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Resistance to Myclobutanil in Populations of *Venturia inaequalis* in Winchester, Virginia

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Abstract

Sterol-inhibiting (SI) fungicides are widely used to manage apple scab, caused by *Venturia inaequalis*. However, recent observations indicate that populations of *V. inaequalis* in orchards in Virginia have developed resistance to myclobutanil and other SI fungicides. Little is known about the frequency and distribution of fungicide resistance in apple scab populations in Virginia. Isolates of *V. inaequalis* were collected from three different apple orchards in Winchester, VA in 2006. Orchards were treated with myclobutanil on 12 April, 19 April, 1 May, 30 May, and 7 July. The sensitivity of 87 single-spored isolates of *V. inaequalis* to myclobutanil was determined by monitoring their growth on agar dishes amended with 0, 0.1, 0.5, or 1.0 µg/ml myclobutanil. A relative continuum of fungicide resistance was observed: 16 isolates were resistant, 40 isolates were moderately resistant, and 31 isolates were sensitive to myclobutanil. After 28 days, the mean growth of isolates collected from trees treated with myclobutanil was significantly greater than that of isolates collected from non-treated trees at all concentrations of myclobutanil tested in vitro. High levels of fungicide resistance found in populations of *V. inaequalis* suggest that replacement programs may need to be developed to manage apple scab in Virginia.

Introduction

Fungicide resistance poses a significant threat to crop production worldwide (20). This stable, inheritable adjustment in pathogen populations to a fungicide limits the efficacy and lifetime use of the fungicide (5,6,7,11). Fungicide resistance can lead to an entire class of fungicides being abandoned (6), with replacement programs usually leading to higher control costs or decreased efficacy.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter, is a devastating disease of apple (*Malus domestica*) worldwide (13). The disease results in unsightly lesions on fruit, which significantly decrease their commercial value (13). Myclobutanil, a sterol-inhibiting (SI) fungicide, has been one of the common fungicides used to control apple scab because it provides adequate control of apple scab when applied within several days following infection (23). In Virginia, SI fungicides have been used commonly in commercial orchards for 15 or more years as pre-bloom and immediate post-bloom sprays to manage scab, powdery mildew (25), cedar-apple rust, and quince rust (24). The number of applications made annually in individual orchards is related to varietal susceptibility to these diseases and the intended market of the fruit as fresh or processed. In some cases these have been routine applications, planned as part of the early season disease management program; in other situations they are applied only on an "as needed" basis as supplements for after-infection control.

There have been published reports of resistance to myclobutanil in populations of *V. inaequalis* in New York (9,23) and Michigan (9). There is also evidence of resistance to myclobutanil in Canada (9) and other regions of the world (3,15,19). Recently, Yoder (26) observed that myclobutanil and other SI fungicides were losing effectiveness for controlling apple scab in Virginia. An increased knowledge of the frequency and distribution of fungicide resistance in populations of *V. inaequalis* in Virginia is important for developing innovative, rational, and informed approaches to managing apple scab (10,18,20,21). Should resistance to myclobutanil persist in apple scab populations, alternative disease management strategies for apple scab must be developed.

Based on data collected by Yoder (26) and reports of apple scab resistance to the SI fungicides in the northeastern United States (9), we hypothesized that (i) populations of *V. inaequalis* in Winchester, VA orchards show various levels of resistance to myclobutanil, and (ii) isolates of *V. inaequalis* collected from apple trees treated with myclobutanil are less sensitive to myclobutanil than to isolates collected from non-treated trees. The specific objective of this study was to characterize the relative frequency of resistance to myclobutanil in *V. inaequalis* populations collected in Winchester, VA in 2006. An abstract on a portion of this work has been published (14).

Experimental Fields

Studies were conducted at the Virginia Tech Agricultural Research and Extension Center (AREC), near Winchester, VA in May, July, and August 2006. The AREC is located within 2 km of 300 ha of commercial apple orchards, most of which have been treated with SI fungicides for 15 years or more. Ethylene-bis-dithiocarbamate (EBDC) fungicides were usually combined with the SIs to broaden the disease spectrum and lengthen residual activity in these orchards. For more than 30 years, experimental and registered SI fungicides have been included in AREC fungicide tests directed at early season diseases, broad spectrum season-long management, or specific tests such as post-infection control of scab, rusts, or powdery mildew. This test pattern resulted in SI fungicide applications in some of the AREC orchard blocks nearly every year for 15 to 20 years, although the applications were made to just a limited number of trees totaling less than a hectare. Approximately 15 ha of additional AREC plot areas used for entomological and horticultural research were treated with SIs in combination with an EBDC fungicide, as in a commercial schedule (16), since 1988.

Mature test trees were selected from three orchard blocks on AREC property. Each of the blocks was represented by the following single apple cultivars: Ramey York/M.9 rootstock trees planted in 1999, Fuji/M.9 rootstock trees planted in 1995, or Gala/M.26 rootstock trees planted in 2000. The Fuji block was located 100 m west of the York block, and the Gala block was located 500 m to the south of the York and Fuji blocks. Some trees within these test blocks had SI usage prior to 2006, but this was relatively minimal compared to usage in nearby research and commercial orchards. The Fuji block had received 12 SI applications in 2003-2005, the Gala block had received six SI applications 2001-2005, and the Ramey York block had been treated with SIs only four times, all in 2005. In 2006, all three blocks were treated with the same rate of myclobutanil (Nova 40W) on the same days. The untreated trees were either part of the same row of trees or were included in rows immediately adjacent to treated trees. Myclobutanil was applied with a high pressure handgun sprayer to runoff at a rate of 1.25 oz/100 gal (9.36 g/100 liters, at approximately 935 liters/ha), on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July. Standard maintenance materials (16), applied to all trees in the test blocks on each application date, included esfenvalerate (Asana) oil, streptomycin (Agri-mycin), methoxyfenozide (Intrepid), methomyl (Lannate), phosmet (Imidan), imidacloprid (Provado), thiacloprid (Calypso), and carbaryl (Sevin XLR) and ethephon (Ethrel). No fungicides were applied to the Fuji block from 1995 to 2003.

Collection of Apple Scab Lesions and Culturing of *V. inaequalis*

Four to five leaves containing at least one scab lesion were collected from individual non-treated or treated trees in each block on 16 May, 6 July, and 4 August 2006. Leaves were placed in individual plastic bags, stored temporarily in a cooler, and transferred to the laboratory where they were maintained in refrigerated storage until further processing. In total, leaves were collected from 45 treated and 26 non-treated trees.

One lesion per leaf was removed with a razor blade and streaked across 2% water agar containing 50 µg/ml tetracycline, chloramphenicol, and streptomycin sulfate. Dishes were incubated at 25°C for 24 h, at which time individual germinated conidia were transferred to ¼-strength potato dextrose agar (PDA) dishes. Single-spored isolates were incubated on ¼ PDA at room temperature for 4 to 5 weeks or until the colony was 1.5 cm or larger. A total of 87 single-spored isolates of *V. inaequalis* was collected and used in this study.

Assays for Fungicide Resistance

Sterile 5 ml pipet tips were used to remove 2 mm diameter agar plugs from the margins of fungal colonies growing on ¼ PDA. Individual agar plugs were transferred to the centers of ¼ PDA dishes amended with myclobutanil at 0, 0.1, 0.5, and 1.0 µg/ml. Isolates were incubated at 19°C and the radial growth of colonies was measured weekly for 4 weeks. The diameter of each isolate was measured in two directions at a 90° angle, and the measurements were averaged. The test was replicated three times, and the colony growth for each isolate for each treatment was averaged across all three replications.

Statistical Analyses

Analysis of variance (ANOVA) was used to test for significant differences among fungicide treatments, isolates collected from treated and non-treated trees, and isolate collection date. Data were analyzed using PROC GLM in SAS (Windows, release 8.02, SAS Institute Inc., Cary, NC).

Results of Fungicide Resistance Assays

Mean colony growth on PDA amended with myclobutanil was greater for isolates collected from treated trees than isolates collected from non-treated trees (Table 1). Most of the isolates grew less at higher concentrations of myclobutanil (Table 1). The mean colony growth of isolates was significantly different among fungicide rates measured at 7 ($P < 0.001$), 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 days ($P < 0.001$). The mean colony growth of isolates collected from treated and non-treated trees was also significantly different when measured at 7 ($P = 0.013$), 14 ($P < 0.001$), 21 ($P < 0.001$), and 28 days ($P < 0.001$). However, the mean colony growth of isolates was not significantly different among collection dates when measured at 7 ($P = 0.764$), 14 ($P = 0.703$), 21 ($P = 0.401$), and 28 days ($P = 0.169$).

Table 1. Average colony diameter (cm) of 87 isolates of *Venturia inaequalis* on PDA amended with varying levels of myclobutanil.

Measurement of colony growth[x]	Myclobutanil concentration and source of isolates							
	0 µg/ml		0.1 µg/ml		0.5 µg/ml		1.0 µg/ml	
	Treated							
	No[y]	Yes[z]	No[y]	Yes[z]	No[y]	Yes[z]	No[y]	Yes[z]
7 days	0.588	0.458	0.495	0.422	0.479	0.442	0.432	0.417
14 days	1.034	0.850	0.693	0.658	0.685	0.517	0.542	0.583
21 days	1.514	1.438	0.955	0.992	0.937	0.833	0.710	0.742
28 days	2.000	2.338	1.312	1.483	1.263	1.175	0.986	1.067

[x] The radial growth of colonies of *V. inaequalis* was measured at 7 days, 14, 21, and 28 following dish inoculation.

[y] Isolates were collected from 26 trees that had not been treated with myclobutanil during the sampling year.

[z] Isolates were collected from 45 trees that had been treated with myclobutanil (Nova 40W) at a rate of 1.25 oz/100 gal dilute, applied to runoff. Fungicide treatments were applied on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July.

Isolates were classified as resistant, moderately resistant, or sensitive based on the percent growth reduction defined as the difference between colony growth on media amended with 0 and 1.0 µg/ml myclobutanil at 28 days. Isolates with equal growth across all fungicide treatments were classified as resistant, and isolates with minimal growth at 0.1, 0.5, and 1.0 µg/ml were sensitive (Fig. 1). Isolates were classified as resistant if the percent growth reduction was less than 25%, moderately resistant if the percent growth reduction was between 25 and 50%, and sensitive if the percent growth reduction was greater than 50% (Table 2). Thirty-one isolates were classified as sensitive, 40 isolates were moderately resistant, and 16 were resistant (Table 2). The frequency of resistant isolates was greater from treated trees (87.5%) than non-treated trees (12.5%).

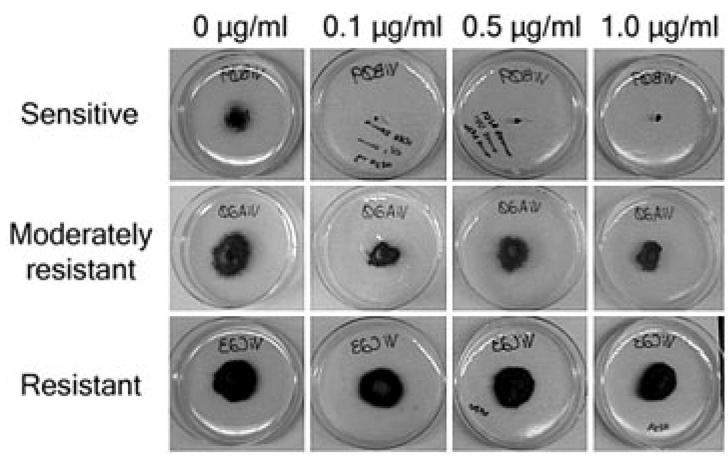

Fig. 1. Classification of fungicide resistance to myclobutanil in three representative isolates of *Venturia inaequalis* from Virginia. Isolates were classified as sensitive (top row), moderately resistant (middle row), or resistant (bottom row) to myclobutanil at four different myclobutanil concentrations in agar.

Table 2. Classification of fungicide resistance of 87 isolates of *Venturia inaequalis* based on percent growth reduction between 0 and 1.0 µg/ml myclobutanil at 28 days.

Classification[x]	**Total number of isolates**	**Percentage of isolates from treated trees**[y]	**Percentage of isolates from non-treated trees**[z]
Sensitive	31	28% (17/61)	54% (14/26)
Moderately resistant	40	49% (30/61)	38% (10/26)
Resistant	16	23% (14/61)	8% (2/26)

[x] Isolates were classified as resistant if the percent growth reduction was less than 25%, moderately resistant if the percent growth reduction was betwen 25 and 50%, and sensitive if the percent growth reduction was greater than 50%.

[y] Isolates were collected from 45 trees that had been treated with myclobutanil (Nova 40W) at a rate of 9.36 g/100 liters dilute at approximately 935 liters/ha (1.25 oz/100 gal dilute at approximately 100 gal/A), applied to runoff. Fungicide treatments were applied on 12 April (tight cluster-pink), 19 April (50% bloom), 1 May (petal fall), 30 May (first cover), and 7 July.

[z] Isolates were collected from 26 trees that had not been treated with myclobutanil during the sampling year.

Discussion

The findings of this study demonstrate that isolates of *V. inaequalis* from the AREC near Winchester, VA vary in their resistance to myclobutanil. Though we observed a continuum of fungicide resistance in our populations, the majority of the isolates were classified as either moderately resistant or resistant to myclobutanil, with the remainder classified as sensitive. Colony growth varied among isolates, fungicide rates, and between replicate growth tests. This is the first detailed report of fungicide resistance to myclobutanil in populations of *V. inaequalis* in Virginia, and to our knowledge, the first report of SI resistance in apple scab from orchards in the southeastern United States. This study represents an important baseline for monitoring fungicide resistance to myclobutanil in apple scab populations in Virginia, and can contribute to the knowledge of fungicide resistance in apple scab populations on a global basis (3,9,15,19,23).

Our data support our hypothesis that populations of *V. inaequalis* in Virginia show varying levels of fungicide resistance to myclobutanil. The percent growth reduction of 87 isolates ranged from 6 to 82 percent on 1.0 µg/ml myclobutanil. Our data also support our hypothesis that isolates of *V. inaequalis* collected from apple trees treated with myclobutanil have higher levels of resistance compared to isolates collected from non-treated trees. The majority of isolates classified as resistant to myclobutanil (87.5%) were collected from treated trees; 12.5% of isolates from non-treated trees were classified as resistant.

Fungicide resistance has been determined using sensitivity assays on agar media amended with varying levels of fungicide (8,9,10), using microscopic analysis of conidiophore formation, and measurements of germ tube length (12). Kunz et al. (12) compared microscopic analysis to other methods frequently used to evaluate fungicide sensitivity, and concluded that all of the methods were indistinguishable from each other when baseline sensitivities were determined. Thus, our results can be compared to others that are using similar methodologies to assay for fungicide resistance.

The frequency of myclobutanil-resistant isolates of *V. inaequalis* collected from treated trees was higher than in isolates collected from non-treated trees. This might be due, at least in part, to the exposure of isolates from treated trees to treatments of myclobutanil over the course of the growing season. Köller and Wilcox (10) observed a similar trend with the SI fungicide fenarimol. They showed that the frequency of fenarimol-resistant isolates significantly increased over three consecutive growing seasons in a New York apple orchard following 12 fungicide applications. If the selection pressure for myclobutanil resistance is similar to fenarimol resistance, then 12 or more applications of myclobutanil or myclobutanil-based sprays may select for higher levels of fungicide resistance. As part of our ongoing research program, we are examining the most appropriate time to select scab lesions that have been treated with myclobutanil, but were least controlled. This would be the most likely way to detect a resistant population, given our limited ability to forecast the onset of scab fungicide resistance beyond an individual growing season.

We found no evidence that the collection date influenced fungicide resistance in our populations. Consequently, resistance assays may be conducted at any time of the year. The lack of an observed increase in fungicide resistance may be due to the aerial transport of spores from adjacent orchard blocks (1,2), or recirculation of spores from baseline inoculum within the sampled block (4). In addition, it is possible each collection date consisted of both older and newer lesions (13), since all lesions were not removed from a tree and older sampled lesions were not labeled as such. The selection pressure also may not have been high enough to favor one resistant genotype over another.

The majority of the *V. inaequalis* isolates collected for this study were classified as either moderately resistant or resistant to myclobutanil. Since fungicide resistance can persist in apple scab populations, alternative disease management strategies for apple scab may be needed. Restricting the use of myclobutanil and other SIs for a period of years may help lower *V. inaequalis* resistance in orchards with an extensive myclobutanil history, but to our knowledge, detailed studies examining this phenomenon have not been conducted. However, SIs have also been highly effective and widely used in Virginia for control of powdery mildew, cedar-apple rust and quince rust, and these uses would continue to exert selective pressure on the fungal population.

Future research may help to identify new tools (e.g., on-site real-time PCR) to rapidly detect and monitor fungicide resistance in populations of *V. inaequalis* (17,22). Additional knowledge about the frequency, timing, and selective processes contributing to SI resistance will be necessary to identify effective and appropriate management strategies for apple scab in the future.

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