SENSITIVITY OF *DIDYMELLA BRYONIAE* TO DMI AND SDHI FUNGICIDES AND THE RELATIONSHIP BETWEEN FUNGICIDE SENSITIVITY AND CONTROL OF GUMMY STEM BLIGHT IN WATERMELON

by

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(Under the Direction of KATHERINE L. STEVENSON and DAVID B. LANGSTON, JR)

ABSTRACT

Baseline isolates of *Didymella bryoniae* were tested for sensitivity to penthiopyrad, tebuconazole and difenoconazole using mycelia growth assay. Based on the sensitivity distribution, a discriminatory concentration of 3.0 µg/ml was selected for future monitoring of shifts in sensitivity. Cross-resistance between boscalid and penthiopyrad was identified in field isolates of *D. bryoniae* and a potential for cross-resistance between DMIs is observed based on baseline sensitivity profile. Field experiments were conducted to establish a relationship between frequency of resistance and fungicide efficacy in managing gummy stem blight (GSB). Tebuconazole and chlorothalonil were proved to be most effective in managing GSB. A consistent negative association was observed between the frequency of resistance and disease control in the field. Isolation of *D. bryoniae* resistant to thiophanate-methyl from commercial seedlots helped explain the unpredictable development of fungicide resistance in watermelon fields. Results from this study will help manage GSB with efficient use of fungicides.

INDEX WORDS: Cross-resistance, *Didymella bryoniae*, Difenoconazole, DMIs, Frequency of resistance, Fungicide resistance, Gummy stem blight, Penthiopyrad, Tebuconazole, Thiophanate-methyl, Watermelon.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Long-term goals

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae,* is one of the most destructive and widespread diseases of watermelon in the southeastern United States. Management of GSB requires an integration of both cultural practices and chemical application. Although cultural practices help to manage the disease to some extent, fungicide application is by far the most effective method for the management of GSB. Many fungicides have been labeled for use in cucurbits against GSB. *D. bryoniae* has a remarkable ability to adapt and become resistant to most of the fungicides developed to control it. Resistance management strategies must be followed to prolong the effective life of these fungicides. Since *D. bryoniae* has a history of developing resistance to fungicides, it is very important to establish the baseline sensitivity of *D. bryoniae* to effective fungicides to facilitate fungicide resistance monitoring. The buildup of resistance is greatly favored by the sustained solo use of fungicides with a specific mode of action. The effective life of a fungicide can be prolonged by avoiding the solo use of site specific fungicide by rotating/ mixing it with fungicides with a multi-site mode of action or with other fungicides that are not cross-resistant to the existing effective fungicide. This current recommendation for fungicide resistance management assumes that fungicide-resistant isolates arise within the field. An introduction of fungicide resistant isolate can overcome the current resistance management efforts and will render them ineffective. Introduction of a resistant isolate through infested seeds or airborne ascospores can make the current resistance management strategies ineffective. A better understanding of the sources of inoculum and the frequency of fungicide resistance in the initial inoculum population and the relationship between

frequency of resistance and fungicide efficacy will help us in designing better management programs to control GSB effectively. The overall goal of this project was to successfully monitor the development of fungicide resistance in the field and to manage GSB by the efficient use of effective fungicides.

Background and literature review

Watermelon (*Citrullus lanatus*) is a member of the family *Cucurbitaceae*, which also includes some other economically important vegetables like cucumber, pumpkin and other gourd vegetables and melons like muskmelon, honeydews etc. The United States of America is one of the world's leading producers and consumers of watermelon. Production of watermelon in the U. S. has steadily increased over the past few years and in 2010, watermelon production was worth a total of $492 million (51). Percapita consumption of watermelon was 14.9 lbs in 2009 and has increased to 15.5 lbs in 2010 (51). Ideal growing conditions for watermelon include warm, frost free conditions. Thus, commercial watermelon production is located primarily are in the southern part of the country, in the states of Florida, California, Texas, Georgia and Arizona (5). Georgia is the second most important watermelon producing state in the United States and accounted for more than 30 % of the total production in the country over the last two years (51).

Most watermelons produced in the southeastern United States are grown from transplants. Growers prefer the use of transplants over direct-seeding as it offers several advantages. Transplants provide good seed use efficiency, which is especially important for hybrid seeds which are quite expensive. Transplants can be produced in greenhouses when conditions are not favorable for good crop establishment in the field. Use of transplants helps to avoid problems associated with poor germination and seedling diseases in the field. Transplants provide a more uniform crop stand and rapid establishment in the field and also facilitate early

harvest. Early harvest of fruits not only offers a marketing advantage but also helps in avoiding diseases that are favored by warmer temperatures that occur later in the season.

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rehm (anamorph *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.) is the most destructive disease of watermelon in greenhouses (1,52) and in the major watermelon-producing areas of the Southeastern U. S. (47,48). GSB can result in an average yield loss of 43% in non-sprayed plots (22,19), primarily due to reduced number, size and quality of fruits resulting from the loss of foliage.

GSB can affect all above-ground parts of the watermelon plant. In the seedling stage, symptoms of GSB are characterized by the appearance of water-soaked lesions on cotyledons. Fungal hyphae from these lesions later can invade the hypocotyl and crown resulting in total girdling of the seedling (45). On a mature watermelon plant, symptoms include lesions on the leaves and petioles, crown blight, stem cankers and extensive defoliation. GSB also can cause lesions on fruits. But fruit infection is not commonly observed. *D. bryoniae* produces pycnidia, the asexual fruiting body, on infected leaves and stems. The conidia are hyaline, cylindrical with rounded ends, non- or monoseptate, and 6-13 µm long (47). The fungus can overwinter on plant debris and produce pseudothecia, the sexual fruiting body. Ascospores produced in pseudothecia are 14-18 × 4-6 µm in size, hyaline, monosepatate with a constriction at the septum and have round ends. The upper cell is usually wider than the lower one (47).

Epidemiology of GSB is not very well understood. It has been shown that the fungus can overwinter as dormant mycelia on host tissue as long as the debris is not decomposed (19) and can produce both pycnidia and pseudothecia on the debris (52,23). Wind-borne ascospores originating from the pseudothecia that form on watermelon debris on the soil may be responsible

for the primary infection of the crop. The conidia produced on watermelon debris left in the watermelon field may also have a role as the primary source of infection (52). There are some reports suggesting that the fungus can survive as dormant mycelia in the seed and this infested seed can act as a potential source for primary inoculum (30). *D. bryoniae* has been isolated from testa, perisperm and cotyledons of cucumber, pumpkin and watermelon seeds (30,41). Seed transmission of *D. bryoniae* has been reported in case of cantaloupe and watermelon seeds (50,41). Presence of pathogen in the seed can easily go undetected, especially if the fungus is deep-seated, and can serve as an important source of inoculum.

GSB can occur both in the transplant production houses and in the fields. Outbreaks of GSB in transplant production houses were reported from California (26) have been observed in Georgia as well. Sources of inoculum for GSB in the transplant production houses could be seed-borne or ascospores arising from the crop debris left outside or inside the greenhouse (30,52). Warm, humid conditions inside the transplant production houses favor disease development and overhead irrigation in transplant production houses facilitates rapid spread of this disease.

Management of GSB requires an integration of both cultural practices and chemical methods in both the transplant production house and the field. Sources for genetic resistance against GSB have been identified (10), but no resistant variety has been released yet. Another method to manage gummy stem blight is by reducing the primary sources of inoculum. Deep-turning of infected debris from the previous season will promote the rapid breakdown of debris and thereby the amount of primary inoculum (22,23). Crop rotation with wheat-soybean double cropping was found to be effective in reducing disease severity in the subsequent season (17). Incorporation of cabbage residue followed by soil solarization can also be effective in reducing the development of disease in the following season (17). Seed treatment prior to planting can

reduce the amount of inoculum associated with seeds. Seed treatment with 1600 µg/ml of peroxyacetic acid for 30 min was found to be effective in preventing seed transmission of GSB to watermelon seedlings (12). Proper ventilation in the greenhouse will reduce the leaf wetness duration and will help reduce disease development to some extent. GSB can be managed in the greenhouse by application of protectant fungicides. Application of plant defense activators like acibenzolar-S-methyl (ABM) to young watermelon seedlings can reduce the incidence of primary crown lesions and thereby the secondary spread of the pathogen (11). Management of irrigation to avoid splash dispersal of the pathogen and periods of prolonged leaf wetness can also help to reduce disease severity.

Cultural practices can reduce the incidence and severity of GSB only to some extent. The most effective option for the management of GSB is the application of protectant and curative fungicides. Protectant fungicides have a multisite mode of action and are associated with low risk for resistance development. The most commonly used protectant fungicides are Bravo (chlorothalonil), belonging to chloronitrile group and Dithane (mancozeb), belonging to ethylenebisdithiocarbamate (EBDC) group (18). Among these two fungicides, chlorothalonil has been found to be more effective against GSB owing to its better retention capacity on the foliage (16,18) but this fungicide can cause phytotoxicity to mature watermelon rinds, if applied late in the season (18). Because of the potential for the explosive spread of GSB under conducive environmental conditions, use of systemic fungicides is usually necessary to manage the disease in the field.

Systemic fungicides labeled for GSB control in the U.S. include Topsin M (thiophanate-methyl) in the methyl benzimidazole (MBC) group, Quadris (azoxystrobin) in the quinone outside inhibitor (QoI) group, Pristine (pyraclostrobin in the QoI group + boscalid in the

succinate dehydrogenase Inhibitor (SDHI) group), Folicur (tebuconazole), Inspire Super (difenoconazole in the demethylation inhibitor (DMI) group + cyprodinil in the anilinopyrimidine group) and Endura (boscalid) in the SDHI group (Georgia Pest Management Handbook 2011-http://www.ent.uga.edu/pmh,48). These systemic fungicides have a site-specific mode of action. Thiophanate-methyl binds to beta-tubulin, thereby preventing the formation of microtubules and disrupting chromosome migration during cell division (6). QoI fungicides block electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (complex III) in the mitochondrial respiration chain. Fungicides in the SDHI group bind to the ubiquinone binding site (Q-site) of the mitochondrial complex II and thus inhibit fungal respiration (4). DMIs inhibit a cytochrome P-450 mono-oxygenase enzyme and thus inhibit the C-14 demethylation reaction in the ergosterol biosynthesis pathway.

D. bryoniae has shown a remarkable ability to adapt and become resistant to most of the effective fungicides developed to control it. The MBC fungicide thiophanate-methyl provided good control of GSB until resistance was observed in early 1990s (21). Azoxystrobin provided excellent control of gummy stem blight (23) and was granted a section 18 Emergency Exemption in the 1998 growing season in Georgia to control GSB (21). A full section 3 national label was granted for azoxystrobin use on cucurbit crops in 1999, which led to intensive use of this chemical for management of GSB and other foliar diseases. However, *D. bryoniae* isolates that were insensitive to azoxystrobin were found in Georgia, Delaware and Maryland within 2 years of its first commercial use (38,48). After development of resistance to azoxystrobin, a new fungicide, Pristine, a formulated mixture of pyraclostrobin and boscalid, showed good efficacy against GSB in the field (46). Isolates of *D. bryoniae* and other fungal pathogens that showed resistance against azoxystrobin were found to be sensitive to Pristine (33,40). Thus, even

pathogens that have developed resistance to azoxystrobin can be controlled by the boscalid component of Pristine (2). Resistance to boscalid had been reported in many pathogens within few years of its use (3,35,37). Loss of efficacy in the field performance of Pristine in managing GSB was observed in Georgia in 2007 and resistance to the boscalid component of Pristine was reported in *D. bryoniae* in 2008, which greatly limited its use as a management tool against GSB (48).

DMI fungicides, introduced in the 1970s, have a broad spectrum of activity against many different fungal pathogens and are being used for the management of a number of plant diseases (6). Although DMI fungicides have been used on other crops for many years they were labeled only recently for use on cucurbits. The DMI fungicides tebuconazole (labeled in 2008) and difenoconazole (labeled in 2010), are the only two registered systemic fungicides to which resistance has not yet been reported in *D. bryoniae*. However, resistance to DMIs has been reported in many other ascomycete fungi including *Monilinia fructicola*, *Venturia inaequalis*, *Mycosphaerella graminicola*, and several powdery mildew pathogens (8,28,34,39). Increased reliance on DMI fungicides for management of GSB will increase selection pressure and the risk of resistance development in populations of *D. bryoniae.* And since *D. bryoniae* has a history of rapidly developing resistance to systemic fungicides (19,38,48,49), it is important to establish the baseline sensitivity of *D. bryoniae* to effective fungicides and initiate monitoring programs to detect any significant change in pathogen sensitivity that might lead to a disease control failure.

The buildup of resistance is generally favored by the sustained solo use of systemic fungicides with a site-specific mode of action. However, unpredicted development of resistance to boscalid was observed in a watermelon field in Quincy, Florida. This field had not previously been planted with watermelon and had no history of SDHI fungicide use, yet isolates of *D.*

bryoniae collected from this field were already resistant to boscalid. There was another report from Greece (32) of similarly unpredicted development of resistance to benomyl in cucurbits grown in the greenhouse. They noticed resistance to benomyl after only one year of use for managing GSB on cucurbits. This rapid and unpredicted development of fungicide resistance suggests that fungicide-resistant isolates may have been introduced into watermelon production greenhouses and fields either through infested seeds or as ascospores arising from fungicide treated watermelon fields. Very few airborne ascospores were detected at sampling locations near watermelon production fields and transplant production greenhouses in Georgia from 2008 till 2010, but GSB was very severe in Georgia during these years (unpublished data). Absence of airborne ascospores also was reported from South Carolina (20). Absence of airborne ascospores points to infested seeds as the most likely potential source of fungicide-resistant inoculum.

Knowledge about the occurrence of cross-resistance between fungicides is very important in selecting the combination of fungicides for resistance management programs. Occurrence of cross-resistance has been reported among fungicides within the same FRAC group. For example cross-resistance has been observed between DMI fungicides in *Cercospora beticola* (15), *Fusicladium effusum* (42), *Sclerotinia homoeocarpa* (13), *Venturia inaequalis* (29), and *Erysiphe necator* (9). Cross resistance between the MBC fungicides thiophanate-methyl and benomyl has been reported in *Helminthosporium solani* and *D. bryoniae* (7,19). Cross-resistance between boscalid and penthiopyrad in the SDHI group has been reported in *Alternaria alternata*, *Corynespora cassiicola* and *Podosphaera xanthii* (2,14). Strong positive correlation between the sensitivity to QoI fungicides was reported in *A. solani* (40) and cross-resistance among the QoIs has been reported in many plant pathogens in the field (40,25). However occurrence of cross-resistance is not universal. A lack of cross-resistance has been reported between SDHIs boscalid

and fluopyram in *A. alternata* (3), *Corynespora cassiicola* and *Podosphaera xanthii* (14). Similarly, a lack of cross-resistance between DMI fungicides has been reported in *Monilinia oxycocci* (36), *Mycosphaerella graminicola* (34), *Ramulispora herpotrichoides* (44), *S. homoeocarpa* (13) and *Tapesia acuformis* (31).

Baseline sensitivities and discriminatory concentrations have been established in *D. bryoniae* for boscalid, but there is no information available regarding the sensitivity of *D. bryoniae* to penthiopyrad, tebuconazole or difenoconazole or the potential for cross-resistance between fungicides within the same class. And although fungicide sensitivity monitoring is helpful for detecting the frequency of resistant isolates in a population or detecting shifts in sensitivity, it is difficult to predict the relative efficacy of a fungicide for control of GSB based solely on results of in vitro sensitivity assays. There are no threshold levels of frequency of resistance established for individual fungicides to help decide whether to withdraw or continue using a fungicide once resistance has been reported. Therefore, the research outlined in this proposal is designed to provide critical information about the baseline sensitivity of effective fungicides, the potential for cross-resistance between them, and the relationship between the frequency of resistant isolates and the efficacy of fungicides. Results from this research will be used to monitor fungicide sensitivity, detect significant shifts in fungicide sensitivity, and to design more effective fungicide spray programs to reduce selection pressure on the pathogen population and avoid the development of fungicide resistance and thus manage GSB in watermelon more effectively.

The specific objectives of this research were as follows:

1. Determine the baseline sensitivity of *D. bryoniae* to penthiopyrad, tebuconazole and difenoconazole and select a discriminatory concentration for routine sensitivity

monitoring and to assess the potential for cross-resistance between (1) tebuconazole and difenoconazole, (2) and between penthiopyrad and boscalid in *D. bryoniae*.

2. Investigate the cross-resistance pattern between boscalid and penthiopyrad in isolates of *D. bryoniae* collected from commercial watermelon production fields in 2009, where boscalid failed to control GSB incidence.
3. Determine the relationship between frequency of resistance and fungicide efficacy in managing gummy stem blight of watermelon in Georgia.
4. Determine if seed is a source for fungicide resistant inoculum.

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., Morgan, D. P., and Michailides, T. J. 2008. Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. Plant pathology 57:135-140.

3. Avenot, H. F., Sellam, A., Karaoglanidis, G., and Michailides, T. J. 2008. Characterizations of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. Phytopathology 98:736-742.

4. BASF Corporation. 2003. Endura Technical Information Bulletin. NVA 02-10-201-3001, BASF Corporation, Research Triangle Park, NC.

5. Borris, H., Brunke, H., and Kreith, M. 2006. Commodity profile: melons. Agricultural marketing resource center.

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

7. Cunha, M. G., and Rizzo, D. M. 2003. Development of fungicide cross-resistance in *Helminthosporium solani* populations from California. Plant Dis. 87:798-803.

8. Délye, C., Laigret, F., and Corio-Costef, M. F. 1997. A mutation in the 14 alpha demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl. Environ. Microbiol. 63:2966-2970.

9. Erickson, E. O., and Wilcox, W. F. 1997. Distributions of sensitivities to three sterol demethylation inhibitor fungicides among populations of *Uncinula necator* sensitive and resistant to triadimefon. Phytopathology 87:784-791.

10. Gusmini, G., Song, R., and Wehner, T. C. 2005. New sources of resistance to gummy stem blight in watermelon. Crop Sci. 45:582-588.

11. Hopkins, D. L. 2002. Control of gummy stem blight of watermelon with plant defense activators combined with fungicides. Proc. Fla. State Hort. Soc. 115:183-186.

12. Hopkins, D. L., Thompson, C. M., Hilgren, J., and Lovic, B. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seed-borne diseases of watermelon. Plant Dis. 87:1495-1499.

13. Hsiang, T., Yang, L., and Barton, W. 1997. Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides in Ontario isolates of *Sclerotinia homoeocarpa*. European Journal of Plant Pathology 103:409-416.

14. Ishii, H., Miyamoto, T., Ushio, S., and Kakishima, M. 2011. Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. Pest Manag. Sci. 67(4):474-482.

15. Karaoglanidis, G. S., and Thanassoulopoulos, C. C. 2003. Cross-resistance patterns among sterol biosynthesis inhibiting fungicides (SBIs) in *Cercospora beticola*. European Journal of Plant Pathology 109:929-934.

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. 1996. Soil amendment with cabbage residue and crop rotation to reduce gummy stem blight and increase growth and yield of watermelon. Plant Dis. 80:564-570.

18. Keinath, A. P. 2000. Effects of protectant fungicide application schedules on gummy stem blight epidemics and marketable yield of watermelon. Plant Dis. 84: 254-260.

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

20. Keinath, A. P. 2008. Survival of *Didymella bryoniae* in infected muskmelon crowns in South Carolina. Plant Dis. 921:1223-1228.

21. 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.

22. Keinath, A. P., and Duthie, J. A. 1998. Yield and quality reduction in watermelon due to anthracnose, gummy stem blight and black rot. Recent Research Developments in Plant Pathology, Vol. 2:77-90. Research Signpost, Trivandrum, India.

23. Keinath, A. P., May, W., III and Du Bose, V. 1999. Effects of number of sequential Quadris applications and companion fungicide on foliar disease of watermelon, 1998. Fungic. Nematicide Tests 54:279.

24. 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.

25. 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.

26. Koike, S. T. 1997. First report of gummy stem blight, caused by *Didymella bryoniae*, on watermelon transplants in California.

27. Köller, W., Parker, D. M., and Reynolds, K. L. 1991. Baseline sensitivities of *Venturia inaequalis* to sterol demethylation inhibitors. Plant Dis. 75:726-728.

28. Köller, W., and Wilcox, W. F. 2001. Evidence for the predisposition of fungicide-resistant phenotypes of *Venturia inaequalis* to a preferential selection for resistance to other fungicides. Phytopathology 91:776-781.

29. Köller, W., Wilcox, W. F., Barnard, T., Jones, A. L., and Braun, G. 1997. Detection and quantification of resistance of *Venturia inaequalis* populations to sterol demethylation inhibitors. Phytopathology 87:184-190.

30. Lee, D. H., Mathur, S. B., and Neergard, P. 1984. Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedlings of cucumber and pumpkin. Phytopathol. Z. 109:301-308.

31. Leroux, P., Chapeland, F., Arnold, A., and Gredt, M. 2000. New cases of negative cross-resistance between fungicides, including sterol biosynthesis inhibitors. Journal of General Plant Pathology 66:75-81.

32. Malathrakis, N. E., and Vakalounakis, D. J. 1983. Resistance to benzimidazole fungicides in the gummy stem blight pathogen *Didymella bryoniae* on cucurbits. Plant Pathology 32:395-399.

33. Markoglou, A., Malandrakin, A., Vitoratos, A., and Ziogas, B. 2006. Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides. European journal of Plant Pathology 115:149-162.

34. Mavroeidi, V. I., and Shaw, M. W. 2005. Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. Crop Protection 24:259-266.

35. McGrath, M. T. 2008. Fungicide sensitivity in *Podosphaera xanthii* and efficacy for cucurbit powdery mildew in NY, USA, in 2003-2006. Journal of Plant Pathology 90:S2.90.

36. McManus, P. S., Best, V. M., Voland, R. P., and Leininger, B. L. 1999. Sensitivity of *Monilinia oxycocci* to fenbuconazole and propiconazole in vitro and control of cranberry cottonball in the field. Plant Dis. 83:445-450.

37. Miyamoto, T., Ishii, H., Seko, T., Kobri, S., and Ogawara, T. 2009. Occurrence of *Corynespora cassicola* isolates resistant to boscalid on cucumber in Ibaraki prefecture, Japan. Plant Pathol. 58:1144-1151.

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

39. Parker, D. M., Zhang, N., Smart, C. D., and Köller, W. D. 2006. Polymorphism of 14 alpha-demethylase gene (CYP51) in brown rot pathogen *Monilinia fructicola* from a resistant orchard in New York State. Phytopathology. 96:S90.

40. Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Dis. 89:269-278.

41. Rankin, H. W. 1954. Effectiveness of seed treatment for controlling anthracnose and gummy-stem blight of watermelon. Phytopathology 44: 675-680.

42. Reynolds, K. L., Brenneman, T. B., and Bertrand, P. F. 1997. Sensitivity of *Cladosporium caryigenum* to propiconazole and fenbuconazole. Plant Dis. 81:163-166.

43. Richardson, M. J. 1979. An annotated list of seed-borne diseases. Third Ed. Int. Seed Test. Ass., Zurich, Switzerland. Page 56.

44. Robbertse, B., Holz, G., and Crous, P. W. 1996. Sensitivity of South African *Ramulispora herpotrichoides* isolate to carbendazim and ergosterol biosynthesis inhibitors. Plant Pathology 45:270-275.

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

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

47. Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

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

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

50. Sudisha, J., Niranjana, S. R., Umesha, S., Prakash S. H., Shekhar Shetty, H. 2006. Transmission of seed-borne infection of muskmelon by *Didymella bryoniae* and effects of seed treatments on disease incidence and fruit yield. Biological Control 32:196-205.

51. U.S. Department of Agriculture. Economics, Statistics and Market Information System. 2011. Albert R. Mann Library, Cornell University.

52. Van Steekelenburg, N. A. M. 1983. Epidemiological aspects of *Didymella bryoniae*, the cause of stem and fruit rot of cucumber. Neth. J. Plant Pathol. 89:75-86.

CHAPTER 2

BASELINE SENSITIVITY AND CROSS-RESISTANCE TO SUCCINATE-DEHYDROGENASE-INHIBITING FUNGICIDES AND DEMETHYLATION-INHIBITING FUNGICIDES IN *DIDYMELLA BRYONIAE*

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Abstract

Didymella bryoniae, the causal agent of gummy stem blight (GSB) of watermelon, has a history of developing resistance to fungicides, most recently the SDHI fungicide boscalid. To facilitate fungicide resistance monitoring, baseline sensitivity distributions were established for DMI fungicides tebuconazole and difenoconazole and the SDHI fungicide penthiopyrad that were recently introduced or are being evaluated for GSB control. In all, 71 isolates with no prior exposure to SDHIs or DMIs were tested using a mycelial growth assay to determine the effective concentration at which mycelial growth was inhibited by 50% (EC_{50}). EC_{50} values for boscalid, penthiopyrad, tebuconazole and difenoconazole ranged from 0.018 to 0.064, 0.015 to 0.057, 0.062 to 0.385 and 0.018 to 0.048 µg/ml with median values of 0.032, 0.026, 0.118 and 0.031 µg/ml. There was a significant positive correlation between the sensitivity to penthiopyrad and boscalid ($P<0.0001$, $r = 0.75$) and between tebuconazole and difenoconazole ($P<0.0001$, $r = 0.59$), indicating a high potential for cross-resistance between these compounds. In 2009, 103 isolates collected from fungicide-treated watermelon fields were tested for resistance to boscalid and penthiopyrad using a discriminatory concentration of 3.0 µg/ml. Of the isolates tested, 82 were resistant and 14 were sensitive to both fungicides. Because of the significant potential for cross-resistance, growers will be advised not to use both the SDHI fungicides and both the DMIs in the same fungicide spray program.

Introduction

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rehm (anamorph *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.) is the most destructive disease of watermelon in greenhouses (1,42) and in the major watermelon-producing areas of the southeastern U. S. (37,40). The disease can spread rapidly and cause significant yield reductions

in warm, wet weather conditions (1). Management of GSB requires an integration of both cultural practices and chemical methods; however, cultural practices have limited effectiveness for GSB management. The most effective means of managing GSB is the frequent application of fungicides. The protectant fungicide chlorothalonil is effective for GSB control, but it can be applied only in the early part of the season because it can cause phytotoxicity on mature watermelon rinds (17). Because of the potential for the explosive spread of GSB under conducive environmental conditions, use of systemic fungicides is usually necessary for the control GSB in the field.

During the first few years following the introduction of systemic fungicides for use on watermelons in the U. S., they provided excellent control of GSB. However, *D. bryoniae* has shown a remarkable ability to adapt and become resistant to several of these systemic fungicides. *D. bryoniae* can be considered a high risk pathogen for the development of resistance as it has a short life cycle and abundant sporulation which in turn demands a frequent application of fungicides for its management (5). Thiophanate-methyl, a fungicide in the methyl benzimidazole carbamate (MBC) fungicide class, provided good control of GSB until resistance was observed in early 1990s (16). In the late 1990s, the quinone-outside inhibitor (QoI) fungicide azoxystrobin provided excellent control of GSB(19) and was granted section 18 Emergency Exemption in the 1998 growing season in Georgia to control GSB (18). However, within 2 years of first commercial use of azoxystrobin, *D. bryoniae* isolates that were insensitive to azoxystrobin were found in Georgia, Delaware and Maryland (31,40). After development of resistance to azoxystrobin, a new fungicide, Pristine, which is a mixture of the QoI fungicide pyraclostrobin and boscalid, in the succinate dehydrogenase inhibitor (SDHI) class, showed good efficacy against GSB in the field (36). Isolates of *D. bryoniae* and other fungal pathogens that showed

resistance against azoxystrobin were found to be sensitive to Pristine (26,33). Thus, even pathogens that have developed resistance to azoxystrobin could be controlled by the boscalid component of Pristine (3). Pristine worked well against *D. bryoniae* until resistance to boscalid was observed in 2007 (40). A new SDHI fungicide penthiopyrad is currently being evaluated for its effectiveness in managing gummy stem blight in experimental plots in Georgia (David Langston, Personal communication).

DMI fungicides, introduced in the 1970s have a broad spectrum of activity against different fungal pathogens and are being used for the management of a number of plant diseases (5). Although DMI fungicides have been around for a while they were labeled for use on cucurbits only recently in 2008. Tebuconazole (labeled in 2008) and difenoconazole (labeled in 2010), belonging to the DMI group are the only two registered systemic fungicides against which no resistance has been reported from Georgia so far in *D. bryoniae*. However, resistance to DMIs has been reported in many other ascomycete fungi including *Monilinia fructicola*, *Venturia inaequalis*, *Mycosphaerella graminicola*, and several powdery mildew pathogens (8,21,27,32). Increased reliance on DMI fungicides for management of GSB will increase selection pressure and the risk of resistance development in populations of *D. bryoniae.* Also since *D. bryoniae* has a history of rapidly developing resistance to introduced fungicides (16,31,40,39), it is important to establish the baseline sensitivity of *D. bryoniae* to effective fungicides and initiate monitoring programs to detect any significant change in pathogen sensitivity that might lead to a disease control failure.

Knowledge about the occurrence of cross-resistance among fungicides is important in selecting appropriate combinations of fungicides for GSB management programs. Cross-resistance between same groups of fungicides has been reported previously in many fungi

(3,12,15,34,11,20). Penthiopyrad belongs to the same cross-resistant group as boscalid and since cross-resistance to boscalid and penthiopyrad has been reported previously in other fungi (3,12), it is important to evaluate the effectiveness of penthiopyrad in controlling boscalid resistant isolates of *D. bryoniae* in Georgia. Tebuconazole and difenoconazole (one component of Inspire super) belongs to the same triazole class of DMI fungicides and since these two are the only effective systemic fungicides available to the watermelon growers, it is important to evaluate the feasibility of using these two fungicides as rotation partners for managing GSB. This study was designed to provide critical information about the baseline sensitivity of *D. bryoniae* and the potential for development of cross resistance between them. The objectives were to (i) determine the baseline sensitivity of *D. bryoniae* to penthiopyrad, tebuconazole and difenoconazole and select a discriminatory concentration for routine sensitivity monitoring, (ii) assess the potential for cross-resistance between (1) tebuconazole and difenoconazole, (2) and between penthiopyrad and boscalid in *D. bryoniae*, (iii) investigate the cross-resistance pattern between boscalid and penthiopyrad in isolates of *D. bryoniae* collected from commercial watermelon production fields in 2009, where boscalid failed to control GSB.

Materials and Methods

Determination of baseline fungicide sensitivity. Seventy one single-lesion isolates of *D. bryoniae* that were never exposed to either DMI or SDHI fungicides were used to determine the baseline sensitivity to penthiopyrad, tebuconazole and difenoconazole. These isolates were originally obtained from watermelon leaves with symptoms of GSB collected in 2001 or 2002 from different counties in Georgia (Table 2.1). The isolates were stored on filter paper at -20℃ until needed. Stored isolates were recovered by placing a piece of filter paper with fungal mycelium on a fresh plate of PDA and incubating at 25℃ for 7 days in preparation for fungicide

sensitivity assays. Technical grade boscalid (98.4% a.i.; BASF Corporation, Research Triangle Park NC), penthiopyrad (99% a.i.; E. I. du Pont de Nemours & Co., Wilmington DE), tebuconazole (97.5% a.i.; Bayer Corporation, Kansas City MO), difenoconazole (95% a.i.; Syngenta Crop Protection, Greensboro NC) were dissolved in acetone to obtain stock solutions of 30 mg/ml. Serial dilutions of the stock solution of each fungicide were made in acetone and added to autoclaved PDA, cooled to 55°C to obtain desired concentrations of 0, 0.0001, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 µg/ml. The final concentration of acetone in fungicide-amended and non-amended medium (acetone only) was 0.1% by volume.

Sensitivity of each isolate to tebuconazole, difenoconazole, penthiopyrad and boscalid was determined by using an in vitro mycelial growth assay on fungicide-amended and non-amended (acetone only) PDA. Mycelial plugs of 6 mm diameter, taken from the margin of a 1-week-old culture on PDA, were placed upside down in the center of fungicide-amended and non-amended PDA plates. Two replications of each isolate and fungicide concentration were prepared. After 4 days of incubation in the dark at 25°C, the diameter of each colony was measured and corrected by subtracting the diameter of the mycelial plug. Relative growth (RG) was calculated as the ratio between the corrected colony diameter on fungicide-amended medium and the corrected colony diameter on non-amended medium.

Evaluation of cross resistance. Watermelon leaves with symptoms of GSB were collected from fungicide-treated and non-treated watermelon fields in Georgia, North Carolina and South Carolina in 2009. A small section of tissue, approximately 0.25 cm^2, was cut from the margin of one lesion on each leaf. The tissue pieces were surface-disinfested with 0.6% NaOCl, rinsed in sterile water, and placed onto PDA amended with antibiotics (50 µg/ml each of tetracycline, chloramphenicol, and streptomycin) and incubated at 25º C for 5 days and subsequently

transferred to PDA to obtain a pure culture. Fungal colonies were identified as *D. bryoniae* based on the morphological characteristics of the colony on PDA. A total of 103 single-lesion isolates of *D. bryoniae* was obtained in this way and tested for sensitivity to boscalid and penthiopyrad.

Sensitivity of the isolates to boscalid and penthiopyrad was determined using an in vitro mycelial growth assay on PDA amended with fungicides at a discriminatory concentration of 3.0 µg/ml. Technical grade boscalid (BASF, Research Triangle Park NC) was dissolved in acetone to obtain a stock solution of 30 mg/ml. A 10-fold dilution of the stock solution was prepared in acetone and added to autoclaved PDA cooled to 55º C to obtain the desired concentration of 3.0 µg/ml in the medium. Control medium was prepared by adding acetone to autoclaved and cooled PDA such that the final concentration of acetone in fungicide-amended and non-amended medium (acetone only) was 0.1% by volume. Isolates were transferred to fungicide-amended and non-amended medium, incubated, and measured as described above. Isolates with an RG value greater than 0.2 were considered resistant to the respective fungicide. A contingency table was prepared to record the number of sensitive and resistant isolates to both the fungicides.

Statistical analysis. The EC_{50} value for each isolate was estimated based on linear regression of probit-transformed relative inhibition (1- RG) on $\log_{10}$-transformed fungicide concentration. The frequency distribution of EC_{50} for each fungicide was tested for normality using the Shapiro-Wilk test (PROC UNIVARIATE) in SAS (version 9.2, SAS Institute Inc., Cary NC). Paired *t*-tests were performed to compare the mean $\log_{10}$-transformed EC_{50} values among experimental repeats. The coefficient of variability (standard error/mean) of $\log_{10}$-transformed EC_{50} values for individual isolates among all experimental repeats was calculated as a measure of assay reproducibility. A discriminatory concentration for each fungicide was selected based on the frequency distribution of EC_{50} values. A concentration at which complete

inhibition of the growth of a sensitive isolate was observed was selected as the discriminatory concentration for penthiopyrad. A concentration at which a significant difference in relative growth could be detected between a sensitive and a reduced sensitive isolate was selected as discriminatory concentration for the DMI fungicides. A concentration closer to the mean EC_{50} could be chosen to detect small shift in sensitivity as this small shift in sensitivity usually does not necessarily translate to a loss of control in the field, a discriminatory concentration well above the mean EC_{50} was chosen as the discriminatory concentration for monitoring purpose in this study (38). Simple linear correlation coefficients were calculated (PROC CORR) to determine the relationship between the sensitivity to (1) boscalid and penthiopyrad, and (2) tebuconazole and difenoconazole, and to evaluate the potential for cross-resistance between them. Fisher's exact test was performed to test for a positive relationship between sensitivity to boscalid and penthiopyrad.

Results

Baseline fungicide sensitivity distributions. Coefficients of variation of log_{10}-transformed EC_{50} values of individual isolates among experimental repeats ranged from 0.7 to 9.0% for penthiopyrad, 0.2 to 11.0% for boscalid, 1.2 to 19.0% for tebuconazole and 0.8 to 9.4 % for difenoconazole (Table 2.2). The coefficient of variation was less than 20% for all fungicides tested, which indicates that the log_{10}-transformed EC_{50} values for individual isolates were consistent among the experimental repeats. Therefore, data from individual experimental repeats were combined to determine the mean EC_{50} value for each isolate and fungicide (Table 2.2). Frequency distributions of mean EC_{50} values were log-normal for penthiopyrad ([Pr<W] =0.87), boscalid ([Pr<W] =0.95) and difenoconazole ([Pr<W] =0.43), but was not log-normal for tebuconazole ([Pr<W] <0.0001). EC_{50} values for isolates exposed to penthiopyrad ranged from

0.015 to 0.057 µg/ml and the median EC_{50} value was 0.026 µg/ml (Fig. 2.1, Table 2.2). For boscalid, the EC_{50} values ranged from 0.018 to 0.064 µg/ml and the median EC_{50} value was 0.032 µg/ml (Fig. 2.2, Table 2.2). For tebuconazole, EC_{50} values ranged from 0.062 to 0.385 µg/ml and the median EC_{50} value was 0.118 µg/ml (Fig. 2.3, Table 2.2). For difenoconazole, EC_{50} values ranged from 0.018 to 0.048 µg/ml and the median EC_{50} value was 0.031 µg/ml (Fig. 2.4, Table 2.2). A discriminatory concentration of 3.0 µg/ml was chosen for penthiopyrad, boscalid, tebuconazole and difenoconazole fungicides for use in future sensitivity monitoring studies (Table 2.2).

There were significant positive correlations of EC_{50} values between penthiopyrad and boscalid and between tebuconazole and difenoconazole among the baseline isolates in all experimental repeats. The correlation coefficients ranged from 0.59 to 0.72 for penthiopyrad and boscalid and from 0.39 to 0.66 for tebuconazole and difenoconazole. The relationship between sensitivity to the two SDHIs (Fig. 2.5) and DMIs (Fig. 2.6) for the combined data is provided.

Cross-sensitivity assay. Out of the 103 single-lesion isolates tested, 86 isolates were resistant to both boscalid and penthiopyrad, 12 isolates were sensitive to both fungicides and 6 isolates were resistant only to boscalid. Fisher's exact test (P <0.0001) indicated a strong positive association between the sensitivity to penthiopyrad and boscalid.

Discussion

Determining the baseline sensitivity is the first step in initiating monitoring programs to detect significant shifts in pathogen sensitivity to a fungicide, to ensure efficacy of current fungicide spray programs, to recommend proper resistance management practices, and to monitor the effectiveness of the recommended practices. This study provides the first report of sensitivity of baseline populations of *D. bryoniae* to the SDHI fungicide penthiopyrad and the

DMI fungicides tebuconazole and difenoconazole. Penthiopyrad is a new SDHI fungicide that has not yet been labeled for use on cucurbits. Tebuconazole and difenoconazole were labeled recently for use on cucurbits and are being widely used by watermelon growers for the management of GSB in Georgia.

Baseline sensitivity to penthiopyrad has not been documented in many fungi as it is a relatively new SDHI fungicide. *D. bryoniae* isolates exhibited a relatively narrow range of EC_{50} values for penthiopyrad (0.015-0.057 µg/ml) as opposed to the broader range of EC_{50} values (0.002-0.30 µg/ml) previously reported in *Ascochyta rabiei* (41). EC_{50} values were estimated for the baseline isolates to boscalid to determine the relationship between sensitivities to boscalid and penthiopyrad. The range of EC_{50} values for boscalid was relatively narrow and is consistent with the previous report in *D. bryoniae* (39). The ranges of EC_{50} values were very similar for both penthiopyrad and boscalid (Table 2.2). The range of EC_{50} values for tebuconazole was small and comparable to ranges of EC_{50} values reported in *Colletotrichum cereale* (43), *Botryosphaeria dothidea* (24) and *Pyrenophora tritici-repentis* (4) and unlike the wider distributions reported in *Sclerotinia homoeocarpa* (28), *Sclerotium rolfsii* (10) and *Botrytis cinerea* (44). The distribution of difenoconazole sensitivity in *D. bryoniae* isolates was narrow when compared to the distribution reported in *Phoma ligulicola* (13) and was similar to that reported in *Alternaria* spp. (31), *Cercospora beticola* (15) and *Colletotrichum coccodes* (30). These relatively narrow ranges of EC_{50} values indicate that there is limited variation within the unexposed population with respect to sensitivity to these fungicides. This may also be due to the small number of isolates that we tested for sensitivity collected from only a few counties in South Georgia.

The rapid development of resistance to successively introduced fungicides with different modes of action supports Köller's report of predisposition of fungicide-resistant isolates to a preferential selection for resistance to other fungicides (21). Monitoring for shifts in sensitivity of *D. bryoniae* isolates to the effective fungicides should be a high priority because isolates resistant to a fungicide may be prone to accelerated adaptation to another fungicide.

Cross resistance is common between fungicides belonging to the same chemical class that share a similar mode of action, but is not true in all cases. For example, in the case of SDHI fungicides, a lack of cross-resistance to fluopyram and occurrence of cross-resistance to penthiopyrad in boscalid-resistant isolates was reported in *A. alternata* (2), *Corynespora cassiicola* and *Podosphaera xanthii* (12). Cross-resistance among DMI fungicides also is not universal. Positive cross-resistance among some DMI fungicides has been observed in *Cercospora beticola* (15), *Cladosporium caryigenum* (34), *Sclerotinia homoeocarpa* (11) and *Venturia inaequalis* (20) However, a lack of cross-resistance among some DMIs has been reported in *Monilinia oxycocci* (25), *Mycosphaerella graminicola* (27), *Ramulispora herpotrichoides*(35), *S. homoeocarpa* (11) and *Tapesia acuformis* (23). Previous reports of inconsistent relationship between sensitivities to fungicides with a similar mode of action make it clear that we cannot assume the existence of a positive cross-resistance between fungicides of same chemical class.

Results from this study showed a significant and positive correlation between sensitivities to boscalid and penthiopyrad within the baseline population. In vitro sensitivity assay of boscalid-resistant isolates to penthiopyrad revealed a high degree of cross-resistance between these two SDHI fungicides and hence the use of penthiopyrad for the management of GSB in Georgia, where widespread boscalid-resistance is present (Unpublished) is not likely to be a

viable option. Significant and positive correlations between sensitivities to tebuconazole and difenoconazole were observed for baseline isolates in this study. However, *Didymella bryoniae* isolates resistant to tebuconazole have not been observed in Georgia and so cross-resistance between tebuconazole and difenoconazole could not be determined. Jones and his colleagues reported a wider range of EC_{50}s for difenoconazole (0.04-13.8 µg/ml) in isolates of *Phoma ligulicola* that were previously exposed to tebuconazole but not to difenoconazole (13). This is indirect evidence that cross-resistance exists between these two DMI fungicides in *P. ligulicola*.

Mechanisms responsible for reduced sensitivity to DMIs include alterations in sterol biosynthesis, alterations in the target binding site (CYP51 gene) and fungicide uptake and efflux pump (14). Alteration in the CYP51gene (substitution of isoleucine with valine at codon 381) is reported as one of the mechanisms responsible for reduced sensitivity to azole fungicides in *M. graminicola* and *Candida albicans* (6,7,9). The study conducted by Fraaije at al. showed that reduced sensitivity to tebuconazole was largely driven by I381V in the CYP51 gene in *M. graminicola* population. This study also showed that tebuconazole and difenoconazole differentially selected isolates with this type of substitution (I381V) (9). Since evidence of cross-resistance between tebuconazole and difenoconazole has been reported in two other closely related fungi, there is a high probability for cross-resistance between these two DMIs in *D. bryoniae*. Use of these two DMI fungicides in the same spray program may not be a good practice as it may lead to an increased selection of isolates with reduced sensitivity.

Difenoconazole has a 4-fold higher intrinsic activity than tebuconazole on mycelial growth of *D. bryoniae* in vitro. This higher intrinsic activity may delay the shift towards reduced sensitivity if difenoconazole is used in the field for the management of GSB. The effectiveness of this strategy will depend on the field application rates and the physical and biochemical

properties of these two chemicals. A slight shift in sensitivity to tebuconazole in vitro has been observed in isolates of *D. bryoniae* collected from Florida (unpublished data). Since there are not many other effective systemic fungicides available for the management of GSB, farmers should be cautious about the use of DMIs. Performance of this fungicide in the field and shifts in the in vitro sensitivity of the pathogen population should be closely monitored to avoid potential disease control failures. Use of protectant fungicides like chlorothalonil and mancozeb as rotation partners with the DMI fungicide will help in reducing the selection of isolates less sensitive to DMIs.

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. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop protection 29:643-651.

3. Avenot, H., Morgan, D. P., and Michailides, T. J. 2008. Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. Plant pathology 57:135-140.

4. Beard, C., Loughman, R., Smith, A., and Speijers, J. 2009. Baseline sensitivity to three triazole fungicides in *Pyrenophora tritici-repentis*. Australasian Plant Pathology 38:168-172.

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

6. Cools, H. J., Fraaije, B. A., and Lucas, J. A. 2005. Molecular examination of *Septoria tritici* isolates with reduced sensitivities to triazoles. Pages 103-114 in: Modern Fungicides and Antifungal Compounds. H-W. Dehne, U. Gisi, K. H. Kulk, P. E. Pussel, and H. H. Lyr, eds. Alton, UK.

7. Cools, H. J., Fraaije, B. A., and Lucas, J. A. 2005. Molecular mechanisms correlated with changes in triazole sensitivity in isolates of *Mycosphaerella graminicola*. Pages 267-274 in: Proc. BCPC cong.-Crop Science and Technology. BCPC, Alton, UK.

8. Délye, C., Laigret, F., and Corio-Costef, M. F. 1997. A mutation in the 14 alpha demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl. Environ. Microbiol. 63:2966-2970.

9. Fraaije, B. A., Cools, H. J., Kim, S-H., Motteram, J., Clark, W. S., and Lucas, J. A. 2007. A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. Molecular Plant Pathology 8(3):245-254.

10. Franke, M. D., Brenneman, T. B., Stevenson, K. L., and Padgett, G. B. 1998. Sensitivity of isolates of *Sclerotium rolfsii* from peanut in Georgia to selected fungicides. Plant Dis. 82:578-583.

11. Hsiang, T., Yang, L., and Barton, W. 1997. Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides in Ontario isolates of *Sclerotinia homoeocarpa*. European Journal of Plant Pathology 103:409-416.

12. Ishii, H., Miyamoto, T., Ushio, S., and Kakishima, M. 2011. Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. Pest Manag. Sci. 67(4):474-482.

13. Jones, S., Pethybridge, S., Hay, F., Groom, T., and Wilson, C. 2007. Baseline sensitivity of Australian *Phoma ligulicola* isolates from pyrethrum to azoxystrobin and difenoconazole. J. Phytopathology 155:377-380.

14. Joseph-Horne, T., and Hollomon, D. W. 1997. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol. Lett. 49:141-149.

15. Karaoglanidis, G. S., and Thanassoulopoulos, C. C. 2003. Cross-resistance patterns among sterol biosynthesis inhibiting fungicides (SBIs) in *Cercospora beticola*. European Journal of Plant Pathology 109:929-934.

16. 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.

17. Keinath, A. P. 2000. Effects of protectant fungicide application schedules on gummy stem blight epidemics and marketable yield of watermelon. Plant Dis. 84: 254-260.

18. 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.

19. Keinath, A. P., May, W., III and Du Bose, V. 1999. Effects of number of sequential Quadris applications and companion fungicide on foliar disease of watermelon, 1998. Fungic. Nematicide Tests 54:279.

20. Köller, W., Parker, D. M., and Reynolds, K. L. 1991. Baseline sensitivities of *Venturia inaequalis* to sterol demethylation inhibitors. Plant Dis. 75:726-728.

21. Köller, W., and Wilcox, W. F. 2001. Evidence for the predisposition of fungicide-resistant phenotypes of *Venturia inaequalis* to a preferential selection for resistance to other fungicides. Phytopathology 91:776-781.

22. Köller, W., Wilcox, W. F., Barnard, T., Jones, A. L., and Braun, G. 1997. Detection and quantification of resistance of *Venturia inaequalis* populations to sterol demethylation inhibitors. Phytopathology 87:184-190.

23. Leroux, P., Chapeland, F., Arnold, A., and Gredt, M. 2000. New cases of negative cross-resistance between fungicides, including sterol biosynthesis inhibitors. Journal of General Plant Pathology 66:75-81.

24. Ma, Z., Morgan, D. P., Felts, D., and Michailides, T. J. 2002. Sensitivity of *Botryosphaeria dothidea* from California pistachio to tebuconazole. Crop Protection 21:829-835.

25. Mc Manus, P. S., Best, V. M., Voland, R. P., and Leininger, B. L. 1999. Sensitivity of *Monilinia oxycocci* to fenbuconazole and propiconazole in vitro and control of cranberry cottonball in the field. Plant Dis. 83:445-450.

26. Markoglou, A., Malandrakin, A., Vitoratos, A., and Ziogas, B. 2006. Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides. European journal of Plant Pathology 115:149-162.

27. Mavroeidi, V. I., and Shaw, M. W. 2005. Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. Crop Protection 24:259-266.

28. OK, C.-H., Popko, J. T., Jr., Campbell-Nelson, K., and Jung, G. 2011. In vitro assessment of S*clerotinia homoeocarpa* resistance to fungicides and plant growth regulators. Plant Dis. 95:51-56.

29. Olaya, G., Bounds, R., and Tally, A. 2008. Sensitivity to azoxystrobin, difenoconazole and cyprodinil of *Alternaria* spp. isolates causing Alternaria leaf spot on almonds. Phytopathology 98:S116.

30. Olaya, G., Cochran, A., and Gudmestad, N. 2010. Difenoconazole baseline sensitivity distribution of *Colletotrichum coccodes* isolates from potatoes. Phytopathology 100:S93.

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

32. Parker, D.M., Zhang, N., Smart, C. D., and Köller, W. D. 2006. Polymorphism of 14 alpha-demethylase gene (CYP51) in brown rot pathogen *Monilinia fructicola* from a resistant orchard in New York State. Phytopathology. 96:S90.

33. Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Dis. 89:269-278.

34. Reynolds, K. L., Brenneman, T. B., and Bertrand, P. F. 1997. Sensitivity of *Cladosporium caryigenum* to propiconazole and fenbuconazole. Plant Dis. 81:163-166.

35. Robbertse, B., Holz, G., and Crous, P. W. 1996. Sensitivity of South African *Ramulispora herpotrichoides* isolate to carbendazim and ergosterol biosynthesis inhibitors. Plant Pathology 45:270-275.

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

37. Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

38. Smith, F. D., Parker, D. M., and Köller, W. 1991. Sensitivity distribution of *Venturia inaequalis* to sterol demethylation inhibitor flusilazole: Baseline sensitivity and implications for resistance monitoring. Phytopathology 81:392-396.

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

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

41. Thaher, N. H., Gossen, B. D., and McDonald, M. 2010. Baseline sensitivity of *Ascochyta rabiei* to penthiopyrad, a new SDHI fungicide. Phytopathology 100:S125.

42. Van Steekelenburg, N. A. M. 1983. Epidemiological aspects of *Didymella bryoniae*, the cause of stem and fruit rot of cucumber. Neth. J. Plant Pathol. 89:75-86.

43. Wong, F. P., and Midland, S. L. 2007. Sensitivity distributions of California populations of *Colletotrichum cereale* to the DMI fungicides propiconazole, myclobutanil, tebuconazole, and triadimefon. Plant Dis. 91:1547-1555.

44. Zhang, C., Zhu, J., Wei, F., Liu, S., and Zhu, G. 2007. Sensitivity of *Botrytis cinerea* from green house vegetables to DMIs and fenhexamid. Phytoparasitica 35(3):300-313.

Table 2.1. Source of baseline isolates of *Didymella bryoniae* originally collected in 2001 or 2002 from watermelon fields in Georgia used to establish baseline sensitivities to boscalid and penthiopyrad

Isolate	GA County	Isolate	GA County
Gate-5	Telfair	Gs15-23	Cook
Gate-6	Telfair	Gs15-25	Cook
Gate-10	Telfair	Gs15-28	Cook
Gs2-1	Dooly	Gs15-33	Cook
Gs2-2	Dooly	Gs15-34	Cook
Gs2-3	Dooly	Gs6-1	Worth
Gs2-4	Dooly	Gs6-2	Worth
Gs2-5	Dooly	Gs6-3	Worth
Gs2-6	Dooly	Gs6-4	Worth
Gs2-10	Dooly	Gs6-5	Worth
Gs2-11	Dooly	Gs6-6	Worth
Gs2-12	Dooly	Gs6-7	Worth
Gs4-1	Cook	Gs6-9	Worth
Gs4-3	Cook	Gs6-10	Worth
Gs4-6	Cook	Gs9-1	Tift
Gs4-7	Cook	Gs9-2	Tift
Gs4-8	Cook	Gs9-3	Tift
Gs4-9	Cook	Gs9-4	Tift
Gs4-10	Cook	Gs9-5	Tift
Gs4-12	Cook	Gs9-6	Tift
Gs12-1	Decatur	Gs9-7	Tift
Gs12-4	Decatur	Gs9-9	Tift
Gs12-5b	Decatur	Gs9-10	Tift
Gs12-10a	Decatur	Gs9-11	Tift
Gs12-30	Decatur	Gs9-12	Tift
Gs14-4	Tift	Gs9-13	Tift
Gs14-5	Tift	Gs9-14	Tift
Gs14-6	Tift	Gs9-16	Tift
Gs14-8	Tift	Gs9-17	Tift
Gs14-20	Tift	Gs9-19	Tift
Gs14-21	Tift	Gs9-20	Tift
Gs14-37	Tift		
Gs15-5	Cook		
Gs15-6	Cook		
Gs15-8	Cook		
Gs15-11	Cook		
Gs15-12	Cook		
Gs15-14	Cook		
Gs15-21	Cook		
Gs15-22	Cook		

Table 2.2. Range, mean and median EC_{50} values and coefficient of variability based on log_{10}-transformed EC_{50} values of baseline isolates of *Didymella bryoniae* for each fungicide along with the discriminatory concentration for each fungicide

		EC_{50} (µg/ml)				
Fungicide	**Experiment**	**Range**	**Mean**	**Median**	**Coefficient of variability[x]**	**Discriminatory concentration (µg/ml)**
Penthiopyrad	1	0.016-0.064	0.033	0.030	0.007-0.093	3.0
	2	0.010-0.085	0.027	0.026		
	3	0.011-0.061	0.027	0.026		
	Combined	0.015-0.057	0.028	0.026		
Boscalid	1	0.017-0.098	0.041	0.040	0.002-0.112	3.0
	2	0.015-0.087	0.033	0.029		
	3	0.015-0.079	0.033	0.032		
	Combined	0.018-0.064	0.034	0.032		
Tebuconazole	1	0.084-0.388	0.143	0.134	0.012-0.186	3.0
	2	0.060-0.483	0.128	0.109		
	3	0.031-0.306	0.113	0.109		
	Combined	0.062-0.385	0.124	0.118		
Difenoconazole	1	0.016-0.078	0.044	0.045	0.008-0.094	3.0
	2	0.013-0.055	0.031	0.029		
	3	0.012-0.057	0.025	0.024		
	Combined	0.018-0.048	0.032	0.031		

[x] Coefficient of variability is the absolute value of (standard error of log_{10} EC_{50} values)/ (mean of log_{10} EC_{50} values)

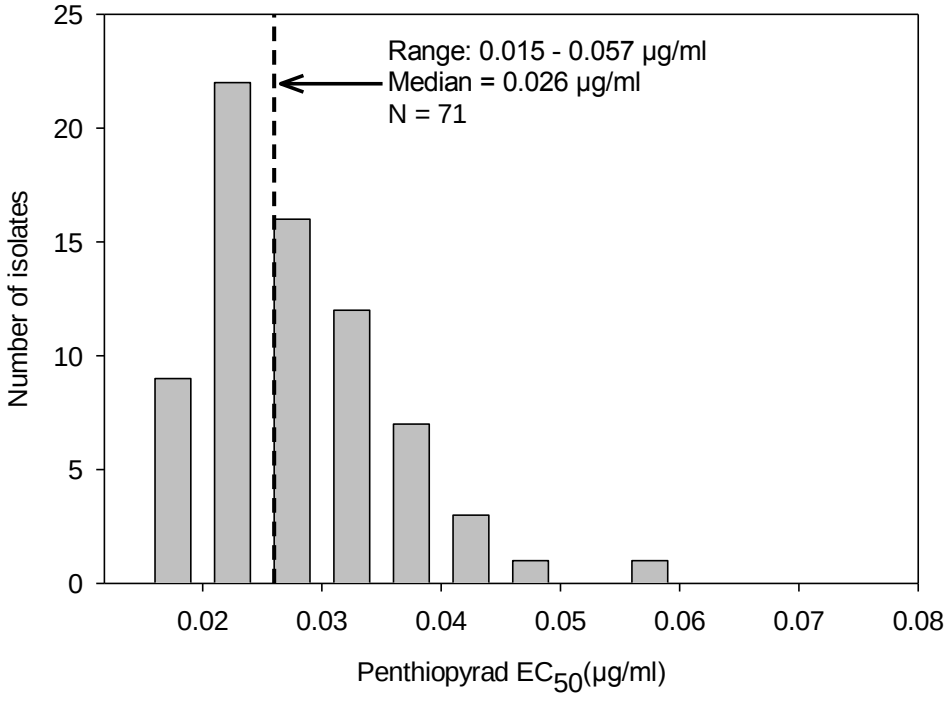

Fig. 2.1. Frequency distribution of EC_{50} values for isolates of *Didymella bryoniae* to penthiopyrad.

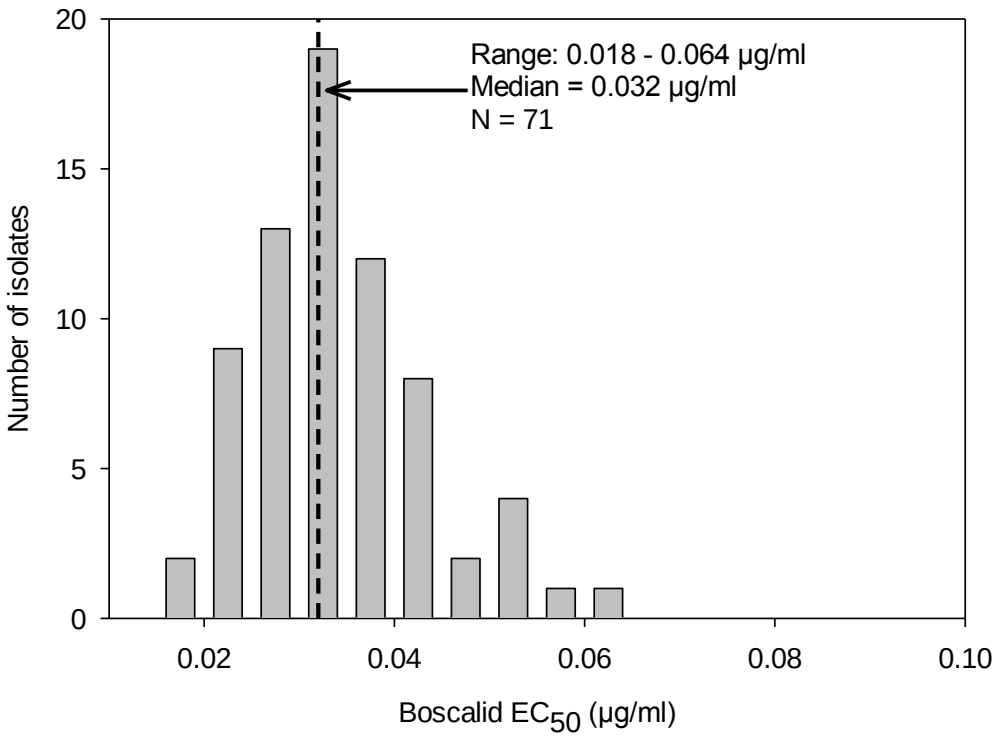

Fig. 2.2. Frequency distribution of EC_{50} values for isolates of *Didymella bryoniae* to boscalid.

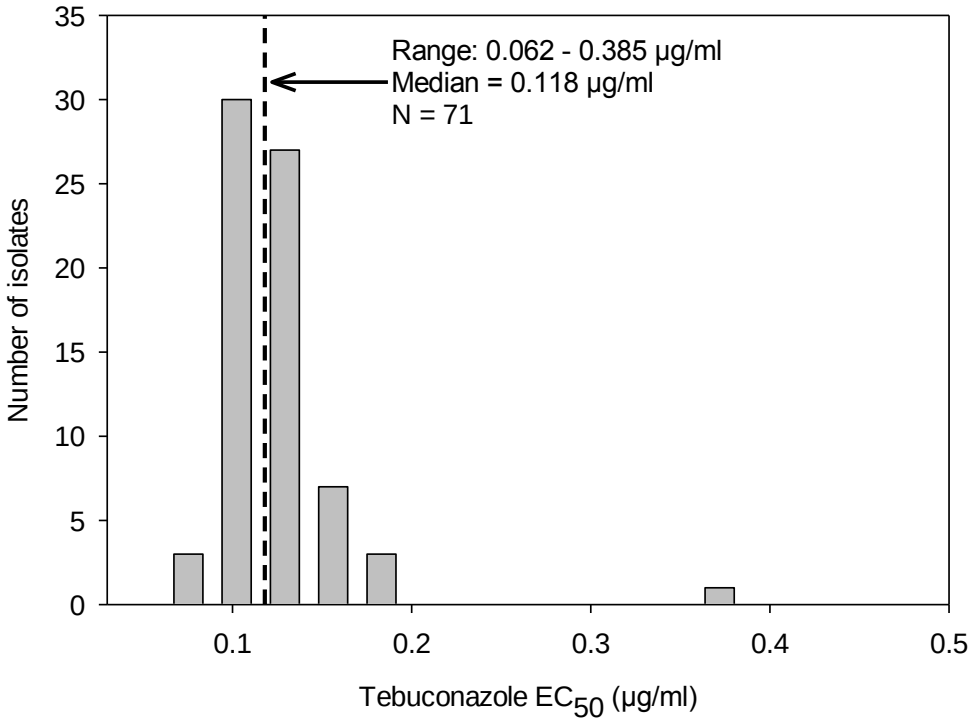

Fig. 2.3. Frequency distribution of EC_{50} values for isolates of *Didymella bryoniae* to tebuconazole.

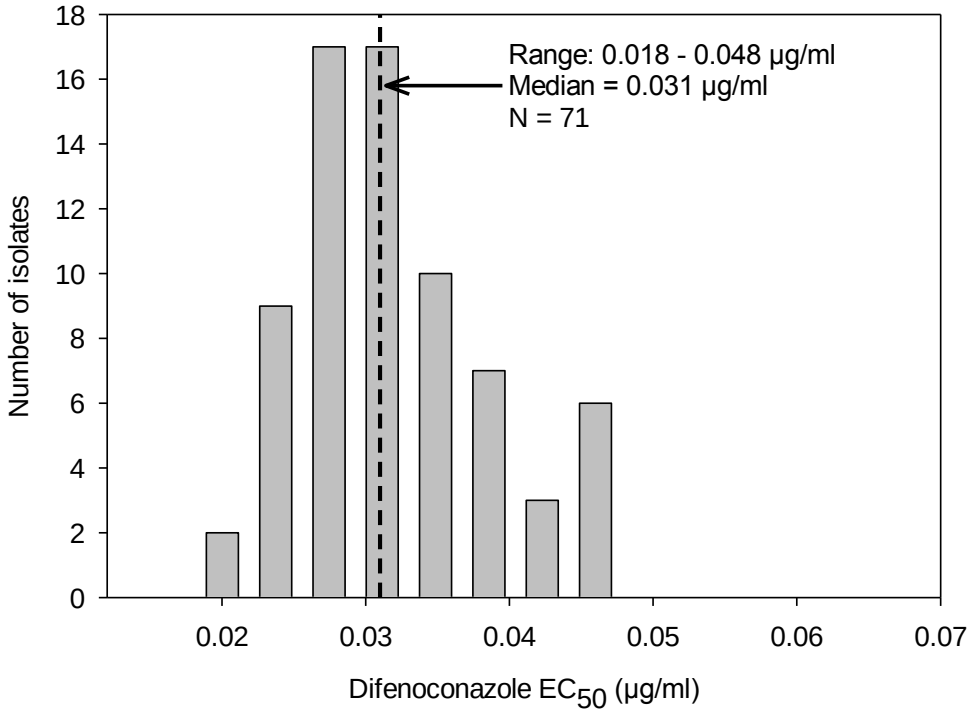

Fig. 2.4. Frequency distribution of EC_{50} values for isolates of *Didymella bryoniae* to difenoconazole.

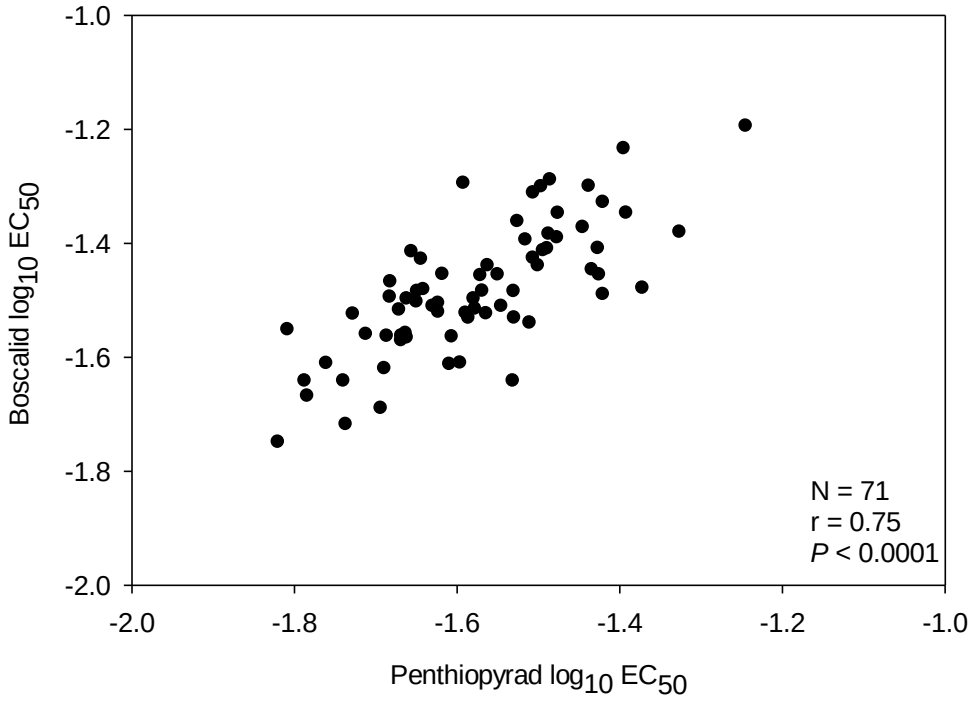

Fig. 2.5. Correlation between $\log_{10}$-transformed EC_{50} values of isolates of *Didymella bryoniae* to penthiopyrad and boscalid.

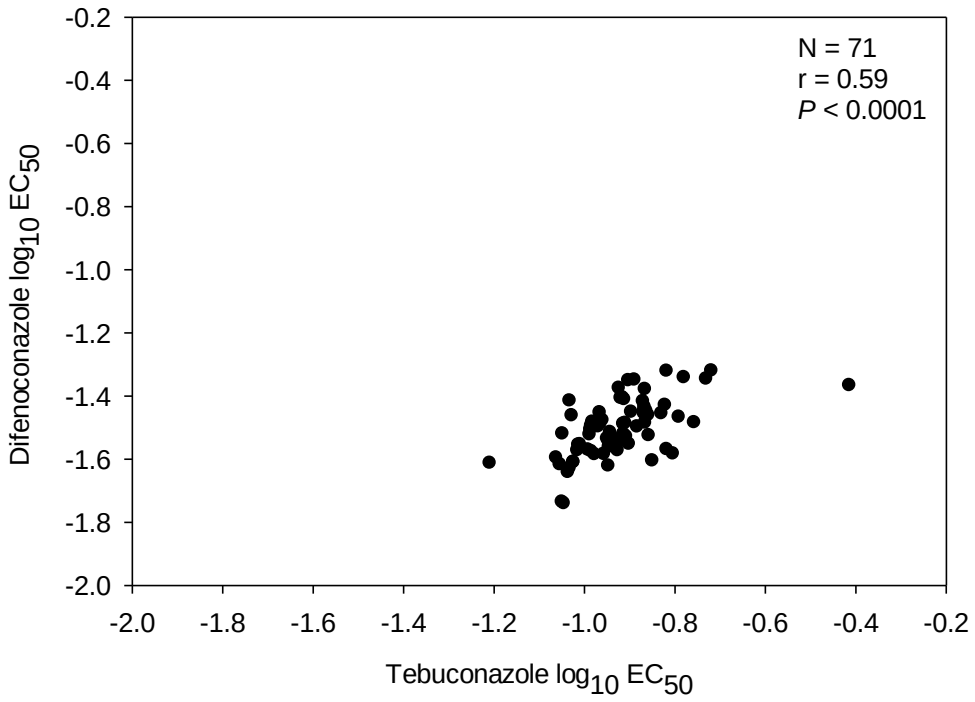

Fig. 2.6. Correlation between log_{10}-transformed EC_{50} values of isolates of *Didymella bryoniae* tebuconazole and difenoconazole.

CHAPTER 3

RELATIONSHIP BETWEEN FUNGICIDE SENSITIVITY AND CONTROL OF GUMMY STEM BLIGHT OF WATERMELON UNDER FIELD CONDITIONS

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Abstract

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae*, is the most destructive disease of watermelon and is managed mainly with fungicides. *D. bryoniae* has developed resistance to many fungicides that were once very effective, including azoxystrobin, boscalid and thiophanate-methyl. Field experiments were conducted in Tifton (TN) and Reidsville (RV), GA in 2009 and 2010 to establish a relationship between frequency of fungicide resistance based on in vitro assays and its efficacy in the management of GSB. Frequency of resistance to boscalid, thiophanate-methyl and azoxystrobin was >0.82 in isolates collected from nontreated plots in both locations in both the years. All isolates collected after six applications of boscalid, thiophanate-methyl or azoxystrobin were resistant to that fungicide. All isolates collected from treated and nontreated plots were sensitive to tebuconazole. GSB severity was assessed on weekly basis from 63 days after planting. GSB severity in plots treated with boscalid, thiophanate-methyl or azoxystrobin was not significantly lower than the nontreated plots (45 %, TN and 16%, RV). GSB severity and frequency of resistance to boscalid and azoxystrobin in tebuconazole-treated plots (14%, TN and 4%, RV) were significantly lower than the nontreated control. There was a consistent negative association between frequency of fungicide resistance and disease control in the field. Thus, knowledge of the frequency of fungicide resistance in the pathogen population will be helpful in selecting the most effective fungicides for the management of GSB in watermelon fields.

Introduction

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rehm (anamorph *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.) is the most destructive disease of watermelon in greenhouses (1,25) and in the major watermelon-producing areas of the

southeastern U. S. (22,24). The disease can spread rapidly and cause significant yield reductions in warm and wet weather conditions (20,1). It has been reported that GSB can result in an average yield loss of 43% in non-sprayed plots (8,10). Management of GSB requires an integration of both cultural practices and chemical methods; however, cultural practices have limited effectiveness for GSB management. And because watermelon varieties with a commercially acceptable level of genetic resistance to GSB are not yet available, the most effective means of managing GSB is the frequent application of both protectant and systemic fungicides. Unfortunately, *D. bryoniae* has shown a remarkable ability to adapt and become resistant to the effective systemic fungicides within few years of their introduction into management programs.

Thiophanate-methyl, a fungicide in the methyl benzimidazole carbamate (MBC) fungicide class, provided good control of GSB until the early 1990s. A loss of efficacy was observed in the field and the resistance to thiophanate-methyl was confirmed in 1995 (10). In the late 1990s, the quinone-outside inhibitor (QoI) fungicide azoxystrobin provided excellent control of GSB (9) and was granted section 18 Emergency Exemption in the 1998 growing season in Georgia to control GSB (7). However, within 2 years of the first commercial use of azoxystrobin, *D. bryoniae* isolates that were insensitive to azoxystrobin were found in Georgia, Delaware and Maryland (16,24). After development of resistance to azoxystrobin, a new fungicide, Pristine, which is a mixture of the QoI fungicide pyraclostrobin and boscalid, in the succinate dehydrogenase inhibitor (SDHI) class, showed good efficacy against GSB in the field (21). Isolates of *D. bryoniae* and other fungal pathogens that showed resistance against azoxystrobin were found to be sensitive to Pristine (14,18). Thus, even pathogens that have developed

resistance to azoxystrobin could be controlled by the boscalid component of Pristine (2). Pristine worked well against *D. bryoniae* until resistance to boscalid was observed in 2007 (23).

Demethylation-inhibiting (DMI) fungicides, introduced in the 1970s, have a broad spectrum of activity against different fungal pathogens and are used to manage a number of plant diseases (3). Although DMI fungicides have been widely used for managing a number of diseases, DMI fungicides have only recently been labeled for use on cucurbits in 2008. Folicur (labeled in 2008) and Inspire Super, a formulated mixture of difenoconazole (DMI) and cyprodinil (anilinopyrimidine) (labeled in 2010) are the only two registered and affordable systemic fungicides against which no resistance has been reported in *D. bryoniae*. However, we have noticed a significant shift towards reduced sensitivity to tebuconazole in some field isolates collected from a commercial watermelon field in Florida in 2010 (unpublished data). Reduced sensitivity to DMI fungicides has been reported in many fungal pathogens (4,12,15,17). Tebuconazole and difenoconazole belongs to the same chemical group of triazoles within the DMI class and we have shown that there is potential for cross-resistance between these two fungicides (Chapter 2).

Genetic resistance to gummy stem blight has been identified recently in South Carolina (5), but GSB-resistant watermelon cultivars are not yet commercially available. With no GSB-resistant watermelon cultivars and limitations of cultural practices in the management of GSB, grower's reliance on the use of chemical fungicides is likely to continue. Therefore, it is essential that farmers have a variety of chemical classes to choose from and to use in rotation to avoid the buildup of resistance to individual fungicides, especially the DMIs. Commercial use of older fungicides thiophanate-methyl, azoxystrobin and boscalid has decreased either because of reduced efficacy or because of the introduction of more efficacious systemic fungicides for the

management of GSB. Unfortunately threshold levels of frequency of resistance have not yet been established for individual fungicides to help decide whether to withdraw or continue using a fungicide once resistance has been reported. The objective of this study was to determine the relationship between the frequency of resistance to individual fungicides and their efficacy in managing GSB.

Materials and Methods

Field experiment and fungicide treatments. Field experiments were conducted at the University of Georgia research farms in Tifton and Reidsville, Georgia in 2009 and 2010. Field location, season, watermelon cultivars, plot size and spacing used for conducting the experiment are presented in Table 3.1. All experiments were arranged in a completely randomized block design with 6 treatments including the untreated control replicated five (Tifton) or six times (Reidsville). Approximately 4-week-old watermelon seedlings of a susceptible cultivar were transplanted onto raised beds covered with black polyethylene mulch. Treatments consisted of the application of following fungicides: boscalid (Endura 70 WG, 6.5oz/a), azoxystrobin (Quadris 2.08 SC, 12.4 fl oz/a), tebuconazole (Folicur 3.6 SC, 8 fl oz/a), thiophanate-methyl (Topsin 4.5 F, 10 fl oz/a) and chlorothalonil (Bravo Weatherstik 6 SC, 2 pt/a) were applied using a Lee Spider Spray Trac with TX-18 hollow cone nozzles calibrated to deliver 40 gal/A at 75-80 psi to each treatment plot on a weekly basis following the recommended rates on the label. During the first year of the study, no measures were taken to avoid the movement of inoculum between treatments except for the 4.5-m unplanted area between plots. In the second year of the study, in addition to the spacing between plots in the east-west direction, the treatments were separated by two rows of sweet corn on either side of the plots to reduce inoculum dispersal among plots.

Watermelon leaves showing typical symptoms of gummy stem blight were collected from all plots except those treated with chlorothalonil. A small section of infected tissue, approximately 0.09 cm^2, was cut from the margin of one lesion on each leaf. The tissue pieces were surface-disinfested with 0.6% NaOCl, rinsed in sterile water, and placed onto PDA amended with antibiotics (50 μg/ml each of tetracycline, chloramphenicol, and streptomycin) and incubated at 25°C for 5days and subsequently transferred to PDA to obtain a pure culture. Twenty isolates, collected from each treatment were tested for sensitivity to boscalid, tebuconazole, difenoconazole, thiophanate methyl and azoxystrobin. A discriminatory concentration of 3 μg/ml was used for boscalid, tebuconazole and difenoconazole and 100 μg/ml was used for thiophanate-methyl (10). Sensitivity to the above listed fungicides was conducted following an in vitro mycelial growth assay.

Fungicide sensitivity assays. Technical grade boscalid (98.4% a.i.; BASF Corporation, Research Triangle Park NC), tebuconazole (97.5% a.i.; Bayer Corporation, Kansas City MO) and difenoconazole (95% a.i.; Syngenta Crop Protection, Greensboro NC) were dissolved in acetone to obtain stock solutions of 30 mg/ml. Serial dilutions of the stock solution of each fungicide were made in acetone and added to autoclaved PDA cooled to 55°C to obtain the desired concentration of 3.0 μg/ml. The final concentration of acetone in fungicide-amended and non-amended medium (acetone only) was 0.1% by volume. Technical grade thiophanate-methyl (95% a.i.; United Phosphorus Inc., King of Prussia PA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 10 mg/ml. Ten milliliters of this stock solution was added to autoclaved PDA cooled to 55°C to obtain the desired concentration of 100 μg/ml. The final concentration of DMSO in fungicide-amended and non-amended medium (DMSO only) was 1.0% by volume.

Sensitivity of each isolate to tebuconazole, difenoconazole, boscalid and thiophanate-methyl was determined using an in vitro mycelial growth assay on fungicide-amended and non-amended PDA. Mycelial plugs of 6 mm diameter, taken from the margin of a 1-week-old pure culture on PDA were placed upside down in the center of fungicide-amended and non-amended PDA plates. Two replications of each isolate and fungicide concentration were prepared. After 4 days of incubation in the dark at 25°C, the diameter of each colony was measured and corrected by subtracting the diameter of the mycelial plug. Relative growth was calculated as the ratio between the corrected colony diameter on fungicide-amended medium and the corrected colony diameter on non-amended medium. Isolates showing a relative growth of more than 0.25 were considered resistant to the respective fungicides and the frequency of resistance to each fungicide was calculated.

Sensitivity to azoxystrobin. Technical grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to make a stock solution of 10 mg/ml. Aliquot of stock solution was added to autoclaved water agar cooled to 55°C, to obtain a final concentration of 10 μg/ml azoxystrobin such that the concentration of acetone was 0.1 % in both fungicide amended and non-amended medium. The medium was also amended with 100 μg/ml salicylhydroxamic acid (SHAM) to inhibit the alternative respiratory pathway in the fungus that can affect the activity of the fungicide.

Sensitivity to azoxystrobin was tested using a spore germination assay. Isolates of *D. bryoniae* were grown on quarter strength PDA for 2 weeks at 25°C under 12:12 dark: light photoperiod to induce sporulation. Conidial suspensions of each isolate were prepared by flooding the plates with 2 ml of sterile water plus Tween 20 solution and gently scraping the surface of the mycelia with a glass rod to dislodge the conidia. Ten to fifteen micro liters of

conidial suspension from individual isolate was transferred and spread out onto fungicide-amended and non-amended plates. Two replicate plates of each isolate and treatment combination were prepared. After incubating at 25°C for 24 h, 50 spores per plate were examined microscopically and the percentage of germination was recorded. A conidium was considered germinated if the length of the germ tube was at least half the length of the conidium. Relative germination was calculated as the ratio between percentage of germination on fungicide-amended and on non-amended medium. An isolate was considered resistant if the relative germination was less than 0.5 (Stevenson et al. 2004). Thus the number of isolates resistant to azoxystrobin was estimated and the frequency of resistance to azoxystrobin was calculated.

Statistical analysis. Disease severity in each plot was assessed visually and expressed as the percentage of diseased leaf area on a weekly basis after the observance of the first symptom for 3 to 5 weeks. Fewer assessments were made when disease pressure was low (Reidsville) or heavy defoliation due to downy mildew incidence (Black shank, 2010) or due to early decline of vines as the experiment was conducted in fall instead of summer. The area under disease progress curve (AUDPC) was calculated for each plot. Data were analyzed using PROC MIXED of SAS (version 9.2, SAS Institute Inc., Cary NC), with fungicide treatment included as a fixed effect and block as a random effect in the model. Least squares means were compared using the PDIFF option in SAS.

Results

Gummy stem blight was severe in Tifton in the fall of 2009 and 2010, and was considerably less severe in Reidsville in fall, 2010. In Tifton in 2009, symptoms of GSB were first observed in 2 weeks after transplanting and the disease progressed rapidly and resulted in

nearly complete defoliation in the nontreated control plots within 6 weeks after first appearance of symptoms. In 2010, in Reidsville, the onset of disease was delayed when compared to Tifton plots and disease development was slower. This might have been due to the dry weather that lasted throughout the growing season in Reidsville. An adjacent muskmelon field that was heavily infected with GSB may have served as a source of inoculum that contributed to the early onset and severe epidemic of GSB in Tifton in 2010. Downy mildew, caused by *Pseudoperonospora cubensis*, contributed to severe necrotic symptoms similar to that caused by *D. bryoniae* and caused heavy defoliation in both treated and nontreated plots in 2010 in both Tifton and Reidsville. Experimental plots in Tifton were more severely affected by downy mildew when compared to Reidsville.

Maximum disease severity (Figs. 3.1, 3.2) and AUDPC values (Table 3.2) were significantly lower in tebuconazole- and chlorothalonil-treated plots when compared to all other treatments in both 2009 and 2010 in both experimental sites. Maximum disease severity in all other treatments was comparable to that of nontreated plots except for the azoxystrobin treatment in Reidsville in 2010. Maximum disease severity was significantly higher in azoxystrobin-treated plots when compared to all other treatments (Table 3.2). AUDPC values were significantly higher for azoxystrobin-treated plots in Tifton and Reidsville in 2009 and 2010 and also for thiophanate-methyl-treated plots in Tifton in 2010 when compared to the nontreated control (Table 3.2).

All isolates collected from both experimental sites in both years were sensitive to tebuconazole (Table 3.2). Frequency of resistance to boscalid, azoxystrobin and thiophanate-methyl in isolates collected from nontreated plots, representing the initial population, was higher than 80% in both locations and years (Table 3.2). All isolates collected at the end of the season

after exposure to seven fungicide applications were resistant to these fungicides (Table 3.2). In fall 2009, in Tifton, the frequency of resistance to azoxystrobin was significantly lower in tebuconazole treated plots when compared to all other treatments. Frequency of resistance to the other fungicides was not significantly affected by the treatments in Tifton. In 2010, frequency of resistance to all fungicides was not significantly affected by any of the treatments in Tifton (Table 3.2). Frequency of resistance to boscalid was significantly lower in tebuconazole-treated plots in Reidsville when compared to all other treatments. Frequency of resistance to the other fungicides was not significantly affected by any of the treatments in Reidsville (Table 3.2).

Discussion

Chlorothalonil and tebuconazole provided superior disease suppression when compared to all other fungicide treatments in both years and locations. This study is in agreement with a previous report on the effectiveness of chlorothalonil and DMIs in managing GSB (19). These fungicides were effective in managing GSB regardless of the amount of disease pressure or the time of onset of disease. It has been reported previously that chlorothalonil can cause phytotoxicity on mature watermelon rinds and hence is not advisable to use it towards the end of the season (24).

This study was aimed at establishing the relationship between frequency of fungicide resistance in *D. bryoniae* based on in vitro assays and fungicide efficacy for GSB management in watermelon fields. With the development of resistance to all systemic fungicides that were once effective, watermelon growers in Georgia are currently relying heavily on DMI fungicides for managing GSB. So it is very important to monitor and manage the development of resistance to these DMI fungicides as this is the only group of effective systemic fungicides that is available to the growers in Georgia. Lack of new systemic fungicides with different modes of action

motivated us to consider older fungicides as potential rotation partners. Since the initial population was already highly resistant to all the older fungicides, a lower threshold for frequency of resistance could not be established to see if the fungicide could still be effective in managing GSB if the frequency of resistance is low. Despite a high level of resistance to multiple fungicides in the initial population, there is no evidence of a fitness cost associated with survival. The fitness cost associated with resistance to different fungicides has not been investigated in *D. bryoniae*. The only inference with respect to the relationship between frequency of resistance and fungicide efficacy that can be drawn from this study is that there is a negative relationship between frequency of resistance and fungicide efficacy; the higher the frequency of resistance to an individual fungicide, the lower the efficacy in managing GSB. From this study it is clear that if the frequency of resistance is more than 0.9, 0.98 and 0.83, to boscalid, azoxystrobin and thiophanate-methyl, respectively in the initial population, then these fungicides will be ineffective against GSB.

In the field experiments conducted in Tifton and Reidsville in 2009 and 2010, the frequency of resistance to boscalid, azoxystrobin and thiophanate-methyl was more than 0.8 among the isolates collected from nontreated plots, indicating that the initial population was already highly resistant to these fungicides. The relative epidemiological importance of different sources of initial inoculum for GSB epidemics is not very well understood, therefore we can only speculate as to the origin of these highly resistant populations. Reported sources of initial inoculum include ascospores produced on watermelon debris from the previous season (20,25). However, very few ascospores were detected in Georgia in 2008 to 2010 prior to epidemic onset in the field or greenhouse and GSB was very severe in Georgia in these years (unpublished data). The disease can also be initiated by splash dispersed conidia produced in pycnidia formed on

watermelon debris from the previous season (6). It has also been shown that GSB can be seed-borne (13). The widespread resistance to systemic fungicides points to ascospores or infested seed as the primary sources of inoculum. However, failure to detect ascospores in Georgia prior to disease outbreaks does not support the hypothesis of ascospores as the primary source of inoculum. Reports of GSB in transplant production greenhouses points infested seed as the primary source (11,23) for fungicide-resistant inoculum. However, a detailed study of the role of ascospores, conidia and infested seed as primary sources of inoculum is needed to explain the origin of fungicide-resistant inoculum.

In this study, it was observed that treatment with azoxystrobin exacerbated GSB, resulting in significantly higher final disease severity compared to nontreated plots. This increased disease severity could be because of the competitive advantage of resistant isolates in presence of azoxystrobin. A detailed study of the fitness cost associated with resistance to azoxystrobin is needed to be done before drawing further conclusions. Another possible explanation for the poor performance of azoxystrobin could be that its broad spectrum of activity is non-differentially inhibiting the beneficial microbes, and thus provides a better environment for azoxystrobin-resistant isolates to survive.

An interesting outcome of this study is that tebuconazole treatment seems to have some effect on isolates resistant to boscalid or azoxystrobin. In Tifton, in 2009, 100% of the isolates collected from the nontreated plots were resistant to azoxystrobin and none of the treatments, except for tebuconazole, reduced the proportion of azoxystrobin resistance significantly. A similar effect was observed in the case of boscalid resistance in Reidsville in 2010. A similar trend was observed for azoxystrobin resistance in Reidsville, but the reduction in frequency was not statistically significant. This might be because of the small sample size from one replicate

plot. Because the overall disease incidence was low in tebuconazole-treated plots, only one isolate was obtained from one of the replicate plots and that isolate was sensitive to azoxystrobin. This contributed to the increased variation among replications and failure to detect significant differences among treatments. Further analysis revealed that that tebuconazole treatment significantly reduced the frequency of resistance to azoxystrobin when this one isolate was omitted from the data set. Based on this study, tebuconazole treatment reduced the frequency of resistance to azoxystrobin by 13% and boscalid by 26%. One possible explanation for this phenomenon is that boscalid- and azoxystrobin-resistant isolates that are otherwise highly fit may have some fitness cost associated with them when they are exposed to tebuconazole. This result is particularly important in light of the high level of resistance to these fungicides in Georgia. Based on these results, inclusion of tebuconazole in a fungicide spray program for GSB management may reduce the frequency of resistance to azoxystrobin and boscalid over time. However, the underlying mechanism involved in this interaction is unknown and needs further investigation.

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., Morgan, D. P., and Michailides, T. J. 2008. Resistance to pyraclostrobin, boscalid and multiple resistance to Pristine (pyraclostrobin + boscalid) fungicide in *Alternaria alternata* causing alternaria late blight of pistachios in California. Plant pathology 57:135-140.
3. Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? Fungicide Resistance Action Committee Monograph No.1.
4. Délye, C., Laigret, F., and Corio-Costef, M. F. 1997. A mutation in the 14 alpha demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl. Environ. Microbiol. 63:2966-2970.

5. Gusumini, G., Song, R., and Wehner, T. C. 2005. New sources of resistance to gummy stem blight in watermelon. Crop Sci. 45:582-588.

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

7. 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.

8. Keinath, A. P., and Duthie, J. A. 1998. Yield and quality reduction in watermelon due to anthracnose, gummy stem blight and black rot. Recent Research Developments in Plant Pathology, Vol. 2:77-90. Research Signpost, Trivandrum, India.

9. Keinath, A. P., May, W., III and Du Bose, V. 1999. Effects of number of sequential Quadris applications and companion fungicide on foliar disease of watermelon, 1998. Fungic. Nematicide Tests 54:279.

10. 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.

11. Koike, S. T. 1997. First report of gummy stem blight, caused by *Didymella bryoniae,* on watermelon transplants in California. Plant Dis. 81:1331.

12. Köller, W., Wilcox, W. F., Barnard, T., Jones, A. L., and Braun, G. 1997. Detection and quantification of resistance of *Venturia inaequalis* populations to sterol demethylation inhibitors. Phytopathology 87:184-190.

13. Lee, D. H., Mathur, S. B., and Neergard, P. 1984. Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedlings of cucumber and pumpkin. Phytopathol. Z. 109:301-308.

14. Markoglou, A., Malandrakin, A., Vitoratos, A., and Ziogas, B. 2006. Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides. European journal of Plant Pathology 115:149-162.

15. Mavroeidi, V. I., and Shaw, M. W. 2005. Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. Crop Protection 24:259-266.

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

17. Parker, D.M., Zhang, N., Smart, C. D., and Köller, W. D. 2006. Polymorphism of 14 alpha-demethylase gene (CYP51) in brown rot pathogen *Monilinia fructicola* from a resistant orchard in New York State. Phytopathology. 96:S90.

18. Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Dis. 89:269-278.

19. Sanders, F. H., Jr., and Langston, D. B., Jr., 2009. Evaluation of fungicide sprays on gummy stem blight of watermelon in Georgia I, 2009. Plant Disease management Reports 4, Vol. 152:1.

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

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

22. Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

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

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

25. Van Steekelenburg, N. A. M. 1983. Epidemiological aspects of *Didymella bryoniae*, the cause of stem and fruit rot of cucumber. Neth. J. Plant Pathol. 89:75-86.

Table 3.1. Year, field location, watermelon cultivar, plot size and spacing used for evaluation of efficacy of fungicides for management of gummy stem blight under field conditions

Season, year	Location	Cultivar	Plot size (m)	Spacing (m)
Fall 2009	Tifton	Summer Flavor 800	1.8 × 6	1.8 × 0.6
Fall 2010	Reidsville	Crimson sweet	5.4 × 7.5	1.8 × 0.9
Fall 2010	Tifton	Crimson sweet	9 × 7.5	1.8 × 1.2

Table 3.2. Area under disease progress curve (AUDPC), maximum disease severity and frequency of resistance to boscalid, azoxystrobin, thiophanate-methyl and tebuconazole in Tifton in 2009 and Tifton and Reidsville in 2010[x]

				Frequency of resistance[y] to:			
Location, year	**Treatment**	**Max. disease severity (%)**	**AUDPC**	**Boscalid**	**Azoxystrobin**	**Thiophanate-methyl**	**Tebuconazole**
Tifton, 2009[z]	Nontreated	39.0 abc	388.5 a-d	1.00 a	1.00 a	0.83 a	0.00 a
	Chlorothalonil	28.0 def	261.8 fg	----	----	----	----
	Boscalid	42.0 abc	390.5 a-d	1.00 a	1.00 a	0.79 a	0.00 a
	Azoxystrobin	43.0 ab	430.5 ab	1.00 a	1.00 a	0.91 a	0.00 a
	Thiophanate-methyl	45.0 a	360.5 cd	0.89 a	1.00 a	1.00 a	0.00 a
	Tebuconazole	27.0 ef	230.3 g	1.00 a	0.87 b	0.80 a	0.00 a
Tifton, 2010	Nontreated	45.0 a	399.0 b	0.90 a	0.98 a	0.90 a	0.00 a
	Chlorothalonil	09.0 b	142.1 c	----	----	----	----
	Boscalid	45.0 a	374.5 b	1.00 a	1.00 a	0.90 a	0.00 a
	Azoxystrobin	55.0 a	521.5 a	0.95 a	1.00 a	0.90 a	0.00 a
	Thiophanate-methyl	54.0 a	535.5 a	0.90 a	1.00 a	1.00 a	0.00 a
	Tebuconazole	14.0 b	210.0 c	0.90 a	0.95 a	0.95 a	0.00 a
Reidsville, 2010	Nontreated	15.8 b	175.0 b	0.90 a	0.95 a	0.97 a	0.00 a
	Chlorothalonil	01.0 c	007.6 c	----	----	----	----
	Boscalid	22.5 b	227.5 b	1.00 a	0.83 a	0.79 a	0.00 a
	Azoxystrobin	32.5 a	313.7 a	0.95 a	1.00 a	1.00 a	0.00 a
	Thiophanate-methyl	21.7 b	184.9 b	1.00 a	1.00 a	1.00 a	0.00 a
	Tebuconazole	4.2 c	67.1 c	0.67 b	0.70 a	0.83 a	0.00 a

[x] Within each location and year, values within a column followed by the same letter are not significantly different based on comparison of least squares means (α=0.05)
[y] Frequency of resistance = (Number of isolates resistant to a fungicide)/ (total number of isolates collected from each treatment). Frequency of resistance to chlorothalonil was not determined.
[z] Results shown in the table are a part of a larger field experiment. Data from treatments of our interest are shown here.

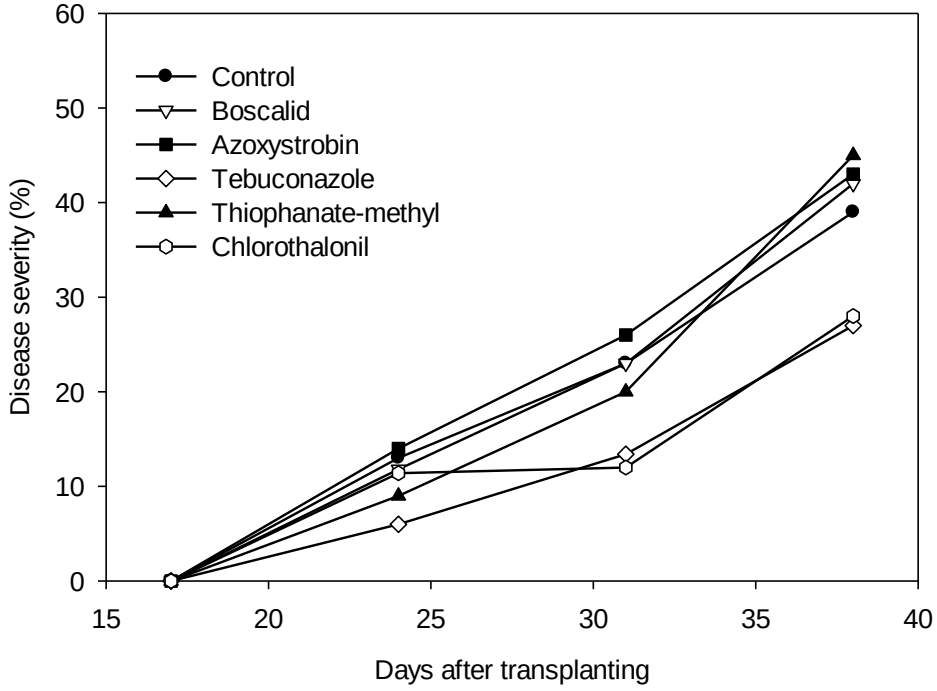

Fig. 3.1. Effect of weekly fungicide applications on progress of gummy stem blight epidemics, caused by *Didymella bryoniae*, in field experiments conducted in fall 2009 in Tifton, GA.

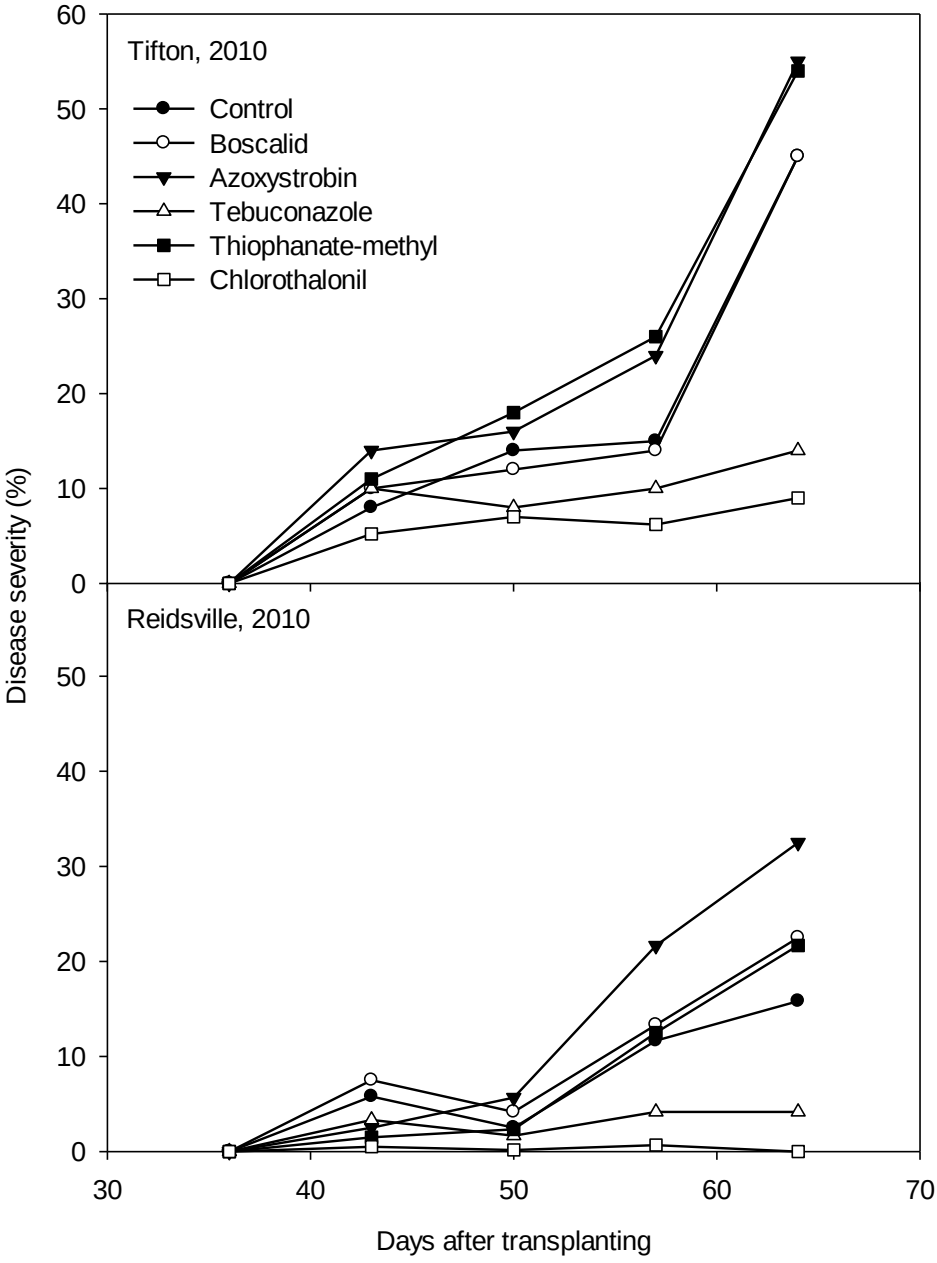

Fig. 3.2. Effect of weekly fungicide applications on progress of gummy stem blight epidemics, caused by *Didymella bryoniae*, in field experiments conducted in fall 2010 in Tifton and in Reidsville, GA.

CHAPTER 4

ROLE OF SEED AS A SOURCE OF FUNGICIDE-RESISTANT INOCULUM FOR GUMMY STEM BLIGHT OF WATERMELON

[1]Thomas, A., Langston, D. B. Jr., Walcott, R. R, Gitaitis, R. D., Stevenson, K. L. 2011. To be submitted to Seed Science and Technology

Abstract

Didymella bryoniae, which causes gummy stem blight (GSB) of watermelon, has a history of developing resistance to fungicides used to manage the disease. Management of fungicide resistance has become a challenge now as *D. bryoniae* developed resistance to most of the systemic fungicides, limiting the availability of different chemistries for rotation purpose. Rapid and unpredictable development of resistance raised the possibility of introduction of fungicide-resistant isolates; either through seeds or through infected and fungicide treated transplants or through ascospores originating from fields treated with systemic fungicides. Selection of resistant isolates is not likely to occur on transplants as the use of systemic fungicide is not recommended for GSB management in transplant production houses. Airborne ascospores also seem to have limited role as primary inoculum as only a few ascospores were detected in Georgia when GSB severity was high in the state. Using direct plating, sweat box and blotter assays, the pathogen was obtained from 3 out of 5800 commercial watermelon seeds. The isolates were tested in vitro for sensitivity to different fungicides and found to be resistant to thiophanate-methyl, demonstrating that seed can harbor fungicide-resistant *D. bryoniae*, which may help explain the unpredictable development of fungicide resistance in some watermelon fields.

Introduction

Gummy stem blight (GSB), caused by the fungus *Didymella bryoniae* (Auersw.) Rehm (anamorph *Phoma cucurbitacearum* [Fr.:Fr.] Sacc.) is a major disease of cucurbits in greenhouses (1,15,7) and in warm humid climates worldwide (7,14) and is the most destructive and widespread disease of watermelon in the southeastern United States (11,12). GSB can cause 100% yield loss if the infected field is left unmanaged (David Langston, Personal

communication). Gummy stem blight can be managed by avoiding the introduction of the pathogen into transplant production houses and subsequently into the field by using pathogen free seeds and transplants. Once introduced into the field, management of GSB requires an integration of both cultural practices and chemical applications. Although new sources of genetic resistance to GSB have been identified recently (2), watermelon cultivars with a satisfactory level of genetic resistance to GSB are not yet available commercially.

Lack of GSB-resistant cultivars, limitations of cultural practices and the explosive nature of this disease force growers to rely heavily on the application of fungicides for the management of GSB. Many systemic and contact fungicides with different modes of action have been labeled for use in cucurbits against GSB. Unfortunately, *D. bryoniae* has a remarkable ability to adapt and become resistant to most of the systemic fungicides used to manage GSB. All the resistance management practices followed currently are based on the assumption that resistance build up is mostly favored by the sustained solo application of systemic fungicides. However, failure of an otherwise effective fungicide in managing GSB in a field that was never planted with watermelon before raises the possibility of introduction of already resistant isolates into the field. If this is the case, then we will have to come up with new recommendations for the management of fungicide resistance.

Introduction of a fungicide resistant isolate into a field could occur through several ways: 1) through infected seeds produced in fields treated with systemic fungicides, 2) through infected transplants treated with systemic fungicides in the transplant production houses, 3) or by wind-dispersed ascospores originating from a distant field that had been treated with systemic fungicides. Since the use of systemic fungicides is not recommended for the management of GSB in transplant production houses, selection of resistant isolates is not likely to occur in the

transplant production houses. Airborne ascospores of *D. bryoniae* were not detected in the air in Georgia in 2008, 2009 and 2010 (Stevenson et al. Unpublished), yet there were reports of severe epidemics of GSB in the state (Thomas et al. Unpublished). This particular observation suggests that airborne ascospores have limited role as primary inoculum. However, there is experimental evidence that GSB can be seed-borne (6). But the role of seed-borne inoculums as primary source of infections in the field is not studied.

Therefore, the objective of this study was to detect and isolate *D. bryoniae* from watermelon seeds and seedlings and to test the isolates for fungicide resistance. Results from this study will help us understand the origin of fungicide resistance in individual fields and thus, will help us in developing/ modifying current resistance management strategies.

Materials and Methods

Seedlots and isolation of pathogen. In 2009 and 2010, watermelon seeds and symptomatic seedlings from an unknown seed lot (seed lot A) were collected from a commercial grower's transplant house in South Georgia, where outbreaks of GSB had occurred. Watermelon seeds were obtained from commercial seed companies that were marked as seed lots B, C, D, E, and F. Information on the location of the seed production fields or fungicide use history in these fields were not provided. Some seeds were also extracted from broken fruits collected from commercial watermelon fields with severe GSB epidemics. Seeds were tested for the presence of *D. bryoniae* following magnetic capture hybridization (MCH) PCR, direct plating on PDA, seedling grow-out assays in a sweat box and on blotter paper.

MCH PCR: DNA was extracted from a seed sample following the method as previously described (3). Approximately 500 seeds were washed in a sterile side-arm flask in 500 ml of sterile PBS buffer. Using a magnetic stir bar and stir plate, seeds were continuously agitated for 1

h with the application of vacuum. The vacuum was interrupted briefly every 15 min to facilitate the extraction of pathogen propagules from under the seed coat. The seed extract was strained by passing through cheese cloth and concentrated by centrifugation at 8000 rpm for 15 min, and the pellet was resuspended in 2 ml of lysis buffer. DNA was extracted from the pellet using a bead beater as reported previously (Biospecs Products, Bartlesville, OK) (3). Crude DNA extract was later purified using sodium acetate and isopropanol precipitation as previously described (3). This purified DNA was resuspended in DIGEASY hybridization buffer and subjected to magnetic capture hybridization using streptavidin-coated magnetic beads (SCBs) coated with biotinylated hybridization capture probes, AACCAPRW and DBCAPRW to capture single-stranded target DNA as described by Ha et al. (3). Captured DNA was then released from the SCBs by incubation at 95ºC for 10 min followed by centrifugation at 15000 rpm for 5 s (3). The DNA thus obtained was amplified using a commercial PCR master-mix (Bio-Rad iQ Supermix; Bio-Rad) and primers and probes specific for *D. bryoniae* that were kindly provided by Dr. Walcott (University of Georgia). The following thermal profile was used for PCR amplification: denaturation at 95ºC for 120 s, followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 55ºC for 30 s, and elongation at 72ºC for 30 s. A baseline of 30 fluorescence units was set as the background threshold and reactions in which Texas Red- based fluorescence exceeded this value were considered positive for *D. bryoniae* (3).

Direct plating on media: Watermelon seeds were surface-sterilized for 2 min using 0.6% NaOCl and rinsed with sterile deionized water. The seeds were placed on a sterilized paper towel to remove excess moisture and air-dried in a laminar air flow hood, then carefully placed on potato dextrose agar (PDA) medium. Some seeds were also dissected and plated as this would allow more surface area of contact with the media if the pathogen is deep-seated within the seed.

The seeds were dissected longitudinally and separated into components of seed coat, perisperm layer and cotyledons. These three parts of the seed were then placed carefully on potato dextrose agar (PDA) medium and incubated at 25°C. These plates were examined daily and mycelium growing out of the seeds or seed parts was transferred to quarter-strength PDA (QPDA). Colonies were identified as *D. bryoniae* based on colony characteristics and conidia morphology. *D. bryoniae* colonies produce sparse white aerial mycelia with a dark olive-green appearance to the substrate from below on QPDA (5). Conidia are hyaline, cylindrical with rounded ends and are non- or monoseptate (5).

Sweat box assay: Aluminum pans with a clear plastic lid were used to provide high humidity and prolonged leaf wetness conditions favorable for disease development. The pan was filled with autoclaved potting medium to a depth of approximately 8 cm and the seeds were planted at a uniform depth of 3 cm and spacing of 3 × 3cm. Covered pans were maintained in the greenhouse to simulate warm humid conditions that exist in transplant production houses. The seedlings were observed daily for symptom development.

Blotter assay: Watermelon seeds were surface-sterilized in 0.6% NaOCl for 2 min and rinsed with sterile water. Fifty seeds were then evenly placed on moistened sterile blotter paper in a clear plastic box. The seeds were incubated for 10 days in complete darkness and were then placed under continuous light for 1week at 25ºC. The seedlings were observed daily from the 10^{th} day onwards for another week for symptoms and signs of GSB including water-soaked lesions and the presence of pycnidia. The pathogen was isolated from symptomatic seeds or seedlings and maintained in pure culture on PDA.

Fungicide sensitivity assays: Technical grade boscalid (98.4% a.i.; BASF Corporation, Research Triangle Park NC), tebuconazole (97.5% a.i.; Bayer Corporation, Kansas City MO)

and difenoconazole (95% a.i.; Syngenta Crop Protection, Greensboro NC) were dissolved in acetone to obtain stock solutions of 30 mg/ml. Serial dilutions of the stock solution of each fungicide were made in acetone and added to autoclaved PDA cooled to 55°C to obtain the desired concentration of 3.0 µg/ml. The final concentration of acetone in fungicide-amended and non-amended medium (acetone only) was 0.1% by volume. Technical grade thiophanate-methyl (95% a.i.; United Phosphorus Inc., King of Prussia, PA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 10 mg/ml. Ten milliliters of this stock solution was added to autoclaved PDA cooled to 55°C to obtain the desired concentration of 100 µg/ml. The final concentration of DMSO in fungicide-amended and non-amended medium (DMSO only) was 1.0% by volume.

Sensitivity of each isolate of *D. bryoniae* to tebuconazole, difenoconazole, boscalid and thiophanate-methyl was determined using an in vitro mycelial growth assay on fungicide-amended and non-amended PDA. Mycelial plugs of 6 mm diameter, taken from the margin of a 1-week-old pure culture on PDA were placed upside down in the center of fungicide-amended and non-amended PDA plates. Two replications of each isolate and fungicide concentration were prepared. After 4 days of incubation in the dark at 25°C, the diameter of each colony was measured and was corrected by subtracting the diameter of the mycelial plug. Relative growth was calculated as the ratio of the corrected colony diameter on fungicide-amended medium to the corrected colony diameter on non-amended medium. Isolates showing a relative growth of more than 0.2 were considered resistant to the respective fungicides.

Sensitivity to azoxystrobin: Technical grade azoxystrobin (Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone to make a stock solution of 10 mg/ml. One milliliter of stock solution was added to 1 liter of autoclaved water agar cooled to 55°C to obtain a final

concentration of 10 µg/ml. The final concentration of acetone was 0.1 % in both fungicide amended and non-amended medium. The medium was also amended with 100 µg/ml salicylhydroxamic acid (SHAM) to inhibit the alternative respiratory pathway in the fungus that may affect the activity of the fungicide.

Sensitivity to azoxystrobin was tested using a spore germination assay. Isolates of *D. bryoniae* were grown on QPDA for 2 weeks at 25ºC under 12:12 dark: light photoperiod to induce sporulation. Conidial suspensions of each isolate were prepared by flooding the plates with 2 ml of sterile water plus a few drops of Tween 20 and gently scraping the surface of the mycelium with a glass rod to dislodge the conidia. Ten to fifteen microliters of conidial suspension from each individual isolate was transferred to fungicide-amended and non-amended plates. Two replicate plates of each isolate and treatment combination were prepared. After incubating at 25°C for 24 h, 50 spores per plate were examined microscopically and the percentage of germination was recorded. A conidium was considered germinated if the length of the germ tube was at least half the length of the conidium. Relative germination was calculated as the ratio of the percentage germination on fungicide amended to percentage germination on non-amended media. An isolate was considered resistant if the relative germination was greater than 0.5 (13).

Results

D. bryoniae was not detected in the sample from the commercial seed lot-A using MCH PCR. However, seed dissection and plating of seeds resulted in isolation of one *D. bryoniae* isolate (Wsd-21) from seed lot-A. This isolate was found to be sensitive to boscalid, azoxystrobin and tebuconazole and resistant to thiophanate-methyl (Table 4.1). *D. bryoniae* was not successfully isolated from the seedlings in the sweat box assay. Germination of seeds was

poor in this assay, maybe due to excess moisture. A total of 4200 seeds was tested following the blotter assay and two *D. bryoniae* (R-1 and R-2) isolates were obtained from the commercial seed lot-B. These two isolates were found to be sensitive to boscalid, azoxystrobin and tebuconazole and resistant to thiophanate-methyl (Table 4.1).

Discussion

Results from this study confirmed earlier reports that *D. bryoniae* can be seed-borne on watermelon (9,10). Isolation of *D. bryoniae* from naturally infested cucumber and pumpkin seeds has been reported (6,8), but not from naturally-infested watermelon seed. All the previous studies on detection, localization and management of *D. bryoniae* in watermelon seed were conducted using artificially-infested seeds (3,4,9). This is the first report of detection of *D. bryoniae* from commercial seed lots. The level of infestation was 0.13% and 0.05% in seed lot-A and B, respectively. Since *D. bryoniae* has the potential to spread rapidly under favorable weather conditions, this level of infestation is high enough to cause a serious epidemic in transplant production houses and watermelon fields.

Didymella bryoniae isolates obtained from watermelon seeds were found to be resistant to thiophanate-methyl, an MBC fungicide, demonstrating that fungicide-resistant inoculum can survive on seeds. Therefore, fungicide-resistant inoculum can be introduced into the transplant production houses and subsequently into the watermelon production fields through infested seeds. Introduction of fungicide-resistant inoculum through seeds was suspected before when resistance to the benzimidazole fungicide, benomyl was detected in greenhouses in Greece after only one year of benomyl use for management of GSB on cucurbits (7). However, the authors ruled out the introduction of resistant isolates through infested seeds because little was known about the seed-borne nature of this pathogen at the time. Therefore, the current fungicide

resistance management strategies are based on the assumption that fungicide resistance arise within the field as a result of repeated exposure to systemic fungicides. Introduction of fungicide resistant isolates into watermelon field can negatively affect the current management strategies and signals the need for new fungicide resistant management practices.

Isolation of *D. bryoniae* from fungicide-treated seeds points to the likelihood of a deep-seated infection, rather than an external contamination of seeds. There are reports of the isolation of *D. bryoniae* from the seed coat, perisperm and cotyledons of cucumber and pumpkin seeds (6), but the mode of entry of pathogen into watermelon seeds is still unknown. Rankin in 1954 showed that watermelon seeds can be infected during the seed extraction process from the fruit if pathogen propagules are present in the fruit pulp. The pathogen can gain entry into the fruit if the fruit is split open in a heavily infected field. Rankin demonstrated that *D. bryoniae* can invade the epidermis, cotyledon and embryo of watermelon seeds even if the seeds are contaminated during the extraction process. He suggested that the pathogen may have gained entry into the seed through the hilum during the extraction process since the pathogen can penetrate the hilum when it is wet (9). It has been shown that *D. bryoniae* can enter the seed through flower infection in cucumber leading to an internal fruit rot (8). However, internal fruit rot is not a common symptom of GSB in watermelon (David Langston, personal communication) unlike the reports from Denmark (8).

Fungicide seed treatment should be enough to remove the external contamination. But if the pathogen is gaining entry into the seed during the extraction process or through flower infection leading to a deep-seated inoculum, care should be taken to identify the infected seeds and more regulations should be in place to avoid the release of a contaminated seed lot. A study on the mechanism and sources of seed infection may help us to avoid and also to manage seed

infestation. Currently seed companies are testing only a few seeds per seed lot for the presence of GSB and if these seeds are free of pathogen the whole seed lot will be released (Ronald Walcott, personal communication). Since the introduction of fungicide resistant inoculum through seeds can overwhelm the current efforts for fungicide resistance management, strict measures must be taken to avoid the introduction of fungicide resistant inoculum into watermelon fields by properly following the seed health assays.

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. Gusmini, G., Song, R., and Wehner, T. C. 2005. New sources of resistance to gummy stem blight in watermelon. Crop Sci. 45:582-588.

3. Ha, Y., Fessehaie, A., Ling, K. S., Wechter, W. P., Keinath, A. P., and Walcott, R. R. 2009. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. Phytopathology 99:666-678.

4. Hopkins, D. L., Thompson, C. M., Hilgren, J., and Lovic, B. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. Plant Dis. 87:1495-1499.

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

6. Lee, D. H., Mathur, S. B., and Neergard, P. 1984. Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedlings of cucumber and pumpkin. Phytopathol. Z. 109:301-308.

7. Malathrakis, N. E., and Vakalounakis, D. J. 1983. Resistance to benzimidazole fungicides in the gummy stem blight pathogen *Didymella bryoniae* on cucurbits. Plant Pathology 32:395-399.

8. Neergaard, E. D. 1989. Studies of *Didymella bryoniae* (Auersw.) Rehm: Development in the host. J. Phytopathology 127:107-115.

9. Rankin, H. W. 1954. Effectiveness of seed treatment for controlling anthracnose and gummy-stem blight of watermelon. Phytopathology 44: 675-680.

10. Richardson, M. J. 1979. An annotated list of seed-borne diseases. Third Ed. Int. Seed Test. Ass., Zurich, Switzerland. Page 56.

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

12. Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

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

14. Sudisha, J., Niranjana, S. R., Umesha, S., Prakash S. H., Shekhar Shetty, H. 2006. Transmission of seed-borne infection of muskmelon by *Didymella bryoniae* and effects of seed treatments on disease incidence and fruit yield. Biological control 32 (2):196-205.

15. Van Steekelenburg, N. A. M. 1983. Epidemiological aspects of *Didymella bryoniae*, the cause of stem and fruit rot of cucumber. Neth. J. Plant Pathol. 89:75-86.

Table 4.1. Sensitivity of *Didymella bryoniae* isolates obtained from watermelon seeds to boscalid, azoxystrobin, tebuconazole and thiophanate-methyl

Isolate	Boscalid	Azoxystrobin	Tebuconazole	Thiophanate-methyl
Wsd-21	Sensitive	Sensitive	Sensitive	Resistant
R-1	Sensitive	Sensitive	Sensitive	Resistant
R-2	Sensitive	Sensitive	Sensitive	Resistant

CHAPTER 5

SUMMARY

Gummy Stem blight (GSB), caused by the fungus *Didymella bryon*iae is the most destructive disease of watermelon in the southeastern United States. Management of GSB requires the integration of both cultural practices and fungicide applications. Although cultural practices can be beneficial, application of fungicides is by far the most effective method for managing GSB (3). Recently two demethylation inhibiting (DMI) fungicides, tebuconazole and difenoconazole were labeled on cucurbits for managing GSB. Another SDHI fungicide, penthiopyrad, is currently under field evaluation for GSB management. *Didymella bryoniae* has shown a remarkable ability to adapt and become resistant to systemic fungicides that were used for its management in the past (1,2,4,5). Therefore, determination of discriminatory concentration for monitoring shifts in sensitivity to effective fungicides and determination of cross-resistance between fungicides of same chemical class were of prime importance to monitor and manage fungicide resistance development. Baseline sensitivity to tebuconazole, difenoconazole and penthiopyrad was determined following a mycelial growth assay using seventy one single-lesion isolates that were never exposed to DMIs or SDHIs. The EC_{50} values for tebuconazole, difenoconazole and penthiopyrad ranged from 0.062 to 0.385 µg/ml, 0.018 to 0.048 µg/ml and 0.015 to 0.057 µg/ml. Based on the baseline sensitivity distribution, a discriminatory concentration of 3.0 µg/ml was selected for monitoring sensitivity to these fungicides. A positive correlation between sensitivity to the two DMIs and the two SDHIs in the baseline isolates showed a potential for cross-resistance between these fungicides. Cross-resistance assay using field isolates exposed to boscalid, revealed high level of cross-resistance between boscalid and penthiopyrad. Results from the field experiment conducted to establish a

relationship between frequency of resistance and fungicide efficacy showed a consistent negative association between frequency of resistance and disease control in the field. Also tebuconazole and chlorothalonil were proved to be most effective in managing GSB. An interesting outcome of this study was that the tebuconazole treatment significantly reduced the frequency of resistance to azoxystrobin and boscalid in two locations, but the underlying mechanisms are unknown and need further investigation.

Based on the results from baseline sensitivity study, fungicide resistance development can be routinely monitored using the discriminatory concentrations determined. And also evidence of cross-resistance between the SDHIs fungicides and potential for cross-resistance between the two DMIs indicates that use of the two DMIs and the two SDHIs in the same fungicide spray program should be avoided. Based on the field experiment results, a spray program including tebuconazole and chlorothalonil as rotation partners would be most effective in managing GSB and this program may even help in managing boscalid and azoxystrobin resistance over time. Detection of fungicide resistant isolates from watermelon seeds will help explain the unpredictable development of resistance in watermelon fields. Also introduction of resistant isolates through seeds signals the need for an entirely new approach to fungicide resistance management in this pathogen. In summary, the results from this study will be essential for the development of effective integrated disease management programs for GSB that minimize development of fungicide resistance.

Literature Cited

1. 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.

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

3. Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: Compendium of Cucurbit Diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.

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

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