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Baseline Sensitivities of *Corynespora cassiicola* to Thiophanate-methyl, Iprodione and Fludioxonil.

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Justin Stewart Clark
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DEDICATION

I would like to dedicate this thesis to my parents, Dennis and Kathy Clark, for instilling in me the love and interest of the outdoors and natural world the good Lord has given us. I would also like to dedicate this thesis to Liz Engel and countless friends and family who have provided love and support that keeps me going everyday.

"Those who dwell among the beauties and mysteries of the earth are never alone or weary of life."

- Rachel Carson

"I know of no more encouraging fact than the unquestionable ability of man to elevate his life by conscious endeavor."

- Henry David Thoreau

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ABSTRACT

Corynespora cassiicola, causal agent of Corynespora leaf spot, causes severe epidemics in African violet production facilities. Recent concerns over loss of fungicide efficacy has led to this investigation of baseline sensitivity distributions to thiophanate-methyl, iprodione and fludioxonil fungicides and temperature sensitivity study to evaluate fitness of high and low sensitivity groups. During a disease outbreak, 325 single lesion isolates were collected and 40 isolates were selected randomly for an *in vitro* assay to determine EC_{50} (the point at which 50% of mycelial growth was inhibited) values on potato dextrose agar (PDA) or PDA amended with thiophanate-methyl, iprodione and fludioxonil. EC_{50} values for iprodione and thiophanate-methyl ranged from 0.0833 to 0.6478 μg/ml and 0.0157 to 0.1539 μg/ml with mean values of 0.2828 μg/ml (Figure 1-1, see Chapter 2 appendix) and 0.0553 μg/ml (Figure 1-2), respectively. Fludioxonil EC_{50} values ranged from 0.0013 to 0.0103 μg/ml and the mean value was 0.0075 μg/ml (**Figure 1-3**). A resistance factor for each fungicide was calculated by dividing the least sensitive isolate's EC_{50} value to the mean EC_{50} for that fungicide. A lower resistance factor for fludioxonil-amended plates (1.37) than for iprodione (2.39) or thiophanate-methyl (2.78) (**Table 1-1**) indicates a tendency toward less insensitivity to this fungicide in this population. Correlation coefficients were calculated to determine cross-sensitivity between fungicides. Of the three fungicides, iprodione and fludioxonil had a moderately significant correlation (r = 0.38686) (P = 0.0125) (**Table 1-2**), indicating moderate cross-resistance.

To determine fitness ranges of isolates to each fungicide, three isolates least sensitive to each fungicide along with three isolates most sensitive to each fungicide were used with mean radial growth area recorded every three days for twelve days across 10, 15, 20, 25, 30 and 35° C temperatures. Across all fungicide sensitivity groups, growth did not occur at 10º C and was very limited at 35º C. Optimum growth for all isolates within sensitivity groups was 25º C (**Figure 2-1** to **Figure 2-3**, see Chapter 3 appendix) across all fungicide and observation times. Differences in growth area (mm^2) between the least and most sensitive isolate groups did not differ for the thiophanate-methyl isolate group ($P = 0.2246$), the iprodione isolate group ($P = 0.0512$), or the fludioxonil isolate group ($P = 0.6070$) based on linear mixed model analysis. Even though significant differences did not exist in this analysis, temperature sensitivity information is an important part of fungicide resistance management. Developing baseline and temperature sensitivity data is the first step in determining fungal population sensitivity shifts for better resistance management strategies.

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1. Literature Review

Introduction

Nursery and greenhouse ornamental crop production has become one of the largest crop segments in United States agriculture (43). The 2004 estimated value of the U.S. greenhouse and nursery crops industry exceeded $26 billion (15). The green industry has a profound impact on Tennessee's economy and is valued at more than $500 million (15).

A large greenhouse production facility located in middle Tennessee produces more than 10 million African violets (*Saintpaulia ionantha* H. Wendl.) annually. Recently, this producer has experienced epidemics of Corynespora leaf spot caused by *Corynespora cassiicola* (Berk & Curt.) Wei. Corynespora leaf spot is a relatively new pathogen of African violets that is characterized by rapidly expanding, water soaked lesions on the leaf surface and petioles (Fig. 1-1, see appendix) (30). *C. cassiicola* infections occur in all stages of production and can result in thousands of plants discarded daily (30).

Because little is known about the African violet/pathogen interaction, fungicide applications have been relied upon for control. Preliminary experiments found that Cleary 3336 (thiophanate-methyl) and Daconil (chlorothalonil) successfully controlled *C. cassiicola* on African violet (42). During outbreak periods, fungicide concentrations and application intervals have been increased to provide sufficient control (Mark Windham, personal communication). Resistance in *C. cassiicola* populations to thiophanate-methyl

has previously been documented and has lead to management difficulties and increased economic losses in Japan on tomato (9).

The Host: *Saintpaulia ionantha*

The genus *Saintpaulia* Wendl. is in the kingdom Plantae, phylum Magnoliophyta, order Lamiales, and family Gesneriaceae (39). Gesneriaceae is a tropical family that is divided into two subfamilies: the Gesnerioideae, which has epigynous flowers with four stamens, and the Cyrtanddroideae which has hypogynous flowers with two stamens (1). *Saintpaulia* is classified into the Cyrtandroideae subfamily.

African violets, *Saintpaulia ionantha*, are native to Tanzania and Kenya and grow at high and low altitudes (1, 34). The species grow as rosettes or trailers that are shaded by large trees around cliffs or rock outcrops (34). In 1884, Sir John Kirk recorded the first collection of *Saintpaulia* with the specimen labeled "off the coast of Zanzibar" and sent the plant to Royal Botanic Gardens, Kew (Kew Gardens) in England (1). Accompanied with the poor specimen was a note which read: "From Sir J. Kirk. Zanzibar April 1884. A lovely blue flower that will quite equal the Sultani Balsam if I can get it home." Unfortunately, the specimen only consisted of one inflorescence with two small flowers and could not be identified (1).

In 1891, a German regional commissioner, Baron Adalbert Emil Walter Redcliffe Le Tanneux von Saint-Paul-Illaire, was stationed in Tanga. He collected flowers in the Easter Usambara Mountains and sent them to his father, Hofmarschal Baron Ulrich von Saint Paul von Fischbach, president of the German Dendrological Society. In 1893, Herman Wendland, director of the Botanic Garden at Herrenhausen in Hannover,

honored the father and son by naming the genus *Saintpaulia* (1). Ionantha was used as the species name because "ion" is greek for violet. Wendland proposed the German species name of "Usambara Veilchen" and was translated to "Usambara violet" (1). The well-known English translation of "African violet" is currently used.

Today, the African violet (*S. ionantha*) is a very popular house plant in the U.S. and generated retail sales of $50 million in 2006. Popularity of *S. ionantha* is due to its need of limited attention and ability to thrive in most homes. This adaptability makes it an easy selection as a center piece or specimen plant in households (19).

In commercial wholesale operations, greenhouse facilities are required to provide control of temperature, humidity and light intensity (19). Under production conditions, daytime temperature of 77º F along with night time temperatures of 68-70º F achieves the highest growth rate in *S. ionantha* (19). Desirable relative humidity is around 50-70% (19). Higher winter light intensities of around 1,200 foot candles and lower summer light intensities of around 800 foot candles are needed for optimum growth (19). Production managers in large commercial greenhouses are able to maintain rigid production schedules by manipulating temperatures and light intensities to induce maximum production of flower quality and desirable foliage forms (16). The optimal growing conditions used to increase productivity are also conducive to infection by *Corynespora cassiicola* (10, 16).

The Pathogen: *Corynespora cassiicola*

Corynespora cassiicola, the causal pathogen of Corynespora leaf spot or target spot, has been documented worldwide (28). *C. cassiicola* is a pathogen on more than 70

hosts used in either greenhouses or field production (32, 39). Major economic hosts are cotton (*Gossypium* L. sp.), rubber tree [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.], tomato (*Lycopersicon esculentum* L.), tobacco (*Nicoteana tabacum* L.), soybean (*Glycine max* L. Merr.), and zebra plant (*Aphelandra squarrosa* Nees) (25, 31).

Taxonomy

The genus *Corynespora* is in the kingdom Fungi, phylum Ascomycota, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales and family Corynesporascaceae (39).

In 1896, Cooke described an unknown species of *Cercospora* found in melon pits and named it *C. melonis* (7). He later proposed the new genus Corynespora because he observed a hyaline isthmus between the conidiophore and conidium and the conidia were formed in chains (**Figure 1-2**) (14). He changed the name to *Corynespora mazei* but in 1910, Lindau renamed the pathogen *Corynespora melonis* (Cooke) Lindau (22).

In 1936 and 1939, *Cercospora vignicola* Kawamura was identified as a pathogen on soybean (*Glycine max* (L.) and cowpea *Vigna unguiculata* (L.) Walp. in China (36, 37 41). Liu later identified the same fungus as *Helminthosporium vignae*t on cowpeas in Japan (23). In 1945, Olive et al. described *H. vignae's* presence on cowpea and soybean within the U.S. (27). After comparing isolates from China and Japan with *Corynespora melonis*, Olive et al. and Wei concluded that *Cercospora melonis*, *Cercospora vignaicola*, and *Helminthosporium vignae* were all species of *Corynespora* (27, 41).

Wei (41) also examined *H. cassiicola* Berkeley & Curtis which is present in tropical countries and he concluded *H. cassiicola* was morphologically identical to *Corynespora*. Wei later proposed the following synonyms of *C. cassiicola*:

Helminthosporium cassiicola Berk. & Curt. (4), *Cercospora melonis* Cooke (7), *Corynespora melonis* (Cooke) Lindau (22), *Corynespora mazei* Güssow (14), *Helminthosporium vignae* Olive (27), *Helminthosporium papayae* H. Sydow (35), and *Cercospora vignicola* Kawamura (17).

Signs, Symptoms and Disease Cycle

In 1973, McRitchie and Miller documented zebra plant as the first foliage plant infected by *C. cassiicola*. Wounding was determined to be necessary for infection of zebra plants, but not usually required for *C. cassiicola* or other hosts. *C. cassiicola* causes irregular, brown lesions on *S. ionantha* (10). On a majority of hosts, the pathogen demonstrates high virulence and is characterized by rapidly expanding necrotic lesions that are rarely surrounded by a halo (6, 26, 30). Lesions become apparent in 7-14 days after inoculation and measure up to 20 mm in diameter (6, 25). Disease development is favored by warm temperatures, high moisture and humidity (5, 9). All phases of *S. ionantha* production are vulnerable to this disease, from propagation material up to finished mature plants (30, 42). Little is currently known about the disease cycle of *C. cassiicola* on *S. ionantha* but for the majority of agricultural hosts, *C. cassiicola* can survive in crop debris for up to two years and also within soybean seeds (10, 26).

Control

Reducing humidity and leaf wetness are recommended for reducing infections of *C. cassiicola*. Preliminary in vitro experiments revealed that Cleary 3336 (thiophanate-methyl) and Daconil (chlorothalonil) achieved sufficient control of *C. cassiicola* on *S. ionantha* (42). Both fungicides are in different chemical classes. Chlorothalonil is a

commonly used multi-site (contact) fungicide and thiophanate-methyl is a site-specific (systemic) fungicide (2).

Fungicide Resistance

Fungicide resistance is a stable, inheritable adjustment by a fungus to a fungicide, resulting in reduced sensitivity of the fungus to the fungicide (25). Prior to the 1970s, fungicide resistance was rarely a problem because most fungicides were multi-site inhibitors with protectant activity (12). If resistance occurred, it was likely due to nonspecific causes like detoxification or decreased uptake of the fungicide (18). Today, if resistance occurs to site-specific fungicides it may be caused by a reduced uptake of the fungicide or by evolutionary metabolic changes in the pathogen, such as an increase in the target enzyme or reduced attraction of the target site (38). Once mutations occur, fungicide applications effectively control sensitive isolates and allow resistant isolates to become dominant in pathogen populations under selection pressure of fungicide use over time (25).

Ecological fitness of resistant isolates determines their persistence once they are selected (25). In many instances, frequencies of resistant isolates will decrease once fungicide use ceases because resistant isolates have lower fitness and can not survive in the absence of fungicide selection pressure (25). On the other hand, benzimidazole and strobilurin resistant isolates have shown to be as fit as sensitive isolates and persist without continued applications of the fungicide (3, 20).

Successful management of Corynespora leaf spot includes the use of both site-specific (systemic) and multi-site (contact) fungicides. These fungicides are assigned to

classes according to their mode of action. Contact fungicides act as a protective coating to prevent infection. Systemic fungicides have become increasingly popular due to their ability to prevent and stop early infections (40). Systemic fungicides are taken up by the plant and may be transported to other parts of the plant.

The term fungicide is typically used to describe all chemicals that protect plants from fungal diseases. A more accurate term is fungistat because they work by inhibiting the growth of a fungus such as preventing spore germination and/or mycelium growth (40). Examples of fungistats are the benzimidazoles, such as benomyl and thiophanate methyl, the demethylation inhibitors (DMIs), such as propiconazole and myclobutanil, and the dicarboximides, such as iprodione and vinclozolin (2). Fungistats can be applied to minimize disease by preventing infection and arresting the growth of the pathogen to allow the infected plant to recover. This inhibits the spread of the pathogen to healthy plants (21). For the remainder of this paper, all fungicides and fungistats will be referred to as fungicides (17, 27, 40).

Benzimidazoles

Resistance first became a large-scale problem due to the widespread use of the class of systemic fungicides called benzimidazoles (e.g., thiophanate-methyl) (11, 12). At their introduction, they represented a ground-breaking class of fungicides that not only protected plants from disease, but also controlled certain diseases by arresting colonization of plant tissue when applied shortly after infection (33). However, unlike the earlier multi-site fungicides, the site-specific mode of action of benzimidazoles was quickly overcome by several fungal pathogens (33). Resistance quickly occurred when point mutations in the β-tubulin gene resulted in altered amino acid sequences at the

binding site (24). Like many site-specific fungicides, benzimidazoles do not inhibit spore germination, but rather severely stunt germ-tube growth (38).

Dicarboximides

Introduced in the mid-1970s, dicarboximides (e.g., iprodione) were very effective in controlling some pathogenic fungi on vines, vegetables, fruit and ornamentals (38, 44). Within 10 years of introduction, resistant isolates of *Botrytis cinerea* and loss of efficacy were observed in New Zealand, Israel and England (38, 44). Although the exact mode of action is still unknown, significant disorder to fungal cells and morphological changes to hyphae and germ tubes is observed when treated with the fungicide (38). Strains resistant to this fungicide have proven to be less fit for survival than sensitive strains (8).

Phenylpyrroles

The phenylpyrrole (e.g., fludioxonil) class of fungicides is derived from the antifungal antibiotic pyrrolnitrin and has a wide range of fungal controls. Phenylpyrroles are used primarily to control *Ascomycetes* and *Basidiomycetes* and can be used as seed fungicides to control *Fusarium, Tilletia* as well as other seed born pathogens (38). These fungicides are considered to be at low risk of developing resistance and there has been no report of pathogen resistance. It is hypothesized that an abnormal accumulation of glycerol is a result of phenylpyrroles interference with the fungi's osmotic signal transduction pathway (21, 29). This activity results in inhibition of spore germination and hyphal growth (21).

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Appendix



Figure 1-1. African violet symptomatic of *Corynespora cassiicola*.

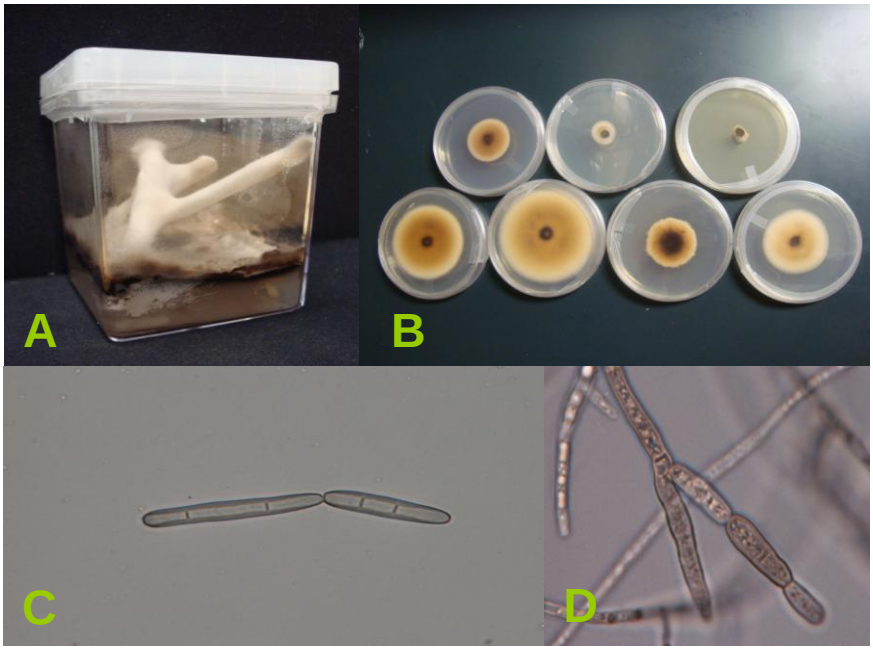


Figure 1-2. *Corynespora cassiicola* (A) In storage on cotton stems/PDA (B) Growth at varying amounts of fungicide (C) Conidial chain (D) Conidial chain produced on conidiophore.

1. Baseline sensitivity of isolates of *Corynespora cassiicola* to thiophanate-methyl, iprodione and fludioxonil fungicides

Abstract

Corynespora cassiicola, causal agent of Corynespora leaf spot, can cause devastating epidemics in African violet production facilities. Because of phytotoxicity and visible residue issues with some fungicides such as chlorothalonil, production facilities have relied on thiophanate-methyl, a site-specific fungicide that possesses a high risk for pathogen populations developing of resistance. During a disease outbreak, 325 isolates of *C. cassiicola* were collected and 40 isolates were selected randomly to be tested in vitro to determine EC_{50} values on agar amended with concentrations of thiophanate-methyl, iprodione and fludioxonil. EC_{50} values for thiophanate-methyl and iprodione ranged from 0.0157 to 0.1539µg/ml and 0.0833 to 0.6478µg/ml with mean values of 0.0553µg/ml (Figure 1-1, see Chapter 2 appendix) and 0.2828µg/ml (Figure 1-2), respectively. Fludioxonil EC_{50}s ranged from 0.0013 to 0.0103µg/ml and the mean value was 0.0075µg/ml (**Figure 1-3**). A resistance factor for each fungicide was calculated by taking the ratio of the least sensitive isolate's EC_{50} value to the mean EC_{50} for that fungicide. A lower resistance factor for fludioxonil-amended plates (1.37) than for iprodione (2.39) or thiophanate methyl (2.78) (**Table 1-1**, see Chapter 2 appendix) indicates a tendency toward less insensitivity to this fungicide in this population. Correlation coefficients were calculated to determine cross-sensitivity between fungicides. Among the three fungicides, iprodione and fludioxonil had a moderately significant correlation (r = 0.38686) (p = 0.0125) (**Table 1-2**).

Developing baseline sensitivity and resistance probability data is the first step in determining fungal population sensitivity shifts.

Introduction

Nursery and greenhouse ornamental crop production has become one of the largest segments in United States agriculture (4). The 2004 estimated value of the U.S. greenhouse and nursery crops industry was more than $26 billion (3). The green industry has a profound impact on Tennessee's economy and is valued at more than $500 million (3).

One large greenhouse facility, located in TN, produces more than 10 million African violets (*Saintpaulia ionantha*) annually. This large producer has experienced epidemics of Corynespora leaf spot caused by *Corynespora cassiicola* (Berk & Curt.) Wei for more than five years. Corynespora leaf spot is characterized by water soaked lesions that expand rapidly on leaf surface and petioles (13). *C. cassiicola* occurs in all stages of production and can result in thousands of plants discarded daily (13). Most outbreaks occur from May through September (19). Because little is known about the African violet/pathogen interaction, cultural controls are not available and fungicide applications have been relied upon for control.

Preliminary experiments found that thiophanate-methyl (Cleary 3336; W.A. Cleary Corporation, Summerset, NJ) and chlorothalonil (Daconil; Syngenta Crop Protection, Greensboro, NC) were successful in controlling *C. cassiicola* on African violet but chlorothalonil is not used due to phytotoxicity and residue issues (19). Because of chlorothalonil's phytotoxicity problems, thiophanate-methyl has been solely relied upon for disease control. Iprodione and fludioxonil were selected for this assay because of their use on many greenhouse and field crops that serve as hosts for *C. cassiicola*.

Thiophanate-methyl is a systemic, site-specific benzimidazole fungicide developed in the late 1960s that stops hyphal growth by inhibiting tubulin polymerization (7). Frequent repeat applications of benzimidazole fungicide treatments can lead to a reduction in fungicide efficacy, management difficulties, and increased economic losses (21). Because of heavy grower reliance on the site-specific mode of action of thiophanate-methyl and history of benzimidazole resistance in *C. cassiicola*, it is important to establish a fungal population profile from this continuous selection exposure.

Fludioxonil, a newly developed pyrronitrin-derived chemical, has become widely used as a fungicide, partly due to its broad spectrum activity toward Ascomycetes, Basidiomycetes, and members of the *Fungi Imperfecti* (5, 22). It is hypothesized that an abnormal accumulation of glycerol is a result of phenylpyrroles interference with the fungi's osmotic signal transduction pathway (7, 10). This disruption results in inhibition of spore germination and hyphal growth (5, 7). The fungicide is considered to have a low risk for developing resistance, although loss of sensitivity to fludioxonil has been reported in *Botryotinia fuckeliana* (de Bary) Whetzel (5).

Iprodione, a dicarboximide fungicide, is another widely used greenhouse fungicide that is effective on a wide variety of fungi. Dicarboximides and aromatic hydrocarbons are the most widely used and available class of fungicides. Their primary mode-of-action involves inhibition of lipid synthesis and metabolism. Although exact molecular details of inhibition are unknown, spore germination and mycelium growth can be inhibited, and mycelium may exhibit swelling that progresses to cell rupturing (17).

This disorder is due to the artificial peroxidation of polyunsaturated fatty acids in phospholipids resulting in a disturbance of lipid function rather than biosynthesis (6).

Inadequate disease control during spring and summer months in recent years has caused African violet growers concern about possible fungal populations becoming less sensitive to thiophanate-methyl. Because of these concerns, a study was conducted to determine baseline sensitivity of isolates of *C. cassiicola* to thiophanate-methyl, iprodione, and fludioxonil.

Materials and Methods

Isolate Collection and Isolation:

Leaves of African violets symptomatic for Corynespora leaf spot were collected at a large African violet production facility during a disease outbreak. Symptomatic leaves were individually placed into sterile polyethylene bags and labeled with the number of greenhouse and location within each house. Bags were stored in an ice chest without ice until they were returned to the lab.

Excised lesions were placed in a solution of 10% sodium hypochlorite and 5% ethanol for 30 seconds and then rinsed in sterile DI water for 10 seconds (1). Surface disinfested tissues were placed in Petri dishes (8.5-cm inside diameter) containing 25 ml of potato dextrose agar (PDA; Difco Laboratories, Detroit) amended with chlortetracycline hydrochloride and streptomycin sulfate (8 μg/ml). Plates were placed in an incubator at 23 ±1ºC with a diurnal 12 hour light and dark cycle for 48 hours as described by Silva et al. (16). After incubation, 5-mm-diameter plugs of mycelium growing from the leaf tissue were cut using a sterile cork borer and plugs were transferred to unamended PDA. Cultures were incubated as previously described until sufficient growth occurred for cultures to be identified using colony and conidial morphology (16).

Each of 325 collected isolates were stored on 75ml PDA and sterile cotton stems in magenta boxes (Magenta Corporation, Chicago, IL.) and maintained at 23 ±1ºC with alternating 12 hour light and dark cycles. Forty isolates were selected randomly to assess their sensitivity to a group of fungicides.

Fungicides:

Technical grade Thiophanate-methyl (97% a.i., W.A. Cleary Corporation, Summerset, 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 20mg/ml. Acetone concentration was corrected and was the same between each dilution. Fungicides were then stored in complete darkness at 23ºC to preserve and maintain fungicidal activity. To ensure a homogenous fungicide concentration, the stock solutions were mixed with a magnetic stir bar for 30 minutes prior to dispensing.

Technical grade thiophanate-methyl, iprodione, and fludioxonil were added as amendments to partially cooled PDA so that the a.i. of each fungicide was 0, 0.01, 0.1, 1, 10, or 100 μg a.i. / ml. Mycelial plugs were cut using a 5-mm-diameter cork borer from the margins of actively growing twelve day old cultures. The mycelium was placed upside down on PDA amended with fungicide or un-amended media (16). The study was repeated with two runs and each fungicide concentration/ isolate combination was replicated three times. Cultures were incubated for 11 days at 23 ±1ºC in complete darkness.

Mean colony diameter was obtained for each culture by taking two perpendicular measurements, at right angles, with the original mycelial plug diameter subtracted from each measurement (8, 9). Preliminary linear mixed model analysis indicated no significant difference ($P \leq 0.05$) between the two runs; therefore, runs were combined for each isolate/fungicide concentration to determine EC_{50} (effective concentration that reduces mycelial growth by 50%) values. Fungicide concentrations were logarithmically

($\log_{10}$) transformed and data was analyzed using nonlinear regression analysis with the NLIN procedure of SAS (version 9.2; SAS Institute, Cary, NC). The following log-logistic dose-response function was used, where *y* is the response, *x* is the dose, *C* is the lower limit, *D* is the upper limit, *b* is the slope, and EC_{50} is the dose giving 50% response (15, 18).

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

The log-logistic dose-response curve is commonly used in dose-response studies where dose (i.e. concentration) ranges from no effect to complete inhibition (15, 18). Estimation of the effective concentration required to reduce growth by 50% (EC_{50}) (18) was provided by the nonlinear least squares method of the NLIN procedure. Lack-of-fit tests were also conducted to evaluate fit of the nonlinear model, as well as a simple linear regression model, to the data for each fungicide and isolate. Lack-of-fit tests compare the fit of a model to the mean responses at each level of the explanatory variable (i.e. concentration). In this study, simple linear regression models provided a poor fit while the nonlinear models (log-logistic dose-response curve) provided a more acceptable fit.

A resistance factor for each fungicide was calculated by taking the ratio of the least sensitive isolate's EC_{50} value to the mean EC_{50} for that fungicide (11). Sample size was determined through previous studies of similar size and scope as recommended by Russell (14). To determine cross-sensitivity relationships between fungicides, Pearson

correlation coefficients (*r*) were calculated for each pair of fungicides using the EC_{50} values for each isolate.

Results and Discussion

Fungal baseline sensitivity surveys are important management techniques because they allow the subsequent detection of exact levels of population sensitivity shifts (14). To the best of our knowledge, this study represents the first report of unexposed sensitivity of greenhouse collected isolates of *C. cassiicola* to dicarboximides and phenlpyrrole fungicides. A true baseline distribution is not represented within this study for thiophanate-methyl because of the previous reliance on this chemistry for control of *C. cassiicola* at the production facility where fungal isolates were collected. Frequency distributions of these 40 isolates provide a range with which future isolates within this population can be compared, for determining fungal population shifts in sensitivity.

Sensitivity to thiophanate-methyl:

The range of EC_{50} values for isolates exposed to thiophanate-methyl was 0.0157 and 0.1539µg/ml and the mean value was 0.0553µg/ml (Figure 1-1). Even though there has not been a significant loss of sensitivity to thiophanate-methyl within this population, resistance has recently been confirmed in isolates of *C. cassiicola* from tomato in Japan which grew at thiophanate-methyl concentrations of >1600 µg/ml (2). This distribution of sensitivities to thiophanate-methyl may not be a true baseline due to previous exposure of the population to this fungicide, but may be useful as a basis for comparison for future sensitivity studies. Widespread use of thiophanate-methyl in virtually all agronomic production, its classification as a high resistance risk fungicide and reported resistance to it within this pathogen create urgency for producers and university professionals to find alternative controls once populations begin to shift.

Sensitivity to iprodione:

In this study, iprodione failed to prove its validity as an effective control of *C. cassiicola* due to the fungicides slightly elevated sensitivity profile. Iprodione EC_{50}s ranged from 0.0833 to 0.6478µg/ml and the mean value was 0.2828µg/ml (Figure 1-2). This sensitivity range is somewhat similar to a report by Myresiotis et al. of iprodione resistant populations of *Botrytis cinerea* of 1 µg/ml (8). *C. cassiicola* range from this study does not approach resistance levels in populations of *Monilinia fructicola* of between 3 and 218 µg/ml reported by Elmer and Gaunt or 25 µg/ml reported by Ritchie (3, 12). Due to the unknown introduction or previous dicarboximide exposure of *C. cassiicola* in this greenhouse facility, one explanation could be that these isolates were previously exposed to iprodione before outbreaks at this facility began. In some fungi, iprodione resistant isolates have been shown to be as fit as sensitive isolates (8). This slightly elevated sensitivity could persist within a population for several years and could be a result of previous exposure or the fungi's natural affinity to this chemistry.

In this study, iprodione failed to prove its validity as an effective control of *C. cassiicola* due to the fungicides slightly elevated sensitivity profile. Iprodione EC_{50} values ranged from 0.0833 to 0.6478µg/ml and the mean value was 0.2828µg/ml (Figure 1-2). This sensitivity range is similar to other reports of iprodione's baseline sensitivity populations of *Botrytis cinerea* (9) and exposed populations of *Monilinia fructicola* (20). However, the same study concluded that *B. cinerea* isolates with EC_{50} values greater than 1 µg/ml were considered to be resistant to iprodione (9). Due to the unknown introduction or previous dicarboximide exposure of *C. cassiicola* in this greenhouse facility, one explanation could possibly be that these isolates were previously exposed to

iprodione before outbreaks at this facility began. In some fungi, iprodione resistant isolates have been shown to be as fit as sensitive isolates (9). This slightly elevated sensitivity could persist within a population for several years and could be a result of previous exposure or the fungi's natural affinity to this chemistry.

Sensitivity to fludioxonil:

In this study, fludioxonil controlled *C. cassiicola* most effectively and possessed the narrowest sensitivity profile of the three fungicides assayed. Fludioxonil EC_{50} values ranged from 0.0013 to 0.0103µg/ml with a mean value of 0.0075µg/ml (**Figure 1-3**). In general, isolates were more sensitive to fludioxonil than thiophanate-methyl or iprodione. As with iprodione sensitivities, previous exposure of these isolates to fludioxonil is not expected but true previous exposure is not known. Sensitivity distributions for fludioxonil and iprodione in this study are probably very similar to wild type isolates but this cannot be known for sure because both chemistries have been on the market for several years.

The resistance factor shows a relative distance between the least sensitive isolate and the mean EC_{50} of the population. A higher resistance factor indicates presence of isolates that are less sensitive to the chemical than the average isolate in the population. Thiophanate-methyl (2.78) had the largest resistance factor which was similar to that of iprodione (2.29) (**Table 1-1**). Fludioxonil possessed the lowest resistance factor of 1.37. Resistance factors reported from this research are similar to those previously reported for true baselines in other studies (5).

For the three fungicides tested, correlation analysis of the EC_{50} values of the 40 isolates showed a significant moderate correlation (r = 0.38686) (p = 0.0125) between iprodione and fludioxonil sensitivities (**Table 1-2**). There was no significant correlation

between iprodione and thiophanate-methyl, or fludioxonil and thiophanate-methyl sensitivities to these isolates. Even though correlation between iprodione and fludioxonil sensitivities was moderately correlated in this study, they are still statistically significant and possibly suggest that sensitivities and resistance between these two chemistries may be linked. These results correspond with laboratory-induced reports of fludioxonil resistant isolates also being resistant to iprodione in *B. fuckeliana* (5). Further tests are needed for each individual pathosystem due to substantial differences in resistance patterns of field and laboratory isolates (5).

Laboratory results within this study provide value to studying fungicidal sensitivities and to investigate new strategies for optimum disease control. Further investigations *in vivo* would possibly prolong the efficacy of these chemistries and create a more effective control to limit resistance build-up. Several resistance management strategies to be employed against *C. cassiicola* are to spray single-site fungicides only when necessary, maintain good spray coverage, tank mix protectant fungicides and alternate single-site fungicides with different mode of action to limit selection of resistant isolates within the population. Hopefully, this study and others will help to predict resistance buildup within pathogen populations and prolong the use of these agricultural chemistries.

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Appendix

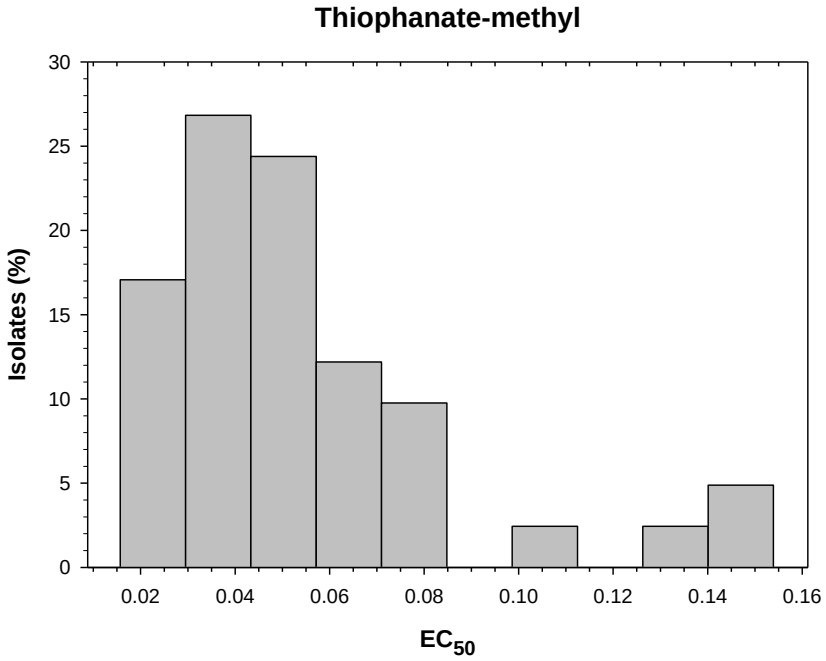


Figure 1-1. Baseline sensitivity distributions of EC_{50} values (µg/ml) for isolates of *Corynespora cassiicola* to iprodione.

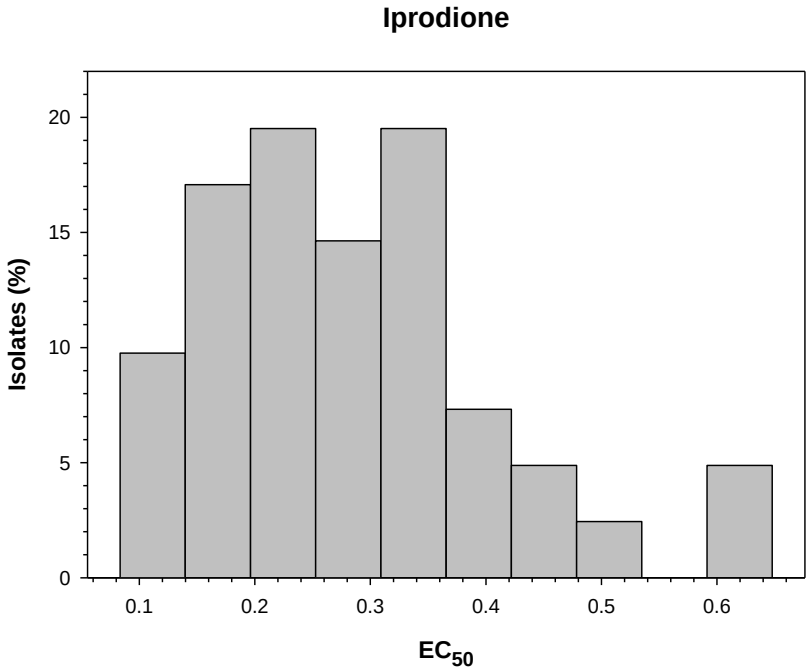


Figure 1-2. Baseline sensitivity distributions of EC_{50} values (µg/ml) for isolates of *Corynespora cassiicola* to iprodione.

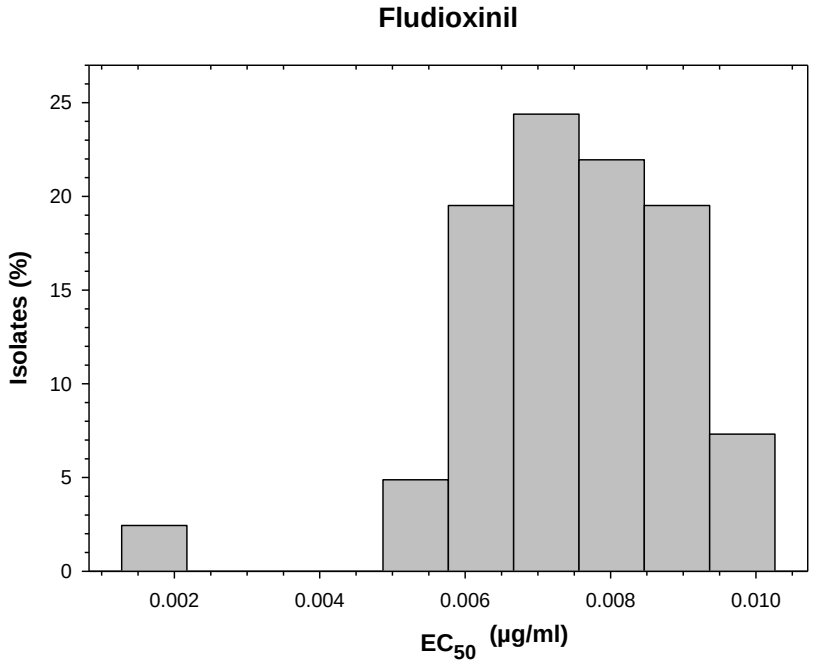


Figure 1-3. Baseline sensitivity distributions of EC_{50} values (µg/ml) for isolates of *Corynespora cassiicola* to fludioxonil

Table 1-1. Baseline distributions and resistance factors of EC_{50} values for thiophanate-methyl, iprodione and fludioxonil to greenhouse collected isolates of *Corynespora cassiicola* from African violet in 2007.

Fungicide	EC_{50} (µg/ml)		Resistance Factor*
	Range	Mean	
Thiophanate-methyl	0.0157 to 0.1539	0.0553	2.78
Iprodione	0.0833 to 0.6478	0.2828	2.29
Fludioxonil	0.0013 to 0.0103	0.0075	1.37

* Resistance Factors = ratio between the least sensitive isolate and the mean EC_{50} of the distribution range.

Table 1-2. Sensitivity correlations of EC_{50} values to thiophanate-methyl, iprodione and fludioxonil from 40 isolates of *Corynespora cassiicola.*

Fungicide	**Thiophanate-methyl**		**Iprodione**		**Fludioxonil**	
	r	***P* value**	***r***	***P* value**	***r***	***P* value**
Thiophanate-methyl	1.0000	-	0.05045	0.7541	-0.13066	0.4155
Iprodione	0.05045	0.7541	1.0000	-	0.38686	0.0125
Fludioxonil	-0.13066	0.4155	0.38686	0.0125	1.0000	-

2. In vitro temperature sensitivity determination of fungicide sensitive and insensitive isolates of *Corynespora cassiicola*.

Abstract

Corynespora cassiicola, causal agent of Corynespora leaf spot, can cause devastating epidemics within African violet production facilities. Recent concerns over loss of fungicide efficacy led to an investigation into baseline sensitivity distributions to thiophanate-methyl, iprodione and fludioxonil fungicides. The emergence of resistant strains leads to questions regarding best management practices and the ability of these strains to persist. Because fungicide resistant isolates may be equally or often more tolerant of temperature extremes (7, 11), this study was conducted to determine temperature sensitivities of isolates that were observed to be most and least tolerant of each fungicide in the previous study. Three isolates that were recorded as being least sensitive to each fungicide along with three isolates recorded as being the most sensitive to each fungicide were grown for twelve days at 10, 15, 20, 25, 30 and 35° C. The mean radial mycelium growth area was measured every three days. Across all fungicide sensitivity groups, growth did not occur at 10º C and was very limited at 35º C. Optimum growth for all isolates within sensitivity groups across all fungicide and observation times was 25º C (**Figure 2-1** to **Figure 2-3**). Differences in growth area (mm^2) between the least and most sensitive isolate groups did not differ for the thiophanate-methyl isolate group ($P = 0.2246$), the iprodione isolate group ($P = 0.0512$), or the fludioxonil isolate group ($P = 0.6070$) based on linear mixed model analysis. Even though significant differences do not exist in this analysis, temperature sensitivity information is an important part of fungicide resistance management. The in vitro studies indicate the

influence of temperature is not likely to be a major factor separating fitness of *C. cassiicola* isolates recovered from commercial African violet production.

Introduction:

Corynespora leaf spot is a relatively new pathogen on African violet that is identified by rapidly expanding, water soaked lesions on leaves and petioles (9). *Corynespora cassiicola* occurs in all stages of African violet production and can result in thousands of plants being discarded daily in production facilities (8).

Ecological fitness of resistant isolates determines their persistence within the environment (5). In many instances, frequencies of resistant isolates decreased once fungicide usage ceased because resistant isolates had lower fitness and could not survive in the absence of fungicide selection pressure (6). However, benzimidazole and dicarboximide resistant isolates have been observed to be as fit as sensitive isolates and persist without the fungicide present (4, 10). Through previous experiments, baseline sensitivity distributions for thiophanate-methyl, iprodione, and fludioxonil were established. Baseline sensitivities of previously tested isolates displayed no measureable shift in population sensitivities to these fungicides. If the growth rate of the most insensitive isolates is higher than or equal to that of the sensitive isolates across a large range of environmental conditions, there is a greater risk of fungicide resistance persisting. This study was conducted to determine if the most fungicide sensitive and insensitive isolates of *C. cassiicola* within this greenhouse population are differentially affected by temperature.

Materials and Methods:

Eighteen isolates of *C. cassiicola* were selected from the 40 isolates tested in a previous study (see chapter 2) on the criteria of the three least and most sensitive isolates to each of the three fungicides. Mycelial plugs, five mm in diameter, were removed from 11 day old isolates and placed on PDA in Petri dishes. The cultures were incubated at 10, 15, 20, 25, 30, and 35º C for 12 days with alternating l2 hour light and dark cycles.

Colony growth diameters were taken every three days over a 12 day incubation period. Mean colony diameters were obtained by taking two perpendicular measurements of the isolate and subtracting the 5-mm plug from each (5). Data were analyzed with linear mixed models using the MIXED procedure of SAS (version 9.2; SAS Institute, Cary, NC) using isolate group (low/high sensitivity) and temperature as main factors and observations taken over time as repeated measures. The experiment was performed twice with three replications of each isolate. Preliminary analysis indicated no significant difference between the two runs; therefore, data from the two runs were combined for analysis.

Results and Discussion:

Fungal fitness surveys are an important component for measuring the current development of fungicide resistance within pathogen populations. Analysis of variance was conducted and indicated that no significant interactions ($\alpha \leq 0.05$) were observed between repetitions or runs and data sets were combined for each high/low sensitivity group to determine significance factors within each fungicide.

Thiophanate-methyl isolates:

Growth area between sensitivity groups of thiophanate-methyl did not differ from zero ($P = 0.2246$), based on the linear mixed model analysis. All isolates within thiophanate-methyl sensitivity groups had no growth at the 10° C treatment and limited growth at 35° C. Optimum growth occurred at 25º C among both sensitivity groups (**Figure 2-1**, see appendix). *C. Cassiicola* isolates possessed a higher resistance factor to thiophanate-methyl than to the other two fungicides. Due to this population's slightly elevated resistance risk factor (Chapter 2) and a previous report of resistance to thiophanate-methyl in *C. cassiicola* (2), fitness determination is especially important. Other studies have shown the ability of several benzimidazole resistant pathogens including *Aspergillus nidulans* (Eidam) G. Winter, *Saccharomyces cerevisiae* Meyen Ex E.C. Hanson and *Monilinia fructicola* (G. Winter) Honey to persist and survive in a broad range of environmental conditions under high and low temperatures (3, 5, 6). In this study, no significant differences between growth of high/low sensitivity groups were detected which is similar to what has been reported for growth patterns of sensitive isolates of *M. fructicola* and *Monilinia laxa* (Aderh. & Ruhland) Honey (7).

Iprodione isolates:

Growth area between sensitivity groups of iprodione did not differ from zero (P = 0.0512). Even though the P value is marginally significant, the average growth area for the least sensitive isolates was 91.3638 mm^2 less than for the most sensitive isolates; thus, the least sensitive isolates grew less than the most sensitive isolates. Comparable to thiophanate-methyl sensitivity groups, all isolates did not grow at 10° C and had very limited growth at 35° C. The largest amount of growth occurred at 25º C among both sensitivity groups across all observation times (**Figure 2-2**).

Fludioxonil isolates:

Fludioxonil sensitivity groups had growth area measurements that did not differ from zero (P = 0.6072), based on linear mixed model analysis. Similar to thiophanate-methyl and iprodione, mean growth area of fludioxonil sensitivity groups was greatest at 25º C across all observation times. Growth was not detected at 10° and limited growth was observed at 35° C (**Figure 2-3**). Sensitivity ranges of C. cassiicola to fludioxonil were comparable to *A. nidulans* and *Botrytis cinerea* Pers. isolates, in which fitness was not reduced or increased across a range of fungicide sensitivities (11).

In all isolates tested, growth did not occur at 10º C and was very limited at 35º C. Optimum growth for all isolates was 25º C (**Figure 2-1**to **Figure 2-3**) at each observation time. No significant differences in mean growth area occurred between any fungicide sensitivity groups in this study. Even though no significant results were found in this study, these results are similar to others that found no difference in mycelium growth in response to temperature when there is not resistance within a population (1, 7, 11). *C.*

cassiicola results differed from some studies by having growth over a wider temperature range than several other fungal species (11). Because of this variation, results from future studies should be studied carefully due to a wide variety of fluctuations in fitness parameters in different pathosystems (11).

Similar to results from Chapter 2, future studies of isolate fitness within this population should compare radial growth results back to this initial trial. Other fitness parameters such as, osmotic or light sensitivity could be used to gain a more accurate and complete understanding of isolates' overall fitness. Information resulting from this study and others has the potential to provide a profound impact on resistance management. By determining a population's fitness and its sensitivity to fungicides, it would be plausible to manipulate environmental conditions and plan fungicide applications at the optimum temperature when/if resistant isolates/populations are most present.

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Appendix

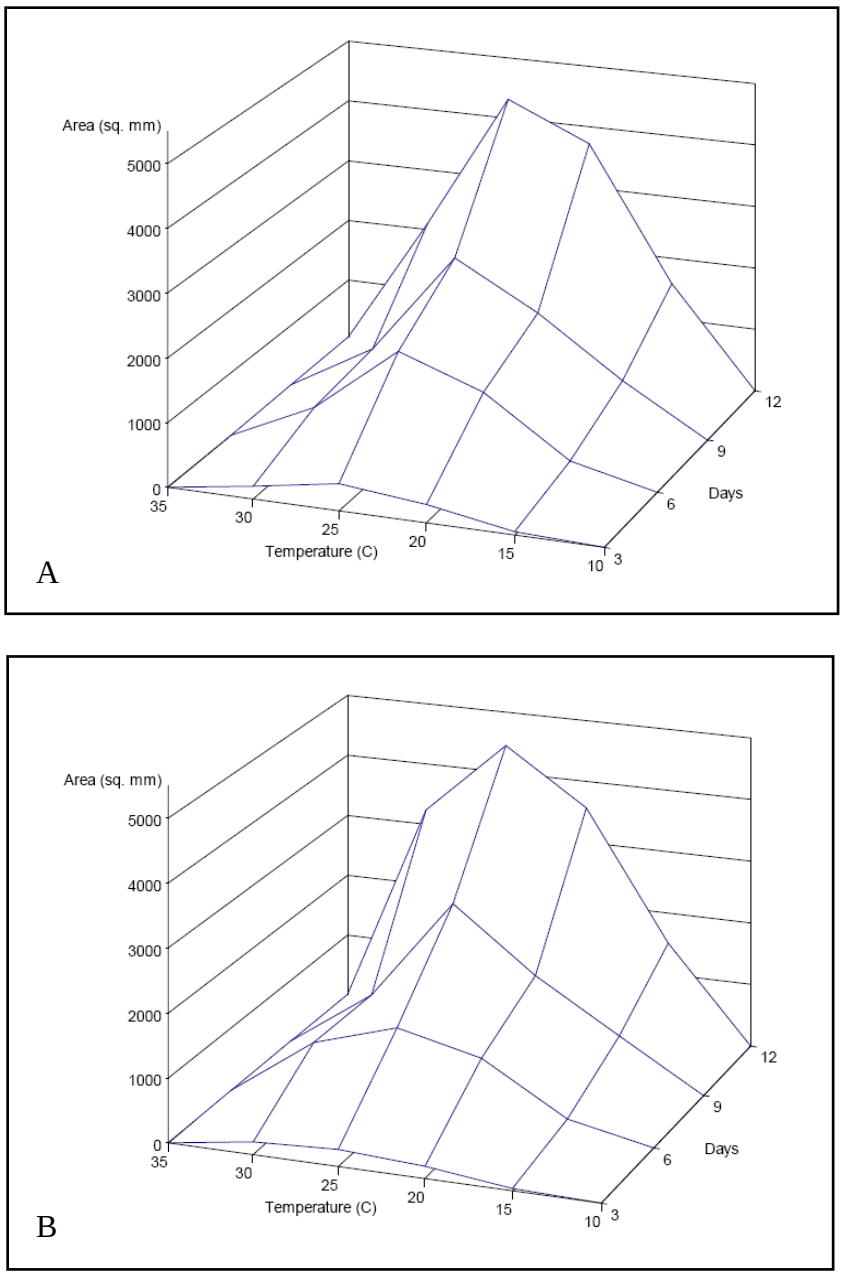


Figure 2-1. Distribution of growth area (mm^2) for the least sensitive isolate group (A) and the most sensitive group (B) of *Corynespora cassiicola* to thiophanate-methyl at 10, 15, 20, 25, 30 and 35° C after 3, 6, 9, and 12 days.

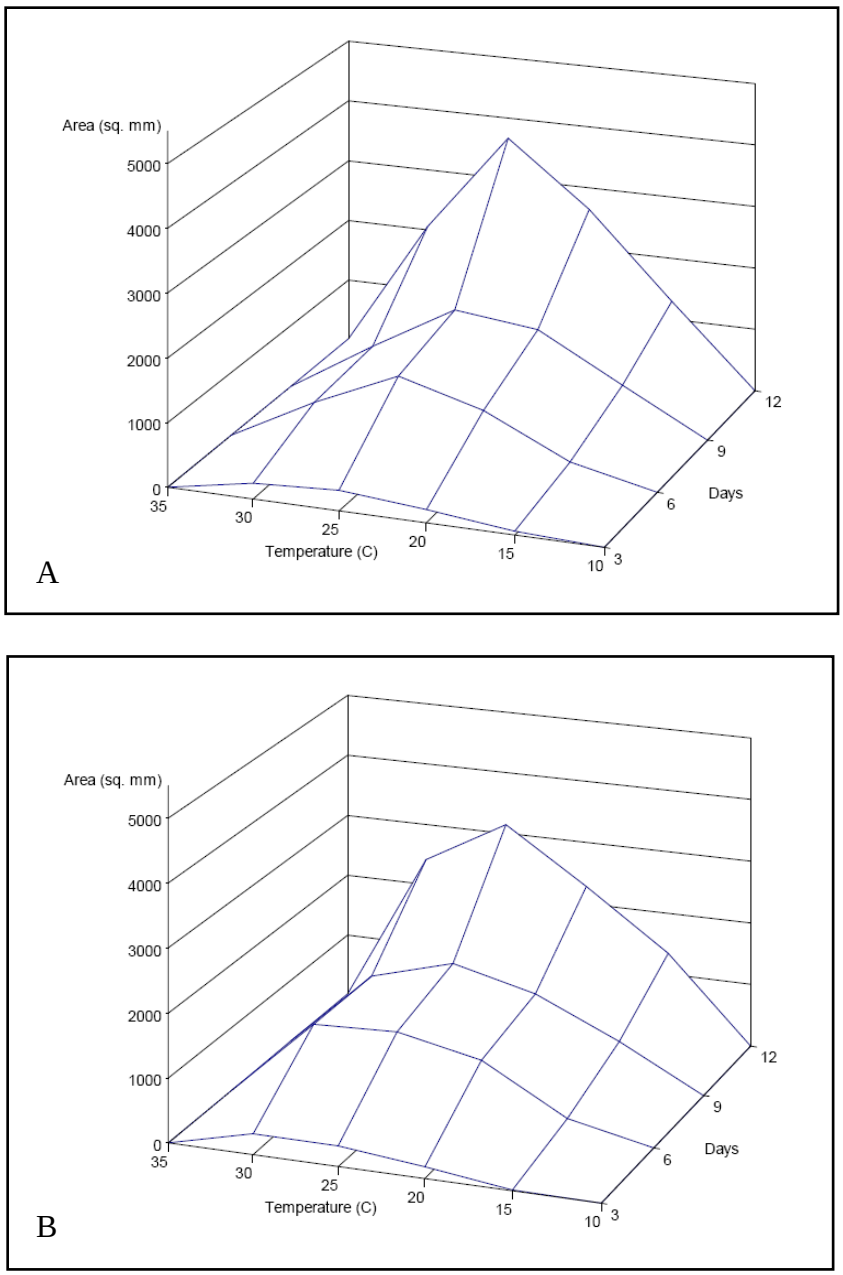
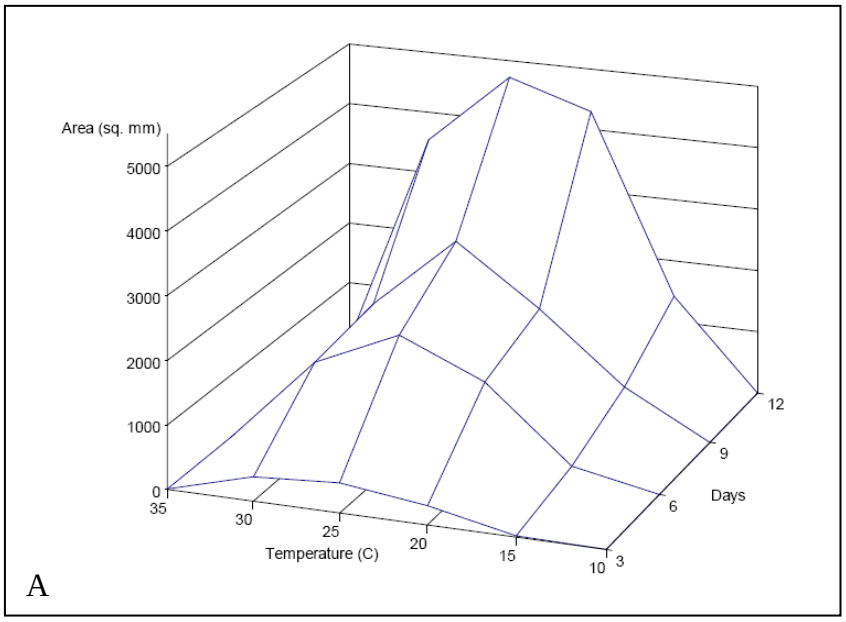


Figure 2-2. Distribution of growth area (mm^2) for the least sensitive isolate group (A) and the most sensitive group (B) of *Corynespora cassiicola* to iprodione at 10, 15, 20, 25, 30 and 35° C after 3, 6, 9, and 12 days.



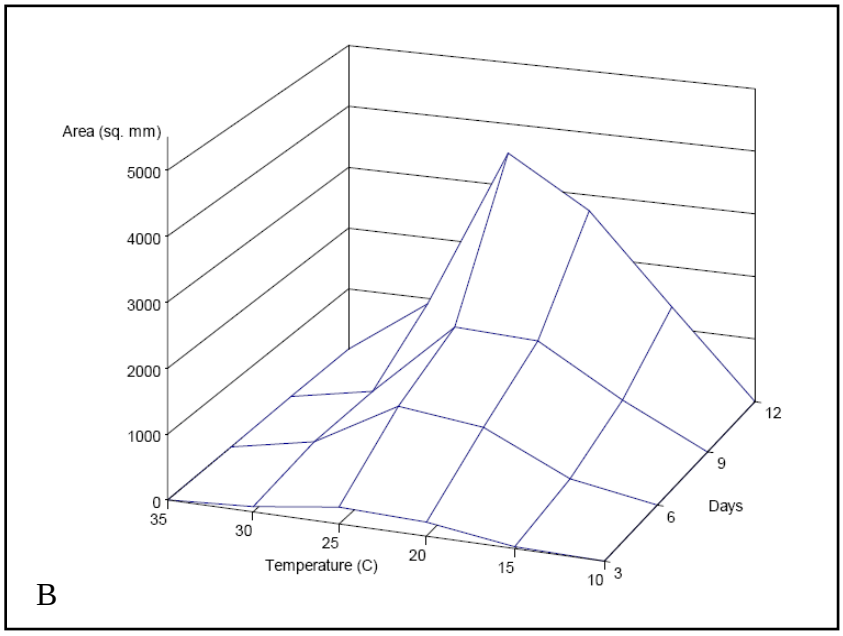


Figure 2-3. Distribution of growth area (mm^2) for the least sensitive isolate group (A) and the most sensitive group (B) of *Corynespora cassiicola* to fludioxonil at 10, 15, 20, 25, 30 and 35° C after 3, 6, 9, and 12 days.

VITA

Justin Stewart Clark was born in Cookeville, TN on February 13, 1982. He was raised on a small family farm in the Old Zion community of White County, TN. He attended White County High School where he received his diploma in 2000. He then attended the University of Tennessee at Knoxville where he received his Bachelor of Science degree in Agriculture in May, 2004. After graduating, he served as an Extension Agent I with the University of Tennessee Extension in Roane County for 18 months before returning to school to further his education. He is currently a student at the University of Tennessee at Knoxville where he is pursuing his Master of Science degree in Entomology and Plant Pathology under the supervision of Dr. Mark Windham. Upon finishing his thesis, Justin plans to attend the University of Georgia to pursue a PhD in plant pathology.