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CHARACTERIZATION OF A NEW *BOTRYTIS* SPECIES AND FUNGICIDE
RESISTANCE IN *BOTRYTIS CINEREA* FROM BLACKBERRY

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Sciences

by
Xingpeng Li
May 2015

Accepted by:
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ABSTRACT

Gray mold caused by the fungus *Botrytis cinerea* is the economically most important pre- and postharvest disease of blackberry. As part of a South Carolina fungicide resistance monitoring program, blackberry fruit were collected to survey for pathogenic fungi. Phylogenetic as well as morphological analysis indicated a new species, described as *Botrytis caroliniana*. A rapid method using polymerase chain reaction was developed to differentiate *B. cinerea* and *B. caroliniana*. A distribution and prevalence study indicated these two species co-existed in four out of six locations investigated. The control of gray mold in commercial fields largely relies on fungicide application. Therefore, survey was conducted to determine the occurrence and prevalence of fungicide resistance. The fungicide resistance profile was described in 198 *B. cinerea* isolates from blackberry. Of these isolates, 72% were resistant to thiophanate-methyl, 59% were resistant to pyraclostrobin, 56% were resistant to boscalid, 11% were resistant to fenhexamid, 10% were resistant to cyprodinil, 8.6% were resistant to iprodione, and 1% were resistant to fludioxonil. A statistical model revealed that multifungicide resistance patterns did not evolve randomly in populations. Resistance to thiophanate-methyl, pyraclostrobin, boscalid, and fenhexamid was based on target gene mutations, including E198A and E198V in β -tubulin, G143A in cytochrome b, H272Y and H272R in SdhB, and F412I in Erg27, respectively. In addition, a new genotype associated with fenhexamid resistance was found in one strain (i.e., Y408H and deletion of P298). Two levels of resistance, low resistance

(LR) and moderate resistance (MR), to fludioxonil were found in three field isolates, and MR was caused by a previously described mutation (R632I) in transcription factor Mrr1. The LR and MR isolates were able to cause lesions and sporulated on detached fruit. The results obtained in this study contribute to our understanding of fungal biology and fungicide resistance development in gray mold fungi and are useful for improving resistance management practices.

DEDICATION

I dedicate this work to my mother Hanming Zheng and my father Youming Li, who both taught me persistence and conscientiousness at my young age. I would also dedicate this work to all the people who helped me to accomplish this work in the last five years.

ACKNOWLEDGMENTS

I very much appreciate my advisor Dr. Guido Schnabel, who is not only an excellent mentor for his students in academia but also inspires their creativity, passion and their interests beyond. I'm so grateful for his guidance in the past five years and felt so fortunate to have such an opportunity to learn from him how to be a good scientist as well as a respected person. I deeply appreciate his wisdom and patience, which gave me a precious experience in Clemson. It was a great joy and honor to work under his supervision.

I would like to bring my thanks to my committee members Dr. Julia Kerrigan, Dr. Hong Luo, Dr. Jeremy Tzeng, and Dr. Hoke Hill, for their support on my committee. I would especially like to thank my lab colleague and good friend Anja Grabke for her support with my experiments and many inspiring conversations she brought up about my research. I also want to thank Dr. Dolores Fernández-Ortuño (Lola), Dr. Mengjun Hu, Dr. Fengping Chen, Dr. Hideo Ishii, and Shuning Chen, who gave genuine advices and suggestions for my projects in the past few years. My thanks also go to Karen Bryson, our nice technician and all my past and present lab members. I would like to acknowledge Dr. Guoqing Li from Huazhong Agricultural University to provide *Botrytis fabiopsis* gene sequences and appreciate Elise L. Schnabel for her helpful comments regarding to the manuscripts of fungicide resistance study. Lastly, I want to thank the loved ones during the past few years for the endless care and support. The research presented in this manuscript was supported by CSREES/USDA, under project number SC-1000642, Clemson

University Distinguished Graduate Fellowship, and the Wade Stackhouse Graduate Fellowship of Clemson University's College of Agriculture, Forestry and Life Science.

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CHAPTER ONE

A REVIEW OF *BOTRYTIS CINEREA* CAUSING GRAY MOLD ON BLACKBERRIES

Botrytis cinerea - The Pathogen of Gray Mold Disease

The fungus *Botrytis cinerea* Pers. Fr. is the causal agent of gray mold disease on over 200 plant species, including lettuce, cucumber, strawberries and blackberries (127). It is an ascomycete which belongs to the subphylum Pezizomycotina, class Leotiomycetes, order Helotiales, and family Sclerotiniaceae (8). Gray mold is considered the second most economically important fungal plant pathogen worldwide, the most important being *Magnaporthe grisea* (20).

Etymology and taxonomy. The name *Botrytis cinerea* means “bunch of grape-like berries” and “gray color ashes”, which refers to the conidiophore and gray color of sporulation of this fungus. The genus *Botrytis* was first named by Pier Antonio Micheli in 1729, who grouped it into the "Nova Plantarum Genera", and was later revised by Hennebert (61) and *Botrytis cinerea* was first described by Persoon (53). The teleomorph is named *Botryotinia fuckeliana* (de Bary) Whetz. also known as *Botryotinia cinerea* (126). However, under the 2013 International Code of Nomenclature the accepted name is *Botrytis cinerea*.

The genus *Botrytis* currently comprises about 25 to 30 species, all of which are considered to be necrotrophic plant pathogens. Since the report of a sequenced-based

phylogeny of 22 recognized species in 2005 (107), several new species have been reported: *B. pseudocinerea* (121), *B. caroliniana* (this manuscript) (82), *B. fabiopsis* (137), *B. sinoallii* (138). There is one hybrid, polyploid species, which arose from hybridization between *B. aclada* and *B. byssoidea* (107). The current classification is largely based on morphological characters and, to a minor extent, on physiology and host range (107). *Botrytis* species were grouped into two distinct phylogenetic clades. The major clade (in terms of number of species), which can be divided into five subclades, contains mainly species from monocot hosts. The other clade contains five species, including *B. cinerea*, that only infect dicot hosts (107, 121).

Biology, Host Range and Distribution. It has been long established that *B. cinerea* is a multinucleate fungus in both hyphal cells and conidia, with numbers for conidia usually in the range 3 to 6 (52, 84). However, the microconidia, which function primarily as male gametes in sexual crosses, are uninucleate (84). Microconidia can be found in ageing cultures of the fungus or those which are contaminated by other organisms, and in association with sclerotia (63). Microconidia develop from germ tubes produced by macroconidia, more mature hyphae, inside empty hyphal cells, and from appressoria and sclerotia (65). Although their sole function is believed to be one of spematization, they may also help the fungus to survive adverse conditions (63). It's been reported that *B. cinerea* is haploid and mostly heterothallic, carrying either the mating type allele *MATI-1* or *MATI-2* (9, 29, 30). The apothecia of *B. cinerea* can be readily obtained in the laboratory following protocols refined by Faretra and Antonacci (28). Observations of apothecia in

nature, however, are extremely rare (8). One was reported from grapevine in Switzerland, and a second from a peach mummy in New Zealand (8), which suggests that sexual reproduction in the field is not common.

Botrytis species have a broad host range which is more than 200 species and able to infect many different plant species and tissues under a wide range of environmental conditions (20). These host plants include nursery plants, ornamentals, and crops, many of which are economically important such as beans, lettuce, cucumber, cabbage, and small fruits like grapes, blueberries, blackberries, and strawberries (107).

Botrytis may have originated from the northern hemisphere, since the host range primarily includes plants in the northern hemisphere. It has been suggested that most of the distribution of *Botrytis* species was through human activity (8). Now *B. cinerea* can be found in almost every continent of the world (127).

Diversity. *Botrytis cinerea* has a high level of diversity in terms of morphology and genetics. Different strains of *B. cinerea* usually expresses itself in various mycelial colors, sporulation capacity, types of aerial mycelia, and number/size of sclerotia (89). A population study based on strains collected from grapes suggested that isolates collected from the same plant always had different haplotypes and up to five different haplotypes were found in spores isolated from a single berry (47). This extensive genotypic diversity indicates limited clonal propagation and a significant role for recombination. However, this is contradictory to the finding that sexual reproduction of *B. cinerea* is rare in the field. This might indicate the high levels of genotypic diversity is not caused by sexual

reproduction. It might be caused by already diversified *B. cinerea* spreading through the population, or, caused by the transposable elements movement which might play a significant role in genotypic diversification (92).

Gray Mold of Blackberries

Blackberry is an important fruit crop in the United States and worldwide (110). The production of blackberry is often threatened by diseases and insect pests. Gray mold caused by *Botrytis* spp. is probably the most important pre- and postharvest disease of blackberry. The genus *Botrytis* Pers. was originally erected by Micheli in 1729 and later validated in 1801 by Persoon (65). The genus *Botrytis* has at times included up to 380 species according to the mycobank records (<http://www.mycobank.org/MycoTaxo.aspx>). But presently includes just over 25 species (107, 137, 138). Most species of *Botrytis* are considered specialists possessing a narrow host range (87), while *Botrytis cinerea* Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* Whetzel) has a much wider host range recorded by the United States Department of Agriculture (USDA) (25).

Knowledge of what species is causing disease is critical for disease control. Different species may have different infection requirements, host tissue preference, and fungicide sensitivity. To our knowledge only two *Botrytis* species, *B. patula* and *B. cinerea*, have been described to cause gray mold of blackberry. While *B. cinerea* is ubiquitous and commonly found on blackberry, few reports of *B. patula* are in the records of the USDA

(United States Department of Agriculture) and this species is now considered a synonym of *Olpitrichum patulum* (Sacc. & Berl.) Hol.-Jech. (62). Raspberry plants comprised of multiple other *Rubus* species have been described by USDA to be attacked by *Botrytis vulgaris* (Pers.) Fr., which is the synonym of *Botrytis cinerea* (42).

Symptoms and Signs. On blackberry, the pathogen may cause soft rots of all aerial plant parts. During early growing season rotting tissue, such as freeze-damaged flowers or fruits, produce abundant gray conidiophores and conidia (127), which are dispersed by wind and rain and can cause secondary infections. The young blossoms are usually very susceptible to infection. One or several blossoms in a cluster may show blasting, in browning and drying, which may extend down the pedicel. Fruit infections usually appear as soft, light brown, and rapidly enlarging areas on the fruit. Then the berry usually dries up and “mummifies”, also covered with a gray, dusty powder, which gives the disease its name “gray mold.”

Survival and Disease Cycle. *Botrytis cinerea* overwinters and survives in the form of sclerotia, chlamydospores, conidia or mycelium in dead plant tissues mummified berries, mulch, and weeds (Fig 1.1)(8). Sclerotia are considered to be the most important structures involved in the survival of *Botrytis* species, because they may withstand adverse environmental conditions, and produce apothecia after sexual recombination (17). Chlamydospores of *B. cinerea* are hyaline cells of extremely variable form and size (115). They are generally found in ageing cultures and commonly occur in the stromatic sectors of cultures of the fungus which are contaminated by other organisms, and in association

with sclerotia. Conidia of *B. cinerea* are generally considered as short-lived propagules in the field and their survival will largely be determined by temperature extremes, moisture availability, microbial activity and sunlight exposure (8). However, Salinas et al. (101) reported that conidia stored dry were able to survive at room temperature for up to 14 months, germinated in vitro, and were pathogenic to flowers. The survival of mycelium of *Botrytis* species under natural conditions is poorly understood. It is often difficult in practice to determine whether survival is based on mycelium, microsclerotia, or chlamydospore structures. Some evidence suggests that the mycelium of certain *Botrytis* species can survive in bulbs, seeds and other vegetative plant parts (17).

The disease cycle starts with sporulation on sclerotia or plant debris. Conidia are considered to be the primary source of inoculum for prebloom infection of flowers and leaves. The conidia are spread by both wind and rain to plant tissue surfaces. Germination can occur when temperatures are between 34-86 °Fahrenheit (or 1-30 °Celsius), and liquid water is present or the relative humidity is at least 90% (96). Fruit infections are initiated near late bloom. Hyphae from germinated spores directly penetrate through susceptible tissues such as senescing blossom parts (stigma, pistil, stamen, etc...), cap scars, and parts of old blossoms and facilitate infection. Later in the season, hyphae will penetrate directly through the epidermis of healthy berries. After infecting the berry, *Botrytis* may stay dormant in the form of mycelia in the plant tissue until the fruit sugar content increases and the acid level decreases enough to support the pathogens growth (27).

Non-Chemical Disease Management. The control of gray mold on blackberry largely relies on the integration of a variety of cultural management practices which include increased ventilation, limiting vegetative growth, and reduction of *Botrytis* spp.'s alternative hosts. The increase of ventilation is to promote air circulation to quicken drying of plant tissue by pruning the plants to open the canopy. In order to create dry conditions for the plant, the use of sites with good air drainage or high tunnels with open sides are also suggested (98). The limited use of nitrogen fertilizer can help reduce vegetative growth. Effective weed control in the field can help reduce the disease incidence by eliminating the gray mold's alternative hosts.

Although blackberries are resistant to most diseases, fruit rots do occur in some years and *B. cinerea* is likely the primary disease-causing post-harvest rot of ripe blackberries (69). Two less-sensitive cultivars, "Kiowa" and "Navaho", were identified (69), but disease incidence can still be high in years favoring fungal development. Due to the limited availability of resistant cultivars in pre-harvest and post-harvest disease control, the primary means of controlling disease in commercial blackberry production is to rely on the application of fungicides.

Biocontrol of gray mold diseases has been extensively investigated over the last 50 years (26). Genera of filamentous fungi, *Trichoderma*, *Gliocladium*, *Ulocladium*, bacteria, *Bacillus* and *Pseudomonas*, and yeasts, *Pichia* and *Candida*, have shown great potential for *Botrytis* disease control (26, 72, 83). Commercial success has been achieved in glasshouse and post-harvest environments where stable environmental conditions are

provided (26). However, environmental conditions are usually unpredictable and may influence the survival, establishment and activity of the biological control agent. Under field conditions, the plant surface is subjected to fluctuating temperatures, vapor pressure deficits, surface wetness, gases and air movement (15), which give limitations to the use of biocontrol agents. (98)

Chemical Control of Gray Mold Disease

Chemical control is the most effective method for controlling gray mold. Seven classes of site-specific fungicides are currently available for the control of gray mold disease in the United States. They include anilinopyrimidines (APs), dicarboximides (DCs), hydroxyanilides (HAs), methyl benzimidazole carbamates (MBCs), phenylpyrroles (PPs), quinone outside inhibitors (QoIs; disease suppression only) and succinate dehydrogenase inhibitors (SDHIs). For blackberry, there are four classes of fungicides recommended, Roval (Iprodione), Elevate (Fenhexamid), Pristine (Pyraclostrobin+Boscalid), and Switch (Cyprodinil+Fludioxonil) (98).

Among these site-specific fungicides, MBCs were introduced in the 1970s and therefore have been used for the longest period of time. The DCs were also introduced in the 1970s but usage dropped in 1999 after the US Environmental Protection Agency in 1999 drastically limited the maximum number of applications because of concerns about dietary exposure. The first QoI, SDHI, AP, and HA fungicides were registered within one

to four years of each other in the US; in 2001, 2003, 2001, and 1999 for disease control of strawberries and 2001, 2003, 2003, and 2002 for disease control of blackberries, respectively (3). While most active ingredients are sold as solo products, some are sold as mixtures. For example, the QoI pyraclostrobin and the SDHI boscalid are sold as Pristine 38 WG (BASF Corporation, Research Triangle Park, NC) or the AP cyprodinil and the PP fludioxonil are sold as Switch 62.5 WG (Syngenta Crop Protection, Inc. Greensboro, NC). Most conventional growers use several different chemical classes of fungicides during the season in mixtures or rotations for resistance management of gray mold disease. Seven chemical classes of fungicides that were registered to against *Botrytis* can be grouped according to their modes of action: fungal respiration inhibitors (QoI and SDHI), anti-microtubule toxicants (MBC), osmoregulatory inhibitors (DC and PP), sterol biosynthesis inhibitors (HA), and methionine biosynthesis inhibitors (AP).

All of these seven classes of fungicides have a different modes of action and different target sites. QoI fungicides inhibit the mitochondrial respiration of fungus by binding to the cytochrome bc1 enzyme complex (complex III) at the Qo site. SDHI fungicides target succinate dehydrogenase (SDH, so-called complex II in the mitochondrial respiration chain), which is a functional part of the tricarboxylic cycle and linked to the mitochondrial electron transport chain. MBC fungicides inhibit fungal tubule function and block the polymerization of tubulin, thus preventing nuclear division of fungal cells. DC and PP fungicides interfere with the osmotic signal transduction pathway, which affects germination of spores and growth of mycelium (with different target enzyme, DC targets

histidine-kinase (OS-1) and PP's target is unknown). HA fungicides target 3-keto reductase (Erg27) in the sterol biosynthesis pathway. AP fungicide inhibit methionine biosynthesis and secretion of hydrolytic enzymes.

Fungicide Resistance and Resistance Management

The definition of the term “fungicide resistance” was first proposed in 1984 following the EPPO (European and Mediterranean Plant Protection Organization) conference on fungicide resistance in Brussels (23). It was defined as stable, inheritable adjustment by a fungus to a fungicide, resulting in a less than normal sensitivity to that fungicide (toxicant). This term is generally used for strains of a sensitive species which have changed, usually by mutation, to be significantly less sensitive to a toxicant (23). The growth or development of the resistant fungus strain is often uncompromised by the fungicide at concentrations which are inhibitory to the original wild-type population (23). Usually, resistance can be observed in field isolates after exposure to the fungicides. In some particular cases, there are isolates with natural resistance to certain kinds of fungicides. It has been reported that *B. pseudocinerea* is naturally resistant to fenhexamid (39), for example.

Mechanisms of Resistance. The molecular basis of resistance has been described for six out of seven classes of fungicides registered against *B. cinerea*. Fungicide resistance is most commonly caused by target gene modifications in form of point mutations (1, 7,

11, 37, 51, 64). The resulting amino acid changes can cause structural changes of the protein, which results in low affinity and thus reduces fungicide efficacy (112). The mechanism of resistance for PP is caused by an increase of fungicide efflux out of cells. It is caused by the over expression of transporter proteins (59, 60, 73, 90, 118). The details of this resistance mechanism will be introduced in the following section.

Multi-Drug Resistance. Multidrug resistance (MDR) is the resistance of organisms to a variety of unrelated toxic compounds. The resistance is caused by a transporter protein located on the membrane, which transports a wide variety of undesired compounds out of the cell. It was first described in mammalian cells resistant to anti-tumor drugs and has been described in various other classes of organisms (77). In fungi, this phenomenon was extensively studied in *Saccharomyces cerevisiae* with the name of pleiotropic drug resistance (PDR). There are two categories of membrane proteins, ATP-Binding Cassette transporters and Major Facilitators Super-family transporter. Both are able to cause multi drug resistance in *B. cinerea* (73, 76, 90).

ABC transporters (ATP-Binding Cassette) are able to bind and hydrolyze ATP and use the energy generated to transport a variety of chemicals across cell membranes. The structural unit of an ABC transporters is composed of two homologous halves, each containing six trans-membrane domains (TMDs) and a conserved nucleotide binding fold (NBF). The majority of ABC transporters are composed of 1300-1600 amino acid residues (109). The complete genome sequence of *B. cinerea* revealed 46 putative ABC proteins (2) and 13 of them have been cloned (59, 118, 133). In this array of ABC transporters, only

AtrB is found to be related to the multi-drug resistance in the field isolates of *B. cinerea* (73, 76). The resistance is caused by point mutations in the gene that encodes transcription factor Mrr1, which leads to the overexpression of *atrB* that encodes AtrB transporter. This type of resistance is also called MDR1. Isolates with the MDR1 phenotype show reduced sensitivity to APs and PPs, as well as tolnaftate, a chemical that has never been sprayed in the field (50, 73).

MFS transporters (Major Facilitators Super-family) facilitate the transport of various compounds using energy from electrochemical gradients across membranes without the hydroxylation of ATP. They are usually composed of 400-800 amino acid residues and share a common topology consisting of two-times six TMDs separated by a large cytoplasmic loop and some of them exhibit two additional TMDs at the C-terminal domain of the protein (109). In *B. cinerea* field isolates, it has been reported that a special re-arrangement of the promoter region of the *mfsM2* gene can cause the overexpression of this MFS transporter, thus leads to the resistance phenotype which is called MDR2 (73, 90). Isolates with MDR2 phenotype show reduced sensitivity to AP, DC, and HA, as well as tolnaftate and cyclohexamid (73). Both tolnaftate and cyclohexamid have not been applied in the field for disease control and can be used to detect the MDR2 phenotype. MDR3 phenotypes possess both MDR1 and MDR2 (73). These isolates have reduced drug sensitivity to all of the chemicals in the spectrum of MDR1 and MDR2 (73).

Resistance Management. The development of fungicide resistance is threatening plant health and yields quality, which eventually bring a serious issue to growers, pesticide

companies, and the environment (122). It may reduce farmers' income by requiring more fungicide applications to control the disease and exposes the environment to more pesticides than necessary. Despite the intensive screening by companies, fungicides with original modes of action, meeting safety requirements, are rarely discovered (122). It is said that it costs 10 years and about \$200 million dollars for the manufacturers to develop a new fungicide product (49). Thus, a successful resistance management strategy is helpful to preserve the efficacy of currently registered fungicides to against the plant pathogen in the fields. In general, the resistance management has two major component: monitoring and anti-resistance strategy.

Fungicide resistance monitoring is a vital component of the resistance management strategies. It provides important information about the resistant profile and evolution of resistance in the field. The information obtained can help to predict the emergence of resistance, develop management strategies, and provide fungal materials to advance the knowledge of fungicide resistance (122).

The other component, anti-resistance strategies, are based on the skillful deployment antifungal compounds to delay resistance. Fungicides may be alternated over time at the seasonal or multiseasonal scale or be used as a mixture of two different chemical classes. However, the ranking of these two strategies (alternation and mixture) based on their efficacy to delay resistance may seem to be a riddle (122). There is evidence showing that a mixed application containing compounds with different selection pressures is associated with longer-term sustainability of pest or pathogen control. And the chemical

mixtures usually outcompete other resistance management strategies because they ensure ‘multiple intragenerational killing’ (100). However, alternations also have advantages under certain circumstances. A simple argument shows that alternation is effective to delay the resistance development only when resistance comes with a fitness cost to the resistant strain in the absence of the selection pressure from the target fungicide. The reasoning behind this is based on the assumption that the resistance to alternative fungicide can be developed in all populations (sensitive or resistant to previous applied fungicide) with the same chance. Without a fitness cost, the fraction of the pathogen population resistant to the fungicide will not change during the time window where the target fungicide is not used (Fig. 1.2.) (24, 103). In that case, it might be true that the resistance developed to one class of fungicide is only relevant to the overall doses of the chemical applied to that field. The alternation would delay the resistance to build up by replacing the usage of certain chemicals with the others. In the situation when resistance does come with a fitness cost, alternation of fungicides is potentially a powerful strategy (103). These results have been also obtained from antibiotic resistance models (10, 12, 24).

Resistance management is in the best interest of the grower and the provider of pesticides. The Fungicide Resistance Action Committee (FRAC) was established in 1981 to “provide fungicide resistance management guidelines to prolong the effectiveness of at risk fungicides and to limit crop losses should resistance occur (www.frac.info)”. FRAC was funded by major fungicide manufacturer companies and aims to: 1, Identify existing and potential resistance problems. 2, Collate information and distribute it to those involved

with fungicide research, distribution, registration and use. 3, Provide guidelines and advice on the use of fungicides to reduce the risk of resistance developing, and to manage it should it occur. 4, Recommend procedures for use in fungicide resistance studies. 5, Stimulate open liaison and collaboration with universities, government agencies, advisors, extension workers, distributors and farmers. FRAC has provide multiple resources for the fungicide management practices including “Mode of Action Code List”, “Pathogen Risk List”, “Recommendations for Fungicide Mixtures”, etc... to help achieve its goal.

As stated above, blackberry is a valuable commercial crop and that IPM practices are needed to control key pests. Gray mold is a key pathogen that can only be controlled effectively with fungicides. However, the safest products are vulnerable to resistance development.

Aim of This Study

The objectives of this study were to:

- identify and determine the *Botrytis* species causing gray mold disease of blackberry in the southeastern United States
- assess the occurrence and distribution of the *Botrytis* species in North and South Carolina blackberry fields
- develop a molecular technique to differentiate co-existing *Botrytis* species in the field

- assess the occurrence and frequency of resistance to seven classes of fungicides in *B. cinerea* from commercial blackberry fields in the Carolinas
- Identify and determine the molecular bases of resistance

The information obtained from this study will help us understand the pathogenic *Botrytis* species in the Carolinas' blackberry fields. Fungicide resistance information will help to determine fungal evolution under fungicide pressure as well as develop management strategies that avoid the selection of resistant phenotypes that would eventually maintain the effectiveness of fungicides and avoid control failure.

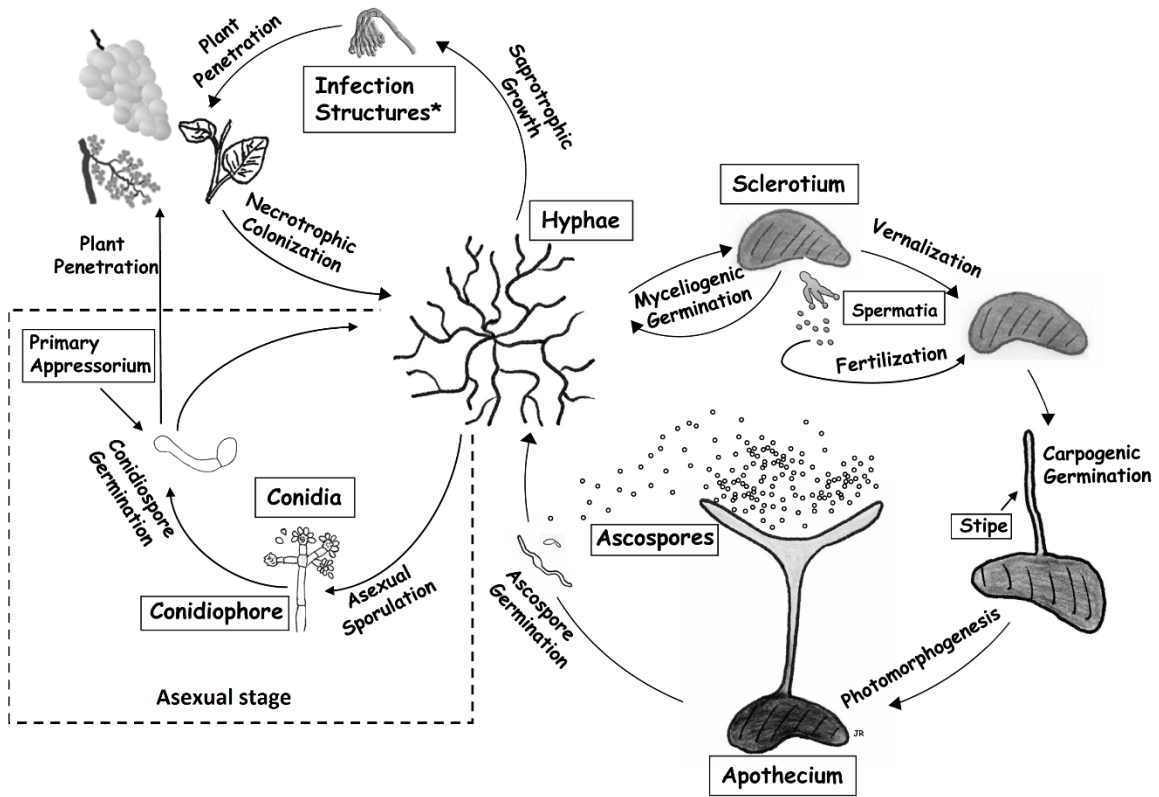


Fig. 1.1. Disease cycle of *Botrytis* gray mold diseases (adapted from Amselem, 2011 (2))

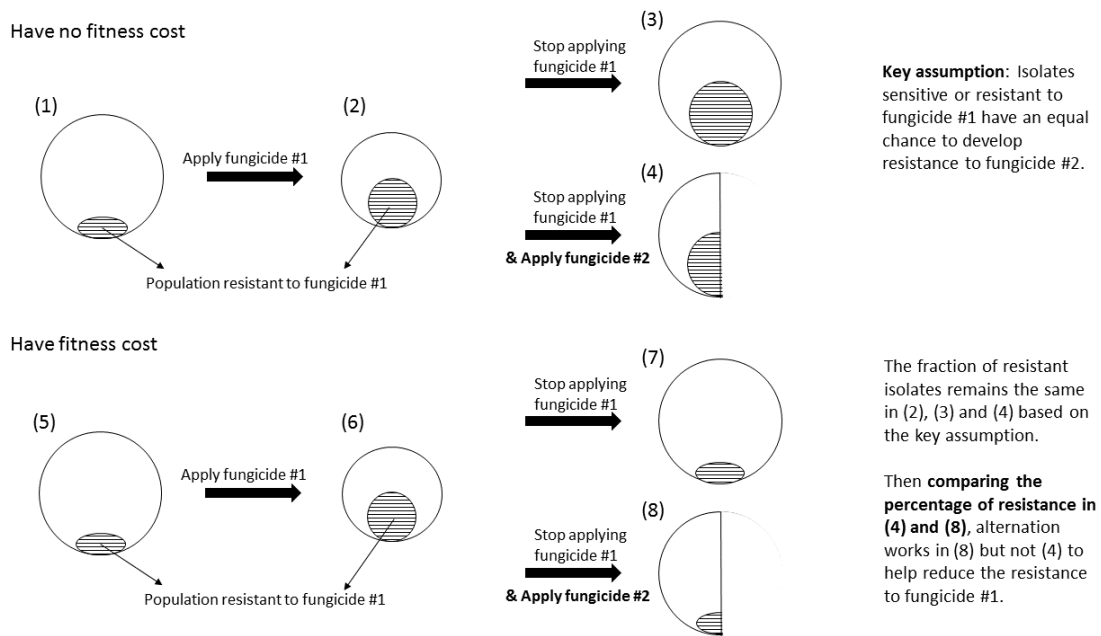


Fig. 1.2. Effect of fitness cost on efficacy of fungicide alternation.

CHAPTER TWO

***BOTRYTIS CAROLINIANA*, A NEW SPECIES ISOLATED FROM BLACKBERRY IN SOUTH CAROLINA**

This work has been published:

X.P. Li, J. Kerrigan, W. Chai and G. Schnabel. 2012. *Botrytis caroliniana*, a new species isolated from blackberry in South Carolina. *Mycologia*. 104(3):650-658.

[Fungal isolates used in this study were collected by Anja Grabke and Xingpeng Li, and single spore isolation was performed by Wendi Chai and Xingpeng Li, and “NEP1/2 sequences of *B. fabiopsis*” were provided by Jing Zhang, Huazhong Agricultural University]

Abstract:

Blackberry fruit symptomatic for gray mold were collected from three commercial blackberry fields located in the northwestern part of South Carolina. Single spore isolates were generated and two distinct phenotypes were discovered in each location; one sporulated on PDA and one did not. One isolate of each phenotype and location (six isolates total) were selected for in depth molecular and morphological characterization. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), heat-shock protein 60 (HSP60) and DNA-dependent RNA polymerase subunit II (RPB2) coding sequence alignment revealed *Botrytis cinerea* as the sporulating phenotype and a new, yet undescribed species as the non-sporulating phenotype. The new *Botrytis* sp., described herein as *Botrytis*

caroliniana, was most closely related genetically to *B. fabiopsis* and *B. galanthina*, the causal agents of gray mold disease of broad bean and snowdrop, respectively. It produces smaller conidia than either *B. fabiopsis* or *B. galanthina* and sequence analysis of genes encoding necrosis and ethylene-inducing proteins (NEPs) also indicated that the *Botrytis* isolates represent a separate and distinct species. The new species is pathogenic on blackberry fruits and broad bean leaves, which distinguishes it further from *B. galanthina*. The new species formed white to pale gray colonies with short, tufted aerial mycelium and produced black sclerotia on PDA at 20 C. To our knowledge this is only the third *Botrytis* species discovered to cause disease on blackberry in the United States.

Introduction

Blackberry (*Rubus fruticosus* and other species) is an important fruit crop in the United States and around the world. In 2005, an estimated 20035 ha of blackberries were commercially cultivated worldwide, 7159 ha in North America and 4818 ha in the United States, which is the second largest producer (110). Blackberries rank highly among fruits for antioxidant strength, particularly due to their dense contents of polyphenolic compounds such as ellagic acid, tannins, ellagitannins, quercetin, gallic acid, anthocyanins and cyanidins (55, 120).

Gray mold caused by *Botrytis* spp. is probably the most important pre- and postharvest disease of blackberry. The genus *Botrytis* Pers. was originally erected by Micheli in 1729 and later validated in 1801 by Persoon (65). The genus has at times

included up to 380 names according to the mycobank records (<http://www.mycobank.org/MycoTaxo.aspx>). But presently includes just over 20 species (107, 137, 138). Most species of *Botrytis* are considered specialists possessing a narrow host range (87), while *Botrytis cinerea* Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* Whetzel) has more than 200 hosts recorded by the United States Department of Agriculture (USDA) (<http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm>; (25). On blackberry, the pathogen may cause soft rots of all aerial plant parts. During early growing season rotting tissue, such as freeze-damaged flowers or fruits, produce abundant gray conidiophores and conidia (127), which are dispersed by wind and rain and can cause secondary infections. To our knowledge only two *Botrytis* species, *B. patula* and *B. cinerea*, have been described to cause gray mold of blackberry. While *B. cinerea* is ubiquitous and commonly found on blackberry, few reports of *B. patula* are in the records of the USDA (United States Department of Agriculture) and this species is now considered a synonym of *Olpitrichum patulum* (Sacc. & Berl.) Hol.-Jech. (62). Raspberry plants comprised of multiple other *Rubus* species have been described by USDA to be attacked by *Botrytis vulgaris* (Pers.) Fr., which is the synonym of *Botrytis cinerea* (42).

As part of a South Carolina fungicide resistance monitoring program, blackberry fruits were collected to survey for pathogenic fungi. During this survey, two species of *Botrytis* were found. This study was undertaken to characterize and identify the species of *Botrytis* using sequence analysis and morphology. Additionally pathogenicity studies were undertaken to understand the role of these species in fruit decay.

Material and Methods

Sample collection, single-spore isolation and selection of isolates. Symptomatic blackberry fruit were collected in Aug 2010 from three commercial farms (GPS coordinates +34°46'27",-83°15'31"; +34°48'43",-82°50'6"; +34°34'39",-82°29'48"; designated CB, CA, and WM) in the north-western part (Piedmont) of South Carolina, USA. The farms were located within 200 miles of each other. Each isolate came from an individual fruit from different plants with at least one buffer plant in between the sampled plants. Each fruit was placed in an individual plastic bag, sealed and stored at 4 C for up to five days. For single spore isolation, conidia were scraped off without touching the fruit using a sterile scalpel and suspended in 1 ml sterile, distilled water with 1% tween-60. Then, 200 µl of the suspension were spread on water-agar (brand) amended with lactic acid (0.1%, v/v) and streptomycin (100 µg/ml) in Petri dishes (90 mm diam). After incubation at 22 C for 24–36 h, three germinated conidia were transferred and placed 2 cm apart in equal distances onto potato dextrose agar (PDA) amended with streptomycin (100 µg/ml) and propiconazole (0.1 µg/ml) and Petri dishes were incubated at 22 C for another 36 h. Only one of the three single-spore colonies was kept for further studies. Upon culturing single-spore isolates from all locations on KMB medium (4), some isolates sporulated after seven days in culture and some never produced conidia in culture. One isolate of each type from each location (six isolates total) were selected for further analysis.

DNA extraction and PCR amplification. Genomic DNA was extracted as described previously (16). An aliquot of 1 μ l of DNA solution (about 50 ng of DNA) was added as template for polymerase chain reaction (PCR) of the ITS region or the partial sequences of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), heat-shock protein 60 (HSP60) and DNA-dependent RNA polymerase subunit II (RPB2). The primer pair ITS1/ITS4 was used to amplify the ITS1-5.8S-ITS2 region (White et al. 1990). Primer pairs G3PDHfor/G3PDHrev, HSP60for/HSP60rev and RPB2for/RPB2rev were used to amplify majority fragments of the G3PDH, HSP60 and RPB2 genes (107). PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, Ohio) following manufacturer's instructions and directly sequenced in both directions at the Clemson University Genomics Institute, Clemson, South Carolina using LiCOR dye-terminator sequencing technology. Some PCR products were cloned prior to sequencing if PCR yield was insufficient for direct sequencing. Briefly, PCR products were extracted from 1.5% agarose gel using the MinElute Gel Extraction Kit (QIAGEN Sciences, Maryland), ligated into the pCR4-TOPO vector (Invitrogen Co., Carlsbad, California) and transformed into competent cells of *Escherichia coli* DH5 α -T1. Positive *E. coli* clones grown on Luria-Bertani agar medium (brand) containing ampicillin (50 μ g/ml) were selected and sequenced in both directions using vector-specific primers. ITS and nuclear gene sequences were analyzed and aligned with DNASTAR sequence analysis software (DNASTAR, Inc., Madison, Wisconsin).

Phylogenetic analysis. G3PDH, HSP60, and RPB2 gene sequences of the selected isolates collected for this study were subjected to phylogenetic analysis separately and as combined datasets and compared to corresponding sequences of other recognized *Botrytis* species (107) using *Monilinia fructigena* strain 9201 as an outgroup. Alignment of the DNA sequences was examined by the neighbor-joining (NJ) method (Kimura's two-parameter distances) in DNAMAN software (Version 6.0.3. Lynnon BioSoft, USA): Pairwise alignment parameters: gap opening penalty 15, gap extension penalty 6.66; multiple alignment parameters: gap opening penalty 15, gap extension penalty 6.66, delay divergent cutoff 30% and 1000 bootstrap replicates were performed. Nucleotide gaps/missing data in the DNA sequences were deleted (111).

Analyses of NEP genes. To further investigate the relationship between *B. fabiopsis*, *B. galanthina*, and the species isolated in this study, the necrosis and ethylene-inducing proteins 1 and 2 (NEP1 and NEP2, respectively) were amplified with primer pairs NEP1for/NEP1revB and NEP2forE/NEP2revE, respectively, as described previously (106). PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, Ohio) following manufacturer's instructions. Purified PCR products were sequenced in both directions as described above. Multiple sequence alignment of NEP1 and NEP2 was conducted and homology trees were produced with DNAMAN software (Version 6.0.3. Lynnon BioSoft, USA). Alignment parameters were identical to the ones described above.

Phenotypic characterization of single-spore isolates. Colony morphology and mycelium growth rate were determined on PDA in Petri dishes. Agar plugs (6 mm diam) containing actively growing mycelium were removed from the colony margin of 2-day-old cultures grown at 20 C using a cork borer and the mycelium side of the plug was placed individually onto the center of Petri dishes (90 mm) containing 30 ml of PDA. The dishes were incubated at 20 C in the dark and the radial growth rate was determined after 24 and 48 h incubation. After 18 days three dishes of three isolates from different location each, were randomly selected to measure sclerotia length and width.

Conidia and conidiophores production. Mature blackberry fruit of an unknown cultivar were purchased from the local grocery store, surface sterilized and used to produce conidia for morphological studies. Fruit were surface sterilized in 5% bleach for 10 min and rinsing twice with sterile water. Agar plugs (6 mm diam) containing actively growing mycelium were removed from 2-day-old cultures grown on PDA and placed individually onto the top of a blackberry fruit. Each inoculated blackberry was placed singly in a Magenta box (60×60×95 mm), which were incubated at 20 C for 7 d at 12 h intervals of fluorescent light and darkness. The infection where the conidia suspension was placed were examed to ensure that no other source of pathogen was involved. Conidiophores and conidia produced by each isolate on where the conidia inoculation settled were examined under a microscope (Olympus BX60F) using Isolution Lite software (Vancouver, British Columbia, Canada). Length and width of 10 conidiophores and 50 conidia per isolate were measured.

Pathogenicity of *Botrytis spp.* isolates on broad bean. Broad beans cv. 'Windsor' were grown from seed in the greenhouse between 21–28 C and 14/10 daylight/night cycle. Leaves were cut off at the stem end with a razor blade from the middle part of 3 to 6-week-old plants. Agar plugs (6 mm diam) containing actively growing mycelium were removed from 2-day-old colonies grown on PDA and placed upside down on the center of detached leaves. For each isolate, five leaves (each from a different plant) were used and each was inoculated with a single plug. Leaves were placed onto moist paper towels in plastic trays (50×24 cm) in a randomized complete block design with 3-tray replicates. The trays were covered with a transparent plastic foil (Polyvinyl-Chloride, Fisher scientific, Pittsburgh, Pennsylvania) to maintain high humidity and incubated at 20 C under fluorescent light and in darkness each for 12 h d⁻¹. Uninoculated bean leaves served as negative controls. After three days, the diameters of developing lesions were measured. All in vitro experiments were replicated once.

Data analysis. Datasets of two independent experiment in vivo were combined after verifying homology of variances using Bartlett's test ($P = 0.221$). All data were analyzed using one way ANOVA and means were separated using Fishers' LSD at $\alpha = 0.05$. All statistical analyses were conducted using SigmaStat version 3.0 (Jandel Co. San Rafael, CA).

Results

The isolates collected from blackberry fruit in commercial orchards from South Carolina were identified as *Botrytis cinerea* and a new undescribed species based on

molecular and morphological characteristics. The new species is designated *Botrytis caroliniana*

Analysis of G3PDH+HSP60+RPB2 gene sequences, NEP1 gene sequences, and the ITS regions. The combined data set of G3PDH+HSP60+RPB2 sequences of the new species was therefore compared to *B. fabiopsis* and *B. galanthina* and eight other closely related *Botrytis* spp. including *B. fabae*, *B. cinerea*, *B. aclada*, etc. A total of 22 taxa of *Botrytis* spp., in addition to *B. fabiopsis* and one of *M. fructigena*, were included in this study and represented 2965 nucleotides. Maximum parsimony (MP) analysis of the combined dataset produced a single most parsimonious tree (Fig. 2.1.). A distinct clade with 100% bootstrap support was formed between all *Botrytis* spp. and the closely related outgroup *M. fructigena*. Within the *Botrytis* clade, *B. cinerea* and *B. fabae* formed a clade distinct from all other *Botrytis* species. Isolates of *B. caroliniana* were clustered in a separate clade with 100% bootstrap support. *B. caroliniana* was most closely related to *B. fabiopsis* and *B. galanthina*. The three gene sequences (G3PDH, HSP60 and RPB2) from *B. caroliniana* isolates CB15, CA3 and WM4 were submitted to GenBank (Table 2.1.)

Nucleotide sequence variation in the NEP1 gene was previously used to distinguish closely related *B. fabiopsis* and *B. galanthina* (106, 137); therefore, this gene was used to further distinguish *B. caroliniana* from *B. fabiopsis* and *B. galanthina*. Comparison of the 749-bp core sequence of the NEP1 gene showed that the three species formed a clade separate from *B. cinerea*. A distinct nucleotide identity difference was also observed between *B. fabiopsis* and *B. caroliniana* (90%) and between *B. caroliniana* and *B.*

galanthina (97%; Fig. 2.2). NEP1 sequences from three *B. caroliniana* isolates were submitted to GenBank (accession nos. JF811593, JF811594 and JF811595). The comparison of the 808-bp core sequence of the NEP2 genes showed 98.9% nucleotide identity between *B. caroliniana* and *B. galanthina*; and 98.4% identity between *B. caroliniana* and *B. fabiopsis* (data not shown).

The complete ITS1-5.8S-ITS2 regions of *B. caroliniana* isolates CB15 and CA3 were 463 bp in length and 100% identical. The same region for isolate WM4 was 464 bp in length and revealed 99.8% identity compared to the above mentioned regions. The ITS sequences of the three isolates CB15, CA3, and WM4 were submitted to GenBank (Accession nos. JF777531, JF777532 and JF777533). In contrast to the above mentioned coding sequences, the ITS sequences were non-informative for delineating *B. caroliniana* from other *Botrytis* spp. There were very few to no ITS nucleotide sequence differences between the new *Botrytis* species and many other *Botrytis* spp. including closely related *B. fabiopsis* and distantly related *B. cinerea* (data not shown).

Morphological analyses. Radial colony growth rates of *B. caroliniana* isolates fell within the values obtained for our *B. cinerea* isolates. Interestingly, our *B. cinerea* isolates appeared to be growing slower compared to published data (137)(Table 2.3.); however a direct comparison was not possible in this study. Cultures of *B. caroliniana* on PDA were white to pale gray with fluffy then matted and tufted aerial mycelium and black sclerotia in concentric rings, but did not produce conidia (Fig. 2.3.). In contrast, *B. cinerea* colonies collected from the same farms were gray-white with less fluffy aerial mycelium and

abundant conidia formation. The sclerotia of *B. caroliniana* were black and thus distinguishable from *B. fabiopsis* (Table 2.3.). On inoculated blackberry fruit the *B. caroliniana* conidiophores were as long as *B. fabiopsis* conidiophores (137) but half the size of *B. cinerea* conidiophores. Conidia of *B. caroliniana* were indistinguishable in size from *B. cinerea* conidia (data not shown) but were significantly smaller than *B. galanthina* or *B. fabiopsis* conidia (137) (Table 2.2).

Pathogenicity. Koch's postulates were fulfilled for *B. caroliniana* on blackberry and broad bean. The fungus was able to infect blackberries via mycelium and spore germination (data not shown), colonize the tissue and produce conidia on the host (Fig. 2.4.). The conidia were reisolated and confirmed to be *B. caroliniana* according to conidiophore and conidia morphology on PDA and the fact that they could not produce spores on PDA. The spores of the *B. caroliniana* from the blackberry were harvested to cause disease on broad beans leaves (Table 2.4.) in the form of necrosis of leaf tissue (data not shown). Mycelia inoculation also performed as an inoculation method and after three days conidia were produced on the leaves, reisolated, and confirmed to be *B. caroliniana*.

Botrytis caroliniana X.P. Li & G. Schnabel sp. nov. Fig. 2.3. – 2.4.

Mycobank MB561754

Etymology, “*caroliniana*” refers to the place, South Carolina, where the fungus was originally isolated.

Coloniis in PDA ad 20 C 9.5–11.5 mm diam d⁻¹ crescentibus, albis et pallidis cinereis, mycelio aereo primo floccoso tum implicito et caespitoso. Sclerotiis in PDA ad 20

C in annulis concentricis dispositis, discretis vel aggregatis, nigris, irregularibus, 0.9–3.7 x 0.9–10.1 mm. Conidiophoris et conidia ex *Rubus fruticosus* fructibus non in PDA. Conidiophoris erectis, septatis, brunneis usque subhyalinis ab basibus usque apicibus, cellula basali inflata contracta usque ad apicem, alternis vel verticillatis ramis ad apicem, 649–1698 × 9.1–18.5µm, cellulis conidiiferis inflatis. Conidiis subhyalinis vel pallidis brunneis, glabris, ellipsoideis, ovoideis vel obovoideis, variabilibus, 7.63–15.53 × 6.03–11.98 µm.

Holotype. USA, SOUTH CAROLINA, from a diseased fruit collected from *Rubus fruticosus*, Aug 2010, collector Xingpeng Li and Guido Schnabel, strains CB15, CA3 and WM4.

Colonies on PDA at 20 C growing 9.5–11.5 mm diam d⁻¹ (Table 2.2.), white to pale-gray, with aerial mycelium at first floccose then matted and tufted (Fig. 2.3.). On PDA at 20 C, sclerotia arranged in concentric rings, discrete to aggregated, black, irregular in shape, 0.9–3.7 x 0.9–10.1 mm (Fig. 2.3.). Conidiophores and conidia produced on *Rubus fruticosus* (blackberry) fruits, not seen in PDA culture. Conidiophores erect, thick-walled, smooth, septate, brown to subhyaline from the base to apex, with swollen basal cell, gradually tapering to the apex, with alternate or whorled branches near the top, 649–1698 × 9.1–18.5 µm, with multiple inflated conidiogenous cells. Conidia subhyaline to light brown, smooth, ellipsoid, ovoid, or obovoid, variable in size, 7.63–15.53 × 6.03–11.98 µm. Teleomorph not seen. Differing significantly from other *Botrytis* spp. in DNA sequence structure.

Discussion

In this study two *Botrytis* species were isolated from blackberry fruit grown in three locations of South Carolina including *B. cinerea*, which is commonly found on blackberry but has not been formally reported on that host in South Carolina, and a new species designated *B. caroliniana*. The new species was genetically and morphologically sufficiently distinct from other described *Botrytis* species and represents a new species and pathogen causing gray mold of blackberry.

Phylogenetic analysis of nucleotide sequences of G3PDH, RPB2 and HSP60 genes clearly distinguished *B. caroliniana* from other *Botrytis* species. Those coding sequences were used previously to characterize and delineate *Botrytis* species (107). *B. caroliniana* was genetically closest to *B. fabiopsis*, a species discovered in China and first reported in 2010 on broad beans (137). Because *B. caroliniana* was genetically closest related to *B. fabiopsis* and *B. galanthina*, we cloned and sequenced the NEP1 gene from *B. caroliniana*, which was used in previous studies to distinguish between *B. fabiopsis* and *B. galanthina*. The alignment of this gene sequence from *B. caroliniana* with those from the two closely related species support the above mentioned phylogenetic analysis of G3PDH, RPB2 and HSP60 genes and further supported the genetic-based distinction between the three species. The ITS regions 1 and 2, which are commonly used to differentiate species and genera in fungi (97) revealed little phylogenetically informative variation between *B. caroliniana* and other *Botrytis* species, which is consistent with earlier reports (106, 107).

The phylogenetic differentiation of *B. caroliniana* from other *Botrytis* species was supported with morphological characteristics. The branching types of conidiophores, conidia size and sclerotia arrangement on PDA were different from *B. cinerea*, *B. fabiopsis*, and *B. galanthina* (137) (Fig. 2.3. – 2.4., Table 2.2. – 2.3.). The conidiophores exhibited whorled branches compared to alternate branches described for *B. fabiopsis* (137). The dimension of conidia produced by *B. galanthina* (114) and *B. fabiopsis* (137) are bigger than conidia produced by *B. caroliniana*. The size of sclerotia of *B. fabiopsis* were reported to be 1.0-29.2×0.9–8.0 mm which is much bigger than sclerotia produced by *B. caroliniana* (0.9×10.1-0.9×3.7 mm) (137). The sclerotia produced by *B. caroliniana* were black, which is also different from the gray sclerotia produced by *B. fabiopsis* (137).

The host range of *B. caroliniana* is not yet known, but in this study we verified it can cause disease on plants from different classes. With the exception of *B. cinerea*, which infects more than 200 eudicot hosts, all other known *Botrytis* species infect only one or few closely related species within the same plant genus (87). *B. fabae*, for example, can infect species of the genera *Vicia*, *Lens*, *Pisum*, and *Phaseolus*, all belonging to the *Fabaceae* (66). All *Botrytis* species with a narrow host range are pathogenic on corolliferous monocotyledons and on members from the four eudicot families *Fabaceae*, *Ranunculaceae*, *Geraniaceae*, and *Paeoniaceae* (66). In this study we established blackberry and broad bean belonging to the plant classes *Eudicots* and *Magnoliopsida*, respectively, as hosts for *B. caroliniana*. This host range differentiated this species even further from *B. galanthina*, which is a pathogen of *Galanthus* spp. (57, 114) but not broad bean (137).

Botrytis cinerea has more than 200 hosts and cause serious yield losses in many agronomically important crops, such as grapevine, tomato, bulb flowers and ornamental crops (25, 66). It has been reported to cause blackberry disease all around the world including Australia, China, New Zealand, South Africa, Norway, and the United States according to the records of USDA. (<http://nt.ars-grin.gov/fungaldatabases/fungushost/FungusHost.cfm>). In the United States, *B. cinerea* was only reported on *Rubus* species in Alaska, California, North Carolina and Washington.

Table 2.1. Origin of *Botrytis* spp isolates used in this study and relevant GenBank

accession numbers of nucleotide sequences

Species	Strain	Origin	GenBank accession numbers				
			ITS	G3PDH	HSP60	RPB2	NEP1
<i>B. cinerea</i>	CB17	Long Creek, SC	JN672673	JN672671	JN672675	JN672677	JN672679
	WM6	Six Mile, SC	JN164269	JN164270	JN164271	JN164272	JN672681
	CA25	Cheddar, SC	JN672674	JN672672	JN672676	JN672678	JN672680
	BC-27	Ba Dong, China	EU563124	EU563107	EU563101	EU563118	
	BC-1	Wuhan, China	EU563120	EU563103	EU563096	EU563113	N/A
	BC-15	Xian Ning, China	EU563123	EU563108	EU563099	EU563114	N/A
	MUCL87 (ex type)	The Netherlands	N/A	AJ705004	AJ716065	AJ745676	N/A
<i>B. fabae</i>	BC-17	Yi Du, China	EU563119	EU563104	EU563095	EU563112	N/A
	BC-22	Xuan En, China	EU563121	EU563105	EU563102	EU563111	N/A
	BC-25	Xiang Fan, China	EU563125	EU563110	EU563098	EU563116	N/A
	MUCL98 (ex type)	Spain	N/A	AJ705014	AJ716075	AJ745686	N/A
<i>B. fabiopsis</i>	BC-2	Wuhan, China	EU519204	EU519211	EU514482	EU514473	REF
	BC-13	Xian Ning, China	EU563122	EU563109	EU563100	EU563115	REF
	BC-30	Yi Chang, China	EU563126	EU563106	EU563097	EU563117	REF
<i>B. caroliniana</i>	CB15	Long Creek, SC	JF777531	JF811584	JF811587	JF811590	JF811593
	WM4	Six Mile, SC	JF777532	JF811585	JF811588	JF811591	JF811594
	CA3	Cheddar, SC	JF777533	JF811586	JF811589	JF811592	JF811595
<i>B. galanthina</i>	MUCL435	The Netherlands	N/A	AJ705018	AJ716079	AJ745689	AM087057
	MUCL3204	The Netherlands	N/A	AJ705017	AJ716078	AJ745690	AM087067
<i>B. tulipae</i>	BT9001	The Netherlands	N/A	AJ705040	AJ716101	AJ745712	N/A
<i>B. narcissicola</i>	MUCL2120	Canada	N/A	AJ705026	AJ716087	AJ745697	N/A
<i>B. polyblastis</i>	MUCL21492	UK	N/A	AJ705031	AJ716092	AJ745703	N/A
<i>B. croci</i>	MUCL436	The Netherlands	N/A	AJ705009	AJ716070	AJ745681	N/A
<i>B. squamosa</i>	MUCL1107	USA	N/A	AJ705037	AJ716098	AJ745710	N/A
<i>B. aclada</i>	PRI006 ^a	N/A	AJ716295	AJ704993	AJ716051	AJ745665	N/A
<i>M. fructigena</i>	9201	N/A	N/A	AJ705043	AJ716047	AJ745715	N/A

^aDr. P. van den Boogert, Plant Research International, Wageningen.^bN/A = not available; ^cREF. = sequence obtained from the author of the associated reference.

Table 2.2. Conidia size average and range of isolates of *B. caroliniana*, *B. fabiopsis* and*B. galanthina*

Species	Isolate	Average ^a (µm)			Range (µm)	
		Length (L)	Width (W)	L/W	Length	Width
<i>B. caroliniana</i>	CB15	11.3	8.1	1.4	8-14.2	6.3-10.5
	CA3	10.9	8.7	1.3	7.6-14.1	6.1-11.0
	WM4	12.4	8.9	1.4	9.8-15.5	6.0-11.0
<i>B. fabiopsis</i> (Zhang et al. 2010)	BC-2	ND	ND	ND	15.7-24.1	10.8-19.2
	BC-13	ND	ND	ND	15.0-26.2	13.3-21.7
	BC-30	ND	ND	ND	15.0-23.4	10.1-17.8
<i>B. galanthina</i> (Zhang et al. 2010)	/	ND	ND	ND	10.3-21.3	7.8-12.3

^aAverage and range of 50 measurements.

Table 2.3. Radial growth rate (RGR), conidiophores length, and sclerotia color of *Botrytis caroliniana*, *B. cinerea*, and *B. fabiopsis*.

Species	Isolate	RGR (mm/d)	Conidiophore length (μm)	Sclerotia color
<i>B. caroliniana</i>	CB15	11.5b ^z	649-1006	Black
	CA3	10.2bc	784-1319	Black
	WM4	9.5c	569-1698	Black
<i>B. cinerea</i>	CB17	8.8c	2155-3925	Black
	CA25	13.8a	741-2193	Black
	WM6	14.3a	1367-2674	Black
<i>B. fabiopsis</i> (Zhang et al. 2010)	BC-2	12	698-1466	Gray
	BC-13	13	558-1396	Gray
	BC-30	12	530-1326	Gray
<i>B. cinerea</i> (Zhang et al. 2010)	BC-1	17	935-1605	Black
	BC-15	18	712-1745	Black
	BC-27	19	810-1619	Black

^zMean values followed by the same letters within columns indicate no significant difference ($\alpha = 0.05$) according to least significant difference (LSD) test.

Table 2.4. Disease incidence and lesion diameter on detached leaves of broad beaninoculated with mycelia of *Botrytis caroliniana*, *B. cinerea*, *B. fabiopsis*, and *B.**galanthina*

Species	Isolate	Disease incidence (%)	Lesion diameter (mm)
<i>B. caroliniana</i>	CB15	100	8.7bc
	CA3	100	7.6bc
	WM4	100	6.4c
<i>B. cinerea</i>	CB17	100	17.9a
	CA25	100	9.9b
	WM6	100	18.8a
<i>B. fabiopsis</i> (Zhang et al. 2010)	BC-2	100	14
	BC-13	100	11
	BC-30	100	10
<i>B. cinerea</i> (Zhang et al. 2010)	BC-1	100	27
	BC-15	100	26
	BC-27	100	28
<i>B. galanthina</i> (Zhang et al. 2010)	MUCL435	0	0

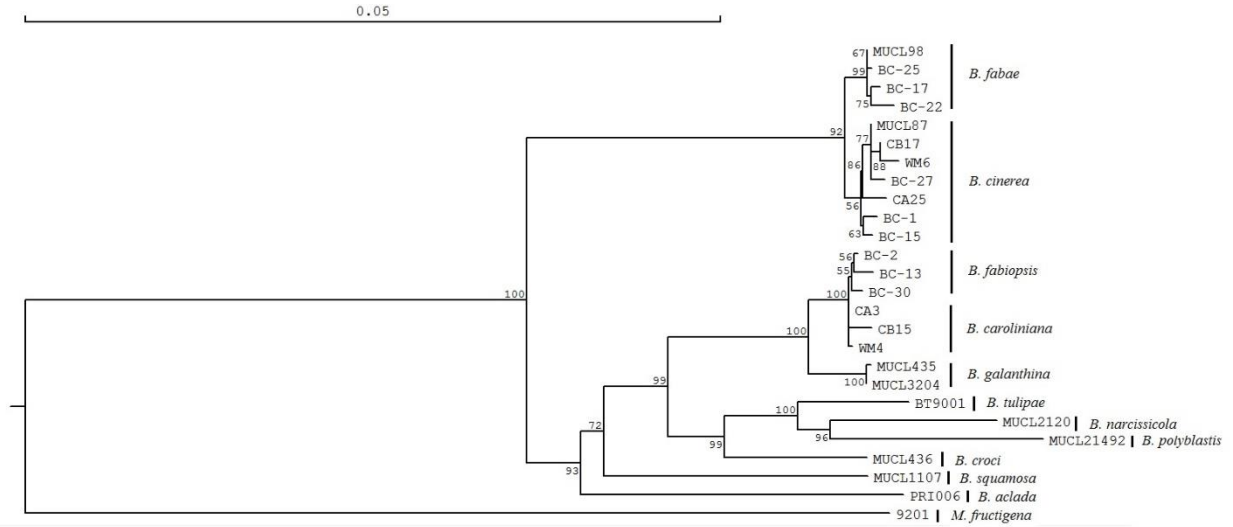


Fig. 2.1. Molecular phylogeny of 24 taxa of presented by a neighbor joining tree inferred from the dataset based on combined DNA sequences of HSP60, RPB2, and G3PDH. The numbers labeled at each node indicate the bootstrap (BS) percentage (N=1000), BS value from the neighbor-joining analysis. Bootstrapping below 50% are not provided. Branch length is proportional to the numbers of nucleotide substitutions as measured by the scale bar (5% sequence divergence).

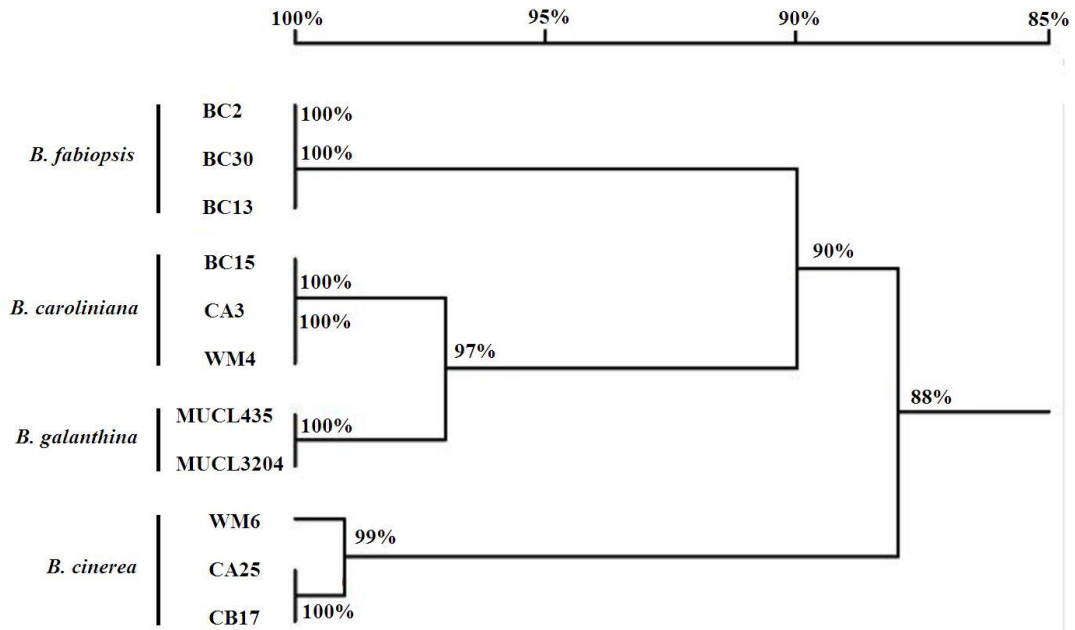


Fig. 2.2. Nucleotide identity (%) of the partial DNA sequences encoding necrosis and ethylene-inducing protein1 NEP1 among *Botrytis caroliniana*, *B. fabiopsis*, *B. cinerea* and *B. galanthina* (nucleotide identity percentage was rounded-up).

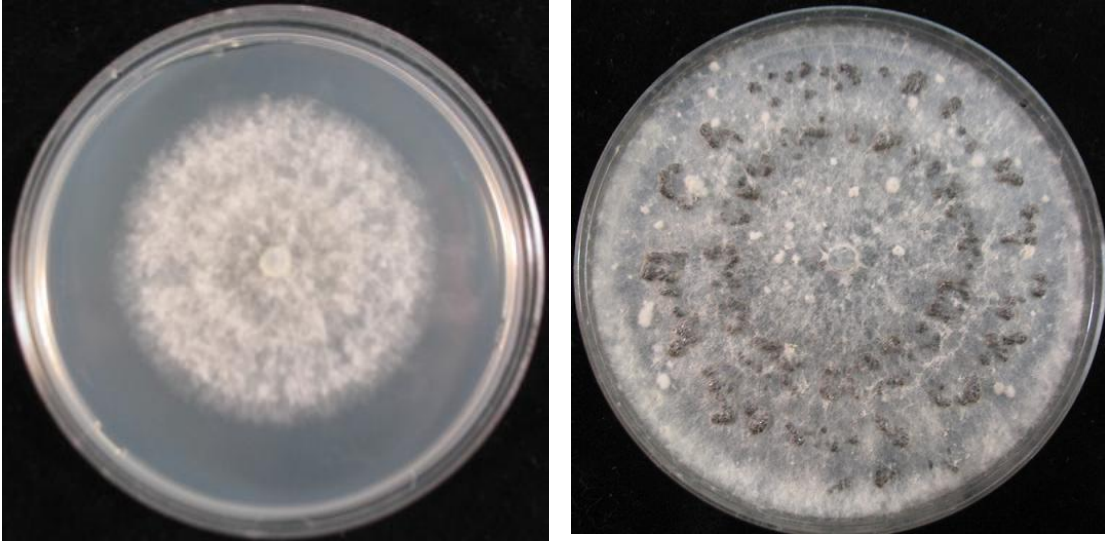


Fig. 2.3. Colony of *Botrytis caroliniana* incubated on PDA for 3 (left) and 18 (right) days at 20°C.

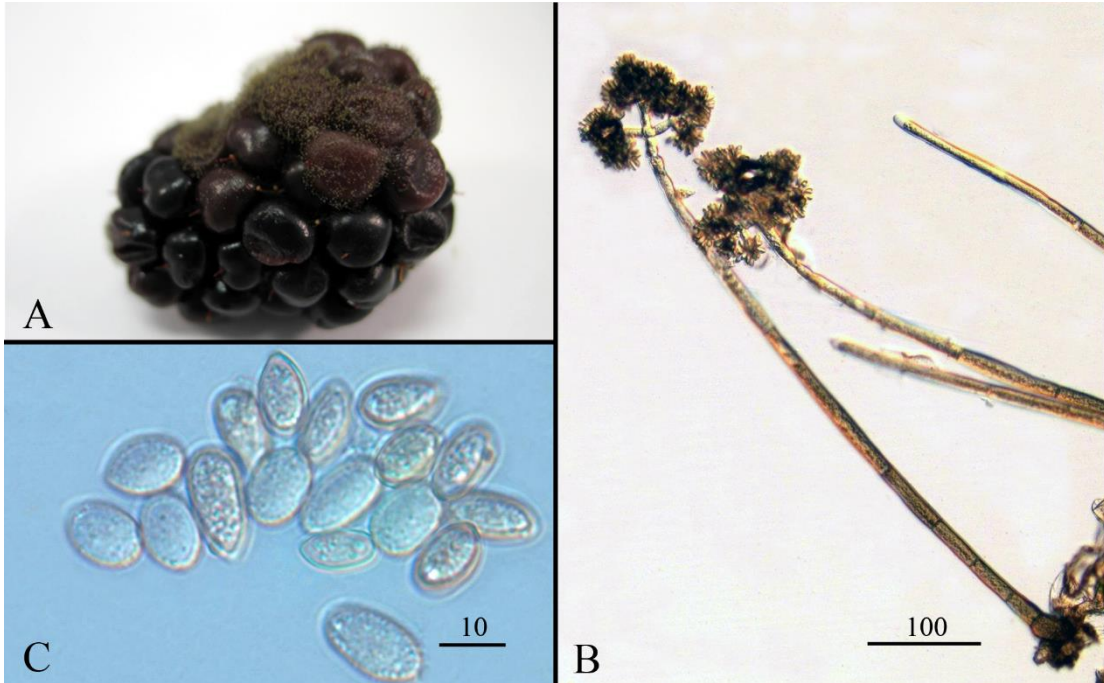


Fig. 2.4. Morphological characteristics of *Botrytis caroliniana* from blackberry. (A) sporulation on blackberry fruit; (B) conidiophores formed on blackberry showing botryose clusters of conidia; and (C) conidia.

CHAPTER THREE

IDENTIFICATION AND PREVALENCE OF *BOTRYTIS* SPP. FROM BLACKBERRY AND STRAWBERRY FIELDS OF THE CAROLINAS

This work has been published:

X.P. Li, D. Fernández-Ortuño, W. Chai, F. Wang and G. Schnabel. 2012. Identification and Prevalence of *Botrytis spp.* from Blackberry and Strawberry Fields of the Carolinas. *Plant Disease*. 96:1634-1637

X.P. Li and G. Schnabel. 2011. First Report of Gray Mold of Blackberry Caused by *Botrytis cinerea* in South Carolina. *Plant Disease*. 95:1592

[Fungal isolates used in this study were collected by Fei Wang, Anja Grabke, and Xingpeng Li, and blackberry isolates` single spore isolation was performed by Wendi Chai and Xingpeng Li, and strawberry isolates` single spore isolation was performed by Dr. Dolores Fernández-Ortuño, and molecular characterization as well as methods development was performed by Xingpeng Li]

Abstract

Gray mold disease of blackberry and strawberry is caused by *Botrytis cinerea* and *B. caroliniana* in the southeastern United States. In this study, methods to distinguish both species were established and their prevalence was determined in commercial blackberry and strawberry fields. Using DNA from *B. cinerea* and *B. caroliniana* reference strains, a

species-differentiating PCR amplification was developed that amplified *G3PDH* gene fragments of two different sizes depending on the species. The PCR is performed with three primers (two species-differentiating forward primers and one universal reverse primer) and amplified a 238 bp product from *B. cinerea* and a 536 bp fragment from *B. caroliniana* reference isolates. A total of 400 *Botrytis* isolates were collected from 6 commercial blackberry and 11 strawberry fields of the Carolinas and identified to the species level with the new PCR method. Both *Botrytis* spp. were identified in blackberry and strawberry fields, but *B. caroliniana* was less common than *B. cinerea*. Only 33 of 202 isolates from blackberry fields were identified as *B. caroliniana* and the majority of these isolates came from two fields in South Carolina. Only 1 of 198 isolates from strawberries was identified as *B. caroliniana* and this isolate was found in central North Carolina. *B. cinerea* but not *B. caroliniana* isolates sporulated on potato dextrose agar and Kings medium B. Our results show that *B. cinerea* and *B. caroliniana* coexist in at least some commercial blackberry and strawberry fields of the Carolinas with *B. cinerea* being the more prevalent species.

Introduction

Among the economically most important small fruit crops in the southeastern United States affected by gray mold disease are strawberries (*Fragaria x ananassa*) and blackberries (*Rubus*). In 2010, more than 950 ha of strawberries were planted in North and South Carolina valued at about \$30 million (116). Other states in the Southeast with

significant strawberry production include Florida, Georgia, and Tennessee. Almost all commercial strawberries are produced with the plasticulture annual cropping system. In the Carolinas plants are typically obtained from nurseries, planted in September or October, and cropped in the spring and early summer of the following year. In contrast, blackberries have biennial canes and perennial roots and the same planting may be cropped for a decade or longer. Often, winter injury and cool temperatures create ideal conditions for gray mold disease in both crops.

Botrytis cinerea Pers. is the causal agent of tissue blight, rots and gray mold disease of over 200 plant species including strawberry and blackberry (127). *B. cinerea* generally overwinters in decaying plant debris or as sclerotia. The pathogen prefers cool and moist weather conditions for infection and for post infection disease development (134). In the plant the pathogen may remain latent if disease development conditions are unfavorable. Eventually, the pathogen triggers host-cell death, which causes the progressive decay of infected petals, leaves, fruit, and various other plant tissues. On the surface of infected tissue it produces abundant conidia, which can be transported by wind over long distances. *B. cinerea* also has a sexual, teleomorphic stage named *Botrytis fuckeliana* (61).

Although *B. cinerea* has been recognized to be a complex of species in Europe (41), it was until recently considered the only species causing gray mold of small fruits in the United States. A new species designated *Botrytis caroliniana* X.P. Li & G. Schnabel was isolated in 2010 from blackberry fruit in South Carolina (82), adding to the *Botrytis* spp. list in North America. Based on phylogenetic and morphological analysis, *B. caroliniana*

is most closely related to *B. fabiopsis* (137), a broad bean (*Vicia faba*) pathogen from Central China (139) and *B. galanthina* (57, 114), a species isolated from snowdrop (*Galanthus* spp.) – a bulbous herbaceous plants. The prevalence and host range of *B. caroliniana* is unknown, but it is becoming increasingly clear that this species causes disease on various small fruits, including blackberry and strawberry (35, 82).

The objective of this study was to develop simple and reliable tools to distinguish *B. caroliniana* from *B. cinerea* and to determine the prevalence of the two species in commercial blackberry and strawberry fields of the Carolinas.

Material and Methods

Origin of isolates. Isolates from symptomatic blackberry fruit were collected in the fall of 2010 and 2011 from commercial fields in Oconee- (isolate code CB), Anderson- (isolate code CA), Pickens- (isolate code WM), Chesterfield- (isolate code MC), and Chesnee County (isolate code CO) in South Carolina, and Cleveland County (isolate code KC) in North Carolina. Isolates collected in 2011 are indicated with an ‘a’ following the isolate code. The isolates from strawberries were collected from commercial fields in Cleveland- (isolate code SBY), Duplin- (isolate code NC), Guilford- (isolate code HP), and Iredell County (isolate code MV) in North Carolina and Aiken- (isolate code GIK), Cherokee- (isolate code JEY), Chesterfield- (isolate code KUD), Florence- (isolate code FLOR), Lexington- (isolate code MER), Saluda- (isolate code WIC) and Spartanburg County (isolate code MOD) in South Carolina. All isolates from blackberry and strawberry

fields came from individual fruits of different plants. Each fruit was placed in an individual plastic bag after harvest and stored at 4°C for up to five days prior to single spore isolation. Three *B. cinerea* isolates (CA25, CB17, and WM6) and three *B. caroliniana* reference isolates (CA3, CB15, and WM4) originally obtained from blackberry fruit (82) were included in this study. They are designated ‘reference isolates’ because they were characterized in a previous study (82)

Development of a species-differentiating PCR assay. Three primers targeting a partial sequence of the glyceraldehydes-3-phosphate dehydrogenase (G3PDH) gene were designed based on sequences published from *B. cinerea* and *B. caroliniana* reference isolates (Table 3.1.). Forward primers G3PDH-F1 and G3PDH-F2 are specific for *B. caroliniana* and *B. cinerea*, respectively, and were used with reverse primer G3PD-R for species-differentiating DNA amplification. Genomic DNA was extracted as described previously (16). PCR amplification was performed in a total reaction volume of 50 µl. The following compounds were added, with final concentrations indicated: 1X ThermoPol reaction buffer (BioLabs, Ipswich, MA), dNTP’s (0.25 mM of each dATP, dGTP, dCTP, dTTP; BioLabs, Ipswich, MA), 0.6 pmol µl⁻¹ of each primer (Table 3.1.), Taq DNA polymerase (1.25 units; BioLabs) and 10 ng – 100 ng of fungal DNA. The following thermocycling protocol was used to amplify the *G3PDH* fragment: 94°C for 3 min (1 cycle); 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min (32 cycles), and 72°C for 5 min (1 cycle). Fragments were separated on a 1% agarose gel, stained with ethidium bromide,

and visualized under UV-light. The fragment size of the PCR product was verified by comparison to a Low Range Plus DNA Ladder (Fisher Scientific).

Single-spore isolation and cultural characterization. For single spore isolation, conidia were scraped off without touching the fruit using a sterile scalpel and suspended in 1 ml sterile, distilled water with 1% Tween-60. Then, 200 µl of the spore suspension were spread on water-agar in petri dishes (90 mm diameter) amended with lactic acid (0.1%, vol/vol) and streptomycin (100 µg/ml). After incubation at 22°C for 24–36 h, three germinated conidia were scooped up by a glass needle under a microscope and placed 2 cm apart in equal distances onto potato dextrose agar (Difco Laboratories, Sparks, MD) amended with streptomycin (100 µg/ml) and propiconazole (0.1 µg/ml) and petri dishes were incubated at 22°C for 36 h. One of the three single-spore colonies was kept for further studies.

To investigate spore production ability *in vitro*, agar plugs (6 mm diameter) containing actively growing mycelium were placed on Kings medium B (KMB: 20g Proteose Peptone No.3 (Difco), 1.5g K₂HPO₄, 0.738g MgSO₄ (Anhydrous), 15g Agar, 10 ml Glycerol and 990 ml water for 1 Liter medium) (4) and PDA with the mycelia side down. KMB was used previously to produce conidia from *B. cinerea* cultures for experimental purposes (40). Plates were incubated at 20°C for 7 d at 12 h intervals of fluorescent light and darkness. Conidiophores and conida were observed on plates with sporulating isolates but not on plates with non-sporulating isolates.

DNA extraction and PCR amplification of *G3PDH*, *HSP60*, and *RPB2* gene fragments. While most *B. cinerea* isolates sporulated heavily on PDA and KMB, isolate WM8a sporulated weakly on both media and was therefore subjected to more vigorous molecular analysis for species verification. It was isolated from a decayed blackberry fruit collected in a field where both species were present. Genomic DNA was extracted as described previously (16). The partial sequence of the glyceraldehydes-3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*) and DNA-dependent RNA polymerase subunit II (*RPB2*) were PCR amplified using primer pairs *G3PDH*for/*G3PDH*rev, *HSP60*for/*HSP60*rev and *RPB2*for/*RPB2*rev, respectively (107). The PCR products were purified using the ExoSAP-IT purification kit (USB Corporation, Cleveland, Ohio) following manufacturers recommendations. Purified products were sequenced at Clemson University Genomic Center. Nucleotide sequences were analyzed and aligned with DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI).

Results

Distinction of *B. cinerea* and *B. caroliniana* using species-differentiating PCR and cultural characteristics. *B. cinerea* reference isolates CA25, CB17, and WM6 produced a 238 bp fragment with primer set *G3PDH*-F1, *G3PDH*-F2, and *G3PDH*-R. The same primer set produced a 536 bp fragment for *B. caroliniana* reference isolates CA3, WM4, and CB15 (Fig. 3.1.). The species-differentiating PCR was further validated by *HSP60* gene sequence analysis. The majority of the *HSP60* gene was sequenced from 10 *B. cinerea* isolates and

10 *B. caroliniana* isolates from different geographic locations and sequence analysis verified that the species-differentiating PCR accurately detected isolates to the species level (data not shown). Using this technique, 33 and 1 additional *B. caroliniana* isolates were identified from blackberries and a single strawberry, respectively (Table 3.2.).

Botrytis cinerea reference isolates CA25, WM6, and CB17 as well as 158 *B. cinerea* isolates from blackberries and 197 isolates from strawberries sporulated on PDA and KMB media. Isolate WM8a was identified by PCR as *B. cinerea* but sporulated only weakly on PDA and KMB (Fig. 3.2.). The culture characteristics therefore are different from all other sporulating isolates. Subsequent sequence analysis of the *G3PDH*, *RPB2*, and *HSP60* genes confirmed that the gene sequences were identical to *B. cinerea* reference isolate sequences available in GenBank (data not shown; accession numbers JN672671, JN672675, and JN672677, respectively for isolate CB17; JN164270, JN164271, and JN164272, respectively for isolate WM6; and JN672672, JN672676, and JN672678, respectively for isolate CA25). Thus, both sporulation phenotypes observed in this collection of 202 isolates corresponded to the PCR-based species differentiation assay.

Prevalence of *B. cinerea* and *B. caroliniana* isolates in blackberry and strawberry fields of the Carolinas. *B. cinerea* was the dominant species in blackberry and strawberry fields of the Carolinas. Only 33 of 202 (16.3%) isolates from blackberry and only 1 of 198 (0.5%) isolates from strawberry were identified as *B. caroliniana* (Table 3.2.). *B. cinerea* and *B. caroliniana* were found to coexist in four of six populations from blackberry fields; three from South Carolina and one from North Carolina. The four fields were located close

to the western border of the Carolinas (Fig. 3.2. A). Although isolates from 10 strawberry fields were included in this study, only one located in the center of North Carolina revealed a *B. caroliniana* isolate (Fig. 3.2. B).

Discussion

B. caroliniana and *B. cinerea* are distinct species of *Botrytis* as determined by phylogenetic analysis of the *HSP60*, *G3PDH* and *RPB2* gene sequences and unique cultural characteristics (82). In this follow-up study we developed and validated tools to more easily distinguish *B. cinerea* from *B. caroliniana*. The molecular distinction based on species-differentiating PCR amplification and the morphological distinction based on sporulation ability on PDA and KMB provided consistent results for the reference isolates and all 400 isolates obtained from strawberries and blackberries for this study. While our data indicate a strong correlation between species and sporulation ability on PDA and KMB, the cultural characteristics should not be used as a sole indicator for a species. That is because sporulation intensity varied among isolates and weak sporulators of the *B. cinerea* spp. may therefore be mistaken for *B. caroliniana*. For example, isolate WM8a sporulated only weakly whereas all other *B. cinerea* isolates sporulated profusely on PDA and KMB media. *HSP60*, *G3PDH*, and *RPB2* gene sequence analysis, however, confirmed this isolate to be *B. cinerea*. Furthermore, it is possible that variability in the population may produce non-sporulating *B. cinerea* and/or sporulating *B. caroliniana* in other locations.

Our results are consistent with other observations identifying *B. cinerea* to be a species complex (41). In the latter study, *B. pseudocinerea* was identified to be a part of this complex (121) in Europe. This species is genetically closely related to *B. cinerea* based on phylogenetic analysis of the *HSP60* and *G3PDH* genes and is therefore only distantly related to *B. caroliniana*, which clusters closest to *B. galanthina* and *B. fabiopsis* (82). Sympatry of *B. cinerea* and *B. caroliniana* was detected in four of six populations, however, it is possible that *B. caroliniana* may have been present in all six populations but escaped collection. Morphologically similar but genetically diverse sub-species of *B. cinerea* (3, 4), *Lophodermium pinastrii* (91), and *Phialocephala fortinii* (54) have been shown to inhabit the same ecological niche. Similarly, species of *Colletotrichum* may co-inhabit certain geographical areas (85). It has also been suggested that *B. cinerea* is a complex of sibling species with similar morphology and a high level of genetic diversity occupying different ecological niches (46). The population genetics analysis revealed a high level of diversity within *B. cinerea*. The present study suggests that *B. cinerea* and *B. caroliniana*, which are morphologically and genetically diverse (82), not only share the same ecological niche; they also share a common host.

B. caroliniana was nearly absent in commercial strawberry fields, which may be related to the strawberry production system. Strawberries are annuals and have been produced in plasticulture systems for the last 15 to 20 years. This system, in contrast to matted row culture, calls for roguing the entire crop after the production season is over and soil fumigation prior to planting. Thus, the field is virtually devoid of gray mold inoculum

at planting. The planting material in form of plugs and bare-root plants derives largely from nurseries in Western North Carolina and Prince Edward Island and Ontario provinces of Canada. While inoculum may migrate into strawberry fields from other crops and weeds, gray mold infections may also originate from latent infections in the planting material. The virtual absence of *B. caroliniana* in strawberry fields indicates that strawberry may not be a preferred host of *B. caroliniana*. Another explanation is that the planting-material produced by nurseries may have been devoid of this species. In contrast, blackberries are cropped for many years with roots being perennial and canes being biennial. The two species of *Botrytis* are therefore much more likely to successfully establish a long-term sympatry in blackberries.

B. caroliniana occurred overwhelmingly in a cluster of commercial fields located at the most western part of South Carolina. This area is characteristic for its diverse vegetation, including woodlands, wildflowers, ornamentals, wild berries and more. Furthermore, the three farms with high *B. caroliniana* prevalence contained at the time of collection a variety of fruit crops, including various berry types, which could have served as additional hosts. If the pathogen has more than one preferred host, it is possible that other, still unknown hosts are more abundant in this region compared to other regions in the Carolinas. This would have resulted in high disease pressure and could explain the higher disease incidence on blackberry in this region.

In conclusion, *B. cinerea* and *B. caroliniana* coexist in commercial blackberry and to a lesser degree in commercial strawberry fields of North and South Carolina. The two

species can be distinguished using species-differentiating PCR amplification and based on sporulation ability on PDA and KMB.

Table 3.1. Nucleotide sequences of primers designed to distinguish *Botrytis cinerea* from

B. caroliniana

Name	Nucleotide sequence (5' - 3')
G3PDH-F1	GGACCCGAGCTAATTTATGTCACGT
G3PDH-F2	GGGTGTCAACAACGAGACCTACT
G3PDH-R	ACCGGTGCTCGATGGGATGAT

Table 3.2. Molecular and cultural distinction of *B. cinerea* and *B. caroliniana* isolates

from blackberry and strawberry

Species	Host	Isolates	No. isolates	PCR fragment size (bp) ^a	Sporulation PDA/KMB
<i>B. cinerea</i>	Blackberry	KC1, KC3-27, KC30-52, WMa1, WMa3-5, WMa8, WMa9, WMa12-14, WMa17, WMa24, WMa26-27, WMa31, WMa33-35, WMa39-41, WMa44-46, WMa48, WMa55, WMa57, WMa61, WMa68, WMa71, WMa75, WM6, MC1, MC5, MC9, MC12-15, MC21-22, MC26-28, MC31-34, COa1, COa3, COa5, COa7, COa9-18, COa20-21, COa23-24, CO2-4, CO6-8, CO10-12, CO15-17, CO20-21, CAa3, CA1, CA11, CA25, CB17, CBa1, CBa3-7, CBa10-14, CBa16-17, CBa19-20, CBa22-23, CBa26-29, CBa31, CBa35-37, CBa40-42, CBa45, CBa47, CBa49, CBa55, CBa57, CBa60-62	169	238	yes
	Strawberry	BC11KUD1-21, BC11MOD2-20, BC11GIK1-6, BC11GIK9-21, BC11JEY1-25, BC11FLOR1-13, BC11MER1-3, BC11WIC1-21, BC11HP1-30, BC11HP32, BC11HP34, BC11MV1-2, BC11MV4-6, BC11MV8-11, BC11Sby1-19, BC11SBY21, BC11SBY23-37	197	238	yes
<i>B. caroliniana</i>	Blackberry	KC29, WM4, WMa6-7, WMa18, WMa22, WMa30, WMa32, WMa47, WMa62, WMa64, WMa67, WMa72, CAa2, CA3, CA8, CA9, CBa2, CBa8-9, CBa24, CBa30, CBa33, CBa34, CBa39, CBa46, CBa48, CBa51-54, CB9, CB15	33	536	no
	Strawberry	BC11HP33	1	536	no

^aPCR fragment was amplified with primers G3PDH-F1, G3PDH-F2, and G3PDH-R in a single reaction.

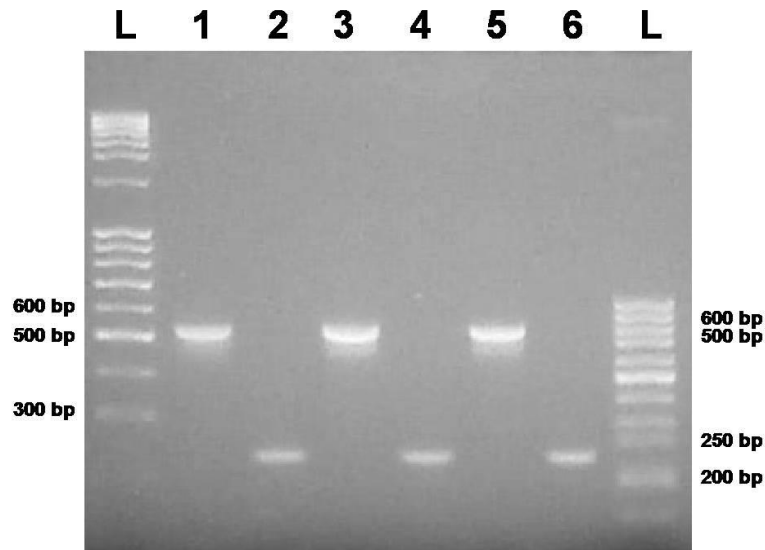


Fig. 3.1. Amplification of the *G3PDH* gene fragment with primer set G3PDH-F1, G3PDH-F2, and G3PDH-R followed by electrophoresis on a 1 % agarose gel. Lanes 1, 3, and 5 are *B. caroliniana* isolates CA3, CB15 and WM4; lanes 2, 4, and 6 are *B. cinerea* isolates CA25, CB17, and WM6; lane L on the left is the Middle Range Plus DNA Ladder (Fisher Scientific, Hampton, NH), lane L on the right is the 50 bp Mini DNA Ladder (Fisher Scientific).

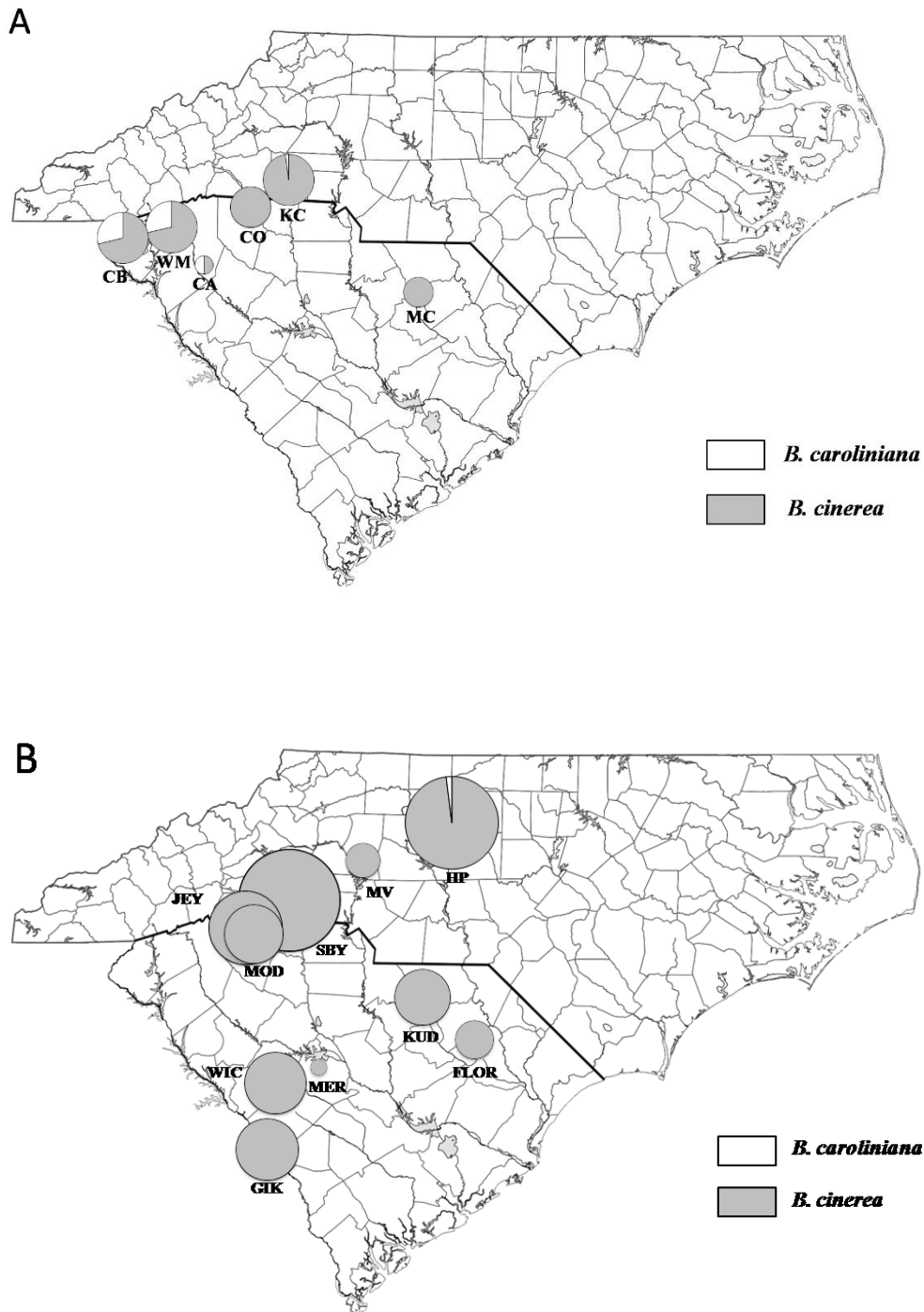


Fig. 3.2. The origin and prevalence of *Botrytis cinerea* and *B. caroliniana* isolates from blackberry (A) and strawberry (B) in the Carolinas. The diameter of each circle is scaled to population size examined.

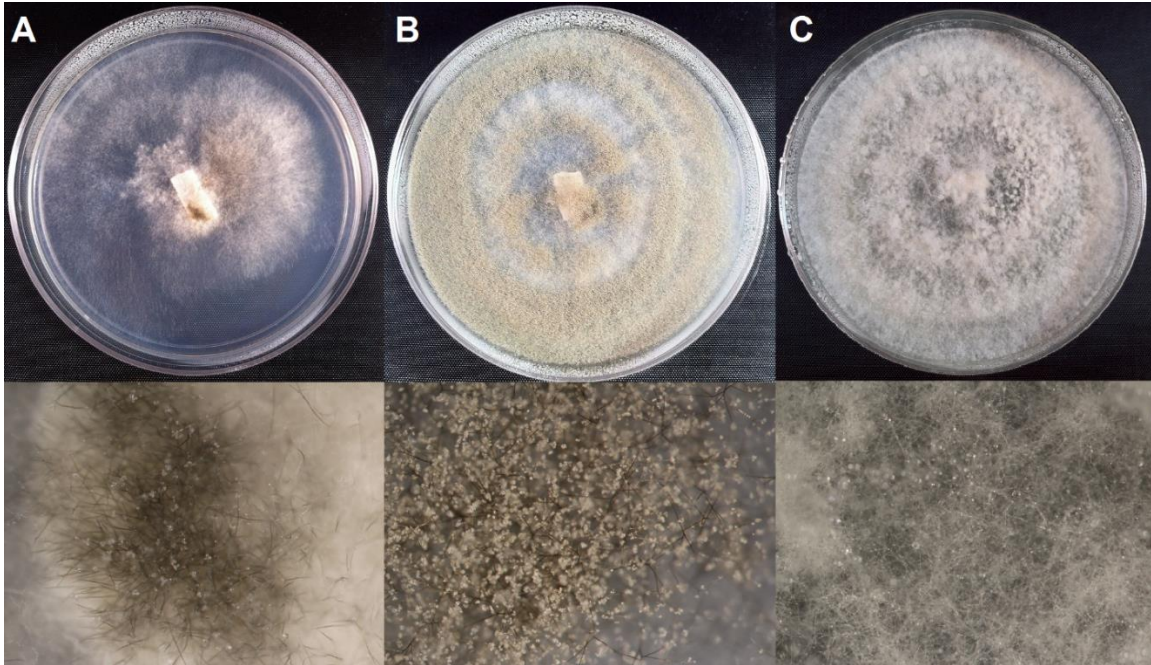


Fig. 3.3. Cultural characteristics of weakly sporulating *Botrytis cinerea* isolate WM8a (A), abundantly-sporulating, representative isolate MC31 (B), and *B. caroliniana* isolate CB15 (C) after 6 days of incubation on PDA at 22°C under discontinuous light exposure.

CHAPTER FOUR

LOCATION-SPECIFIC FUNGICIDE RESISTANCE PROFILES AND EVIDENCE FOR STEPWISE ACCUMULATION OF RESISTANCE IN *BOTRYTIS CINEREA*

This work has been published:

X.P. Li, D. Fernández-Ortuño, S. Chen, A. Grabke, C.X. Luo, W.C. Bridges and G. Schnabel. 2014. Location-Specific Fungicide Resistance Profiles and Evidence for Stepwise Accumulation of Resistance in *Botrytis cinerea*. *Plant Disease*. 98:1066-1074. ["Fungal isolates and culture conditions" of strawberry and blackberry isolates were performed by Dr. Dolores Fernández-Ortuño and Xingpeng Li, respectively; "Sequence analysis of target genes" were performed by Xingpeng Li and Shuning Chen; "Statistical analysis" were performed by Dr. William C. Bridges and Xingpeng Li]

Abstract

The fungicide resistance profiles to seven chemical classes of fungicides were investigated in 198 *Botrytis cinerea* isolates from five blackberry fields and 214 *B. cinerea* isolates from 10 strawberry fields of North and South Carolina. Populations of *B. cinerea* tended to have a single dominant, location-specific resistance profile that consisted of resistance to multiple fungicides in fields sprayed weekly with site-specific fungicides. The most prevalent profile in blackberry fields consisted of resistance to thiophanate-methyl, pyraclostrobin, and boscalid. The most prevalent resistance profile found in conventional

strawberry fields consisted of resistance to thiophanate-methyl, pyraclostrobin, boscalid, and cyprodinil. A statistical model revealed that multifungicide resistance patterns did not evolve randomly in populations from both crops. Instead, strains resistant to thiophanate-methyl were more likely to acquire resistance to pyraclostrobin, the resulting dual-resistant population was more likely to acquire resistance to boscalid, the resulting triple-resistant population was more likely to acquire resistance to cyprodinil, and the resulting quadruple-resistant population was more likely to acquire resistance to fenhexamid (strawberry population only) compared to random chance. Resistance to iprodione and fludioxonil appeared to have evolved by random chance from a pool of strains with different fungicide resistance profiles. Resistance to thiophanate-methyl, pyraclostrobin, boscalid, and fenhexamid in blackberry isolates was without exception based on target gene mutations, including E198A and E198V in β -tubulin, G143A in cytochrome b, H272Y and H272R in SdhB, and F412I in Erg27, respectively. A new genotype associated with fenhexamid resistance was found in one strain (i.e. Y408H & deletion of P298). Fungicide resistant strains were present but rare in an unsprayed blackberry field, where some unique phenotypes, including low and medium resistance to fludioxonil, had emerged in the absence of fungicide pressure. The isolates resistant to fludioxonil had EC_{50} values of 0.16 $\mu\text{g/ml}$ (low resistance) and 0.32 and 0.38 $\mu\text{g/ml}$ (medium resistance) and were also resistant to AP fungicide cyprodinil indicating that this and similar phenotypes will eventually be selected by continued applications of the fludioxonil+cyprodinil premixture Switch. This study shows that multifungicide-resistant phenotypes are common in conventionally

maintained strawberry and blackberry fields and that resistance to multiple fungicides evolved from stepwise accumulation of single resistances.

Introduction

Gray mold is one of the most economically important diseases of commercially produced blackberry and strawberry fruit. In the southeastern United States, the disease is caused by *Botrytis cinerea* Pers. and *B. caroliniana* X.P. Li & G. Schnabel, a species that has only recently been reported in North and South Carolina blackberry fields (65, 82) and a North Carolina strawberry field (35). *Botrytis cinerea* can affect yield in different ways. The pathogen may cause blight on leaf or petal tissues, crown rot, stem cankers, cutting rot, and damping-off (18, 65). The fungus produces germ tubes from conidia that can infect through natural openings or wounds. It is a cool season disease and infection is favored under wet conditions with temperatures below 22°C (65, 105). Besides actively causing disease during the growing season, the fungus is also able to cause latent infections leading to disease after harvest, either during storage, transit, in the store, or after purchase by the consumer (66, 107, 123).

Seven classes of site-specific fungicides are currently available for the control of gray mold disease in the United States. They include anilinopyrimidines (APs), dicarboximides (DCs), hydroxylanilides (HAs), methyl benzimidazole carbamates (MBCs), phenylpyrroles (PPs), quinone outside inhibitors (QoIs; disease suppression only) and succinate dehydrogenase inhibitors (SDHIs). Among these site-specific fungicides, MBCs

were introduced in the 1970s and therefore have been used for the longest period of time compared to other six site specific fungicides mentioned above. The DCs were also introduced in the 1970s but usage dropped after the United States Environmental Protection Agency decided in 1999 to drastically limit the maximum number of applications allowed because of concerns about dietary exposure. A survey among growers in the Carolinas revealed that most had not used DCs at all over the last decade. The first QoI, SDHI, AP and HA fungicides were registered within 1 to 4 years of each other in the US; in 2001, 2003, 2001, and 1999 for disease control of strawberries and 2001, 2003, 2003, and 2002 for disease control of blackberries, respectively (3). While most active ingredients are sold as solo products, some are sold as mixtures. For example, the QoI pyraclostrobin and the SDHI boscalid are sold as Pristine 38 WG (BASF Corporation, Research Triangle Park, NC) or the AP cyprodinil and the PP fludioxonil are sold as Switch 62.5 WG (Syngenta Crop Protection, Inc. Greensboro, NC). Most conventional growers use several different chemical classes of fungicides during the season in mixtures or rotations for resistance management. The use of multiple fungicide products over time, however, may produce pathogen populations with multifungicide resistance (1, 76, 124). Some isolates recovered from strawberries in Germany and France exhibited multifungicide resistance in form of ATP-binding cassette (ABC) transporter and major facilitator super family (MFS) transporter activity (73). But in the southeastern United States, resistance to many fungicide classes, including the MBCs, QoIs, SDHIs and HAs is based on target gene alterations (1, 33, 36, 50). The objective of this study was to investigate phenotypic and

evolutionary patterns of multifungicide resistance in *B. cinerea* isolates collected from commercial blackberry and strawberry farms in the Carolinas.

Materials and Methods

Origin of isolates and conidia production. Single spore isolates of *B. cinerea* were obtained from decayed blackberry (198 isolates) and strawberry (214 isolates) fruits in 2010 and 2011. Isolates were either verified to be *B. cinerea* in a previous study (81) or as part of this study using cultural and molecular methods. The blackberry samples were collected from five commercial fields in Oconee (isolate code CB), Pickens (isolate code WM), Chesterfield (isolate code MC), and Chesnee (isolate code CO) counties in South Carolina, and Cleveland County (isolate code KC) in North Carolina. The strawberry samples were collected from different commercial strawberry fields encoded HP, MV, NC, and SBY of North Carolina and FLOR, GIK, JEY, KUD, MOD, and WIC of South Carolina. All but one blackberry field (CB) had received fungicide applications in years prior to sampling. Growers from locations CO, KC, and MC used MBC, QoI, SDHI, AP, and HA fungicides in rotation or as a mixture on a weekly schedule, making an average of over 12 applications per season. None of the growers interviewed had used DCs over the last decade. Growers from locations in WM sprayed only occasionally prior to rain events and used less than five applications on average per season. Weekly applications of site-specific fungicides were conducted at strawberry locations GIK, JEY, KUD, MOD, and SBY. The spray history from location NC is not known, but locations FLOR, HP, and MV

only received occasional fungicide treatments (less than five per season). Location WIC only received fungicides approved for organic strawberry production. We previously characterized the isolates from strawberry for resistance to APs, HAs, MBCs, QoI, PPs, and SDHIs (32, 33, 36, 51), but their multifungicide resistant phenotypes are reported here for the first time.

For conidia production, all isolates were grown on potato dextrose agar medium (PDA; Difco Laboratories, Sparks, MD) in 9-cm-diameter Petri dishes for one to two weeks at 22°C with 12-h intervals of fluorescent light and darkness. Conidia were gently scraped from the colonies without touching the agar with a sterile, disposable 10- μ l inoculation loop (VWR international LLC, Radnor, PA). Conidia were then suspended in 1 ml of sterile distilled water, and adjusted to 1 to 5 $\times 10^4$ spores/ml up to 2 h prior to use.

Sensitivity of *B. cinerea* isolates from blackberry to cyprodinil, iprodione, fenhexamid, thiophanate-methyl, fludioxonil, pyraclostrobin, and boscalid. The following active ingredients were obtained as formulated products: AP fungicide cyprodinil (Vanguard WG, Syngenta Crop Protection, Greensboro, NC), DC fungicide iprodione (Roval 4 FL, Bayer CropSciences, Research Triangle Park, NC), HA fungicide fenhexamid (Elevate 50 WDG, ArystaLifeScience, Cary, NC), MBC fungicide thiophanate-methyl (Topsin M 70 WP, Ceraxagri, King of Prussia, PA), PP fungicide fludioxonil (Scholar SC, Syngenta), QoI fungicide pyraclostrobin (Cabrio EG, 20% W/W, BASF Corporation, Research Triangle Park, Raleigh, NC) and SDHI fungicide boscalid (Endura 70% W/W, BASF). Sensitivity was assessed using a previously published germination assay (128) that

evaluates fungicide sensitivity based on spore germination on a medium amended with discriminatory doses of fungicides. The experiments were repeated once. Germination was assessed visually under a light microscope (Olympus BX41TF, Olympus Optical Co. Ltd., Japan). There were 12 isolates that germinated at 5 µg/ml iprodione, but were unable to germinate at 50 µg/ml. This phenotype was not described by Weber & Hahn (125) and was described in this study as low-resistance (LR). The LR and sensitive phenotypes for iprodione were merged in Tables 4.1. and 4.2. for simplicity reasons because the low-resistant phenotype has not been associated with field resistance. The distinction between medium resistance (MR) and low resistance to fludioxonil was based on differences in germination at the two discriminatory doses of 0.1 and 10 µg/ml fludioxonil (125). Isolates were assigned to the low resistance category if germ tube growth exceeded 60% at 0.1 µg/ml and 5% at 10 µg/ml compared to the non-fungicide treated control. For the DC and PP groups, where there is no discontinuity in the sensitivity distribution, experimental evidence was provided recently that the ‘resistant’ isolates are pathogenic on fruit treated with field rates of formulated product (50).

The effective doses that inhibit 50% of mycelial growth (EC_{50} values) were investigated in vitro for fludioxonil as described previously (73) with minor modifications. Briefly, 5,000 spores were transferred to 0.1 ml malt extract broth containing a series of dilutions of fungicides in a 96-well microplate. Final concentrations for all compounds were 10, 3, 1, 0.3, 0.1, 0.03, 0.01, and 0 µg/ml. After 48 h of incubation at 22°C in darkness, the optical density at wavelength A_{600} was determined. The experiment was performed

twice.

Molecular identification of point mutations in target genes *erg27*, *sdhB*, *cytb*, and β -tubulin. Nucleic acid sequence alterations in the strawberry isolates associated with the here described resistance phenotypes were reported previously (32, 33, 36, 51). Sequence alterations in target genes of *B. cinerea* isolates from blackberry were assessed in this study. Isolates were cultured on PDA plates at 22°C in darkness. Mycelia was collected from the developing margin of an actively growing colony using a sterile toothpick. Genomic DNA was extracted as described previously (16). The partial 3-keto reductase gene (*erg27*) was amplified and sequenced with primer pair F412_F and F412_R (51). The entire *erg27* gene of isolate KC52 was amplified with primer pair *erg27*Beg and *erg27*End because sequence analysis of the fragment amplified with primers F42_F and F412_R did not show the suspected F412 mutations. Sequencing was conducted with primers *erg27*Beg, *erg27*End, *erg1800*down, and *erg2000*up (39).

Point mutations in the *sdhB* gene were identified as described previously with primer pair IpBcBeg and IpBcEnd2 (79) with minor modifications of the PCR protocol. The initial denaturation was at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH) following manufacturer's instructions and sequenced at the Clemson University Genomics Institute, Clemson, South Carolina using LiCOR dye-terminator sequencing technology.

Nucleotide sequence variation at amino acid (aa) codon 143 of the cytochrome b gene (*cytb*) was detected as described previously using the primer pair Qo13ext and Qo14ext (79). The amplicon was digested with restriction enzyme Fnu4HI (New England Biolabs, Beverly, MA) at 37°C for 2h. The nucleotide variation at aa codon 198 of the β -tubulin gene was identified as described previously (36, 86). The partial β -tubulin gene was amplified with primer pair BCF/BCR (86) and the amplicon was digested with *ThaI* (New England Biolabs, Beverly, MA) at 60°C for 3h. The PCR products were sequenced if the digestion did not reveal the expected fragments. Amplicons were purified prior to sequencing using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH) following manufacturer's instructions. All PCR amplification or digestion products mentioned above were separated on 1.5% agarose gels.

Data analysis. Statistical analysis was performed to determine if the probability resistance ($P(R)_R$) to a fungicide in subpopulations, which were derived from previous R population, differed from the probability of resistance in the over-all population ($P(R)_E$). A chi-square test was used to test the hypothesis $H_0: P(R)_R = P(R)_E$. Not included in this analysis were observations for fungicides fludioxonil and iprodione because of the low numbers of representative isolates. For all other fungicides, if less than 10 isolates represented a subpopulation, the Fisher's exact test was conducted to verify the chi square result. All calculations were performed by SAS, version 8.01. (SAS Institute Inc., Cary, NC), Chi-square test p-value <0.05 were considered evidence of significant differences in the $P(R)$.

Results

Fungicide resistance profiles of *B. cinerea* isolates from blackberry and strawberry fields. A total of 17 different fungicide resistance profiles containing 0 to 5 single resistance phenotypes (0SR to 5SR) were found in blackberry and strawberry fields of North and South Carolina; 15 phenotypes were present in blackberry fields and 11 in strawberry fields (Table 4.1.). All but one location had a predominant resistance profile. For blackberry fields, the majority of isolates in the unsprayed field CB were sensitive to all fungicides and the majority of isolates from occasionally-treated field WM were resistant to thiophanate-methyl. In all other locations a majority of the isolates were resistant to at least three chemical classes: thiophanate-methyl, pyraclostrobin and boscalid. A similar pattern emerged from the strawberry fields. The majority of isolates from the organic farm and from fields sprayed less than six times per season were sensitive to all of the fungicides. However the majority of isolates from most fields sprayed weekly were resistant to four chemical classes of fungicides: thiophanate-methyl, pyraclostrobin, boscalid, and cyprodinil. The two most heavily sprayed strawberry fields were located within 1 km of each other (SBY and MOD) and isolates from these fields had the greatest number of isolates resistant to five fungicides. Interestingly, isolates with resistance to fungicides were found in the organic field WIC, which was geographically isolated (at least 5 km from next strawberry field) from other strawberry or blackberry fields. Two isolates from heavily sprayed, commercial field KC had a very unique phenotype not found anywhere else; they

were resistant to thiophanate-methyl, pyraclostrobin, cyprodinil, iprodione, and fludioxonil. Although from the same location, the two isolates resistant to fludioxonil were genetically different based on nucleotide sequence variations in the *mrr1*, *bos2* and *rrg-1* genes (data not shown). Iprodione-MR isolates were only found in the JEY and SBY locations (Table 4.1.).

Of the 198 blackberry field isolates collected from 5 locations in the Carolinas, 142 (72%) were resistant to thiophanate-methyl, 117 (59%) were resistant to pyraclostrobin, 111 (56%) were resistant to boscalid, 22 (11%) were resistant to fenhexamid, 19 (10%) were resistant to cyprodinil, 17 (8.6%) were resistant to iprodione, and 3 (1%) were resistant to fludioxonil (2 from field KC were medium-resistant and one from field CB was low-resistant; Table 4.1.). A majority of the blackberry isolates resistant to more than two fungicides was from three commercial fields CO, KC, and MC (Fig. 4.1.). Of these isolates, 37% were resistant to 3 classes, 18% to four classes, and 2% were resistant to five classes of fungicides (Table 4.2.). All other isolates from blackberry resistant to at least one fungicide were resistant to 1 (13%), 2 (2%), and 5 (2%) classes of fungicides.

Of the 214 strawberry field isolates, 149 (70%) were resistant to thiophanate-methyl, 142 (66%) were resistant to pyraclostrobin, 133 (62%) were resistant to boscalid, 46 (22%) were resistant to fenhexamid, 101 (47%) were resistant to cyprodinil, and 4 (2%) were resistant to iprodione (Table 4.3.). The majority of resistant isolates from strawberry possessed resistance to three or more fungicide classes (13%, 37%, and 13% resistant to three, four, and five classes, respectively). Most of these were from commercial fields SBY,

JEY and KUD, all located at the border of North and South Carolina, which also possessed the highest frequency of multifungicide resistance. The lowest frequencies of multifungicide-resistant isolates were observed in locations HP, MV and FLOR (Fig. 4.2.).

Among the isolates resistant to three fungicides, 97% and 90% of the blackberry and strawberry isolates respectively, were resistant to thiophanate-methyl, pyraclostrobin, and boscalid. Among isolates resistant to four fungicides, more than half of the blackberry (19 of 36) and more than three quarters of the strawberry (67 of 79) isolates were resistant to thiophanate-methyl, pyraclostrobin, boscalid, and fenhexamid. Most of the other 4SR isolates were resistant to thiophanate-methyl, pyraclostrobin, boscalid, and cyprodinil (15 of 36 for blackberry and 11 of 79 for strawberry; Table 4.3.).

To further characterize the rare occurrence of resistance to fludioxonil, the effective dose that inhibited 50% of mycelial growth (EC_{50}) was determined for the three resistant isolates. The EC_{50} value of isolate CBa82 was 0.16 $\mu\text{g/ml}$ and was categorized as low-resistant. This EC_{50} value was about 16-fold higher than the EC_{50} of the two sensitive isolates investigated (EC_{50} 0.01 $\mu\text{g/ml}$). The other two isolates resistant to fludioxonil (KC25 and KC33) had EC_{50} values of 0.324 and 0.383 $\mu\text{g/ml}$, respectively, and were categorized as medium-resistant. All three isolates were also resistant to iprodione.

A model was introduced to test whether the distribution of susceptible and resistant phenotypes of isolates to a specific fungicide was random or biased. For the blackberry population, the results indicated that the subpopulation resistant to thiophanate-methyl was more likely to have evolved into a subpopulation resistant to thiophanate-methyl and

pyraclostrobin ($p < 0.05$), which was more likely to have evolved into a subpopulation resistant to thiophanate-methyl, pyraclostrobin and boscalid ($p < 0.05$). There was a bias toward developing strains resistant to thiophanate-methyl, pyraclostrobin, boscalid and cyprodinil from the subpopulation resistant to thiophanate-methyl, pyraclostrobin, and boscalid ($p < 0.05$). The low number of isolates ($n=2$) resistant to all five chemical classes including the hydroxylanilides (fenhexamid) did not support significant differences between the S/R ratio that derived from the subpopulation resistant to four chemical classes of fungicides (Fig. 4.3.A). A very similar pattern was observed for strawberry isolates (Fig. 4.3.B). The 5SR phenotype characterized by resistance to thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, and fenhexamid was more likely to have derived from the subpopulation resistant to thiophanate-methyl, pyraclostrobin, boscalid and cyprodinil ($p < 0.05$); the latter phenotype was more likely to have derived from the population resistant to thiophanate-methyl, pyraclostrobin, and boscalid ($p < 0.05$) and the isolates resistant to thiophanate-methyl and pyraclostrobin were more likely to have derived from the subpopulation resistant to thiophanate-methyl ($p < 0.05$).

Detection of target gene mutations. To determine whether resistance was based on single gene resistances, the presence or absence of mutations in genes encoding target proteins for fenhexamid, boscalid, pyraclostrobin, and thiophanate-methyl were investigated for all blackberry isolates with resistance phenotypes. All 23 blackberry isolates resistant to fenhexamid yielded a band approximately 586 bp in size when DNA was amplified with primers F412_F and F412_R. Sequence analysis revealed that 18

isolates possessed the F412S mutation, 4 had the F412I mutation, and one isolate (KC52) had a new mutation Y408H. Because the latter mutation had not been described before, The entire *erg27* gene was sequenced and found that this gene also lacked aa P298 (Table 4.4.). Three fenhexamid-sensitive isolates were randomly chosen and screened for mutations in the *erg27* gene but none were found (data not shown). Primers IpBcBeg and IpBcEnd2 amplified PCR fragments of about 950 bp in length from the *sdhB* gene of 24 blackberry isolates resistant to boscalid. Sequence analysis revealed nucleotide variations in resistant isolates but not the sensitive isolate from “CAC” to “TAC” in 8 isolates and ”CGC” in 16 isolates. This mutation changed the aa at position 272 from histidine (H) to tyrosine (Y) or arginine (R) (Table 4.4.). Primers Qo13ext and Qo14ext amplified the expected 560 bp product from the *cytb* gene of all 93 blackberry isolates resistant to pyraclostrobin and 1 isolate sensitive to pyraclostrobin. Enzyme *Fnu4HI* cut all amplicons from resistant isolates into two fragments of 318 bp and 242 bp in length indicating the presence of the G143A mutation (Table 4.4.). The PCR product from the isolates sensitive to pyraclostrobin remained undigested. A PCR product of approximately 380 bp in size was amplified from 137 blackberry isolates (136-resistant and 1 sensitive to thiophanate-methyl, respectively). The amplicon from 119 blackberry resistant isolates was successfully digested with *ThaI*, indicating the presence of the E198A mutation. The PCR product from the isolates sensitive to thiophanate-methyl remained undigested (data not shown). The remaining 17 blackberry isolates (all from location WM) resistant to thiophanate-methyl were not digestable with *ThaI* and therefore subjected to sequence

analysis. The target gene revealed a nucleotide change from “GAG” to “GTG” at aa position 198, which changed glutamic acid to valine (E198V mutation; Table 4.4.).

Discussion

Current models suggest that fungicide-resistant genotypes pre-exist at a low frequency (about 10^{-9}) in a genetically diverse population (48, 136) prior to the introduction of a fungicide. In the absence of other constraints, one, two, or more independent forces, such as pressure from single or multiple fungicides, could select for single, dual, or greater levels of resistances. This is especially so if fungicides with different modes of action act on targets at different places in the genome. The phenotypic fungicide resistance patterns observed for populations from both crops (Tables 4.2. and 4.3.) did not evolve randomly (Fig. 4.3.A and B). Phenotypes resistant to multiple fungicides were more likely to have evolved from previously resistant subpopulations. The model suggests a stepwise accumulation of single resistance loci in *B. cinerea* populations from both crops. Populations resistant to thiophanate-methyl gave rise to populations resistant to pyraclostrobin, which gave rise to populations resistant to boscalid, which gave rise to populations resistant to cyprodinil and/or fenhexamid. Fungicide pressure narrows the populations gene pool due to targeted selection of genotypes, but evolution in form of progressive changes in the gene pool causes the population to diversify and therefore allows emergence of new phenotypes (58). A simplified version of this standard population genetics theory describing stepwise selection, diversification, and accumulation of

resistances is outlined in figure 4.4. In this model, field applications of fungicides of a single chemical class followed by population recovery selects for genotypes resistant to fungicides of this chemical class and allows for diversification of the population. Diversification allows for emergence of strains with preexisting resistance to the next fungicide. The combined or alternated application of fungicides of the two chemical classes allows for the selection of preexisting genotypes with triple fungicide resistance (Fig. 4.4.).

Populations of *B. cinerea* from blackberry and strawberry fields in the Carolinas were resistant to multiple chemical classes of fungicides. Populations from conventionally sprayed fields (i.e. KC, MC, CO, MOD, SBY, JEY, and KUD) had, with the exception of field SBY, a single-most prevalent resistance phenotype. These phenotypes were a resistance to thiophanate-methyl, pyraclostrobin, and boscalid and a resistance to thiophanate-methyl, pyraclostrobin, boscalid and iprodione in blackberry fields, and resistance to thiophante-methyl, pyraclostrobin, and boscalid and a resistance to thiophanate-methyl, pyraclostrobin, and cyprodinil in strawberry fields. This suggests that weekly applications of alternating fungicides, or mixtures of site-specific fungicides, selected for multifungicide resistant strains. In contrast, the most prevalent phenotype for less frequently sprayed fields, such as WM (blackberry), MV, HP, and FLOR (strawberry), consisted of either only one (1SR) or no (0SR) single-resistances phenotypes. These data support common risk assessments that a reduction in the number of applications per season is an effective strategy to slow resistance development (14). Interestingly, multifungicide-resistant phenotypes existed at low frequency in the organic field, WIC, and many of the

less-frequently sprayed fields (WM, MV, HP, FLOR). This suggests that if growers were to increase their use of fungicides, selection for multifungicide resistance could occur within a few sprays or a single season.

Resistance to four or five chemical classes (4SR and 5SR) was found in almost all blackberry and strawberry fields, raising the question whether these phenotypes are a result of independent selection with small amounts of migration, or successive expanding foci. Some locations were well over 100 km apart suggesting that the multifungicide-resistant genotypes may have evolved independently from another with little migration. Furthermore, recent evidence suggests that strawberry plug plants are a source of *B. cinerea* infections in plastic culture production systems (95). Therefore *B. cinerea* multifungicide-resistant strains may have evolved at the nursery level and then be distributed to producers by way of planting material. This could also explain the presence of multifungicide-resistant strains in the organic field WIC.

With only one exception, all fungicide-resistant isolates from both crops were resistant to MBC fungicide thiophanate-methyl. MBC fungicides were introduced in the 1970s and therefore used more often than the other six chemical classes, most of which were introduced much later. The combination of spray frequency, the existence of naturally resistant strains in the field (128), the qualitative type of resistance (13, 22), and stability of the resistance (21, 113) may have probably caused a shift in the endemic population towards a dominant MBC resistance dominance. Our data suggest that virtually all other resistance phenotypes derived from that dominant population. Dual resistance to QoIs and

SDHIs have been reported in field strains from Europe (7, 79, 88), the United States (70), and Asia (64). The delay in resistance development to AP fungicides may be because this product is mostly used in combination with fludioxonil, a compound that has proven quite resilient to resistance development (141). The delay in resistance to the HA fungicide fenhexamid may be the result of a documented reduced fitness of mutants with point mutations in the *erg 27* gene (11, 19). As for the DC fungicide iprodione, resistance was rare despite the fact that it had been registered for over 30 years. However strawberry producers have not been using iprodione or used it rarely, due to its label restricted use of one application prior to bloom. Although more applications are available to blackberry growers, the compound is not commonly used. In addition, multiple-genes could be involved in the development of resistance to DC fungicides, explaining the slower shift toward resistance (113).

Resistance to fludioxonil has only been described in isolates from grapevines in Germany (76), some apple isolates from apple in Washington State (141), and one isolate from strawberry in Virginia (31). The EC₅₀ values for fludioxonil in resistant isolates ranged from 0.04 to 1.5 µg/ml based on sampling location and assay method: Germany (1.5 µg/ml, germination assay), Washington (0.04 µg/ml, mycelia growth assay), and Virginia (0.26 µg/ml, germination assay). Isolates with resistance to fludioxonil possessed fitness penalties (141), which may explain the lack or low frequency of fludioxonil-resistant isolates in *B. cinerea* populations (32, 76). Both isolates from orchard KC that were resistant to fludioxonil were also resistant to thiophanate-methyl, pyraclostrobin,

cyprodinil, and iprodione. Whether the fludioxonil-resistant strains described in this study are capable of causing significant yield loss following field applications of formulated product (i.e., Switch) is unclear.

Consistent with results published for our strawberry isolates (33, 36, 51), resistance to thiophanate-methyl, pyraclostrobin, boscalid, and fenhexamid was without exception associated with target site mutations. The predominant mutation conferring resistance to fenhexamid in blackberry fields of the Carolinas was F412S. This was consistent with our investigation of *B. cinerea* from strawberry fields in the Carolinas, where F412S was found in 80.6 % of the isolates (51). In both studies the F412I mutation was found rarely. The resistance phenotype associated with the Y408H mutation combined with the P298 deletion is described here for the first time. Other mutations such as the T63I and F412C, found at low frequencies in isolates from strawberries resistant to fenhexamid (51), were not found in the blackberry isolates we examined.

Although many mutations in the *sdhB* gene, such as H272L (79), P225L, and P225F (108), can cause resistance to SDHI fungicides, the predominant mutations in our collection were H272R and H272Y. Both genotypes were also the most prevalent found in our strawberry collection from the Carolinas (33). In both studies, the two mutations appeared at a ratio of 2 to 1. This may indicate a slight fitness advantage of genotype H272R, as described previously (117), or may be evidence that the blackberry and strawberry populations share a common habitat and that there is an exchange of isolates among populations from different hosts. If this is true, it is more likely that the blackberry

populations provide inoculum for the strawberry populations, because blackberries are perennials while most strawberry varieties in the Carolinas are grown as annuals in plastic culture-production systems.

The G143A mutation is the most frequently found and most powerful mutation in the cytochrome b protein conferring resistance to QoI fungicides (37). In this study, all isolates resistant to pyraclostrobin analyzed had the G143A mutation. The same mutation also was responsible for resistance to pyraclostrobin in *B. cinerea* from strawberry (33). Other point mutations, such as G143R were found to contribute to resistance in *Pyrenophora* spp., but were not present in our studies (104). The sole presence of G143A in *B. cinerea* isolates resistant to QoI fungicides further validates further the use of molecular techniques specific for the detection of the mutation in this pathogen.

In our study, 87.5% of the blackberry isolates resistant to thiophanate-methyl contained the E198A mutation, which makes this mutation the most prevalent one in *B. cinerea* from blackberry and strawberry in the Carolinas (36). A total of 12.5% of the blackberry isolates contained the E198V mutation in the β -tubulin gene. Variations at position 198 are commonly found in isolates highly resistant to thiophanate-methyl (6, 86), but the E198V mutation is rather rare. It was first reported in 2008 in isolates from various of hosts (i.e., cucumber, eggplant, tomato and ornamental crops) in Japan (3) and to the best of our knowledge it has never been identified anywhere else.

In conclusion, this study shows that *B. cinerea* populations from blackberry and strawberry fields tend to have a single dominant fungicide resistance profile and that this

profile is represented by multiple fungicides in fields frequently sprayed with site specific fungicides. Multifungicide resistance likely evolved through stepwise selection and accumulation of resistance. The discovery of isolates resistant to fludioxonil and cyprodinil indicates that repeated application of Switch, may eventually select for resistance to both fungicides. The continued use of site-specific fungicides, applied as mixtures or in alternation, will likely increase the prevalence of strains resistant to multiple fungicides. This assumption will decrease the growers` ability to control gray mold in the future. New strategies may have to be implemented to delay the selection of multifungicide resistance. These strategies may include a more frugal use of site-specific fungicides, a more frequent use of multisite inhibitors, and emphasis on clean plant sources and sanitation.

Table 4.1. Phenotypic variation in fungicide resistance of *Botrytis cinerea* isolates from blackberry and strawberry fields in North and South Carolina

No.	Phenotype Resistance to fungicide ^a	Single resistances (SR)	Number of isolates ^b														Total	%	
			Blackberry field					Strawberry field											
			CB ⁰	WM ^{<5}	KC ^{>12}	MC ^{>12}	CO ^{>12}	WIC ⁰	MV ^{<5}	HP ^{<5}	FLOR ^{<5}	NC ^{N/A}	GIK ^{>12}	MOD ^{>12}	SBY ^{>12}	JEY ^{>12}	KUD ^{>12}		
1	None	0SR	51	4	1	0	0	8	8	29	10	5	3	2	0	0	0	121	29.37
2	Py	1SR	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.24
3	Tm	1SR	0	24	0	0	0	0	0	3	0	0	1	0	0	0	0	28	6.80
4	Tm-bo	2SR	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.24
5	Tm-cy	2SR	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	3	0.73
6	Tm-py	2SR	0	2	1	0	0	3	0	0	0	0	2	0	1	0	0	9	2.18
7	Tm-ip-fl	3SR	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.24
8	Tm-py-bo	3SR	0	7	33	3	19	2	1	0	1	0	6	3	3	2	8	88	21.36
9	Tm-py-cy	3SR	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	3	0.73
10	Tm-py-fe	3SR	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.24
11	Tm-py-bo-cy	4SR	0	0	0	4	8	6	2	0	2	3	5	8	11	18	12	79	19.17
12	Tm-py-bo-fe	4SR	0	1	14	0	4	0	0	0	0	1	0	0	8	1	1	30	7.28
13	Tm-py-bo-ip	4SR	0	1	0	7	3	0	0	0	0	0	0	0	1	0	0	12	2.91
14	Tm-py-bo-cy-ip	5SR	0	1	0	1	0	0	0	0	0	0	0	0	1	2	0	5	1.21
15	Tm-py-bo-ip-fe	5SR	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0.24
16	Tm-py-bo-cy-fe	5SR	0	0	2	0	0	0	0	1	0	3	3	5	11	2	0	27	6.55
17	Tm-py-cy-ip-fl	5SR	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	0.49
Total		...	53	42	53	16	34	21	11	33	13	12	21	20	37	25	21	412	100.00

^apy = pyraclostrobin (QoI), tm = thiophanate methyl (MBC), bo = boscalid (SDHI), cy = cyprodinil (AP), ip = iprodione (DC), fl = fludioxonil (PP), fe = fenhexamid (HA).

^bnumbers in superscript following field names indicate the estimated number of sprays with site-specific fungicide per season.

^cthe number of isolates of a majority phenotype is displayed in bold.

Table 4.2. Fungicide-resistant phenotypes in North and South Carolina blackberry fields

Isolates		Phenotype*							
No.	%	Single resistances	Tm	Py	Bo	Cy	Fe	Ip	Fl
56	28.3	0SR	S	S	S	S	S	S	S
1	0.5	1SR	S	R	S	S	S	S	S
24	12.1	1SR	R	S	S	S	S	S	S
1	0.5	3SR	R	S	S	S	S	R	LR
1	0.5	2SR	R	S	R	S	S	S	S
3	1.5	2SR	R	R	S	S	S	S/LR	S
1	0.5	3SR	R	R	S	S	R	S	S
2	1.0	5SR	R	R	S	R	S	R	MR
70	35.4	3SR	R	R	R	S	S	S/LR	S
21	10.6	4SR	R	R	R	S	R	S/LR	S
2	1.0	4SR	R	R	R	S	S	R	S
14	7.1	4SR	R	R	R	R	S	S/LR	S
2	1.0	5SR	R	R	R	R	R	S/LR	S

*Tm = thiophanate methyl (MBC), py = pyraclostrobin (QoI), bo = boscalid (SDHI), cy = cyprodinil (AP), fe = fenhexamid (HA), ip = iprodione (DC), fl = fludioxonil (PP); S = sensitive, R = resistant, LR = low resistance, MR = medium resistance.

Table 4.3. Fungicide-resistant phenotypes in North and South Carolina strawberry fields

Isolates		Phenotype*							
No.	%	Single resistances	Tm	Py	Bo	Cy	Fe	Ip	Fl
65	30.4	0SR	S	S	S	S	S	S	S
4	1.9	1SR	R	S	S	S	S	S	S
3	1.4	2SR	R	S	S	R	S	S	S
6	2.8	2SR	R	R	S	S	S	S/LR	S
3	1.4	3SR	R	R	S	R	S	LR	S
26	12.1	3SR	R	R	R	S	S	S/LR	S
11	5.1	4SR	R	R	R	S	R	S/LR	S
1	0.5	4SR	R	R	R	S	S	R	S
67	31.3	4SR	R	R	R	R	S	S/LR	S
3	1.4	5SR	R	R	R	R	S	R	S
25	11.7	5SR	R	R	R	R	R	S/LR	S

*Tm = thiophanate methyl (MBC), py = pyraclostrobin (QoI), bo = boscalid (SDHI), cy = cyprodinil (AP), fe = fenhexamid (HA), ip = iprodione (DC), fl = fludioxonil (PP); S = sensitive, R = resistant, LR = low resistance.

Table 4.4. Mutations in target genes in blackberry isolates of *Botrytis cinerea* resistant to thiophanate-methyl, boscalid, pyraclostrobin, and fenhexamid

	Amino acid substitutions in target genes							
	<i>β</i> -tubulin (n=136)		SdhB (n=24)		Cytochrome b (n=93)	Erg27 (n=23)		
	E198A	E198V	H272Y	H272R	G143A	F412I	F412S	Y408H & deletion P298
number	119	17	8	16	93	4	18	1
%	87.5	12.5	33.3	66.7	100	17.4	78.3	4.3

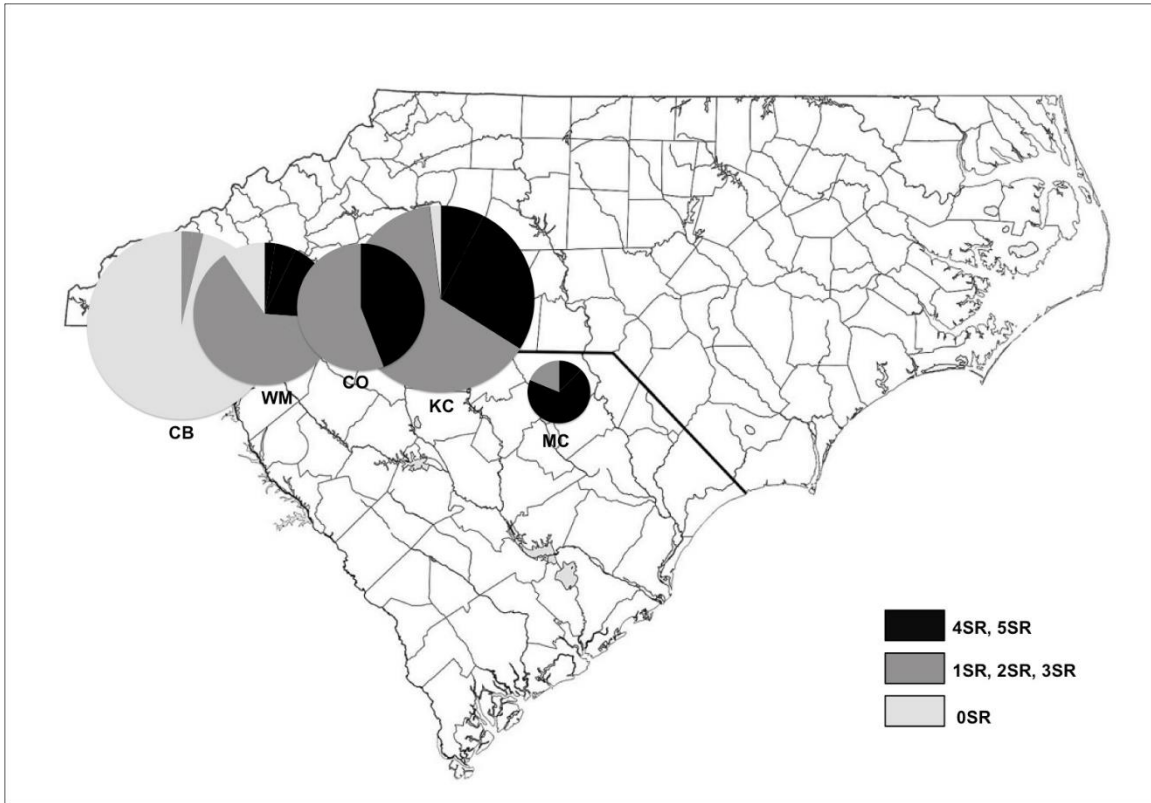


Fig. 4.1. Occurrence and prevalence of single resistances (SR) in *Botrytis cinerea* isolates from blackberry farms in North and South Carolina. The circle diameter corresponds to the number of isolates tested in each location.

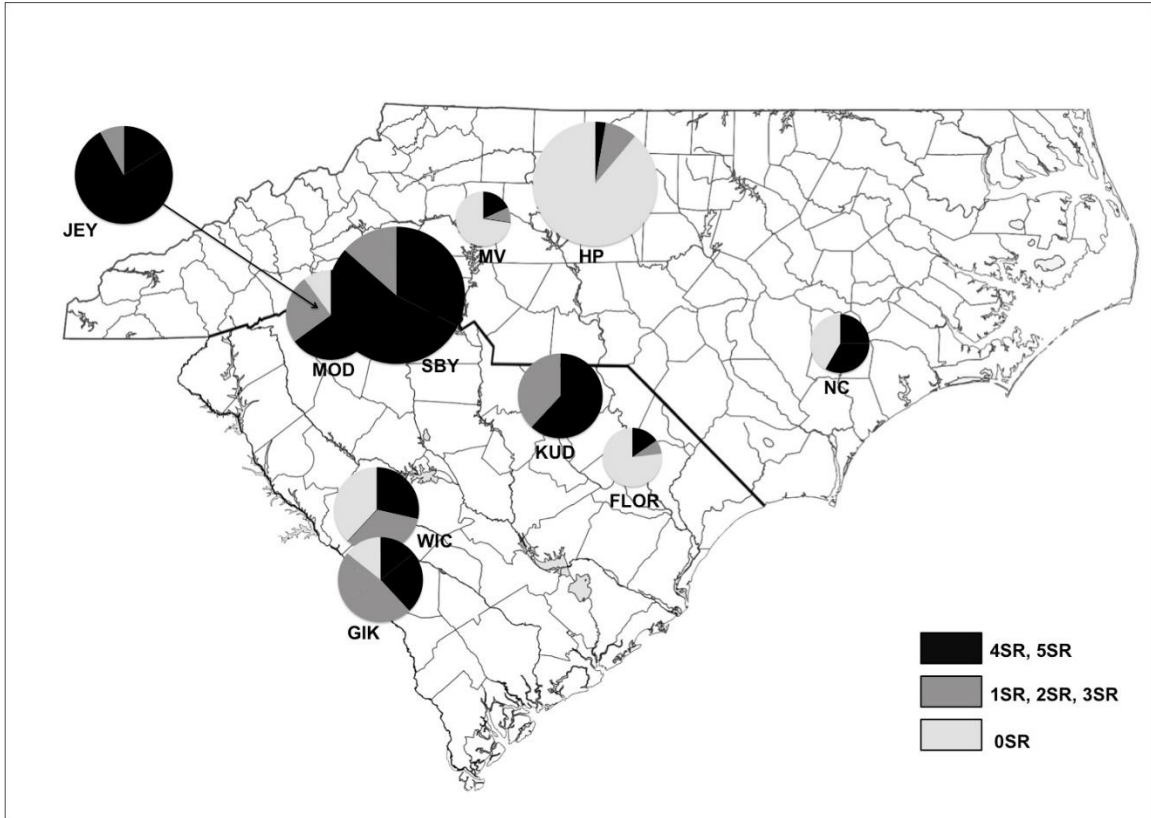


Fig. 4.2. Occurrence and prevalence of single resistance (SR) in *Botrytis cinerea* isolates from strawberry fields in North and South Carolina. The circle diameter corresponds to the number of isolates tested in each location, which is also indicated in parenthesis. The black arrow indicates the center of the circle for commercial field JEY.

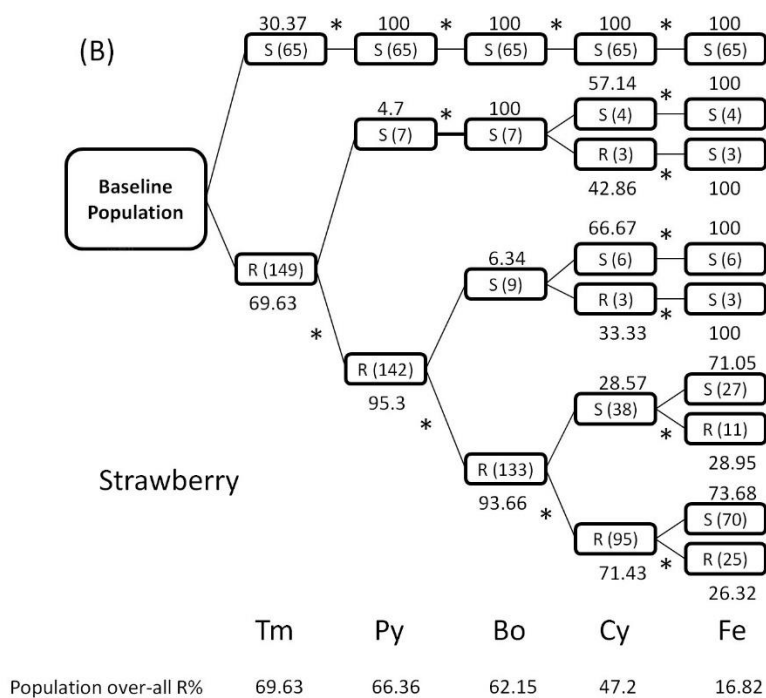
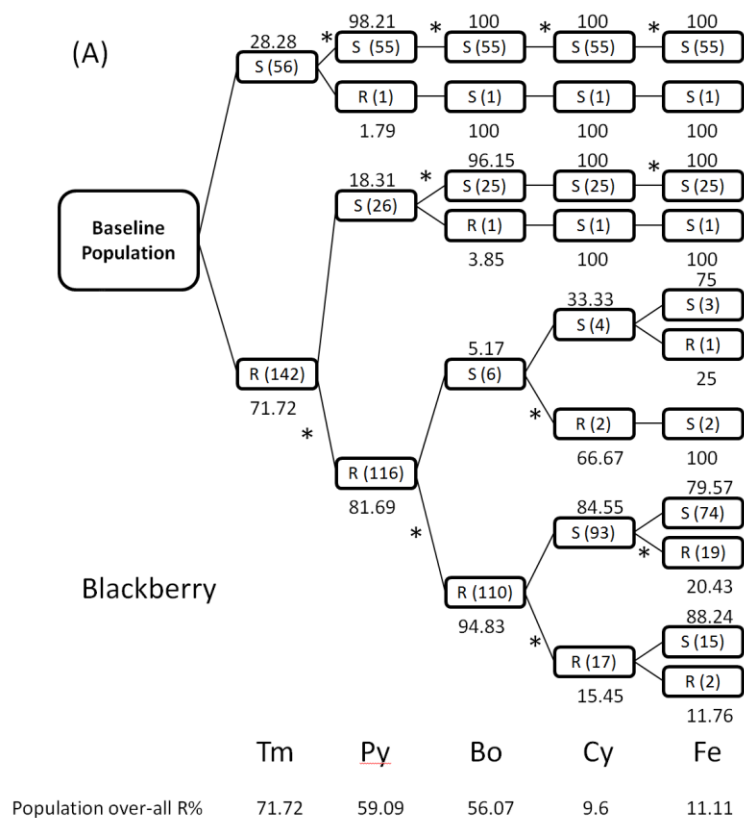


Fig. 4.3. Statistical model indicating bias toward the evolution of phenotypic fungicide resistance patterns in *Botrytis cinerea* from strawberry and blackberry fields. An asterisk (*) indicates the probability of resistance (R) in the subpopulation, which derived from a previous R population is significantly higher (chi-square test; p-value <0.05) than the probability of R in the over-all population for a certain fungicide. The number above or below the box indicates the ratio of that phenotype derived from the previous population. The numbers in parenthesis in the box indicates the isolate number in that group. The bottom line indicates the fungicide (tm = thiophante-methyl, py = pyraclostrobin, bo = boscalid, cy = cyprodinil, and fe = fenhexamid) in each selection stage and below is the resistant ratio in over-all population to that fungicide.

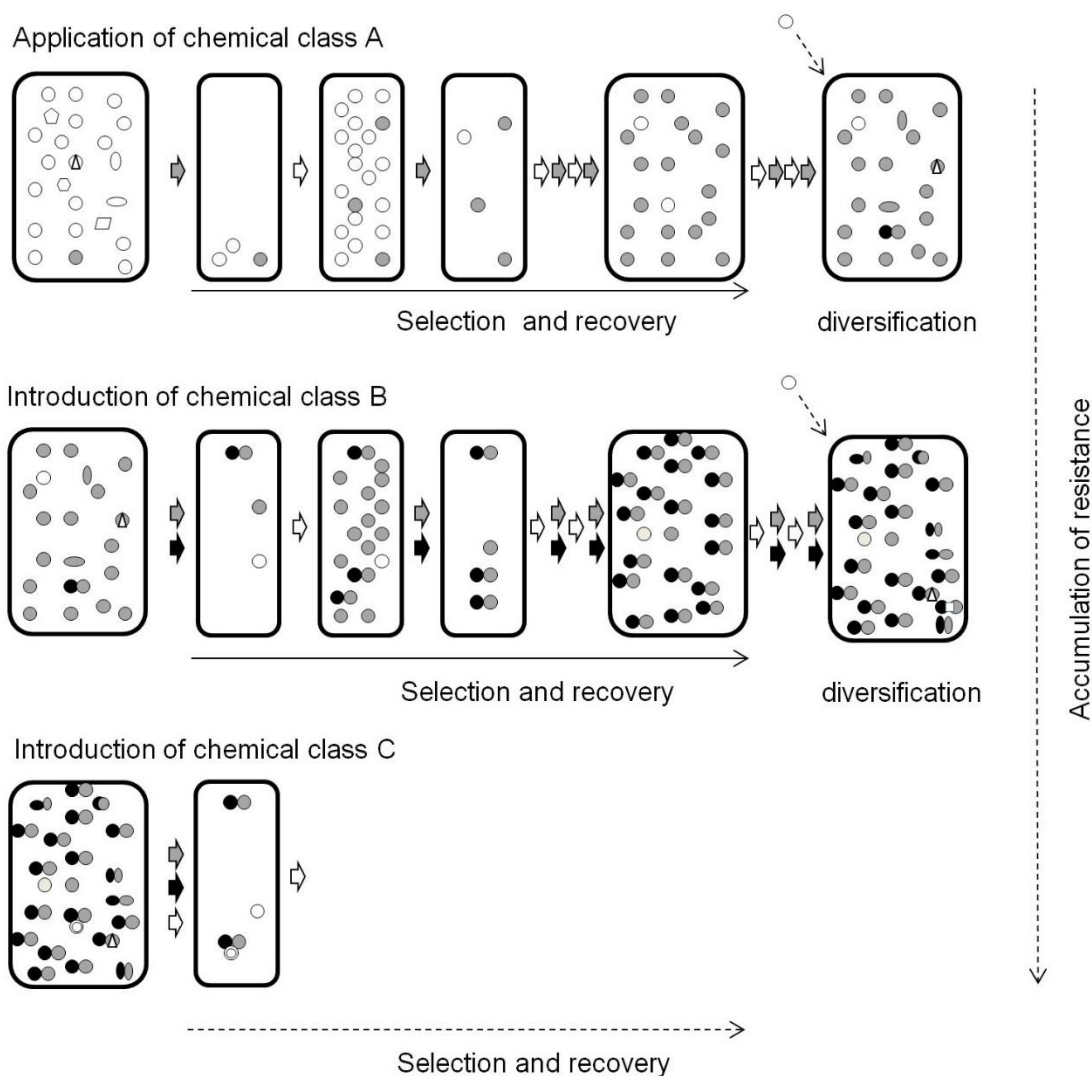


Fig. 4.4. Simplified representation of standard population genetics theory showing stepwise selection and accumulation of single resistances. A genotype (gray circle) with preexisting resistance to chemical class A is emerging from a genetically diverse population. The process of application of fungicides of chemical class A (gray arrow) and recovery (white arrow) selects for genotypes resistant to chemical class A and allows for diversification of the population. The introduction of chemical class B (black arrow) in rotation or mixture with A allows for the selection of pre-existing phenotypes with dual

resistance (combined gray and black circle). The single, empty circle outside the boxes indicates influx of genotypes from the outside.

CHAPTER FIVE

RESISTANCE TO FLUDIOXONIL IN *BOTRYTIS CINEREA* ISOLATES FROM BLACKBERRY AND STRAWBERRY

This work has been published:

X.P. Li, D. Fernández-Ortuño, A. Grabke and G. Schnabel. 2014. Resistance to Fludioxonil in *Botrytis cinerea* Isolates from Blackberry and Strawberry. *Phytopathology*. 104:724-732. ["Fungal isolates and culture conditions" of strawberry and blackberry isolates were performed by Dr. Dolores Fernández-Ortuño and Xingpeng Li, respectively; "RNA extraction and cDNA synthesis" was performed by Xingpeng Li and Anja Grabke]

Abstract

Site-specific fungicides, including the phenylpyrrole fludioxonil, are frequently used for gray mold control but are at risk for the development of resistance. In this study field isolates with low resistance (LR) and moderate resistance (MR) to fludioxonil from blackberry and strawberry fields of North Carolina, South Carolina, and Virginia were characterized. Genes involved in osmoregulation, including *bcsak1*, *BcOS4*, *bos5* and *BRRG-1*, were cloned and sequenced to detect potential target gene alterations, but none were found. A previously described mutation (R632I) in transcription factor Mrr1, which is known to increase the expression of ABC transporter AtrB, was found in MR but not in sensitive (S) or LR isolates. Expression of *atrB* in MR isolates was about 200-fold

increased compared to a sensitive isolate, but 30 to 100-fold overexpression was also detected in LR isolates. Both MR isolates exhibited increased sensitivity to salt stress in form of mycelial growth inhibition at 4% NaCl, indicating a disruption of osmoregulatory processes in those strains. However, the glycerol content was indistinguishable between S, LR and MR isolates with and without exposure to fludioxonil suggesting that the glycerol synthesis pathway may not be a part of the resistance mechanism in LR or MR strains. An investigation into the origin of LR and MR isolates from blackberry revealed two insertions in the *mrr1* gene consistent with those found in the *Botrytis* clade group S. The emergence of strains overexpressing *atrB* in European and now in North American strawberry fields underscores the importance of this resistance mechanism for resistance development to fludioxonil in *B. cinerea*.

Introduction

Fludioxonil is a phenylpyrrole fungicide with broad spectrum activity against fungal plant pathogens among ascomycetes and basidiomycetes (45). Fludioxonil is effective against most diseases of seeds, including seedling blight, stem-base browning, snow mold, and common bunt. It is very effective against *Botrytis cinerea* Pers.:Fr, the causal agent of gray mold disease of many vegetables and small fruits, and inhibits both mycelial growth and spore germination (78). It is registered in the United States against various post-harvest rots of pome fruits, stone fruits, and sweet potato. Fludioxonil was

first registered in 2002 and is listed under Fungicide Resistance Action Committee (FRAC) code 12 with FRAC resistance risk category low to medium.

Resistance in *B. cinerea* to fludioxonil is rarely found in the field (32, 80, 119) worldwide. One isolate from apples grown in Washington State exhibited moderate resistance (MR) to fludioxonil but were impaired in fitness and pathogenicity on apple fruit (141). Isolates with reduced sensitivity to fludioxonil were found in strawberry fields in Virginia (31), Maryland and South Carolina (34). Low resistant (LR) and MR *B. cinerea* isolates were reported from European small fruits, including strawberry (73, 76) and grapevines (73, 75, 90). The EC₅₀ values for isolates with MR to fludioxonil from Germany and Washington ranged from 0.04 to 1.5 µg/ml depending on the evaluation method (76, 141). High levels of resistance to fludioxonil have not been reported in the field, probably due to fitness penalty in such isolates (14, 80).

The precise mode of action of fludioxonil is still unknown, but fludioxonil treatment induced cell death in filamentous fungi by improperly activating the Hog1-type mitogen-activated protein kinase (MAPK) (71, 135, 140), indicating possible disruption of the osmoregulation pathway. High levels of fludioxonil resistance in laboratory mutants are linked to enzymes involved in osmoregulation pathway, such as histidine-kinases (HK) and specifically the group III HK is considered to be a target for fludioxonil in yeast and filamentous fungi (5, 56, 94, 135). Some evidence was provided for fludioxonil resistance to be linked to at least two different genes (119). Genes assumed to be involved in osmoregulation in *B. cinerea* were identified by gene disruption and include *bos1* (), *bcsak1*

(102), *BcOS4* (132), *bos5* (131) and *BRRG-1* (130). Corresponding knock out mutants revealed reduced salt tolerance and increased resistance to fludioxonil. Hypersensitivity to osmotic stress has also been associated with lack of glycerol accumulation and resistance to the fludioxonil (68).

Moderate resistance to fludioxonil in *B. cinerea* is associated with the multidrug resistance phenotypes MDR1 and MDR1h (73, 76). Both phenotypes are associated with overexpression of the ATP-binding cassette (ABC) transporter gene *atrB* and mutations in transcription factor Mrr1 (73, 118). While MDR1 phenotypes express *atrB* about 50- fold, MDR1h phenotypes express *atrB* at even higher levels (76). The overexpression of two other multidrug transporters has been linked to multifungicide resistance in *B. cinerea*, including *mfsM2* and *atrD*. MfsM2 belongs to the major facilitator superfamily (MFS) and overexpression is associated with increased resistance to multiple fungicides (designated MDR2), including low levels of resistance to fludioxonil (17). AtrD is an ATP-binding cassette (ABC) transporter that was linked previously to azole resistance in strains of *B. cinerea* (59, 60).

As part of a region wide fungicide resistance monitoring program involving *B. cinerea* from blackberry and strawberry, we identified several isolates MR and LR to fludioxonil. The goal of this study was to characterize the molecular basis of resistance to fludioxonil in these isolates. Specific objectives were to investigate (i) potential alterations in genes involved in osmoregulation and promoter rearrangements upstream *mfsM2*, (ii) overexpression of *atrB* and *atrD*, (iii) glycerol synthesis and hypersensitivity to salt stress,

and (iv) fitness components and cross resistance with dicarboxamide fungicide iprodione in isolates LR and MR to fludioxonil.

Material and Methods

Isolates and fungicides. *B. cinerea* isolates were isolated from North and South Carolina blackberry fields, a Virginia strawberry field, and obtained from Germany (Table 5.1.). Single spore isolations from symptomatic blackberry and strawberry fruits were conducted as described previously (81). *B. cinerea* strain Do9_K_A31 (designated Do9 in this study) from a strawberry field in Germany was obtained from Dr. Matthias Hahn, University of Kaiserslautern, Kaiserslautern, Germany and used as multi-drug resistance type 1 (MDR1h), group S reference strain (76). Formulated fludioxonil (Scholar SC fungicide, Syngenta, Research Triangle Park, Raleigh, NC; 20.4% a.i. vol/vol) and iprodione (Rovral 4 FL, Bayer CropScience, Research Triangle Park, NC; 41% a.i. vol/vol) were used to determine sensitivities to these fungicides in vitro. Tolnaftate and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO), dissolved in 100% ethanol, and diluted in ultra-purified water before added to the liquid medium. Fungicide stock solutions were adjusted to keep final solvent concentrations below 1.5% (vol/vol). No significant growth inhibition was observed for strains on media with less than 1.5% ethanol (73, 90).

Fungicide sensitivity tests. Fungicide sensitivity categories to fludioxonil and iprodione were determined using a spore germination assay (50, 125). The effective doses

that inhibit 50% of mycelial growth (EC₅₀ values) were determined as described previously (73) with minor modifications. Briefly, 5000 spores were transferred to 0.1 ml 96-microplate cultures. Final concentrations for all compounds were 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 µg/ml. Tests were performed in malt extract broth. After 48 h of incubation at 22°C in darkness, the optical density at wavelength A₆₀₀ was determined. The experiment was repeated.

DNA extraction, amplification and sequencing of *mrr1* and the *mfsM2* promoter regions. DNA was extracted as described previously (16). The *mrr1* gene was amplified in two parts; the upstream region was amplified with primers *mrr1_atg* and *TF1-2_new* and the downstream region was amplified with *TF1-3_new* and *TF1-4* (76). The *mfsM2* promoter region of *B. cinerea* was amplified with primers *Prom_Mfs2_1fw* and *Prom_Mfs2_2rev* (73). Fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV-light. The fragment sizes of PCR products were verified by comparison to a Low Range Plus DNA Ladder (Fisher Scientific, Waltham, MA). The PCR products were purified using the ExoSAP-IT Purification Kit (USB Corporation, Cleveland, Ohio) following manufacturers recommendations. Purified products were sequenced at Clemson University Genomic Center (Clemson, SC). Nucleotide sequences were analyzed, assembled, and aligned with DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI). *B. cinerea* strain T4 genome sequences were obtained from Genbank, accession number FQ790286.1.

RNA extraction and gene expression analysis. Isolates were grown on potato

dextrose agar medium (PDA; Difco Laboratories, Sparks, MD) in 9-cm in diameter Petri dishes for 10 to 14 days at 22°C with 12 h intervals of fluorescent light and darkness. Conidia were gently scraped off with a sterile, disposable 10 µl inoculation loop (VWR International LLC, Radnor, PA) without touching the agar. Then spores were suspended in 2 ml potato dextrose broth (PDB) with final spore concentration between 1 to 5×10^5 in 6 cm diameter Petri dishes. The spores were incubated for 14.5 h on a shaker with 100 rpm at 22°C. The germlings were incubated for another 30 min in the petri-dish either with or without fludioxonil at a final concentration of 1 mg/liter. For RNA isolation, the germlings with the medium were transferred to a 2 ml centrifuge tube and centrifuged for 10,000 rpm for 4 min. The pellet was washed twice with autoclaved, distilled water. Total fungal RNA was isolated using the MasterPure™ Yeast RNA Purification Kit (EpiCentre Pte. Ltd. Madison, WI), the RNA quality was checked and quantified by spectrophotometric analysis with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). The RNA was then reverse transcribed into cDNA (iScript™ Cdna Synthesis Kit, Bio-Rad Laboratories, Inc., Hercules, CA). Quantitative RT-PCR was performed as described previously (50) with the exception that we used primers atrBfor / atrBrev and atrDfor/ atrDrev for *AtrB* and *AtrD* expression analysis, respectively, following published amplification protocols (73). Expression of the genes was calculated according to Pfaffel (99). Transcript levels were normalized against the expression levels of housekeeping genes encoding elongation factor 1α (BC1G_09492.1) and shown as normalized fold-expression levels of non-induced germlings from sensitive (S) strains. Means of at least

two biological replicates are shown. Three technical replicates of PCR reaction were used for each biological replicate.

Salt tolerance and determination of glycerol content in germlings. Salt tolerance was investigated on 4% NaCl-amended minimal medium. A 5-mm-diameter mycelial plug taken from 3-day-old culture grown on PDA was transferred to the center of the amended medium and dishes were incubated at 22 °C in the dark. The colony diameter was measured after 3 days of incubation with three replicates per isolate. Mean colony diameter was used to calculate mycelia growth inhibition rate. The experiment was repeated.

The glycerol content was determined in germlings of S, LR, and MR isolates to assess involvement of osmoregulation in the resistance mechanism. Conidia were suspended in 2 ml of PDB in 6 cm diameter Petri dishes with a final concentration of 1×10^5 per ml. Four dishes per isolate per treatment were incubated for 48 h on a shaker with 100 rpm at 22°C. The germlings were incubated for another 1.5 h in the dishes either with or without fludioxonil at a final concentration of 1 mg/liter. The medium containing the germlings was transferred to a 2 ml impact resistant screw cap tube (USA Scientific, Inc. Orlando, FL) and centrifuged at 12,000 rpm for 10 min. Medium was removed and the pellet was washed twice with autoclaved distilled water. The volume for each sample was adjusted to 1 ml with water and ground at 4 m/s for 40 s using a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH). Samples were then centrifuged for 10 min at 12,000 rpm and the glycerol content in the supernatant was determined using EnzyChrom™ Glycerol Assay Kit following the manufacturer's instruction. After all of

the supernatant was removed, the precipitated cell debris was completely dried in an oven for 24 h at 60 °C. The experiments were repeated two times.

Detection of mutations in the *bcsak1*, *BcOS4*, *bos5* and *BRRG-1* genes. Nucleotide sequences of osmotic regulator genes *bcsak1* (102), *BcOS4* (132), *bos5* (131) and *BRRG-1* (130) were amplified by PCR from fludioxonil S, LR and MR isolates. Two overlapping fragments of *bcsak1* were amplified with primer pairs SAK1F-f (TACCCACTCAACCACCAAC)/SAK1F-r (ATCTTGAATACGGGCGAG) / SAK1B-f (GCTGGTCCTGGAGATACTAAGA) /SAK1B-r (AAGCACAAGAGAGCACTCCT). These two fragments were assembled into one contiguous sequence covering the entire gene. The core sequence of *BcOS4* was amplified with primer pair BOS4C-f (GCTGACGATGAGGAATCA) / BOS4C-r (GTGCTGTAAACACCGACA). The primers were designed based on the *B. cinerea* T4 genome sequences (2). The *bos5* and *BRRG-1* genes were amplified with primer pairs Os5-F1 / Os5-R1 (131) and Rrg-F1 / Rrg-R1, respectively (130).

Assessment of fitness and pathogenicity on fungicide-treated fruit. The following fitness components were investigated for isolates sensitive and resistant to fludioxonil and iprodione. All experiments were repeated.

Mycelial growth on PDA. PDA dishes were inoculated with a 5-mm-diameter mycelial plug taken from 7-day-old culture grown on PDA. The dishes were incubated at 22 °C in the dark. The colony diameter was measured after 3 days of incubation with three replicates per isolate. Mean colony diameter was calculated for growth rate determination.

Pathogenicity on untreated and fungicide-treated detached fruit. Pathogenicity of each isolate was confirmed by determining its ability to infect and sporulate on strawberry fruit. Commercially grown ripe strawberry fruit were rinsed with sterile water three times for 30 s each and allowed to air dry. Then, they were placed into plastic boxes (8 strawberries per box for each of the three replicates). When the fruit surface had dried off, each fruit was punctured at one point to a depth of 9.5 mm using a 26G3/8 9.5-mm beveled syringe tip (Becton Dickson & Co.). The wounds were injected with a 30 µl-droplet of conidia suspension (10^6 spores/ml) prepared in distilled sterile water using the same type of syringe. Most of the conidia suspension formed a droplet on top of the wounded area. After inoculation, the boxes were kept at 22 °C. During the first 24 hrs the boxes were sealed with plastic bags to keep the relative humidity high. After 4 days the lesion diameters were measured.

The ability of isolates resistant to fludioxonil and iprodione to cause disease on fruit treated with fungicides was assessed. Experiments were performed as described above for pathogenicity tests with minor modifications. Strawberry fruit were sprayed 4 h prior to inoculation with the recommended label rate of the fungicides Scholar SC (2.5 ml/L), Switch 62.5WG (1.9 g/L; combination of fludioxonil 25% vol/vol and cyprodinil 37.5% vol/vol) and Rovral 4 Flowable (2.5 ml/L), using a hand mister to run off. Control fruit were sprayed with sterile distilled water. After 4 days of inoculation the absence or presence of sporulating lesions was assessed and the lesion diameters were measured and calculated as percent of the control. The experiment was repeated.

Data analysis. Statistical analysis was performed related to the in vitro and in vivo responses (growth rate, lesion size, disease incidence, control efficacy and relative expression of *atrB* and *atrD* genes) to the experimental factors of experimental replication, isolate or fungicide treatment. Significant differences were determined through Tukey HSD test. All calculations were performed by IBM SPSS Statistics, version 19. (IBM SPSS, Armonk, NY) and all tests were performed with $\alpha = 0.05$ except described otherwise.

Results

Sensitivity of isolates to fludioxonil and iprodione in vitro. In our collection of 412 *B. cinerea* isolates from blackberry and strawberry only four isolates were resistant to fludioxonil. Two (Wland1 and CB82a) were categorized as LR based on their ability to germinate on 1 $\mu\text{g/ml}$ fludioxonil and two (KC25 and KC33) as MR based on their ability to germinate on 1 $\mu\text{g/ml}$ fludioxonil and residual growth on 10 $\mu\text{g/ml}$. EC_{50} values determined in microtiter assays were 0.16 and 0.26 $\mu\text{g/ml}$ for LR isolates and 0.32 and 0.38 $\mu\text{g/ml}$ for MR isolates. Fludioxonil LR/MR but not S isolates were resistant to tolnaftate and all were sensitive to cycloheximide consistent with the MDR1 phenotype. Together with two S isolates, the four LR and MR isolates were also investigated for iprodione sensitivity because phenylpyrroles and dicarboxamides are believed to both target proteins involved in osmotic regulation. All isolates LR and MR to fludioxonil were also resistant (R) to iprodione, but some fludioxonil-sensitive isolates were LR to iprodione (Table 5.2.).

Expression of multidrug transporter genes *atrB* and *atrD* in isolates with different fludioxonil sensitivity phenotypes. The *atrB* genes of MR isolates from blackberry (KC25 and KC33) and MDR1h reference isolate Do9 were expressed at approximately 200-fold the level of the sensitive isolates (Fig. 5.1.). Lower expression levels were found for all other phenotypes. The *atrB* expression levels of LR isolates Wland1 and CB82a were 30 and 100-fold increased compared to the S isolate, respectively (Fig. 5.1.). Fludioxonil exposure prior to RNA extraction increased expression of four of the six isolates regardless of their phenotype. They included S isolate CB3a, LR isolate Wland1, and MR isolates KC33 and MDR1h reference isolate Do9.

Analysis of *atrD* gene expression was conducted because previous reports noted overexpression of this gene in association with multidrug resistance phenotypes (59, 60). In this study, the expression of *atrD* was not different among fludioxonil S, LR and MR isolates in untreated or fludioxonil-treated germlings (Fig. 5.2.). Interestingly, the expression of *atrD* in treated germlings was reduced by more than 50% in all isolates after fludioxonil treatment.

Mutations in the transcription factor *mrr1* and the promoter sequences of *mfsM2* in strains LR/MR to fludioxonil. Certain mutations in transcription factor Mrr1 influence *atrB* expression. Therefore *mrr1* was sequenced in its entirety from strawberry isolate Wland1 and six blackberry isolates representing sensitive and resistant phenotypes. The *mrr1* coding regions (including introns) of two sensitive isolates from blackberry (MC14 and KC20) were 2444 bp in length and encoded identical amino acid (aa) sequences compare

to reference isolate T4 with the exception of two aa variation (L475S and M483V) in isolate MC14 (Fig. 5.3.). Sensitive isolate CB3a had an *mrr1* gene sequence of 2477 bp in length and revealed several aa variations compared to T4. The *mrr1* coding region of isolates with resistance phenotypes were 2444 bp, 2483 bp and 2489 bp in size for isolates Wland1, CB82a, and KC25 and KC33 (both 2489 bp), respectively. Two insertions characteristic for group S isolates, 18 bp and 21 bp in length, were discovered in one S, the LR, and both MR isolates from blackberry (Fig. 5.3.). Group S isolates were considered a special clade of *B. cinerea* and genetically different from the isolates studied (76). In our study, there were only five aa variations among non-group S isolates, but considerably more (about 13 on average) among group S isolates. The most diversity (up to 38 aa variations) among *mrr1* gene sequences was found between non-group S and group S isolates. The MDR1-associated mutation R632I was the only mutation uniquely associated with the fludioxonil MR phenotype (Fig. 5.3.). No point mutations or insertions were detected in the *mfsM2* promoter region of isolates LR or MR to fludioxonil.

Evaluation of salt tolerance and glycerol content. Changes in osmo-regulatory pathways conferring resistance to PP and DCs may also influence osmotic sensitivity (44, 67, 93, 129). Isolates MR to fludioxonil were significantly more susceptible to salt stress compared to fludioxonil LR and S isolates (Fig. 5.4.). The sensitivity varied among S isolates, with a range of 25 to 40% inhibition. One of each fludioxonil sensitivity phenotype (S, LR, and MR) was subjected to a glycerol content analysis. The three representative isolates roughly produced similar amounts of glycerol in germlings (Fig. 5.5.). Exposure

of germings to fludioxonil prior to extraction increased glycerol content about 4-fold in all isolates regardless of the phenotype.

Sequencing of genes involved in the osmotic signal transduction pathway. We sequenced large portions of the *bcsak1* (1669 bp), *bos5* (1880 bp), and *BRRG-1* (1655 bp) genes and the core region of the *BcOS4* (1091 bp) gene for the presence of point mutations in S, LR, and MR isolates. No point mutations were identified that were uniquely associated with the fludioxonil LR or MR phenotype (data not shown). Both MR isolates had a 5 bp deletion in an intron at nucleotide position 1261 of the *bcsak1* gene (data not shown), the sequences from one S and one MR isolate was submitted to Genbank (accession numbers KF964016 (CB3a) and KF964017 (KC25)). Analysis of 45 fludioxonil S isolates that originated from the same site as the MR isolates (location KC), and 11, 16, 4, and 24 isolates from locations MC, CO, CA, and WM, respectively, revealed that the deletion was unique to the MR isolates (data not shown). However, the deletion was absent in the fludioxonil MR reference strain from Germany, suggesting that the deletion is not linked to the MDR1 phenotype.

Fitness components of isolates S, LR, and MR to fludioxonil. Cross resistance between phenylpyrroles and dicarboxamides is common because products of both fungicide classes are believed to be involved in the inhibition of proteins involved in osmotic signal transduction. All isolates LR and MR to fludioxonil were also resistant to iprodione. The fludioxonil S isolates however, revealed various iprodione sensitivity phenotypes (Table 5.2.). There were no significant differences in mycelial growth rates and

lesion size between sensitive isolates and fludioxonil LR and MR isolates, with one exception. Mycelium growth was slower for isolate KC25 compared to most isolates including 4 sensitive and 2 dual resistant isolates (Table 5.2.). The same isolate also produced a significantly smaller lesion ($p < 0.05$) size on inoculated fruit compared to all other isolates. The other MR isolate, KC33, did not suffer a fitness penalty in our study. All sensitive and resistant isolates were able to produce sporulating lesions on detached fruit (Table 5.2.).

The ability of fludioxonil S, LR, and MR isolates to cause disease on fruit sprayed with fungicides was assessed to determine if these isolates can withstand label rates of fungicide. Isolates sensitive to fludioxonil were completely controlled with Scholar SC fungicide, but fludioxonil LR and MR isolates developed disease with sporulating lesions. Both isolates MR to fludioxonil were also able to develop sporulating lesions on fruit treated with Switch 62.5WG, a commercially available combination product of fludioxonil and AP fungicide cyprodinil indicating that the AP fungicide component in this mixture did not prevent disease formation. This is consistent with the *in vitro* resistance to cyprodinil and fludioxonil and the MDR1 phenotype. Isolates resistant to iprodione *in vitro*, including the isolates resistant to both iprodione and fludioxonil, developed disease with sporulating lesions on fruit treated with label rates of Rovral 4 Flowable. The isolates sensitive to iprodione were almost completely controlled in the Rovral 4 Flowable treatment (Table 5.2.).

Discussion

While resistance in *B. cinerea* to many classes of fungicides including benzimidazoles, quinone outside inhibitors, succinate dehydrogenase inhibitors, hydroxyanilides, anilinopyrimidines and dicarboxamides is fairly common (7, 74, 75, 76, 124), resistance to phenylpyrroles including fludioxonil is still rare. In this study we show that resistance to fludioxonil is emerging in small fruit crops in the Eastern United States. Resistance to fludioxonil is likely to increase in light of resistance problems with many other compounds of different modes of action. The low frequencies of isolates LR and MR to fludioxonil in our collection of 412 isolates from blackberry and strawberry is consistent with other studies that found that fludioxonil resistance is either absent or rare in Southeastern strawberry fields (31, 32) and that it is likely not of practical relevance for fruit growers at this time. The low frequency of LR and MR isolates validates that phenylpyrroles are still valuable tools for rotations or mixtures with other site-specific fungicides for effective gray mold control. However, the here documented emergence of such strains in United States berry crop production fields should be considered in disease management strategies meant to delay selection. Examples already exist where large portions of field isolates gained partial resistance to fludioxonil due to fungicide selection (76).

In this study, the EC_{50} values for fludioxonil in MR isolates were comparable to those found in isolates from strawberry (73, 76) and grapevines (73, 75, 90) in Europe. MR to fludioxonil in *B. cinerea* isolates from European grapes was designated MDR1 and

conferred by mutation R632I in transcription factor Mrr1 leading to overexpression of ABC transporter gene *atrB* (73). Both MR isolates analyzed in this study contained the R632I mutation in Mrr1 associated with MDR1, but *atrB* overexpression levels were similar to MDR1h reference isolate Do9 (73, 118). MDR1h isolates revealed higher resistance levels to cyprodinil and fludioxonil with generally higher overexpression of *atrB* compared to MDR1 (76). Our isolates, however, did not possess the MDR1h's signature L497 deletion in Mrr1 (23). This suggests that this deletion may not exclusively be responsible for the high levels of *atrB* overexpression. MR isolates were resistant to tolnaftate, but sensitive to cyclohexamide further confirming the MDR1 phenotype (73). The existence of MDR1 in the United States suggests that this resistance mechanism is important for the adaptation of *B. cinerea* to fludioxonil pressure. Because of the great distance between the two continents, the Atlantic Ocean separating the two land masses, and the significant genetic variation in the *mrr1* gene between the German MDR1h reference strain and the isolates from the USA (data not shown), it is likely that the same resistance mechanism developed independently in the two continents.

The LR phenotype may be caused by overexpression of *atrB* as well, albeit at a lower expression level (up to 100-fold in this study). However, variations associated with the MDR1 or MDR1h phenotypes were not found in the *mrr1* genes of LR isolates. An investigation into genes involved in osmotic regulation, including *bcsak1*, *bos5*, *BcOS4* and *BRRG-1*, revealed no nucleotide variations associated with resistance between S, LR, or MR isolates. Furthermore we found no evidence in MR or LR isolates that would suggest

involvement of the MDR2 genotype, which is characterized by promoter region rearrangements upstream the *mfsM2* promoter region and reduced sensitivity to cycloheximide (data not shown) (73).

The lack of fitness cost of isolate KC33 MR to fludioxonil in this study indicates that overexpression of *atrB* may not necessarily come with a fitness cost. However, MR isolate KC25 did have slower growth rates on fruit, which is consistent with reports for a fludioxonil-resistant *P. expansum* field isolate (80). Laboratory mutants of *B. cinerea* revealed two types of fludioxonil-resistant phenotype; one resistant to osmotic stress (FLDosm/r) and another sensitive to high osmotic stress (FLDosm/s). Only FLDosm/s had fitness cost (142) that was later confirmed in field studies (141). In this study, both MR isolates were hypersensitive to salt in form of exposure to 4% NaCl despite having different fitness phenotypes. Neither salt stress hypersensitivity, nor a linkage to iprodione resistance has been reported for MDR1 and MDR1h strains before, and are not necessarily expected because MDR phenotypes are purely based on efflux transporter overexpression. Whether hypersensitivity to salt stress and MR to fludioxonil are linked is unknown due to the low sample size investigated in this study. If they are linked, it could be exploited for management purposes. The increased sensitivity to salt stress was not associated with mutations in the *bcsak1*, *bos5*, *BRRG-1*, and *BcOS4* genes involved in the osmoregulation pathway (data not shown). It is possible that their expression pattern or other genes involved in osmoregulation are responsible for this phenotype. Increased sensitivity to salt stress was also found in *Botrytis* isolates from apple orchards in Washington State (141).

Numerous studies reported the linkage of the fludioxonil-resistant phenotype and hypersensitivity to salt stress in laboratory mutants with dysfunctional genes involved in osmoregulation in *Neurospora crassa* (43, 44, 93) as well as in *B. cinerea* (38, 102, 130, 131, 132). Previous studies on the mode of actions of phenylpyrrole fungicides in *N. crassa* indicated that this class of fungicides affects the osmotic signal transduction pathway (43) and our results indicate that this mode of action is also true for *B. cinerea*.

All isolates, regardless of fludioxonil resistance phenotype, accumulated glycerol when exposed to fludioxonil, which increases the internal turgor pressure. In other fungi the exposure to fludioxonil increased the intracellular glycerol content for both sensitive and moderately resistant isolates but not highly resistant isolates in *Penicillium digitatum* (68). The authors concluded that the mode of action of fludioxonil in *P. digitatum* is probably the mitogen-activated protein kinase pathway that stimulates glycerol synthesis in S and MR. We saw the same phenomenon in our S, LR, and MR isolates, which confirms the involvement of fludioxonil in the mitogen-activated protein kinase pathway in *B. cinerea*, but also suggests that the mechanism conferring LR and MR may not be directly associated with the regulation of glycerol synthesis.

We focused our *mrr1* gene analysis on characterization of fludioxonil phenotypes and therefore sequenced 3 S, 2 LR (one from blackberry and one from strawberry) and 2 MR isolates. Among those strains, 1 S, 1 LR (blackberry) and 2 MR contained the *B. cinerea* group S signature consistent of two insertions (18 bp and 21 bp) in *mrr1* (Fig. 5.3.), which were first identified in isolates from German strawberry fields (76). Thus far, group

S isolates from Germany have been exclusively found in strawberry fields and are believed to be a result of differential host adaptation and/or reduced genetic exchange (76). The presence of group S and non-group S strains in populations from blackberry in this study would indicate that group S strains are not a result of host adaptation. Group S strains might be sexually isolated from other clades as suggested above, given the large numbers of aa variation in group S compared to the non-group S strains. However, many more isolates and different genes would need to be sequenced to validate this hypothesis.

The LR and MR phenotypes were capable of developing disease on fruit sprayed with label rates of Switch 62.5WG, Scholar SC, or Rovral 4 Flowable. Together with our fitness data, this indicates that these resistance phenotypes are likely being selected for by these fungicides in the field and that they may be able to compete with sensitive isolates in the absence of selection pressure. This finding is consistent with a study from Germany that showed establishment of MDR-like phenotypes in both vineyards and strawberry fields after application of different spray programs containing Signum (pyraclostrobin and boscalid), Teldor (fenhexamid) and Switch 62.5WG (76).

In conclusion, *B. cinerea* strains with resistance to fludioxonil are emerging in strawberry and blackberry fields of the Southern United States. The mechanism of resistance in MR isolates is the same as described in European isolates emphasizing the importance and relevance of this mechanism of resistance for fludioxonil. However, the low number of isolates examined does not allow firm conclusions to what extent this

mechanisms of resistance is present in MR isolates. The role of *atrB* overexpression in LR isolates is still unclear.

Table 5.1. Name, origin, and host of isolates used in this study.

Isolate name	Origin	Host	Year of isolation
CA25	South Carolina, US	Blackberry	2010
CB3a	South Carolina, US	Blackberry	2011
CO5	South Carolina, US	Blackberry	2010
MC14	South Carolina, US	Blackberry	2010
WM14	South Carolina, US	Blackberry	2010
CO3a	South Carolina, US	Blackberry	2011
CB82a	South Carolina, US	Blackberry	2011
Wland1	Virginia, US	Strawberry	2012
KC25	North Carolina, US	Blackberry	2011
KC33	North Carolina, US	Blackberry	2011
Do9_K_A31	N/A, Germany	Strawberry	2009

Table 5.2. Fitness components for *Botrytis cinerea* isolates sensitive (S), low-resistant (LR), moderate resistant (MR), or resistant to fludioxonil or iprodione

Isolates	Phenotype ^x		EC ₅₀ ^y (mg/liter)		Fitness components ^w								
					Mycelial growth (cm)	Lesion size (cm)	Sporulating lesions (%)	Scholar	Switch	Rovral	Scholar	Switch	Rovral
	Lesion size (cm)			Sporulating lesions (%)									
	Fludioxonil	Iprodione	Fludioxonil	Iprodione	3 dai ^z	4 dai		4 dai			4 dai		
CA25	S	S	n/d	n/d	7.3b	3.3b	100.0	0.0a	n/d	0.01a	0.0	n/d	4.2a
CB3a	S	S	0.09	0.21	7.3ab	3.3b	100.0	0.0a	0.0	0.01a	0.0	0.0	2.7a
CO5	S	LR	n/d	n/d	7.6b	3.0b	100.0	0.0a	n/d	2.0bc	0.0	n/d	89.6b
MC14	S	LR	0.06	3.09	7.4b	3.5b	100.0	0.0a	n/d	2.5bcd	0.0	n/d	100.0b
WM14	S	LR	n/d	n/d	7.4b	3.3b	100.0	0.0a	n/d	1.9b	0.0	n/d	100.0b
CO3a	S	HR	n/d	n/d	7.2ab	3.5b	100.0	0.0a	0.0	2.7de	0.0	0.0	100.0b
CB82a	LR	HR	0.16 ^v	6.94	7.5b	3.2b	100.0	1.2b	0.0	2.5cd	100.0	0.0	100.0b
Wland1	LR	HR	0.26 ^v	3.56	7.1ab	2.7a	100.0	1.7c	n/d	2.2bcd	100.0	n/d	100b
KC25	MR	HR	0.32 ^v	>10	6.1a	2.8a	100.0	1.1b	1.8	2.4bcd	100.0	98.3	97.9b
KC33	MR	HR	0.38 ^v	>10	7.3b	3.3b	100.0	1.8c	1.5	3.2e	100.0	100.0	100.0b

^wNumbers in each column followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by analysis of variance (ANOVA). Mean separation was conducted using Tukey test.

^xPhenotypes were determined based on a spore germination assay using fludioxonil at 0.1 and 10 $\mu\text{g/ml}$ and iprodione at 5 and 50 $\mu\text{g/ml}$ (36). The phenotypic characterization (S, LR, MR or HR) for some of the isolates listed was reported in an earlier study from our lab (13).

^yEC₅₀= fungicide concentration that reduced mycelial growth by 50%.

^zDai = Days after inoculation or transfer.

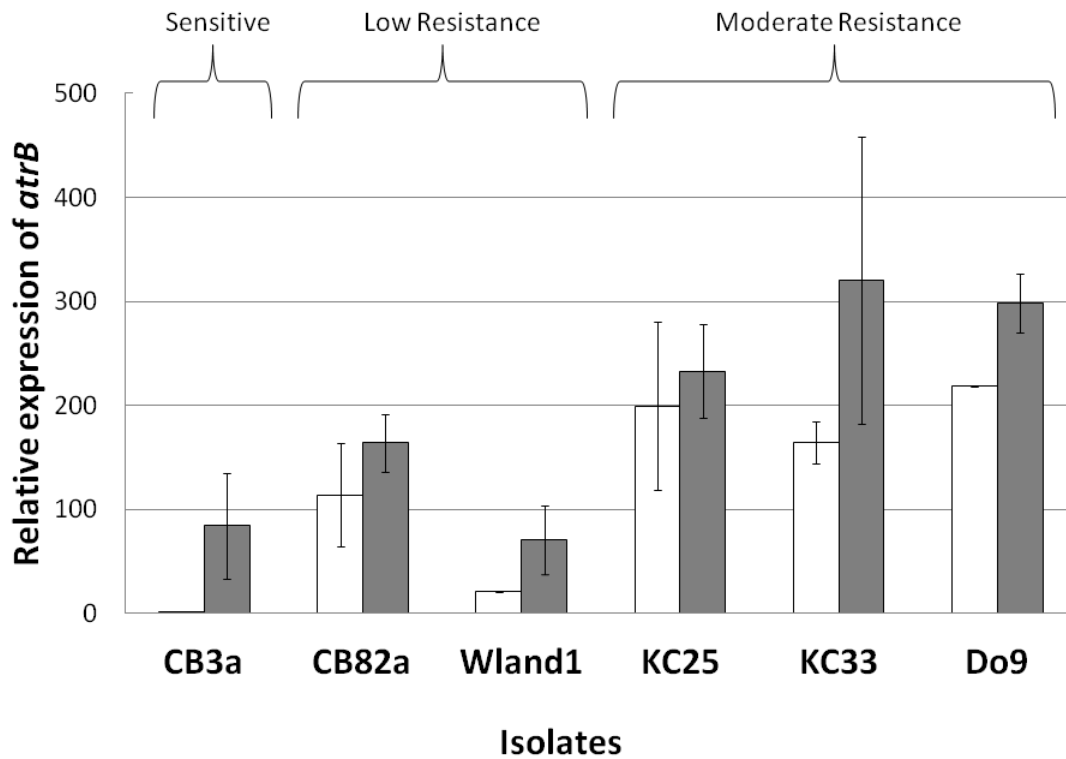


Fig. 5.1. Expression analysis of *atrB* determined by quantitative RT-PCR. Values indicate expression levels relative to the expression of sensitive strain CB3a (which was normalized to 1) with (gray bars) and without (white bars) fludioxonil treatment prior to RNA extraction.

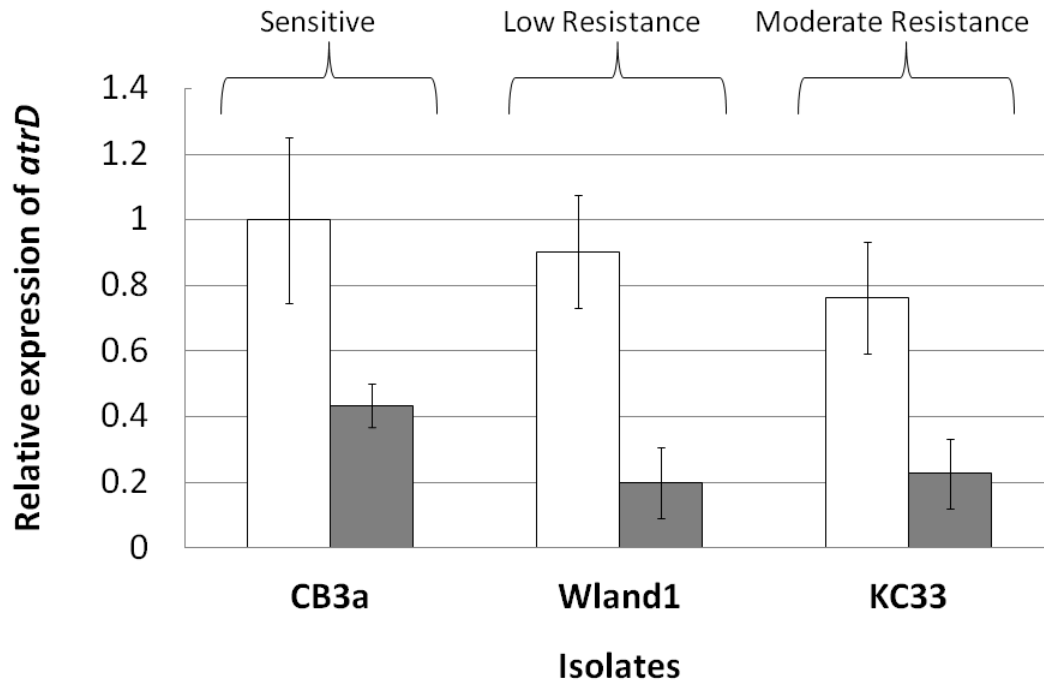


Fig. 5.2. Expression analysis of *atrD* determined by quantitative RT-PCR. Values indicate expression levels relative to the expression of sensitive strain CB3a with (gray bars) and without (white bars) fludioxonil treatment prior to RNA extraction.

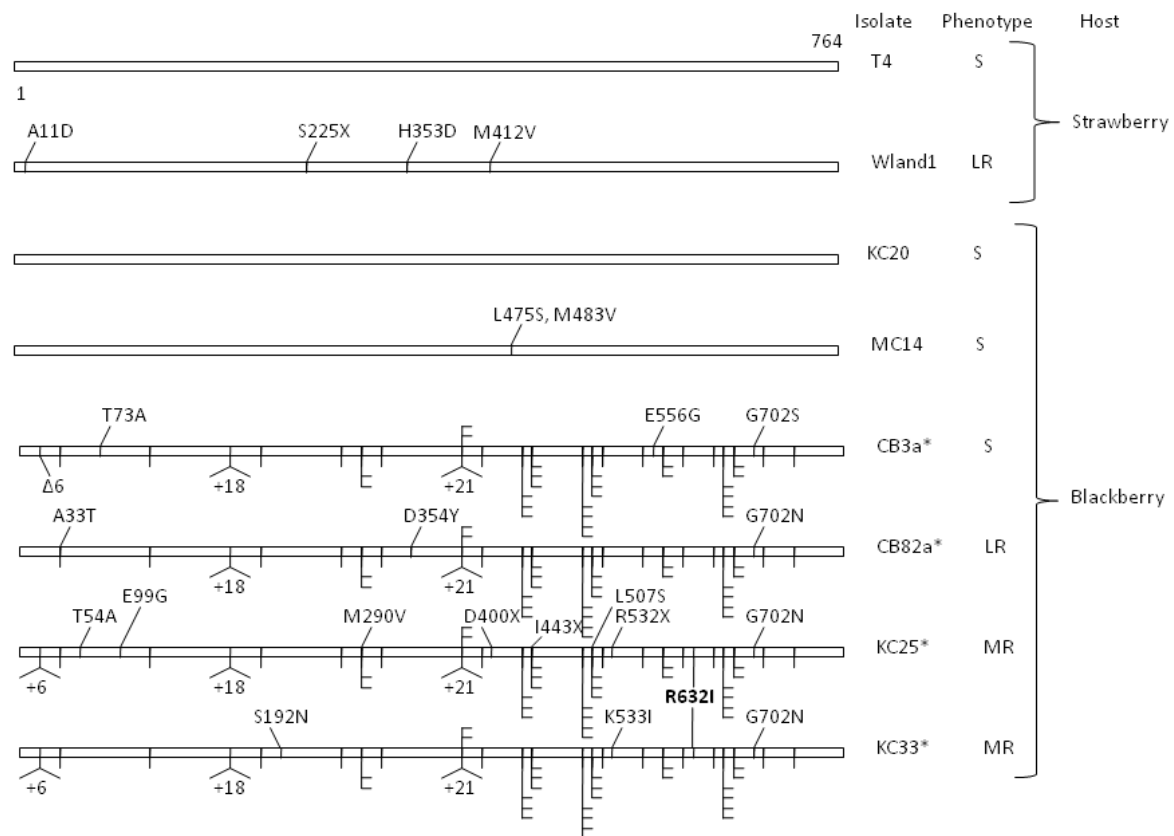


Fig. 5.3. Amino acid variations found in the transcription factor Mrr1 of isolates from strawberry and blackberry with different fungicide resistance phenotypes. Nucleotide insertions are indicated by ‘+’ followed by the number of inserted base pairs. Nucleotide deletions are indicated with a ‘Δ’ followed by the number of missing base pairs.

B. cinerea group S isolates are marked with ‘*’ after the isolate name. Vertical lines indicate aa variations between group S and non-group S isolates. If the change was consistent among all isolates from the group S, the exact aa change is not indicated for simplicity reasons. The number of horizontal lines attached to the bottom of a vertical

line corresponds to the number of aa variations at that position that are different from non-group S isolates. The mutation R632I associated with MDR1 is indicated in bold.

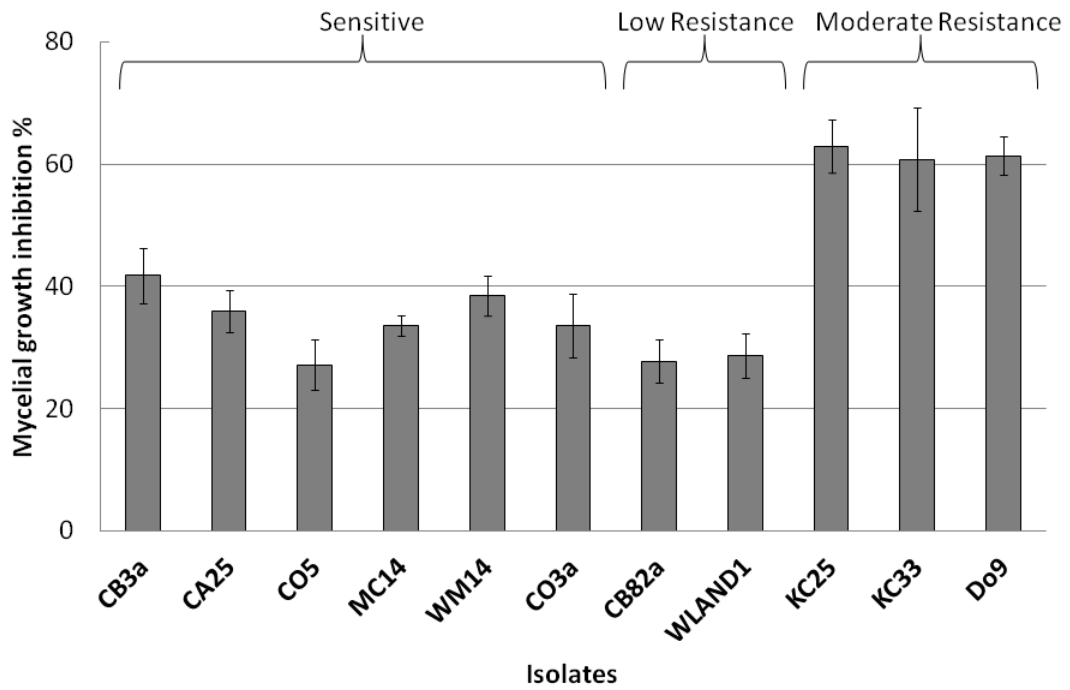


Fig. 5.4. Mycelial growth inhibition of isolates S, LR, and MR to fludioxonil on minimal medium amended with 4% NaCl.

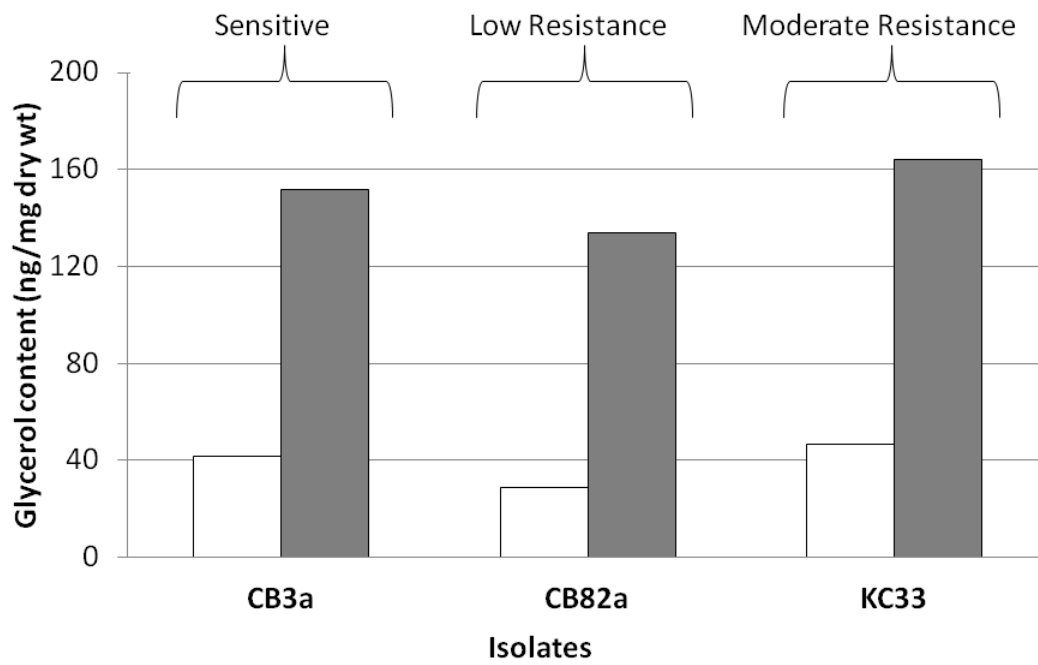


Fig. 5.5. Glycerol content in mycelia of *B. cinerea* isolates S, LR, and MR to fludioxonil.

Mycelia subjected to fludioxonil treatment prior to analysis are indicated in shaded bars.

CHAPTER SIX

CONCLUSION

This study shows that at least two *Botrytis* species exist in blackberry fields of the southeastern United States. *Botrytis cinerea* and *Botrytis caroliniana* co-existed in multiple but not all locations sampled, indicating it is not as widespread as *B. cinerea*. These two species cannot be distinguished based on symptoms on fruit or ITS sequencing. The rapid polymerase chain reaction we developed will facilitate the identification and separation of the two species. Many questions about the new species are still at large, including its host preference, sensitivity to fungicides, adaptability to stress, infection requirement, and life cycle.

Resistance to many classes of fungicides including HA, SDHI, MBC, QoI, and DC fungicides were widespread in *Botrytis cinerea* from blackberry. The most prevalent resistance profile in blackberry fields consisted of resistance to the MBC thiophanate-methyl, the QoI pyraclostrobin, and the SDHI boscalid. A statistical model suggests a stepwise accumulation of resistances in *B. cinerea* in commercial farms.

Resistance to thiophanate-methyl, pyraclostrobin, boscalid, and the HA fenhexamid was based on target gene mutations, including E198A and E198V in β -tubulin, G143A in cytochrome b, H272Y and H272R in SdhB, and F412I in Erg27, respectively. These mutations are known to not be associated with a fitness penalty or reduced competitive ability, indicating that these genotypes are likely to remain in the population in the absence of fungicide selection pressure.

Resistance to the PP fludioxonil was discovered and characterized. The molecular mechanism of resistance was based on a previously described mutation (R632I) in transcription factor Mrr1, which increases the expression of ATP-binding cassette transporter AtrB. This mechanism was first described in European isolates and thus far is the only mechanism known to confer resistance to PP fungicides. The lack of other mechanisms conferring resistance to the PP fungicide fludioxonil indicates that target site mutations may be detrimental to the fungus.

Our studies indicated that isolates with resistance to five and six chemical classes of fungicides are rare but do exist in commercial blackberry fields. Current resistance management practices, such as rotation and mixtures of site-specific fungicides, will continue to select for these isolates. Furthermore, our analysis of fitness of multifungicide resistant isolates indicated very little to no penalties compared to sensitive isolates, which may further accelerate the built up of multifungicide resistant populations. New integrated disease management and resistance management practices will need to be developed to counteract this development. Future resistance management must emphasize on inoculum reduction, reduction of applications/season of site-specific fungicides, and the integration of multi-site fungicides that are not prone to resistance development. In the absence of gray mold resistant varieties, reduction of inoculum may be achieved by cultural methods and the strategic use of biorational fungicides that are not prone to resistant development. Resistance monitoring should be conducted if site-specific fungicides are to be used effectively for disease management.

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