Fungicide Resistance in Botrytis cinerea from Strawberry - Molecular Mechanisms and Management

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FUNGICIDE RESISTANCE IN *BOTRYTIS CINEREA* FROM STRAWBERRY - MOLECULAR MECHANISMS AND MANAGEMENT

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
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Master of Science
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by
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Accepted by:
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Dr. Julia Kerrigan
ABSTRACT

The United States is the largest producer of strawberries worldwide, generating approximately $2.4 billion worth of fruit each year. Strawberry production, however, is often threatened by pathogens. One of the most destructive fungal pathogens is *Botrytis cinerea*, the causal agent of gray mold disease. The control of gray mold in commercial fields is largely dependent on the application of fungicides, including the dicarboximide iprodione and the hydroxyanilide fenhexamid. Because both fungicides are prone to resistance development, a survey was conducted to determine the occurrence and prevalence of fungicide resistance. Single-spore isolates were collected from strawberry fields in Florida (for the dicarboximide study), North Carolina, and South Carolina and subjected to a spore-germination assay that distinguishes sensitive from resistant isolates. Of the isolates collected, 16.8% were resistant to fenhexamid, 2% were moderately resistant to iprodione, and 17.6% had low levels of resistance to iprodione. Resistance to the two fungicides was found in almost every location, indicating that resistance was widespread but only accounted for a low percentage of the population. Resistance to fenhexamid was associated with nucleotide mutations in the target gene *erg27*, resulting in aa changes T63I, F412S, F412C, or F412I. A rapid method was developed based on polymerase chain reaction to specifically detect these mutations. Low and moderate resistance to iprodione was associated with mutations in target gene *bos1* resulting in I365N, I365S or a combination of Q369P and N373S, respectively. The I365N/S mutations were also present in five highly resistant isolates that were included to investigate the molecular mechanism of iprodione resistance, but no mutation or mutation
combinations in bos1 were uniquely associated with the highly resistant phenotype. Detached strawberry fruit assays indicated that field rates of Elevate 50 WDG (fenhexamid) did not control fenhexamid-resistant isolates, and field rates of Rovral 4 Flowable (iprodione) did not control isolates moderately and highly resistant to iprodione. Fitness studies revealed that iprodione-resistant isolates did not differ from sensitive isolates in regard to radial growth rate on artificial medium, sporulation, and pathogenicity on strawberry fruit. The results obtained in this study contribute to our understanding of fungicide resistance development in the gray mold fungus and are useful for improving current resistance management practices.
DEDICATION

I dedicate this work to my mother Monika, my father Wolfgang, and my sister Sabrina. My family supported my interest in science and nature at a young age, and continues to do so today. I also dedicate this work to my grandmother Edith and to the memory of my grandfather Simon, who was a longtime admirer of the beauty of the natural world.
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CHAPTER ONE

A REVIEW OF BOTRYTIS CINEREA CAUSING GRAY MOLD ON STRAWBERRIES

The Fungal Pathogen Botrytis cinerea

The fungus Botrytis cinerea Pers. Fr. is an ascomycete which belongs to the subphylum Pezizomycotina, class Leotiomycetes, order Helotiales, and family Sclerotiniaceae (11). It is a plant pathogen of immense economic importance, and causes serious losses in more than 200 crop species worldwide, both pre- and postharvest (147). In recent years, B. cinerea has become an important model of necrotrophic fungal pathogens due to its worldwide importance as a pathogen and the availability of molecular tools to study it, such as the entire genome sequence (5,147). It is thus not surprising that B. cinerea was deemed the second most important fungal plant pathogen of the world, based on perceived scientific and economic importance (34).

Nomenclature and Systematics. The name Botrytis cinerea is derived directly from the fungus' morphology: "Botrytis" is named after the Greek word for “bunch of grape berries”, describing the grape-like morphology of conidiophores, and "cinerea" refers to the gray color of sporulation (Fig. 1.1). The genus Botrytis was created by Pier Antonio Micheli in 1729, who grouped it into the "Nova Plantarum Genera", and was later revised by Hennebert (70,108). Botrytis cinerea was first described by Persoon and the name was accepted by the Swedish botanist Magnus Fries, who, together with Linnaeus, created the foundation of fungal systematics (67). The teleomorph or sexual form of B. cinerea is named Botryotinia fuckeliana (de Bary) Whetz. (145).
The genus *Botrytis* contains 22 species and one hybrid, and is closely related to the genus *Sclerotinia* (5,128). Former classification based mainly on morphology and host range was later revised by a molecular approach using multiple gene analyses. This classification revealed that the genus may be separated into two clades, with one containing four species colonizing dicotyledons including *B. cinerea*, and another which is compromised of 18 species colonizing both monocotyledons and dicotyledons. The comparison of pathogen and host phylogeny suggests that the two have not coevolved, but that the genus *Botrytis* diversified by acquiring new pathogenicity factors that allowed it to parasitize new hosts (128).

**Biology, Geographical Distribution, and Host Range.** *Botrytis cinerea* is an unspecialized fungus with a necrotrophic life style, and derives its nutrients from dead host cells, but is also capable of a saprotrophic lifestyle. It has a wide host range of over 280 plant species that contain mainly dicotyledonous species including wild flowers and multiple cultivated plants. Many of these plants are involved in the production of important protein, oil, fiber, and crops. Nursery plants and ornamentals, as well as vegetables such as beans, cabbage and lettuce, and small fruits like blackberries, grapes, and strawberries are most severely affected (147).

The genus *Botrytis* presumably originated in the northern hemisphere as host-specific taxa are primarily associated with northern hemisphere, temperate-zone hosts. It has been suggested that it was distributed though human activity (11). Today, *B. cinerea* is a cosmopolitan and ubiquitous fungus, and can be found wherever its host is grown, for example on strawberries in Alaska, and on cucumbers in Israel (7,147,152).
Hyphal cells and conidia of *B. cinerea* are multinucleate with numbers usually in the range of 1-12 nuclei, while microconidia, which primarily function as male gametes, are uninucleate (11,66,125). *Botrytis cinerea* is haploid and mostly heterothallic, carrying either the mating type allele *MAT1-1* or *MAT1-2* (13,36,47,48). Sexual reproduction supposedly occurs in winter, and is a result of the fusion of microconidia produced by mycelium with female gametes produced in sclerotia. Apothecia and ascospores can be produced in vitro in three to six months after contact between the two sexually opposite gametes (46). Observations of apothecia in nature, however, are extremely rare (11,147). One was reported from grapevine in Switzerland (11,65), and a second from a peach mummy in New Zealand (11), which suggests that sexual reproduction is uncommon. Molecular markers, however, indicate the existence of high levels of recombination (11,62). In strawberry fields, no apothecia of the teleomorph have been found, though apothecia were produced in vitro from spermatized sclerotia of isolates from strawberry (Fig. 1.1). This suggests the possibility of sexual reproduction of *B. cinerea* in strawberry fields (21).

**Diversity and Adaptability.** *Botrytis cinerea* is known for its extreme genetic diversity, great adaptability, and high morphological variability, which visibly expresses itself in various mycelial colors, differences in spore production, form of aerial mycelia, and sclerotia formation (105). The cause of such high diversity still needs to be determined, but recent research has led to the development of several theories, including haploidy, heterokaryosis, heteroploidy such as aneuploidy, high reproductive rate, mycoviruses, sexual recombination, and transposable elements (11).
Traditionally, heterokaryosis has been considered to play a major role in the diversity of *B. cinerea*, and variability between offspring from the same parent caused by heterokaryosis has been shown to exist under laboratory conditions. It is, however, still unknown to what extent it accounts for significant variation in the field (11,12). Molecular markers indicate high levels of recombination, which probably result from sexual recombination rather than heterokaryosis, and suggests that sexual recombination may play a more important role than formerly believed (11,62,94). Two transposable elements called *Boty* and *Flipper* are also frequently found in *B. cinerea*, as are mycoviruses, which consist of mostly double-stranded and a few single-stranded RNA viruses. These transposable elements and mycoviruses may contribute to genetic diversity (11,28,39,72,73,93). Recent studies suggest that heteroploidy (including aneuploidy) may also be important (11). Additionally, *B. cinerea* produces high numbers of conidia, which not only pose a serious threat to susceptible crops such as strawberries, but may also contribute to its high adaptability (42). The extreme genetic diversity in *B. cinerea* plays an important role in the fungus' potential to readily adapt to control strategies and fungicide treatments, and explains the constant need for researchers to investigate and assess new management strategies to control this successful pathogen.

**Gray Mold of Strawberries**

Strawberries (*Fragaria × ananassa*) are an important commercial fruit crop worldwide. The strawberry crop in the United States alone is worth 2.4 billion dollars, with approximately 2.9 billion pounds of fruit produced each year on 56,990 acres (112).
California is by far the largest producer of strawberries in the United States, though strawberries are also an important part of the economy in the southeast, and are worth 366 million dollars in Florida, 27 million dollars in North Carolina, and 13 million dollars in South Carolina (112,127). Strawberry production is often threatened by diseases and insect pests. One of the most destructive and economically important diseases of strawberries is gray mold, also known as Botrytis fruit rot or ash mold. The disease is caused by the fungal pathogen *B. cinerea*, and can occur wherever strawberries are grown, but chiefly develops under cool and humid conditions. It is not only a major problem in preharvest, but also in postharvest disease management, when seemingly healthy berries can suddenly develop gray mold during storage, transport, or at the retailer (Fig. 1.1). The cost of *B. cinerea* damage is very difficult to determine, as it occurs at several stages of the production and distribution chain (34). Previous reports, however, indicate that preharvest yield losses can exceed 50% when environmental conditions are favorable for disease development and can be up to 90% in severe cases (44). *Botrytis cinerea* can cause preharvest losses of up to 15% of the fruit in Florida, even when fungicides are applied on a weekly basis (85).

**Symptoms and Signs.** Symptoms are most commonly observed on ripening and ripened fruit, but may also appear on blossoms or on green, white, and pink fruit of the early developmental stages (Fig. 1.1) (130). Blossom blight usually appears under cold and wet conditions, and can be severe when frost injury occurs (76). Petals and pedicels of affected blossoms become brown and eventually die (130). Rot can be found on any part of the strawberry fruit, but is most frequently observed on the calyx end or on sides
of fruits touching affected tissues (76). Lesions are light brown in color and usually remain firm, thus little leakage is associated with this rot, in contrast to *Rhizopus* rot (130). The absence of a distinct border between diseased and healthy tissues aids in the discrimination of gray mold disease from anthracnose fruit rot, another frequently encountered strawberry disease (130). Lesions in green and white fruit enlarge slowly, and result in deformation of the developing fruit, which may die before maturity (130). Typically, the affected tissue appears grayish and fuzzy because of the abundant layer of conidiophores with conidia on the fruit surface (Fig. 1.1). However, under extremely humid conditions, the fungus can also produce a mass of white mycelium (130). Whole rotten fruit eventually dry, become tough in texture and mummify (Fig. 1.1) (130).

**Disease Cycle and Epidemiology.** *Botrytis cinerea* overwinters in the form of sclerotia and dormant mycelium predominantly in dead strawberry debris, but also in mummified berries, straw mulch, and weeds (Fig. 1.2) (21,22,129,131). Under suitable conditions in spring, the fungus grows mycelium and large amounts of conidia are produced, which serve as the primary source of inoculum (Fig. 1.1 and Fig. 1.2) (21,131). Conidia are released from conidiophores in response to vibrations or a rapid decline in relative humidity, and are disseminated by air currents and water splashes (75). Flowers and wounds are the main entrance of *B. cinerea* infections (Fig. 1.2) (24,147). Conidial germ tubes readily penetrate the host's epidermis on petals, stamens, and pistils (Fig. 1.2) (24,130). Once the fungus is established in the flower parts, it invades the developing fruit, and usually remains quiescent until the fruit ripens or is damaged (76,77). The growth of mycelia from the infected flower into the receptacles results in the fruit's
characteristic calyx-end rot (76,130). Spore production starts rapidly when berries ripen, and is continuous throughout the fruiting season. This serves as the secondary source of inoculum (Fig. 1.2) (130). *Botrytis cinerea* usually does not produce symptoms on strawberry leaves, but can form quiescent infections on young leaves (Fig. 1.1) (7,20,76,147). It then colonizes senescent tissue and produces conidia during periods of high humidity or leaf wetness, which serves as another inoculum source (Fig. 1.1 and Fig. 1.2) (7,20).

*Botrytis cinerea* favors moderate temperatures (15-25°C), but is able to grow at temperatures as low as 0°C, making it an important pathogen of stored products (25,26,146). In addition to moderate temperatures, long periods of high relative humidity or surface wetness are important for disease development (26,130). These conditions promote spore production, conidial germination, and penetration of the host. Thus, frequent rains and overhead irrigation increase disease incidence. Gray mold infection and dissemination is increased by bird, hail, mechanical, and insect damage, as well as narrow planting, in which infected strawberries can easily come in contact with healthy tissue (76,130,147). Everbearing cultivars with multiple flowering and harvest cycles also promote fruit rot due to dispersal of conidia from affected fruit to newly opened flowers (130).

**Management and Control.** The control of gray mold disease is difficult due to the pathogen's variety of infection strategies, wide host range, genetic diversity, and survival as conidia, mycelia, and sclerotia (21,39,147). The best way to control gray mold is thus by the integration of a variety of cultural management practices with the application of
fungicides or biological agents (86). Sanitation practices, such as the removal of infected debris and berries, drip- instead of overhead-irrigation, and ventilation, minimize the source of inoculation and surface wetness, and thus reduce the incidence of disease (7,147). The use of mulch can assist in burying leaf litter and in inducing microbial breakdown of inoculum, while shelters and clear plastic tunnels reduce problems caused by excessive rainfall during blossom (147,149). Wider plant spacing allows proper ventilation and reduces gray mold incidence, but may result in reduced total yield (86). As transplants are a potential source of \textit{B. cinerea} introduction into a newly planted field, transplants can be treated with hot water or fungicides to suppress the initial build-up of the fungus (130). The fertilization of plants with nitrogen should be limited, since nitrogen encourages rapid vegetative growth and increases disease incidence by 60-80% in weather suitable for gray mold development (130). The control of insect pests that wound plants or vector \textit{B. cinerea} spores can also help control secondary infection (147). Disease forecast systems, which use leaf wetness and local temperature to predict disease outbreaks, have shown success in reducing serious crop damage and in minimizing the number of fungicides applied per season (14,103).

Biological control strategies have been developed for gray mold management as an alternative to chemical fungicides (41). Biological agents frequently used in formulations include activators of systemic acquired resistance such as acibenzolar, filamentous fungi like \textit{Gliocladium roseum}, and \textit{Trichoderma harzianum}, as well as yeasts such as \textit{Aureobasidium pullulans}, \textit{Candida oleophila} and \textit{Rhodotorula glutinis}, or bacteria like \textit{Bacillus subtilis} and \textit{Pseudomonas syringae} (41,80,97,116,147,153).
Biological control agents act as competition for nutrients and space on the surface of the plant, as inhibitors of germination or sporulation, as mycoparasites, as stimulators of host plant defense mechanisms, or secrete fungicidal toxins (40,41). Most biological control agents are sprayed on the plants, but research has shown that bees with inoculation trays in hives can possibly be used to distribute *Gliocladium roseum*, and *Trichoderma harzianum* (18,19,80,116). Biological control agents have shown some potential in controlling *B. cinerea* on strawberries, but are usually not part of conventional management programs due to inconsistent control success, their restricted temperature and humidity range, and their possible inhibition by natural populations of other microbes (107,123,147).

Some strawberry cultivars are less susceptible to gray mold disease than others, however no cultivar is resistant (126,130). Breeding for resistance against *B. cinerea* has been unsuccessful in most crops (147) except for some substantial advances made in tomato breeding, where quantitative trait loci for *B. cinerea* resistance were identified (58,59). Due to the absence of *B. cinerea*-resistant strawberry cultivars and reliable and stable biological control agents, the primary means of controlling gray mold disease in commercial strawberry fields has been by the application of fungicides beginning at or before bloom.

**Chemical Control of *Botrytis cinerea***

Chemical control remains the most effective method for controlling gray mold. It was estimated that fungicides specifically targeting *B. cinerea* cost around 540 million
euro annually, representing 10% of the world fungicide market (34). Fungicide applications are most effective during the flowering period in commercial strawberry fields (22,146), but sprays are usually applied weekly starting at bloom up to harvest in the southeastern United States because the flowering period generally extends into the production season.

Various fungicides are used for control. Multi-site inhibitors such as captan and thiram are chemicals affecting multiple chemical pathways in the pathogen. They suppress conidial germination by inhibiting several thiol-containing enzymes involved in respiration, but show little activity against mycelial growth. Multi-site fungicides act as a protective layer only on the plant surface, and therefore need frequent applications at high doses (88). The risk of resistance to these fungicides is generally low, and control failure based on resistance development has rarely been reported (88,147). In the last decade, researchers have developed a number of site-specific fungicides that have stronger activities against B. cinerea and are applied at substantially lower doses per acre. The production of high quality strawberries relies on the use of these site-specific fungicides (124). Many of them were categorized as "reduced risk" fungicides by United States Environmental Protection Agency (EPA) due to their "low-impact on human health, low toxicity to non-target organisms (birds, fish, and plants), low potential for groundwater contamination, lower use rates, low pest resistance potential, and compatibility with Integrated Pest Management" (134). "Reduced risk" fungicides include anilinopyrimidines (APs), hydroxyanilides (HAs), methyl benzimidazole carbamates (MBCs), phenylpyrroles (PPs), quinone outside inhibitors (QoIs), and succinate
dehydrogenase inhibitors (SDHIs) (124). These six chemical classes of fungicides, as well as dicarboximides (DCs), represent seven important chemical classes of site-specific fungicides currently available for gray mold control in the southeastern United States (Table 1.1). These chemical classes of fungicides can also be classified according to their modes of action: (i) fungal respiration inhibitors, (ii) anti-microtubule toxicants, (iii) osmoregulatory inhibitors, (iv) sterol biosynthesis inhibitors, and (v) methionine biosynthesis inhibitors (88). The advantages of low toxicity and high effectiveness of these fungicides, however, come with the cost of high vulnerability to resistance development due to their site-specific mode of action. In fact, resistance emerged soon after the introduction of these chemical groups (133).

**Fungicide Resistance of *Botrytis cinerea***

Fungicide resistance is a key challenge in modern strawberry protection (23,68) and can be defined as a "stable, inheritable adjustment by a fungus to a fungicide, resulting in less than normal sensitivity to that fungicide" (113). In other words, strains of a sensitive species change, usually due to point mutations, and become significantly less sensitive to a fungicide. The growth of these resistant strains is then uninhibited or slightly inhibited by the fungicide at concentrations that were inhibitory to the original population (113). Fungicide resistance can be observed in field isolates, or can be created under laboratory conditions. Isolates that show fungicide resistance in the laboratory, however, do not necessarily have the ability to cause control failure in the field. The loss of field control as a result of a shift in sensitivity in a population is described by the term
'practical resistance' or 'field resistance', and has been reported for over 100 diseases (84,113). Acquired resistance is another term used for fungicide resistance acquired though genetic modifications which are transmittable to progeny (124). Natural resistance is distinguished from acquired resistance through the preexistence of resistance in the population before the fungicides are introduced, as is the case with *Botrytis pseudocinerea*, which is naturally resistant to fenhexamid (15).

The development of resistance in the field is an evolutionary process. A naturally diverse population is subjected to selection pressure due to fungicide applications. This results in the selection of advantageous genotypes that are adapted to the current environmental conditions. The frequency of those resistant genotypes can increase in the population over time, resulting in poor control (138).

The fungal pathogen *B. cinerea* is recognized by the Fungicide Resistance Action Committee (FRAC) as a pathogen at high risk of fungicide resistance development due to numerous characteristics, including large population size, long distance dissemination of conidia by air currents, high genetic variability, the ability to reproduce sexually and asexually, abundant sporulation, polycyclic disease cycle, and wide host range (90). These factors may explain why fungicide resistance has been found with all site-specific fungicides registered for gray mold control (88,124,138). In contrast, the risk of resistance development to multi-site inhibitors is considered to be low, and though some studies report resistance development to this group, it has been used over a long period without substantial control failure in the field (88,147).
Resistance to multiple chemical classes of site-specific fungicides has developed in *B. cinerea* worldwide. Despite multiple applications of fungicides, the appearance of gray mold has been reported by southeastern strawberry growers, increasing concerns of fungicide resistance in regional strawberry production. In fact, most of the chemical classes of fungicides, including APs, MBCs, QoIs, and SDHIs, have encountered specific resistance in the southeastern United States, with variable frequencies in populations, and possible consequences for fungicide efficacy in the field (Table 1.1) (51,52). However, no comprehensive study of the occurrence and prevalence of dicarboximide or hydroxyanilide resistance has been conducted for isolates from strawberry fields or any other crop in the southeastern United States.

**Mechanisms of Resistance.** The determination of the molecular basis of resistance helps to explain the development and evolution of fungicide resistance. It is also an essential prerequisite for the development of molecular monitoring techniques. Fungicide resistance is most commonly caused by point mutations in the target gene that alter the structure of the protein which the fungicide attacks (90). These alterations in protein structure can reduce the affinity and thus the efficacy of the fungicide. Specific single nucleotide polymorphisms have been associated with resistance to several chemical classes of fungicides, and may eventually result in control failure (133). Apart from point mutations in the target gene, four other mechanisms are known to confer resistance in fungal pathogens. One of them is the overexpression of the target protein. An example of this mechanism is the overexpression of the MfCYP51 gene based on the presence of a 65-bp genetic element located upstream of the gene which confers resistance to the sterol
demethylation inhibitor (DMI) fungicides in *Monilinia fructicola* (100). Fungicides can also be detoxified by metabolic enzymes, including the metabolism of edifenphos by *Pyricularia orizae* or the fenhexamid detoxification in *Botrytis pseudocinerea* (15,83,138). Another mechanism of resistance is the activation of an alternative metabolic pathway, such as the activation of alternative oxidase which compensates for the inhibition of the respiration complex III by strobilurin fungicides (148). The fifth mechanism consists of an increase of fungicide efflux out of cells. This is caused by the overexpression of membrane transport proteins (82).

**Multi-Drug Resistance.** Multi-drug resistance (MDR) is the simultaneous resistance to several unrelated toxic compounds (88). It was first described in the human pathogen *Candida albicans* (110) and in mammalian cancer cells resistant to drugs (63). MDR is caused by an overexpression of membrane transport proteins which excrete a variety of toxic molecules (82,88). This efflux is created either by ABC transporters which have an ATP-binding cassette and require energy in the form of ATP, or by the major-facilitator superfamily (MFS) which uses electrochemical gradients across membranes as an energy source (82,88). MDR has been detected in the following phytopathogenic fungi in recent years: *Mycosphaerella graminicola*, the causal agent of septoria leaf blotch of rye (92,119), *Oculimacula yallundae*, the causal agent of eyespot of wheat (91), *Penicillium digitatum*, the causal agent of green mold of citrus (69,111), and *B. cinerea* (82). In all cases, MDR is characterized by resistance to multiple fungicides with different modes of action, and by a relatively low resistance factor compared to resistance created by point mutations in the target gene (82,138).
Three different types of MDR (MDR1, MDR2, and MDR3) were described for *B. cinerea*, and can be distinguished through their resistance spectra. Isolates with the MDR1 phenotype show mainly reduced sensitivity to anilinopyrimidines and phenylpyrroles, while isolates with the MDR2 phenotype are less sensitive to anilinopyrimidines, dicarboximides, and hydroxyanilides. MDR3 is a combination of MDR1 and MDR2, which results in a hybrid of those two phenotypes. All MDR phenotypes are highly resistant to tolnaftate, a sterol squalene epoxidase used in medicine, which clearly discriminates them from non-MDR strains (82,138). The molecular mechanism of MDR has been identified. MDR1 is associated with the ABC transporter BcAtrB. Several different mutations and a deletion in the transcription factor *mrr1* were shown to induce the overexpression of this ABC transporter (82). The MDR2 phenotype is associated with the overexpression of the MFS-transporter BcmfsM2, the result of a unique insertion in its promotor region. The MDR3 phenotype, therefore, contains both types of genetic rearrangement.

**Resistance Management**

The development of practical resistance threatens high yields and crop quality, and is thus a serious issue for growers, scientists, and manufacturers. It not only reduces the grower's and manufacturer's income, but also has consequences for the environment (e.g. more fungicide applications are necessary to control the pathogen) and human health (e.g. mycotoxin production and high levels of fungicide residue) (138). In spite of the intensive screening conducted in companies, the discovery of new fungicides with
different modes of action that meet the high safety requirement is rare and new chemical classes are introduced every ten years on average. Additionally, the current cost of bringing a new fungicide to the market is extremely high at an estimated US$250 million (84,102). Successful resistant management is therefore vital in order to delay resistance development, to protect the effectiveness of currently registered fungicides, and to maintain the number of modes of action available to growers.

An important prerequisite for the development of effective resistance management strategies is the monitoring of fungicide resistance in *B. cinerea* populations. The purpose of monitoring programs is to investigate resistance in the field, to predict the emergence of resistance problems, to develop management strategies, to follow the evolution and dynamics (e.g. spread, distribution, and sensitivity shifts) of resistant populations, and to advance our understanding of fungicide resistance (23,124).

Since the risk for resistance development is extremely high when a product is applied repeatedly, it is generally recommended to use mixed spray programs in which each spray is from a different mode of action. This not only reduces the risk of field resistance development, but it also aids in keeping residue levels for each chemical class below the permitted maximum (138,147). Apart from alternation, fungicide mixtures may also help to delay resistance. Mixtures have the advantage of combining the specific characteristics (i.e. curative plus protective activity) of two fungicides with different modes of action (23). This strategy may not be useful in all situations, particularly when resistance to one of the fungicide in the mixtures has already been detected. In this case, a mixture would only select for dual resistant isolates (51,138). There is no clear evidence
indicating whether alternations or mixtures are more suitable for resistance management, and the decision should be made on a case-by-case basis (138). Another management strategy is the change of the fungicide dose. For example, multifungicide resistant isolates can be controlled with a full dose application, as they usually have low levels of resistance. In other cases, a full dose application may promote resistance development, since higher doses of some chemical classes have been shown to increase selection for resistance (135, 138). Resistance may vary from one field to another based on differences in spray history, and location-specific resistance monitoring should therefore be used for the development of precise management strategies (124). It is also vital for resistance management to estimate the risk of distribution and persistence of resistance in *B. cinerea* populations, as resistance can sometimes lead to reduced fitness of the pathogen (6). This can eventually help to develop a suitable control strategy for these isolates (6, 81).

A case-by-case control strategy is necessary for the establishment of an effective resistance management. This program should be based on the monitoring results of *B. cinerea* populations, which provide vital information on the evolution of resistance to this fungicide in the field. The key elements of resistance management of *B. cinerea* and strategies adapted to specific resistance situations are shown in Fig. 1.3. Developed by Walker et al. this figure was derived from experience in French vineyards.

The Fungicide Resistance Action Committee (FRAC) plays another important role in the management of resistance. This organization was established in 1981 in recognition of the threat of fungicide resistance development. It is based on and funded by industry, and tries to prolong the effectiveness of fungicides. The purpose of FRAC is
to identify and discuss existing and potential resistance problems, and to provide guidelines on how to best use fungicides in order to avoid resistance development and to manage existing resistance issues so that crop losses can be avoided. FRAC is trying to achieve this goal by providing educational material such as the "Mode of Action Code List", and by collaborating with universities, government agencies, extension workers, and growers (84).

Based on the overview above, we conclude that (i) strawberries are a valuable commercial crop worldwide, (ii) gray mold disease caused by \textit{B. cinerea} is one of the most important pathogens of strawberries, (iii) fungicide applications are important tools to control gray mold disease, (iv) \textit{B. cinerea} develops resistance rapidly to multiple chemical classes of site-specific fungicides, (v) resistance is emerging in the Southeast, (vi) a comprehensive study is needed to determine dicarboximide and hydroxyanilide fungicide resistance problems, and (vii) monitoring of resistance is vital to develop management strategies that prolong the effectiveness of fungicides.

**Aims of this Study**

The objectives of this study were to:

- assess the occurrence and frequency of dicarboximide- and hydroxyanilide resistance in \textit{B. cinerea} from commercial strawberry fields in the southeastern United States
- identify the genetic basis of resistance
- determine fitness and pathogenicity of resistant isolates
• develop a molecular technique to detect resistant isolates in the field

The knowledge acquired from this study will help to develop management strategies that avoid the selection of resistant phenotypes, and thus protect and maintain the effectiveness of fungicides and reduce control failure associated with practical resistance. The information provided will also eventually aid in determining the dynamics, spread, and stability of resistance in the field under fungicide pressure.
Table 1.1. Fungicides used for gray mold control in the southeastern United States, their properties, resistance frequencies, and mechanisms of resistance.

<table>
<thead>
<tr>
<th>Mode of action (inhibition)</th>
<th>Chemical class</th>
<th>Fungicide</th>
<th>Target site</th>
<th>Common amino acid changes</th>
<th>Resistance frequency in NC and SC in 2011/2012</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane sterol biosynthesis</td>
<td>Hydroxy-anilides</td>
<td>Fenhexamid</td>
<td>3-keto-reductase (Erg27)</td>
<td>F412I/S/V and others</td>
<td>N/A this study</td>
<td>(33,51)</td>
</tr>
<tr>
<td>Methionine biosynthesis</td>
<td>Anilino-pyrimidines</td>
<td>Cyprodinil, pyrimethanil</td>
<td>Unknown</td>
<td>Unknown</td>
<td>47%</td>
<td>(33,51)</td>
</tr>
<tr>
<td>Mitosis and cell division</td>
<td>Methyl benzimidazole carbamates</td>
<td>Thiophanate-methyl</td>
<td>β-tubulin assembly</td>
<td>E198A/K/V, F200Y</td>
<td>70%</td>
<td>(33,51)</td>
</tr>
<tr>
<td>Respiration</td>
<td>Succinate dehydrogenase inhibitors</td>
<td>Boscalid, pentiopyrad</td>
<td>Complex II</td>
<td>P225L/F/T, H272Y/R/L, N230I in SdhB, H132R in SdhD</td>
<td>62%</td>
<td>(33,52)</td>
</tr>
<tr>
<td>Respiration</td>
<td>Quinone outside inhibitors</td>
<td>Azoxystrobin, pyraclostrobin</td>
<td>Complex III</td>
<td>G143A</td>
<td>66%</td>
<td>(33,52)</td>
</tr>
<tr>
<td>Osmotic signal transduction</td>
<td>Dicarboximides</td>
<td>Iprodione</td>
<td>Histidine-kinase (Bos1)</td>
<td>I365N/S/R, Q369H/P, N373S, and others</td>
<td>N/A this study</td>
<td>(33,51)</td>
</tr>
<tr>
<td>Osmotic signal transduction</td>
<td>Phenylpyrroles</td>
<td>Fludioxonil</td>
<td>Histidine-kinase (Bos2)</td>
<td>N/A</td>
<td>0%</td>
<td>(33,51)</td>
</tr>
</tbody>
</table>
Fig. 1.1. Signs, symptoms, and morphology of the gray mold fungus *Botrytis cinerea* on strawberries: A) Gray mold affecting fruit in various stages of development, B) Gray masses of spores on severely infected fruit, C) *B. cinerea* cultured on potato-dextrose agar medium, D) Sporulation on mummified strawberry fruit, E) Apothecia produced in the laboratory, F) Conidiophore and conidia, G) Conidia, H) Quiescent hypha in an epidermal cell of a strawberry leaf, I) Sporulation on a strawberry leaf injured by frost, J) Postharvest development of gray mold (adapted from Sutton, 1998 (130)).
Fig. 1.2. Disease cycle of *Botrytis* gray mold diseases (adapted from Agrios, 2005 (1)).
Fig. 1.3. Decision tree for strategies for managing fungicide resistance in *Botrytis cinerea* populations. Scheme valid for synthetic and natural antifungal agents. In all cases, resistance monitoring is a prerequisite for the identification of the right management strategy, and its adaptation, if resistance continues to evolve. In all situations, prophylactic measures help to delay resistance. aMixtures can be proposed to manage resistance only when active ingredients not displaying positive cross-resistance and/or not already encountering resistance are available and can be used at an appropriate dose. bMDR on its own generally induces low to moderate RFs. Nevertheless, when this mechanism is associated with other resistance mechanisms (for example, specific resistance due to target alteration), RFs may be greater for a mode of action. RF: resistance factor (adapted from Walker et al., 2013 (138)).
CHAPTER TWO

FENHEXAMID RESISTANCE IN *BOTRYTIS CINEREＡ* FROM STRAWBERRY
FIELDS IN THE CAROLINAS IS ASSOCIATED WITH FOUR TARGET GENE
MUTATIONS

This work has been published:
*Botrytis cinerea* from strawberry fields in the Carolinas is associated with four target

["Fungal isolates and culture conditions", "Sensitivity of *B. cinerea* isolates to
fenhexamid", and "Detached fruit assay" were performed by Dr. Dolores Fernández-
Ortuño; "Analysis of erg27 gene sequences", and "Development of the allele-specific
PCR assays" were performed by Anja Grabke.]

Abstract

*Botrytis cinerea*, the causal agent of gray mold disease, is one of the most
important pathogens of strawberry. Its control in commercial strawberry fields is largely
dependent on the application of fungicides during bloom and fruit maturation. The
hydroxyanilide fenhexamid is one of the most frequently used fungicides in the Southeast
of United States for gray mold control. It inhibits the 3-ketoreductase (Erg27) of the
ergosterol biosynthesis pathway and, due to this site-specific mode of action, is at-risk for
resistance development. Single-spore isolates were collected from 11 commercial
strawberry fields in North and South Carolina and subjected to a conidial germination
assay that distinguished sensitive from resistant phenotypes. Of the 214 isolates collected,
16.8% were resistant to fenhexamid. Resistance was found in three of four locations from North Carolina and in four of seven locations from South Carolina indicating that resistance was widespread. Mutations in Erg27 (T63I, F412S, F412C, and F412I) were associated with resistance with F412S the predominant and most widespread mutation. In this study mutations T63I and F412C in field isolates of B. cinerea are described for the first time. Detached fruit studies showed that field rates of Elevate 50 WDG (fenhexamid) controlled sensitive but not resistant isolates carrying any of the four mutations. Resistant isolates produced the same lesion size and number of sporulating lesions on fruit sprayed with Elevate 50 WDG as on untreated controls showing the fungicide's loss of efficacy against those isolates. A rapid polymerase chain reaction method was developed to quickly and reliably distinguish isolates sensitive or resistant to fenhexamid in the Carolinas and to determine the mutation associated with resistance. The presence of fenhexamid-resistant strains in B. cinerea from strawberry fields in the Carolinas must be considered in future resistance management practices for sustained gray mold control.

Introduction

Gray mold is one of the most economically important diseases of cultivated strawberry (Fragaria × ananassa), and a significant threat to the United States' 2.4 billion dollar strawberry crop (112). In the southeastern United States, the disease is caused by Botrytis cinerea Pers.:Fr., but berries can also be affected by B. caroliniana X.P. Li & G. Schnabel sp. nov. (53,54,96). Crop losses resulting from gray mold disease are especially
severe under moist weather conditions, and occur not only during the crop growing season, but also after harvest and during storage and transit.

The control of gray mold in commercial strawberry fields is largely dependent on the application of fungicides during bloom and fruit maturation. Several site-specific fungicides with different modes of action are currently available for gray mold management, including anilinopyrimidines, benzimidazoles, dicarboximides, hydroxyanilides, quinone outside inhibitors (QoIs), phenylpyrroles, and succinate dehydrogenase inhibitors (SDHIs). However, effective control of gray mold is threatened due to the emergence of fungicide resistance. For example, *B. cinerea* from strawberry fields in California, North Carolina, and South Carolina has developed resistance to QoIs and SDHIs indicating the emergence of resistance to site-specific fungicides (52,106).

The hydroxyanilide fenhexamid is a site-specific fungicide with a broad activity spectrum that includes *Botrytis* spp. and related fungi such as *Monilinia* sp. and *Sclerotinia* sp. (120,132) and has become a key component of gray mold disease management in the United States. This fungicide specifically inhibits the 3-ketoreductase involved in the C-4 demethylation during ergosterol biosynthesis (35). Resistance to this fungicide was reported in *B. cinerea* isolates from North American grapevine, the herbaceous perennial *Heuchera* and from strawberry fruit in California and Germany (60,106,109,121,141), but it is unclear if resistance has emerged on the east coast of the United States.

Four fenhexamid-resistant phenotypes named HydR1, HydR2, HydR3−, and HydR3+ have been described in *Botrytis* field isolates (2,3,57,90). Mycelium of isolates
classified as HydR1 or HydR2 are able to grow in the presence of high or moderate levels of fenhexamid, respectively, but their germinating spores remain susceptible (9,124,132). All HydR1 isolates were *Botrytis pseudocinerea* from grapevine in France whereas HydR2 isolates were *B. cinerea* from France and Japan (2,3,9,61). HydR3 isolates show moderate (HydR3⁺) or high (HydR3⁻) resistance during both mycelial growth and germ tube elongation (2,3,57,90). HydR3 isolates are thus the greatest cause of concern for disease management and have been regularly found in commercial fields since 2004 (2,45,57).

HydR3 phenotypes are associated with genetic modifications in the target gene *erg27* resulting in alterations of the 3-ketoreductase (15). Several aa substitutions in Erg27 were reported in field isolates of *B. cinerea* conferring different levels of resistance to this fungicide. For example, HydR3⁻ isolates were found to contain point mutations in the *erg27* gene resulting in a variety of predicted aa changes (F26S, L195F, V309M, A314V, S336C, N369D, L400F/S, Y408S, R496T, and a deletion at P238) (33,45,57). In HydR3⁺ isolates alterations at aa positions 496 (T496R) and 412 (F412S, V, or I) were found (2,57).

The objectives of this study were to (i) assess the occurrence and frequency of fenhexamid-resistance in *B. cinerea* from commercial strawberry fields in North and South Carolina, (ii) identify mutations in the *erg27* gene and potentially associated fitness penalties in resistant isolates, and (iii) develop a PCR assay to identify mutations associated with fenhexamid resistance.
Materials and Methods

Fungal isolates and culture conditions. A total of 214 single-spore isolates of *Botrytis cinerea* obtained from strawberry fruit with gray mold symptoms in 2011 from commercial fields HP, MV, NC, and SBY in North Carolina and FLOR, GIK, JEY, KUD, MER, MOD, and WIC in South Carolina were used in this study. Fenhexamid was used in all locations, except for organic field WIC. The same collection minus 2 isolates from SBY and 1 isolate from MV was used in previous studies to characterize the isolates for QoI and SDHI resistance (52). Single-spore isolation was described by Fernández-Ortuño et al. (52). Mycelium of each isolate was stored at -80°C in 80% (v/v) glycerol (Fisher Scientific, New Jersey, USA).

Sensitivity of *B. cinerea* isolates to fenhexamid. A conidia germination assay using discriminatory doses of 1 and 50 mg/liter fenhexamid (Elevate 50 WDG, Arysta LifeScience, Cary, NC) was used to distinguish isolates with different levels of resistance to fenhexamid (142). For each isolate, a 40 µl drop of spore suspension (4 x 10^5 spores/ml) was streaked out across the length of a 1% malt extract agar plate amended with or without fungicide. After 12-14 h of incubation, the germ-tube growth of 50 conidia per isolate was visually assessed, using a microscope (OLYMPUS BX41TF, Olympus Optical Co. Ltd., Japan). Isolates with a fully grown germ-tube on 1 and 50 mg/liter were classified as resistant, while sensitive isolates displayed no germ-tube development. The lower dose was added to the screening to potentially identify isolates with low levels of resistance. This experiment was replicated three times. Determination of 50% effective concentration (EC_{50}) values for resistant isolates was not attempted.
because mycelium grew uninhibited at 50 mg/liter. Therefore EC_{50} values can be considered >50 mg/liter.

**Analysis of erg27 gene sequences from sensitive and resistant isolates.** Genomic DNA was extracted from mycelia as previously described by Chi at al. (29). The oligonucleotides used in this study are listed in Table 2.1. The primer pair erg27Beg and erg27End was used to amplify the erg27 gene from *B. cinerea* DNA. PCR reactions were performed in an iCycler™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a final volume of 50 µl containing 50 ng of fungal template DNA, 10 pmol of each primer, 2.5 mM of each dNTP, 5 µl of 10x LA PCR buffer II and 2.5 U of Takara LA Taq Polymerase (Takara Bio Inc., Japan). The following PCR run parameters were used: 3 min at 95°C, followed by 40 cycles 95°C for 30 s, 60°C for 30 s, and 68°C for 1.5 min, with a final elongation at 68°C for 5 min. PCR products were purified with the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH, USA) using the manufacturer's instructions and sequenced with the primers erg27Beg, erg27End, erg1800down and erg2000up at the Clemson University Genomics Institute (Clemson, SC, USA) using BigDye dye-terminator sequencing technology.

**Development of the allele-specific PCR assays.** Variations at aa position 63 (T63I) and 412 (F412S/I/C) of Erg27 were observed in fenhexamid-resistant isolates. Primers were designed to screen for these aa variations (Fig. 2.1). Six primers (Table 2.1) were used in a PCR cocktail to simultaneously screen for isolates with the T63 or F412 alterations. Primer pair T63_F and T63_R amplified a 584 bp fragment from all isolates and internal primer T63_int in combination with T63_R amplified a single 354 bp
fragment only from isolates carrying the T63 wildtype allele. F412_F and F412_R amplified a 586 bp fragment from all isolates but internal primer F412_int in combination with F412_F amplified a 421 bp fragment only from isolates carrying the F412 wildtype allele (Fig. 2.1; Fig. 2.2). The 584 bp and 586 bp amplicons appeared as a single band for all isolates after electrophoresis on the agarose gel, due to their size similarities (Fig. 2.2). The larger fragments verified the functionality of the PCR assay in the potential absence of the 354 bp and 421 bp fragments, which could occur if an isolate possessed nucleotide variations at both aa positions (63 and 412). PCR reactions were performed in a final volume of 50 µl containing 50 ng of fungal template DNA, 10 pmol of primers F412_F, F412_int, T63_R and T63_int, 6 pmol of primers F412_R and T63_F, 2.5 mM of each dNTP, 5 µl of 10x LA PCR buffer II and 2.5 U of Takara LA Taq Polymerase (Takara Bio Inc., Japan). PCR was conducted with an initial preheating for 3 min at 95°C, followed by 40 cycles 94°C for 30 s, 64°C for 30 s, and 68°C for 35 s, with a final elongation at 68°C for 5 min. The PCR products were separated on ethidium bromide-stained 1.8% (w/v) agarose gels (Thermo Scientific, Rockford, IL, USA) in 1 x TBA and exposed to UV-light to visualize DNA fragments. Taq DNA Polymerase (New England BioLabs, Ipswich, MA, USA) can be used instead of Takara LA Taq Polymerase if the 6-primer PCR is done in two steps (data not shown). The first detects the F412 mutations with primers F412_int, F412_F, and F412_R, the second detects the T63 mutations with primers T63_int, T63_F, and T63_R. Then each PCR needs to be conducted with a slightly changed protocol: 3 min at 95°C, followed by 35 cycles 94°C for 30 s, 63°C for 30 s, and 72°C for 40 s, with final elongation at 72°C for 5 min (data not shown).
A second assay (F412 allele-specific PCR) was designed, to distinguish between the F412S, F412C, and F412I mutations. Primers F412_F and F412_R were used in combination with internal primer F412S_int, F412C_int, or F412I_int to detect the F412S, F412C, and F412I mutations, respectively (Fig. 2.1). For each isolate reactions with each of the three allele-specific primers was performed. The PCR conditions were the same as described above, except that 10 pmol of each primer were used.

**Detached fruit assay.** Commercially grown, mature but still firm strawberry fruit were rinsed with sterile water three times for 30 s each and allowed to air dry. The dried fruit were placed into plastic boxes (8 strawberries per box for each of three replicates per treatment) and sprayed 4 h prior to inoculation with Elevate 50 WDG at 3.6 g/liter to runoff using a hand mister. Untreated fruit were sprayed with distilled sterile water. The fruit was again allowed to dry for 4 h at 20 to 22°C before each fruit was stabbed at three equidistant points to a depth of 9.5 mm using a 26G3/8 9.5-mm beveled syringe tip (Becton Dickson & Co., Rutherford, NJ). Immediately thereafter, the wounds were inoculated with a 30 µl-droplet of conidia suspension prepared in distilled, sterile water (10⁶ spores/ml). Untreated strawberry fruit inoculated with sterile water was used as negative control. After inoculation, the boxes were wrapped in plastic bags to keep the relative humidity at 98 to 100% and kept at 22°C for 24 h. After that, the fruit were uncovered and disease incidence was scored after 4 days visually by assessing the presence or absence of gray mold signs and symptoms. The diameter of the lesions was measured with a standard ruler to the nearest millimeter. The entire experiment was replicated twice.
Data Analysis. A statistical model was developed that related the detached fruit assay responses (percent sporulating lesions and lesion size) to the experimental factors of experimental replication, isolate, fungicide treatment and their combination. The method of least squares was used to estimate the model terms associated with the factors, and analysis of variance was used to test for a significant effect of the factors on the response means. If a factor was found to be significant, mean separation (using student’s t test) was used to further determine the nature of the effect of the factor on the responses. All calculations were performed using the statistical package JMP version 9.0.0 (2010 SAS Institute Inc., Cary, NC) and all tests were performed with $\alpha = 0.05$.

Results

Sensitivity of *B. cinerea* isolates to fenhexamid. Among the 214 field isolates, 36 (17%) were resistant to fenhexamid and grew at 1 and 50 mg/liter. Resistance was found in three of four locations from North Carolina and in four of seven locations from South Carolina indicating that resistance was widespread but may not have been present in every location. The highest percentage of resistant isolates was obtained from location SBY (54%) in North Carolina. At all other locations 33% or fewer isolates were resistant (Table 2.2; Fig. 2.3).

Amino acid sequence variations in the *erg27* gene of fenhexamid-resistant isolates. Because aa changes in the Erg27 protein had previously been associated with fenhexamid resistance, we sequenced the majority of the *erg27* gene from three fenhexamid-sensitive (GIK1, MV4, and NC11) and one fenhexamid-resistant isolate
A change from TTC to TCC in codon 412 was present in the resistant isolate resulting in the replacement of phenylalanine with serine (F412S). The remaining resistant isolates were screened for the F412S allele by PCR (data not shown) and while most of the isolates possessed the F412S allele, seven did not. Sequencing of erg27 from these seven isolates revealed additional mutant alleles. In three isolates (SBY10, SBY21 and SBY27) a change from ACA to ATA was found in codon 63 causing a substitution of isoleucine for threonine (T63I). The other four isolates had mutations in codon 412 (TTC to TGC or ATC) resulting in an aa change from phenylalanine to cysteine (F412C; isolates NC6, NC7 and NC12) or isoleucine (F412I; isolate SBY20), respectively. One aa variation at position 238 (substitution of proline by serine) was subject to change in resistant and sensitive isolates and thus did not correlate with resistance. The four different alleles associated with resistance were submitted to Genbank (JX436968, JX436969, JX436970, JX436971).

Development of an assay to screen for erg27 mutations present in fenhexamid resistant strains in the Carolinas. A multiplex PCR assay able to differentiate between isolates with wild type codons at T63 and F412 and those with a T63I or F412/S/I/C allele was developed to allow for rapid screening of B. cinerea isolates. The assay simultaneously amplifies two regions of the erg27 gene (586 and 584 bp) from all isolates as a positive control, a 354 bp region primed from the T63 wild type codon, and a 421 bp fragment primed from the F412 wild type codon. A single, unpredicted and non-specific band of about 1200 bp in size was amplified when using the 6-primer PCR cocktail (data not shown). The ability of the assay to differentiate between representative
wild type, T63I and F412S isolates is shown in Fig. 2.2. The assay also detects isolates with the F412C and F412I alleles (data not shown). An allele-specific PCR assay to discriminate between the F412 alleles was also developed (Fig. 2.4).

All of the isolates collected in 2011 were subjected to both PCR assays (Table 2.2). Modifications of F412 were more common than the T63I mutation and the predominant F412 variation (n=29) was F412S. This mutation was found in all locations where fenhexamid-resistant phenotypes were present. The F412C mutation was observed in three isolates collected from a single location (NC) in North Carolina, whereas the F412I mutation was found in only one isolate at location SBY in North Carolina. The three isolates possessing the T63I mutation were also found at location SBY (Fig. 2.3). Whether these were clonal isolates or whether they derived from different genetic backgrounds was not investigated in this study.

**Detached fruit assay.** To assess the performance of formulated fenhexamid *in vivo* against resistant isolates, fruit treated with Elevate 50 WDG at label rates were challenged with *B. cinerea* isolates GIK1, SBY21, NC12, SBY20, and SBY36 carrying, respectively, the *erg27* wild type, T63I, F412C, F412I, and F412S allele in detached fruit assays. No treatment by experimental replication, isolate by experimental interaction, or treatment by isolate by experimental interaction (all *P* > 0.89) were observed and thus the data of the independent experimental replications were combined. Gray mold symptoms on the fruit sprayed with distilled sterile water began at the site of inoculation as a small, light brown lesion which enlarged quickly, and became covered with a gray, fuzzy mass of spores after four days of inoculation. Field rates of Elevate 50 WDG controlled isolates
carrying the erg27 wild type allele but not isolates carrying either one of the four Erg27 aa mutations (F412S/C/I and T63I) (Table 2.3).

Discussion

Fenhexamid is one of the most frequently used site-specific fungicides for gray mold control in commercial strawberry fields in the Carolinas and other states in the Southeast. This study documents widespread resistance to fenhexamid in the Carolinas based on the presence of fenhexamid-resistant isolates in 7 of 11 strawberry fields sampled. All producers (except the one operating location WIC) indicated having used Elevate for gray mold disease control suggesting that producers have selected for resistance over time. By far the highest percentage of resistant isolates was obtained from commercial strawberry field SBY in North Carolina, but it is unknown whether this producer used Elevate 50 WDG more frequently compared to others which would potentially have selected more aggressively for resistant strains. The same location had the greatest diversity of resistance alleles which may also be an indicator of higher selection pressure, but it is also possible that the genetic diversity was simply higher in this location. Another explanation for the detection of multiple genotypes only in this location may be that the sample size was greater than that of other locations, making it more likely to find infrequent genotypes. Fenhexamid resistance was absent in locations FLOR, MER, MV and WIC, which might have been a result of lower selection pressure or may have been a consequence of smaller sample sizes (FLOR, MER, and MV represented the lowest sample sizes with 13, 3, and 10 isolates, respectively). The WIC
location was the only organic farm we sampled and thus Elevate 50 WDG or any other site-specific fungicide was not used there for at least the last three years. Therefore, in this location the absence of selection pressure can explain the absence of resistant genotypes on sampled fruit.

The frequency of fenhexamid-resistant isolates obtained from commercial strawberry fields in North and South Carolina was low (16.8% on average) compared to the frequency in other studies, which may indicate that Carolina populations are still in the early stages of selection for resistance. The overall frequency of fenhexamid-resistant isolates of *B. cinerea* in Californian and Northern German strawberry production areas was 38% and 45%, respectively (106,141). Frequencies of isolates from German and French vineyards highly-resistant to fenhexamid varied from less than 30% to more than 50% (57), while 35% of *B. cinerea* isolates from Chilean table grapes were highly-resistant (45). Interestingly, all Carolina isolates resistant to fenhexamid were also resistant to the QoI azoxystrobin and the SDHI boscalid (52). This emergence of strains with resistance to multiple fungicides and lack of reduced fitness poses a serious threat for our efforts to manage fungicide resistance in strawberry fields.

As has been found in other *Botrytis* isolates with high resistance to fenhexamid, mutations in the *erg27* gene were present in resistant Carolina isolates. The F412S mutation of Erg27 has been the most common mutation conferring resistance to fenhexamid in *B. cinerea* populations from strawberry fields in France, Germany and Chile, and was also most common mutation found in the Carolinas. Fenhexamid resistance in our isolates was associated with three other aa substitutions in the Erg27
protein, i.e. T63I, F412I, and F412C. Mutations of F412 (F412S, F412I, and F412V) were first observed in field isolates from French and German vineyards (57) and later described in fenhexamid-resistant laboratory mutants (122). The F412S and F412V mutations were also found in Chilean isolates from table grapes (45). Proof of association of these mutations with the resistant phenotype was provided using isogenic lines carrying the mutant alleles (16). Two novel alleles, F412C and T63I, were present in our collection. Both are strongly associated with the resistant phenotype because isolates carrying these mutations did not contain any of the other mutations associated with resistance and none of the sensitive isolates had those mutations.

The F412 mutations are located in the carboxylic end of the putative transmembrane domain of the 3-ketoreductase and probably modify the affinity of the 3-ketoreductase for fenhexamid (16,124). For example, a hydrophobic aa such as a serine residue at position 412 seems to change the structure of the Erg27 protein resulting in a decrease in affinity for fenhexamid (16). The variation at aa position 63 found in this study for the first time in field isolates was described previously in laboratory mutants resistant to fenhexamid (122,124). This confirms the validity of making and analyzing laboratory mutants in anticipation of such mutants being produced in the field. The aa T63 is located in a domain of still unknown function.

Isolates carrying the T63I or F412S/C/I mutations displayed not only fenhexamid resistance in vitro but also caused gray mold disease on detached fruit treated with the label rate of Elevate 50 WDG. These results demonstrate the significance of these mutations in commercial strawberry production. A previous study of isogenic strains
carrying the F412S, F412I, or F412V found that the mutants grew more slowly in extreme conditions and displayed variations in the production of conidia and susceptibility to freezing (16). However, no significant difference was detected under optimal growth conditions, which is in line with our findings. Taken together, isolates with F412S, F412I, or F412V mutations are likely to successfully compete with wildtype isolates during the fruit production season, however, during winter strains carrying one of the mutations may experience a disadvantage, which may reduce the frequency of resistant strains in the total population for the next season. Whether these isolates carrying F412S have a competitive advantage in the field compared to other fenhexamid-resistant isolates is not known.

We developed two PCR assays that quickly and reliably distinguish isolates from the Carolinas sensitive or resistant to fenhexamid and that determine the mutation associated with resistance. Billard et al. had previously developed a real-time PCR method called the allele-specific probe and primer amplification assay (17). This method can quantify the three single-nucleotide polymorphisms resulting in the aa substitutions F412S/I/V. However, F412C and T63I cannot be identified with this assay. In addition, a real-time PCR cycler is needed whereas only a standard PCR cycler is required for the two assays developed in this study. The PCR assay developed in this study will be useful to study the prevalence and spread of these specific genotypes in the southeastern United States. However, due to the specificity of the PCR assay for the specific alleles found in this study, the assay may not necessarily be able to detect other potential alleles at these aa positions.
In conclusion, fenhexamid resistance is widespread in the Carolinas but most often accounted for only a small percentage of the local population. Interestingly, resistance was without exception based on one of four different target site mutations. Our study strongly suggests that fenhexamid resistance in *B. cinerea* needs to be monitored in commercial strawberry fields of the Carolinas. Growers need to implement resistance management strategies such as rotating chemical classes of fungicides or tank mixing fenhexamid with other products from different chemical classes for gray mold disease management.
Table 2.1. Name, sequence, direction, and location of oligonucleotides in the \textit{erg27} gene used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>DNA strand direction</th>
<th>Location in the \textit{erg27} gene (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>erg27Beg</td>
<td>TGGGATTACCACCATGGGAGACAAGTG</td>
<td>Forward</td>
<td>2-28</td>
<td>(15)</td>
</tr>
<tr>
<td>erg27End</td>
<td>CAATGGTTCGCATTTCAGGCTCCC</td>
<td>Reverse</td>
<td>1556-1582</td>
<td>(15)</td>
</tr>
<tr>
<td>erg1800down</td>
<td>CCGCACTTATTTCCGACAGATTT</td>
<td>Forward</td>
<td>531-553</td>
<td>(15)</td>
</tr>
<tr>
<td>erg2000up</td>
<td>TCGGAGGGTTTGCGTTTGTGTTT</td>
<td>Reverse</td>
<td>813-834</td>
<td>(15)</td>
</tr>
<tr>
<td>T63_F</td>
<td>TGGGAGACAAGTGAGAGCCAG</td>
<td>Forward</td>
<td>16-36</td>
<td>This paper</td>
</tr>
<tr>
<td>T63_int</td>
<td>CACCTCTGAGAAGACACCATTCACA</td>
<td>Forward</td>
<td>246-268</td>
<td>This paper</td>
</tr>
<tr>
<td>T63_R</td>
<td>CGCCTCAGACCCCCCTTTC</td>
<td>Reverse</td>
<td>580-599</td>
<td>This paper</td>
</tr>
<tr>
<td>F412_F</td>
<td>GACATTACGTTCTCGCACACG</td>
<td>Forward</td>
<td>918-938</td>
<td>This paper</td>
</tr>
<tr>
<td>F412_int</td>
<td>CTTCCCATCCTTACAGGTAGAA</td>
<td>Reverse</td>
<td>1313-1338</td>
<td>This paper</td>
</tr>
<tr>
<td>F412_R</td>
<td>CAACCAGGAACCTCGGTCG</td>
<td>Reverse</td>
<td>1484-1503</td>
<td>This paper</td>
</tr>
<tr>
<td>F412S_int</td>
<td>CTTCCCATCCTTACAGGTAGG</td>
<td>Reverse</td>
<td>1314-1338</td>
<td>This paper</td>
</tr>
<tr>
<td>F412I_int</td>
<td>CTTCCCATCCTTACAGGTAGAT</td>
<td>Reverse</td>
<td>1313-1338</td>
<td>This paper</td>
</tr>
<tr>
<td>F412C_int</td>
<td>CTTCCATCCTTACAGGTAGCA</td>
<td>Reverse</td>
<td>1313-1338</td>
<td>This paper</td>
</tr>
</tbody>
</table>
**Table 2.2.** Sensitivity to fenhexamid and presence or absence of mutations in the 3-ketoreductase (Erg27) of *Botrytis cinerea* isolates collected in North and South Carolina

<table>
<thead>
<tr>
<th>Isolates</th>
<th>N° isolates</th>
<th>Fenhexamid sensitivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Erg27 alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLOR1-13, GIK1-9, GIK13-21, HP1-15, HP17-32, HP34, JEY1-4, JEY6-16, JEY18-23, JEY25, KUD1-9, KUD11-21, MER1-3, MOD1-10, MOD12, MOD14, MOD17-18, MOD20, MV1-5, MV7-11, NC1-4, NC8-11, SBY1, SBY4-5, SBY7-9, SBY12, SBY15, SBY17-19, SBY23-25, SBY28-29, WIC1-21</td>
<td>178</td>
<td>S</td>
<td>none</td>
</tr>
<tr>
<td>SBY10, SBY21, SBY27</td>
<td>3</td>
<td>R</td>
<td>T63I</td>
</tr>
<tr>
<td>NC6-7, NC12</td>
<td>3</td>
<td>R</td>
<td>F412C</td>
</tr>
<tr>
<td>SBY20</td>
<td>1</td>
<td>R</td>
<td>F412I</td>
</tr>
<tr>
<td>GIK10-12, HP16, JEY5, JEY17, JEY24, KUD10, MOD11, MOD13, MOD15-16, MOD19, NC5, SBY2, SBY6, SBY11, SBY13-14, SBY22, SBY26, SBY30-37</td>
<td>29</td>
<td>R</td>
<td>F412S</td>
</tr>
</tbody>
</table>

<sup>a</sup> S = sensitive; R = resistant. Isolate sensitivity was assessed according to Weber and Hahn (142).
### Table 2.3. Efficacy of Elevate 50WDG treatment on detached strawberries on field isolates of *Botrytis cinerea* carrying the T63I, F412C, F412I, or F412S mutations

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sporulating lesions (%)</th>
<th>Lesion size (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 dai&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3 dai</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Elevate</td>
</tr>
<tr>
<td><strong>GIK1&lt;sup&gt;野生型&lt;/sup&gt;</strong></td>
<td>19 ab&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0 c</td>
</tr>
<tr>
<td>SBY2&lt;sup&gt;T63I&lt;/sup&gt;</td>
<td>15 ab</td>
<td>8 bc</td>
</tr>
<tr>
<td>NC12&lt;sup&gt;F412C&lt;/sup&gt;</td>
<td>17 ab</td>
<td>8 bc</td>
</tr>
<tr>
<td>SBY20&lt;sup&gt;F412I&lt;/sup&gt;</td>
<td>31 a</td>
<td>13 ab</td>
</tr>
<tr>
<td>SBY36&lt;sup&gt;F412S&lt;/sup&gt;</td>
<td>19 ab</td>
<td>13 ab</td>
</tr>
</tbody>
</table>

<sup>y</sup>dai = days after inoculation.

<sup>z</sup>Numbers in control and Elevate treatment columns for each dai section separately followed by the same letter are not significantly different at α = 0.05 as determined by analysis of variance (ANOVA). Mean separation was conducted using student’s t test.
Fig. 2.1. Schematic representation of the phenotype-differentiating PCR assay strategy distinguishing isolates sensitive and resistant to fenhexamid. Gray boxes indicate coding sequences of the *erg27* gene, the line between represents the position of a single intron, and the primer locations and directions are indicated with black arrows. The star indicates the position of primers F412S_int, F412C_int, and F412I_int used for the F412 allele-specific PCR.
Fig. 2.2. PCR amplification of *erg27* gene fragments with universal primers (T63_F, T63_R, F412_F, and F412_R) in combination with allele-specific primers T63_int and F412_int to distinguish between *erg27* alleles. Lane 1, no template control; lanes 2-4, amplifications from isolates JEY7\(^*_{\text{wildtype}}\), SBY31\(^{F412S}\), SBY27\(^{T63I}\); M, GeneRuler\(^\text{TM}\) 100 bp DNA Ladder (Fermentas Inc., Glen Burnie, MD, USA). PCR products were run on 1.8 % agarose gels.
Fig. 2.3. Occurrence and frequency of Erg27 amino acid genotypes associated with resistance to fenhexamid in *Botrytis cinerea* isolates from the Carolinas. Sensitive isolates are represented in gray and resistant isolates with mutations F412C, F412I, F412S, and T63I are represented with patterns checker board, black and white stripes, solid black, and waived lines, respectively. The circle diameter correspond to the number of isolates collected in each location, which is indicated below the locations' name. The white arrow indicates the center position of the JEY circle.
Fig. 2.4. F412 allele-specific PCR distinguishing the F412S, F412I, and F412C mutations in *B. cinerea*. Primers F412S$_\text{int}$ (A), F412I$_\text{int}$ (B) and F412C$_\text{int}$ (C) in combination with primer F412$_\text{F}$ amplified each a 421-bp fragment from isolates SBY11$^{F412S}$, NC6$^{F412I}$, and SBY20$^{F412C}$ (lanes 3-5, respectively) but not from isolate JEY13$^{\text{wildtype}}$ (lane 2). M, 50-bp Mini DNA ladder (Fisher Scientific, Pittsburgh, PA, USA). Products were electrophoresed on 1.8 % agarose gels.
CHAPTER THREE
CHARACTERIZATION OF IPRODIONE RESISTANCE IN *BOTRYTIS CINEREA* FROM STRAWBERRY AND BLACKBERRY

This work has been published:

["Fungal isolates and culture conditions" of strawberry isolates from Florida, strawberry isolates from the Carolinas, and blackberry isolates were performed by Dr. Achour Amiri, Dr. Dolores Fernández-Ortuño, and Xingpeng Li, respectively; "Sensitivity to iprodione and fludioxonil" of strawberry isolates from Florida, strawberry isolates from the Carolinas, and blackberry isolates were determined by Anja Grabke, Dr. Dolores Fernández-Ortuño, and Xingpeng Li, respectively; "Sensitivity to tolnaftate" was performed by Anja Grabke; "RNA extraction and cDNA synthesis" was performed by Xingpeng Li and Anja Grabke; "Analysis of *bos1* gene sequences" and "Expression analyses" were performed by Anja Grabke; "Evaluation of fitness components and in vivo sensitivity to fungicides" was performed by Dr. Dolores Fernández-Ortuño; "Data analysis" was performed by Xingpeng Li.]

Abstract

Gray mold, caused by the fungal pathogen *Botrytis cinerea*, is one of the most destructive diseases of strawberries. Control of the disease in commercial fields is largely
dependent on the application of fungicides, including the dicarboximide iprodione. Single-spore isolates were collected from strawberry fields in Florida, North Carolina, and South Carolina and subjected to an assay using conidial germination that distinguished sensitive (S) isolates from isolates with various levels of resistance to iprodione. Of the 245 isolates, one was highly resistant (HR), 5 were moderately resistant (MR), and 43 had low resistance (LR) to iprodione. LR and MR strains were found in the Florida population and in nine of eleven locations from North Carolina and South Carolina, indicating that resistance was widespread but only accounted for a relatively small percentage of the *B. cinerea* population. Sequence analysis of the target gene *bos1* which codes for a class III histidine kinase revealed that the MR phenotype was associated with Q369P and N373S mutations, and that the LR phenotype was associated with either a I365S or a I365N mutation. The I365S and I365N mutations were also present in five additionally included HR isolates from Carolinian blackberry fields and one HR isolate from a Virginia strawberry field, but no mutation or mutation combinations in *bos1* were uniquely associated with the HR phenotype. Expression analysis of *bos1* in S and HR isolates did also not reveal convincing evidence of the gene's involvement in HR resistance. The six HR isolates had three different phenotypes with respect to their sensitivity to fludioxonil; two were S, two were LR, and two were MR. The fludioxonil LR and MR isolates were also resistant to tolnaftate, an indication of multidrug efflux pump activity. These data suggest that in addition to point mutations in *bos1*, drug efflux pump activity and potentially a third mechanism of resistance may be
contributing to the iprodione HR phenotype. Detached fruit studies showed that field rates of Rovral 4 Flowable (iprodione) did not control iprodione MR and HR isolates.

**Introduction**

Gray mold, also known as Botrytis fruit rot, is one of the most destructive diseases of cultivated strawberries (*Fragaria x ananassa*) in the United States and in other production areas worldwide. Flowers are infected first, mostly from conidia germinating on petals, stamens, and pistils, and infections remain latent until weather conditions become favorable and symptoms are eventually expressed on green and ripe fruit. Secondary infections occur through contact of healthy fruit with infected plant material or via conidia that are spread from symptomatic to healthy fruit via wind and rain splash (147). In the southeastern United States, the disease is mainly caused by the plant pathogenic fungus *Botrytis cinerea* Pers.:Fr. but fruit can also be affected by *B. caroliniana* X.P. Li & G. Schnabel (53,54,95,96). Preharvest crop loss resulting from infections by *B. cinerea* is especially severe under moist conditions and temperatures around 20°C.

Chemical control during bloom and fruit maturation remains the main approach to control gray mold in commercial strawberry fields. In addition to the multi-site fungicides captan and thiram, site-specific fungicides of seven different chemical classes are currently available for gray mold management in the southeastern United States, including anilinopyrimidines (APs), dicarboximides (DCs), hydroxyanilides (HAs), methyl benzimidazole carbamates (MBCs), quinone outside inhibitors (QoIs),
phenylpyrroles (PPs), and succinate dehydrogenase inhibitors (SDHIs). Many of the site-specific fungicides are considered reduced health risk fungicides due to their generally higher efficacy, lower toxicity, and lower application rates but these advantages are somewhat offset by their vulnerability to resistance development. *B. cinerea* is known for its ability to develop resistance quickly (90) and in fact, resistance in *B. cinerea* from strawberry fields in the southeastern United States has been reported to AP, HA, MBC, SDHI, and QoI fungicides (4,51,52,55,64).

The dicarboximide iprodione is a site-specific fungicide which has, compared to many other classes of fungicides, a relatively narrow spectrum of activity that includes *Botrytis* spp. and the closely related genera *Monilinia* and *Sclerotinia* (37,150). The fungicide has been used to control *B. cinerea* since the late 1970s and resistance has emerged on different crops in many areas where dicarboxamides had been used frequently (79,89,90,99,139). For example, iprodione-resistant *B. cinerea* isolates from strawberries were found in Australia (140), Germany (141), Israel (43), New Zealand (10), and Spain (38). Reports of resistance from the Southeastern and Eastern U.S. are rare. Resistance to dicarboximides was reported in *B. cinerea* isolates from three strawberry nurseries in North Carolina, several strawberry farms in Louisiana and Oregon, and greenhouse-grown ornamental crops in South Carolina (27,51,78,143,144,151); however, no comprehensive study of the occurrence and prevalence of iprodione resistance or mechanisms of resistance has been conducted for isolates from strawberry fields of the Eastern United States.
Isolates of *B. cinerea* resistant to iprodione may possess low, moderate, or high levels of resistance. Low to moderate levels are typically found in field isolates, whereas high levels of resistance have been found in laboratory mutants and are rarely seen in field isolates. Cross resistance between isolates resistant to PPs and DCs was observed in laboratory mutants, but has rarely been found under field conditions (49,89,141). Genetic analysis of dicarboximide-resistant *B. cinerea* isolates revealed that resistance was usually linked to a single gene *bos1* (also called *BcOS1* or *Daf1*), but a second gene (*daf2*) was involved in conferring resistance in laboratory mutants (49). *Bos1* codes for a class III histidine kinase which is involved in the adaptation to adverse environmental conditions such as osmotic and oxidative stress. This osmosensing histidine kinase is also important for the macroconidation and pathogenesis of *B. cinerea* which may explain the low frequency of highly resistant field strains (98,101,136).

The objectives of this study were to (i) determine the occurrence and frequency of iprodione resistance in *B. cinerea* isolates from commercial strawberry fields in Florida, North Carolina, and South Carolina, (ii) identify mutations in the *bos1* gene corresponding with the resistance phenotypes, and (iii) examine fitness and pathogenicity of isolates with low, moderate and high levels of resistance to iprodione.

**Materials and Methods**

**Fungal isolates and culture conditions.** A total of 245 single-spore isolates of *B. cinerea* were collected from strawberry fruit with gray mold symptoms; 30 from Florida, 90 from North Carolina, 124 from South Carolina, and one (isolate Wland1)
from Virginia (Table 3.1). Most isolates from strawberry fields HP, MV, NC, and SBY in North Carolina and FLOR, GIK, JEY, KUD, MER, MOD, WIC in South Carolina were used in previous studies to assess the sensitivities and molecular bases of resistance to QoIs, SDHIs, APs, PPs, and HAs (51,52,64). Single-spore isolation was performed as previously described (52,53).

For more thorough investigation of the molecular mechanism of iprodione resistance, isolates from blackberry were included; five with high resistance (CBa82, COa3, CO20, KC25, and KC33), one with low resistance (MC14), and two sensitive (CA25 and CBa3) to iprodione. the origin of these isolates was reported previously (95). Each isolate had been stored as dried mycelium on filter paper (Whatman Inc., Maidstone, UK) at -20°C as previously described (154) before it was grown on PDA for experimental use.

**Sensitivity of** B. cinerea **isolates to iprodione, fludioxonil, and tolnaftate.** The sensitivity of the B. cinerea isolates to fludioxonil and iprodione was assessed using a conidial germination assay that distinguishes sensitive isolates from isolates with various levels of resistance (142). Discriminatory concentrations of 5 and 50 mg/liter iprodione formulated as Rovral 4 Flowable (Bayer CropScience, Durham, NC, USA) permitted a distinction between highly sensitive (SS), sensitive (S), moderately resistant (MR), and highly resistant (HR) isolates. Germ tubes of conidia of SS isolates were completely inhibited at 5 and 50 mg/liter; germ tubes of S isolates showed residual growth (10-25% of the rate of the control) at 5 mg/liter but were completely inhibited at 50 mg/liter; germ tubes of MR isolates grew 50 to 80% at 5 mg/liter and 10 to 40% at 50 mg/liter; isolates
with a fully developed germ tube at 5 mg/liter and a germ tube longer than 50% at 50 mg/liter were categorized as HR. A new resistance category named low resistant (LR) was assigned to isolates with germ tubes displaying growth of more than 80% at 5 mg/liter but completely inhibited at 50 mg/liter.

Discriminatory doses of 0.1 and 10 mg/liter of fludioxonil were used to distinguish SS, S, MR, and R isolates. Germination of conidia of SS isolates was completely inhibited at both concentrations; germ tubes of S isolates grew 30 to 50% at 0.1 mg/liter but were inhibited at 10 mg/liter; germ tubes of MR isolates grew more than 80% at 0.1 mg/liter, and 10 to 20% at 10 mg/liter; and germ tubes of R isolates grew without inhibition at 0.1 mg/liter and more than 25% at 10 mg/liter.

Effective inhibitory concentrations (EC₅₀; mg/liter) for tolnaftate were assessed as previously described by Kretschmer et al. (82) using 1,000 spores in 0.1 ml 96-microplate cultures and a three-fold drug dilution series. Tests were performed in malt extract broth with final concentrations of 30, 10, 3, 1, 0.3, 0.1, and 0.03 mg/liter of tolnaftate. After 48 h of incubation in the dark at 22°C, light absorption at A₆₀₀ nm was determined. Isolates with EC₅₀ values <2.5 mg/liter were designated sensitive and isolates with EC₅₀ values >10 mg/liter were designated resistant.

Analysis of *bos1* gene sequences from isolates with different iprodione resistance phenotypes. To investigate molecular mechanisms of resistance, nucleotide sequences of the *bos1* gene from sensitive and resistant isolates were compared. Genomic DNA was extracted from actively growing mycelia using either the MasterPure™ Yeast DNA Purification Kit (Epicentre, Madison, WI, USA) or a method published previously by Chi
et al. (29). Five primer pairs (BF1 + BR1, BF2 + BR2, BF3 + BR3, BF4 + BR4, and BF5 + BR5) previously published by Ma et al. (101) were used to amplify the complete bos1 gene from *B. cinerea* isolates listed in Table 3.2. In addition, a fragment containing previously described key point mutations in bos1 including I365N/S/R, Q369H/P, and N373S (8,31,90,114,115) that correlate with resistance to iprodione was amplified from LR isolates 10-43, JEY2, JEY25, KUD19, MOD4, SBY1, SBY4, SBY7, and SBY10 with primer pair BF2 and BR2. PCR reactions were performed in a 25 µl volume containing 25 ng of fungal genomic DNA, 5 mM of each dNTP, 10 pmol of each primer, 2.5 µl of 10x Thermo PolTM Buffer, and 0.625 U of Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA). PCR was carried out with an iCyclerTM Thermal Cycler (Biorad Laboratories Inc., Hercules, CA, USA) programmed as follows: 3 min at 95°C, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. All amplified PCR products were purified using the ExoSAP-IT purification kit (USB Corporation, Cleveland, OH, USA) and sequenced at the Clemson University Genomics Institute (Clemson, SC, USA).

**RNA extraction and expression analyses.** For analysis of bos1 expression in *B. cinerea*, 2 ml of potato dextrose broth in polystyrene Petri dishes (60 × 15 mm) were inoculated with 5 × 10⁶ conidia and incubated for 14.5 h at RT and 150 rpm. After 14.5 h the germlings were incubated for another 30 min either with or without 10 ml/liter iprodione formulated as Rovral 4 Flowable (41.6% (w/v) active ingredient; Bayer CropScience, Durham, NC, USA). For RNA isolation, the liquid culture was transferred into a 2-ml tube and centrifuged for 4 min at 10000 rpm. The pellet was washed twice
with ice-cold water followed by centrifugation. Total fungal RNA was extracted using the MasterPure™ Yeast RNA Purification Kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. RNA concentration and purity was determined using agarose gel electrophoresis and spectrophotometric analysis with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). One microgram of RNA was reverse-transcribed into cDNA with the iScript cDNA Synthesis Kit (Biorad Laboratories Inc., Hercules, CA, USA), according to the manufacturer's instructions.

Quantitative RT-PCR was performed in an iCycler™ Thermal Cycler with an iQ™5 Multicolor Real-Time PCR Detection System (Biorad Laboratories Inc., Hercules, CA, USA). Each reaction was prepared in a total volume of 20 µl containing 1 µl of cDNA, 250 nM of each primer, and 10 µl of the iQ™ SYBR Green Supermix (Biorad Laboratories Inc., Hercules, CA, USA). The primer pair Bos1F (5′-TCGAACGCGTAGCAGCTCTC-3′) and Bos1R (5′-ACCTTCTGTGCCGACCTCTC-3′) was used for the analysis of bos1 expression. The primer set was verified by standard curves for amplification efficiencies ranging from 90 to 95% and for absence of non-specific amplicons. The cycle conditions were as follows: 3 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 56°C for 30 s, and elongation at 72°C for 25 s. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing in steps of 0.5°C every 30 s. The expression of genes was calculated according to Pfaffl (117). Expression levels of bos1 were normalized against the expression levels of the housekeeping gene actA with primer pair Act_RT_fw/ Act_RT_rev (87), and shown as normalized fold-expression relative to
levels of non-induced germlings from the sensitive isolate CBa3. Means and standard deviations were calculated from two biological replicates of each treatment. Three technical replicates of the PCR reaction were used for each biological replicate.

Evaluation of fitness components and in vivo sensitivity to fungicides. Isolates with various resistance phenotypes were assessed for their in vitro and in vivo fitness, and in vivo fungicide sensitivity in comparison to wild-type isolates. Analyses of mycelial growth rate on PDA, in vitro spore production, in vivo pathogenicity, in vivo sensitivity to fungicides, and in vivo spore production were conducted exactly as previously described (51) with the exception that PDA was used instead of wateragar, and Rovral 4 Flowable (41.6% (w/v) active ingredient; Bayer CropScience, Durham, NC, USA) and Scholar SC (20.4% (w/v) active ingredient; Syngenta Crop Protection, Greensboro, NC, USA) formulated products were used at 2.5 ml/liter for the assessment of in vivo pathogenicity and sensitivity to fludioxonil and iprodione in detached fruit assays. In order to reduce the potential influence of multiple fungicide resistance in the fitness study, we chose SS and S isolates to iprodione that were either sensitive to all seven classes of fungicides (isolate GIK2) or isolates that only differed in their resistance to iprodione but had otherwise the same resistance profiles related to other chemical classes. Specifically, MR isolates JEY6 and SBY9 were resistant to APs, MBCs, SDHIs, and QoIs, just like SS and S isolates JEY2, JEY8, and SBY24. SS isolate JEY21 resembled in its phenotypic resistance profile iprodione LR isolate GIK3 (both were resistant to MBCs, SDHIs, and QoIs), and S isolate GIK10 resembled in its resistance profile iprodione LR isolates JEY24, and SBY26 (all were resistant to APs, HAs, MBCs, SDHIs, and QoIs).
Data analysis. A statistical model related the experimental responses of the fitness test to replication, isolate, fungicide treatment, and their combination. The method of least squares was used to estimate the model terms connected with the factors. The data of the two independent experiments for each experimental response were combined and 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 B. cinerea isolates to iprodione. Most of the 245 field isolates from strawberry fruit were either SS (38%) or S (41%) to iprodione. Five isolates or 2% (isolates 12-402 from Florida, SBY9 and SBY23 from North Carolina, and JFY4 and JFY6 from South Carolina) were MR, and 44 or 18% were LR to iprodione (Table 3.1). The isolate Wland1 from Virginia was HR to iprodione. Most isolates LR or MR to iprodione were observed in North Carolina (18%) and South Carolina (23%). A majority of isolates (27 of 30) from Florida were either SS of S whereas only 3% of the isolates were LR or MR to iprodione. Isolates LR to iprodione were found in two of four locations in North Carolina and in all seven locations in South Carolina, indicating that this phenotype was widespread but not present in every location in the Carolinas. Most LR and MR isolates were obtained from locations MOD, JFY, and SBY, which clustered in the western part of the Carolinas.
Amino acid variations in Bos1 of isolates with varying levels of resistance to iprodione. The bos1 gene from one SS, one S, six LR, five MR, and one HR isolates from strawberry fruit was sequenced in its entirety, because amino acid (aa) changes in the Bos1 protein had previously been associated with resistance to iprodione. Two S, one LR, and five HR B. cinerea isolates from blackberry fruit were included in this sequence analysis. A combination of two point mutations in bos1 was present in all MR isolates resulting in the replacements of glutamine with proline at amino acid position 369 (Q369P; CAG to CCG) and asparagine with serine at aa position 373 (N373S; AAC to AGC) (Table 3.2). All LR and HR isolates had a mutation at codon 365 (ATC to AGC or AAC), resulting in aa change from isoleucine to either serine (I365S) or asparagine (I365N). Isolates containing the I365N mutation had an additional mutation at codon 127 (TTT to TCT), causing a substitution of phenylalanine to serine (F127S). Four out of six HR isolates and one LR isolate with the I365S mutation (MOD8, CBa82, KC25, KC33, and Wland1) contained an additional change from valine to isoleucine at aa position 1136 (V1136I; GTC to ATC). A fragment of bos1 was sequenced from nine additional LR isolates (10-43, JEY2, JEY25, KUD19, MOD4, SBY1, SBY4, SBY7, and SBY10) to investigate whether the mutation I365S/N was characteristic for isolates LR to iprodione. The same isolates were also screened for the F127S and V1136I mutations by allele-specific PCR and restriction length polymorphism, respectively (data not shown). The combination of F127S and I365N mutations, which had been found in isolates 12-104, COa3, and CO20 (Table 3.2), was also present in LR isolates 10-43, SBY1, and SBY10. The combination of I365S and V1136I mutations, which had been found in isolates
MOD8, CBa82, KC25, KC33, and Wland1 (Table 3.2), was also present in LR isolates JEY2, JEY25, KUD19, MOD4, SBY4, and SBY7. No single aa change or any aa change combination in Bos1 was associated with the HR phenotype, indicating that mutations in bos1 were not responsible for the highly resistant phenotype.

Some isolates contained uncommon nucleotide changes leading to aa variations. The D757L mutation was present in one (isolate MC14) and the P972L mutation in three LR isolates (GIK21, JEY1, and JEY23). The same isolates also contained the I365S mutation associated with LR resistance, it is thus unclear if D757L and P972L contribute to the LR phenotype. Two aa variations at position 1259 (substitution of threonine by proline or alanine) were found in resistant and sensitive isolates, thus, did not correlate with resistance. Interestingly, two isolates (MOD8, and 12-104) categorized as sensitive to iprodione did contain the aa substitution I365S/N characteristic for LR confirming our observations that the distinction between S and LR isolates was not always easy to make in our sensitivity assay.

**Analysis of bos1 expression levels.** Because point mutations in target gene bos1 could not explain the HR phenotype, we investigated its expression in germlings with and without iprodione exposure prior to RNA extraction. Constitutive expression of bos1 was consistently lowest in the two S isolates and HR isolates CBA82 and Wland1, but twice as high in HR isolates KC25 and COa3. With the exception of isolate KC25, iprodione exposure slightly but significantly induced bos1 expression levels in S and HR isolates (Fig. 3.1).
Sensitivity of *B. cinerea* isolates to fludioxonil and tolnaftate. Cross resistance between dicarboximides and phenylpyrroles is a well-known phenomenon in laboratory mutants. We found that our SS, S, LR and MR field isolates were sensitive to fludioxonil (Table 2). The six HR isolates had three different phenotypes with respect to their sensitivity to fludioxonil. Two were S (COa3 and CO20), two were LR (CBa82 and Wland1), and two were MR (KC25 and KC33) to fludioxonil. None of the aa changes found in Bos1 or any aa combination was distinctly associated with resistance to fludioxonil, including the fludioxonil-LR and MR phenotypes. The four isolates resistant to fludioxonil were also the only ones resistant to tolnaftate, an indication that multidrug resistance activity based on drug efflux pumps may be involved.

Evaluation of fitness components and in vivo sensitivity to fungicides. Isolates with various resistance phenotypes to iprodione were assessed for their in vitro and in vivo fitness, and in vivo fungicide sensitivity. LR, MR, and HR isolates to iprodione grew as fast on PDA and produced as many spores in vitro as the S and SS isolates (Table 3.3). Although some isolates developed significantly different lesion sizes on nontreated strawberry fruit no correlation was observed between lesion size and resistance phenotype. Field rates of Rovral 4 Flowable controlled the sensitive isolates but not the LR, MR isolates, or the one HR isolate included in this study. There were no significant differences in lesion size and sporulating lesions among and between MR and LR isolates, but LR isolates had significantly smaller lesion sizes and sporulated less on strawberry fruit treated with Rovral 4 Flowable compared to the HR isolates. Only the
isolate LR to fludioxonil (Wland1) produced a sporulating lesion on fruit treated with Scholar SC fungicide (Table 3.3).

Discussion

This study documents that resistance to iprodione in B. cinerea from strawberry fields in North and South Carolina is widely distributed but low in prevalence. Isolates resistant to iprodione were found in the majority (9 of 11) of strawberry fields examined. The frequency of LR and MR isolates obtained from fields in Florida, North Carolina, and South Carolina was low (3%, 18%, and 23%, respectively) compared with the frequencies reported in previous studies. The overall frequency of B. cinerea isolates resistant to dicarboximides from strawberry fields in Australia, Louisiana, New Zealand, Oregon, and Spain was 45%, 55%, 25%, 41%, and 44%, respectively (10,38,43,140,141,143,144). The relatively low frequency of resistance observed in our study may be due to limited exposure of B. cinerea isolates in strawberry fields to dicarboximides in recent years. In the U.S., use of dicarboximides was restricted to one application per season prior to bloom after 1998 due to human exposure concerns (134). This not only dramatically reduced the number of permitted sprays per season in the U.S. for strawberries, it also prevented farmers from using this product during the peak time of infection. This change in use pattern could also explain why fewer HR isolates were collected from strawberry fields (one of 215 isolates) compared to blackberry fields (five of 202 isolates) where four iprodione applications are still permitted up to harvest.
Mutations in *bos1* were present in all of the resistant *B. cinerea* isolates, but aa changes could only be associated distinctly with the LR and MR phenotypes. The I365S/N/R mutations were associated with low levels of resistance to dicarboximides in *B. cinerea* isolates from England, France, Israel, Italy, Japan, New Zealand, Switzerland, and USA (30,32,90,101,115). Consistent with these published studies, mutations I365S and I365N were associated with the LR phenotype in our collection. The MR phenotype was associated with a combination of aa changes Q369P and N373S in our study, which was consistent with other reports (8,30,101,114). However, none of the mutations or mutation combinations in *bos1* was uniquely associated with the HR phenotype. In previous studies, highly resistant laboratory mutants contained point mutations in *bos1* resulting in various aa alterations including G278, G232C, G357N, G415D, G446S, G493T, and P742T (32,56,124). The presence of mutations in *bos1* associated with LR in our HR field mutants indicates that additional mechanisms of resistance to iprodione may be involved. Multi-drug resistance based on overexpression of gene *mfsM2* encoding a major facilitator transporter has been shown to confer low levels of resistance to iprodione (82). We investigated the expression of this gene both in iprodione S and HR isolates, but no significant differences in expression were found (data not shown). However, four of the six HR isolates that carried the mutations I365S or I365N associated with LR and were also resistant to fludioxonil and tolnaftate, which could be an indication for a different multi-drug efflux pump activity (82). The other two HR isolates carried the mutations F127S and I365N associated with LR, but were sensitive to tolnaftate and fludioxonil. That suggests in addition to mutations in *bos1*, drug efflux
pump activity and a third mechanism of resistance may be involved in the development of HR phenotypes in *B. cinerea* field isolates. The expression level of *bos1* was analyzed in this study as a possible third mechanism of resistance. Though two isolates showed a 2 to 2.5-fold overexpression of *bos1* in the absence of iprodione, this evidence is insufficient to conclude that overexpression of *bos1* results in resistance to iprodione.

Isolates displaying iprodione resistance in vitro also caused gray mold disease on detached fruit treated with the recommended label rate of Rovral 4 Flowable, which demonstrates the significance of isolates with different resistance phenotypes for commercial strawberry production. Isolates with LR, MR, and HR phenotypes were pathogenic on strawberry fruit treated with Rovral 4 Flowable. MR and HR isolates were equally aggressive, but LR isolates had smaller lesion sizes and sporulated less compared to the HR isolate. Considering the relatively harsh inoculation method we used to induce infection (stab inoculation to simulate insect damage and injection of spores), especially LR but possibly also MR and HR isolates may still be controlled in the field with DC fungicides. Further studies are needed to investigate whether LR, MR and HR isolates are able to cause disease under field conditions when insects are sufficiently controlled. Previous fitness studies found that dicarboximide-resistant *B. cinerea* field isolates did not differ from sensitive isolates in regard to radial growth rate on artificial medium, sporulation, and pathogenicity, which is in line with our findings (10,30,114), however, one study reports that sclerotia of isolates resistant to DCs survived less well compared to sensitive isolates (118). A previous study also showed reduced in vitro growth rate, sporulation rate, and lesion development in laboratory mutants with HR phenotype (56).
However, we established that the molecular basis of resistance in our isolates is different (not based solely on target gene mutations) and that our HR isolate did not suffer a competitive disadvantage compared to sensitive isolates in our fitness study. Resistance management programs should therefore not rely on the exploitation of reduced fitness as a tool to control MR and HR mutants.

Cross resistance between dicarboximides and phenylpyrroles is a common phenomenon in fungal pathogens, as both classes of fungicides interfere with the osmoregulatory pathway. For example, field isolates of *Alternaria brassicicola* and laboratory mutants of *Aspergillus parasiticus* had previously shown high levels of cross resistance between those two fungicides (74,104). In *B. cinerea*, cross resistance between dicarboximides and phenylpyrroles is frequently observed in laboratory strains, but rarely found under field conditions (49,56,71,89,155), which is in line with our findings. The majority of our isolates (two HR, and all of MR and LR isolates to iprodione) were sensitive to the phenylpyrrole fludioxonil; only four of the six HR isolates to iprodione were also resistant to fludioxonil. In laboratory mutants, cross resistance between dicarboximides and phenylpyrroles was associated with mutations in *bos1*, but analysis of crosses between field isolates resistant to DCs and PPs suggested that different genes regulate resistance to these two fungicides (49,56,137). No mutation in *bos1* was associated with resistance to fludioxonil in our field isolates. Further studies are needed to investigate mechanisms of fludioxonil resistance, including multi-drug resistance activity, which has previously been shown to confer resistance to fludioxonil and in some cases also to iprodione (82).
In conclusion, iprodione resistance is widespread but only accounts for a relatively small percentage of the *B. cinerea* population of the southeastern United States. Interestingly, only LR and MR phenotypes were associated with target gene mutations, indicating that the HR phenotype in field strains is caused by different, still unknown mechanisms. While the commercial significance of the LR phenotype has not yet been established, MR and HR phenotypes were not controlled on detached fruit sprayed with label rates of Rovral and resistance should be monitored in commercial strawberry fields.
Table 3.1. Origin and sensitivity to iprodione of *Botrytis cinerea* isolates collected from strawberry fields in Florida, North Carolina, South Carolina, and Virginia.

<table>
<thead>
<tr>
<th>Iprodione sensitivity&lt;sup&gt;z&lt;/sup&gt;</th>
<th>Isolates</th>
<th>N° of isolates</th>
<th>State&lt;sup&gt;y&lt;/sup&gt;</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>01-37, 12-19, 12-87, 12-178, HP1 to 15, HP17 to 34, MV1, MV5, MV8 to 10, SBY8, SBY15, SBY25, SBY28, FLOR1 to 5, FLOR7 to 13, GIK2, GIK4, GIK6, GIK7, GIK13, GIK15, JEY8, JEY21, KUD1, KUD3, KUD5, KUD13, KUD16, KUD17, KUD20, MER2, MER3, MOD3, MOD9, MOD10, MOD18, WIC2, WIC4 to 6, WIC8, WIC10 to 13, WIC16 to 21</td>
<td>48</td>
<td>SC</td>
<td>2011</td>
</tr>
<tr>
<td>S</td>
<td>05-26, 12-26, 12-32, 12-39, 12-55, 12-64, 12-73, 12-83, 12-84, 12-91, 12-98, 12-106, 12-108, 12-125, 12-129, 12-138, 12-152, 12-156, 12-165, 12-369, 12-397, 12-409, 12-427, MV2 to 4, MV7, MV11, NC1 to 10, NC12, SBY2, SBY11, SBY12, SBY17, SBY19 to 22, SBY24, SBY27, SBY29, SBY31, SBY34 to 37, GIK1, GIK5, GIK8 to 12, GIK14, GIK16, GIK17, GIK19, GIK20, JEY2, JEY5, JEY7, JEY9 to 16, JEY18 to 20, JEY22, KUD2, KUD4, KUD6 to 12, KUD14, KUD15, KUD18, KUD21, MER1, MOD1, MOD2, MOD11, MOD12, WIC3, WIC9</td>
<td>32</td>
<td>NC</td>
<td>2011</td>
</tr>
<tr>
<td>LR</td>
<td>10-43, 12-104, NC11, SBY2, SBY4 to 7, SBY10, SBY13, SBY14, SBY18, SBY26, SBY30, SBY32, SBY33, FLOR6, GIK3, GIK8, GIK18, GIK21, JEY1, JEY3, JEY17, JEY23 to 25, KUD19, MOD4 to 8, MOD13 to 17, MOD19, MOD20, WIC1, WIC7, WIC14, WIC15</td>
<td>27</td>
<td>SC</td>
<td>2011</td>
</tr>
<tr>
<td>MR</td>
<td>12-402, SBY9, SBY23, JEY4, JEY6</td>
<td>1</td>
<td>FL</td>
<td>2010</td>
</tr>
<tr>
<td>HR</td>
<td>Wland1</td>
<td>1</td>
<td>VA</td>
<td>2012</td>
</tr>
</tbody>
</table>

<sup>z</sup> SS = highly sensitive; S = less sensitive; LR= low-resistant; MR = moderate-resistant; HR = high-resistant. Isolate sensitivity was assessed with a germination assay using iprodione at 5 and 50 mg/liter (142).

<sup>y</sup> FL = Florida; NC = North Carolina; SC = South Carolina, VA = Virginia.
Table 3.2. Sensitivity of *Botrytis cinerea* isolates with different iprodione resistance phenotypes to fludioxonil and tolnaftate and presence or absence of mutations in the Class III Histidine-Kinase Bos1.

<table>
<thead>
<tr>
<th>Iprodione resistance phenotype</th>
<th>Isolate</th>
<th>Host</th>
<th>AA substitution in Bos1</th>
<th>Sensitivity</th>
<th>Fludioxonil</th>
<th>Tolnaftate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F127&lt;sup&gt;v&lt;/sup&gt;</td>
<td>I365&lt;sup&gt;w&lt;/sup&gt;</td>
<td>Q369&lt;sup&gt;x&lt;/sup&gt;</td>
<td>N373&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>SS</td>
<td>MER2</td>
<td>Strawberry</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>S</td>
<td>CA25</td>
<td>Blackberry</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>CBa3</td>
<td>Blackberry</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>MV8</td>
<td>Strawberry</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>LR</td>
<td>MC14</td>
<td>Blackberry</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>12-104</td>
<td>Strawberry</td>
<td>S</td>
<td>N</td>
<td>S</td>
<td>S</td>
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<tr>
<td></td>
<td>GIK21</td>
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<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td></td>
<td>JGY1</td>
<td>Strawberry</td>
<td>S</td>
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<td>S</td>
<td>S</td>
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<tr>
<td></td>
<td>MOD8</td>
<td>Strawberry</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
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<tr>
<td></td>
<td>SBY11</td>
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<td>S</td>
<td>S</td>
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<tr>
<td>MR</td>
<td>12-402</td>
<td>Strawberry</td>
<td>P</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>JGY4</td>
<td>Strawberry</td>
<td>P</td>
<td>S</td>
<td>S</td>
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<td></td>
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<td>Strawberry</td>
<td>P</td>
<td>S</td>
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<td></td>
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<td>S</td>
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<td></td>
<td>SBY23</td>
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<td>P</td>
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<tr>
<td>HR</td>
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<td>Blackberry</td>
<td>S</td>
<td>I</td>
<td>LR</td>
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<td></td>
<td>COA3</td>
<td>Blackberry</td>
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<td>N</td>
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<td></td>
<td>Wland1</td>
<td>Strawberry</td>
<td>S</td>
<td>I</td>
<td>LR</td>
<td>R</td>
</tr>
</tbody>
</table>

<sup>v</sup>Mutation was identified in this study for the first time.

<sup>w</sup>Mutations were previously described (30,90,101,115).

<sup>x</sup>Mutations previously described (8,30,101,114).

<sup>y</sup>SS = highly sensitive; S = sensitive; LR = low-resistant; MR = moderate-resistant; R = resistant.

Fludioxonil sensitivities were assessed according to Weber and Hahn (142) and tolnaftate sensitivities according to Kretschmer et al. (82).

<sup>z</sup>Sensitivity to fludioxonil was previously reported for isolate Wland1 (50) and all other isolates (51) except for 12-402 and 12-104.
Table 3.3. Fitness components for *Botrytis cinerea* isolates with different sensitivity to iprodione.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Iprodione</th>
<th>Fludioxonil</th>
<th>Mycelial growth (Ø in cm)</th>
<th>Spores x 10⁴ per ml</th>
<th>Lesion size (Ø in cm)</th>
<th>Sporulating lesions (%)</th>
<th>Rovral</th>
<th>Scholar</th>
<th>Rovral</th>
<th>Scholar</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIK2</td>
<td>SS</td>
<td>S</td>
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Numbers 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 Student’s t test.

Differentiation between isolates that are S and MR to fludioxonil was based on a spore germination assay using fludioxonil at 0.1 an 10 mg/liter; differentiation between iprodione SS, S, LR, MR, and HR isolates was based on the same assay with iprodione at 5 and 50 mg/liter (142).

$dai =$ days after inoculation.
**Fig. 3.1.** Analysis of *bos1* expression in germlings with and without exposure to iprodione prior to RNA extraction. The values were normalized with housekeeping gene (beta)-actin and indicate fold-expression relative to the expression levels of the lowest expressor (isolate CBa3). White bars indicate expression without iprodione exposure, gray bars with iprodione exposure. Mean values and standard deviations were calculated from two biological replicates.
This study shows that both fenhexamid and iprodione resistance are widespread in the southeastern United States, but often accounted for only a small percentage of the local populations. Fenhexamid resistance was based on one of four different target site mutations in the 3-ketoreductase of the ergosterol biosynthesis pathway. Two of the mutations, T63I and F412C, were described for the first time from field isolates in this study. A rapid polymerase chain reaction was developed to quickly detect the four mutations found in the Carolinas. This can help to monitor fenhexamid resistant genotypes in future research. Interestingly, in the event of iprodione resistance, only low resistant and moderately resistant phenotypes were associated with target gene mutations, indicating that the highly resistant phenotype in field strains is caused by a different mechanism. Four of the six isolates highly resistant to iprodione were also resistant to tolnaftate, which could be an indication of multidrug efflux pump activity. The only described multi-drug resistance that confers resistance to iprodione is MDR type 2, which is based on overexpression of the gene \textit{mfsM2}. However, investigation of the \textit{mfsM2} expression showed no significant differences between sensitive and resistant isolates (Xingpeng Li, unpublished data). In addition to point mutations in the target gene \textit{bosI}, these results suggest that a yet unknown drug efflux pump activity, or potentially a different mechanism of resistance, may be contributing to iprodione resistance. Further research is necessary to unravel and describe the molecular basis of the highly resistant
phenotype to iprodione. Isolates displaying iprodione resistance in vitro also caused gray mold disease on detached fruit treated with the recommended label rates of Rovral 4 Flowable, while isolates resistant to fenhexamid in vitro caused gray mold disease on detached fruit treated with the recommended label rates Elevate 50 WDG. This implies that the resistant strains detected may be capable of causing resistance despite fungicide applications and demonstrates the significance of resistance monitoring in commercial strawberry fields. Growers need to implement resistance management strategies such as the rotation or mixture of chemical classes for which resistance has not been detected. This will help avoid control failure and help protect and maintain the effectiveness of fungicides.

Future efforts should be focused on long-term monitoring for resistance to all fungicides registered for gray mold control. The information gained would help detect the emergence of new resistant phenotypes and genotypes, and would eventually aid in determining the dynamics of resistance in strawberry fields under fungicide pressure. Long term monitoring will also address questions concerning the spread and stability of resistance, the influence of fitness on resistant isolates, and the evolution of fungicide resistance in local populations over time. This knowledge would help to ultimately establish a sustainable gray mold control program for strawberry growers in the United States. Such a program could possibly reduce the number of fungicidal applications per season, which reduces costs for growers and minimizes the impact on consumer, farm workers, and the environment.
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