatoes, Augusta, GA). Tomato plants were utilized for the in vivo study because of this cultivar's susceptibility to early blight. Also, as opposed to potatoes, their compact size allowed for adequate replication while evaluating multiple fungicides and fungicide concentrations across several *A. solani* isolates. Five tomato seeds were sown in each 10-cm^2 pot, in Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA) without additional fertilizer. When the first true leaves were nearly fully expanded, plants were removed to obtain three uniformly sized plants per pot. The three plants were treated with commercial formulations of azoxystrobin (Quadris), pyraclostrobin (Headline), or trifloxystrobin (Gem). Tenfold fungicide concentrations of each active ingredient were applied to plants (0, 0.1, 1.0, 10.0, and 100.0 µg/ml) to obtain a dose response curve for each Q_oI fungicide. Each fungicide was applied to runoff 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 prewetted with distilled water applied using a hand sprayer and then inoculated with a 2.0×10^5 conidia per ml suspension from 12- to 14-day-old cultures of an *A. solani* isolate using a Preval paint-spray gun (Preval Sprayer Division, Precision Valve Corporation, Yonkers, NY). The inoculated tomato plants were held in individual mist chambers (>95% relative humidity [RH], 22 to 24°C, 16-h photoperiod) for 24 h, after which plants were transferred to confinement chambers (plastic chambers with open ceilings) on greenhouse benches. The plants were watered once per day, and the temperature in the isolation chambers was maintained at approximately 24 ± 2°C.

The experiment was a split-plot randomized complete block design. Confinement chambers, used to reduce the potential for cross contamination among *A. solani* isolates, were treated as the main blocking factor. Fungicides at each concentration were treated as the split-plots. Seven to 14 days postinoculation disease severity was rated visually by estimating percent infected leaf area of the first two true leaves (two subsamples) and recorded as percent diseased tissue. Three replications (three pots) and three samples (three plants per pot) were tested for each isolate × fungicide concentration. The experiment was performed three times. Levene's test (8) was conducted to test for homogeneity of variance among the three independent experiments at each fungicide concentration and between the two isolate groups (sensitive and azoxystrobin reduced-sensitive). For each fungicide concentration, disease severity data were transformed to percent disease control for further statistical analysis using the formula: [1 – (% diseased tissue/% diseased tissue in untreated plants) × 100]. The ANOVA was performed separately for each fungicide × isolate group combination at each fungicide concentration using the Statistical Analysis System, and *t* tests were used on the combined data to detect differences at each fungicide concentration.

Table 1. Mean in vitro conidial germination of *Alternaria solani* isolates in the presence and absence of salicylhydroxamic acid (SHAM) at 100 µg/ml

Isolate group	% Germination[a]	
	Without SHAM	With SHAM
1998	96.25 (9.30)[b]	97.94 (4.05)
2001	98.59 (3.01)	97.89 (3.01)

[a] No significant differences in % germination were observed between the SHAM and without SHAM treatments with 1998 (n = 7) (P = 0.3500) or 2001 isolate groups (n = 24) (P = 0.1581).

[b] Values in parentheses denote standard deviation.

RESULTS

In vitro assessment of azoxystrobin sensitivity. Among the 31 *A. solani* isolates tested, it was determined that 100 µg/ml SHAM had no deleterious effects on spore germination (Table 1). Therefore, SHAM was used at the rate of 0.1% vol/vol (100 µg/ml) in all subsequent in vitro Q_oI fungicide experiments.

Analysis of each in vitro azoxystrobin sensitivity experiment separately produced the error mean squares necessary to complete the *F* test for homogeneity of variance; therefore, the data were combined for further analysis. Significant differences in EC_{50} values were detected among *A. solani* isolates grouped by year (Fig. 1). Nearly all of the isolates examined in the azoxystrobin assessment could be clearly classified into two groups. One group of 76 isolates, with EC_{50} values less than 0.10 µg/ml, was clearly sensitive to azoxystrobin and was designated "azoxystrobin sensitive." Azoxystrobin sensitive isolates were collected in 1998, 1999, 2000, and 2001 (Table 2). A total of 100 *A. solani* isolates from 2000 and 2001 had EC_{50} values above 1.450 µg/ml and were designated as "azoxystrobin reduced-sensitive" and are hereby referred to frequently as reduced-sensitive for the purpose of clarity. One *A. solani* isolate collected in 1999 with an EC_{50} value of 0.250 µg/ml and three 2001 *A. solani* isolates with EC_{50} values of 0.775, 0.700, and 0.510 µg/ml were in a transition group between the obviously sensitive and reduced-sensitive groups (Table 2).

Sensitivity of *A. solani* to azoxystrobin among the 32 baseline isolates, expressed as EC_{50} values, ranged from 0.011 to 0.090 µg/ml (Table 2), with a mean EC_{50} value of 0.038 µg/ml (SD = 0.023) (Fig. 1). EC_{50} values obtained for the 21 *A. solani* isolates collected in 1998 after 30 July from states where emergency use registration of azoxystrobin was granted ranged from 0.020 to 0.077 µg/ml (Table 2), with a mean of 0.039 µg/ml (SD = 0.016), which is numerically higher than, but not significantly different from, the mean EC_{50} of the baseline isolates collected before azoxystrobin registration (Fig. 1). Eleven isolates collected from the 1999 growing season had a range of EC_{50} values from 0.031 to 0.250 µg/ml (Table 2), with a mean of 0.057 µg/ml (SD = 0.064), which also does not differ significantly from either the true baseline isolate group or the

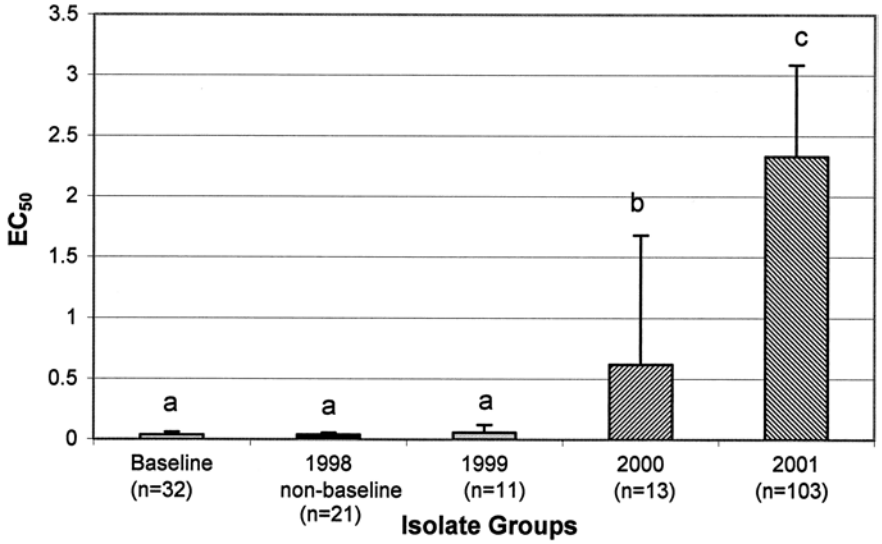

Fig. 1. Mean EC_{50} values (effective fungicide concentration that inhibited spore germination by 50%) for *Alternaria solani* isolate groups obtained from the in vitro azoxystrobin assessment with mean separation based upon the least significant difference (LSD). Columns with the same letter are not significantly different (P = 0.05). Vertical bars indicate standard deviation for all tests performed on each isolate group.

nonbaseline 1998 isolate group (Fig. 1). The 13 *A. solani* isolates tested from the 2000 growing season had a range of EC_{50} values from 0.032 to 2.550 µg/ml (Table 2), with a mean of 0.621 µg/ml (SD = 1.062), which is significantly higher than the mean EC_{50} values obtained for the baseline, nonbaseline 1998, and 1999 isolate groups (Fig. 1). Among 100 *A. solani* isolates assayed from 2001, the EC_{50} values ranged from 1.210 to 3.400 µg/ml (Table 2), with a mean of 2.333 µg/ml (SD = 0.755), which is significantly higher than any other group of *A. solani* isolates tested (Fig. 1). Three *A. solani* isolates obtained in 2001 were obtained from commercial potato fields in North Dakota in which azoxystrobin was effectively controlling early blight disease. These three isolates had EC_{50} values of 0.028, 0.037, and 0.041 µg/ml, within the range of baseline EC_{50} values reported in this study.

Table 2. Collection data and azoxystrobin assessment of EC_{50} values (effective fungicide concentration that inhibited spore germination by 50%) of *Alternaria solani* isolates collected from fields in the 1998, 1999, 2000, and 2001 growing seasons from several states

State of origin of *A. solani* isolates	No. of isolates	Azoxystrobin exposures	Mean EC_{50} (µg/ml)	EC_{50} range
1998 Baseline group[a]				
Minnesota	12	0	0.04 (0.02)[b]	0.01-0.08
Nebraska	14	0	0.03 (0.01)	0.02-0.05
North Dakota	1	0	0.03	
Colorado	1	0	0.09	
Idaho	2	0	0.05 (0.01)	0.04-0.05
South Dakota	1	0	0.03	
Texas	1	0	0.03	
1998 Nonbaseline group[c]				
Minnesota	14	2-3	0.04 (0.02)	0.02-0.08
Nebraska	3	2-3	0.04 (0.01)	0.03-0.04
North Dakota	2	2-3	0.04 (0.01)	0.04-0.05
Wisconsin	2	0-1	0.04 (0.02)	0.04-0.06
1999 Group				
Minnesota	8	6-8	0.07 (0.08)	0.03-0.25
North Dakota	3	6-8	0.04 (0.00)	0.03-0.04
2000 Group				
Nebraska	3	12-16	2.37 (0.24)	2.10-2.55
North Dakota	10	9-11	0.04 (0.01)	0.03-0.05
2001 Group				
Minnesota	8	13-14	2.91 (0.35)	2.45-3.35
Nebraska	76	15-18	2.28 (0.47)	0.51-2.85
North Dakota	19	13-14	2.43 (1.13)	0.03-3.40

[a] Isolates collected in subsequent years were not necessarily collected from the same sites as baseline isolates.
[b] Values in parentheses denote standard deviation.
[c] Isolates of *A. solani* collected after azoxystrobin received emergency registration in 1998 (~7/30/98) in North Dakota, Nebraska, Minnesota, and Wisconsin.

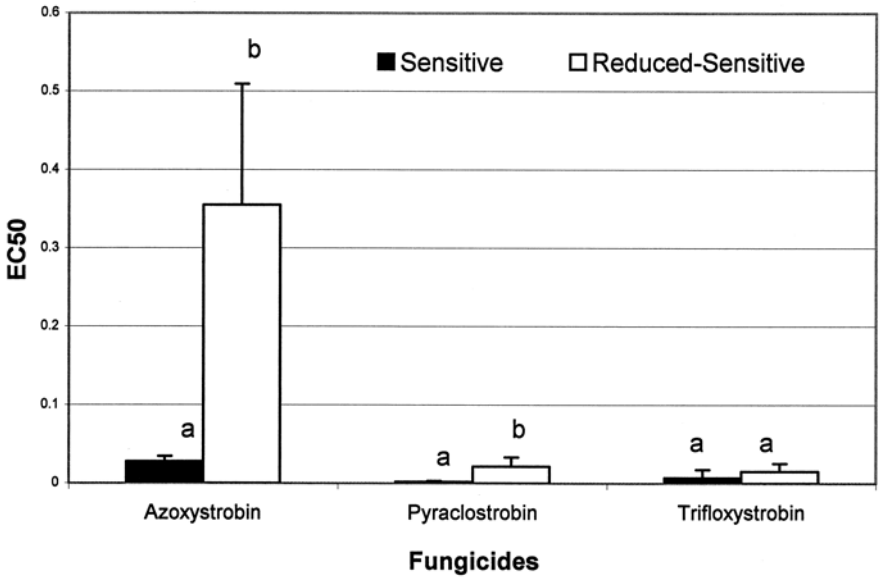

Fig. 2. Mean EC_{50} values (effective fungicide concentration that inhibited spore germination by 50%) for sensitive and reduced-sensitive *Alternaria solani* isolates obtained from the in vitro cross-sensitivity assessment of azoxystrobin, pyraclostrobin, and trifloxystrobin. Mean separation provided by Student's *t* tests ($P = 0.05$). Within fungicides, columns with the same letter are not significantly different. Vertical bars indicate standard deviation for all tests performed on each isolate group.

In vitro cross-sensitivity assessment. Highly significant differences ($P = 0.001$) were detected between the two isolate groups, sensitive and reduced-sensitive, among the three fungicides. The mean EC_{50} of the azoxystrobin reduced-sensitive isolates were nearly 13-fold and 10-fold higher than *A. solani* isolates sensitive to azoxystrobin and pyraclostrobin, respectively, resulting in significant differences between the two isolate groups in response to these two Q_oI fungicides (Fig. 2). The shift in sensitivity to trifloxystrobin between sensitive and reduced-sensitive *A. solani* isolates was only twofold and not significant, resulting in a significant isolate group × fungicide interaction ($P = 0.0001$).

Correlation of EC_{50} values for isolates exposed to azoxystrobin and pyraclostrobin were strong and highly significant (Fig. 3A). Correlations between EC_{50} values for azoxystrobin and trifloxystrobin (Fig. 3B) and between EC_{50} values for trifloxystrobin and pyraclostrobin were also significant but not as strong (Fig. 3C).

Effect of isolate sensitivity to Q_oI fungicides on disease control. Levene's test for homogeneity of variance indicated that among the 225 variances estimated (five isolates × three fungicides × five fungicide concentrations × three trials), all but 18 were homogeneous, and therefore data were combined for further analysis.

There were significant differences in disease control among the fungicides at all fungicide concentrations except 0.1 µg/ml ($P > 0.05$) (Fig. 4). Dose response curves indicate that sensitive *A. solani* isolates were controlled similarly at concentrations of 1.0, 10.0, and 100.0 µg/ml azoxystrobin and pyraclostrobin, which was substantially higher than the control provided by trifloxystrobin (Fig. 4). For all fungicides, disease control of reduced-sensitive isolates of *A. solani* was significantly less than the control provided by azoxystrobin and pyraclostrobin on sensitive isolates (Fig. 4). At higher fungicide concentrations (10.0 and 100.0 µg/ml) disease control of reduced-sensitive isolates with pyraclostrobin was significantly greater than disease control provided by azoxystrobin (Fig. 4). The amount of disease control provided by trifloxystrobin was not significantly different for sensitive and reduced-sensitive *A. solani* isolates at any concentration (Fig. 4).

DISCUSSION

Q_oI fungicides are inhibitors of spore germination (10); therefore, a spore germination assay was developed to determine the baseline sensitivity of *A. solani* to azoxystrobin, pyraclostrobin, and trifloxystrobin. It has been suggested that in vitro spore germination assays cannot be used to

determine sensitivities of fungal pathogens to these chemistries (9,16) and that in vivo assays are much more sensitive. In vitro spore germination assays have been successfully utilized (1), however, and it is apparent from the data reported here that in vitro spore germination assays were sufficiently sensitive to detect shifts in sensitivity to Q_oI fungicides in *A. solani*. Initial studies performed by our laboratory demonstrated approximately a 100-fold shift in mean EC_{50} values between azoxystrobin sensitive (0.011 to 0.091 µg/ml) and azoxystrobin reduced-sensitive isolates (1.45 to 3.40 µg/ml). However, with improved technique development during our studies, including the omission of Tween 20, and the drying and parafilming of the petri plates, we now know that the sensitivity of the baseline population is much more narrow (0.019 to 0.036 µg/ml) and that the shift in sensitivity within the azoxystrobin reduced-sensitive *A. solani* population is of much less magnitude, approximately 10-fold (0.145 to 0.625 µg/ml). With the use of an in vitro spore germination assay, we report here the first shift in sensitivity to Q_oI fungicides of a fungal plant pathogen with only an anamorphic stage and no known teleomorphic stage. To our knowledge, this is the first full report of a plant pathogen with no known sexual cycle developing resistance or reduced-sensitivity to Q_oI chemistry (11).

The in vitro spore germination assay used in these studies was also sensitive enough to detect cross-sensitivity of the three Q_oI fungicides investigated. It was interesting to note that baseline *A. solani* isolates were nearly 10 times more sensitive to pyraclostrobin and trifloxystrobin than they were to azoxystrobin, which supports the use of in vitro spore germination assays to successfully monitor populations for resistance development in *A. solani.* However, the greater intrinsic activity of pyraclostrobin on *A. solani* compared with azoxystrobin has not translated into increased disease control in the field. Previously conducted field experiments have demonstrated no difference in disease control between pyraclostrobin and azoxystrobin on sensitive isolates, while both fungicides were superior to trifloxystrobin (N. C. Gudmestad, *unpublished*). Clearly, intrinsic activity alone is not an adequate means to assess relative fungicide efficacy, as has been previously discussed with other Q_oI fungicides (16,17).

Cross-sensitivity tests demonstrated that isolates with reduced-sensitivity to azoxystrobin also possess reduced-sensitivity to trifloxystrobin and pyraclostrobin, despite the fact that these fungicides were not registered on potato until 2001 and 2002, respectively. The relationship of cross-sensitivity between azoxystrobin and pyraclostrobin is much stronger than it is between trifloxystrobin and either of the other two Q_oI fungicides. This may be due to the fact that azoxystrobin and pyraclostrobin are more closely related Q_oI fungicides and have been classified by the Fungicide Resistance Action Committee (FRAC) (information available online) into the methoxy-acrylate and methoxy-carbamate groups, respectively, while trifloxystrobin belongs to the oximino-acetate group. Additionally, the relationships between in vitro sensitivity of the sensitive and reduced-sensitive *A. solani* isolates to each Q_oI fungicide is reflected in the results of the in vivo studies. Azoxystrobin and pyraclostrobin provided similar levels of control of early blight disease caused by sensitive *A. solani* isolates, which was significantly superior to the control provided by trifloxystrobin, consistent with the aforementioned results in the

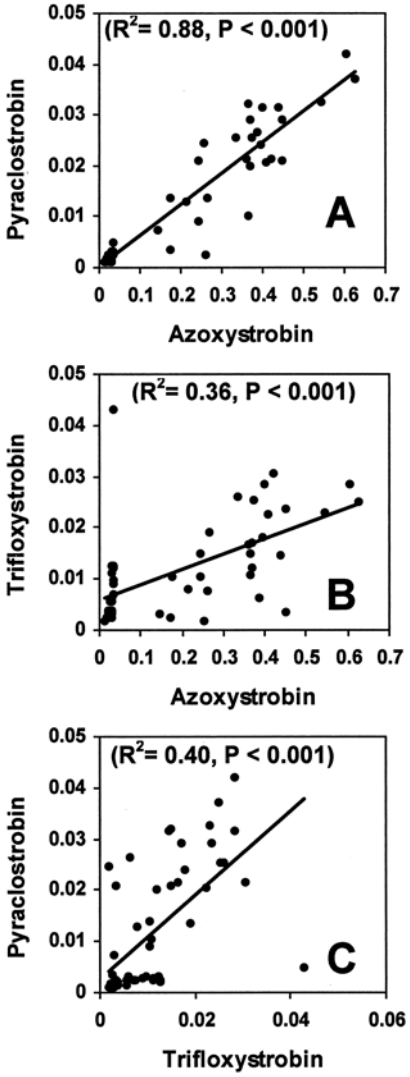

Fig. 3. Relationship between **A,** in vitro azoxystrobin and pyraclostrobin sensitivity, **B,** in vitro azoxystrobin and trifloxystrobin sensitivity, and **C,** in vitro trifloxystrobin and pyraclostrobin sensitivity of the 54 *Alternaria solani* isolates tested in the cross-sensitivity assessment. X and Y axes represent mean EC_{50} values (effective fungicide concentration that inhibited spore germination by 50%) in µg/ml for the respective fungicides.

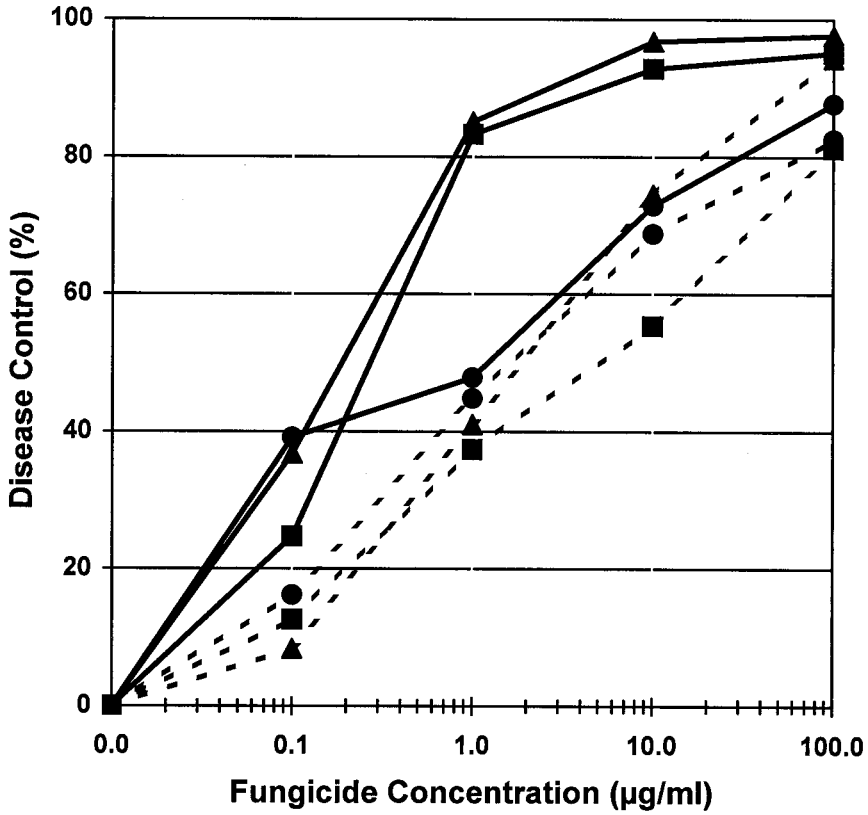

Fig. 4. Mean in vivo percent disease control for two sensitive (—) and three reduced-sensitive (---) isolates of *Alternaria solani* to azoxystrobin (■), pyraclostrobin (▲), and trifloxystrobin (●) at each fungicide concentration.

field (N. C. Gudmestad, *unpublished*). Azoxystrobin and pyraclostrobin provided significantly less control of disease caused by *A. solani* isolates with reduced-sensitivity to azoxystrobin compared with the level of control of disease caused by sensitive isolates. However, at higher concentrations, pyraclostrobin consistently had an advantage in controlling disease caused by reduced-sensitive isolates, perhaps due to its greater intrinsic activity. The shift in sensitivity was less pronounced in vitro with trifloxystrobin, and these results are reflected also in the in vivo studies. Disease control of reduced-sensitive isolates provided by trifloxystrobin was not significantly different than the level of control this fungicide provided for sensitive isolates.

We have been careful in our choice of terminology in reference to the shift in sensitivity of *A. solani* to Q_oI fungicides. In this specific case, we prefer to use the term "reduced-sensitivity" rather than resistance to reflect the shift in sensitivity. For example, in vivo assays performed demonstrated approximately a 40 to 50% loss of early blight disease control at 1.0 µg/ml when using azoxystrobin and pyraclostrobin compared with the level of disease control of a sensitive population at the same fungicide concentration. In our view, resistance to this fungicide class would equate to a 100% loss of disease control, at every concentration we tested, rendering the fungicide of no value to the potato producer. However, from the data reported here, the shift in sensitivity we detected does not result in a total loss of disease control, hence the use of the term "reduced-sensitivity" to describe the shift in sensitivity of the *A. solani* population in response to Q_oI chemistry.

Resistance to Q_oI fungicides has been reported previously in a number of plant pathogens. The cytochrome b gene has been sequenced for a number of these Q_oI-resistant plant pathogenic fungi, including *Venturia inaequalis* (18), *Erysiphe (Blumeria) graminis* f. sp. *tritici* (14), *Pyricularia* (*Magnaporthe*) *grisea* (7), *Pseudoperonospora cubensis* (6), *Podosphaera fusca* (6), and *Mycosphaerella fijiensis* (13). Comparing the DNA sequence of sensitive and Q_oI-resistant isolates of *E. graminis*, *P. cubensis*, *P. fusca*, and *M. fijiensis* revealed a single-point mutation leading to an amino acid change at position 143 from glycine (G) to alanine (A). Interestingly, six Q_oI-resistant isolates of *P. grisea* were found to possess the G143A mutation, while two isolates, which were not fully resistant to Q_oI fungicides in vivo, possessed a mutation resulting in an amino acid change at position 129 from phenylalanine (F) to leucine (L) (7,15). From the 180 *A. solani* isolates tested for sensitivity to azoxystrobin in this study, six isolates (521-1, 521-2, 523, 526-1, 526-2, 815-B) collected in 2001 and one baseline isolate (13-1) collected in 1998 prior to commercial use of azoxystrobin had the cytochrome b gene sequenced by researchers at Syngenta Crop Protection. None of the isolates tested had undergone the G143A mutation. However, all six isolates collected in 2001 had undergone the F129L mutation, while the baseline isolate had not (G. Olaya, Syngenta Crop Protection, *personal communication*). As observed with *P. grisea* (15), these isolates are not totally resistant to Q_oI fungicides in vivo, which supports our reference to the shift in sensitivity to azoxystrobin in *A. solani* as reduced-sensitivity rather than resistance. *A. solani* may be the only plant pathogenic fungus reported to date in which a disruptive shift in sensitivity is due solely to the F129L mutation rather than the G143A mutation. Interestingly, cross-sensitivity between Q_oI fungicides (azoxystrobin, kresoxim-methyl, trifloxystrobin) and other Q_oI fungicides such as famoxadone and fenamidone has been reported with a number of plant pathogenic fungi possessing the G143A mutation (1,4). We do not know if Q_oI reduced-sensitive isolates of *A. solani* possessing the F129L mutation are cross-sensitive with famoxadone and fenamidone, but those studies are warranted since registration of these Q_oI fungicides on potato is pending with the EPA.

We also do not know the extent to which the shift in Q_oI sensitivity in *A. solani* has occurred in other regions of the United States. Isolates in our collection were recovered primarily from the north central United States, from North Dakota to Nebraska. Early blight disease pressure in midwestern potato production areas is high primarily because of the frequent formation of dew at night. Dew formation, which provides the alternating wet and dry periods necessary for conidial formation, provides an environment that is generally conducive for disease development and hence frequent fungicide application. The shift in sensitivity of *A. solani* to azoxystrobin occurred within the first 2 years of use in the Upper Midwest, and occurred in potato fields in which the early blight fungus was exposed to approximately 12 to 16 azoxystrobin applications.

The first field observation suggesting a lack of early blight disease control with azoxystrobin was made by one of the authors in potato fields in an isolated potato production area of Nebraska during the summer of 2000. In these specific cases, five to six applications of azoxystrobin had been made to potato fields in that production area over the course of each of the 1999 and the 2000 growing seasons (10 to 12 total azoxystrobin exposures), with six being the maximum number of applications allowed by the label per growing season. Fungicide records maintained by the grower indicated that label recommendations were never compromised; i.e., no sequential applications, rotation with other fungicide chemistries, and full label rates used during each application (113 g a.i./ha). However, we cannot discount the possibility that the Section 18 emergency exemption for azoxystrobin that EPA granted four states in 1998 (Nebraska, North Dakota, Minnesota, Wisconsin) may also have played a detrimental role in the development of reduced-sensitivity in *A. solani* to this chemistry. Applications of azoxystrobin were made during the latter one-third of the growing season (after 30 July) to a potato crop in which early blight disease pressure was extremely high, hence the Section 18 request. Resistance management strategies frequently recommend that fungicides be applied preventatively, and the applications made to the potato crop late in the 1998 growing season were not consistent with this recommendation. Nonetheless, as a result of this disruptive shift in sensitivity to Q_oI fungicides in *A. solani*, a significant tool in the management of early blight on potato has been seriously compromised. Since Q_oI fungicides represent a premium-priced fungicide group, where the cost of the chemical was justified initially by the excellent early blight disease control that was derived from their use, it is now questionable whether or not the loss of efficacy in certain areas of the north central United States due to the presence of reduced-sensitive *A. solani* isolates can further justify their use. Potato producers in the Midwest, where early blight disease pressure is high, may be forced to more closely evaluate cost:benefit ratios before they apply a Q_oI fungicide in situations where early blight represents their only significant foliar disease.

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