DETECTION, STABILITY, AND RELATIVE FITNESS OF FUNGICIDE–RESISTANT ISOLATES OF THE GUMMY STEM BLIGHT PATHOGEN, *DIDYMELLA BRYONIAE*

by

MAVIS J. FINGER

(Under the Direction of KATHERINE L. STEVENSON and PINGSHENG JI)

ABSTRACT

The sensitivity of conidial germination and mycelial growth of *Didymella bryoniae* to azoxystrobin was compared to determine if mycelial growth assays can be used as an alternative to conidial germination fungicide sensitivity assays. Based on the dose response of mycelial growth, the azoxystrobin concentration of 1.0 µg/ml was selected as a discriminatory concentration in mycelial growth assays. An allele-specific PCR assay was also developed to rapidly detect azoxystrobin resistance in *D. bryoniae*. Resistance to azoxystrobin was determined to be stable. Additionally, the relative fitness of azoxystrobin-, boscalid-, penthiopyrad-, and thiophanate-methyl-sensitive and –resistant isolates was similar. However, thiophanate-methyl-resistant isolates grew significantly less than thiophanate-methyl-sensitive isolates at certain temperatures suggesting a fitness cost may be sustained. The results of this study will be used to rapidly and efficiently monitor fungicide resistance and to improve current fungicide resistance management recommendations.

INDEX WORDS: Allele-specific PCR, Azoxystrobin, Boscalid, *Didymella bryoniae*, Fungicide resistance, G143A, Gummy stem blight, Penthiopyrad, QoI, Relative fitness, Stability, Thiophanate-methyl, Watermelon

DETECTION, STABILITY, AND RELATIVE FITNESS OF FUNGICIDE-RESISTANT ISOLATES OF THE GUMMY STEM BLIGHT PATHOGEN, *DIDYMELLA BRYONIAE*

by

MAVIS J. FINGER

BS, Louisiana State University, 2010

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

DETECTION, STABILITY, AND RELATIVE FITNESS OF FUNGICIDE-RESISTANT ISOLATES OF THE GUMMY STEM BLIGHT PATHOGEN, *DIDYMELLA BRYONIAE*

by

MAVIS J. FINGER

Major Professor:	Katherine L. Stevenson Pingsheng Ji
Committee:	David B. Langston, Jr. Ronald Walcott

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2013

DEDICATION

This work is dedicated to the memory of my loving mother, Ellen, whom I owe so dearly for all that I am and all that I hope to become.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisors, Dr. Katherine L. Stevenson and Dr. Pingsheng Ji, for their support throughout my time at UGA. Without their shared wisdom and continued guidance, this thesis would not have been possible. Additionally, I extend gratitude to my committee members, Dr. David B. Langston, Jr. and Dr. Ronald Walcott, for offering their expertise to this project. I would also like to express thanks to Dr. Venkat Parkunan for his help in all molecular studies. A special thank you is extended to Bonnie Evans, Kippy Lewis, and Michael Purvis for the indispensable technical support they provided. My sincerest gratitude is also extended to my fellow graduate students for offering moral support when needed. Finally, I wish to thank my family for their endless love and support.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Gummy stem blight (GSB), caused by the ascomycete fungus *Didymella bryoniae*, is an important disease of cucurbits in the southeastern United States and worldwide (1,20,36). In the southeastern United States, GSB is particularly destructive and widespread on watermelon (20,36). Management of the disease is achieved through a combination of cultural practices and chemical controls. Chemical controls are currently the most effective strategy for managing GSB. However, with the introduction and subsequent heavy reliance on fungicides with site-specific modes of action, rapidly developing resistance to these fungicides has been observed in *D. bryoniae* populations. In an attempt to better manage fungicide resistance and GSB development, it is important to understand the population biology of the pathogen. In particular, determining whether fitness is affected in fungicide-resistant members of the pathogen population could provide information necessary to develop better strategies to limit fungicide resistance development. The overall goal of this research project was to develop rapid and reliable fungicide resistance monitoring techniques and to determine if the mutations that result in fungicide resistance in *D. bryoniae* have altered the relative fitness of the fungus.

BACKGROUND AND LITERATURE REVIEW

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai), a member of the family Cucurbitaceae, is an economically important horticultural crop in the United States.

Watermelons thrive in well-drained, light textured soils in areas with hot days and warm nights (16). The primary commercial producers of watermelon in the United States include Florida, Georgia, California, Texas, and South Carolina (38). In Georgia, production has steadily increased over the last several years. In 2012, 25,000 acres of watermelons were harvested in Georgia and valued at $81 million USD (38).

Among the diseases that affect watermelon, gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rhem, is the most destructive in Georgia and other southeastern U.S. states (20,36). Yield losses in watermelon due to GSB can be the result of a reduced number or weight of fruit, increased fruit rot, or sunburn on fruit caused by increased exposure to the sun due to foliage loss (21). On average, GSB can cause greater than 40% yield loss in watermelon under favorable environmental field conditions with high inoculum pressure (21). Complete yield loses can occur when GSB epidemics are severe. In Brazil, 100% incidence of GSB has been reported in research plots even during the dry season (10).

Didymella bryoniae can infect watermelon at any growth stage. Symptoms may appear on all aboveground plant parts. Symptoms on seedlings appear first as water-soaked areas that eventually develop into necrotic lesions. Seedlings can become girdled once hyphae from these lesions invade the crown (34). Symptoms on older plants appear as dark brown, irregularly shaped lesions with a concentric ring pattern that occurs near the leaf margins and in the inter-veinal regions. Extensive defoliation may occur. Lesions may also be found on stems and may expand and girdle the main stem creating a canker. Stem cracking can occur and gummy ooze may exude from the canker (29). Small black pycnidia or pseudothecia may develop on infected leaves, stems, and fruit.

Although the epidemiology of GSB is not fully understood, infested seed, airborne ascospores, and infested crop debris are thought to be important sources of primary inoculum (23). Small amounts of initial inoculum can result in disease epidemics when environmental conditions are conducive for GSB because it is a polycyclic disease (23). During the growing season, conidia arising in pycnidia are splash dispersed and serve as secondary inoculum (34).

Gummy stem blight is managed through a combination of cultural practices and chemical controls. Eliminating sources of primary inoculum is important in the management of this disease. Seed treatments are also employed. Cultural practices, such as deep plowing, are used to promote the decay of crop debris and reduce the amount of initial inoculum that survives from one season to the next (22). Rotation away from watermelon with non-host crops for 2 to 3 years has been shown to be effective in reducing initial inoculum levels (22,23). Irrigation practices that limit water splashing and leaf wetness periods are important in reducing GSB incidence. It has been reported that one hour of prolonged leaf wetness is sufficient for *D. bryoniae* to infect plant tissue (1). Although these cultural practices are important in managing GSB, they have limited effectiveness when used alone. Fungicides with protective and curative action are critical for successful management of GSB.

Fungicides are effective in managing GSB in field and greenhouse production. Transplants grown in greenhouses are at a particularly high risk for GSB because planting densities and moisture levels in these structures are generally high. As of 2012, protectant fungicides registered for commercial use on watermelon in Georgia included mancozeb, maneb, chlorothalonil, cuprous oxide, copper hydroxide, Bordeaux mixture, and copper hydroxide + mancozeb (2013 Georgia Pest Management Handbook, http://www.ent.uga.edu/pmh/Com_Vegetable.pdf). Although chlorothalonil has been shown to

be effective in controlling GSB when used alone or in combination with benomyl, application on mature plants with exposed fruit can result in sunburn of the fruit rind. Hence, this fungicide is not recommended after fruit set (20,36). Systemic fungicides registered for watermelon in Georgia as of 2013 include azoxystrobin + difenoconazole, thiophanate-methyl, tebuconazole, boscalid + pyraclostrobin, cyprodinil + difenoconazole, cyprodinil + fludioxonil, azoxystrobin, pyraclostrobin, and fluopyram + tebuconazole (2013 Georgia Pest Management Handbook, http://www.ent.uga.edu/pmh/Com_Vegetable.pdf). Mixtures containing both systemic and protectant fungicides such as mefenoxam + chlorothalonil, azoxystrobin + chlorothalonil, and potassium phosphate + chlorothalonil are also registered for commercial use in watermelon (2013 Georgia Pest Management Handbook, http://www.ent.uga.edu/pmh/Com_Vegetable.pdf).

With the introduction and subsequent heavy reliance on systemic fungicides with site-specific modes of action, disease control failures related to fungicide resistance have been observed for several fungi (31). In particular, the rapid evolution of fungicide-resistant isolates of *D. bryoniae* to the quinone outside-inhibitor (QoI) fungicide, azoxystrobin, and to the succinate-dehydrogenase-inhibiting (SDHI) fungicide, boscalid, was reported within a few years of registration of the fungicides for use in commercial watermelon production (9,36). Heavy disease pressure and environmental conditions conducive for disease development have resulted in the frequent use of these fungicides because, unlike protectant fungicides, fungicides with site-specific modes of action have been shown to have some curative action (17).

Benomyl and thiophanate-methyl were used to control GSB and other foliar diseases on watermelon until resistance was observed in the southeastern United States in the 1990s (25). In response, a Section 18 emergency exemption was granted so that azoxystrobin could be used to control GSB on watermelon in 1997 and 1998 (24,36). Azoxystrobin was officially labeled for

commercial use on cucurbits in 1999 and initially provided excellent control of GSB; however, reduced control of GSB was observed in research plots and commercial fields that same year (24,36). The boscalid component in the fungicide Pristine (BASF Corporation, Research Triangle Park, NC), which is a mixture of boscalid and pyraclostrobin, was effective in controlling GSB after widespread resistance to azoxystrobin was observed. However, resistance to boscalid in *D. bryoniae* was confirmed in 2007 (35).

The excessive and extended use of azoxystrobin and boscalid has resulted in strong selection pressure that has led to fungicide-resistant members of the pathogen population (3). It has been observed that azoxystrobin and boscalid resistance frequencies in initial field populations of *D. bryoniae* were higher than 80% in non-fungicide treated plots (37). After application of azoxystrobin or boscalid, resistance frequencies increased to 100% in most cases (37). Once the frequency of fungicide-resistant isolates increases, systemic fungicides have limited effectiveness. Interestingly, disease severity is often higher in azoxystrobin or boscalid treated plots where high resistance frequencies have been observed than in non-treated control plots (37).

Quinone outside-inhibitor fungicides, such as azoxystrobin, inhibit mitochondrial respiration by binding to the outer, quinone oxidizing pocket of the cytochrome bc1 enzyme complex which, in turn, blocks electron transport and results in energy loss in the fungus (15). The rapid development of resistance to QoI fungicides in fungal plant pathogens has been attributed to a substitution of alanine for glycine at amino acid position 143 (15). Substitution of leucine for phenylalanine at amino acid position 129 in some fungi also confers resistance to QoI fungicides (27). However, the F129L mutation has been associated with a lower level of

fungicide resistance and does not seriously affect field performance of QoI fungicides (8,27). In *D. bryoniae*, the rapid development of QoI fungicide-resistant isolates has been attributed to the G143A mutation (14,36).

Traditionally, resistance to QoI fungicides has been monitored using in vitro conidial germination fungicide sensitivity assays. Conidial germination is particularly sensitive to inhibition by QoI fungicides because it is highly energy demanding as compared to other developmental stages (6). Therefore, it is an ideal indicator of sensitivity to QoI fungicides. A limitation of in vitro conidial germination based QoI sensitivity assays is that some plant pathogenic fungi can circumvent the activity of respiration inhibiting fungicides by activating an alternative respiratory pathway (14,22,28). However, salicylhydroxamic acid (SHAM) may be added to QoI fungicide-amended medium to inhibit the alternative respiratory pathway in the fungus that interferes with the activity of the fungicide (13).

To better manage fungicide resistance, several studies have been conducted to determine the fitness of fungicide-sensitive and fungicide-resistant isolates. Fitness is defined as the survival and reproductive success of an allele, individual, or group (32). In the presence of fungicides, resistant isolates have a higher fitness relative to sensitive isolates and, in turn, they occur at higher frequencies (31). In the absence of fungicides, however, the fitness of sensitive and resistant isolates is less predictable.

Differences in fitness may be the result of fitness costs associated with mutation(s) that confer(s) fungicide resistance. A fitness cost would make a pathogen less competitive compared to wild-type isolates in the absence of the selection pressure imposed by the fungicide. This is important in fungicide resistance management because fitness costs may significantly increase the efficacy of fungicide rotation strategies. When the use of a particular fungicide is

discontinued, there is less selection pressure towards isolates resistant to that fungicide (31). The absence of selection pressure towards fungicide-resistant isolates allows for competition between fungicide-sensitive and –resistant isolates and may allow sensitive isolates to outcompete resistant isolates.

The G143A mutation has been observed in several fungi and oomycetes that are highly resistant to QoI fungicides. Interestingly, even after this mutation, no significant effect on enzyme activity has been observed (15). This suggests that there is no fitness cost associated with the G143A mutation. Studies that have observed fitness parameters, other than enzymatic activity, have further supported the suggestion that no fitness costs are sustained as a result of this mutation. For example, no significant difference was observed in colony size, conidia production, pathogenicity, or virulence in G143A mutants of *Magnaporthe grisea* as compared to wild-type isolates (5). In *Alternaria alternata*, no significant differences between G143A fungicide-resistant or -sensitive isolates were observed in mycelial growth, in vitro and in vivo sporulation, or incubation period (19). Furthermore, G143A fungicide-resistant isolates of *A. alternata* were significantly more aggressive than fungicide-sensitive isolates (19). In *Plasmopara viticola*, no significant differences were observed in latent period or spore production between G143A fungicide-resistant isolates and –sensitive isolates (12). The G143A fungicide-resistant isolates of *P. viticola* were also able to infect grapevine leaves at a significantly higher rate than fungicide-sensitive isolates (12). However, significant differences in conidia production and virulence were reported between azoxystrobin-resistant G143A mutants and azoxystrobin–sensitive *M. oryzae* isolates (27). The G143A mutants of *M. oryzae* were less virulent and produced more secondary inoculum than fungicide-sensitive isolates (27). Although these results suggest fitness costs may occur as a result of the G143A mutation, only

two isolates, one resistant G143A mutant and one sensitive wild-type, were used in the study. Other studies on fitness have reported large variation among isolates within the same sensitivity class (6,12,18,19). A larger number of isolates must be compared to confirm this relationship.

Although limited, the majority of evidence suggests that no fitness costs are observed in QoI fungicide-resistant G143A mutants. This suggests that no fitness costs will be observed in azoxystrobin-resistant *D. bryoniae* isolates. It is worth noting that a study comparing the fitness of QoI fungicide-resistant F129L mutants to wild-type sensitive isolates of *A. solani* determined that the resistant mutant could incite greater disease severity on the plant but conidia produced from the mutant had a lower germination rate (30).

Several point mutations in the succinate dehydrogenase iron sulfur gene have been associated with SDHI fungicide resistance in various fungi. In *D. bryoniae*, high levels of resistance to boscalid have been attributed to two different single point mutations at amino acid position 277 (4). A substitution of tyrosine for histidine at position 277 corresponds with resistance to both boscalid and penthiopyrad in *D. byroniae* (3,4,35). The second mutation which substitutes arginine for histidine at position 277 leads to *D. bryoniae* individuals that are highly resistant only to boscalid (4). While the mechanisms of resistance to SDHIs have been identified in some fungi, few studies have been done to determine the fitness of SDHI fungicide-resistant individuals. In *A. alternata*, no significant differences in sporulation, mycelial growth rates, conidia germination percentages, or virulence were observed between boscalid-resistant and –sensitive isolates collected from pistachio orchards in California (2). It has been previously reported that there were no differences in mycelial growth rates between boscalid-resistant and –sensitive isolates of *D. bryoniae* and, it may be further hypothesized that no differences in fitness will be observed between boscalid-resistant and –sensitive isolates of *D. bryoniae* (35).

In many plant pathogenic fungi, resistance to benzimidazole fungicides has been attributed to several point mutations in the beta-tubulin gene (28). Although the presence of these mutations has not been confirmed in *D. bryoniae*, they may play a role in resistance to benzimidazoles in *D. bryoniae*. Studies regarding the fitness of benzimidazole-resistant fungi are limited. It has been reported that benomyl-resistant isolates of *Botrytis cinerea* grew less than wild-type isolates (26), suggesting that a fitness cost may accompany resistance to benzimidazoles. However, *B. cinerea* isolates that were also resistant to dicarboximide fungicides grew as well as wild-type isolates (26). Additionally, there was no significant difference in the growth of thiophanate-methyl-resistant and –sensitive isolates of *Venturia inaequalis* (11). Although limited, the majority of evidence suggests that fitness costs do not accompany benzimidazole resistance. Therefore, it may be hypothesized that the fitness of thiophanate-methyl-resistant and –sensitive isolates of *D. bryoniae* will be similar.

Fitness costs have been reported in fungicide-resistant isolates of *B. cinerea*, *Fusarium graminearum*, *Cercospora beticola*, *M. oryzae*, and *A. solani* (7,18,27,30,33); however, it cannot be assumed that fitness costs accompany resistance because no consistent pattern of fitness costs has been associated with fungicide resistance (31). No fitness cost was reported when *A. alternata*, *B. cinerea*, *M. grisea*, and *Monilinia fructicola* developed fungicide resistance (2,5,6,13,33). These findings suggest that fitness costs associated with fungicide resistance are specific to fungal species, fitness parameters, and the fungicide (13). For example, no differences in fitness were observed in *M. grisea* G143A fungicide-resistant mutants, however, G143A fungicide-resistant mutants of *M. oryzae* produced more conidia and were less virulent than wild-type isolates (5,27). Propiconazole-resistant isolates of *M. fructicola* initially had similar fitness characteristics as sensitive isolates but, after undergoing several consecutive transfers on potato

dextrose agar, there was a significant decrease in spore germination and spore production of resistant isolates (13). In *B. cinerea*, sclerotia of isolates with dicarboximide resistance survived significantly less in soil than did sclerotia of sensitive isolates (33). However, anilinopyrimidine-resistant *B. cinerea* isolates had similar fitness characteristics as sensitive isolates (6).

Competitive ability of a pathogen may also be affected when fungicide resistance develops. The establishment of resistance in a population is determined by the competition between sensitive and resistant isolates (27). If a fitness cost is associated with fungicide resistance, resistant isolates may be less competitive than sensitive isolates in the absence of fungicides. This could have important implications in disease management because the frequency of fungicide resistance would decline overtime in the absence of the fungicide. The pathogen population could theoretically be restored to complete sensitivity. In contrast, if no fitness cost is associated with fungicide resistance, resistant isolates may be equally or more competitive than sensitive isolates. From a disease management standpoint, this suggests that mixtures with chemically unrelated fungicides or alternations of fungicides with different modes of action are critical in delaying the development of fungicide resistance (6).

The rapid evolution of fungicide resistance to newer generation systemic fungicides with site-specific modes of action has made plant disease management challenging. A better understanding of the evolution of fungicide resistance in plant pathogens is critical for extending the performance of these important fungicides. Fitness of fungicide-resistant plant pathogens has been evaluated for several fungi but it has not been studied in *D. bryoniae*. Determining whether differences in fitness exist between fungicide-resistant isolates and fungicide-sensitive isolates will provide important information that can be used to better manage fungicide resistance. This study will first determine whether mycelial growth of *D. bryoniae* differs in sensitivity to the QoI

fungicide azoxystrobin as compared to conidial germination. This study will also determine if azoxystrobin resistance is stable in *D. bryoniae* and whether fitness is affected by mutations that lead to fungicide resistance. The molecular mechanism of resistance to QoI fungicides will be examined in order to design a molecular-based assay for detection of azoxystrobin resistance in *D. bryoniae*. Results obtained from this study will be used to rapidly and efficiently detect fungicide resistance. Also, the results will be useful in designing better fungicide spray programs that emphasize resistance management and prolong the efficacy of fungicides.

The specific objectives of the study are as follows:

1. Compare the sensitivity of different growth stages of *D. bryoniae* to the quinone outside-inhibitor (QoI) fungicide azoxystrobin.
2. Evaluate the stability of azoxystrobin resistance and the fitness of fungicide-resistant and –sensitive field isolates of *D. bryoniae.*
3. Design an allele-specific PCR assay for detection of azoxystrobin resistance in *D. bryoniae.*

LITERATURE CITED

1. Arny, C. J., and Rowe, R. C. 1991. Effects of temperature and duration of surface wetness on spore production and infection of cucumbers by *Didymella bryoniae*. Phytopathology 81:206-209.

2. Avenot, H. F., and Michailides, T. J. 2007. Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. Plant Dis. 91:1345-1350.

3. Avenot, H. F., and Michailides, T. J. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Prot. 29:643-651.

4. Avenot, H. F., Thomas, A., Gitaitis, R. D., Langston, D. B., Jr., & Stevenson, K. L. 2012. Molecular characterization of boscalid- and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. Pest Manag. Sci. 68:645-651.

5. Avila-Adame, C., and Köller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Curr. Genet. 42:332-338.

6. Bardas, G. A., Karaoglanidis, G. S., and Myresiotis, C. K. 2008. Stability and fitness of anilinopyrimidine-resistant strains of *Botrytis cinerea*. Phytopathology 98:443-450.

7. Becher, R., Deising, H. B., Wirsel, S. G. R., Hettwer, U., and Karlovsky, P. 2010. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. Phytopathology 100:444-453.

8. Borris, H., Brunke, H., and Kreith, M. 2006. Commodity profile: melons. UC Agricultural Issues Center, University of California. Online publication.

9. Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograph No. 1.

10. Café-Filho, A. C., Santos, G. R., and Laranjeira, F. F. 2010. Temporal and spatial dynamics of watermelon gummy stem blight epidemics. Eur. J. Plant Pathol. 128:473-482.

11. Chapman, K. S., Sundin, G. W., and Beckerman, J. L. 2011. Identification of resistance to multiple fungicides in field populations of *Venturia inaequalis*. Plant Dis. 95:921-926.

12. Corio-Costet, M. F., Dufour, M. C., Cigna, J., Abadie, P., and Chen, W. J. 2011. Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 129:315-329.

13. Cox, K. D., Schnabel, G., and Bryson, P. K. 2007. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. Phytopathology 97:448-453.

14. Fungicide Resistance Action Committee (FRAC). 2011. List of pathogens with field resistance towards QoI fungicides. Accessed on 06/23/13. Available at www.frac.info.

15. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manag. Sci. 58:859-867.

16. Hall, K. D., Holloway, R. L., and Smith, D. 2000. Texas crop profile: watermelon. Texas A&M AgriLife Extension Service, Texas A&M University. Online publication.

17. Karadimos, D. A., Karaoglanidis, G. S., and Tzavella–Klonari, K. 2005. Biological activity and physical modes of action of the Qo inhibitor fungicides trifloxystrobin and pyraclostrobin against *Cercospora beticola*. Crop Prot. 24:23-29.

18. Karaoglanidis, G. S., Ioannidis, P. M., and Thanassoulopoulos, C. C. 2001. Fitness of *Cercospora beticola* field isolates-resistant and -sensitive to demethylation inhibitor fungicides. Eur. J. Plant Pathol. 107:337-347.

19. Karaoglanidis, G. S., Luo, Y., and Michailides, T. J. 2011. Competitive ability and fitness of *Alternaria alternata* isolates resistant to QoI fungicides. Plant Dis. 95:178-182.

20. Keinath, A. P. 1995. Fungicide timing for optimum management of gummy stem blight epidemics on watermelon. Plant Dis. 79:354-358.

21. Keinath, A. P. 2001. Effect of fungicide applications scheduled to control gummy stem blight on yield and quality of watermelon fruit. Plant Dis. 85:53-58.

22. Keinath, A. P. 2002. Survival of *Didymella bryoniae* in buried watermelon vines in South Carolina. Plant Dis. 86:32-38.

23. Keinath, A. P. 2008. Survival of *Didymella bryoniae* in infested muskmelon crowns in South Carolina. Plant Dis. 92:1223-1228.

24. Keinath, A. P. 2009. Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. Pest Manag. Sci. 65:1090-1096.

25. Keinath, A. P., and Zitter, T. A. 1998. Resistance to benomyl and thiophanate-methyl in *Didymella bryoniae* from South Carolina and New York. Plant Dis. 82:479-484.

26. Korolev, N., Mamiev, M., Zahavi, T., and Elad, Y. 2011. Screening of *Botrytis cinerea* isolates from vineyards in Israel for resistance to fungicides. Eur. J. Plant Pathol. 129:591-608.

27. Ma, B., and Uddin, W. 2009. Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. Plant Dis. 93:1044-1049.

28. Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.

29. Paret, M. L., Dufault, N. S., and Olson, S. M. 2011. Management of gummy stem blight (black rot) on cucurbits in Florida. Institute of Food and Agricultural Sciences, University of Florida. Online publication.

30. Pasche, J. S., and Gudmestad, N. C. 2007. Prevalence, competitive fitness and impact of the F129L mutation in *Alternaria solani* from the United States. Crop Prot. 27:427-435.

31. Peever, T. L., and Milgroom, M. G. 1995. Fungicide resistance---lessons for herbicide resistance management? Weed Technol. 9:840-849.

32. Pringle, A., and Taylor, J. W. 2002. The fitness of filamentous fungi. Trends Microbiol. 10:474-481.

33. Raposo, R., Melgarejo, P., Urrutia, T., and Gomez, V. 2000. Fitness of *Botrytis cinerea* associated with dicarboximide resistance. Phytopathology 90:1246-1249.

34. Schenck, N. C. 1968. Epidemiology of gummy stem blight (*Mycosphaerella citrullina*) on watermelon: Ascospore incidence and disease development. Phytopathology 58:1420-1422.

35. Stevenson, K. L., Langston, D. B., and Sanders, F. 2008. Baseline sensitivity and evidence of resistance to boscalid in *Didymella bryoniae*. Phytopathology 98:S151.

36. Stevenson, K. L., Seebold, K. W., and Langston, D. B., Jr. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. Plant Health Progress doi: 10.1094/PHP-2004-1207-01-RS.

37. Thomas, A., Langston, D. B., Jr., Sanders, H. F., and Stevenson, K. L. 2012. Relationship between fungicide sensitivity and control of gummy stem blight of watermelon under field conditions. Plant Dis. 96:1780-1784.

38. USDA. 2013. Vegetables 2012 summary. USDA, National Agricultural Statistics Service. Online publication.

CHAPTER 2

COMPARATIVE SENSITIVITY OF CONIDIAL GERMINATION AND MYCELIAL GROWTH OF *DIDYMELLA BRYONIAE* TO AZOXYSTROBIN

Finger, M. J., Ji, P., and Stevenson, K. L. 2013. To be submitted to Plant Disease.

ABSTRACT

The sensitivity of conidial germination and mycelial growth of *Didymella bryoniae* to azoxystrobin (AZO) was compared using 11 AZO-sensitive (AZO-S) and 13 AZO-resistant (AZO-R) isolates. The effective concentration of AZO at which conidial germination (C-EC_{50}) or mycelial growth (M-EC_{50}) was inhibited by 50% was estimated. For AZO-S isolates, M-EC_{50} values were significantly greater than C-EC_{50} values. For AZO-R isolates, M-EC_{50} and C-EC_{50} values were >10.0 µg/ml. Relative growth (RGr) of AZO-S isolates was significantly lower than relative germination (RG) at AZO concentrations ≤0.3 µg/ml. At concentrations ≥1.0, RGr was significantly higher than RG. At 10.0 µg/ml, RGr was 0.44 and 0.42 in trials 1 and 2, respectively, but conidial germination of AZO-S isolates was completely inhibited. Mycelial growth and spore germination of AZO-R isolates were slightly inhibited at AZO concentrations ≤0.1 µg/ml; no significant differences between RGr and RG were observed. RGr of AZO-R isolates was significantly lower than RG at AZO concentrations ≥0.3 µg/ml in trial 1 and ≥3.0 µg/ml in trial 2. Salicylhydroxamic acid (100 µg/ml) increased the sensitivity of mycelial growth of AZO-S isolates. The AZO concentration of 1.0 µg/ml was selected as a discriminatory concentration for mycelial growth sensitivity assays which are faster and easier to perform than the currently recommended conidial germination assays.

INTRODUCTION

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rhem, is an important disease of cucurbits in the southeastern U.S. and worldwide. In the southeastern U.S., GSB is particularly destructive and widespread on watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai). On average, GSB can cause greater than 40% yield loss in watermelon under

favorable environmental field conditions with high inoculum pressure (9). Effective management of the disease involves a combination of cultural practices and chemical controls. Fungicides with protective and systemic properties are of particular importance, as they are generally the most effective in controlling GSB.

Of the systemic fungicides labeled for GSB control, the quinone-outside inhibiting (QoI) fungicide azoxystrobin first entered the commercial market in the late 1990s. QoI fungicides, such as azoxystrobin, work by inhibiting mitochondrial respiration by binding to the outer, quinone oxidizing pocket of the cytochrome bc1 enzyme complex which, in turn, blocks electron transport and results in energy loss in the fungus (7). Initially, azoxystrobin provided excellent GSB control. However, resistance to azoxystrobin was noted within a year of registration of the fungicide for use in commercial watermelon production (11,20).

Currently, azoxystrobin resistance monitoring in *D. bryoniae* is accomplished using in vitro conidial germination fungicide sensitivity assays (10,12,20). Conidial germination is particularly vulnerable to inhibition by QoI fungicides because it is a relatively energy demanding developmental stage as compared to the mycelial growth stage (6). Therefore, QoI sensitivity assays are usually based on spore germination rather than mycelial growth. Additionally, it has been shown, in vitro, that some plant pathogenic fungi can circumvent the activity of respiration inhibiting fungicides by activating an alternative respiratory pathway (14,22,28). However, the alternative respiratory pathway does not play a significant role in field resistance to QoI fungicides (13,28). Salicylhydroxamic acid (SHAM) can be added to QoI fungicide-amended medium to inhibit the alternative respiratory pathway in the fungus that interferes with the activity of the fungicide (13). The addition of SHAM to fungicide-amended medium has been shown to increase the sensitivity of some plant pathogenic fungi to QoI

fungicides (2,3,5,8,23). However, it has also been observed that SHAM has no effect on the sensitivity of certain developmental stages of some plant pathogenic fungi to QoI fungicides (17).

While conidial germination assays are useful in azoxystrobin resistance screening, they are time consuming because *D. bryoniae* may take up to 2 weeks to sporulate. Also, measuring conidial germination is a tedious process that requires a microscope. Mycelial growth assays are also useful in azoxystrobin resistance screening (15,24) but they are not currently recommended for *D. bryoniae*. Mycelial growth assays are desirable in that they can be completed in as few as 4 days. Also, mycelial growth can be easily measured without the need of special instrumentation.

The objectives of this study were to compare the sensitivity of conidial germination and mycelial growth stages of *D. bryoniae* to azoxystrobin and to determine the effect of SHAM on the sensitivity of mycelial growth to azoxystrobin. The results of this study will be used to determine if mycelial growth assays are useful in determining the sensitivity of *D. bryoniae* to azoxystrobin.

MATERIALS AND METHODS

Fungal isolates. Twenty-four single-lesion isolates of *D. bryoniae* were used to determine the sensitivities of germinating conidia and mycelia to azoxystrobin. The isolates were selected from a larger set of isolates collected in 2009 and 2010 from watermelon plants showing symptoms of GSB in different counties in Georgia (Table 2.1). Eleven isolates were characterized as azoxystrobin-sensitive (AZO-S) and 13 isolates were characterized as azoxystrobin-resistant (AZO-R), based on in vitro conidial germination assays using the

discriminatory azoxystrobin concentration of 10.0 µg/ml (21). Isolates exhibiting >50% relative germination on fungicide-amended medium were considered AZO-R and isolates exhibiting relative germination values <50% were considered AZO-S (21). All isolates were stored on filter paper at -20°C.

Sensitivity of conidial germination to azoxystrobin. Technical grade azoxystrobin (98.4% a.i.; Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to obtain a stock solution of 30 mg/ml. The stock solution was serially diluted in acetone and added to autoclaved water agar (WA) that was cooled to 55°C to obtain azoxystrobin concentrations of 0, 0.0001, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 µg/ml. The final concentration of acetone in fungicide-amended and non-amended media was 0.1% by volume. Azoxystrobin-amended and non-amended media was also amended with 100 µg/ml of SHAM to inhibit an alternative respiratory pathway in the fungus that can interfere with the activity of the fungicide. *Didymella bryoniae* isolates were recovered by placing a small (~25 mm^2) piece of infested filter paper on one-quarter-strength potato dextrose agar (QPDA). The plates were incubated at 24°C for 2 weeks with a 12-h photoperiod provided by cool white fluorescent light (Philips Electronics, Somerset, NJ) and wide-spectrum fluorescent plant and aquarium light (F40PL/AQ-ECO, General Electric, Fairfield, CT) to encourage conidial production. The QPDA plates were flooded with 2 ml of a solution of sterile water and Tween 20 (1 drop of Tween/100 ml water) and swirled to dislodge the conidia. Using a hemocytometer, the concentration of conidia in each suspension was adjusted to approximately 10^6 conidia/ml. Conidial suspension (15 µl) of each isolate was spread uniformly across the surface of azoxystrobin-amended and non-amended media. Two replications per isolate and fungicide concentration were prepared. After incubation at 24°C for 24 h, the number of germinated conidia was determined by examining 50 arbitrarily

selected conidia per plate. A conidium was considered germinated if the length of the germ tube was equal to or greater than half the length of the conidium. Relative conidial germination was calculated as the ratio of the percentage of germinated conidia on azoxystrobin-amended medium to the percentage of germinated conidia on non-amended medium. The assay was conducted twice.

Sensitivity of mycelial growth to azoxystrobin. Technical grade azoxystrobin (98.4% a.i.; Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to obtain a stock solution of 30 mg/ml. The stock solution was serially diluted in acetone and added to autoclaved QPDA that was cooled to 55°C to obtain desired azoxystrobin concentrations as described above for WA. SHAM (100 µg/ml) was added to azoxystrobin-amended and non-amended media. Isolates of *D. bryoniae* were recovered by placing a small (~25 mm^2) piece of infested filter paper on potato dextrose agar (PDA) and incubating at 24°C. After 7 days, 6-mm diameter mycelial plugs were removed from the margin of an actively growing culture of each isolate and transferred to azoxystrobin-amended and non-amended QPDA. Two replications per isolate and fungicide concentration were prepared. After incubation in the dark at 24°C for 7 days, colony diameter was measured. The colony diameter was corrected by subtracting the diameter of the agar plug from the colony diameter measurement. Relative mycelial growth was calculated as the ratio of the corrected colony diameter on azoxystrobin-amended medium to the corrected colony diameter on non-amended medium. The assay was conducted twice.

Effects of SHAM on sensitivity of mycelial growth to azoxystrobin. Azoxystrobin-amended and non-amended QPDA was prepared as previously described, both with and without 100 µg/ml SHAM. A subset of 10 isolates of *D. bryoniae* was arbitrarily selected to determine the effects of SHAM on the sensitivity of mycelial growth to azoxystrobin (Table 2.1). The

subset of isolates included 5 AZO-S and 5 AZO-R isolates. The isolates were recovered by placing a small piece (~25 mm^2) of infested filter paper on PDA and incubating at 24°C. After 7 days, 6-mm diameter mycelial plugs were removed from the margins of the actively growing culture of each isolate and transferred to azoxystrobin-amended or non-amended QPDA plates, both with and without SHAM. Two replications per isolate, fungicide concentration, and SHAM treatment were prepared. After incubation in the dark at 24°C for 6 days, colony diameter was measured and corrected by subtracting the diameter of the agar plug. Relative mycelial growth was calculated as the ratio of the corrected colony diameter on azoxystrobin-amended medium to the corrected colony diameter on non-amended medium. Relative mycelial growth was calculated for each azoxystrobin concentration, with or without SHAM. The entire experiment was conducted five times; however, three of the experimental repeats included only the "with SHAM" treatment.

Data analysis. The effective concentration at which conidial germination or mycelial growth was inhibited by 50% (EC_{50}) value for each isolate was estimated based on linear regression of probit-transformed relative inhibition (1- [relative conidial germination] or 1- [relative mycelial growth]) on log_{10}-transformed fungicide concentration. Differences in log_{10}-transformed EC_{50} values for each isolate between conidial germination and mycelial growth assays and effect of SHAM on mycelial growth sensitivity to azoxystrobin were evaluated using PROC GLIMMIX in SAS 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Comparison of sensitivity of conidial germination and mycelial growth to azoxystrobin. The dose response of conidial germination encompassed the full range of

responses for AZO-S isolates. Inhibition of conidial germination of AZO-S isolates ranged from 0-100% in both experimental trials (Fig. 2.1). Mycelial growth of AZO-S isolates was only slightly inhibited by azoxystrobin. Inhibition of mycelial growth of AZO-S isolates ranged from 30-58% in both experimental trials (Fig. 2.1). The dose responses of conidial germination and mycelial growth of AZO-R isolates were similar. Slight to no inhibition (0-3%) of conidial germination of AZO-R isolates was observed in both experimental trials (Fig. 2.2). Inhibition of mycelial growth of AZO-R isolates ranged from 0-32% in both experimental trials (Fig. 2.2).

For AZO-S isolates, conidial germination EC_{50} (C-EC_{50}) values ranged from 0.29 to 0.93 µg/ml in trial 1 and 0.33 to 0.96 µg/ml in trial 2 (Table 2.2). Mycelial growth EC_{50} (M-EC_{50}) values ranged from 0.25 to 3.83 µg/ml in trial 1 and 0.34 to 2.60 µg/ml in trial 2 (Table 2.2). In both trials, M-EC_{50} values were significantly higher than C-EC_{50} values (Table 2.2). For AZO-R isolates, relative growth and relative germination values were greater than 0.5 at all azoxystrobin concentrations tested in both trials. Therefore, M-EC_{50} and C-EC_{50} values of AZO-R isolates could not be accurately estimated, but were assumed to be greater than 10.0 µg/ml, the highest concentration tested.

Due to significant resistance group by assay type interactions, differences between relative germination and relative growth values are shown separately for each trial and fungicide concentration in Table 2.3. For AZO-S isolates, relative growth was significantly lower than relative germination at azoxystrobin concentrations of 0.3 µg/ml or less; however, at concentrations of 1.0 µg/ml or greater, relative growth was significantly higher than relative germination (Table 2.3). At 10.0 µg/ml, the highest concentration tested, relative growth was 0.44 and 0.42 in trials 1 and 2, respectively, but conidial germination was completely inhibited. For AZO-R isolates, there was little or no inhibition of conidial germination or mycelial growth

at the lowest azoxystrobin concentrations tested (0.1 µg/ml or less), and there was no significant difference between relative mycelial growth and relative germination values. However, relative growth of AZO-R isolates was significantly lower than relative germination at azoxystrobin concentrations of 0.3 µg/ml or greater in trial 1 and at concentrations of 3.0 µg/ml or greater in trial 2 (Table 2.3).

Differences in relative mycelial growth were observed between AZO-S and AZO-R isolates at all azoxystrobin concentrations tested (Fig. 2.3). The greatest separation between AZO-S and AZO-R isolates was observed at azoxystrobin concentrations ranging from 0.03 to 3.0 µg/ml (Fig. 2.3). Less separation between the relative mycelial growth of AZO-S and AZO-R isolates was observed at azoxystrobin concentrations of 0.01 and 10.0 µg/ml (Fig. 2.3). At 1.0 µg/ml, relative mycelial growth of AZO-S isolates ranged from 45-56% and the relative mycelial growth of AZO-R isolates ranged from 90-99% (Fig. 2.3). Additionally, there was low variation in relative mycelial growth among AZO-S and AZO-R isolates at 1.0 µg/ml. Therefore, the azoxystrobin concentration of 1.0 µg/ml was selected as a discriminatory concentration for azoxystrobin sensitivity mycelial growth assays in *D. bryoniae*.

Effect of SHAM on sensitivity of mycelial growth to azoxystrobin. SHAM increased the sensitivity of AZO-S isolates to azoxystrobin (Fig. 2.4). However, SHAM did not increase the sensitivity of AZO-R isolates to azoxystrobin (Fig. 2.4). For AZO-S isolates, relative growth (RG) values of isolates on QPDA with SHAM were significantly lower than RG values of isolates on QPDA without SHAM at all azoxystrobin concentrations (Table 2.4). For AZO-R isolates, slight but significant differences were observed at four azoxystrobin concentrations. RG

values of AZO-R isolates on QPDA without SHAM were significantly lower than RG values of AZO-R isolates on QPDA with SHAM at azoxystrobin concentrations of 0.01, 0.03, and 0.1 µg/ml and significantly higher at 1.0 µg/ml (Table 2.4).

DISCUSSION

The dose responses of conidial germination and mycelial growth differ to a large degree in AZO-S isolates of *D. bryoniae*. In this study, the dose response of conidial germination encompassed the full range of responses for AZO-S isolates. Inhibition of conidial germination of AZO-S isolates ranged from 0 to 100%. However, mycelial growth of AZO-S isolates was only slightly inhibited by azoxystrobin. Inhibition of mycelial growth of AZO-S isolates ranged from 30 to 58%. It was previously reported that the mycelial growth stage of several Ascomycete fungi was less sensitive to QoI fungicides than the conidial germination stage (16,18,22,26,27). The mycelial growth stage of the oomycete pathogen, *Phytophthora cactorum*, was also determined to be less sensitive to azoxystrobin than the zoospore germination stage (17). These results are consistent with previous reports that described the spore germination stage as particularly sensitive to the QoI fungicides in comparison to other growth stages (4,25). In contrast, mycelial growth of *Monilinia fructicola* was reported to be as sensitive to azoxystrobin as conidial germination (1). For AZO-R isolates of *D. bryoniae*, the dose responses of conidial germination and mycelial growth differed slightly. Slight inhibition of mycelial growth of AZO-R isolates was observed at the two highest azoxystrobin concentrations tested.

Alternative respiration has been shown to be responsible for the decreased sensitivity of mycelial growth to QoI fungicides in some fungi in vitro (14,28). The activation of alternative respiration allows the fungus to circumvent the cytochrome b target site of the fungicide (14).

SHAM is a specific inhibitor of alternative respiration (28). In order to counteract the inhibitory action of alternative respiration on fungicidal activity, SHAM is routinely added to media containing QoI fungicides at 100 ppm. In this study, SHAM was added at 100 ppm to both the QPDA used to assay the sensitivity of mycelial growth to azoxystrobin and the WA used to measure the sensitivity of conidial germination to azoxystrobin. Interestingly, even at the highest concentration of azoxystrobin tested, 10.0 µg/ml, mycelial growth was not completely inhibited. Complete inhibition of mycelial growth was achieved in other fungi when 100 µg/ml SHAM was added to the fungicide medium. For example, complete inhibition of mycelial growth of *Colletotrichum graminicola* was achieved at an azoxystrobin concentration of 10.0 µg/ml with the addition of 100 ppm SHAM (2). Mycelial growth of *Botrytis cinerea* was also completely inhibited at an azoxystrobin concentration of 25.0 µg/ml with the addition of 50 ppm SHAM (3). The concentration of 100 ppm of SHAM may not be high enough to completely inhibit mycelial growth of *D. bryoniae.* Although concentrations of SHAM higher than 100 ppm were not tested in this study, they may further reduce growth as observed in *Sclerotinia sclerotiorum* (5).

The addition of 100 ppm of SHAM did significantly increase the sensitivity of mycelial growth of AZO-S isolates of *D. bryoniae* to azoxystrobin. SHAM alone has been shown to be toxic to some fungi (19) but inhibition from SHAM alone did not exceed 30% in this study. Relative growth was analyzed in this study rather than EC_{50} values because inhibition of mycelial growth did not reach 50% and, therefore, EC_{50} values could not be accurately estimated. The increase in sensitivity of mycelial growth to QoI fungicides when SHAM was added to the medium has been reported for several other fungi (2,5,8,17,26). For AZO-R isolates, SHAM did not increase the sensitivity of mycelial growth to azoxystrobin. The increase in sensitivity of mycelial growth of AZO-S isolates of *D. bryoniae* to azoxystrobin when SHAM was added to

the medium suggests that alternative respiration may play a role in the reduced sensitivity of mycelial growth to azoxystrobin. However, because cellular respiration was not measured in this study, firm conclusions cannot be drawn.

This study focused on determining if mycelial growth assays can be used as an alternative to conidial germination assays in azoxystrobin resistance screening in *D. bryoniae*. Mycelial growth assays are currently used for azoxystrobin resistance screening in other fungi (15,24). Mycelial growth assays are desirable because they can be completed faster than conidial germination assays and special instrumentation is not required. Although complete inhibition of mycelial growth of *D. bryoniae* was not observed even at the highest concentration of azoxystrobin tested, a clear distinction was observed between the relative growth values of AZO-S and AZO-R isolates at most azoxystrobin concentrations tested (Fig. 2.3). An azoxystrobin concentration of 1.0 µg/ml was suggested for a discriminatory concentration for mycelial growth assays to determine azoxystrobin resistance in *D. bryoniae*. The recommended discriminatory concentration for conidial germination assays of 10.0 µg/ml of azoxystrobin was not selected because the separation between relative growth values of AZO-S and AZO-R isolates at this concentration was less clear than at the lower concentrations tested.

Although mycelial growth assays are useful for identifying AZO-S and AZO-R isolates of *D. bryoniae*, this assay has limitations. For example, when the effect of SHAM on mycelial growth sensitivity to azoxystrobin was tested, inhibition of mycelial growth did not reach 50% and EC_{50} values could not be calculated. Additionally, because alternative respiration is suspected to play a role in the reduced sensitivity of mycelial growth to azoxystrobin, EC_{50} values calculated in the initial mycelial growth sensitivity assays may be abnormally high. Therefore, EC_{50} values for azoxystrobin sensitivity in *D. bryoniae* should be estimated using

conidial germination assays. In summary, the use of mycelial growth assays in azoxystrobin resistance screening in *D. bryoniae* provides a quick and reliable method for accurately identifying sensitive and resistant isolates.

LITERATURE CITED

1. Amiri, A., Brannen, P. M., and Schnabel, G. 2010. Reduced sensitivity in *Monilinia fructicola* field isolates from South Carolina and Georgia to respiration inhibitor fungicides. Plant Dis. 94:737-743.

2. Avila-Adame, C., Olaya, G., and Köller, W. 2003. Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin-related QoI fungicides. Plant Dis. 87:1426-1432.

3. Banno, S., Yamashita, K., Fukumori, F., Okada, K., Uekusa, H., Takagaki, M., Kimura, M., and Fujimura, M. 2009. Characterization of QoI resistance in *Botrytis cinerea* and identification of two types of mitochondrial cytochrome b gene. Plant Pathol. 58:120-129.

4. Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2004. Review: The strobilurin fungicides. Pest Manag. Sci. 60:309-309.

5. Duan, Y., Liu, S., Ge, C., Feng, X., Chen, C., and Zhou, M. 2012. In vitro inhibition of *Sclerotinia sclerotiorum* by mixtures of azoxystrobin, SHAM, and thiram. Pestic. Biochem. Physiol. 103:101-107.

6. Fernandez-Ortuno, D., Tores, J. A., de Vincente, A., and Perez-Garcia, A. 2010. The QoI fungicides, the rise and fall of a successful class of agricultural fungicides. in: In Tech, O. Carisse, ed., Spain. doi: 10.5772/13205

7. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manag. Sci. 58:859-867.

8. Jin, L., Chen, Y., Chen, C., Wang, J., and Zhou, M. 2009. Activity of azoxystrobin and SHAM to four phytopathogens. Agri. Sci. China 8:835-842.

9. Keinath, A. P. 2001. Effect of fungicide applications scheduled to control gummy stem blight on yield and quality of watermelon fruit. Plant Dis. 85:53-58.

10. Keinath, A. P. 2009. Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. Pest Manag. Sci. 65:1090-1096.

11. Langston, D. B., Jr., Lewis, K. L., and Jennings, W. T. 2000. Evaluation of fungicides and spray programs for control of gummy stem blight on watermelon. Fungic. Nematicide Tests 55:291.

12. Olaya, G., and Holm, A. 2001. Sensitivity of *Didymella bryoniae* isolates to azoxystrobin. Phytopathology 91:S67.

13. Olaya, G., and Köller, W. 1999. Baseline sensitivities of *Venturia inaequalis* populations to the strobilurin fungicide kresoxim-methyl. Plant Dis. 83:274-278.

14. Olaya, G., and Köller, W. 1999. Diversity of kresoxim-methyl sensitivities in baseline populations of *Venturia inaequalis*. Pestic. Sci. 55:1083-1088.

15. Olaya, G., Pearsaul, D., Zhang, G., and Bradley, C. 2011. Evaluation of *Cercospora sojina* isolates sensitive and resistant to azoxystrobin using a mycelial growth inhibition assay. Phytopathology 101:S132.

16. Olaya, G., Zheng, D., and Köller, W. 1998. Differential responses of germinating *Venturia inaequalis* conidia to kresoxim-methyl. Pestic. Sci. 4:230-236.

17. Rebollar-Alviter, A., Madden, L. V., Jeffers, S. N., and Ellis, M. A. 2007. Baseline and differential sensitivity to two QoI fungicides among isolates of *Phytophthora cactorum* that cause leather rot and crown rot on strawberry. Plant Dis. 91:1625-1637.

18. Reuveni, M., and Sheglov, D. 2002. Effects of azoxystrobin, difenoconazole, polyoxin b (polar) and trifloxystrobin on germination and growth of *Alternaria alternata* and decay in red delicious apple fruit. Crop Prot. 21:951-955.

19. Seyran, M., Brenneman, T. B., and Stevenson, K. L. 2010. In vitro toxicity of alternative oxidase inhibitors salicylhydroxamic acid and propyl gallate on *Fusicladium effusum*. J Pestic. Sci. 83:421-427.

20. Stevenson, K. L., Seebold, K. W., and Langston, D. B., Jr. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. Plant Health Progress doi:10.1094/PHP-2004-1207-01-RS.

21. Thomas, A., Langston, D. B., Jr., Sanders, H. F., and Stevenson, K. L. 2012. Relationship between fungicide sensitivity and control of gummy stem blight of watermelon under field conditions. Plant Dis. 96:1780-1784.

22. Vincelli, P., and Dixon, E. 2002. Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Dis. 86:235-240.

23. Wise, K. A., Bradley, C. A., Pasche, J. S., Gudmestad, N. C., Dugan, F. M., and Chen, W. 2008. Baseline sensitivity of *Ascochyta rabiei* to azoxystrobin, pyraclostrobin, and boscalid. Plant Dis. 92:295-300.

24. Wong, F. P., Midland, S. L., and de la Cerda, K. A. 2007. Occurrence and distribution of QoI-resistant isolates of *Colletotrichum cereale* from annual bluegrass in California. Plant Dis. 91:1536-1546.

25. Ypema, H. L., and Gold, R. E. 1999. Kresoxim-methyl: Modification of a naturally occurring compound to produce a new fungicide. Plant Dis. 83:4-19.

26. Zhang, C. Q., Liu, Y. H., Ding, L., and Zhu, G. N. 2011. Shift of sensitivity of *Botrytis cinerea* to azoxystrobin in greenhouse vegetables before and after exposure to the fungicide. Phytoparasitica 39:293-302.

27. Zhang, C. Q., Liu, Y. H., Wu, H. M., Xu, B. C., Sun, P. L., and Xu, Z. H. 2012. Baseline sensitivity of *Pestalotiopsis microspora*, which causes black spot disease on chinese hickory (*Carya cathayensis*), to pyraclostrobin. Crop Prot. 42:256-259.

28. Ziogas, B. N., Baldwin, B. C., and Young, J. E. 1997. Alternative respiration: A biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. Pestic. Sci. 50:28-34.

Table 2.1. Source of *Didymella bryoniae* isolates that were originally collected from watermelon fields in Georgia and used in this study[x]

Isolate	GA County	Year Collected	Azoxystrobin Sensitivity[y]
R606-3-2	Tattnall	2010	R
C1-1	Tift	2010	R
C1-10*	Tift	2010	R
C1-2*	Tift	2010	R
C1-3*	Tift	2010	R
C1-4*	Tift	2010	R
C1-5*	Tift	2010	R
C1-7	Tift	2010	R
C1-8	Tift	2010	R
C1-9	Tift	2010	R
C2-1	Tift	2010	R
C2-10	Tift	2010	R
C2-2	Tift	2010	R
C2-8	Tift	2010	S
R204-1-1*	Tattnall	2010	S
R501-3-1*	Tattnall	2010	S
R501-3-2*	Tattnall	2010	S
R501-3-4*	Tattnall	2010	S
R606-3-1*	Tattnall	2010	S
UR3-4	Tattnall	2010	S
UR3-5	Tattnall	2010	S
UR4-2	Tattnall	2010	S
3C-13	Tift	2009	S
B204-3-4	Tift	2010	S

[x] Isolates used to determine effects of SHAM on sensitivity of mycelial growth to azoxystrobin are indicated with an asterisk (*).

[y] Previously determined using in vitro fungicide sensitivity assay by Thomas et al. (21). Isolates exhibiting >50% relative germination (RG) on azoxystrobin-amended medium (10 µg/ml) were considered resistant (R). Isolates exhibiting <50% RG were considered sensitive (S).

Table 2.2. Comparison of effective concentration of azoxystrobin that reduces growth or germination by 50% (EC_{50}) for azoxystrobin-sensitive isolates of *Didymella bryoniae*

	EC_{50} value (µg/ml)			
	Trial 1		Trial 2	
Isolate	Mycelial growth[x]	Conidial germination[y]	Mycelial growth	Conidial germination
3C-13	0.40	0.29	0.60	0.37
B204-3-4	0.25	0.54	0.55	0.33
C2-8	0.53	0.48	0.75	0.35
R204-1-1	0.47	0.93	0.67	0.96
R501-3-1	1.25	0.59	2.43	0.77
R501-3-2	1.71	0.68	1.68	0.60
R501-3-4	0.45	0.77	1.18	0.77
R606-3-1	0.30	0.89	0.34	0.53
UR3-4	3.83	0.35	1.15	0.49
UR3-5	0.92	0.63	2.60	0.47
UR4-2	3.40	0.47	0.45	0.59

[x] Isolates were incubated in the dark at 24°C for 7 days on one-quarter-strength potato dextrose agar amended with azoxystrobin.

[y] Isolates were incubated in the dark at 24°C for 24 h on water agar amended with azoxystrobin.

Table 2.3. Comparison of relative mycelial growth and conidial germination of azoxystrobin-sensitive and -resistant isolates of *Didymella bryoniae* on azoxystrobin-amended medium[x]

Azoxystrobin concentration (µg/ml)	Trial 1				Trial 2			
	Sensitive		Resistant		Sensitive		Resistant	
	Relative growth[y]	Relative germ.[z]	Relative growth	Relative germ.	Relative growth	Relative germ.	Relative growth	Relative germ.
0.01	0.66 a	0.97 b	1.00 a	0.99 a	0.70 a	0.99 b	1.00 a	0.99 a
0.03	0.58 a	0.99 b	1.00 a	0.99 a	0.60 a	1.00 b	0.99 a	0.97 a
0.1	0.54 a	0.99 b	0.99 a	0.99 a	0.57 a	0.97 b	0.99 a	0.99 a
0.3	0.51 a	0.87 b	0.97 a	0.99 b	0.55 a	0.83 b	0.99 a	0.98 a
1.0	0.48 a	0.19 b	0.94 a	0.99 b	0.52 a	0.17 b	0.98 a	0.97 a
3.0	0.47 a	0.04 b	0.87 a	0.98 b	0.50 a	0.04 b	0.92 a	0.97 b
10	0.44 a	0.00 b	0.68 a	0.97 b	0.42 a	0.00 b	0.70 a	0.98 b

[x] Relative mycelial growth or conidial germination on fungicide-amended medium expressed as a proportion of the non-amended control. For sensitive isolates, values are the least squares means of 2 replications of 11 isolates. For resistant isolates, values are the least squares means of 2 replications of 13 isolates. Values within the same trial, sensitivity group and fungicide concentration followed by the same letter are not significantly different ($P>0.05$).

[y] Isolates were incubated in the dark at 24°C for 7 days on one-quarter-strength potato dextrose agar amended with azoxystrobin.

[z] Isolates were incubated in the dark at 24°C for 24 h on water agar amended with azoxystrobin.

Table 2.4. Comparison of relative mycelial growth of azoxystrobin-sensitive and -resistant isolates of *Didymella bryoniae* on azoxystrobin-amended medium with and without SHAM[w]

Azoxystrobin concentration (µg/ml)	Relative growth[x]			
	Sensitive		Resistant	
	+ SHAM[y]	- SHAM[z]	+ SHAM[y]	- SHAM[z]
0	0.95 a	0.98 b	1.04 a	1.02 a
0.01	0.63 a	0.72 b	1.05 a	1.02 b
0.03	0.55 a	0.67 b	1.02 a	0.99 b
0.1	0.51 a	0.68 b	1.03 a	1.00 b
0.3	0.49 a	0.67 b	1.02 a	1.00 a
1	0.49 a	0.70 b	0.97 a	1.01 b
3	0.46 a	0.69 b	0.87 a	0.89 a
10	0.40 a	0.69 b	0.71 a	0.72 a

[w] Isolates used listed in Table 2.1.

[x] Isolates were incubated in the dark at 24°C for 6 days on one-quarter-strength potato dextrose agar amended with azoxystrobin. Relative mycelial growth on fungicide-amended medium expressed as a proportion of the non-amended control. Values within the same sensitivity group and fungicide concentration followed by the same letter are not significantly different (P>0.05).

[y] Values are the least squares means of 2 replications of 5 isolates and 5 trials.

[z] Values are the least squares means of 2 replications of 5 isolates and 2 trials.

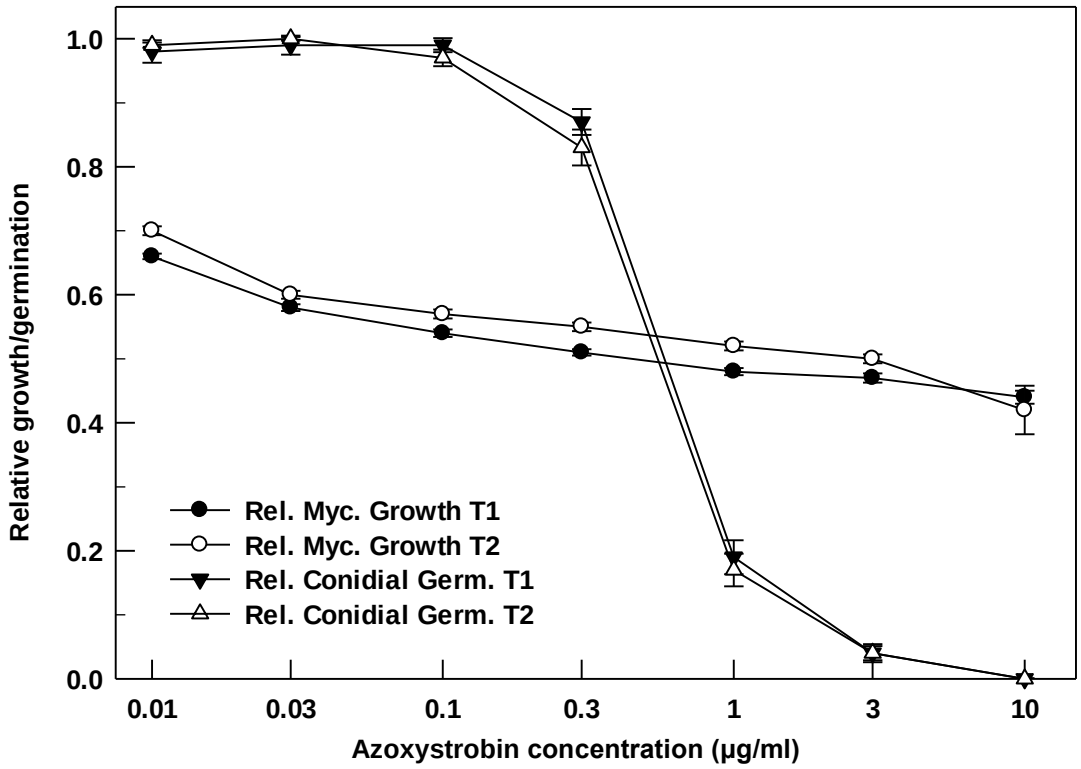

Fig. 2.1. Dose response of mycelial growth and conidial germination of azoxystrobin (AZO)-sensitive isolates of *Didymella bryoniae* to AZO. Sensitivity to AZO was previously determined by Thomas et al. (21). Isolates exhibiting <50% relative germination on AZO-amended medium (10 µg/ml) were considered sensitive. Data points represent the least squares means of 2 replications of 11 isolates. Error bars represent the standard error of the mean.

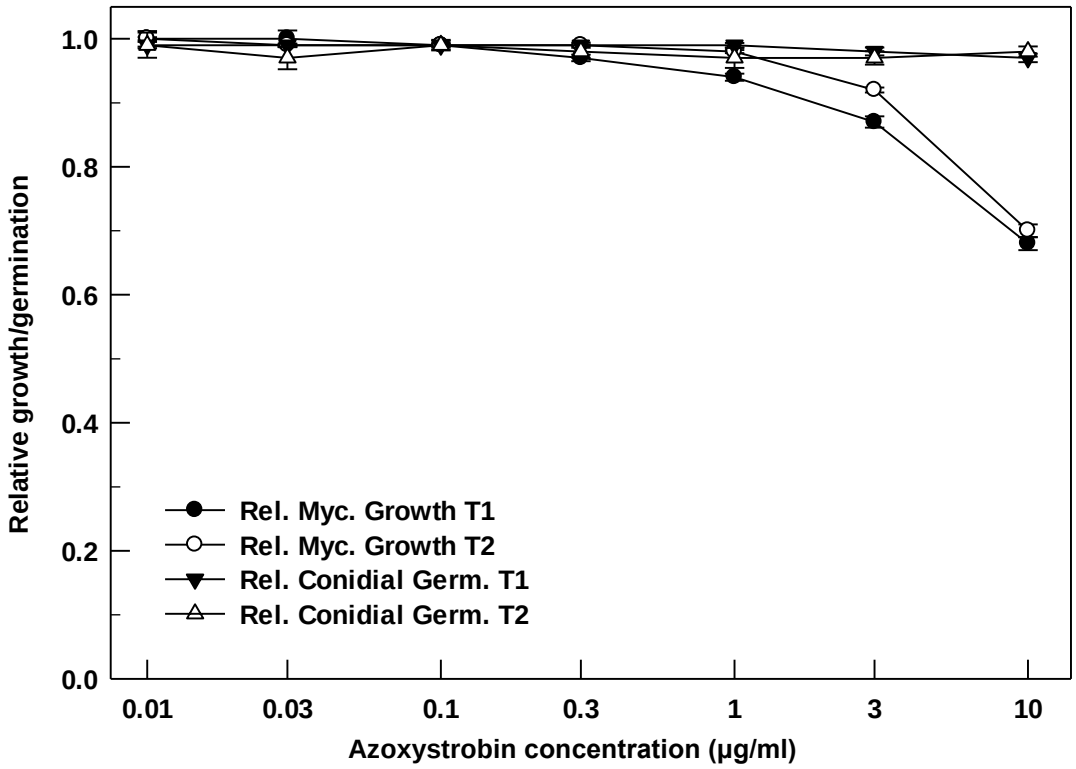

Fig. 2.2. Dose response of mycelial growth and conidial germination of azoxystrobin (AZO)-resistant isolates of *Didymella bryoniae* to AZO. Sensitivity to AZO was previously determined by Thomas et al. (21). Isolates exhibiting >50% relative germination on AZO-amended medium (10 µg/ml) were considered resistant. Data points represent the least squares means of 2 replications of 13 isolates. Error bars represent the standard error of the mean.

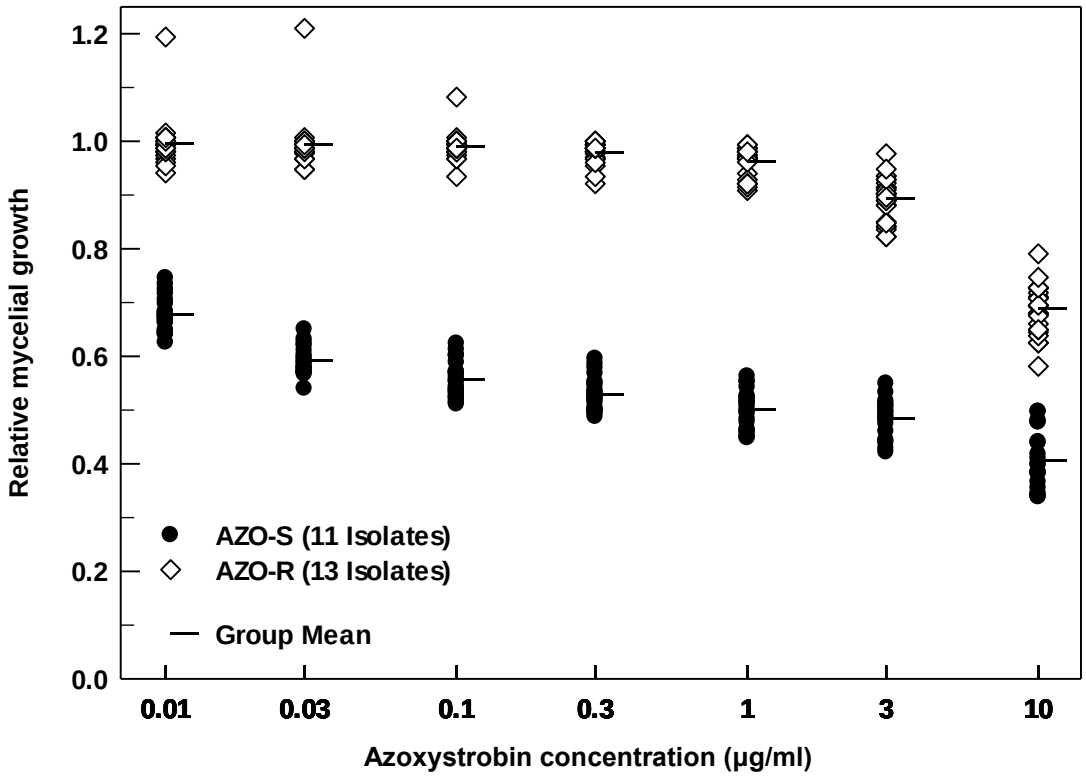

Fig. 2.3. Sensitivity of mycelial growth of azoxystrobin-sensitive (AZO-S) and -resistant (AZO-R) isolates of *Didymella bryoniae* to AZO. Sensitivity to AZO previously determined by Thomas et al. (21). Isolates exhibiting >50% relative germination (RG) on AZO-amended medium (10 µg/ml) were considered AZO-R. Isolates exhibiting <50% RG were considered AZO-S. Data points for the AZO-S treatment are the means of 2 replications of 11 isolates for 2 trials. Data points for the AZO-R treatment are the means of 2 replications of 13 isolates for 2 trials.

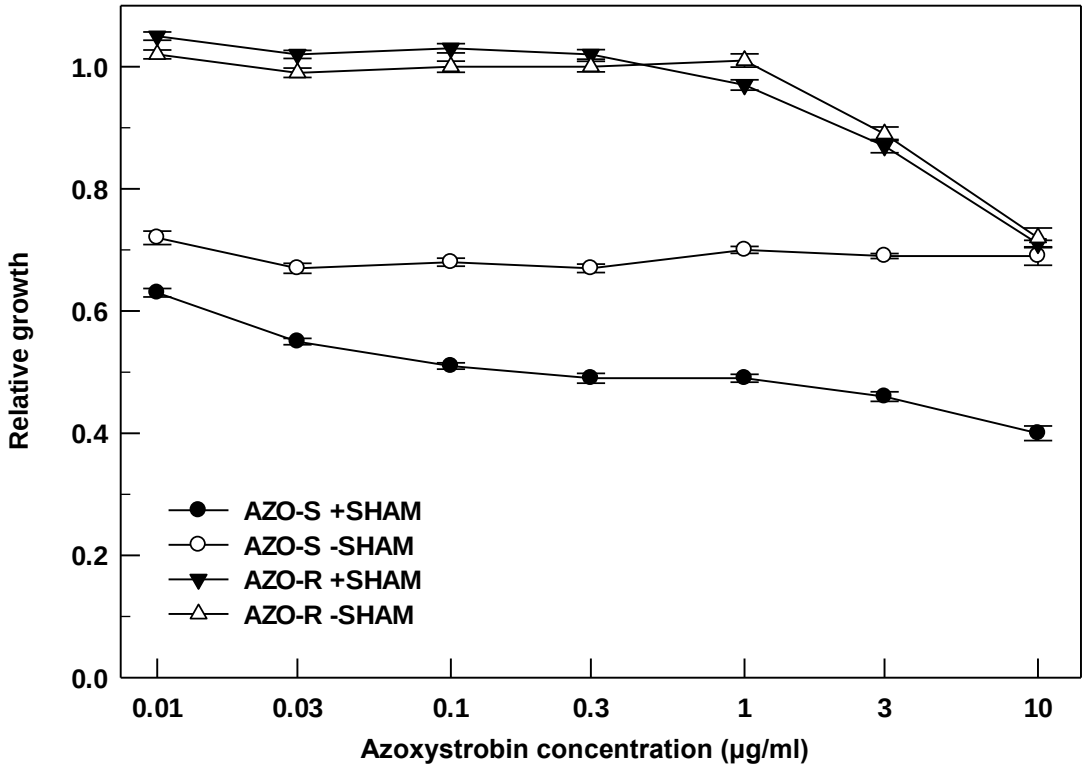

Fig. 2.4. Effect of salicylhydroxamic acid (SHAM) (100ppm) on sensitivity of mycelial growth of azoxystrobin-sensitive (AZO-S) and -resistant (AZO-R) isolates of *Didymella bryoniae* to AZO. Sensitivity to AZO was previously determined by Thomas et al. (21). Isolates exhibiting >50% relative germination (RG) on AZO-amended medium (10 µg/ml) were considered AZO-R. Isolates with RG <50% were considered AZO-S. Data points for the +SHAM treatments are the least squares means of 2 replications of 5 isolates and 5 trials. Data points for the -SHAM treatments are the least squares means of 2 replications of 5 isolates and 2 trials. Error bars represent the standard error of the

CHAPTER 3

STABILITY OF AZOXYSTROBIN RESISTANCE AND RELATIVE FITNESS OF FUNGICIDE-SENSITIVE AND –RESISTANT ISOLATES OF *DIDYMELLA BRYONIAE*

Finger, M. J., Ji, P., and Stevenson, K. L. 2013. To be submitted to Plant Disease.

ABSTRACT

Stability of azoxystrobin (AZO) resistance and relative fitness of fungicide-sensitive (S) and –resistant (R) isolates of *Didymella bryoniae* were investigated using isolates with different sensitivities to AZO, boscalid (BOS), penthiopyrad (PEN), and thiophanate-methyl (TPM). To determine AZO resistance stability, isolates were allowed to undergo four consecutive reproductive cycles on one-quarter-strength potato dextrose agar. Conidial germination on AZO-amended (10 µg/ml) and non-amended media was measured after each cycle. AZO-S isolates remained sensitive and AZO-R isolates remained highly resistant after up to four cycles. Fitness components measured included mycelial growth at 14, 20, 24 and 28°C, spore production, spore germination, and virulence. No significant differences in mycelial growth were observed between AZO-S and AZO-R or BOS/PEN-S and BOS/PEN-R isolates at any temperature. TPM-R isolates grew significantly less than TPM-S isolates at some temperatures. No significant differences in conidial germination were observed among isolates. Virulence was determined by measuring lesion length on inoculated petioles of watermelon seedlings. There were no significant differences in lesion length among isolates. These results suggest that AZO resistance is stable and fitness of AZO-R and AZO-S isolates and BOS/PEN-R and BOS/PEN-S isolates is similar while TPM-R isolates are less fit than TPM-S isolates in terms of mycelial growth.

INTRODUCTION

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rhem, is considered the most destructive disease of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai) in Georgia as well as in other southeastern states of the U.S. (16,33). The disease is managed through a combination of cultural practices and chemical controls. Although cultural practices play an important role in GSB management, they are ineffective when used alone.

Therefore, fungicides with protective and systemic properties are essential for the successful management of GSB. Typically, when environmental conditions are conducive for disease development and disease pressure is high, six to seven fungicide sprays are applied in a growing season because the risk of economic loss is high (16,30).

The systemic fungicides, benomyl and thiophanate-methyl, were used to control GSB and other foliar diseases on watermelon until resistance was observed in the southeastern U.S. in the 1990s (18). In response, a Section 18 emergency exemption was granted so that azoxystrobin could be used to control GSB on watermelon in 1997 and 1998 (17,33). Azoxystrobin, which was initially highly effective in GSB control, was officially labeled for commercial use on cucurbits in 1999; however, reduced efficacy was observed in research plots and commercial fields that same year (17,21,33). The boscalid component in the fungicide Pristine (BASF Corporation, Research Triangle Park, NC), which is a mixture of boscalid and pyraclostrobin, was effective in controlling GSB after widespread resistance to azoxystrobin was observed. However, resistance to boscalid in *D. bryoniae* was observed in 2007 (31,32). The excessive and extended use of thiophanate-methyl, azoxystrobin, and boscalid has resulted in the selection of fungicide-resistant members of the pathogen population (2).

In other fungi, resistance to benzimidazole fungicides such as benomyl and thiophanate-methyl has been attributed to mutations in the beta-tubulin gene (23). Although these mutations have not been confirmed in *D. bryoniae*, they may play some role in the resistance of the fungus to benomyl and thiophanate-methyl. Resistance to the quinone-outside inhibiting fungicides, such as azoxystrobin, has been attributed to a substitution of alanine for glycine at the amino acid position 143 (G143A) in several fungi (12). This mutation is responsible for resistance to

azoxystrobin in *D. bryoniae* (10). Finally, a substitution of tyrosine for histidine at amino acid position 277 confers resistance to both boscalid and penthiopyrad in *D. bryoniae* (2,3,32).

To better manage fungicide resistance, studies have been conducted to determine the fitness of sensitive and resistant members of the pathogen population. Fitness may be defined as the survival and reproductive success of an allele, individual, or group (28). In the presence of fungicides, resistant isolates have a higher fitness relative to the sensitive isolates and, in turn, their frequency is increased (27). In the absence of fungicides, however, fitness of sensitive and resistant isolates is less predictable.

Differences in fitness may be the result of fitness costs resulting from the mutation(s) that confer(s) fungicide resistance. A fitness cost would make a pathogen less fit to compete with wild-types of the pathogen in the absence of the selection pressure imposed by the fungicide. This is important in fungicide resistance management because fitness costs may significantly increase the effectiveness of fungicide rotation strategies by selecting against resistant members of the pathogen population when use of the fungicide is discontinued (27). It has been reported that fitness is affected in some fungicide-resistant pathogens (5,6,14,22,26,29). However, a generalization cannot be made that fitness costs accompany resistance evolution because a consistent pattern of fitness costs has not been associated with the emergence of fungicide resistance regardless of fungicide mode of action or the mechanism of resistance (27). It has also been reported that there is no effect on fitness when pathogens develop fungicide resistance (1,4,9,15). These findings suggest that fitness costs associated with fungicide resistance are specific to fungal species, fitness component, fungicide mode of action, and the mechanism of resistance (9).

The fitness of fungicide-resistant plant pathogens has not been studied in *D. bryoniae*. Determining whether differences in fitness exist between fungicide-resistant and -sensitive isolates can provide important information that could improve fungicide resistance management. The objectives of this study were to evaluate the stability of azoxystrobin resistance in *D. bryoniae*, to compare the relative fitness of isolates of *D. bryoniae* that are resistant and sensitive to thiophanate-methyl, azoxystrobin, boscalid, and penthiopyrad, and to determine if the number of fungicides an isolate is resistant to affects mycelial growth at different temperatures.

MATERIALS AND METHODS

Fungal isolates. Eight single-lesion isolates of *Didymella bryoniae*, previously characterized by Thomas et al. (34) for sensitivity to the fungicides azoxystrobin (AZO), boscalid (BOS), penthiopyrad (PEN), and thiophanate-methyl (TPM), were used to determine the stability of azoxystrobin resistance and relative fitness of *D. bryoniae* isolates. Isolates resistant to BOS were cross-resistant to PEN. An additional ten isolates were used to measure mycelial growth on potato dextrose agar (PDA) at various temperatures. The isolates were originally collected in 2009 and 2010 by Thomas et al. (34) from field-grown watermelon plants showing symptoms of GSB in different counties in Georgia (Table 3.1). All isolates were stored on filter paper at -20°C until use.

Stability of azoxystrobin resistance. Technical grade AZO (98.4% a.i.; Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to obtain an AZO concentration of 10.0 mg/ml and added to autoclaved water agar that was cooled to 55°C to obtain final concentrations of 0 and 10.0 µg/ml. The final concentration of acetone in fungicide-amended and non-amended media was 0.1% by volume. AZO-amended and non-amended media were also amended with

100 µg/ml of salicylhydroxamic acid (SHAM) to inhibit an alternative respiratory pathway in the fungus that can interfere with the activity of the fungicide. Four AZO-sensitive (AZO-S) and 4 AZO-resistant (AZO-R) isolates of *D. bryoniae* were used to measure the stability of azoxystrobin resistance. The isolates were stored on filter paper at -20°C. The isolates were recovered by placing a small (~25 mm^2) piece of infested filter paper on PDA and incubating for 7 days at 24°C. A 6-mm mycelial plug of each isolate was removed from the margins of the actively growing region of the culture and transferred to the center of one-quarter-strength PDA (QPDA) plates. The plates were incubated at 24°C for 2 weeks with a 12-h photoperiod provided by cool white fluorescent light (Philips Electronics, Somerset, NJ) and wide-spectrum plant and aquarium fluorescent light (F40PL/AQ-ECO, General Electric, Fairfield, CT) to encourage conidial production. The QPDA plates were flooded with 2 ml of a sterile solution of water and Tween 20 (1 drop of Tween/100 ml water) and gently scraped using a glass rod to dislodge conidia. The concentration of each conidial suspension was adjusted to approximately 10^6 conidia/ml using a hemocytometer. Fifteen microliters of each spore suspension was then transferred to the center of fresh QPDA plates and incubated under conditions described above to encourage conidial production. The process of harvesting conidia, adjusting the conidial suspension concentration, transferring the conidial suspension to fresh QPDA plates, and incubating the QPDA plates was repeated after each reproductive cycle. Each isolate was initially assayed for in vitro sensitivity to AZO and again after the first, second, third, and fourth consecutive reproductive cycle. To measure AZO sensitivity, 15 µl of conidial suspension of each isolate, collected as previously described, was transferred to AZO-amended and non-amended media. Five replications per isolate and fungicide concentration were prepared. After incubation at 24°C for 24 h, the number of germinated conidia was determined by

microscopically examining fifty arbitrarily selected conidia per plate. A conidium was considered germinated if the length of the germ tube was equal to or greater than half the length of the conidium. Relative conidial germination was calculated as the ratio of the percentage of germinated conidia on azoxystrobin-amended medium to the percentage of germinated conidia on non-amended medium. The experiment was conducted twice.

Fitness components. The following fitness components were determined for azoxystrobin-resistant and -sensitive isolates of *D. bryoniae*: (i) mycelial growth on PDA at various temperatures, (ii) spore production in vitro, (iii) spore germination rate, and (iv) virulence.

Mycelial growth. Eighteen single-lesion isolates of *D. bryoniae* were used to measure mycelial growth at different temperatures. The isolates stored on filter paper at -20°C were recovered by placing a small (~25 mm^2) piece of infested filter paper on PDA and incubating for 7 days at 24°C. A 6-mm mycelial plug of each isolate was removed from the margins of the actively growing region of the culture and transferred to the center of PDA plates. The plates were incubated in the dark at 14, 20, 24 and 28°C for 4 days. Colony diameter was measured and corrected colony diameter was obtained by subtracting the diameter of the agar plug from the colony diameter measurement. Five replications were made for each isolate. The experiment was conducted twice.

Spore production. Eight single-lesion isolates of *D. bryoniae* were used to measure spore production in vitro. The isolates stored on filter paper at -20°C were recovered as described above. A 6-mm mycelial plug of each isolate was removed from the margins of the actively growing region of the culture and transferred to the center of QPDA plates, which were incubated under the conditions described above to encourage conidial production. The QPDA

plates were flooded to dislodge conidia as described above. Conidia were counted using a hemocytometer. Five replications were made for each isolate. The experiment was conducted three times.

Spore germination. Conidial suspensions of each isolate collected from the spore production in vitro experiment were used. Twenty-five microliters of each spore suspension was spread on 4% water agar plates. The plates were incubated at 24°C for 24 h. Fifty conidia per plate were examined for germination, which was determined as described above. The experiment was conducted twice.

Virulence. Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai) cv. Stargazer was seeded in 10-cm pots in 60% peat and 40% vermiculite potting mix in a greenhouse. Three-week-old seedlings were inoculated using a micro-centrifuge tube technique described by Gulya et al. (13) and Urs et al. (35). Virulence was determined using eight single-lesion isolates of *D. bryoniae*. Isolates stored at -20°C were recovered as described above. Inoculation tubes were prepared by soaking cotton in an aqueous solution of 10% glycerin in water, inserting the cotton into the 1.5-ml micro-centrifuge tube, and compressing the cotton up to the 0.5-ml mark of the micro-centrifuge tube. Tubes were autoclaved and stored at 4°C until inoculation. Prior to inoculation, an 8-mm mycelial plug, from a 7-day-old culture of each isolate was placed inside each sterile micro-centrifuge tube. Ten replications were made for each isolate. Control inoculation tubes were prepared in the same manner as described above but an 8-mm plug containing only PDA was inserted into the micro-centrifuge tube. The leaf petiole was cut 55 mm away from the base. The micro-centrifuge tube containing the mycelial plug or PDA plug

was inserted onto the cut end of the petiole so that the fungus was in contact with the host plant tissue. The length of necrotic area on the inoculated petiole was measured after 4 days of incubation in the greenhouse. The experiment was conducted twice.

Data analysis. Differences in stability of azoxystrobin resistance, mycelial growth at different temperatures, spore production, spore germination, and virulence were evaluated using PROC GLIMMIX in SAS 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Stability of azoxystrobin resistance. The four AZO-R isolates remained highly resistant and four AZO-S isolates remained sensitive to azoxystrobin after up to four consecutive reproductive cycles. Mean relative germination (RG) values for AZO-R isolates ranged from 0.98 to 1.00 after up to four consecutive reproductive cycles. Conidial germination was not observed in AZO-S isolates after up to four consecutive reproductive cycles (RG= 0).

Fitness components. No significant differences in mean radial mycelial growth were observed between the 7 AZO-S and 11 AZO-R isolates or the 10 BOS/PEN-sensitive (BOS/PEN-S) and the 8 BOS/PEN-resistant (BOS/PEN-R) isolates at any temperature (Table 3.2). However, the 10 TPM-resistant (TPM-R) isolates grew significantly less than the 8 TPM-sensitive (TPM-S) isolates at 24 and 28°C in trial 1 and at all temperatures in trial 2 (Table 3.3). Significant trial by temperature and temperature by TPM sensitivity phenotype interactions were observed, therefore, TPM results of trials 1 and 2 are reported separately. For AZO-S, AZO-R, BOS/PEN-S, and BOS/PEN-R isolates, radial growth increased with increasing temperature until reaching a maximum at 24°C (Fig. 3.1). Radial growth of TPM-S isolates increased with increasing temperature until reaching an optimum at 28°C in both experimental trials (Fig. 3.2).

However, the radial growth of TPM-R isolates increased until reaching a maximum at 24°C then declining with increasing temperature (Fig. 3.2). Although differences between trials were observed, there was no consistent effect of the number of fungicides to which an isolate was resistant to on mycelial growth at any temperature (Table 3.4).

Although variability in spore production, spore germination, and virulence among individual isolates was observed, no significant differences in spore production, spore germination, or virulence were observed between 4 AZO-S and 4 AZO-R isolates, 5 BOS/PEN-S and 3 BOS/PEN-R isolates, or 5 TPM-S and 3 TPM-R isolates (Table 3.5).

DISCUSSION

In this study, the stability of AZO resistance was evaluated and the relative fitness of fungicide-sensitive and –resistant isolates of *D. bryoniae* was compared. There are limited studies on stability of resistance to QoI fungicides in fungal plant pathogens and there are no previously published studies on the stability of AZO resistance in *D. bryoniae*. In other fungal species, stability of fungicide resistance was measured by performing consecutive mycelial subcultures at certain time intervals and evaluating fungicide sensitivity after a specific number of transfers using mycelial growth fungicide sensitivity assays (9,19,24). Spore germination assays are generally recommended for determining sensitivity to AZO and other QoI fungicides (11,25). Therefore, a method similar to that described by Avila-Adame et al. (4), which utilized conidial germination fungicide sensitivity assays to measure the stability of AZO resistance in *Magnaporthe grisea*, was used. The results of the present study indicated that after up to four consecutive reproductive cycles, AZO-R isolates remained highly resistant to AZO. This result is in agreement with reports for *M. grisea* and *Botrytis cinerea*, where resistance to QoI fungicides

was considered stable (4,19). In contrast, resistance to QoI fungicides, pyraclostrobin and AZO, decreased in laboratory mutants of *B. cinerea* and *Ustilago maydis,* respectively, after isolates were consecutively subcultured (24,36). The difference in findings in those studies compared to the current study may be attributed to differences of fungal species used and the tendency of laboratory mutants to differ biologically, particularly in terms of survivability, from field collected resistant isolates.

The G143A mutation, which has been identified in many fungi that are highly resistant to QoI fungicides, was previously implicated in AZO resistance in *D. bryoniae* (21) and was confirmed to be present or absent in the eight isolates used to measure fitness components in this study (Chapter 4). The G143A mutation was not confirmed in the additional 10 isolates used to measure mycelial growth at different temperatures. Furthermore, the mutations responsible for resistance to BOS and PEN in *D. bryoniae,* a substitution of tyrosine for histidine at the amino acid position 277 (2,3,32), and TPM, mutations in the beta-tubulin gene (23), were not investigated in the isolates. However, fungicide resistance phenotypes were confirmed in all isolates used in this study.

In this study, the fitness of AZO-S and -R isolates was determined to be similar in regard to the fitness components tested because no significant differences in mycelial growth at different temperatures, sporulation, germination, or virulence were observed. A similar result was reported in *M. grisea*, where no significant difference in colony size, conidia production, or virulence was observed between G143A mutants and wild-type, or QoI-sensitive, isolates (4). Also, *B. cinerea* isolates resistant to the QoI fungicide, pyraclostrobin, were not significantly different from pyraclostrobin-sensitive isolates in mycelial growth, spore production, conidial germination, or virulence (19). There was also no significant difference in spore production

between femoxadone-sensitive and –resistant isolates of *Plasmopara viticola* (8). Additionally, no significant differences in mycelial growth or conidial production were reported between kresoxim-methyl-sensitive and –resistant isolates of *Venturia inaequalis* (7). In contrast, while there were no differences in conidial germination between *M. oryzae* AZO-R mutants and AZO-S isolates, AZO-R mutants produced significantly more conidia than wild-type isolates (22). Although the results for *M. oryzae* suggest that G143A mutants are more fit than the wild-type isolates, only two isolates, one resistant G143A mutant and one sensitive wild-type, were used in the study and other studies on fitness have reported large variation among isolates even within the same sensitivity class (8,15). Our findings also differ from previous reports in which AZO-R mutants of *Alternaria alternata* were more virulent than AZO-S isolates (15). Difference of fungal species used and different methods used in inoculation and virulence measurement between studies may have contributed to this difference in results.

We also investigated the relative fitness of BOS/PEN-S and -R isolates. We observed no significant differences in mycelial growth at any temperature, spore production, spore germination, or virulence and fitness was therefore determined to be similar in BOS/PEN-S and –R isolates of *D. bryoniae*. Similar findings which indicated no significant differences in mycelial growth, spore production, spore germination, or virulence between BOS-S and –R isolates were reported in *A. alternata* and *B. cinerea* (1,19).

TPM-R isolates grew significantly slower than TPM-S isolates at certain temperatures. This finding suggests that a fitness cost may be associated with TPM resistance in *D. bryoniae*. This result is in agreement with a previous study in *B. cinerea* that indicated that benomyl-resistant isolates grew significantly slower than wild-type, benomyl-sensitive, isolates at 20°C (20). Interestingly, the authors of that study also found that *B. cinerea* isolates that were also

resistant to dicarboximide fungicides did not exhibit any differences in fitness as compared to wild-type isolates (20). An additional contrasting result was reported for *V. inaequalis* (7). In both studies in which contrasting results were reported, mycelial growth was measured at a single temperature, that was optimal for growth, and this may account for the difference in findings between those studies and the current study.

Finally, the results of this study indicated that there was no obvious association between the number of fungicides to which an isolate of *D. bryoniae* is resistant to and its rate of growth at certain temperatures. The results varied greatly between the two experimental trials, but no clear association was observed. Similarly, there were no significant differences in mycelial growth between *V. inaequalis* isolates resistant to multiple fungicides (kresoxim-methyl, TPM, dodine, and myclobutanil) and isolates resistant to only one fungicide (7).

To our knowledge, this is the first comparative study of the relative fitness of AZO-, BOS-, PEN-, and TPM-R and –S isolates of *D. bryoniae*. The lack of apparent fitness costs accompanying resistance to AZO, BOS, and PEN in *D. bryoniae* provides important information in regard to fungicide resistance management. Isolates resistant to these fungicides may be equally or more competitive than sensitive isolates. Therefore, the frequency of resistant isolates is unlikely to decrease even in the absence of fungicide use. These results support the current recommended fungicide resistance management practice of alternating between two chemically unrelated fungicides and/or tank mixing chemically unrelated fungicides to prevent resistance development (27). Additionally, the presence of an apparent fitness cost in TPM-R isolates of *D. bryoniae* suggests that TPM-R isolates may be at a competitive disadvantage as compared to TPM-S isolates. It can be hypothesized that the removal of benzimidazoles from gummy stem blight disease management programs where TPM resistance has been reported may reduce the

frequency of resistance to TPM over time. Further research is needed to confirm whether the presence or absence of fitness costs affects the competitive ability of isolates of *D. bryoniae* that are resistant to AZO, BOS, PEN, and TPM.

LITERATURE CITED

1. Avenot, H. F., and Michailides, T. J. 2007. Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. Plant Dis. 91:1345-1350.

2. Avenot, H. F., and Michailides, T. J. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Prot. 29:643-651.

3. Avenot, H. F., Thomas, A., Gitaitis, R. D., Langston, D. B., Jr., and Stevenson, K. L. 2012. Molecular characterization of boscalid- and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. Pest Manag. Sci. 68:645-651.

4. Avila-Adame, C., and Köller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Curr. Genet. 42:332-338.

5. Bardas, G. A., Karaoglanidis, G. S., and Myresiotis, C. K. 2008. Stability and fitness of anilinopyrimidine-resistant strains of *Botrytis cinerea*. Phytopathology 98:443-450.

6. Becher, R., Deising, H. B., Wirsel, S. G. R., Hettwer, U., and Karlovsky, P. 2010. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. Phytopathology 100:444-453.

7. Chapman, K. S., Sundin, G. W., and Beckerman, J. L. 2011. Identification of resistance to multiple fungicides in field populations of *Venturia inaequalis*. Plant Dis. 95:921-926.

8. Corio-Costet, M.-F., Dufour, M.-C., Cigna, J., Abadie, P., and Chen, W.-J. 2011. Diversity and fitness of *Plasmopara viticola* isolates resistant to QoI fungicides. Eur. J. Plant Pathol. 129:315-329.

9. Cox, K. D., Schnabel, G., and Bryson, P. K. 2007. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. Phytopathology 97:448-453.

10. FRAC (Fungicide Resistance Action Committee). 2011. List of pathogens with field resistance towards QoI fungicides. Available from www.frac.info.

11. FRAC (Fungicide Resistance Action Committee). 2012. List of monitoring methods. Available from www.frac.info.

12. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manag. Sci. 58:859-867.

13. Gulya, T. J., Rama Raje Urs, N. V., and Lukatch, J. R. 1996. A simple inoculation method for evaluating *Sclerotinia* head rot resistance. Proceedings of the 18th Sunflower Research Group Workshop Sponsered by National Sunflower Association:18-23.

14. Karaoglanidis, G. S., Ioannidis, P. M., and Thanassoulopoulos, C. C. 2001. Fitness of *Cercospora beticola* field isolates-resistant and -sensitive to demethylation inhibitor fungicides. Eur. J. Plant Pathol. 107:337-347.

15. Karaoglanidis, G. S., Luo, Y., and Michailides, T. J. 2011. Competitive ability and fitness of *Alternaria alternata* isolates resistant to QoI fungicides. Plant Dis. 95:178-182.

16. Keinath, A. P. 1995. Fungicide timing for optimum management of gummy stem blight epidemics on watermelon. Plant Dis. 79:354-358.

17. Keinath, A. P. 2009. Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. Pest Manag. Sci. 65:1090-1096.

18. Keinath, A. P., and Zitter, T. A. 1998. Resistance to benomyl and thiophanate-methyl in *Didymella bryoniae* from South Carolina and New York. Plant Dis. 82:479-484.

19. Kim, Y. K., and Xiao, C. L. 2011. Stability and fitness of pyraclostrobin- and boscalid-resistant phenotypes in field isolates of *Botrytis cinerea* from apple. Phytopathology 101:1385-1391.

20. Korolev, N., Mamiev, M., Zahavi, T., and Elad, Y. 2011. Screening of *Botrytis cinerea* isolates from vineyards in Israel for resistance to fungicides. Eur. J. Plant Pathol. 129:591-608.

21. Langston, D. B., Jr., Lewis, K. L., and Jennings, W. T. 2000. Evaluation of fungicides and spray programs for control of gummy stem blight on watermelon. Fungic. Nematicide Tests 55:291.

22. Ma, B., and Uddin, W. 2009. Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. Plant Dis. 93:1044-1049.

23. Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.

24. Markoglou, A. N., Malandrakis, A. A., Vitoratos, A. G., and Ziogas, B. N. 2006. Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides. Eur. J. Plant Pathol. 115:149-162.

25. Olaya, G., and Holm, A. 2001. Sensitivity of *Didymella bryoniae* isolates to azoxystrobin. Phytopathology 91:S67.

26. Pasche, J. S., and Gudmestad, N. C. 2007. Prevalence, competitive fitness and impact of the F129L mutation in *Alternaria solani* from the United States. Crop Prot. 27:427-435.

27. Peever, T. L., and Milgroom, M. G. 1995. Fungicide resistance---lessons for herbicide resistance management? Weed Technol. 9:840-849.

28. Pringle, A., and Taylor, J. W. 2002. The fitness of filamentous fungi. Trends Microbiol. 10:474-481.

29. Raposo, R., Melgarejo, P., Urrutia, T., and Gomez, V. 2000. Fitness of *Botrytis cinerea* associated with dicarboximide resistance. Phytopathology 90:1246-1249.

30. Seebold, K. W., and Langston, D. B. 2005. Evaluation of fungicide programs for control of gummy stem blight in watermelon. 2004. Fungic. Nematicide Tests 60:V128.

31. Seebold, K. W., and Langston, D. B., Jr. 2004. Evaluation of fungicides for control of gummy stem blight of watermelon. Fungic. Nematicide Tests 59:140.

32. Stevenson, K. L., Langston, D. B., and Sanders, F. 2008. Baseline sensitivity and evidence of resistance to boscalid in *Didymella bryoniae*. Phytopathology 98:S151.

33. Stevenson, K. L., Seebold, K. W., and Langston, D. B., Jr. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. Plant Health Progress doi: http://dx.doi.org/10.1094/PHP-2004-1207-01-RS

34. Thomas, A., Langston, D. B., Jr., Sanders, H. F., and Stevenson, K. L. 2012. Relationship between fungicide sensitivity and control of gummy stem blight of watermelon under field conditions. Plant Dis. 96:1780-1784.

35. Urs, R. R., Mayhew, D. E., and Roberts, P. D. 2004. Hypovirulence of *Didymella bryoniae* associated with dsRNA. J. Phytopathol. 152:55-59.

36. Ziogas, B. N., Markoglou, A. N., and Tzima, A. 2002. A non-mendelian inheritance of resistance to strobilurin fungicides in *Ustilago maydis*. Pest Manag. Sci. 58:908-916.

Table 3.1. Source of *Didymella bryoniae* isolates that were originally collected from watermelon fields in Georgia and used in this study[x]

	GA	Year	Fungicide Sensitivity[y]			
Isolate	County	Collected	BOS	PEN	TPM	AZO
UR3-5*	Tattnall	2010	S	S	S	S
R501-3-1*	Tattnall	2010	S	S	S	S
R204-1-1*	Tattnall	2010	R	R	S	S
C2-8*	Tift	2010	S	S	R	S
UR5-9*	Tattnall	2010	S	S	S	R
B105-3-3*	Tift	2010	S	S	R	R
C2-1*	Tift	2010	R	R	S	R
C5-1*	Tift	2010	R	R	R	R
R501-3-2	Tattnall	2010	S	S	S	S
R606-3-4	Tattnall	2010	S	S	R	S
R606-3-19	Tattnall	2010	S	S	R	S
16R2-3	Decatur	2009	S	S	R	R
B402-2-1	Tift	2010	S	S	R	R
B302-3-1	Tift	2010	R	R	S	R
B401-3-1	Tift	2010	R	R	S	R
C5-6	Tift	2010	R	R	R	R
R606-3-2	Tattnall	2010	R	R	R	R
C5-5	Tift	2010	R	R	R	R

[x] Eight isolates used to determine azoxystrobin (AZO) stability, spore production, spore germination, and virulence are indicated with an asterisk (*).

[y] Previously determined by Thomas et al. (34). Isolates exhibiting >50% relative germination (RG) on AZO-amended medium at a discriminatory concentration (DC) of 10 µg/ml were considered resistant (R). Isolates exhibiting <50% RG were considered sensitive (S). Isolates exhibiting >25% relative mycelial growth (RGr) on boscalid (BOS)- or penthiopyrad (PEN)-amended medium at a DC of 3 µg/ml and on thiophanate-methyl (TPM)-amended medium at a DC of 100 µg/ml were considered R. Isolates exhibiting <25% RGr were considered S. Isolates R to BOS were cross-R to PEN.

Table 3.2. Radial growth of *Didymella bryoniae* isolates sensitive or resistant to the fungicides azoxystrobin, boscalid, and penthiopyrad cultured on potato dextrose agar after four days incubation at different temperatures

Fungicide sensitivity[y]	# isolates	Radial growth (mm)[x]							
		14°C		20°C		24°C		28°C	
AZO-S	7	13.85	a	24.20	a	28.94	a	28.42	a
AZO-R	11	13.97	a	24.20	a	29.07	a	28.96	a
BOS/PEN-S	8	13.59	a	23.73	a	28.71	a	28.28	a
BOS/PEN-R	10	14.34	a	24.79	a	29.41	a	29.36	a

[x] Radial growth measurements are the least squares means of five replications of each isolate of 2 trials. Means within a column and a fungicide group followed by the same letter are not significantly different ($P>0.05$).

[y] AZO-S = azoxystrobin-sensitive, AZO-R = azoxystrobin-resistant, BOS/PEN-S = boscalid- and penthiopyrad-sensitive, BOS/BEN-R = boscalid- and penthiopyrad-resistant. Isolates resistant to BOS are cross-resistant to PEN.

Table 3.3. Radial growth of *Didymella bryoniae* isolates sensitive or resistant to the fungicide thiophanate-methyl cultured on potato dextrose agar after four days incubation at different temperatures

			Radial growth (mm)[x]							
Trial	Fungicide sensitivity[y]	# isolates	14°C		20°C		24°C		28°C	
1	TPM-S	8	14.59	a	25.56	a	31.16	a	32.34	a
	TPM-R	10	13.73	a	24.77	a	28.65	b	27.28	b
2	TPM-S	8	14.71	a	24.66	a	29.49	a	30.50	a
	TPM-R	10	12.96	b	22.16	b	27.30	b	25.92	b

[x] Radial growth measurements are the least squares means of five replications of each isolate. Means within a trial and a column followed by the same letter are not significantly different (P>0.05).

[y] TPM-S = thiophanate-methyl-sensitive, TPM-R = thiophanate-methyl-resistant.

Table 3.4. Radial growth of *Didymella bryoniae* isolates sensitive or resistant to one or more of the following fungicides: azoxystrobin, boscalid, penthiopyrad, and thiophanate-methyl

		Mean radial growth (mm)[x]							
# fungicides[y]	# isolates	14°C		20°C		24°C		28°C	
0	3	14.30	ab	24.83	a	30.37	a	31.38	a
1	4	13.05	b	23.26	b	27.85	b	27.16	b
2	4	14.13	ab	24.10	ab	28.55	b	26.99	b
3	3	14.92	a	24.95	a	30.77	a	32.45	a
4	4	13.58	ab	24.19	ab	28.34	b	27.25	b

[x] *D. bryoniae* isolates were cultured on potato dextrose agar and incubated at the described temperatures for 4 days. Means within a column followed by the same letter are not significantly different ($P>0.05$).

[y] Number of fungicides to which isolate is resistant.

Table 3.5. Relative fitness of *Didymella bryoniae* isolates sensitive or resistant to the fungicides azoxystrobin (AZO), boscalid (BOS), penthiopyrad (PEN), and thiophanate-methyl (TPM)[u]

		Fitness component[w]		
Fungicide sensitivity[v]	# isolates	Spore production[x]	Spore germination[y]	Virulence[z]
AZO-S	4	7.82 a	98.42 a	26.24 a
AZO-R	4	7.83 a	98.95 a	26.60 a
BOS/PEN-S	5	6.85 a	98.62 a	26.74 a
BOS/PEN-R	3	7.59 a	98.80 a	25.88 a
TPM-S	5	8.23 a	98.73 a	26.02 a
TPM-R	3	6.52 a	98.60 a	27.08 a

[u] Isolates used listed in Table 3.1.

[v] S = sensitive, R = resistant. Isolates resistant to BOS are cross-resistant to PEN.

[w] Means within a column and fungicide group followed by the same letter are not significantly different (P>0.05).

[x] Number spores × 10^3 per square millimeters of colony surface area. Values are the least squares means of 5 replications per isolate of 3 trials.

[y] Percent germination. Values are the least squares means of 5 replications per isolate of 2 trials.

[z] Lesion length in millimeters. Values are the least squares means of 10 replications per isolate of 2 trials.

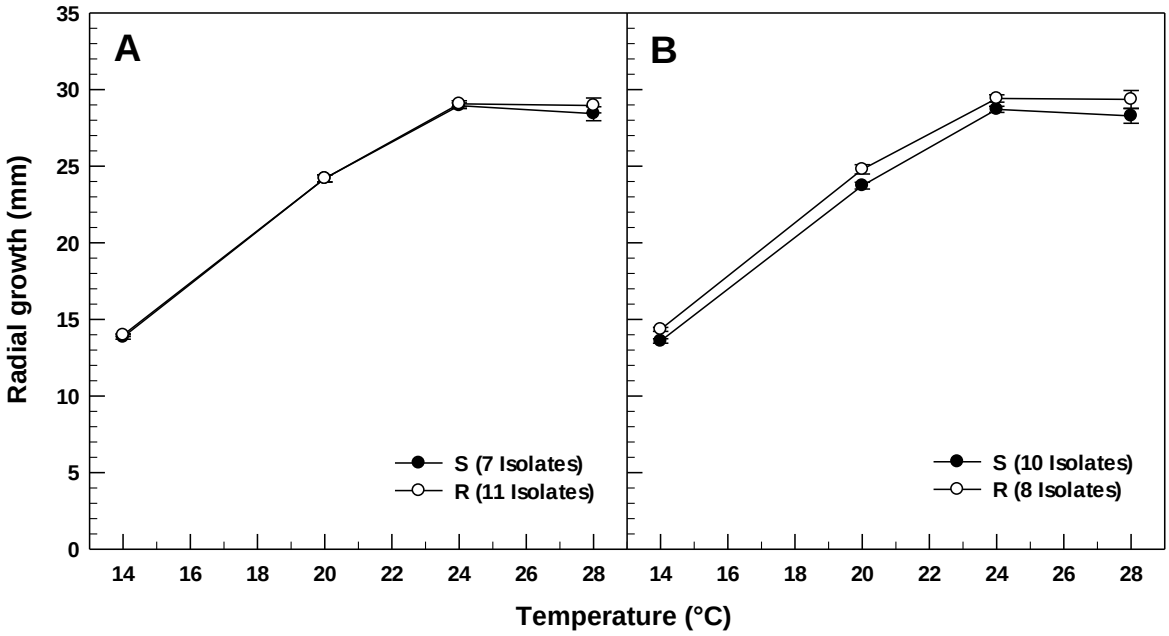

Fig. 3.1. Mycelial growth of *Didymella bryoniae* isolates resistant (R) or sensitive (S) to the fungicides **A**, azoxystrobin and **B**, boscalid and penthiopyrad on potato dextrose agar after 4 days of incubation at different temperatures. Data points represent the least squares means of 5 replications per isolate and 2 trials. Error bars represent the standard error of the mean.

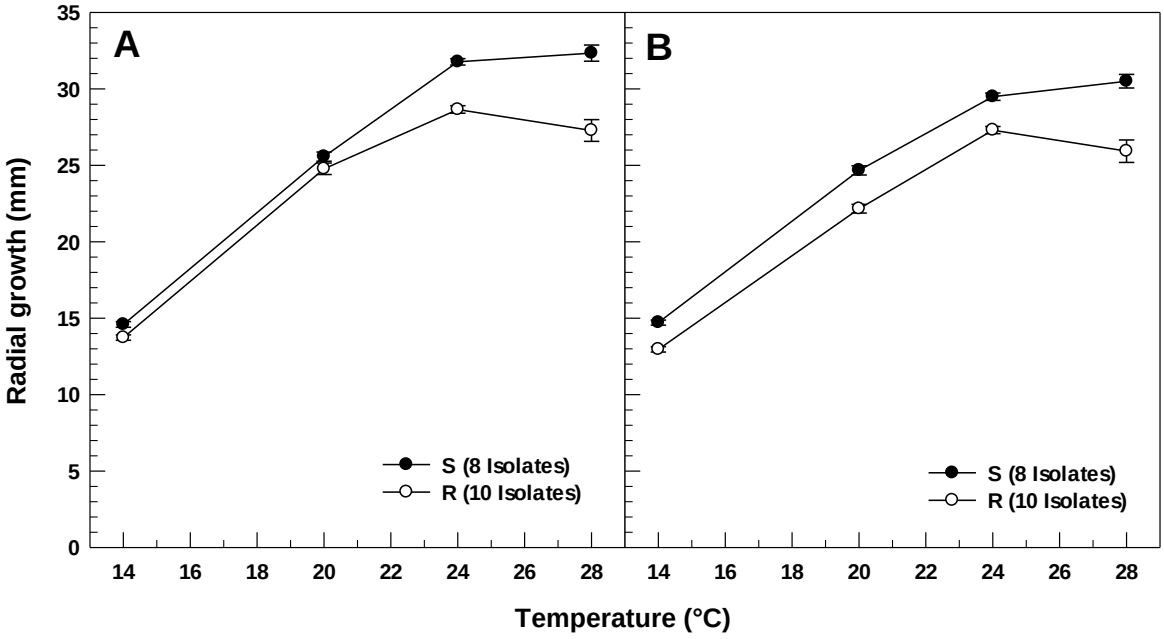

Fig. 3.2. Mycelial growth of *Didymella bryoniae* isolates resistant or sensitive to the fungicide thiophanate-methyl on potato dextrose agar after 4 days of incubation at different temperatures in **A,** trial 1 and **B,** trial 2. Data points represent the least squares means of five replications per isolate. Error bars represent the standard error of the mean.

CHAPTER 4

ALLELE-SPECIFIC PCR FOR THE DETECTION OF AZOXYSTROBIN RESISTANCE IN *DIDYMELLA BRYONIAE*

Finger, M. J., Parkunan, V., Ji, P., and Stevenson, K. L. 2013. To be submitted to Plant Disease.

ABSTRACT

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae*, is considered the most widespread and destructive disease of watermelon in the southeastern U.S. The quinone outside-inhibiting (QoI) fungicide, azoxystrobin (AZO), which inhibits mitochondrial respiration by binding to the outer, quinone oxidizing pocket of the cytochrome bc1 (*cyt b*) enzyme complex, was initially very effective in controlling GSB. However, resistance to azoxystrobin has been observed in *D. bryoniae* in many watermelon-producing regions. In this study, the genetic sequences of partial *cyt b* genes of four AZO-resistant (AZO-R) and four AZO-sensitive (AZO-S) isolates of *D. bryoniae* confirmed the amino acid substitution of glycine by alanine at the 143 codon (G143A) in the AZO-R isolates tested. Allele-specific primer pairs Dbcytb-F4/DbcytbR-R and Dbcytb-F4/DbcytbS-R were designed which amplified a 389-bp PCR product from complementary DNA of the AZO-R and AZO-S isolates, respectively. Additionally, primer pairs gCytBF1/DbcytbR-R and gCytBF1/DbcytbS-R were developed which amplified a 165-bp PCR product from genomic DNA of the AZO-R and AZO-S isolates, respectively. The primer pairs gCytBF1/DbcytbR-R and gCytBF1/DbcytbS-R did not amplify DNA from other pathogens tested in the study. The results indicated that the PCR assays developed in the study were specific in differentiating AZO-R and AZO-S isolates and could facilitate azoxystrobin resistance detection in *D. bryoniae*.

INTRODUCTION

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rhem, is considered the most widespread and destructive disease of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai) in Georgia and other southeastern U.S. states (8,21). GSB is

primarily controlled using a combination of cultural practices and chemical controls. Although cultural practices play an important role in GSB management, they are less efficacious when used alone. When disease pressure is high, fungicides with protective and systemic properties are essential for the successful management of GSB.

With the introduction and subsequent heavy reliance on systemic fungicides with site-specific modes of action, disease control failures related to fungicide resistance have been noted in several fungi (19). In particular, rapid evolution of fungicide-resistant isolates of *D. bryoniae* to the quinone outside inhibitor (QoI) fungicide azoxystrobin was reported within a year of registration of the fungicide for use in commercial watermelon production (11,21). Most recently, resistance frequencies of up to 100% have been reported in untreated control plots (22).

QoI fungicides, such as azoxystrobin, inhibit mitochondrial respiration by binding to the outer, quinone-oxidizing pocket of the cytochrome bc1 (*cyt b*) enzyme complex, which, in turn, blocks electron transport and results in energy loss in the fungus (5). The rapid development of resistance to QoI fungicides in fungal plant pathogens has been attributed to a substitution of alanine for glycine at amino acid position 143 (5). Substitution of leucine for phenylalanine at amino acid position 129 (F129L) and a substitution of arginine for glycine at amino acid position 137 (G137R) also confer resistance to QoI fungicides in some fungi (3). However, the F129L and G137R mutations have been associated with a lower level of fungicide resistance and do not seriously affect field performance of QoI fungicides (3). In *D. bryoniae*, the rapid development of QoI fungicide-resistant isolates has been attributed to the G143A mutation (4,12,21).

QoI resistance monitoring can be accomplished using traditional fungicide sensitivity assays or through molecular techniques. Currently, the detection of azoxystrobin resistance in *D. bryoniae* is accomplished using conidial germination assays on fungicide-amended medium.

While conidial germination assays are useful in resistance monitoring, these assays are time consuming and labor intensive. Molecular based detection of resistance would significantly decrease the time and labor required to monitor azoxystrobin resistance development. Therefore, the objectives of this study were to confirm the mutation in the *cyt b* of *D. bryoniae* isolates resistant to azoxystrobin and develop a rapid allele-specific PCR assay to detect azoxystrobin resistance.

MATERIALS AND METHODS

Fungal Isolates. Eight single-lesion isolates of *D. bryoniae* were used in this study. The isolates were previously collected in 2010 by Thomas et al. (22) from field-grown watermelon plants showing symptoms of GSB in different counties in Georgia (Table 4.1). Each isolate was previously characterized as azoxystrobin-resistant (AZO-R) or azoxystrobin-sensitive (AZO-S) using a conidial germination assay with a discriminatory azoxystrobin concentration of 10.0 µg/ml (22). Isolates exhibiting greater than 50% relative germination on fungicide-amended medium were considered AZO-R (22). All isolates were stored on filter paper at -20°C. Before use, the isolates were assayed again using methods described by Thomas et al. (22) to confirm sensitivity to azoxystrobin.

Total RNA extraction and cDNA synthesis. *Didymella bryoniae* isolates were grown in 50 ml potato dextrose broth at 24°C for 4 days with continuous shaking (100 rpm). The mycelia was harvested (100 to 200 mg fresh weight), frozen in liquid nitrogen, and then ground into a fine powder using mortar and pestle. Total RNA was extracted from each isolate using RNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All RNA samples were treated with DNase I, Amplification Grade (Life Technologies, Carlsbad, CA).

RNA was then converted to complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA) following manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to measure cDNA concentrations. The cDNA concentration of each isolate was adjusted to 25 ng/µl and stored at -20°C until further use.

Genomic DNA extraction. *Didymella bryoniae* isolates were grown in 50 ml potato dextrose broth at 24°C for 4 days with continuous shaking (100 rpm). Mycelia was harvested (about 100 to 200 mg fresh weight), frozen in liquid nitrogen, and then ground into a fine powder using mortar and pestle. Genomic DNA (gDNA) was extracted from each isolate using DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. DNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and adjusted to 50 ng/µl. DNA was stored at -20°C until further use.

Partial sequencing of the cDNA *cyt b* of *D. bryoniae*. Preliminary screening of *D. bryoniae* isolates was accomplished using previously published primer sets based on conserved regions of the *cyt b* containing the amino acid codon 143 in other closely related Ascomycetes (10,13,16,18). The primers KES115 and KES126 (Table 4.2), designed at BASF (Gerd Stammler, personal communication), successfully amplified a fragment of the *cyt b* in cDNA of *D. bryoniae* containing the amino acid codon 143. The PCR conditions were modified from those developed at BASF (Gerd Stammler, personal communication). All PCR amplifications were performed in a 25-µl volume containing 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP, 1.5 µl each of the 10 µM forward and reverse primers, 0.2 µl (1 unit) *Taq* polymerase, and 2 µl of template DNA (25 ng/µl). All PCR reactions were run in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions were as follows: an initial preheat for 3

min at 95°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The purified PCR products were sent for direct sequencing in both directions (Eurofins MWG Operon, Huntsville, AL). The sequences obtained from the four AZO-R and four AZO-S isolates were compared using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Partial sequencing of the gDNA *cyt b* of *D. bryoniae*. The previously published primer sets (10,13,16,18) described above were again used to screen the *cyt b* in *D. bryoniae* gDNA. The primers FG143 and RG143 (Table 4.2), designed previously by Patel et al. (18), amplified a fragment of the cyt *b* containing the amino acid 143 codon in all eight isolates of *D. bryoniae*. The PCR was performed in a 25-µl volume containing 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP, 1.5 µl each of the 10 µM forward and reverse primers, 0.2 µl (1 unit) *Taq* polymerase, and 2 µl of template DNA (50 ng/µl). All PCR reactions were run in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions were modified from those reported by Patel et al. (18) as follows: an initial preheat for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified PCR products were sent for direct sequencing in both directions (Eurofins MWG Operon, Huntsville, AL). The sequences obtained from the four AZO-R and four AZO-S isolates were compared using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Development of *D. bryoniae* allele-specific PCR primers for detection of azoxystrobin resistance using cDNA. The primer sets Dbcytb-F4/DbcytbS-R and Dbcytb-F4/DbcytbR-R (Table 4.2) were designed in this study and used to amplify a specific allele by matching the desired allele and mismatching the other allele at the 3′ end of the primer. The reverse primers DbcytbR-R and DbcytbS-R were designed so that they contained a C or G nucleotide, respectively, at the first position at the 3′ end.

All PCR amplifications were performed in a 25-µl volume containing 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP, 2 µl each of the 10 µM forward and reverse primers, 0.2 µl (1 unit) *Taq* polymerase, and 2 µl of template DNA (25 ng/µl). The PCR conditions were as follows: an initial preheat for 3 min at 95°C, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 53°C for 15 s, extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. PCR products were examined by electrophoresis in 1% agarose gel in Tris-borate-EDTA (TBE) buffer. The gel was amended with 1X GelRed nucleic acid gel stain (Biotium, Inc., Hayward, CA) and visualized directly in a Bio-Rad Chemi XRS Gel Documentation system (Bio-Rad, Hercules, CA).

Development of *D. bryoniae* allele-specific PCR primers for detection of azoxystrobin resistance using gDNA. The primer sets gCytBF1/DbcytbS-R and gCytBF1/DbcytbR-R (Table 4.2) were designed and used to amplify a specific allele by matching the desired allele and mismatching the other allele at the 3′ end of the primer. The reverse primers DbcytbR-R and DbcytbS-R were designed so that they contained a C or G nucleotide, respectively, at the first position at the 3′ end. A third primer set, DbComF/DbComR (Table 4.2), was designed to amplify both alleles.

To confirm species specificity of the primer sets gCytBF1/DbcytbS-R, gCytBF1/DbcytbR-R, and DbComF/DbComR, the eight *D. bryoniae* isolates and other fungal species isolated from watermelon including *Pythium ultimum*, *Phytophthora capsici*, *Fusarium oxysporum* f. sp. *niveum*, *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pseudoperonospora cubensis* were tested. Genomic DNA was extracted from all species as described above.

All PCR amplifications were performed in a 25-µl volume containing 2.5 µl 10X PCR buffer, 0.5 µl 10 mM dNTP, 2 µl each of the 10 µM forward and reverse primers, 0.2 µl (1 unit) *Taq* polymerase, and 2 µl of template DNA (50 ng/µl). The PCR conditions were as follows: an initial preheat for 3 min at 95°C, followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, extension at 72°C for 15 sec, and a final extension at 72°C for 3 min. PCR products were examined by electrophoresis in 1% agarose gel in TBE buffer. The gel was amended with 1X GelRed nucleic acid gel stain (Biotium, Inc., Hayward, CA) and visualized directly in a Bio-Rad Chemi XRS Gel Documentation system (Bio-Rad, Hercules, CA).

RESULTS

Analysis of partial *cyt b* sequence of *D. bryoniae* cDNA. The primers KES115 and KES126 amplified a single 530-bp DNA fragment in the four AZO-R and four AZO-S isolates of *D. bryoniae* tested. Sequence analysis revealed the presence of a single nucleotide substitution of cytosine for guanine (GGT to GCT) at the amino acid codon 143 in all four AZO-R isolates. This nucleotide substitution was not present in any of the AZO-S isolates tested (Fig. 4.1).

Analysis of partial *cyt b* sequence of *D. bryoniae* gDNA. The primer pair KES115/KES126, used previously to amplify cDNA *cyt b* of *D. bryoniae*, did not amplify *D. bryoniae* gDNA. The primers FG143 and RG143 amplified a single ~265-bp DNA fragment in

the four AZO-R isolates and the four AZO-S isolates of *D. bryoniae* tested. Sequence analysis revealed the presence of a single nucleotide substitution of cytosine for guanine (GGT to GCT) at the amino acid codon 143 in all AZO-R isolates (Fig. 4.1). This nucleotide substitution was not present in any of the AZO-S isolates tested (Fig. 4.1).

Comparison of partial *cyt b* cDNA and gDNA *D. bryoniae* sequences. When the partial *cyt b* sequences of all tested isolates of *D. bryoniae* cDNA and gDNA were aligned, only a small fragment including amino acid codons 132 through 146 aligned in both cDNA and gDNA (Fig. 4.1).

cDNA allele-specific PCR. The AZO-R allele-specific primer pair Dbcytb-F4/DbcytbR-R consistently amplified a 389-bp PCR product from four AZO-R isolates of *D. bryoniae* but not from any of the four AZO-S isolates tested (Fig. 4.2). The AZO-S allele-specific primer pair Dbcytb-F4/DbcytbS-R consistently amplified a 389-bp PCR product from four AZO-S isolates of *D. bryoniae* but not from any of the four AZO-R isolates tested (Fig. 4.3).

gDNA allele-specific PCR. The AZO-R allele-specific primer pair gCytBF1/DbcytbR-R consistently amplified a 165-bp PCR product from four AZO-R isolates of *D. bryoniae* but not from any AZO-S isolate (Fig. 4.4). The AZO-S allele-specific primer pair gCytBF1/DbcytbS-R consistently amplified a 165-bp PCR product from four AZO-S isolates of *D. bryoniae* but not from any of the four AZO-R isolates tested (Fig. 4.5). Both alleles were consistently amplified with primer sets DbComF and DbComR (Fig. 4.6). DNA of *P. ultimum*, *P. capsici*, *F. oxysporum* f. sp. *niveum*, *S. rolfsii*, *R. solani*, or *P. cubensis* was not amplified by primer pairs gCytBF1/DbcytbR-R (Fig. 4.4), gCytBF1/DbcytbS-R (Fig. 4.5), or DbComF/DbComR (Fig. 4.6).

DISCUSSION

In this study, a fragment of the *cyt b* gene containing the G143A site was amplified in both cDNA and gDNA of *D. bryoniae*. The highly conserved nature of the *cyt b* region containing the G143A site (6) allowed us to use previously published primer sets for closely related Ascomycetes to screen the *cyt b* gene of *D. bryoniae*. Prior to this study, there were no deposited *cyt b* sequences for *D. bryoniae* in the NCBI GenBank. In all AZO-R isolates tested, the G143A mutation was detected in both cDNA and gDNA. The G143A mutation was not detected in either cDNA or gDNA of AZO-S isolates. These results are in agreement with previous reports that the G143A mutation is present in and responsible for resistance to QoI fungicides in *D. bryoniae* (4,12). These findings also further validate the results obtained from the conidial germination assays used to characterize isolates as either AZO-R or AZO-S, as the G143A mutation was present in all AZO-R phenotypes and absent in all AZO-S phenotypes.

When the partial *cyt b* sequences containing the G143A site of cDNA and gDNA were compared in this study, only a small region encompassing amino acid codons 132-146 aligned in both cDNA and gDNA of *D. bryoniae*. This finding suggests that introns may be present before the amino acid codon 132 and after the amino acid codon 146 in gDNA of *D. bryoniae*. The presence of introns in the *cyt b* has been documented in some plant pathogenic fungi (6,16,20,23). More specifically, an intron was observed in *Pestalotiopsis longiseta* between amino acid codons 131 and 132 (23). The presence of introns on either side of the *cyt b* region containing the G143A site may explain why the primers KES115 and KES126, which amplified cDNA, did not amplify gDNA of *D. bryoniae*.

Several qualitative and quantitative molecular techniques have been used to identify resistance to QoI fungicides in a number of plant pathogenic fungi. Of those techniques, allele-

specific PCR has been used for both qualitative and quantitative detection of QoI resistance in *Blumeria graminis* f. sp. *tritici*, *Alternaria* spp., *Venturia inaequalis*, and *Botrytis cinerea* (1,2,7,14,15). In the present study, allele-specific PCR was used to detect azoxystrobin resistance in both cDNA and gDNA of *D. bryoniae*. In the case of cDNA, two primer sets were designed that amplified AZO-R and AZO-S alleles. In addition to two primers sets designed to amplify the AZO-R and AZO-S alleles in gDNA, a third primer set was designed that amplified both allele types as a control. All gDNA allele-specific primer sets developed in this study were determined to be specific to *D. bryoniae,* as they did not amplify any other fungal species tested.

To our knowledge, this is the first report in which molecular-based detection has been developed to identify resistance to azoxystrobin in *D. bryoniae*. Traditionally, conidial germination assays have been employed for azoxystrobin resistance detection in *D. bryoniae* (9,17,21,22). While conidial germination assays are useful in azoxystrobin resistance detection, these assays can be time-consuming and laborious. The allele-specific PCR assays significantly decrease time and labor required to detect azoxystrobin resistance in *D. bryoniae*. The results of this study also provide the basis for development of a quantitative molecular assay that will be useful in studying epidemiological aspects of GSB. However, further research is needed in order to confirm that the allele-specific PCR protocols developed in this study can be used to successfully detect azoxystrobin resistance in GSB-infected plant samples.

LITERATURE CITED

1. Fontaine, S., Remuson, F., Fraissinet-Tachet, L., Micoud, A., Marmeisse, R., and Melayah, D. 2009. Monitoring of *Venturia inaequalis* harbouring the QoI resistance G143A mutation in French orchards as revealed by PCR assays. Pest Manag. Sci. 65:74-81.

2. Fraaije, B. A., Butters, J. A., Coelho, J. M., Jones, D. R., and Hollomon, D. W. 2002. Following the dynamics of strobilurin resistance in *Blumeria graminis* f.sp.*tritici* using quantitative allele-specific real-time PCR measurements with the fluorescent dye SYBR green I. Plant Pathol. 51:45.

3. FRAC (Fungicide Resistance Action Committee). 2006. Mutations associated with QoI-resistance. Accessed on 06/25/13. www.frac.info.

4. FRAC (Fungicide Resistance Action Committee). 2012. List of pathogens with field resistance towards QoI fungicides. Accessed on 06/25/13. http://www.frac.info.

5. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manag. Sci. 58:859-867.

6. Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. Pest Manag. Sci. 62:465-472.

7. Jiang, J., Ding, L., Michailides, T. J., Li, H., and Ma, Z. 2009. Molecular characterization of field azoxystrobin-resistant isolates of *Botrytis cinerea*. Pestic. Biochem. Phys. 93:72-76.

8. Keinath, A. P. 1995. Fungicide timing for optimum management of gummy stem blight epidemics on watermelon. Plant Dis. 79:354-358.

9. Keinath, A. P. 2009. Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. Pest Manag. Sci. 65:1090-1096.

10. Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. Phytopathology 93:891-900.

11. Langston, D. B., Jr., Lewis, K. L., and Jennings, W. T. 2000. Evaluation of fungicides and spray programs for control of gummy stem blight on watermelon. Fungic. Nematicide Tests 55:291.

12. Langston, D. B., Jr. 2002. Quadris resistance in gummy stem confirmed. Georgia Extension Vegetable News 2:1-2.

13. Ma, Z., Felts, D., and Michailides, T. J. 2003. Resistance to azoxystrobin in *Alternaria* isolates from pistachio in California. Pestic. Biochem. Phys. 77:66-74.

14. Ma, Z., and Michailides, T. J. 2004. An allele-specific PCR assay for detecting azoxystrobin-resistant *Alternaria* isolates from pistachio in California. J. Phytopathol. 152:118-121.

15. Michalecka, M., Malinowski, T., Broniarek-Niemiec, A., and Bielenin, A. 2011. Real-time PCR assay with SNP-specific primers for the detection of a G143A mutation level in *Venturia inaequalis* field populations. J. Phytopathol. 159:569-578.

16. Miessner, S., and Stammler, G. 2010. *Monilinia laxa*, *M. fructigena* and *M. fructicola*: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome b gene sequences. J. Plant Dis. Protect. 4:162-167.

17. Olaya, G., and Holm, A. 2001. Sensitivity of *Didymella bryoniae* isolates to azoxystrobin. Phytopathology 91:S67.

18. Patel, J. S., Meinhardt, S. W., Sierotzki, H., Stammler, G., Gudmestad, N. C., and Adhikari, T. B. 2011. A two-step molecular detection method for *Pyrenophora tritici-repentis* isolates insensitive to QoI fungicides. Plant Dis. 95:1558-1564.

19. Peever, T. L., and Milgroom, M. G. 1995. Fungicide resistance---lessons for herbicide resistance management? Weed Technol. 9:840-849.

20. Samuel, S., Papayiannis, L. C., Leroch, M., Veloukas, T., Hahn, M., and Karaoglanidis, G. S. 2011. Evaluation of the incidence of the G143A mutation and cytb intron presence in the cytochrome bc-1 gene conferring QoI resistance in *Botrytis cinerea* populations from several hosts. Pest Manag. Sci. 67:1029-1036.

21. Stevenson, K. L., Seebold, K. W., and Langston, D. B., Jr. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. Plant Health Progress doi: 10.1094/PHP-2004-1207-01-RS.

22. Thomas, A., Langston, D. B., Jr., Sanders, H. F., and Stevenson, K. L. 2012. Relationship between fungicide sensitivity and control of gummy stem blight of watermelon under field conditions. Plant Dis. 96:1780-1784.

23. Yamada, K., and Sonoda, R. 2012. Characterization of moderate resistance to QoI fungicides in *Pestalotiopsis longiseta* and polymorphism in exon–intron structure of cytochrome b gene. J. Gen. Plant Pathol. 78:398-403.

Table 4.1. Source of *Didymella bryoniae* isolates that were originally collected from watermelon fields in Georgia and used in this study[x]

Isolate	GA County	Year Collected	AZO Sensitivity[y]
C2-8	Tift	2010	S
R204-1-1	Tattnall	2010	S
UR3-5	Tattnall	2010	S
R501-3-1	Tattnall	2010	S
C2-1	Tift	2010	R
B105-3-3	Tift	2010	R
UR5-9	Tattnall	2010	R
C5-1	Tattnall	2010	R

[x] Isolates collected by Thomas et al. (23)

[y] Previously determined using in vitro fungicide sensitivity assay by Thomas et al. (23). Isolates exhibiting >50% relative germination (RG) on azoxystrobin (AZO)-amended medium at a discriminatory concentration of 10.0 µg/ml were considered resistant (R). Isolates exhibiting <50% RG were considered sensitive (S).

Table 4.2. Oligonucleotide primers used in this study

Primer name	Sequence	Feature[x]	Reference
KES115	5′ GAG TTT GCA TTG GAT TAG CCA 3′	Partial *cyt b* covering codon 143	BASF
KES126	5′ GAG GTT TAT ATT ACG GAT CAT ATA GAG 3′	Partial *cyt b* covering codon 143	BASF
FG143	5′ GCA GCT TTA GCC CTT GGT AA 3′	Partial *cyt b* covering codon 143	Patel et al.
RG143	5′ CTG CGC TAT TTT TAA TAT AGG TTC CTG 3′	Partial *cyt b* covering codon 143	Patel et al.
Dbcytb-F4	5′ TCA CCT AAT ACG TTA GGC AT 3′	Allele specific forward in cDNA	This study
gCytBF3	5′ CGT ATA GTC TCT GAG GAA CC 3′	Allele specific forward in gDNA	This study
DbCytbS-R	5′ GCA GAT GTC ATT ATG AGG 3′	AZO-R allele specific	This study
DbcytbR-R	5′ GCA GAT GTC ATT ATG AGC 3′	AZO-S allele specific	This study
DbComF	5′ CAG ATG TCA TTA TGA GGT GCT ACA 3′	Both alleles	This study
DbComR	5′ CCG CTT CAC AAA GGC CTA TAA 3′	Both alleles	This study

[x] *cyt b* = cytochrome b gene, cDNA = complementary DNA, gDNA = genomic DNA, AZO-R = azoxystrobin-resistant, AZO-S = azoxystrobin-sensitive

Fig. 4.1. Partial alignment of the cytochrome b sequences from complementary DNA (cDNA) and genomic DNA (gDNA) of azoxystrobin-resistant (AZO-R) and –sensitive (AZO-S) isolates of *Didymella bryoniae*. The vertical box indicates the nucleotide change at amino acid position 143 that confers resistance to azoxystrobin. The shaded region indicates a similar region in cDNA and gDNA.

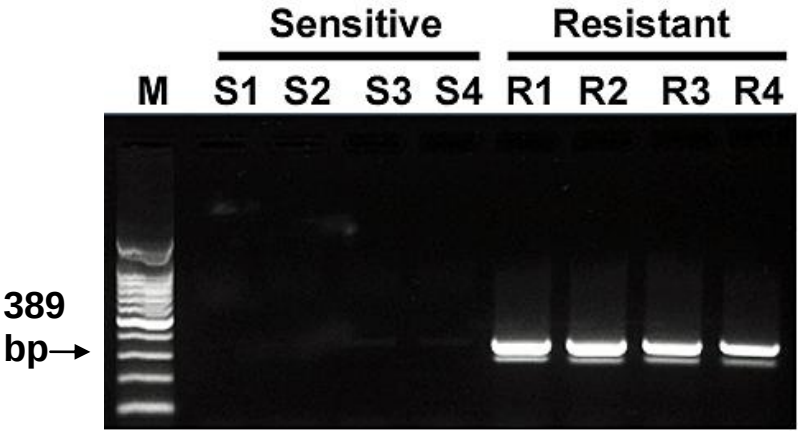

Fig. 4.2. Allele-specific PCR using complimentary DNA with primer pair Dbcytb-F4/DbcytbR-R for the detection of azoxystrobin-resistant (AZO-R) isolates of *Didymella bryoniae*. Azoxystrobin-sensitive isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. AZO-R isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. M = 100 bp DNA ladder.

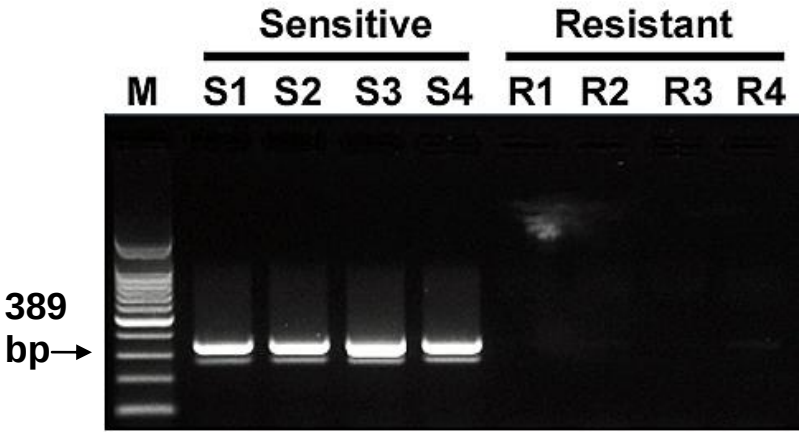

Fig. 4.3. Allele-specific PCR using complimentary DNA with primer pair Dbcytb-F4/DbcytbS-R for the detection of azoxystrobin-sensitive (AZO-S) isolates of *Didymella bryoniae*. Azoxystrobin-sensitive isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. AZO-R isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. M = 100 bp DNA ladder.

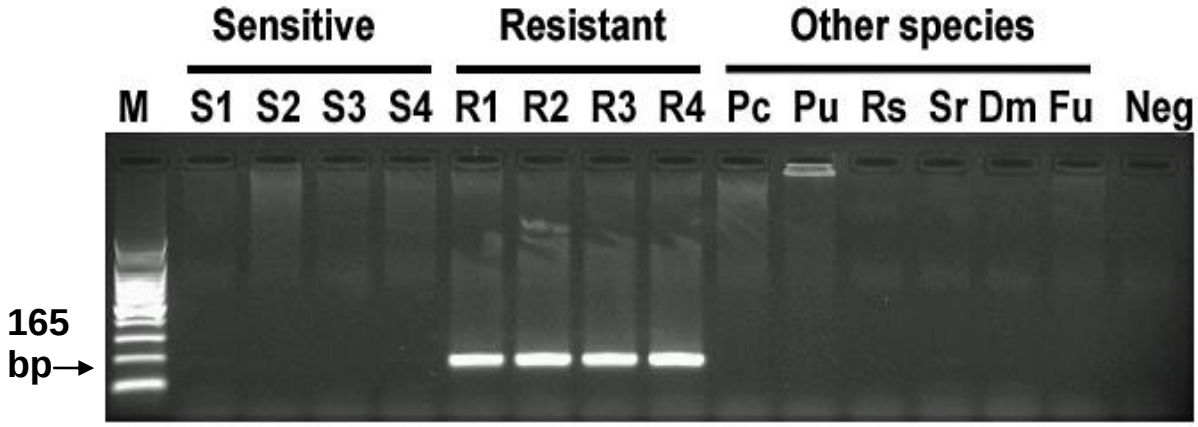

Fig. 4.4. Allele-specific PCR using genomic DNA with primer pair gCytBF1/DbcytbR-R for the detection of azoxystrobin-resistant (AZO-R) isolates of *Didymella bryoniae*. Azoxystrobin-sensitive isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. AZO-R isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. Other species: Pc = *Phytophthora capsici*, Pu = *Pythium ultimum*, Rs = *Rhizoctonia solani*, Sr = *Sclerotium rolfsii*, Dm = *Pseudoperonospora cubensis*, Fu = *Fusarium oxysporum* f. sp. *niveum*. Neg = negative control. M = 100 bp DNA ladder.

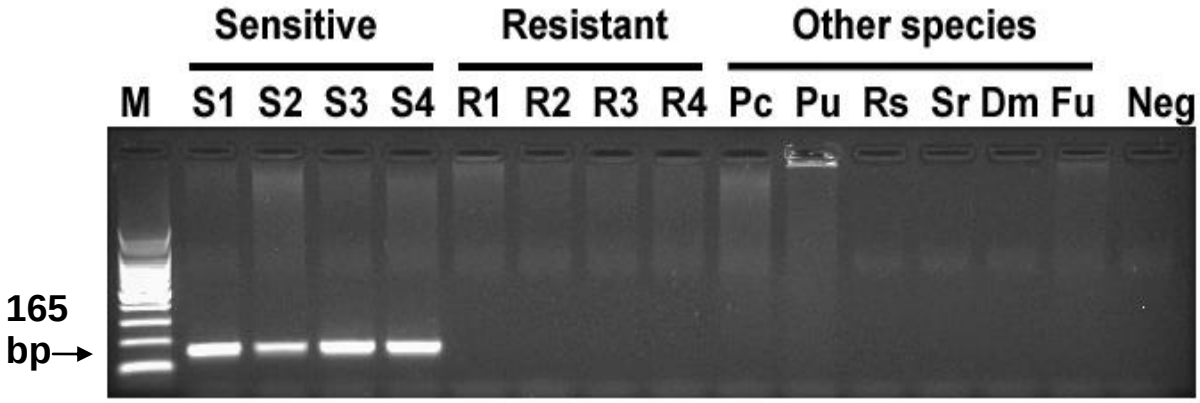

Fig. 4.5. Allele-specific PCR using genomic DNA with primer pair gCytBF1/DbcytbS-R for the detection of azoxystrobin-sensitive (AZO-S) isolates of *Didymella bryoniae*. AZO-S isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. Azoxystrobin-resistant isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. Other species: Pc = *Phytophthora capsici*, Pu = *Pythium ultimum*, Rs = *Rhizoctonia solani*, Sr = *Sclerotium rolfsii*, Dm = *Pseudoperonospora cubensis*, Fu = *Fusarium oxysporum* f. sp. *niveum*. Neg = negative control. M = 100 bp DNA ladder.

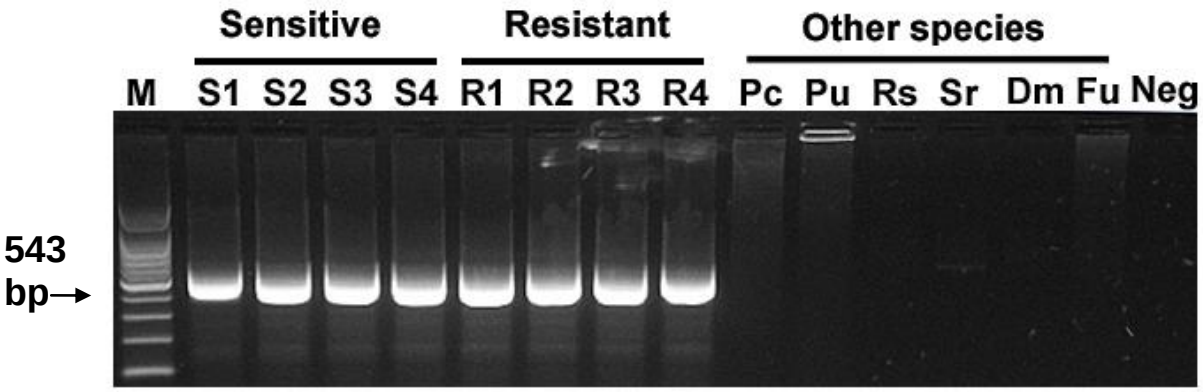

Fig. 4.6. Allele-specific PCR using genomic DNA with primer pair DbComF/DbComR for the detection of azoxystrobin-sensitive (AZO-S) and -resistant (AZO-R) isolates of *Didymella bryoniae*. AZO-S isolates: S1 = UR3-5, S2 = R501-3-2, S3 = C2-8, S4 = R204-1-1. Azoxystrobin-resistant isolates: R1 = C2-1, R2 = C5-1, R3 = B105-3-3, R4 = UR5-9. Other species: Pc = *Phytophthora capsici*, Pu = *Pythium ultimum*, Rs = *Rhizoctonia solani*, Sr = *Sclerotium rolfsii*, Dm = *Pseudoperonospora cubensis*, Fu = *Fusarium oxysporum* f. sp. *niveum*. Neg = negative control. M = 100 bp DNA ladder.

CHAPTER 5

SUMMARY

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rhem, is considered the most widespread and destructive disease of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nikai) in the southeastern U.S. (2,3). Although cultural practices play an important role in GSB management, they provide little effectiveness when used alone. Therefore, fungicides with protective and systemic properties are essential in the successful management of GSB. The heavy reliance on fungicides, particularly systemic fungicides, has created issues with disease control due to fungicide resistance development in *D. bryoniae*. In the case of azoxystrobin and other quinone outside-inhibiting (QoI) fungicides, resistance is typically monitored using in vitro conidial germination assays (1). As an alternative, a mycelial growth assay was designed for a faster method for detecting azoxystrobin resistance in *D. bryoniae*. The dose responses of conidial germination and mycelial growth differ to a large degree in azoxystrobin (AZO) –sensitive (S) isolates of *D. bryoniae*. Inhibition of conidial germination of AZO-S isolates ranged from 0 to 100%. However, inhibition of mycelial growth of AZO-S isolates ranged from 30-58%. For AZO-resistant (R) isolates, slightly different dose responses were observed for conidial germination and mycelial growth. Slight inhibition of mycelial growth of AZO-R isolates did occur at the two highest AZO concentrations tested. The addition of 100 ppm salicylhydroxamic acid (SHAM), a known inhibitor of alternative respiration, to the medium did increase the sensitivity of mycelial growth of AZO-S isolates to AZO but did not affect the sensitivity of mycelial growth of AZO-R isolates. Alternative respiration may play a role in the reduced sensitivity of mycelial growth to AZO but further research is needed to

confirm this. An AZO concentration of 1.0 µg/ml was suggested to serve as a discriminatory concentration for mycelial growth assays to determine AZO resistance in *D. bryoniae.* At this AZO concentration, isolates exhibiting relative mycelial growth values greater than 90% would be considered AZO-R and isolates exhibiting relative mycelial growth values less than 70% would be considered AZO-S. Additionally, an allele-specific PCR assay was designed to qualitatively detect AZO resistance in both complementary DNA and genomic DNA of *D. bryoniae*. The allele-specific PCR assay provides a powerful tool for the rapid and highly specific qualitative detection of AZO resistance in *D. bryoniae*.

Another focus of this study was to determine the relative fitness of *D. bryoniae* isolates resistant to the fungicides AZO, boscalid (BOS), penthiopyrad (PEN), and thiophanate-methyl (TPM). Fitness components included mycelial growth at different temperatures, spore production, spore germination, and virulence. There were no differences in fitness among AZO-, BOS-, or PEN-S or –R isolates. However, TPM-R isolates grew significantly less than TPM-S isolates at some temperatures tested. There was also no obvious association between the number of fungicides to which an isolate of *D. bryoniae* is resistant to and its rate of growth at certain temperatures. The lack of apparent fitness costs accompanying resistance to AZO, BOS, and PEN and the possible presence of a fitness cost accompanying resistance to TPM in *D. bryoniae* provide important information in regard to fungicide resistance management. The frequency of isolates resistant to AZO, BOS, or PEN is unlikely to decrease even in the absence of fungicide use because they may be equally or more competitive than wild-type isolates. Additionally, resistance to AZO was determined to be stable. Furthermore, care must be taken to alternate chemically unrelated fungicides and/or tank mix chemically unrelated fungicides to prevent resistance development in areas where resistance has not been reported. The presence of an

apparent fitness cost in TPM-R isolates of *D. bryoniae* suggests that TPM-R isolates may be at a competitive disadvantage as compared to TPM-S isolates. The removal of benzimidazoles from gummy stem blight disease management programs where TPM resistance has been reported may reduce the frequency of resistance to TPM over time. Further research is needed in order to confirm whether the presence or absence of fitness costs affects the competitive ability of isolates of *D. bryoniae* that are resistant to these fungicides. In summary, the results of this study provide information critical for the continued monitoring of fungicide resistance and the development of better fungicide resistance management strategies.

LITERATURE CITED

1. FRAC (Fungicide Resistance Action Committee). 2012. List of monitoring methods. Accesed on 06/19/13. Available at www.frac.info.

2. Keinath, A. P. 1995. Fungicide timing for optimum management of gummy stem blight epidemics on watermelon. Plant Dis. 79:354-358.

3. Stevenson, K. L., Seebold, K. W., and Langston, D. B., Jr. 2004. Resistance to azoxystrobin in the gummy stem blight pathogen documented in Georgia. Plant Health Progress doi: 10.1094/PHP-2004-1207-01-RS