DETECTION AND MECHANISMS OF RESISTANCE TO STEROL DEMETHYLATION INHIBITING FUNGICIDES IN *CERCOSPORA ARACHIDICOLA*

by

JIA QIU

(Under the Direction of ALBERT CULBREATH and KATHERINE STEVENSON)

ABSTRACT

Isolates of *Cercospora arachidicola* were tested for sensitivity to the demethylation-inhibiting (DMI) fungicide tebuconazole using a microtiter plate assay and a new micro-colony assay. Sensitivities were determined based on EC_{50} values. Mutation of the target *CYP51* gene, overexpression of *CYP51*, and ABC transporter activity as the possible mechanisms were investigated. Based on real-time PCR assays, there was no significant difference in *CYP51* expression between tebuconazole-sensitive and resistant isolates, indicating that tebuconazole resistance was not associated with over-expression of *CYP51*. Except for one resistant isolate that became more sensitive, there was no apparent increase in tebuconazole sensitivity in the presence of ABC transporter inhibitors, suggesting that ABC transporter activity was not responsible for tebuconazole resistance in these isolates. However, alterations at codons 453 or 461 were revealed in *CYP51* in four tebuconazole-resistant isolates. This is the first report of mutations in the *CYP51* gene associated with DMI resistance in *C. arachidicola*.

INDEX WORDS: tebuconazole, DMI, fungicide resistance, *Cercospora arachidicola*, peanut early leaf spot, fungicide sensitivity, sterol inhibitor, fungicide resistance monitoring

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JIA QIU

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JIA QIU

Major Professor:	Katherine Stevenson
	Albert Culbreath
Committee:	Scott Gold
	Robert Kemerait

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Peanut leaf spot diseases and their management

The peanut plant, *Arachis hypogaea L*., is an annual, self-pollinating legume, that is thought to have originated in Paraguay (55). Remnants of peanut dating from 1500 to 1800 B.C. have been found on the northern coast of Peru (50). Because peanut has a high oil content (50%) (50) and protein (25%) content (55) it has become an important commercial crop worldwide. In the United States, 70% of the production is used in domestic food products, such as peanut butter, salted nuts and confections (43). About 1.7 million acres of peanut are harvested in the United States and the value can exceed one billion dollars (50). The states of Alabama, Florida, Georgia, North Carolina, Texas, Virginia and Oklahoma account for 98% of the total U.S. peanut production (55). In Georgia, peanut production increased from 1.6 billion pounds in 2007 to 2.3 billion pounds in 2008 (http://www.gapeanuts.com/growerinfo/usdacropreports/2008/011108ga_crppress.pdf).

The quality and quantity of peanut yield can be significantly reduced by disease. Most of the peanut diseases are caused by fungi, representing 50 different genera (50). Early leaf spot, a foliar disease caused by *Cercospora arachidicola* (teleomorph = *Mycosphaerella arachidicola*) and late leaf spot, caused by *Cercosporidium personatum* can cause severe defoliation and more than 50% yield loss (66), if not controlled. According to the data from University of Georgia Cooperative Extension (39, 46-48), the mean cost of damage of leaf spot, including peanut early leaf spot and peanut late leaf spot caused by different pathogens in Georgia is about 4.5 million dollars from 2005-2008, and the mean cost of control is about 34.5 million dollars from 2005 to 2008. *C. arachidicola* damages the peanut by formation of foliar lesions, which interfere with the photosynthetic ability of peanut. Infection can result in premature defoliation, and reduced seed yield. Symptoms of early leaf spot are characterized by circular lesions surrounded by a chlorotic

halo that appear dark brown on the adaxial surface of the leaf with lighter shade of brown on the adaxial leaf surface (70). The life cycle of *C. arachidicola* starts from the dispersal of conidia by wind, rain splash and insects. Peak dispersal occurs in the early morning hours coinciding with the onset of the rainfall (55). Lesions form within 10-14 days under the temperatures from 16 to 25° C in a wet environment (50). Early leaf spot was more prevalent in Virginia, North Carolina; however, it has become more prevalent in Georgia since 2000 (11).

Different methods are applied to control peanut early spot. Rotation of peanut with crops such as cotton or corn helps to reduce the disease. The cv. Georgia Green, has good yield but is susceptible to *C. arachidicola* (2). In recent years, several new breeding lines have been developed that have greater levels of resistance to early leaf spot pathogen than Georgia Green. These include Florida MDR-98 and C-99R, released in 1998 and 1999, respectively, with better resistance to *C. arachidicola* (6, 51). However, none of these commercial cultivars in the US are highly resistant to either *C. arachidicol*a or *C. personatum*. The primary method use to control this disease is still multiple applications of fungicides. Fungicides for the control of leaf spot include chlorothalonil, tebuconazole, propiconazole plus chlorothalonil, propiconazole plus trifloxystrolin, azoxystrobin, pyraclostrobin, boscalid and prothioconazole, which are available as solo products and various formulated mixtures (65). The first spray is suggested no more than 35 days after planting, followed by additional sprays at 14-day intervals, resulting in seven or more applications per season (28).

DMI fungicides and fungicide resistance

In the late 1960s, benzimidazole fungicides were first introduced for peanut and proved very effective for controlling foliar diseases, including early leaf spot and late leaf spot (59).

However, in the early 1970s, following development of benzimidazole resistance in *Venturia inaequalis* in Germany (36) and in *C. beticola* in Greece (22), it became evident that benzimidazoles were highly prone to the development of resistance. In 1980's, benzimidazole resistance in peanut early leaf spot was reported in the southeastern US (17). Studies in Alabama also confirmed the *in vitro* benzimidazole resistance of *C. arachidicola* (11). Chlorothalonil became the standard fungicide for early leaf spot control after 1970s (11). Chlorothalonil usually provides excellent control of early leaf spot, and is considered to be low risk for resistance, however, it is dangerous to have only one effective product available for chemical control (3). In 1994, additional choices became available for early leaf spot control with registration of the demethylation inhibitor (DMI) fungicides tebuconazole and propiconazole for use on peanut (11). The new DMI fungicides provided peanut growers with wider options for disease control. A major advantage of DMIs is activity against soilborne diseases, especially southern stem rot (*Sclerotium rolfsii*). In addition, DMIs have low toxicity to other organisms, and are safer to the environment than other chemicals.

DMIs are the largest subgroup of the Sterol biosynthesis inhibiting (SBI) fungicides that disrupt the biosynthesis of ergosterol, the primary sterol in most fungi that is essential for normal function of fungal cell membranes and cell survival (14, 31). The SBI fungicides are divided into four different classes based on the specific biochemical target in the ergosterol biosynthetic pathway: DMIs (Class I), amines (Class II), hydroxyanilides (Class III), and squalene epoxidase inhibitors (Class IV). Class I DMI fungicides include triazoles, pyrimidines, pyridines, piperazines, and imidazoles (38). The specific target of the DMI fungicides is the sterol 14α demethylase enzyme that catalyzes the 14α demethylation of eburicol (24-methylenedihydrolanosterol), a precursor of ergosterol (57-58). When the DMI fungicide binds

to the enzyme, demethylation is effectively prevented, leading to an accumulation of ergosterol precursors and lower ergosterol production.

Despite the advantages of DMIs, these single-site fungicides are more vulnerable to the development of fungicide resistance than multi-site compounds. For DMIs, a gradual decrease in sensitivity has been observed over the last 20 years for many pathogen species (37). This was first observed in the cucumber powdery mildew pathogen, *Sphaerotheca fuliginea*, in the Mediterranean region (30) and in Dutch glasshouses (63). Unlike resistance to benzimidazoles, which appears to be controlled by a single gene with great effects on sensitivity, resistance to DMIs typically appears in several small genetic steps (29, 63, 72). Loss of DMI fungicide activity is usually not complete, and in some cases, it can be overcome by shortening the intervals between treatments. Because the change in sensitivity is usually gradual, it is more difficult to monitor DMI resistance and to relate monitoring data to product performance in the field.

Mechanisms of DMI resistance

At the molecular and genetic level. Several molecular and genetic mechanisms of resistance to DMI fungicides have been identified in a number of different plant pathogens. These include mutations in the target gene (14-α demethylase gene, *CYP51*), overexpression of the *CYP51* gene and up-regulation of ATP-binding cassette (ABC) transporter genes. With DMIs, resistance often is due to the combination of several different mechanisms in a population (23).

A. Mutations in the target gene (CYP51 and ERG11). Mutations in the target gene encoding the sterol 14α-demethylase can alter the affinity of the essential enzyme for DMI inhibitors. Single-point mutations in the *CYP51* and *ERG11* genes have been observed in the

human pathogen *Candida albicans* conferring resistance to DMIs. Four mutations (Y132H, S405F, G464S and R467K) were identified in azole-resistant isolates of *C. albicans* by functional analysis in *Saccharomyces cerevisiae* (61). They are located at amino acid positions 132, 405, 464, and 467 (42). Biochemical assays showed that Y132H and R467K changed the affinity of *CYP51* for azole inhibitors (61).

In *Uncinula necator*, the fungus that causes powdery mildew of grape, a single mutation results in replacement of phenylalanine (F) by tyrosine (Y) at position 136 of the *CYP51* gene conferring resistance to triadimenol (18). This same Y136F mutation was also responsible for observed DMI resistance in *Erysiphe graminis* (19).

In *V. nashicola*, the causal agent of Japanese pear scab, an alteration was found at position 133 in a highly conserved region of the *CYP51* gene that is associated with DMI resistance (10). Numerous mutations in the *CYP51* gene of *Mycosphaerella graminicola* isolates have been identified, a number of which are at orthologous positions to those found in azole-resistant *C. albicans* strains (9). In *M. graminicola*, a valine instead of an isoleucine at position 381 of *CYP51*, in combination with the alterations at codons 459, 460, and 461, determined the highest resistance levels to triflumizole, fluquinconazole and tebuconazole but not prochloraz (40).

Two closely related cereal eyespot fungi, *Tapesia yallundae* and *T. acuformis*, show different sensitivities to DMIs. *T. yallundae* is sensitive to both triazole and imidazole DMIs, but *T. acuformis* is naturally resistant to triazoles and sensitive to the imidazoles (1). DNA sequence analysis of the *CYP51* gene of *T. acuformis* showed that a conserved phenylalanine residue at codon position 180, which was also found in *T. yallundae* and in all known *CYP51* proteins from

other filamentous fungi, was substituted by a leucine in *T. acuformis*. Therefore, the leucine at 180 position of *CYP51* is likely responsible for the resistance of *T. acuformis* to triazoles (1).

In addition, several other amino acid alterations in the *CYP51* gene were found to be associated with DMI resistance in *Penicillium italicum* (32) and *Ustilago maydis* (4). Interestingly, single mutations are not usually associated with high resistance factors; combined mutations effect greater reductions in DMI sensitivity than single ones, proving that different *CYP51* mutations can combine to increase the resistance level. The combined amino acid changes can increase resistance factors up to 64 (61). In *Aspergillus fumigatus CYP51* gene which encodes the target of azole antifungal agents, the residue Ile301 was mutated to Ala in resistant strains and is proposed to hydrogen bond with the sterol substrate, leading to the resistance to fluconazole (20). In *M. fijiensis*, six mutations that could be related to the loss in sensitivity to propiconazole were found: Y136F, A313G, Y461D, Y463D, Y463H and Y463N. These changes in the sequence of *CYP51* gene were found that have been described in other fungi as being correlated with resistance to azole fungicides (5). However, studies on *V. nashicola* (10) and *T. yallundae* (74) confirmed that not all DMI resistance in field isolates are associated with amino acid changes in the *CYP51* proteins. Mechanisms other than mutations in the target gene can be associated with DMI resistance.

B. Overexpression of target gene (CYP51). In addition to the mutations in the target gene of fungi of DMI fungicides, the expression level of the gene also affects the sensitivity of DMI fungicides. Increased intracellular levels of the demethylase enzyme have been associated with gradual development of DMI resistance. Several mechanisms have been known to increase *CYP51* expression in fungi. Increased *CYP51* expression due to an increased copy number of the *CYP51* was responsible for DMI resistance in the human fungal pathogen *C. glabrata* (45). In

isolates of the plant pathogen *P. digitatum*, azole resistance in the field was also associated with the overexpression of *CYP51* (25). Amino acid sequences of *CYP51* gene from six isolates of *P. italicum* were identical (three DMI resistant and three DMI sensitive). However, a 126-bp sequence in the promoter region of the *CYP51* gene was tandem repeated five times in resistant isolates compared to only one copy of the sequence in sensitive isolates. After the *CYP51* gene (including the promoter region) was transformed from a resistant isolate into a sensitive isolate, transformants showed a higher level of *CYP51* expression than the sensitive isolates and were DMI-resistant. When the tandem repeats in resistant isolates were reduced from five to two, both DMI resistance and *CYP51* expression level decreased. The above results revealed that the 126-bp repeats enhanced *CYP51* expression, and resulted in DMI resistance in *P. digitatum*. A similar phenomenon was also observed in *Blumeriella jaapii*, a pathogen of cherry (44). *CYP51* expression in DMI-resistant isolates was 5- to 12-fold higher than that in DMI-sensitive isolates. A 2120- to 5585-bp insert in the promoter region of *CYP51* was found in all of the DMI-resistant isolates, but not in the DMI-sensitive ones. Expression of *CYP51* in DMI resistant isolates was not correlated with the size of the upstream insert. In the apple scab pathogen *V. inaequalis*, overexpression of *CYP51* in DMI-resistant isolates was associated with the presence of a 553-bp insertion located upstream of the *CYP51* gene (64). In France, researchers found high constitutive levels of the *CYP51* transcript in field isolates of *M. graminicola* with an intermediate level of resistance to DMIs (68). However, overexpression of *CYP51* was not found in more recently collected DMI-resistant isolates of *M. graminicola* (8). In *C. beticola*, study of transcriptional levels of the *CYP51* gene showed that overexpression was strongly associated with the highly DMI-resistant phenotype (53).

C. Overexpression of ATP-binding cassette (ABC) encoding gene. In nature, successful survival and reproduction of microorganisms is contingent upon the ability to resist the noxious effects of toxic compounds. Microorganisms have a membrane efflux pump system, which is localized in the cytoplasmic membrane of all kinds of cells. In eukaryotic cells, the existence of efflux pumps has been known since the discovery of p-glycoprotein in 1976 by Juliano and Ling (33). Efflux pumps are one of the major causes of anticancer drug resistance in eukaryotic cells. The function of the efflux pump system is to protect the microorganisms by reducing the concentration of toxin in certain cell compartments of the cytoplasm. This efflux pump system is involved in sensitivity and multidrug resistance to fungicides in many fungi, including *S. cerevisiae* (15), *A. nidulans* (16), *A. fumigatus* (71), *Botrytis cinerea* (26), and *M. graminicola* (75).

Efflux systems function via an energy-dependent mechanism (active transport) to pump out unwanted toxic substances through specific efflux pumps. They are active transporters meaning that they require a source of chemical energy to perform their function. Some are primary active transporters utilizing adenosine triphosphate hydrolysis as a source of energy, while others are secondary active transporters (uniporters, symporters or antiporters) in which transport is coupled to an electrochemical potential difference created by pumping out hydrogen or sodium ions outside the cell.

There are two major classes of transporters: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters. The role of ABC transporters in fungicide sensitivity and resistance has been well established. ABC transporters are able to bind and hydrolyze nucleotide triphosphates (mainly ATP), which is an energy source to perform the function. The energy generated in this process is used to transport solutes across cell membranes.

Because of this, ABC transporters are considered as primary active transporter systems. Most efflux pumps that are capable of excluding of a great number of endogenous and exogenous toxicants belong to this group (28). In contrast, MFS transporters do not hydrolyze ATP, and are considered to be active in secondary transport systems (41). Compounds transported by MFS transporters over membranes are controlled by the proton-motive force (28). De Waard (12) predicted that the natural function of the transporters in plant pathogens is the secretion of endogenous factors and the exogenous plant defense compounds. This postulation has been confirmed during the past few years (14).

In the human pathogen *C. albicans*, efflux pump systems received much attention due to the severe problem of drug resistance in clinical treatment (73). Five Candida drug resistance genes (*CDR1*, *CDR2*, *CDR3*, *CDR4*, and *CDR5*) encoding ABC transporters have been identified (73). *CDR1* plays a major role in resistance to common azoles, such as fluconazole and miconazole (56, 63). *CDR2* also confers resistance to several azole drugs (62). Evidence has indicated that ABC transporters play a role in azole resistance in other *Candida* species, including *C. krusei* (*ABC1*) (34), *C. glabrata* (*CgCDR1*) (60), and *C. dubliniensis* (*CdCDR1*) (54).

Five ABC transporter genes from the wheat pathogen *M. graminicola* have been identified as *Mgatr1-Mgatr5*. Two single copies of *Mgatr1* and *Mgatr2* have been cloned (75). The expression of these five genes in mutants showed different resistance levels and indicated that ABC transporters might play a role in sensitivity and resistance of *M. graminicola* to DMIs. The putative role of these genes in DMI resistance is still under investigation.

In *B. cinerea*, a pathogen of tomatoes, strawberries, and many other hosts, the ABC transporter genes *BcatrA*, *BcatrB* and *BcatrD* have been cloned. Functional analysis showed that

disruption of *BcatrA* did not result in a clear phenotype with regard to DMI fungicide sensitivity. However, disruption of *BcatrB* resulted in increased fungicide sensitivity (26). For this reason, *BcatrB* seems to play a crucial role in regulation of fungicide sensitivity. Two separate studies have shown that the ABC transporter gene *BcatrD* was a determinant of the sensitivity of *B. cinerea* to DMIs (27). The function of *BcatrD* gene-replacement and overexpression mutants was analyzed. *BcatrD*-replacement mutants demonstrated a higher sensitivity to DMI fungicides compared to the parental isolates. The accumulation of DMI fungicides in gene replacement mutants was relatively high compared to wild type. *BcatrD*-overexpression mutants showed increased levels of basal and DMI-induced expression of *BcatrD*. Mutants with the highest expression level displayed the lowest DMI sensitivity and accumulated a relatively low concentration of fungicide compared to wild type.

Research has shown that the ABC transporter gene *PMR1* plays an important role in resistance of *P. dititatum* to DMIs. Sequence analysis of *PMR1* in *P. digitatum* revealed significant amino acid homology between the PMR1 and the CDR1 proteins encoded by ABC transporter gene *CDR1* in *C. albicans*. Disruption of the *PMR1* gene of a DMI-resistant *P. digitatum* isolate restored DMI sensitivity to almost wild-type level. A northern blot assay demonstrated that basal transcript levels of *PMR1* were several fold higher in DMI-resistant isolates than in DMI- sensitive isolates in the absence of fungicide, proving that *PMR1* was responsible for DMI resistance of the fungus (52).

At the biochemical and physiological level. The physiology of energy-dependent efflux pumps that results in a differential accumulation of DMIs in wild type and DMI-resistant fungi was elucidated before the molecular basis of ABC transporters was described (13, 15). Mohr and Gisi (23) found that higher efflux rate in mycelium of *M. graminicola* isolates may reflect the

mechanism of resistance to DMIs. In addition, the role of ABC transporters in DMI resistance was verified by biochemical assay with different inhibitors of ABC transporters. The primary DMI resistance mechanism in *Candida* spp. has been attributed to a diminished binding affinity of the target enzyme encoded by the *CYP51* gene. However, in *C. krusei*, an efflux pump was proposed by Katiyar and Edlind (34) as an alternative resistance mechanism. To test their hypothesis, the researchers used CCCP (carbonyl cyanide 3-chloro-phenylhydrazone), an efflux-pump inhibitor, which reduced the minimum inhibitory concentration (MIC) of multiple DMI-resistant strains. Five different DMIs were applied to 32 DMI-resistant isolates, but only one isolate showed a two-fold decrease in MIC to fluconazole when CCCP was added (24). Although evidence for the contribution of CCCP-inhibited efflux pumps in DMI resistance is limited, other efflux pump inhibitors should be studied.

The genetic changes in the fungus that confer resistance to a fungicide may also confer resistance to other fungicides with the same mode of action. This phenomenon is referred to as cross resistance. Different DMIs have different levels of intrinsic activity. However, cross-resistance occurs among the major DMIs, including pyridines, pyrimidines, imidazoles, and triazoles (23). Cross-resistance among DMIs is common, but there are some notable exceptions. For example, in *M. graminicola,* no correlation was found between sensitivity to fluquinconazole and prochloraz. Kendall et al. (35) examined cross-resistance patterns in the pathogen *Rhynchosporium secalis* and reported that cross-resistance occurred between triadimenol, propiconazole and tebuconazole but not between these fungicides and prochloraz.

At the population level. The goal of DMI-resistance studies at the population level is focused on understanding the frequency and geographic distribution of DMI resistance in plant pathogens worldwide. Over several years, isolates of *M. graminicola* collected from wheat fields

in France, Germany, and Great Britain were tested for DMI sensitivity. In 2004, resistant phenotypes were detected in European *M. graminicola* populations (49). Comparison of the CYP51-haplotypes of 615 *M. graminicola* isolates from different European regions for their *in vitro* sensitivity towards the triazole epoxiconazole nevertheless showed that the influence of the CYP51-haplotypes on sensitivity is limited. There was no correlation between *in vitro* sensitivity or CYP51-haplotype pattern and field performance of epoxiconazole at different trial sites (67). In *B. graminis* (*E. graminis*) and other pathogens, isolates with decreased DMI sensitivity have been reported and the sensitivity tended to decline in succeeding years (21). The selection of DMI resistance seemed to be directional and resulted in populations with stable sensitivity distributions (7).

In general, mechanisms responsible for DMI resistance are complex and vary among different plant pathogens. The primary molecular mechanisms are associated with the alteration of the target gene (*CYP51*) and the genes encoding the ABC transporters. However, the composition of *CYP51* genotypes in field populations changed significantly over the last two decades, and molecular probes for specific mutations in *CYP51* may not detect the mutants with the varied *CYP51* genotypes developed during these two years.

Research Objectives

Early leaf spot of peanut caused by fungus *C. arachidicola* can significantly reduce both yield and quality of the peanut production. DMI fungicides are essential for the production of crops having satisfactory quality and yields. Recently, peanut growers in Georgia and neighboring states have reported that the DMI fungicide tebuconazole seemed to be less effective

than in previous years, although early leaf spot still can be controlled by this DMI (11). The shift sensitivity to tebuconazole was confirmed in laboratory assays (69).

This research was undertaken to investigate the mechanisms responsible for resistance to DMIs in *C. arachidicola*. The first objective of this study was to (i) develop an in vitro fungicide sensitivity assay using a colony-diameter-based rapid assay as an alternative to the standard microtiter plate assay, (ii) evaluate the correlation between results of these two assays, and (iii) use this rapid assay to identify fungicide-resistant *C. arachidicola* isolates for further research. Using a set of DMI-sensitive and DMI-resistant isolates of *C. arachidicola*, the second objective of the study was to determine if DMI resistance in *C. arachidicola* is attributable to (i) mutation of the target *CYP51* gene , by comparing sequences of the *CYP51* gene, (ii) overexpression of *CYP51*, by quantifying the expression level of *CYP51* gene by real-time PCR, or (iii) active efflux mediated by ABC transporters, by examining the effects of known inhibitors of ABC transporters on tebuconazole sensitivity *in vitro*. Results of these investigations will provide timely results and information to researchers and growers for fungicide resistance monitoring aspect, increase the knowledge in better understanding DMI fungicide resistance in *C. arachidicola* and could provide new clues for management of fungicide-resistant pathogens.

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CHAPTER 2

DEVELOPMENT OF A RAPID ASSAY FOR DETECTION OF DMI RESISTANCE IN THE PEANUT EARLY LEAF SPOT PATHOGEN *CERCOSPORA ARACHIDICOLA*

[1]Qiu, J., Stevenson, K. L., and Culbreath, A. K. 2010. To be submitted to Plant Disease.

Abstract

Commercial production of peanut (*Arachis hypogaea*) in the southeastern U.S. relies on DMI fungicide applications to control early leaf spot, caused by *Cercospora arachidicola*. These fungicides include propiconazole, tebuconazole, and prothioconazole. During periods of disease pressure, many applications may be made per season and the potential development of fungicide resistance is a major concern. A rapid method was developed to determine fungicide sensitivity of tebuconazole based on measurement of micro-colony transverse diameter. This method uses conidia transferred directly from lesions to fungicide-amended media. Sensitivities were determined for isolates from one organically managed field without any previous history of tebuconazole exposure and four different fields with a history of tebuconazole use. *In vitro* fungicide resistance was detected in 108 isolates, 12 from the organic field, and 96 from the locations with tebuconazole spray history. The new method enabled detection of fungicide resistance in 72 hrs by measuring transverse diameters of micro-colonies of *C. arachidicola*. To compare this novel method, sensitivities of isolates were also tested by the standard microtiter plate assay. Using the micro-colony assay, EC_{50} values of 21 isolates ranged from 0.39 to 6.17 μg/ml in 2008. EC_{50} values of 78 isolates ranged from 0.36 to 9.73 μg/ml in 2009. Using the microtiter plate assay, EC_{50} values of 29 isolates ranged from 0.017 to 4.65 μg/ml in 2008. EC_{50} values of 58 isolates ranged from 0.025 to 5.56 μg/ml in 2009. A significant positive correlation between EC_{50} values from the two assays was demonstrated based on combined data for both years.

Introduction

Peanut early leaf spot, caused by *Cercospora arachidicola*, is one of the most common and damaging foliar disease of peanut. If not well-controlled, yield loss due to early and late leaf spot (*Cercosporidium personatum*) can reach 50% (15). The mean cost of damage by peanut leaf spot from 2005 to 2008 in Georgia is about 4.5 million dollars, with over 20 million dollars spent on control (9, 11-13). The disease can be partially controlled by several different cultural practices, such as rotation with other crops, earlier planting, or growing peanut cultivars that have modest levels of resistance to early leaf spot. However, early leaf spot management relies heavily on multiple fungicide applications. Typically the first application is made 30-40 days after planting, and subsequent applications are made every 14 days or according to a prescription schedule based on Peanut Rx (http://www.caes.uga.edu/commodities/fieldcrops/peanuts/2009peanutupdate/peanutrx.html). A total of seven sprays are usually applied per season to control leaf spot (2).

Demethylation inhibitors (DMIs) labeled for use on peanut in the U.S. include tebuconazole, propiconazole, and prothioconazole. When they were first introduced, the DMIs, especially tebuconazole, were very widely used because they were highly effective against both peanut leaf spot and soilborne diseases (1). Four-spray blocks (sprays 3-6) of tebuconazole provided excellent control of peanut early leaf spot (4). The Fungicide Resistance Action Committee (FRAC) guidelines indicate that tebuconazole has a medium resistance risk (6). Resistance to DMI fungicides is quantitative, and typically develops slowly in the field. A gradual decrease in DMI sensitivity was observed over the last 20 years for many pathogen species, such as *Sphaerotheca fuliginea* (powdery mildew of cucumber) (7), *Uncinula necator* (powdery mildew of grape) (5), and *Blumeriella jaapii* (leaf spot of cherry) (10). Tebuconazole

was first labeled for use on peanut in 1994 (1). In 1996, the baseline sensitivity to tebuconazole was established for *C. arachidicola* and *C. personatum* (18). Decline in performance of tebuconazole for leaf spot control in research plots was first reported in 2003 (3). Isolates of the pathogens were collected from Georgia, Alabama, South Carolina, Oklahoma and tested for sensitivity in 2005. Results provided evidence that tebuconazole sensitivity had shifted significantly from 1996 to 2005, although the disease was still effectively controlled by tebuconazole in many locations (18).

Typically, DMI resistance develops slowly and is difficult to detect at first. Resistant strains may have reduced fitness; therefore, resistance management may effectively change the resistant populations back toward sensitivity. However, this is not always the case, and fungicides with resistance problems cannot be successfully reintroduced into areas where resistant strains are highly fit. For example, resistant strains of *C. arachidicola* are still present in the southeastern U.S. where benomyl resistance was a problem years after use of benomyl was discontinued (3). Unfit strains only compete well under the selection pressure of the fungicide. Thus, a resistance problem may be at least partially reversible when the selection pressure of the fungicide is removed or minimized by using resistance management. Monitoring resistance levels in pathogen populations is essential for assessing risk and evaluating management practices. Baseline sensitivities (EC_{50} values) are being determined for some pathogens and crops where widespread DMI fungicides. A microtiter plate assay has been used for monitoring fungicide sensitivity of *C. arachidicola* for many years. However, because *C. arachidicola* grows extremely slowly in culture, several months are required to complete the assay. Because of time required for this method, it cannot provide timely results for growers. In addition, the risk for contamination of assay cells is high. This research was undertaken to develop a rapid method

to determine the fungicide sensitivity based on the measurement of the transverse diameter of *C. arachidicola* micro-colonies at different fungicide concentrations. This new approach is much faster and time-efficient and is a modification of a similar assay developed by Seyran for assessing fungicide resistance in the pecan scab pathogen, *Fusicladium effusum* (14).

The objectives of this study were to (i) develop an in vitro fungicide sensitivity assay using a colony-transverse diameter-based rapid assay as an alternative to the standard microtiter plate assay, (ii) evaluate the correlation between results of these two assays, and (iii) use this rapid assay to identify fungicide-resistant *C. arachidicola* isolates for further research.

Materials and methods

Sampling locations. Peanut leaves with actively sporulating early leaf spot lesions were collected from six different locations in southern Georgia in 2008 and 2009. Twenty-nine isolates were collected and tested in 2008, from fields with a history of tebuconazole use. Seventy-eight isolates were collected and tested in 2009. Of these 108 isolates, 12 isolates were from a non-treated field with no previous history of DMI exposure, and 96 isolates were from fields with a history of DMI use.

Micro-colony growth assay. For conidia suspensions, a solution of sterile deionized water with antibiotics (streptomycin sulfate, chloramphenicol, and tetracycline, 50 µg/ml for each) and Tween 20 (one drop per 100 ml) was prepared. Three hundred microliters of the solution were transferred to 1.5-ml microcentrifuge tubes. Only early leaf spot lesions bearing visible conidia were selected for testing. Conidia suspensions from individual lesions were made by transferring 9 µl of the antibiotic solution to the surface of a sporulating lesion with a pipette, and pumping the solution up and down several times until most of the conidia were dislodged

and suspended in the solution. Then the solution with conidia was transferred back to the tubes. This step was repeated twice for each lesion.

Technical grade tebuconazole (97.5%) (Bayer Corporation, Kansas City MO) was dissolved in acetone to obtain a stock solution containing 30,000 µg/ml and then serially diluted in acetone to obtain fungicide concentrations of 3,000, 1,000, 300, 100, 10, 1, 0.1 µg/ml. One milliliter of each fungicide concentration was added to 1 liter of autoclaved molten PDA (60°C) to obtain the final target concentrations of 3, 1, 0.3, 0.1, 0.01, 0.001, and 0.0001µg/ml in the medium. One milliliter of acetone was added to 1 liter of PDA for the non-amended controls. The final concentration of acetone in the prepared medium was 1% (v/v) for all fungicide concentrations and the non-amended control.

Twelve microliters of each conidial suspension were spread onto two replicates of fungicide-amended PDA plates or control plates. After incubation at room temperature (23 to 25°C) for 72 h, colony transverse diameters were measured using an integrated microscope camera system (MoticCam 2300, 3.0 MPixel USB 2.0, Motic Inc. Richmond, British Columbia, Canada). Plates were examined microscopically at a magnification of 32X, and the images were transferred to a computer. For each replication, 10 single, well separated colonies were arbitrarily selected and the colony transverse diameter of each was measured using the Motic Images Plus 2.0 software provided by the camera manufacturer (Fig. 2.1). Relative growth inhibition for each replication of each isolate and each fungicide concentration was determined according to the following formula:

$$\text{Growth inhibition} = 1 - (DF_{10}/DC_{10}),$$

where DF_{10} = mean colony transverse diameter of ten colonies on fungicide-amended medium, and DC_{10} = mean colony transverse diameter of ten colonies on non-amended medium (control).

The EC_{50} value (the effective fungicide concentration that inhibits growth by 50%) was estimated for each isolate by regressing the probit-transformed relative growth inhibition on the logarithm of the fungicide concentration using SAS PROC REG (SAS ver. 9.1, SAS Institute, Cary NC).

Microtiter plate assay. After the micro-colonies were measured, three distinct micro-colonies of each isolate were arbitrarily selected and transferred from the non-amended control plates to fresh PDA. Colonies were incubated at 24°C for at least 2 months, until they were large enough to use. A 2-mm mycelial plug of each isolate was transferred to a tube containing 3 ml potato dextrose broth (PDB) and incubated at 24°C for about 20 days. After 20 days, the mycelial plug was homogenized with a tissuemizer (IKA Labortechnik T25 Basic, IKA Works, Inc. Wilmington, NC) to obtain a uniform suspension of fine mycelial fragments.

To prepare the fungicide-amended medium, a stock solution of technical grade tebuconazole was prepared and serially diluted in acetone as described above. Eighty microliters of each fungicide concentration was added to 60 ml of autoclaved and cooled PDB. The final concentrations of fungicide-amended PDB were 3, 1, 0.3, 0.1, 0.01, 0.001, and 0.0001 μg/ml after mycelial suspensions were added into microtiter plate. Eighty microliters of acetone was added to 60 ml PDB for the non-amended controls. The final concentration of acetone in the prepared medium was 1% (v/v) for all fungicide concentrations and the non-amended control.

Fifty microliters of mycelial suspension for each isolate and one hundred fifty microliters of fungicide-amended or non-amended medium were added to each well of 96-well microtiter plates using a multi-channel pipette. In 2008, three replicate plates were prepared for each isolate and fungicide concentration. The initial amount of mycelium in each well on day 0 was estimated based on light absorbance at a wavelength of 405 nm as measured by an automated

plate reader (Multiskan Plus, Fisher Scientific, Pittsburgh, PA). To avoid contamination, only one replicate plate was opened and read immediately after it was prepared, and the data were used as the initial growth measurement for the other two replicate plates. The remaining two replicate plates were incubated at 24°C on a shaker set at 100 rpm for 14 days and the final amount of mycelium in each well was estimated using an automated plate reader as described above. In 2009, only two replicate plates were prepared for each isolate and fungicide concentration and the amount of mycelium in each well was estimated twice for each plate, on day 0 and day 14. Absolute mycelial growth in each well was calculated as the difference in light absorbance values as measured on day 0 and day 14. Relative growth inhibition for each replication of each isolate and fungicide concentration was determined according to the following formula:

$$\text{Growth inhibition} = 1 - (GF/GC),$$

where GF = absolute growth in the well containing fungicide-amended medium, and GC = absolute growth in the well containing non-amended medium (control). EC_{50} values were estimated for each isolate by regressing probit-transformed relative growth inhibition on the logarithm of the fungicide concentration as previously described.

To investigate if there is a significant linear relationship between the micro-colony $\log_{10}$-transformed EC_{50} (LEC_{50}) and the microtiter $\log_{10}$-transformed EC_{50} (LEC_{50}), SAS PROC MIXED (SAS ver. 9.1, SAS Institute, Cary, NC) was applied.

Results

Micro-colony growth assay. In 2008, EC_{50} values of all 21 isolates ranged from 0.39 to 6.17 µg/ml with a mean EC_{50} value of 1.35 µg/ml (Fig. 2.2). The EC_{50} values of 20 isolates ranged from 0.29 to 2.19 µg/ml; 1 isolate was considerably less sensitive than others, with an

EC_{50} value of 6.17 µg/ml. In 2009, EC_{50} values of 78 isolates ranged from 0.36 to 9.73 µg/ml with a mean EC_{50} value of 2.73 µg/ml (Fig. 2.3). EC_{50} values of 70 isolates ranged from 0.36 to 4.96 µg/ml; 8 isolates were more resistant than others. EC_{50} values ranged from 6.04 to 9.73 µg/ml.

Microtiter plate assay. In 2008, EC_{50} values of 29 isolates ranged from 0.017 to 4.65 µg/ml with a mean EC_{50} value of 0.80 µg/ml (Fig. 2.4). Among these 29 isolates, EC_{50} values of 27 isolates ranged from 0.017 to 1.68 µg/ml; 1 isolate was moderately resistant compared to the 27 isolates, and EC_{50} value was 2.71 µg/ml. One isolate was highly resistant with an EC_{50} value of 4.65 µg/ml (Fig. 2.4). In 2009, EC_{50} values of 58 isolates ranged from 0.025 to 5.56 µg/ml with a mean EC_{50} value of 0.74 µg/ml (Fig. 2.5). EC_{50} values of 57 isolates ranged from 0.025 to 3.18 µg/ml. Two isolates were considered highly resistant, with EC_{50} values of 4.66 µg/ml and 5.56 µg/ml (Fig. 2.5).

Comparison of EC_{50} values from the two assay methods. An analysis was performed on $\log_{10}$-transformed EC_{50} (LEC_{50}) values from the micro-colony and microtiter plate assays for 19 isolates from 2008 and 58 isolates from 2009. Results provided evidence of a significant linear relationship between the micro-colony LEC_{50} and the microtiter LEC_{50}; however, the relationship was best described by separate regression lines for each year, with a common slope (=0.2401) and significantly different intercepts ($P<0.0001$) (Fig. 2.6). The regression equations for 2008 and 2009 were

$$\text{For 2008: } y = 0.0748 + 0.2401\ x$$

$$\text{For 2009: } y = 0.3351 + 0.2401\ x$$

where, y = micro-colony LEC_{50}, and x = microtiter plate LEC_{50}. The common slope indicates a similar relationship between the assay results in both years and the different intercepts indicate that micro-colony LEC50s were significantly higher in 2009 than in 2008.

Discussion

foliar and soil-borne diseases. Reduced sensitivity to the DMI fungicides has been reported in the peanut leaf spot pathogens (18). However, DMIs are still effective for leaf spot control in many locations. Therefore, DMI sensitivity monitoring can provide very important information for selecting fungicides for leaf spot control programs. The early leaf spot pathogen, *C. arachidicola*, grows extremely slowly in culture, making it difficult to monitor sensitivity to DMI fungicides using in vitro assays. A microtiter plate assay has been used for determining fungicide sensitivity of *C. arachidicola*, but this assay requires several months to complete. In this study, a new micro-colony growth assay was developed and evaluated as a more rapid alternative to the microtiter plate assay. Isolates collected in two different years from different locations in Georgia were used to compare tebuconazole sensitivity using both micro-colony growth assay and microtiter plate assay.

EC_{50} values from the micro-colony growth assay were consistently higher than those from microtiter plate assay in both 2008 and 2009. This result may be due to inhibitory effects of microbial contaminants on growth of *C. arachidicola*. For the micro-colony growth assay, spores were taken directly from peanut leaf surfaces and then spread on PDA plates. Other microorganisms present on peanut leaf surfaces can be transferred along with the conidia and may grow as contaminants on the PDA. This is most likely to occur on the control plates that do not contain tebuconazole. Some contaminants may produce antifungal substances to hinder

growth of the micro-colonies of *C. arachidicola* (8). This may result in colony transverse diameter measurements that are smaller than expected. If the same contaminants are inhibited by tebuconazole, then the inhibition of growth of micro-colonies of *C. arachidicola* by the contaminants may be greatly reduced in the fungicide-amended medium. EC_{50} values are calculated based on relative growth values. Therefore smaller micro-colony transverse diameters on the control plate would result in larger relative growth values on tebuconazole-amended plates and consequently, an overestimation of EC_{50} values. Unlike the micro-colony growth assay, the measurements from microtiter plate assay were not affected by contamination because the mycelial suspensions for this assay were prepared from pure, monoconidial cultures.

The second possible explanation for the higher EC_{50} values from the micro-colony assay compared to the microtiter plate assay may be related to the rate of depletion of ergosterol in the germinating conidia. Tebuconazole, like other DMI fungicides, inhibits the synthesis of ergosterol, which is essential for fungal growth and development. Since *C. arachidicola* conidia contain a constitutive level of sterol, DMI fungicides cannot inhibit the initial conidial germination. They begin to inhibit fungal development only after the fungus has depleted the sterol that is initially present in the conidium. In a previously published study, DMI fungicide resistance was monitored in the late leaf spot pathogen, *C. personatum,* by measuring the elongation of the primary hyphal growth of after 6 days incubation on PDA plates (16). In our micro-colony growth assay, the colony transverse diameters of *C. arachidicola* were measured after only 3 days (72 h), primarily to prevent potential interference from contaminants. Both of these leaf spot pathogens grow extremely slowly in culture. Therefore, 3 days incubation may not be long enough for all the initial sterol in the conidium to be depleted. In that case, the micro-colonies of *C. arachidicola* would not be inhibited by the amended tebuconazole and the EC_{50}

would be over-estimated. In the microtiter plate assay, *C. arachidicola* isolates were incubated in the presence of tebuconazole for 2 weeks, which should be sufficiently long to deplete the initial supply of sterol in the fungal mycelium.

The microtiter plate method was performed on individual pure cultures of monoconidial isolates, whereas the micro-colony method was performed on a collection of conidia from a single lesion. Genetic and biological variation among the conidia from the same lesion could affect the rates of spore germination and individual colony growth on both fungicide-amended and non-amended PDA. This variation in germination and growth rates could be responsible for the variation in transverse diameter measurements of individual colonies observed in the micro-colony assay. However, relative growth was calculated based on the mean transverse diameters of ten colonies, arbitrarily selected from the control plate and the tebuconazole-amended PDA plates, which would likely reduce the effects of variation in individual colony size on relative growth values.

Based on the data from 2008 and 2009, the EC_{50} values are different from both assays. Modifications of the rapid screening method described might improve its EC_{50} measurement. For example, using a semi-selective medium instead of PDA to grow *C. arachidicola* micro-colonies may reduce the influence of contamination (17). Using a semi-selective medium, the plates could be incubated for more than 3 days to ensure that all the constitutive ergosterol in the conidium is depleted. In spite of these, micro-colony assay still save lots of time in testing the sensitivity of *C. arachidicola* isolates compared to microtiter plate assay. Additional work is needed to modify the micro-colony assay method and verify that the new assay provides estimates of EC_{50} values that are similar to those obtained from the standard microtiter plate assay.

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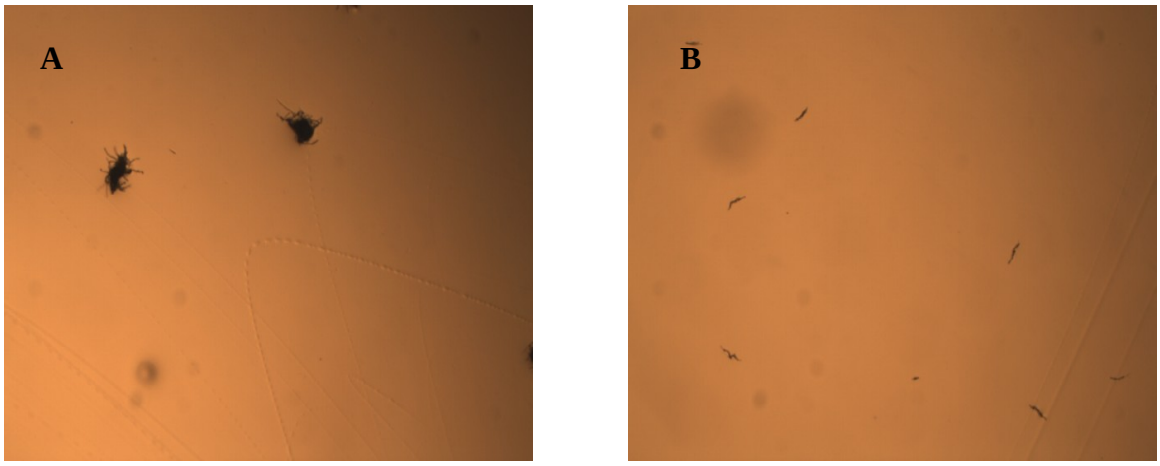

Fig. 2.1. A) Micro-colonies of a resistant isolate of *Cercospora arachidicola* after 3 days incubation on tebuconazole-amended PDA, and B) germinated conidia of a sensitive isolate of *Cercospora arachidicola* after 3 days incubation on tebuconazole-amended PDA.

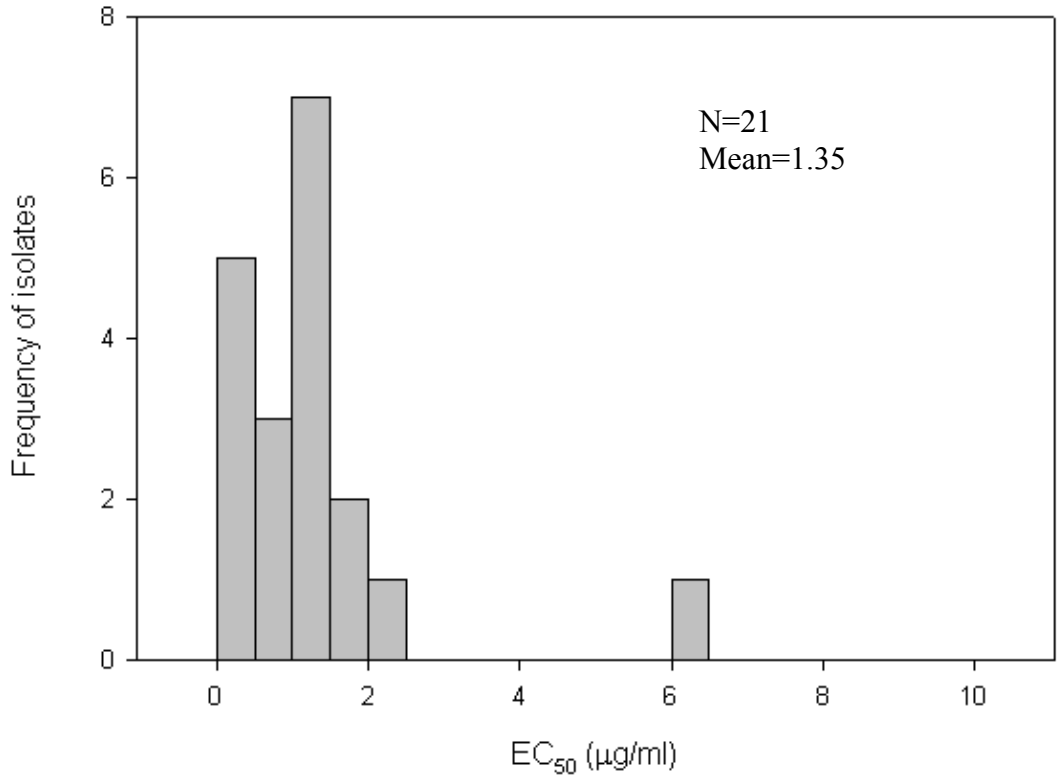

Fig. 2.2. Frequency distribution of tebuconazole EC_{50} values for *Cercospora arachidicola* isolates collected in 2008 based on a micro-colony growth assay.

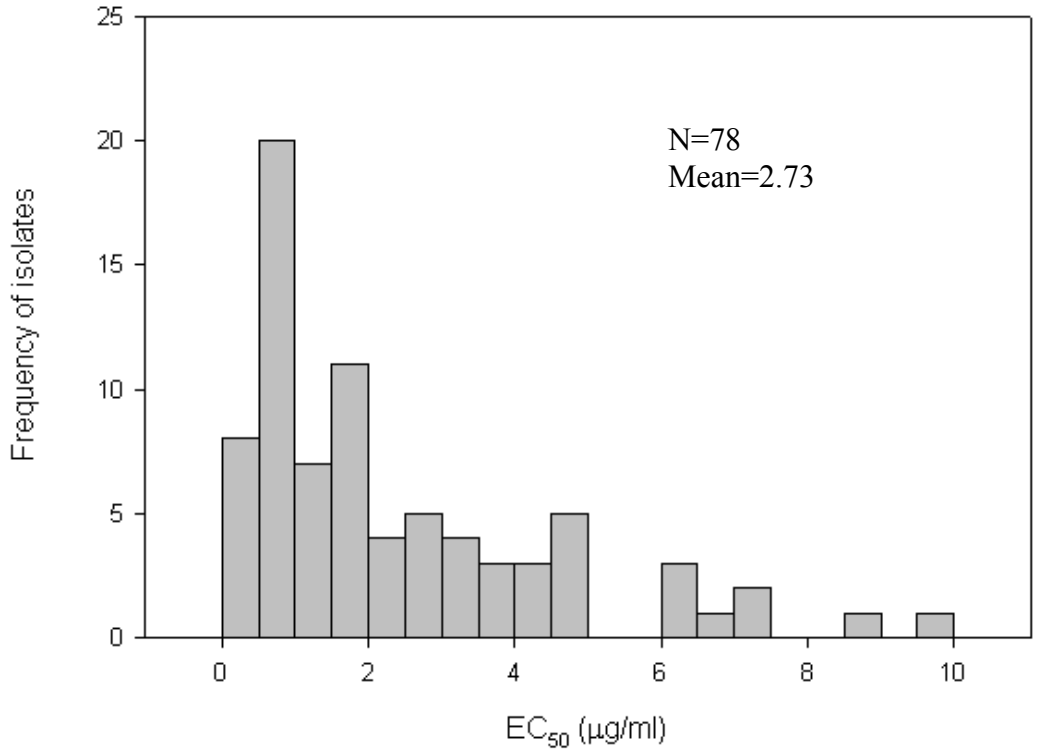

Fig. 2.3. Frequency distribution of tebuconazole EC_{50} values for *Cercospora arachidicola* isolates collected in 2009 based on a micro-colony growth assay.

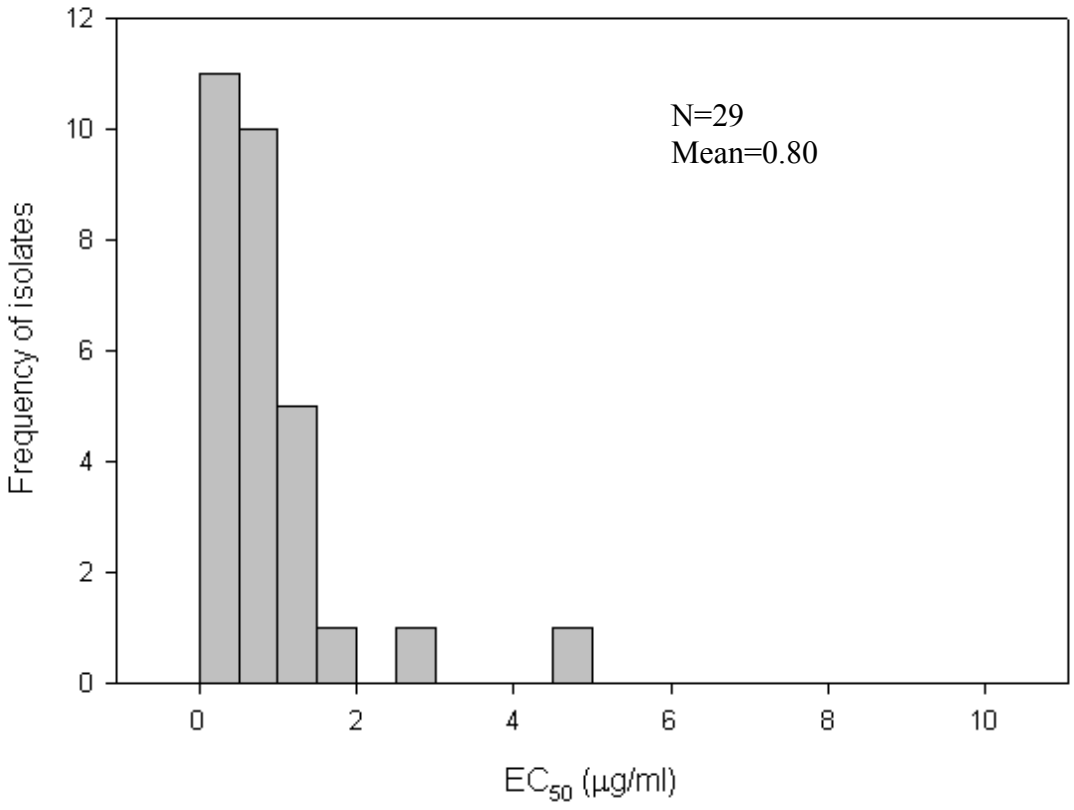

Fig. 2.4. Frequency distribution of tebuconazole EC_{50} values for *Cercospora. arachidicola* isolates collected in 2008 based on a microtiter plate assay.

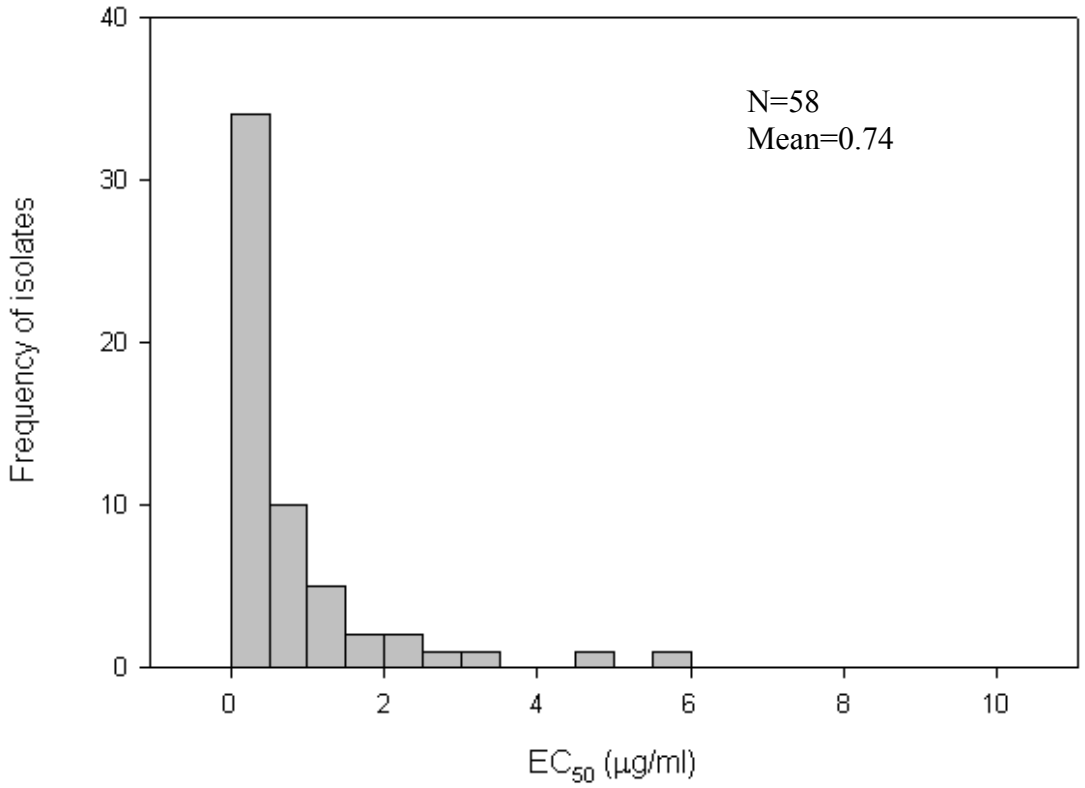

Fig. 2.5. Frequency distribution of tebuconazole EC_{50} values for *Cercospora arachidicola* isolates collected in 2009 based on a microtiter plate assay.

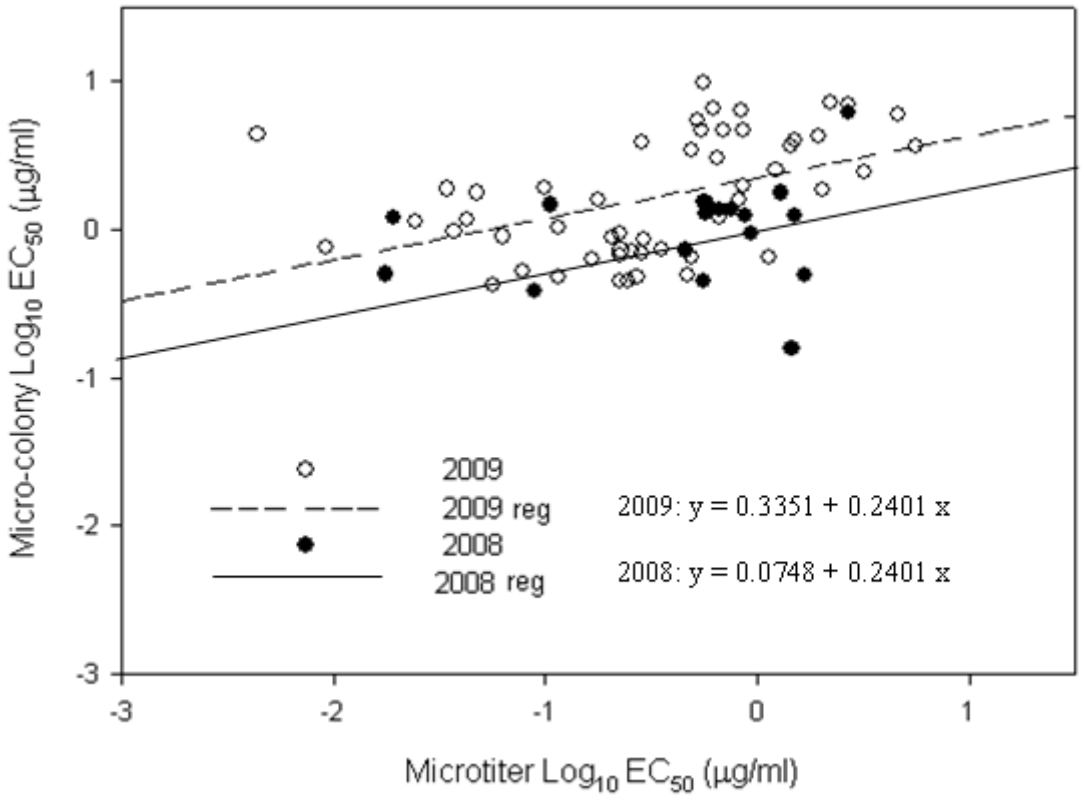

Fig. 2.6. Correlation between tebuconazole EC_{50} value from the micro-colony growth assay and microtiter plate assay from *Cercospora arachidicola* isolates collected in 2008 and 2009.

CHAPTER 3

MECHANISMS OF DMI FUNGICIDE RESISTANCE IN FIELD ISOLATES OF *CERCOSPORA ARACHIDICOLA*

[1]Qiu, J., Stevenson, K. L., and Culbreath, A. K. 2010. To be submitted to Pest Management Science.

Abstract

In the early 2000s, a significant decline in performance of tebuconazole for leaf spot control was reported in Georgia and neighboring states. This decline in performance was associated with a significant decrease in tebuconazole sensitivity based on laboratory assays of isolates collected from field sites. Mechanisms of DMI resistance reported in other fungi include mutations of the *CYP51* gene, which encodes the fungicide target 14 alpha-demethylase necessary for sterol biosynthesis, over-expression of *CYP51*, and active efflux of fungicide mediated by ATP-binding cassette (ABC) transporters. However, mechanisms of resistance to DMIs in *Cercospora arachidicola* have not been previously studied. The objectives of this study were to investigate the potential mechanisms of DMI resistance in *C. arachidicola*. Sequencing of the *CYP51* gene from DMI-resistant and DMI-sensitive field isolates of *C. arachidicola* revealed alterations at codons 453 or 461 (G453S, Y461D, Y461N) in 4 of the 10 DMI-resistant isolates. This is the first report of mutations in the *CYP51* gene associated with DMI resistance in *C. arachidicola*. However, based on a real-time PCR assay, *CYP51* expression in DMI-resistant isolates of *C. arachidicola* was not different from that in DMI-sensitive isolates. Except for one resistant isolate that became more sensitive to tebuconazole when promazine was added, there was no apparent increase in tebuconazole sensitivity in the presence of ABC transporter inhibitors flavanone or promazine. Although there was no evidence to indicate the mechanism of resistance in most of the tebuconazole-resistant isolates evaluated, this study indicates that mutations in the *CYP51* gene are associated with reduced sensitivity to tebuconazole in some of the *C. arachidicola* isolates. These possible investigations of mechanisms increase our understanding of the development of DMI fungicide resistance, lay the groundwork for the

development of a PCR-based detection method for DMI resistance in *C. arachidicola* and provide new clues for the management of peanut early leaf spot.

Introduction

Management of peanut early leaf spot, caused by *Cercospora arachidicola* (teleomorph = *Mycosphaerella arachidicola*), relies heavily on multiple fungicide applications. Demethylation inhibiting fungicides (DMIs), especially tebuconazole, have been very widely used over the past 15 years because of their efficacy against both peanut leaf spot and soilborne diseases (3). However, a significant decline in performance of tebuconazole for leaf spot control in research plots was observed in 2003 (8). Isolates collected from Georgia, Alabama, South Carolina, and Oklahoma were tested for sensitivity in 2005, and results indicated that sensitivity of isolates collected in 2005 were less sensitive than those from 1996. However, in many cases, leaf spot was still effectively controlled with tebuconazole (30).

Several mechanisms of resistance to DMI fungicides have been identified in a number of plant pathogens, and resistance often is due to the combination of several different mechanisms (14). Mutations in the target gene encoding the sterol 14α-demethylase can alter the affinity of the essential enzyme for DMIs. In *Uncinula necator*, the fungus that causes powdery mildew of grape, a single mutation resulting in replacement of phenylalanine (F) by tyrosine (Y) at position 136 of the *CYP51* gene confers resistance to triadimenol (12). In *Venturia nashicola*, the causal agent of Japanese pear scab, an alteration was found at position 133 in a highly conserved region of the *CYP51* gene that is associated with DMI resistance (7). In *Mycosphaerella graminicola*, a valine instead of an isoleucine at position 381 of *CYP51*, in combination with alterations at codons 459, 460, and 461, were associated with the highest resistance levels to triflumizole, fluquinconazole and tebuconazole, but not prochloraz (19). Similarly, in *Mycosphaerella*

fijiensis, six mutations in the CYP51 gene were found in isolates of that fungus with reduced sensitivity to the DMI fungicide propiconazole (5).

In addition to the mutations in the target gene of fungi of DMI fungicides, the expression level of the gene also affects the sensitivity to DMI fungicides. In *Blumeriella jaapii*, the cherry leaf spot pathogen, a 126-bp sequence in the promoter region of the *CYP51* gene was tandem repeated five times in resistant isolates compared to only one copy of the sequence in sensitive isolates. *CYP51* expression in DMI-resistant isolates was 5- to 12-fold higher than that in DMI-sensitive isolates (21). In the apple scab pathogen *Venturia inaequalis*, overexpression of *CYP51* in DMI-resistant isolates was associated with the presence of a 553-bp insertion located upstream of the *CYP51* gene (26). In France, researchers found high constitutive levels of the *CYP51* transcript in field isolates of *M. graminicola* with an intermediate level of resistance to DMIs (29).

Efflux systems function via an energy-dependent mechanism (active transport) to pump out unwanted toxic substances through specific efflux pumps. They are active transporters, meaning that they require a source of chemical energy to perform their function. There are two major classes of transporters: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters. The role of ABC transporters in fungicide sensitivity and resistance has been well established (9). ABC transporters are able to bind and hydrolyze nucleotide triphosphates (mainly ATP), which is an energy source to perform the function. The energy generated in this process is used to transport solutes across cell membranes.

The physiology of energy-dependent efflux pumps that results in a differential accumulation of DMIs in wild type and DMI-resistant fungi has been described (9-10). Mohr and Gisi (13) found that a higher efflux rate in the mycelium of DMI-resistant *M. graminicola*

isolates may be responsible for resistance to DMIs. In addition, the role of ABC transporters in DMI resistance was verified by biochemical assay with different inhibitors of ABC transporters. In *M. graminicola*, some ABC transporter inhibitors increased the sensitivity to cyproconazole based on the *in vitro* assay by measuring the growth on PDA plates (25).

Our objective in this study was to determine if DMI resistance in *C. arachidicola* is attributable to one or more of these known mechanisms. Using a set of DMI-sensitive and DMI-resistant isolates of *C. arachidicola*, the *CYP51* gene of *C. arachidicola* was sequenced and compared, the expression level of *CYP51* gene was quantified by real-time PCR, and the role of ABC transporters in DMI-resistance in *C. arachidicola* was investigated by examining the interactive effects of known inhibitors of ABC transporters and the DMI fungicide tebuconazole on fungal growth *in vitro*.

Materials and methods

Fungal isolates and DMI sensitivity assays. A total of 108 isolates of *C. arachidicola* were obtained from peanut leaves with actively sporulating early leaf spot lesions were collected from six different locations in southern Georgia in 2008 and 2009. Among these 108 isolates, 12 isolates were from an organic peanut field at the University of Georgia Ponder Farm in Tift Co. GA with no previous history of direct DMI applications, and 96 were collected from 4 other peanut fields with a history of DMI use. Sensitivity of each isolate to tebuconazole was assessed based on two different assay methods (see Chapter 2). Based on results of the microtiter plate and micro-colony growth assays, five of the most resistant isolates, and five of the most sensitive isolates from each year were selected for this study (Table 3.1).

Amplification and sequencing of the CYP51 gene. For each of the 20 selected isolates of *C. arachidicola*, 0.5-cm diameter mycelial plugs were removed from 6-week-old cultures on

PDA, transferred to glass tubes containing 3 ml potato dextrose broth (PDB), and incubated at room temperature (23-25°C) for 20 days. Genomic DNA was extracted from mycelium by mortar and pestle using a SDS lysis buffer (0.5 M NaCl, 0.01 M EDTA pH 8.0, 0.2 M Tris pH 7.5, 1% SDS). Degenerate primers CYP51-DEG-F6 and CYP51-DEG-R6 (Table 3.2), based on a conserved region of the fungal *CYP51* gene, were used to amplify a fragment of the *CYP51* gene. PCR reactions were carried out in a total volume of 50 µl containing 5 µl 10X buffer, 150 pmol each of CYP51-DEG-F6 and CYP51-DEG-R6 200 µM dNTPs, 1.25 U Takara Ex Taq Polymerase (Takara, Tokyo, Japan) and approximately 20 ng *C. arachidicola* genomic DNA. PCR cycle conditions were 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 2 min 30 sec and 72°C for 2 min; 72°C for 10 min. An appropriately sized band was cut and purified by a QIAquick gel extraction kit (Qiagen, Germantown, MD) before sequencing.

The sequenced *CYP51* gene fragment from *C. arachidicola* was further used to design six specific primers for the amplification of upstream and downstream genomic regions of this gene with the high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) method (20). Three nested sequence-specific primers CYP51-HI-UP1, CYP51-HI-UP2, CYP51-HI-UP3 (Table 3.2), and two arbitrary primers LAD3 and AC1 (Table 3.2) were used to amplify the upstream region of the CYP51 fragment. Another three sequence-specific primers CYP51-HI-D1, CYP51-HI-D2, and CYP51-HI-D3 (Table 3.2) with the same two arbitrary primers were used to amplify the downstream region of the *CYP51* fragment. hiTAIL-PCR conditions were the same as those reported by Liu (20). The band containing the amplified product from the tertiary PCR step was cut from the gel, purified with a QIAquick gel extraction kit (Qiagen, Germantown, MD), and then sequenced (Davis Sequencing, Davis, CA). Sequencing results were analyzed by Bioedit (Bioedit, Stockport, UK) and ContigExpress (Invitrogen, Carlsbad, CA). Sequences of resistant

and sensitive isolates were compared. To verify the presence of mutated sequences in some isolates, allele-specific primers Mut-F, Mut-R1, and Mut-R2 (Table 3.3) were designed to amplify the specific fragment encompassing the mutant region of *CYP51* gene from more resistant *C. arachidicola* isolates. PCR reactions were conducted at an annealing temperature of 58°C and a primer concentration of 0.1 μM.

Quantitative expression of the CYP51 gene. Five relatively DMI-sensitive isolates, APP3, APP12.2, R7, R8, R13 and five relatively DMI-resistant isolates, APP7, APP8, BS4, BS11 and BS19 were grown in liquid PDB at room temperature for 20 days with continuous shaking. Mycelium was frozen in liquid nitrogen and crushed to a fine powder with a mortar and pestle. RNA was extracted with a Spectrum Plant Total RNA Kit (Sigma, St. Louis, MO) according to the user guide. For each RNA sample, 5 ng RNA was used for reverse transcription with Superscript III First-Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Real-time PCR was performed in a thermocycler (SmartCycler II, Cepheid, Sunnyvale, CA) using SYBR Green I fluorescent dye. Reactions were carried out in a total volume of 25 μl containing 1 μl cDNA, 1 μl of each of the forward and reverse primers QRT-3F, QRT-3R (10 pmol) **(**Table 3.4**)**, and 12.5 μl SYBR Green Master Mix (Applied Biosystems, Foster, CA). Ten serial dilutions of cDNA of each isolate were used to create the standard curves. Two replicates of each sample were prepared. Real-time PCR was performed with the following conditions: preheat at 50°C for 2 min, 95°C for 10 min, and then 40 cycles at 95°C for 15s and 60°C for 1 min. PCR amplification of the β-tubulin gene of *C. arachidicola* by primers β – tub F and β – tub R (10 pmol) (Table 3.4) was done at the same time to serve as a endogenous control for each sample. Data were collected by using SmartCycler II software (Cepheid, Sunnyvale, CA). The

double standard curve method was used to analyze the data (31). One standard curve was established from RNA of known serial concentrations of *CYP51* gene. This curve was then used as a reference standard for extrapolating quantitative information for unknown concentrations of *CYP51* gene. Another standard curve of β-tubulin gene was established and used as a reference standard for extrapolating quantitative information for unknown concentrations of β-tubulin gene by using same method. Since expression of the β-tubulin gene should be constant across all samples being analyzed, it standardizes the amount of sample RNA added into reaction. Based on these two standard curves and known Ct values, the concentrations of target *CYP51* gene and β-tubulin gene of each isolate were calculated. The ratio between concentration of target *CYP51* and concentration of β-tubulin gene was considered as the relative *CYP51* expression level.

ABC transporter inhibitor assay. Five most resistant and five most sensitive isolates were tested in 2008 and 2009. A 2-mm diameter mycelial plug of each isolate was transferred to a tube containing 3 ml PDB and incubated at 24°C for about 20 days. After 20 days, the mycelial plug was homogenized with a tissuemizer (IKA Labortechnik T25 Basic, IKA Works, Inc. Wilmington, NC) to obtain a uniform suspension of fine mycelial fragments. Promazine and flavanone were used as the inhibitors in this assay (25). A stock solution of each inhibitor was prepared and serially diluted in methanol to obtain final concentrations of each inhibitor in PDB of 100, 50, 25, 10, 1, or 0 µg/ml after mycelial suspensions and fungicide-amended medium were added to the wells of a 96-well microtiter plate. A stock solution of technical grade tebuconazole was prepared and serially diluted in acetone to obtain final concentrations of 1, 0.1, 0.01, or 0µg/ml. Fifty microliters of mycelial suspension of each isolate were added to each well of 96-well microtiter plates using a multi-channel pipette. Treatments consisted of all possible combinations of 6 concentrations of one of the inhibitors and 4 concentrations of tebuconazole.

Each treatment combination was repeated 4 times on each plate, and two replicate plates were prepared for each isolate. Absolute mycelial growth in each well was calculated as the difference in light absorbance values measured on day 0 and day 14, using an automated plate reader. Relative growth inhibition for each replication of each isolate and fungicide concentration was determined according to the following formula:

$$\text{Growth inhibition} = 1 - (GF/GC),$$

where GF = absolute growth in the well containing fungicide-amended medium, and GC = absolute growth in the control well containing non-amended medium (no-fungicide and no-inhibitor). EC_{50} values were estimated by regressing probit-transformed relative growth inhibition on the logarithm of the inhibitor concentrations.

Absolute growth was analyzed using SAS PROC MIXED (SAS ver. 9.1, SAS Institute, Cary, NC) to determine the effects of fungicide and ABC transporter inhibitors on growth of each isolate. The entire experiment was repeated using the 2009 isolates only.

Results

Mutation of the CYP51 gene. The sequenced *CYP51* gene was approximately 1.68kb long, and one intron was predicted and confirmed (MIT Genescan, http://genes.mit.edu/GENSCAN.html) (Fig 3.1). The 1.63 kb coding region encodes 543 amino acids. There are six conserved regions in *CYP51* of *C. arachidicola* based on the *CYP51* sequence of *C. arachidicola* and other fungi. Alignment was done based on the amino acid sequences of *M. graminicola*, *M. fijiensis*, and *C. arachidicola* (Fig. 3.2). Based on the amino acids of *CYP51*, the similarity between *C. arachidicola* and *M. graminicola* is 77%, the similarity between *C. arachidicola* and *M. fijiensis* is 74%. No mutations were found in *CYP51*

from the five resistant isolates collected in 2008. *CYP51* sequences of tebuconazole-resistant isolates were not different from those of sensitive isolates. In 2009, four out of five resistant isolates carried a mutation at amino acid position 453 or 461 (nucleotide sequence 1410 and 1434). APP7 showed a serine (S) instead of glycine (G) at position 453. APP8 and BS4 had an aspartic acid (D) instead of tyrosine (Y) at position 461. BS19 had an asparagine (N) at position 461 instead of tyrosine (Y). *CYP51* sequences of five sensitive isolates are as same as those from all 2008 isolates (Table 3.5). For the allele-specific PCR, primers Mut-F+Mut-R1 and Mut-F+Mut-R2 consistently amplified a 423 bp fragment and a 459 bp fragment, respectively, from four out of five DMI-resistant isolates collected in 2009. No PCR products were amplified from DMI-sensitive isolates collected in 2009.

Overexpression of CYP51 gene. In 2009, the relative expression of *CYP51* in each isolate was determined by using real-time PCR (Fig. 3.3). The relative expression levels were close to 1.0 for all isolates tested (Fig. 3.3). The relative expression for the five sensitive isolates ranged from 0.575 to 0.813 and from 0.603 to 0.849 in the five resistant isolates. The results from the *t*-test indicated that there was no significant difference (P=0.6786) in the *CYP51* gene relative expression level between sensitive isolates and resistant isolates.

ABC transporter activity. In 2008 and 2009, there was no significant interactive effect of tebuconazole and the inhibitor flavanone on growth of resistant or sensitive isolates of *C. arachidicola*. In 2008, there was a significant promazine by tebuconazole interaction effect (P=0.0085) on growth of resistant isolate PL2 (EC_{50}=2.56 µg/ml), which means that the growth of *C. arachidicola* in response to fungicide was affected significantly by adding promazine. For PL2, growth decreased when promazine was added (for 0, 0.01, and 0.1 µg/ml tebuconazole**).** Growth was not affected by promazine at 1.0 µg/ml tebuconazole**,** except when 10 µg/ml

promazine was added, and growth increased. However, promazine decreased sensitivity to tebuconazole (Fig. 3.4)**.** In 2008, resistant isolate PL10 (EC_{50}=2.00 µg/ml) was found a significant interactive effect (P = 0.0469) on growth of tebuconazole and promazine (Fig. 3.5). For this isolate, promazine significantly reduced growth (P < 0.0001) at all fungicide concentrations. Tebuconazole did not have a significant effect on growth (P = 0.729), which confirmed that PL10 is a resistant isolate. Without promazine, the EC_{50} value of tebuconazole was 2.0 µg/ml. The EC_{50} value decreased to 1.34 µg/ml when tebuconazole was combined with 50 µg/ml promazine. When the promazine concentration was increased to 100 µg/ml, the fungus grew very little regardless of tebuconazole concentration (Fig. 3.5)**.** In 2009, there was a significant promazine by tebuconazole interaction effect on growth of resistant isolate APP7 (EC_{50}=2.03 µg/ml). When the assay was repeated in 2009, APP7 showed consistently significant interactions for both repeats (P=0.0096 in first repeat, P=0.0192 in second repeat). The growth decreased and sensitivity of fungus increased by adding 10, 50 µg/ml promazine (Fig. 3.6, 3.7).

Discussion

Mechanisms of resistance to the demethylation inhibiting (DMI) fungicides in the early leaf spot pathogen, *C. arachidicola*, were investigated. In this study, G453S, Y461D, and Y461N mutations were detected in the *CYP51* gene from DMI resistant isolates of *C. arachidicola* collected from two locations in Georgia approximately 135 km apart. Positions Y461D and Y461N were in the YGYG region. Based on the alignment results for the *CYP51* amino acid sequence of *M. graminicola*, *M. fijiensis*, and *C. arachidicola*, this YGYG region is highly conserved. In previous research, mutations have been found in this YGYG region in DMI-resistant isolates of *M. graminicola* and *M. fijiensis* (5, 28). Investigations conducted by Cools et

al. (6) have shown that resistance to DMI fungicides in *M. graminicola* was generally associated with changes in this YGYG region. Similar alterations at positions in the YGYG region were reported in DMI-resistant isolates of *M. graminicola* from several different European countries (6, 28). In *M. fijiensis*, four different types of mutations associated with loss of propiconazole sensitivity were found in the YGYG region: Y461D, Y463D, Y463H and Y463N. (5). These alterations in a highly conserved region of fungal C14α- demethylase proteins were located just upstream from the heme-binding site (6). These indicated that the YGYG region in *CYP51* genes of different fungi may be very important for the affinity between DMI fungicide and C14α-demethylase. APP7, tebuconazole-resistant isolate of *C. arachidicola* devoid of mutations at YGYG region showed the substitution G453S, which has not been previously reported in a plant pathogen. The observation suggests that this position could be associated with substrate interaction unique to *C. arachidicola*. These three different types of mutations found in the *CYP51* gene of *C. arachidicola* indicated that mutation of *CYP51* is associated with DMI resistance in *C. arachidicola*. However, there may be other mutations in *CYP51* that are associated with resistance that have not yet been identified. Detection of resistant mutants is important in monitoring development of fungicide resistance in pathogen populations. Identification of these specific *CYP51* mutations can be used to design primers for quick detection of DMI-resistant mutants of *C. arachidicola* by PCR.

Overexpression of the *CYP51* gene was not associated with DMI resistance in the isolates of *C. arachidicola* that were included in this study. Similarly, characterization of propiconazole resistant isolates of *M. fijiensis* provided no indication that overexpression of the *CYP51* gene was associated with DMI resistance in that pathogen. However, this mechanism of DMI resistance in *C. arachidicola* still cannot be ruled out. Overexpression of the *CYP51* gene was

found to be associated with DMI resistance in *B. jaappi* (21), *P. digitatum* (15), and *C. beticola* (24). Additional resistant *C. arachidicola* isolates should be collected and investigated for possible overexpression of *CYP51*.

It is clear that efflux transporters are involved in multidrug resistance (4, 16-18, 23, 29, 32). ABC transporters are thought to contribute to fungicide resistance in different plant-pathogenic fungi (11, 29). In *M. graminicola*, the causal agent of leaf blotch of wheat, the roles of five different ABC transporters in fungicide resistance have been studied (32). In the citrus green mold pathogen, *P. digitatum*, disruption of the ABC transporter gene *PMR1* demonstrated that this transporter was an important determinant of DMI resistance (23), and in the gray mold pathogen, *B. cinerea*, the ABC transporter BcatrD synergized the MFS transporter Bcmfs1 to mediate DMI resistance (18).

In the present study, ABC transport inhibitors promazine and flavanone, did not increase the activity of tebuconazole against most tebuconazole-resistant isolates of *C. arachidicola* except for PL10. Flavanone did not significantly affect sensitivity to tebuconazole. Promazine did have significant effects on tebuconazole sensitivity of several of the isolates tested, but there was no significant increase in tebuconazole sensitivity of the resistant isolates, except for isolate PL10. The sensitivity to tebuconazole of PL10 increased significantly in the presence of promazine at 25 μg/ml (EC_{50}=1.71 μg/ml) and 50 μg/ml (EC_{50}=1.34 μg/ml). However, growth was not inhibited completely even at the highest concentration of tebuconazole tested (1.0 μg/ml). Various models have been described to explain the efflux activity mediated by ABC transporters (2). One proposed mechanism is inhibitor binding to a site on the transporter protein, and results in blocking transport (25). Different ABC transporter genes were found in association with multi-drug resistance, for example, the *BcatrD* in *B. cinerea* (16), *PMR1* in *P. digitatum*

(23). In addition to flavanone and promazine, there are several compounds described in previous studies as inhibitors of efflux pumps, such as amitriptyline, diethylstilbestrol, phenothiazines, chlorpromazine, and thioridazine (27). Different inhibitors increased the activity of DMI fungicides against different fungi. One possible explanation for the lack of significant effects of these ABC transporter inhibitors (promazine and flavanone) on tebuconazole sensitivity with one exception is that these inhibitors were not able to bind to the specific binding sites on ABC transporter proteins in *C. arachidicola*. However, this does not rule out the possible involvement of other inhibitors. The second possibility for the lack of significant effects of these ABC transporter inhibitors (promazine and flavanone) on tebuconazole sensitivity with one exception is that ABC transporters may not be the transporters involved in DMI fungicide resistance in *C. arachidicola*. MFS transporter MgMfs1 was described as a potent transporter of azole fungicide in *M. graminicola* (25). The ABC transporter inhibitors tested are not described in the literature as the inhibitors of MFS transporters. Additional studies are needed to investigate the effects of other known ABC transporter inhibitors on DMI sensitivity in *C. arachidicola*.

In conclusion, reduced DMI sensitivity of *C. arachidicola* was associated with mutations at nucleotide sequence positions 1410 and 1434 (amino acid 453 and 461) in the *CYP51* gene encoding the C14α-demethylase in four of ten resistant isolates tested. This is the first report of an association of mutations in the *CYP51* gene with resistance to DMI fungicides in this pathogen. Further research is needed to determine that these alterations are indeed responsible for DMI resistance in *C. arachidicola*. The involvement of mutations in DMI resistance has been confirmed in similar research on the human pathogen, *C. albicans*, by demonstrating that the cloned *ERG11* (ortholog to *CYP51*) genes from DMI-resistant isolates were expressed in *S. cerevisiae* (1, 22). Additional structural information will lead to a better understanding of how

the amino acid substitutions influence the binding of tebuconazole and other DMI fungicides, and the affinity of the fungicide for the enzyme. Overexpression of *CYP51* gene was not involved in DMI resistance in *C. arachidicola* based on the limited number of isolates we investigated. This mechanism has been demonstrated in *B. jaapii* (21), *P. digitatum* (15), and *C. beticola* (24), and it may be found to be involved in DMI resistance in *C. arachidicola*, but characterization of more isolates is needed. The role of ABC transporters in DMI fungicide resistance in *C. arachidicola* remains unclear. Clear evidence of a significant increase in DMI sensitivity in the presence of an ABC transporter inhibitor was observed in only one isolate (PL10). This mechanism cannot be proved conclusively until the role of ABC transporters, including which, if any, transporters are involved in DMI resistance in *C. arachidicola* is more fully understood. Future research involving cloning, sequencing, and disruption of the ABC transporter genes from *C. arachidicola* would help to further our understanding. In addition, the analysis of the regulation of expression of ABC transporter genes is necessary to clarify the mechanism of DMI resistance in *C. arachidicola* by ABC transporters.

Finally, this is the first time that we report *CYP51* mutations (G453S, Y461D, and Y461N) are associated with DMI resistance in *C. arachidicola*. These results not only address the possible mechanisms involved in DMI resistance in *C. arachidicola*, but provide a rapid PCR detection method in this study will enable farmer to rapidly adjust the fungicide applications in managing peanut early leaf spot. This will provide an insight into the nature of the mechanisms of DMI resistance that occur in the field isolates of *C. arachidicola*, which ultimately lead to better management of peanut early leaf spot.

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Table 3.1. Tebuconazole-sensitive and resistant isolates of *Cercospora arachidicola* were used in investigation of DMI resistance mechanisms in 2008 and 2009.

Year	Isolate	Tebuconazole sensitivity[1]	EC_{50} value [2] (μg/ml)
2008	BF1	S	0.089
	BF4	S	0.069
	SC1 18	S	0.029
	SC1 19	S	0.060
	CC8	S	0.035
	EC5	R	2.70
	PL2	R	2.56
	PL10	R	2.00
	PL14	R	2.03
	PL24	R	5.56
2009	APP3	S	0.079
	APP12.2	S	0.05
	R7	S	0.035
	R8	S	0.037
	R13	S	0.063
	APP7	R	2.03
	APP8	R	3.18
	BS4	R	4.66
	BS11	R	5.58
	BS19	R	2.69

1. S = sensitive, R = resistant.

2. EC_{50} values are from sensitivity test (microtiter plate assay) in Chapter 2.

Table 3.2. Primers used in sequencing the *CYP51* gene from *Cercospora arachidicola*.

Primer	Sequence (5'⟶ 3')
CYP51-DEG-F6	YTNACNACNCCNGTNTTCGG
CYP51-DEG-R6	GCRAAYTGYTCNCCDATRCA
LAD3	ACGATGGACTCCAGAGCGGCCGCHNVNNNCCAC
AC1	ACGATGGACTCCAGAG
CYP51-HI-UP1	AAAGGATGCGTCGAATCGCTCACG
CYP51-HI-UP2	ACGATGGACTCCAGAGCGGTGCCGCTATTGGCAGCGAA CTTCTTG
CYP51-HI-UP3	TCGTTTACGATCAGGGTCACGTAGGACT
CYP51-HI-D1	AGTCAAATCGCCCATGCCAATCGAA
CYP51-HI-D2	ACGATGGACTCCAGAGCGACTCTTATGGGAGCCGCATCG ATGGGA
CYP51-HI-D3	ACCTGGTACCAGCGATGGTGCGGGAA

Table 3.3. Primers used for confirming mutations in the *CYP51* gene of tebuconazole-resistant isolates of *Cercospora arachidicola*.

Primer	Sequence (5’ ⟶ 3’)
Mut-F	TCAGCACTCGTCTTCTGCCAC
Mut-R1	GCGACACCTTCCCGCACCAT
Mut-R2	CTGACGAGACCGTATCCGTAGT

Table 3.4. Primers used in real-time PCR for investigation of the *CYP51* expression level in isolates of *Cercospora arachidicola*.

Primer	Sequence (5'⟶3')
QRT-3F	CTCATGGCTGGTCAGCACTCG
QRT-3R	GAGTGGATTGGAGCGTGGATG
β – tub F	TGCCGGTATGGGTACGCTCTT
β – tub R	AGGTGGTTCAAGTCGCCGTAT

Table 3.5. Summary of mutations in the *CYP51* gene in *Cercospora arachidicola*.

Isolate	Tebuconazole sensitivity[1]	*CYP51* gene sequence (1409 → 1435)	P 450 amino acid sequence
APP3	S	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
APP12.2	S	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
R7	S	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
R8	S	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
R13	S	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
APP7	R	AAGTGTCGCAGAGGAGAAGG AGGACTA	SVAEEKEDY GYG
APP8	R	AGGTGTCGCAGAGGAGAAGG AGGACGA	GVAEEKEDD GYG
BS4	R	AGGTGTCGCAGAGGAGAAGG AGGACGA	GVAEEKEDD GYG
BS11	R	AGGTGTCGCAGAGGAGAAGG AGGACTA	GVAEEKEDY GYG
BS19	R	AGGTGTCGCAGAGGAGAAGG AGGACAA	GVAEEKEDN GYG

1. S = sensitive, R = resistant.


intron

Fig 3.1. Nucleotide sequence of *Cercospora arachidicola CYP51* gene. Intron is included. Mutations were found at position 1410 (G to A) and 1434 (T to G, T to A).

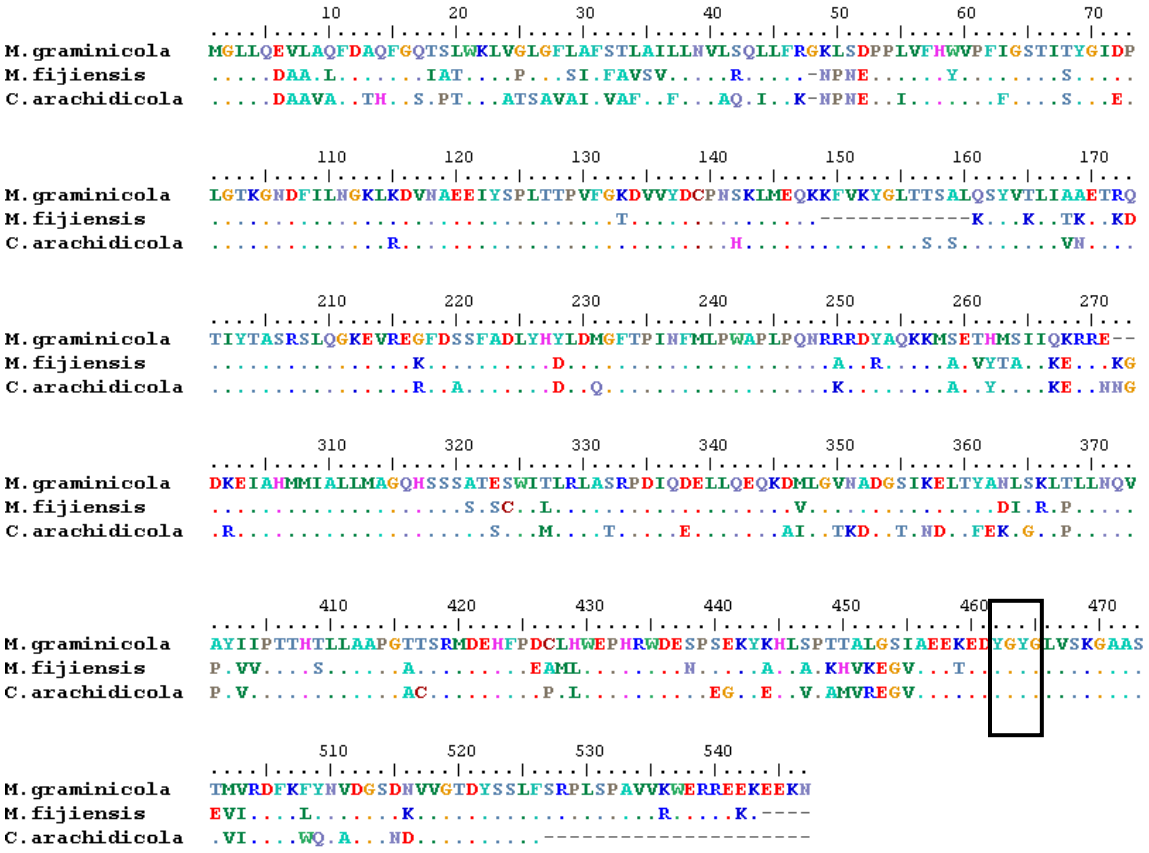

Fig 3.2. Alignment between amino acid sequences of *CYP51* gene from *Mycosphaerella fijiensis*, *Mycosphaerella graminicola* and *Cercospora arachidicola*. YGYG region is highlighted by a rectangle.

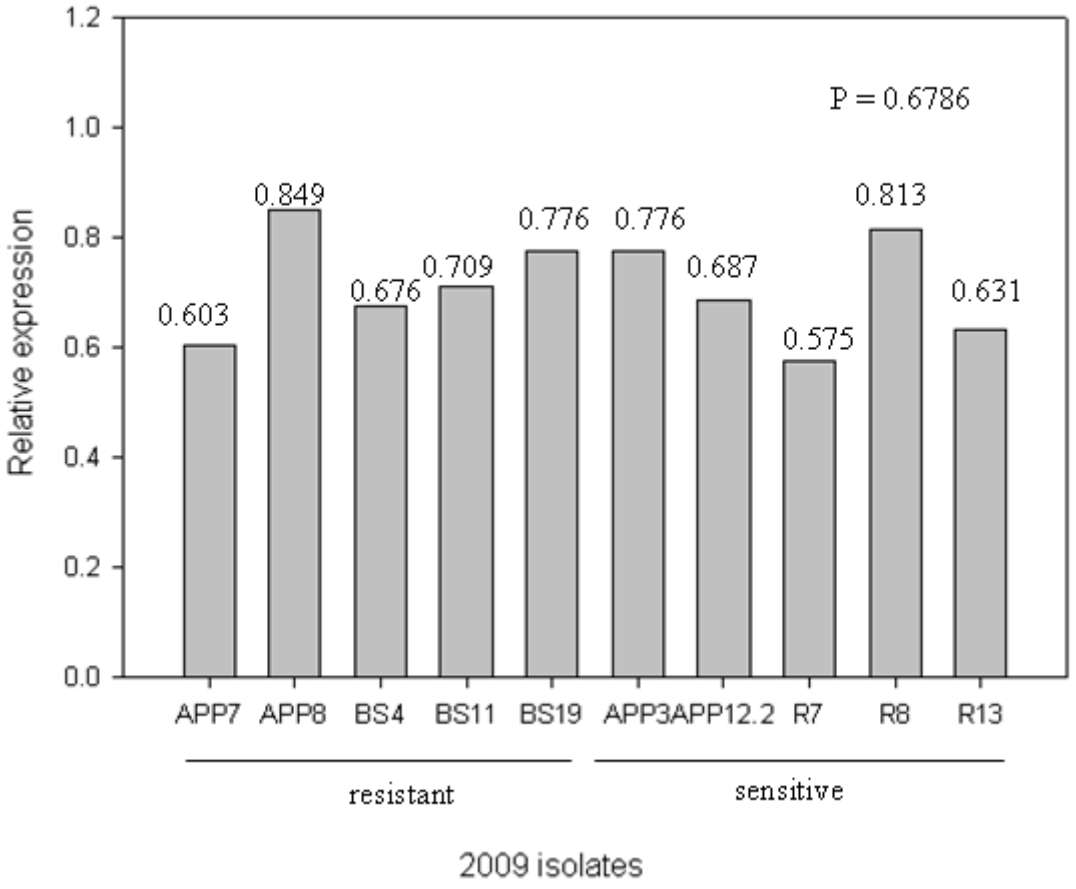

Fig 3.3. Relative expression of the *CYP51* gene in tebuconazole-resistant and sensitive isolates of *Cercospora arachidicola*. Relative expression was quantified by real-time PCR using a double standard curve method.

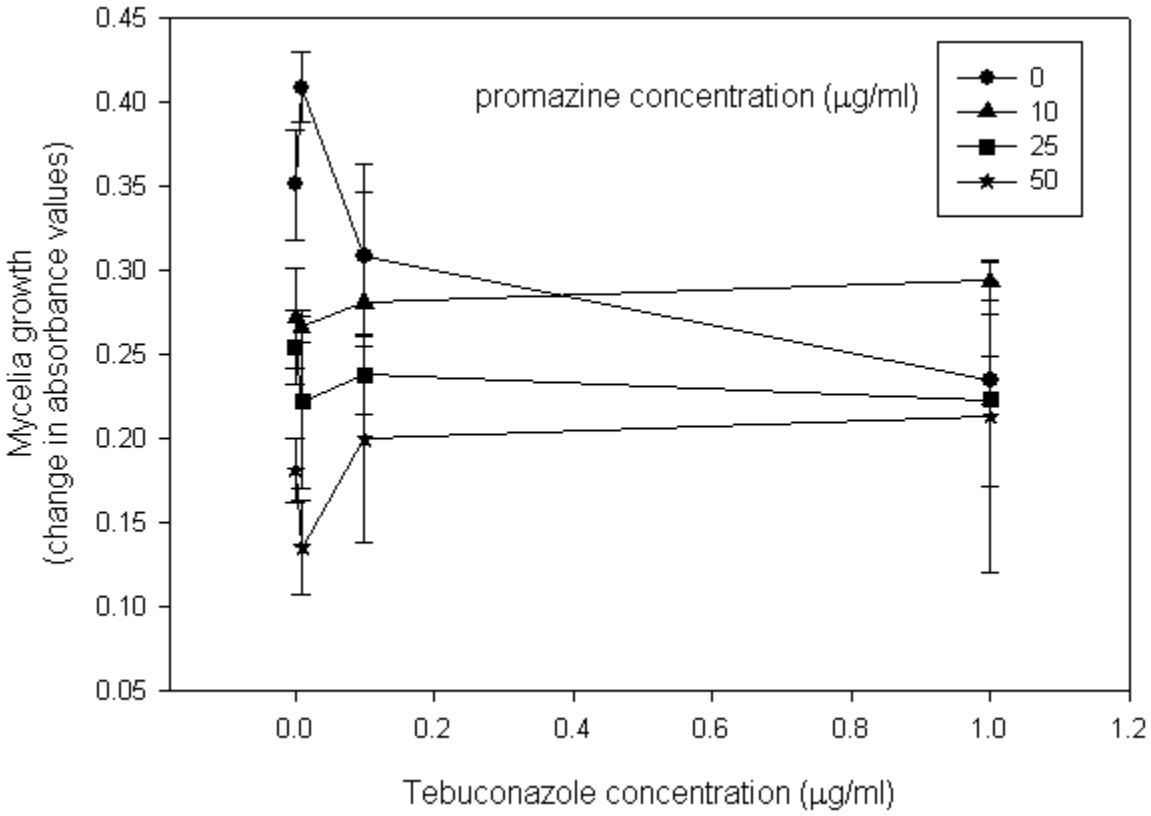

Fig. 3.4. Effect of different concentrations of the ABC transporter inhibitor promazine on mycelia growth of tebuconazole-resistant isolate PL2 of *Cercospora arachidicola* (EC_{50} = 2.56 µg/ml) in response to tebuconazole, as measured by the absolute change in absorbance values.

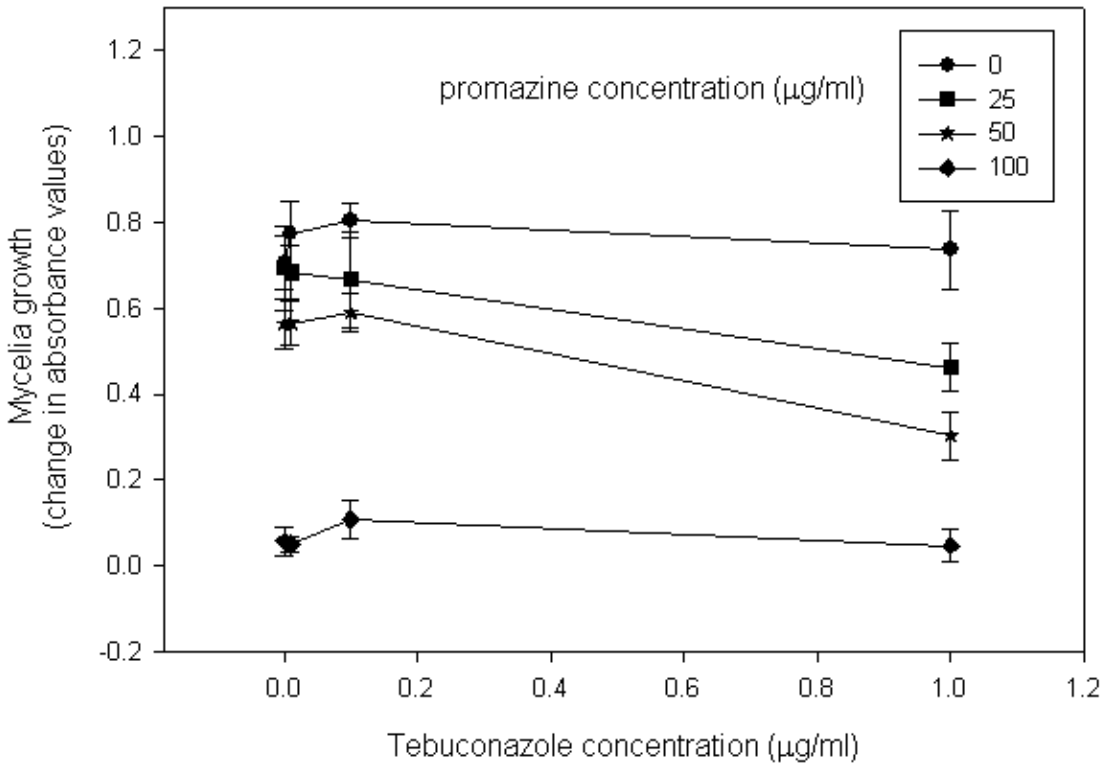

Fig. 3.5. Effect of different concentrations of the ABC transporter inhibitor promazine on mycelia growth of tebuconazole-resistant isolate PL10 of *Cercospora arachidicola* (EC_{50} = 2.0 µg/ml) in response to tebuconazole, as measured by the absolute change in absorbance values.

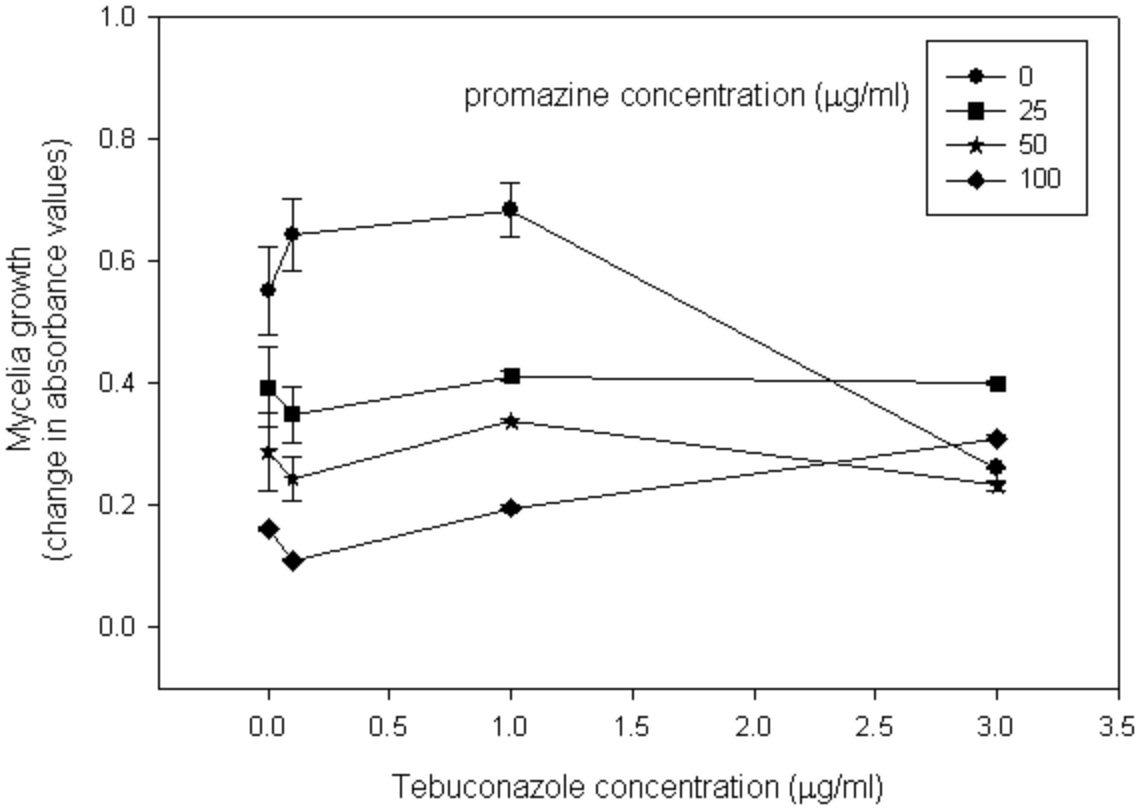

Fig. 3.6. Effect of different concentrations of the ABC transporter inhibitor promazine on mycelia growth of tebuconazole-resistant isolate APP7 (1 st repeat) of *Cercospora arachidicola* (EC_{50} = 2.03 µg/ml) in response to tebuconazole, as measured by the absolute change in absorbance values.

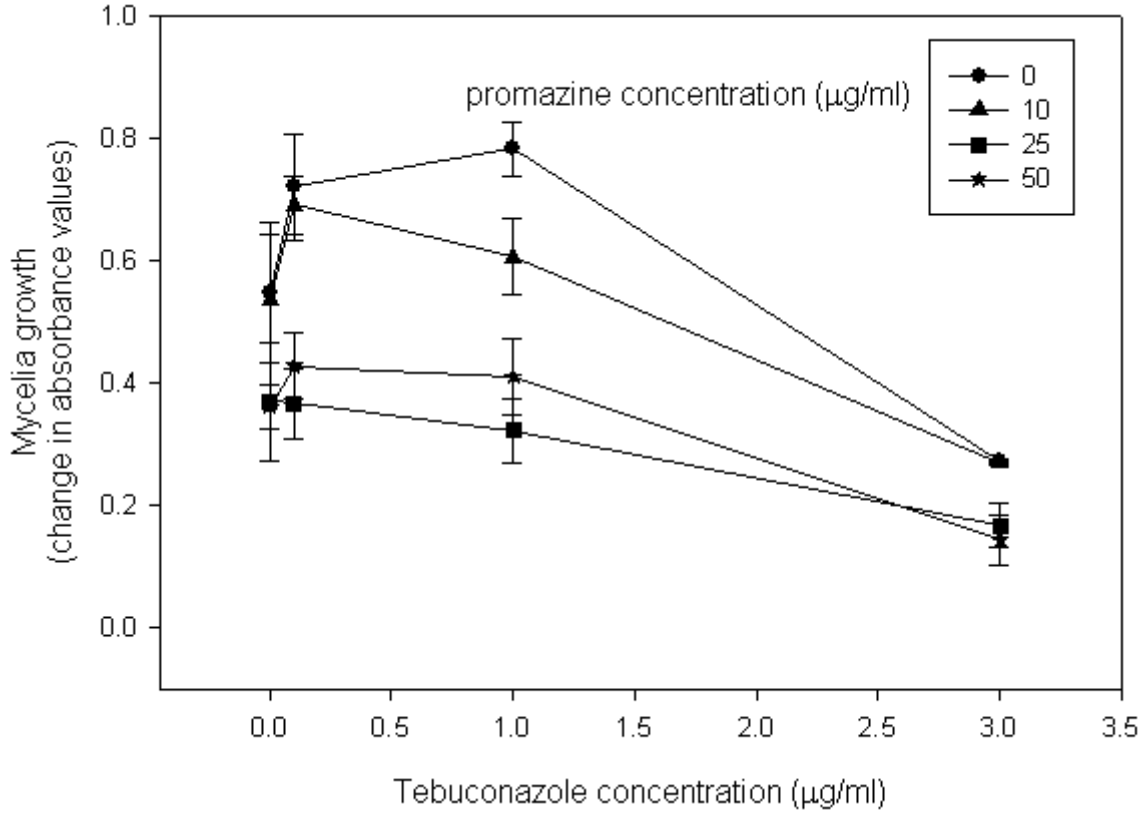

Fig. 3.7. Effect of different concentrations of the ABC transporter inhibitor promazine on mycelia growth of tebuconazole-resistant isolate APP7 (2 nd repeat) of *Cercospora arachidicola* (EC_{50} = 2.03 µg/ml) in response to tebuconazole, as measured by the absolute change in absorbance values.

CHAPTER 4

SUMMARY

Peanut early leaf spot, caused by the fungus *Cercospora arachidicola*, is a major disease of peanut in Georgia. Management of peanut early leaf spot mostly relies on spraying fungicide and usually requires 7 or more fungicide applications per season. The DMI fungicide, tebuconazole, is widely used in Georgia to control early leaf spot of peanut. Recently, reports from Georgia and neighboring states indicated that tebuconazole seemed to be less effective than it used to be, although it still controls early leaf spot (8). The general objectives of this study were to develop a rapid assay to detect tebuconazole resistance in field populations of *C. arachidicola* and to detect the mechanisms of DMI resistance in *C. arachidicola*.

In the new rapid assay that was developed, conidia were transferred directly from lesions to tebuconazole-amended medium and sensitivity was based on transverse diameters of 3-day-old colonies. Isolates were collected in 2008 and 2009 from peanut fields with or without a history of DMI use. EC_{50} values were determined using the new assay and compared to EC_{50} values based on the standard mycelial growth assay in microtiter plates. For the new assay, EC_{50} values ranged from 0.39 to 6.17 µg/ml for 21 isolates in 2008 and from 0.36 to 9.73 µg/ml for 78 isolates in 2009. For the standard assay, EC_{50} values ranged from 0.017 to 4.65 µg/ml for 29 isolates in 2008 and from 0.025 to 5.56 µg/ml for 58 isolates in 2009. EC_{50} values were consistently higher for the new assay compared to the microtiter plate assay. For combined data from both years, there was a significant positive correlation between EC_{50} values from the two assays. The main advantage of the new assay is that it can be completed in 3 days, compared to 2-3 months for the standard microtiter plate assay.

Mechanisms of resistance to the demethylation inhibiting (DMI) fungicides in the early leaf spot pathogen, *C. arachidicola*, were investigated. Based on mechanisms of DMI resistance reported in other fungi, mutations of the *CYP51* gene (2, 4, 7), which encodes the target 14

alpha-demethylase necessary for sterol biosynthesis, over-expression of *CYP51* (3, 6), and active efflux of fungicide mediated by ATP-binding cassette (ABC) transporters (1, 5) were evaluated in relatively DMI-resistant and DMI-sensitive isolates of *C. arachidicola* collected from peanut fields. Sequencing of the *CYP51* gene revealed alterations at codons 453 or 461 in 4 of the 10 DMI-resistant isolates. This is the first report of mutations in the *CYP51* gene associated with DMI resistance in *C. arachidicola*. However, based on a real-time PCR assay, *CYP51* expression in DMI-resistant isolates of *C. arachidicola* was not different from that in DMI-sensitive isolates. Except for one resistant isolate that became more sensitive to tebuconazole when promazine was added, there was no apparent increase in tebuconazole sensitivity in the presence of ABC transporter inhibitors flavanone or promazine. ABC transporter inhibitors may not play an important role in DMI resistance in the isolates tested. However, mutations in the *CYP51* gene were associated with DMI resistance in some *C. arachidicola* isolates, which can be used to develop PCR-based assays for detection of DMI resistance in populations of this pathogen.

In summary, based on my study of the detection and mechanisms of fungicide resistance in the peanut early leaf spot pathogen, the newly developed, fast assay can provide timely results and information to researchers and growers for fungicide resistance monitoring aspect. Mutations of *CYP51* gene in *C. arachidicola*, associated with DMI resistance, was first found and reported. The possible DMI resistance mechanisms investigated in this study increase the knowledge in understanding DMI fungicide resistance in *C. arachidicola* and could provide new clues for management of fungicide-resistant pathogens.

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