Growth Sensitivity of *Corynespora cassiicola* to Thiophanate-methyl, Iprodione, and Fludioxonil

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**Abstract**

An African violet production facility has relied solely on thiophanate-methyl, a site-specific fungicide that possesses a high risk for pathogens to develop resistance, to manage a devastating *Corynespora* leaf spot problem. During a disease outbreak in September 2007, 325 isolates of *C. cassiicola* were collected and 40 isolates were randomly selected to determine the pathogen's sensitivity for mycelium growth on agar amended with various concentrations of thiophanate-methyl, iprodione, or fludioxonil. EC50 values, concentration resulting in a 50% reduction in mycelium growth, were determined and indicate a population that currently is sensitive to all three fungicides. Due to the high risk of the pathogen developing resistance to thiophanate-methyl and iprodione, a moderate risk for cross-sensitivity between iprodione and fludioxonil, and phytotoxicity and visual residue problems with protective fungicides such as chlorothalonil, a fungicide rotation is recommended with fludioxonil as the main chemical selection.

**Introduction**

*Corynespora* leaf spot, caused by *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei, can cause devastating epidemics on African violets (*Saintpaulia ionantha* H. Wendl.). The disease is characterized by water soaked lesions that expand rapidly on leaf surfaces and petioles (21). A large greenhouse facility in Tennessee that produces more than 10 million African violets annually has experienced epidemics of *Corynespora* leaf spot for more than five years. The facility receives cuttings from several overseas operations and distributes to a large number of businesses. As a result, the pathogen's population likely includes an influx and efflux of isolates, typical of many greenhouse and nursery commodities. Outbreaks at the TN facility occur mostly from May through September (31). Symptoms occur on plants in all stages of production and can be severe enough that plants are discarded. In an effort to eliminate inoculum, thousands of plants have been discarded during a single episodic event. Common cultural and sanitation practices alone have not provided adequate control. To date, fungicides are the only tool that has restricted disease development.

Thiophanate-methyl (Cleary 3336; W.A. Cleary Corp., Somerset, NJ) and chlorothalonil (Daconil; Syngenta Crop Protection, Greensboro, NC) were effective in reducing incidence of *Corynespora* leaf spot in experiments conducted at the facility (31). Due to phytotoxicity and visible residue issues resulting from application of chlorothalonil, thiophanate-methyl has been solely relied upon for control of *Corynespora* leaf spot.
Thiophanate-methyl is a methyl benzimidazole carbamate (MBC) fungicide [FRAC code 1 (7)]. MBC chemicals inhibit nuclear division that consequently stops hyphal growth. MBC fungicides have been classified as being high risk for selecting resistance in pathogen populations, and resistance has been documented for a number of pathogens in other crops (11,12,26,29,33).

Continued use of this single fungicide product at a greenhouse operation increases the probability of chemical control failure, and could be a factor in the Tennessee facility. One objective of this research was to establish a thiophanate-methyl sensitivity profile for the *C. cassiicola* population, since it has already been subjected to continuous selection pressure at this production facility. An additional objective was to establish sensitivity profiles for iprodione and fludioxonil, which were selected because these products have not been used on this crop, but are registered for use on many greenhouse and field crops that serve as hosts for *C. cassiicola*. Iprodione is a dicarboximide fungicide (FRAC code 2) that inhibits spore germination and mycelium growth by affecting lipid synthesis and metabolism (28). Fludioxonil is a phenylpyrrole fungicide (FRAC code 12) that inhibits spore germination and mycelium growth by affecting the osmotic signal transduction pathway (8,10,13,18). By establishing sensitivity profiles of these three fungicides for the *C. cassiicola* population, the influence of previous exposure to thiophanate-methyl can be assessed, the similarity of iprodione and fludioxonil efficacy to baseline data of other organisms can be determined, and recommendations for product use patterns can be developed.

**Fungal Isolate Collection**

Leaves of African violets symptomatic for Corynespora leaf spot were collected at a large African violet production facility during a disease outbreak in September 2007. Symptomatic leaves were individually placed into sterile polyethylene bags and transported to the lab. Excised lesions were submerged in a solution of 10% sodium hypochlorite and 5% ethanol for 30 sec, and then rinsed in sterile deionized water for 10 sec (4) and set on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with chlorotetracycline hydrochloride (8 μg/ml) and streptomycin sulfate (8 μg/ml). Cultures were grown in an incubator at 23 ± 1°C with a diurnal 12-h light and dark cycle for 48 h before transferring the leading edge of mycelium to unamended PDA. Isolates of *C. cassiicola* was identified based on colony and conidial morphology (25). Each of 325 collected isolates were grown on sterile cotton stems embedded in 75 ml PDA in GA-7 culture vessels (Magenta Corp., Chicago, IL) at 23 ± 1°C with 12-h light and dark cycles.

**Isolate Sensitivity to Fungicides**

Forty isolates were selected randomly to assess their sensitivity to the selected fungicides. Technical grade thiophanate-methyl (97% a.i.; W.A. Cleary Corp., Somerset, NJ), iprodione (99% a.i.; Bayer Environmental Science, Research Triangle, NC), and fludioxonil (98% a.i.; Syngenta Crop Protection, Greensboro, NC) were dissolved in acetone to provide stock solutions of 20 mg/ml that were added to partially cooled PDA to obtain concentrations of 0, 0.01, 0.1, 1, 10, and 100 μg/ml. Acetone was added to achieve equal concentrations in all dilutions.

Plugs of the leading mycelium edge from twelve-day-old cultures were placed upside down on PDA amended with fungicide or unamended media (25). Each fungicide concentration and isolate combination was replicated three times. Cultures were incubated for 11 days at 23 ± 1°C in complete darkness. Mean colony diameter was obtained for each culture by taking two perpendicular measurements, with the original 5-mm plug diameter subtracted from each measurement (16,17). The experiment was repeated once.

Preliminary linear mixed model analysis indicated no significant difference (*P > 0.1*) between the two runs; therefore, runs were combined for each isolate and fungicide concentration to determine EC50 (effective concentration that reduces mycelial growth by 50%) values. Lack-of-fit tests were also conducted to compare fit of nonlinear and simple linear regression models to the data for each fungicide and isolate. In this study, simple linear regression models...
provided a poor fit \( P \leq 0.05 \), whereas the nonlinear models (log-logistic dose-response curve) provided an acceptable fit \( P > 0.05 \).

Fungicide concentrations were logarithmically \( \log_{10} \) transformed and data were analyzed using nonlinear regression analysis with the NLIN procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). The following log-logistic dose-response function was used,

\[
y = C + \frac{D - C}{1 + e^{b\left(\log_{10}(x) - \log_{10}(EC_{50})\right)}}
\]

where \( y \) is the mean colony diameter growth response, \( x \) is the fungicide dose, \( C \) is the lower growth limit, \( D \) is the upper growth limit, \( b \) is the slope, and \( EC_{50} \) is the dose giving the 50% response \((23,30)\). The log-logistic dose-response curve is used in studies where the dose response ranges from no effect to complete inhibition \((23,30)\).

A resistance factor (RF) for each fungicide was defined as the ratio of the least sensitive isolate’s \( EC_{50} \) value to the mean \( EC_{50} \) for that fungicide \((19)\). Cross-sensitivity relationships between fungicides were calculated using the \( EC_{50} \) values for each isolate to generate Pearson correlation coefficients \((r)\) for each pair of fungicides.

**Sensitivity to Thiophanate-methyl**

A true baseline distribution is not represented within this study for thiophanate-methyl because of the previous reliance on this chemical for control of *C. cassiicola* at the production facility. The range of thiophanate-methyl \( EC_{50} \) values was 0.0157 to 0.1539 \( \mu g/ml \) (Fig. 1) and the mean value was 0.0553 \( \mu g/ml \). Despite the past exposure to thiophanate-methyl, the \( EC_{50} \) range falls within the sensitive grouping for several pathogens \((24,26,33,34)\), and overlaps but extends slightly above the sensitive range for a few pathogens \((28)\). The upper \( EC_{50} \) value (0.1539 \( \mu g/ml \)) is well below the discriminatory doses of 1 \( \mu g/ml \) \((24)\) and 10 \( \mu g/ml \) \((32,34)\) previously used to identify fungal isolates resistant to thiophanate-methyl. The RF value of 2.8 was similar to the RF value for a sensitive population of *Fusicladium effusum* sensitive to thiophanate-methyl \((24)\).
To the best of our knowledge, this study represents the first report of the sensitivity of previously unexposed greenhouse collected isolates of *C. cassiicola* to dicarboximide fungicides. In this study, iprodione EC50 values ranged from 0.0833 to 0.6478 μg/ml (Fig. 2) and the mean value was 0.2828 μg/ml. This sensitivity range is similar to other reports of a baseline sensitivity for iprodione (15,17), and for previously treated fungal populations that were still sensitive to iprodione (1,27), and overlaps but extends above the sensitive range of 0.01 to 0.354 μg/ml reported by Yoshimura et al. (33). The upper EC50 value (0.6478 μg/ml) is below the discriminatory doses of 1 μg/ml (5,33) and 25 μg/ml (15,20) previously used to identify fungal isolates resistant to iprodione. The RF value, 2.3, was similar to the RF value derived for a population of *B. fuckeliana* sensitive to vinclozolin, a dicarboximide (8).
Sensitivity to Fludioxonil

To the best of our knowledge, this study represents the first report of the sensitivity of previously unexposed greenhouse collected isolates of *C. cassiicola* to phenylpyrrole fungicides. In this study, fludioxonil possessed the narrowest sensitivity profile of the three fungicides tested. Fludioxonil EC$_{50}$ values ranged from 0.0013 to 0.0103 $\mu$g/ml (Fig. 3) with a mean value of 0.0075 $\mu$g/ml. The range of EC$_{50}$ values in the current study was within the range of EC$_{50}$ values in other studies for *Alternaria brassicicola*, *Botrytis cinerea*, and *Penicillium digitatum* (2,9,10). The upper EC$_{50}$ value (0.0103 $\mu$g/ml) is below the discriminatory dose of 0.10 $\mu$g/ml used for *Botryotinia fuckeliana* (3) and 1000 $\mu$g/ml used for *Penicillium expansum* (14). The RF value, 1.4, was similar to the RF value derived for a population of *B. fuckeliana* sensitive to fludioxonil (8).
Isolates from the African violet production facility were obtained from the transfer of mycelial plugs rather than from single-spore transfers, because sporulation was sparse. Isolates produced from mass mycelium could potentially be comprised of more than one true isolate, and consequently could increase the probability for recovering fungicide resistant isolates. This bias results because growth of the least sensitive individual is favored on the fungicide-amended medium, thus increasing a bias for higher EC$_{50}$ estimates and RF values. The lack of high EC$_{50}$ or RF values increases confidence in concluding that the \textit{C. cassiicola} isolates from this production site are sensitive to all three fungicides.

**Potential for Cross-Resistance**

Correlation analysis of the EC$_{50}$ values of the 40 isolates showed a significant but moderate correlation ($r = 0.387; P = 0.0125$) between iprodione and fludioxonil sensitivities (Table 1). Cross-resistance to dicarboximides and phenylpyrroles has been previously reported with \textit{A. brassicicola}, \textit{B. fuckeliana}, and \textit{Neurospora crassa} (8,9,18); therefore, the possibility of cross-resistance should be considered in future chemical usage. There was no significant correlation between isolate sensitivity to thiophanate-methyl and iprodione or fludioxonil. The lack of correlations between fungicides may have been the result of the limited range of sensitivities encountered in this study, and should not be construed to indicate that cross-resistance is not a threat.
Table 1. Pearson correlation coefficients between EC\textsubscript{50} values for thiophanate-methyl, iprodione and fludioxonil from 40 greenhouse-collected isolates of *Corynespora cassiicola* from African violet in 2007.

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>Thiophanate-methyl</th>
<th>Iprodione</th>
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<tbody>
<tr>
<td></td>
<td>r</td>
<td>P value</td>
</tr>
<tr>
<td>Iprodione</td>
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<td>0.7541</td>
</tr>
<tr>
<td>Fludioxonil</td>
<td>−0.1307</td>
<td>0.4155</td>
</tr>
</tbody>
</table>

**Resistance Management Strategy**

Fungal baseline sensitivity surveys are an important management technique because they allow the subsequent detection of population sensitivity shifts (22). This laboratory study shows that *C. cassiicola* is still sensitive to thiophanate-methyl. Since less than optimum control of Corynespora leaf spot occurred at the greenhouse facility, other factors such as spray coverage and/or timing of fungicide sprays should be evaluated. Regardless, caution should be taken with frequency of thiophanate-methyl applications. *C. cassiicola* isolates recovered from tomato in Japan grew on media amended with 1600 μg/ml thiophanate-methyl (6). Single-site fungicides, such as thiophanate-methyl, should be limited to only a few applications per year or tank mixed with another fungicide (e.g. thiophanate-methyl and iprodione). Tank mixes are a good general strategy; unfortunately, a broad spectrum, contact fungicide has not yet been shown to be suitable for application on African violets.

Since *C. cassiicola* is sensitive to all three fungicides but each has a different mode of action, a resistance management strategy can be employed involving rotational use of these fungicides with limited applications of each chemical. Of the three chemistries tested, fludioxonil is considered to have the lowest risk for selection of resistance, although loss of sensitivity to fludioxonil has been reported (8, 14). Fludioxonil could be used as the predominate fungicide in the rotation. In addition, other chemical classes such as the demethylation inhibitor (DMI) fungicides (e.g., propiconazole, tebuconazole) and Quinone outside inhibitor (QoI) fungicides (e.g., azoxystrobin, pyraclostrobin, kresoxim-methyl, trifloxystrobin) could be included in the chemical rotation. DMI and QoI fungicides should be used selectively, since they are considered at moderate and high risk, respectively, for selection of resistance from the population. Use of iprodione should be limited, because dicarboximide fungicides are considered at high risk for the selection of pathogen resistance and iprodione has been commonly used on plants produced in greenhouses for decades, which increases potential for previous exposures. Also, a potential for cross-resistance has been shown between iprodione and fludioxonil due to the presence of similar genes affecting osmotic signal reduction of both chemicals (18), even though iprodione and fludioxonil have different FRAC codes. As a result, the two chemistries should not be tank mixed and should not be closely alternated in the chemical rotation.

Fungicide resistance management should include cultural and sanitation practices when possible (22). Practices that may be beneficial include segregation of different age crops to minimize inoculum distribution between houses, crop placement to minimize air flow from crops with greater disease over other crops, use of selective heating in early evening followed by expulsion of moisture laden air to the outside, use of fans to mix horizontal air strata’s and minimize humidity around plants, minimization of worker activity during morning hours in areas with more severe leaf spot symptoms, use of shorter spray intervals when intense worker activity is required in an area, and attention to chemical selection and spray schedules. Since these practices will be difficult to implement in large gutter-connected greenhouse facilities, control efforts should be reviewed quarterly and innovative steps taken, possibly including consultation with a plant pathologist.
Even though these data are not a true baseline, the EC$_{50}$ ranges are representative of a sensitive population and will be useful as a basis for comparison for future sensitivity studies. Future studies could measure changes in the population sensitivity and allow better insight into alternative fungicide use patterns.

**Acknowledgements**

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**Literature Cited**


