ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF *ALTERNARIA* SPP., *BOTRYTIS* SPP., AND *NEOPESTALOTIOPSIS* SPP. ISOLATES ASSOCIATED WITH FRUIT ROTS ON BLUEBERRIES IN GEORGIA

by

MIRZA ASHIKUL BEG

(Under the Direction of JONATHAN E. OLIVER)

ABSTRACT

Georgia is one of the largest producers of blueberries in the United States, but the hot, humid climate provides ideal conditions for disease development. Among other disease issues faced by growers, postharvest diseases caused by different fungal groups are of great concern. The main aim of this research was to isolate, identify and characterize the pathogens associated with postharvest diseases in blueberries. Characterization involved in vitro fungicide sensitivity assessment of the *Alternaria* spp. and *Botrytis cinerea* populations and comparative virulence assessment of the emerging pathogen *Neopestalotiopsis* spp. on blueberries and strawberries. A mycelial growth inhibition assay was utilized to calculate $EC_{50}$ values for *Alternaria* spp. and *B. cinerea* against fungicides commonly utilized in the blueberry production system in Georgia. Among the 46 *Alternaria* spp. isolates tested, all were sensitive to metconazole, fluazinam, fludioxonil, and cyprodinil; however, 21 and 10 isolates, respectively, were identified with resistance to boscalid and pyraclostrobin. In addition, 12 isolates with reduced sensitivity to pydiflumetofen were detected. For *B. cinerea*, all 60 tested isolates were found to be sensitive to fludioxonil, boscalid, and pyraclostrobin based on the determined $EC_{50}$ values; however, 49 and

58 of the isolates, for boscalid and pyraclostrobin, respectively, exhibited minimum inhibitory concentrations exceeding 100 ppm. Although, no highly resistant isolates were identified for fenhexamid and cyprodinil, 12 isolates with moderate resistance to fenhexamid were identified, and one weakly resistant, six low-resistant, and 3 moderately resistant isolates were identified for cyprodinil. Moreover, a significant number of *Neopestalotiopsis* spp. isolates were also recovered from rotting blueberries, among which a small subset was found to be phylogenetically identical to the emerging novel *Neopestalotiopsis* sp. causing disease epidemics in strawberries in the Eastern U.S. Virulence assessments showed that these novel *Neopestalotiopsis* sp. isolates from blueberry are as virulent as *Neopestalotiopsis rosae* isolates in blueberry, relatively more virulent than *N. rosae* in strawberry, but not as virulent on strawberry as a novel *Neopestalotiopsis* sp. isolate from strawberry. Taken together, all of these findings provide valuable information regarding fungal fruit rots of blueberry in Georgia and should aid in the management of these important pathogens.

INDEX WORDS: Blueberry, postharvest disease, *Botrytis cinerea*, *Alternaria* spp., Novel *Neopestalotiopsis* spp., fungicide resistance, $EC_{50}$, virulence, cross-host infection, strawberry, phylogenetic identification

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MIRZA ASHIKUL BEG

B.S., University of Dhaka, Dhaka 1000, Bangladesh, 2018

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MIRZA ASHIKUL BEG

| | |
|---|---|
| Major Professor: | Jonathan Oliver |
| Committee: | Albert Culbreath |
| | Phillip Brannen |
| | Timothy Brenneman |

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2025

# DEDICATION

I dedicate this work to all the oppressed and deprived people around the world.

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I sincerely thank my family — my father, brother, sister-in-law, Banna, Russell bhai, Tahmid, Rusha, Zayan — and all my friends, especially those from Kushtia Zilla School, Dhaka University, and GEB-15. Their encouragement and prayers helped me persevere during the hardest times.

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Earning a PhD is not the end, but the beginning of lifelong learning. I pray for the strength and humility to keep growing, exploring, and serving through knowledge.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

**Blueberry Production in U.S and Worldwide.** Blueberry is a very popular fruit in the United States that has a high demand not only because of its taste but also due to the health benefits that it provides. High levels of antioxidants help improve cognitive performance and reduce the risk of cardiovascular disease and aging-related damage (Hein et al. 2019; Wood et al. 2019). This beneficial fruit has a large impact on the national and global economy. Being the largest producer of blueberries in the world, the United States plays an important role in the global blueberry market. According to the USDA, U.S. production has averaged approximately 300,000 metric tons since 2015, accounting for 36% of global production (USDA-FAS 2021). In 2021, the U.S. exported $135 million in fresh and $75 million in frozen blueberries (USDA 2022), and the U.S. states of California, Florida, Georgia, Michigan, New Jersey, Oregon, Washington are the largest producers of blueberries. Globally, blueberry production increased from 419,050 metric tons to nearly 1,934,400 metric tons (IBO 2022) between 2009 to 2021, and production is forecast to reach 3,000,000 metric tons by 2025 (IBO 2022).

**Types of Blueberries.** There are five major types of blueberries grown in the United States: lowbush, northern highbush, southern highbush, rabbiteye, and half-high. Of these, northern highbush (*Vaccinium corymbosum*) blueberry varieties are the most common types of cultivated blueberries worldwide. These 1.5 to 2.7 meters tall blueberry varieties are native to much of the eastern and northeastern United States. Rabbiteye blueberries (*Vaccinium virgatum*) are native to

the southeastern United States and can tolerate long, hot summers (Finn et al. 2014). Southern highbush blueberries are complex hybrids of *V. corymbosum*, *V. virgatum*, and *Vaccinium darrowii* (Nishiyama et al. 2021). This type was developed to allow blueberry production in low-chill areas. Lowbush blueberries (*Vaccinium angustifolium*) are low-growing shrubs that hardly grow taller than 0.6 meters (Hepler and Yarborough 1991). These are native to the northeastern U.S. and Canada. Half-high blueberries can be grown in the colder areas such as eastern Washington, northeastern Oregon, and Idaho since they can tolerate temperature from -38°C to -43°C. These blueberries were developed by crossing northern highbush and lowbush blueberries (Finn et al. 1990).

In Georgia, three types of blueberries are grown: rabbiteye, southern highbush, and northern highbush. As mentioned, rabbiteye is native to the southeastern U.S. including Georgia. Being native, this type of blueberry has more inherent disease resistance against diseases present in this region (Polashock et al. 2017). Accordingly, rabbiteye blueberries are the most suitable and easiest to grow in Georgia, and once established, rabbiteye bushes can be productive for decades. Unfortunately, a major market disadvantage with rabbiteye is that they ripen later in the season than the southern highbush and the northern highbush. In this regard, southern highbush are more profitable, since they ripen earlier and thereby cover the early demand window. Fruit that reach market earlier in the season typically give farmers better prices, and this has made southern highbush cultivation more popular in Georgia in recent years. Though the low winter chilling requirement of southern highbush blueberries makes them suitable for Georgia, their increased susceptibility to disease can make them more difficult to grow than rabbiteye (Baptista et al. 2004). Accordingly, the productive lifespan of a southern highbush planting may be only 10-15 years. Since it is not as well-adapted to the hotter parts of the state (due to its higher winter chilling hour

requirement and propensity to experience heat stress), northern highbush is not cultivated as extensively as rabbiteye and southern highbush in Georgia. It is primarily cultivated in the northern part of the state.

According to the 2022 USDA report, Georgia ranks first in the U.S. in harvested acres, accounting for 8300 hectares (NASS 2022). Georgia also has a relatively high yield of 4,700 kilograms per hectares. Blueberries have a farmgate value worth around $300 million which accounts for nearly 42% of the total value fruits and nuts produced in Georgia (UGA 2021). Bacon County tops blueberry production in Georgia and the city of Alma boasts itself to be "Georgia's blueberry capital". Over 2800 hectares of land were utilized to produce blueberries in Bacon County, with a farmgate value of around 70 million dollars in this county (UGA 2021). Other Georgia counties that also have significant blueberry production include Appling, Atkinson, Brantley, Clinch, Coffee, Pierce, and Ware. All told, blueberry production is crucial to Georgia's agricultural economy.

**Diseases Affecting Blueberry Production.** Nematodes, fungi, oomycetes, bacteria, and viruses all cause disease on blueberry. Blueberry production is severely affected by the diseases caused by these pathogens. Of these pathogen groups, fungi pose the greatest threat to production and are associated with the greatest losses annually. A significant fungal disease of blueberry called mummy berry is caused by the fungus *Monilinia vaccinii-corymbosi*. This disease, which occurs more frequently in cool, rainy weather, can result in yield reductions of up to 80% under the right conditions (Alvarez Osorio et al. 2022). Botryosphaeria stem canker, caused by *Botryosphaeria corticis* (Phillips et al. 2006) is another serious disease of blueberry in the southeastern United States, while Botryosphaeria stem blight, caused by several fungal species in the family Botryosphaeriacea (Milholland 1972), is also prevalent and destructive in southeastern

Georgia. Some fungi are responsible for causing leaf spots as well as preharvest and postharvest fruit rots on blueberry. *Alternaria* species can cause both a leaf spot and a major postharvest fruit rot on blueberry (Wright et al. 2007; Zhu and Xiao 2015). Circular-to-irregular, light brown-to-gray leaf spots are typical symptoms of blueberry infection with this pathogen. Blight can also develop in some cases. *Colletotrichum acutatum* (Verma et al. 2006) and *C. gloeosporioides* (Hartung et al. 1981) are recognized as the causative agents of anthracnose fruit rot in blueberry. Anthracnose fruit rot is a severe postharvest disease that can cause up to 100 percent postharvest losses (Sharma and Kulshrestha 2015). However, *C. gloeosporioides* and *C. acutatum* have also been found to cause shoot blight and leaf spot in addition to fruit rots in blueberry (Xu et al. 2013; Yoshida and Tsukiboshi 2002). These pathogens cause necrotic lesions and spots on both shoots and leaves, and flower buds die on the blighted shoots. However, shoot blight and leaf spots are not typically as severe as the fruit rots, since fruit are more susceptible to these pathogens. Blueberries and many other flowering plants are also susceptible to the widespread disease Botrytis blight, which is more prevalent during extended periods of high humidity (Elad et al. 2007). Botrytis blight, also known as blueberry blossom blight, is caused by the fungus *Botrytis cinerea* (Abbey et al. 2018). Under favorable conditions, this fungus can infect blossoms, twigs, and fruit. The fruit rot of blueberry caused by the same pathogen is called gray mold.

Bacterial leaf scorch, caused by *Xylella fastidiosa*, has the potential to cause major damage to southern highbush blueberries in the southeastern United States (Chang et al. 2009). Other diseases caused by bacteria such as bacterial canker or crown gall are relatively minor diseases in blueberry. Another very common disease in blueberry is root rot caused by the oomycete *Phytophthora cinnamomi.* This disease occurs in poorly drained sites or in low areas (de Silva et al. 1999) where it causes severe dieback or plant death, resulting in large yield losses.

**Postharvest Fruit Rots in Blueberry.** With a perfect combination of virulent pathogens, susceptible blueberry cultivars, and suitable environmental conditions, any part of a blueberry plant can be affected by disease. However, the small size and soft outer skin of blueberry fruit make them especially vulnerable to diseases. Several studies have shown that postharvest degradation of rabbiteye and southern highbush blueberries is a significant barrier to blueberry production (Romero et al. 2004). Postharvest fruit losses typically vary from 10 to 40% (Abugoch et al. 2016). The primary postharvest fruit rots of blueberries are caused by fungi, and gray mold (*Botrytis cinerea*), Alternaria rot (*Alternaria* spp.), and anthracnose (*Colletotrichum* spp.) are the major contributors. Although these are the most common fungal pathogens causing blueberry fruit decay, other pathogens including *Aureobasidium pullulans* (yeast rot), *Aspergillus* spp. (Aspergillus rot), *Epicoccum nigrum* (Epicoccum rot), *Rhizopus stolonifera* (Rhizopus rot), *Trichoderma* spp. (white mold), *Phomopsis vaccinii* (Phomopsis rot), and *Pestalotia vaccinii* (Pestalotia rot) are also infrequently associated with blueberry fruit rots (Wharton and Schilder 2003). In some cases, the role of these fungi as primary causal agents for fruit rots on blueberry is questionable; nonetheless, they may cause secondary rots when blueberry fruit is vulnerable – following a primary infection by another fruit rotting organism such as *Botrytis* spp., *Colletotrichum* spp., or *Alternaria* spp. for example.

**Botrytis Blossom Blight and Fruit Rot.** The necrotrophic fungus *B. cinerea* causes gray mold disease in several plant species, including blueberry. *B. cinerea* infection results in blossom blight during the bloom period and fruit rot during postharvest handling and storage. This fungus attacks blueberry fruit year-round at temperatures between 15 and 25°C and humidity levels greater than 95%. *Botrytis* infections that result in postharvest decay often happen in the field and can remain latent until storage, when *B. cinerea* can emerge from rotten fruit and degrade nearby

healthy fruit, severely damaging the product and occasionally destroying entire lots. Gray fuzzy mycelium is characteristic of the gray mold caused by this pathogen (**Figure 1.1**). Hyaline conidia (asexual spores) are widely distributed on *B. cinerea* conidiophores, which resemble branching trees. In older cultures, the fungus also creates extremely resilient sclerotia as a form of survival. In the field, this fungus overwinters as intact mycelia or sclerotia, both of which sprout conidiophores in the spring. *Botrytis* conidia are spread by the wind and rain, resulting in new infections. In the summer, *B. cinerea* goes through an asexual cycle. The gray mold fungus prefers a low pH to function efficiently. By exuding organic acids like oxalic acid, *Botrytis cinerea* can acidify its surroundings. Cell wall degrading enzymes (CWDEs) are increased, plant-protection enzymes are suppressed, stomatal closure is unregulated, and pH signaling is mediated to facilitate its pathogenesis when the environment is made more acidic. The most critical period for infection is during bloom. Twig blight, where infection progresses from flower clusters into the main stem, can also occur.

**Alternaria Fruit Rot and Leaf Spot.** Another group of fungi that has devastating consequences for blueberry is *Alternaria* spp. These fungi are saprophytic in nature and can cause leaf spot and fruit rot. *Alternaria* spp. isolates found in rotting blueberry fruit differ greatly from one another, suggesting the involvement of more than one species. *Alternaria alternata, A. tenuissima, A. arborescens* have been reported to cause fruit rots on blueberry (Zhu and Xiao 2015). *Alternaria* spp. can enter the fruit through natural holes, wounds, or directly through a rupture in the host's cuticle. The stem scar present after harvest provides an easy way for the pathogen to get inside the fruit tissue. Dark and depressed lesions on the fruit, white to greenish-gray mycelium development, and green olive conidial growth are all signs of fruit putrefaction caused by this pathogen (**Figure 1.2**). When the temperature is between 20°C and 30°C, *Alternaria*

spp. mycelium can grow at the rate of 9.1 mm per day. The incubation period for colonies of this fungus is between 3 and 5 days, and the colonies are often dark green in color. Conidia have an oval shape, transverse/vertical walls, and, depending on the species, range in size from 13 to 15 μm. Alternaria leaf spot (caused by *Alternaria tenuissima*) is most prevalent during the spring season, which is when spore production is at its peak. Typically, only the bottom leaves are impacted by Alternaria leaf spot; however, there are rare occasions where a severe infection causes the plant to completely defoliate. Leaf symptoms are brown to gray lesions with a red border that might be round or irregular in appearance. Long stretches of excessive humidity encourage the development of disease. Growth of the fungus on blueberry leaves in early spring can lead to significant harm to fruit after harvest due to its significance as a postharvest fruit rot organism.

**Blueberry Postharvest Disease Management.** Blueberry fruit rots can be of two types – one that develops in the field (preharvest) and another that does so after harvest (postharvest). If the picking of ripe fruit is delayed, decaying fruit is typically visible in the field. Fungicides by themselves are insufficient for control of postharvest fruit rots, and correct harvesting and handling practices are crucial. Postharvest losses can be minimized by following certain practices. When picking fruit mechanically, as opposed to by hand, a higher percentage of fruits are destroyed. Losses are also compounded due to the short shelf-life of blueberries. Blueberries typically keep well at room temperature for two to three days, but in a 0-4°C cold storage system, freshly harvested blueberries can last for 1 to 3 weeks. Harvesting during the cooler part of the day and storing as early as possible in cooler temperatures can reduce postharvest losses to a significant extent; therefore, it is critical to cool fruit after harvest with forced-air or partial-vacuum systems that use fans to draw near-freezing air through the pallets of harvested fruit. Pre- and postharvest rots can also be considerably reduced by frequently harvesting all ripe fruit from the bush at each

harvest. Nonetheless, despite doing everything properly, fungus infections may still occur and result in losses of up to 40% in severe cases. Typically, pathogens are carried with the fruit from the field and develop in berries during processing and storage. Therefore, reducing fungal infection in the field (and not allowing fungus-infected berries to be packed with healthy fruit) is essential. To reduce field infections, growers are advised to apply effective fungicides several times during the season beginning at bloom and continuing until just before harvest. In typical southeastern U.S. production systems, fungicides are applied targeting the three major fruit rots in blueberry – anthracnose rot caused by *Colletotrichum* spp., Alternaria rot caused by *Alternaria* spp., and gray mold caused by *Botrytis* spp. (Sial et al. 2025). Two registered fungicide products – trade names Switch® and Pristine® - are rated as excellent against all three of these fruit rots (**Table 1.1**). Switch contains two fungicides, cyprodinil and fludioxonil, with FRAC codes of 9 and 12, respectively. Pristine also contains two fungicides, pyraclostrobin and boscalid, with FRAC codes 11 and 7, respectively. Generally, DMI fungicides (FRAC code 3) are not recommended against *Botrytis*, however, another fungicide, fenhexamid (FRAC code 17), is effective against *Botrytis*, although it is advised to be tank mixed with captan for optimum efficacy. Among strobilurins or QoI fungicides, azoxystrobin and pyraclostrobin are used most often to deal with ripe rot and Alternaria fruit rot, but due to their usage through numerous growing seasons, the potential for resistance development to these fungicides in target fungal species is very high. To avoid resistance development, combinations of QoI fungicides, including azoxystrobin + propiconazole (Quilt Xcel®) and pyraclostrobin + boscalid (Pristine) are often utilized. Quilt Xcel, which is also very effective against mummy berry and Phomopsis, is a relatively new fungicide with broad-spectrum activity. The only DMI fungicide that has excellent efficacy against anthracnose ripe rot and Alternaria fruit rot is metconazole. Fenhexamid is not recommended for use alone against ripe rot

or Alternaria rot, but it can have a fairly good efficacy when applied with captan. Fluazinam (trade name Omega®; FRAC code 29) also has good efficacy against blueberry fruit rots.

**Fungicide Resistance.** In agriculture, fungicide resistance is a major concern. Fungicide resistance is the natural inheritable adjustment in the ability of fungi in a population to survive a fungicide application that would normally provide effective control. With more and more fungi in different crop populations becoming resistant, there is a risk of catastrophic effects on agricultural productivity. The pathogens that cause blueberry fruit rots have already been found to be resistant to some fungicides in different parts of the U.S. In a recent publication, sensitivity profiles of *A. alternata* isolates from blueberry fields to quinone outside inhibitors (QoIs), boscalid, fluopyram, fludioxonil, cyprodinil, and polyoxin D in California were examined (Wang et al. 2022). Out of 143 examined isolates, all (100%) were considered resistant to boscalid and sensitive to fludioxonil and cyprodinil while 32 (22%), 69 (48%), and 42 (29%) isolates were sensitive, low resistant, and resistant to fluopyram, respectively. In addition, 60 out of 143 isolates (42%) were found to be QoI resistant. Fungicide resistance in *C. gloeosporioides*, the pathogen responsible for anthracnose rot, has already been documented in blueberry in Georgia (Ali et al. 2019), with resistance to pyraclostrobin, boscalid, and thiophanate-methyl identified. Resistance in the gray mold-causing pathogen *Botrytis* spp. from blueberry has also been reported in other states including California, Florida, and Washington (Amiri et al. 2018; Saito et al. 2016). In a study conducted in central Florida, 5%, 15%, 24%, 28%, 54%, and 93% of the 432 isolates collected from southern highbush blueberries grown near the vicinity of strawberry fields were resistant to penthiopyrad, cyprodinil, boscalid, fenhexamid, pyraclostrobin, and thiophanate-methyl, respectively (Amiri et al. 2018). In another study, sensitivities to boscalid, cyprodinil, fenhexamid, fludioxonil, and pyraclostrobin, representing five different fungicide classes, were also examined for 249 (California) and 106

(Washington) *B. cinerea* isolates recovered from decayed blueberry fruit or flowers. In California and Washington, 66% and 49% of the isolates were resistant to boscalid, 20% and 29% were moderately resistant to cyprodinil, 29% and 29% were resistant to fenhexamid, and 66% and 55% were found to be resistant to pyraclostrobin. All isolates from California were sensitive to fludioxonil, whereas 70% of the isolates from Washington were shown to have reduced sensitivity to fludioxonil (Saito et al. 2016). Since the number of available fungicides is limited, this ongoing resistance problem is a major concern. Without appropriate resistance management, the number of fungicides currently available to growers will continue to decline, resulting in lower fruit yields and lower fruit quality. In addition, without information regarding fungicide resistance, it is possible that Georgia blueberry growers are making unneeded (and expensive) applications of ineffective fungicides. Therefore, it is urgent that fungicide resistance profiling be conducted for the pathogens causing fruit rots on blueberry in Georgia.

**Emerging Diseases Caused by *Neopestalotiopsis* sp. in Small Fruits**. Isolates of *Neopestalotiopsis* and *Pestalotiopsis* are routinely isolated from blueberry plant samples, including rotting fruit, in the southeastern U.S.; however, it has largely been assumed that these fungi (which spread and overgrow culture media quickly) are secondary opportunistic organisms rather than the primary causes of any disease issues on blueberry (Phil Harmon, personal communication). *Neopestalotiopsis* spp. and *Pestalotiopsis* spp. are not known to cause fruit rots or postharvest disease on blueberry, and only *Neopestalotiopsis clavispora* has been reported to cause twig dieback on highbush blueberries in different parts of the world (Borrero et al. 2018; Jevremovic et al. 2022; Lee et al. 2019; Zheng et al. 2023). In recent years, however, a newly identified species of *Neopestalotiopsis* has emerged in the southeastern U.S. as a major pathogen on another small fruit – strawberries – where it causes a fruit rot as well as a root and crown rot

(Baggio et al. 2021; Rebollar-Alviter et al. 2020). In Florida, during the 2019-2020 growing season, there was a severe disease outbreak that affected 18 commercial strawberry fields (Baggio et al. 2021). In total, an estimated 80 ha of strawberry fields were destroyed resulting in significant economic losses. The pathogen involved was determined to be a possible new *Neopestalotiopsis* sp. that is similar to, but phylogenetically distinct from, *Neopestalotiosis rosae*. Pathogenicity tests with isolates of this pathogen showed that the new species is much more aggressive than *N. rosae* and causes disease on strawberry fruit, leaves, and roots and crowns. To differentiate this new *Neopestalotiopsis* sp. from previously characterized species, phylogenetic analysis of the internal transcribed spacer (ITS), β-tubulin (β-tub), and transcription elongation factor EF-1 alpha (tef1) regions are necessary. In particular, analysis of β-tub plays a crucial role and a mutation at position 318 has been identified which appears to be unique to this aggressive pathogen species. Accordingly, a molecular tool has been developed to detect this specific mutation and is currently being utilized in diagnostic efforts to separate this pathogen from other *Neopestalotiopsis* spp. (Kaur et al. 2023).

Since its initial identification on strawberries in Florida, this emerging *Neopestalotiopsis* sp. has been found on strawberries in multiple states including Georgia, Indiana, North Carolina, Pennsylvania, and Texas (Baggio and Peres 2021) as well as in Canada and Mexico (Goldenhar and Pate 2021; Rebollar-Alviter et al. 2020). Investigative work has indicated that this disease can be spread on transplants and that this pathogen may have been originally disseminated on nursery plants to strawberry fields across the Eastern U.S. and Canada (Baggio and Peres 2021). Other potential hosts or reservoirs of this pathogen are currently being investigated, and unpublished (and unverified) research results by Florida Ag Research (Sances et al. 2020) suggest that this pathogen may also be able to infect blueberry and various other cultivated and wild hosts. Given

the importance of blueberries to Georgia's economy, the close proximity of strawberry fields and blueberry fields in some locations in Georgia, and the frequency with which *Neopestalotiopsis* spp. are isolated from rotting blueberry fruit, additional research efforts in this area are needed.

**Postharvest Pathogens Causing Diseases in Blueberries in GA.** As discussed above, fruit rots in blueberry are a major problem in blueberry production. Moreover, some of the fruit rotting pathogens are also capable of causing postharvest diseases that make storage and shipment difficult. However, there are no recent studies available on fruit rots or postharvest diseases on blueberry in Georgia. As a result, it is unclear which pathogens are most likely causing fruit rots on blueberry in Georgia, making fruit rot management more difficult. Accordingly, we initially conducted a survey to get a clear picture of the fruit rots on blueberry in Georgia. To accomplish this, we collected samples from 6 major blueberry producing counties (Appling, Bacon, Brantley, Clinch, Pierce, Ware) in Georgia. Samples were collected from 46 plantings in these six counties. Sites were chosen based on their past history of fruit rot or current season rot issues. From sampled rotten fruit, the pathogens causing fruit rots were isolated on PDA (Potato Dextrose Agar) medium. After that, their morphological characteristics were evaluated for identification. Molecular identification was also carried out by sequencing the fungal ITS1 and ITS2 regions of the fungal *rRNA* gene with the ITS1/ITS4 primer set.

In total, 836 fungal isolates were collected from six major blueberry producing counties in Georgia (**Table 1.2**). As expected, three of the most abundant and prevalent fungi were the pathogens known to be associated with three common fruit rots of blueberry: Botrytis gray mold, anthracnose rot, and Alternaria fruit rot. *B. cinerea* isolates accounted for 19% (n=163) of the collected fungal isolates. *Colletotrichum* spp., cause of anthracnose rot, were second in abundance at 132 isolates (16%). We also found a significant number of *Alternaria* isolates in our study.

Surprisingly, there was also a large number of *Neopestalotiopsis* spp. and *Aureobasidium* spp. identified from the rotten blueberry samples. Though these organisms are yet to be established as primary causal agents for fruit rots in blueberries, they are frequently found in blueberries as secondary organisms when there is already infection established.

## Justification and Objectives

**Fungicide Sensitivity of *Alternaria* spp. isolates.** Alternaria fruit rot is a major cause of fruit loss in blueberry. Different *Alternaria* species are responsible for this disease, contributing significantly to both preharvest and postharvest losses. Based on Bollenbacher (2023), 7% of the collected isolates were identified as *Alternaria* spp. To identify these *Alternaria* spp. at the species level, a phylogenetic study based on multilocus sequence analysis (MLSA) is essential. As previously mentioned, fungicide resistance in *Alternaria* spp. from blueberries has already been reported in different regions of Georgia. Therefore, it is critical to assess the current fungicide sensitivity status of the fungicides commonly used by growers in Georgia to manage Alternaria fruit rot.

Thus, my first objective is:

1) Screen collected isolates of *Alternaria* spp. for resistance to commonly used fungicides and investigate underlying resistance mechanisms. (Chapter 2)
    a. Identify species of collected isolates and confirm pathogenicity.
    b. Determine $EC_{50}$ values of *Alternaria* spp. isolates for tested fungicides.
    c. Characterize genetic determinants of fungicide resistance in resistant isolates.

**Fungicide Sensitivity of *Botrytis cinerea* isolates.** *Botrytis* spp. are major pathogens causing fruit rots in blueberry. Due to their high sporulation capacity, they can spread rapidly and

are a significant cause of postharvest losses. Their ability to infect fruit both before and after harvest makes them particularly devastating for growers. In our fruit rot survey, we found 19% of the isolates to be *Botrytis* spp. Fungicide resistance in *Botrytis* spp. has become an increasing concern, threatening the effectiveness of commonly used management strategies. Therefore, it is crucial to evaluate the current fungicide sensitivity status of *Botrytis* spp. isolates associated with blueberry fruit rot in Georgia and characterize the genetic determinants of fungicide resistance in resistant isolates.

Thus, the second objective is:

2) Screen collected isolates of *Botrytis* spp. for resistance to commonly used fungicides and investigate underlying resistance mechanisms. (Chapter 3)
    a. Identify species of collected isolates and confirm pathogenicity.
    b. Determine $EC_{50}$ values of *Botrytis* spp. isolates for tested fungicides.
    c. Characterize genetic determinants of fungicide resistance in resistant isolates by mutational and differential expression analyses.

**Characterization of *Neopestalotiopsis* spp. Identified from Blueberries.** As part of our fruit rot survey efforts, we isolated a significant number of *Neopestalotiopsis* spp. from rotting blueberry fruit. This finding drew our attention to this fungus, and the initial assessment of some of these isolates identified at least four isolates from 2021 and six isolates from 2022 that were identical within the ITS, *β-tub*, and *tef1* regions to published sequences from the new aggressive *Neopestalotiopsis* sp. from strawberry in Florida. In particular, these isolates possessed the (supposedly) unique mutation within β-tub currently being used to differentiate the new, aggressive *Neopestalotiopsis* sp. from other species (Kaur et al. 2023). As such, these isolates from blueberry fruit appear to belong to the new aggressive species causing disease on strawberry.

This interesting finding led us to hypothesize: 1. That a sub-population of the isolated *Neopestalotiopsis* spp. from rotting blueberries might be the same population that caused the disease outbreak on strawberries in Florida or has the capability to do it; and 2. As in strawberries, isolates from this *Neopestalotiopsis* sp. might be more aggressive than other *Neopestalotiopsis* spp. and capable of causing disease on blueberries as well.

Accordingly, the final objective of my work is:

3) Characterize *Neopestalotiopsis* spp. isolates identified from rotting blueberries. (Chapter 4 and 5)
    a. Conduct phylogenetic analysis of *Neopestalotiopsis* spp. isolates from blueberry alongside previously characterized isolates from strawberry.
    b. Assess pathogenicity of *Neopestalotiopsis* spp. isolates from blueberry on blueberry and strawberry fruit, leaves, and crown/roots.

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**Table 1.1** List of fungicides that have good to excellent efficacy against different fruit rots according to the 2025 Southeast Regional Blueberry Integrated Management Guide.

| Trade Name | Active Ingredient | Gray Mold | Alternaria Rot | Anthracnose Rot | Type (FRAC Group) |
|---|---|---|---|---|---|
| Abound | azoxystrobin | n.r. | Excellent | Excellent | QoI (11) |
| Quilt Xcel | azoxystrobin<br>propiconazole | n.r. | Excellent | Excellent | QoI (11)<br>DMI (3) |
| Pristine | pyraclostrobin<br>boscalid | Excellent | Excellent | Excellent | QoI (11)<br>SDHI (7) |
| Quash | metconazole | n.r. | Excellent | Excellent | DMI (3) |
| Switch | cyprodinil<br>fludioxonil | Excellent | Excellent | Excellent | Anilopyrimidines (9)<br>Phenylpyrroles (12) |
| Elevate | fenhexamid | Excellent | n.r. | n.r. | Hydroxyanelides (17) |
| Omega | fluazinam | Fair | Good | Good | 2,6-dinitro-anilines (29) |

n.r. = not recommended

## Tables

**Table 1.2.** Fungal isolates collected and identified from blueberries and the prevalence of each genus among sites surveyed in 2021 and 2022 as part of Objective 1.

| **Species** | **Counts (% of All Isolates)** | **Prevalence (% of All Sites)** |
|---|---|---|
| *Botrytis cinerea* | 163 (19%) | 22/46 (48%) |
| *Colletotrichum* spp. | 132 (16%) | 29/46 (63%) |
| *Neopestalotiopsis* spp. | 124 (15%) | 28/46 (61%) |
| *Aureobasidium* spp. | 64 ( 8%) | 17/46 (37%) |
| *Alternaria* spp. | 59 ( 7%) | 23/46 (50%) |
| *Pestalotiopsis* spp. | 58 ( 7%) | 25/46 (54%) |
| *Epicoccum* spp. | 46 ( 6%) | 12/46 (26%) |
| *Cladosporium* spp. | 35 ( 4%) | 13/46 (28%) |
| *Sporidiobolus* spp. | 23 ( 3%) | 8/46 (17%) |
| *Aspergillus* spp. | 22 ( 3%) | 6/46 (13%) |
| *Neofusicoccum* spp. | 19 ( 2%) | 4/46 ( 9%) |
| *Mucor* spp. | 11 ( 1%) | 3/46 ( 7%) |
| *Papiliotrema* spp. | 10 ( 1%) | 4/46 ( 9%) |
| *Penicillium* spp. | 10 ( 1%) | 7/46 (15%) |
| Other identified species | 60 ( 7%) | n.d. |
| **Total** | **836** | **46 sites** |

**Figures**

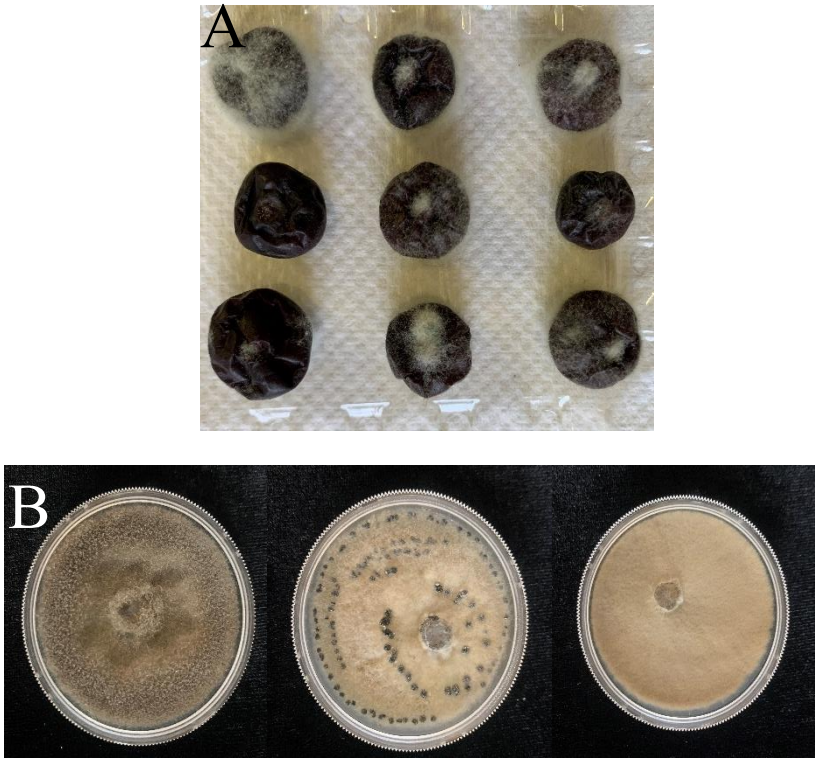


**Figure 1.1.** A) Gray mold on blueberries caused by *Botrytis cinerea.* B) Growth of *B. cinerea* on potato dextrose agar.

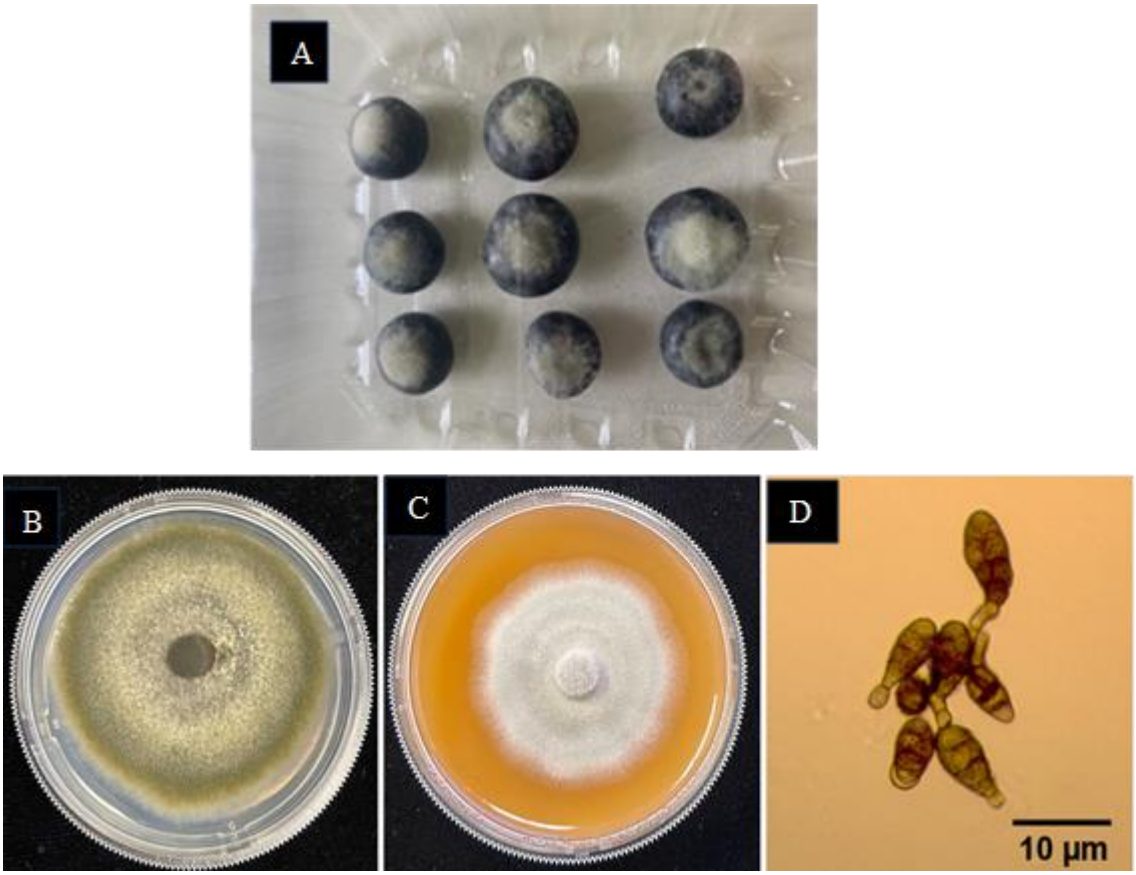


**Figure 1.2**. A) *Alternaria alternata* on blueberries. B) *A. alternata* on potato dextrose agar. C) *A. alternata* on V8 media. D) *A. alternata* conidia.

# CHAPTER 2

## Fungicide resistance profiles of *Alternaria spp.* associated with fruit rot of blueberry in Georgia, USA[1]

[1]Beg, M. A., Aktaruzzaman, M., Lewis, K. J., & Oliver, J. E. (2025). Fungicide resistance profiles of *Alternaria* spp. associated with fruit rot of blueberry in Georgia, USA. *Frontiers in Plant Science*, 16, 1524586. https://doi.org/10.3389/fpls.2025.1524586

**Abstract**

Georgia blueberry growers experience significant losses annually due to fruit rots including Alternaria rot caused by *Alternaria* spp. Fungicide applications from bloom through harvest are typically recommended for management of fruit rots, however fungicide resistance development has the potential to complicate management activities by reducing fungicide efficacy. To evaluate fungicide resistance issues in Georgia, 46 isolates of *Alternaria* spp. from ripe blueberry fruit from four major blueberry-producing counties were collected and identified by morphological and molecular features. The majority of the isolates were *Alternaria alternata* (n=43) but also included *Alternaria tenuissima* (n=1), *Alternaria dumosa* (n=1), and *Alternaria limoniasperae* (n=1). All isolates were assessed for resistance to fungicides which included fludioxonil, fluazinam, metconazole, cyprodinil, pydiflumetofen, boscalid, and pyraclostrobin. For all tested fungicides, with the exception of pyraclostrobin, a mycelial growth inhibition assay was used to determine the $EC_{50}$ values. For pyraclostrobin, a spore germination assay was used. $EC_{50}$ value ranges of *A. alternata* for fludioxonil, fluazinam, cyprodinil, metconazole, pydiflumetofen, boscalid, and pyraclostrobin were 0.037 to 0.234 µg/mL, 0.025 to 0.125 µg/mL, 0.015 to 0.404 µg/mL, 0.125 to 5.729 µg/mL, 0.008 to 1.114 µg/mL, 0.551 to >100 µg/mL, and 0.04 to >100 µg/mL, respectively. These $EC_{50}$ values suggest that all tested *Alternaria* spp. isolates were sensitive to fludioxonil, fluazinam, metconazole, and cyprodinil. However, 12 *Alternaria* spp. isolates showed reduced sensitivity to pydiflumetofen, 21 were resistant to boscalid and 10 were resistant to pyraclostrobin. Among these resistant isolates, 6 were resistant to both of the two latter fungicides. Sequencing portions of the *sdhB*, *sdhC* and *sdhD* genes from boscalid-resistant isolates and the cytochrome b gene from pyraclostrobin-resistant isolates revealed the presence of known resistance mutations in resistant isolates - including H134Q or G79R mutations in the *sdhC* gene or H134R mutations in the *sdhD* gene of some, but not all, boscalid-resistant isolates, and the presence of the G143A

mutation in pyraclostrobin-resistant isolates. Our findings indicate that resistance to boscalid and pyraclostrobin is present in *Alternaria* spp. from Georgia blueberries and suggest that growers utilizing these fungicides in some Georgia locations may experience Alternaria fruit rot control failures.

## Introduction

Blueberry is a very popular fruit in the United States, in high demand not only for its taste but also due to the health benefits that it provides. High levels of antioxidants are considered to help improve cognitive performance and reduce the risk of cardiovascular disease and aging-related damage (Hein et al., 2019; Wood et al., 2019). Globally, blueberry production increased from 419,050 metric tons to nearly 1,934,400 metric tons between 2009 to 2021, and production is forecast to reach 3,000,000 metric tons by 2025 (IBO, 2022). Georgia ranks first in the U.S. with 20,600 harvested acres (NASS, 2022), and blueberries have a farmgate value estimated of $300 million, accounting for over 41% of the total value of fruits and nuts produced in Georgia (UGA, 2021).

The small size and soft outer skin of blueberry fruit make them especially vulnerable to pathogens. Several studies have shown that postharvest degradation of rabbiteye (*Vaccinium virgatum*) and southern highbush (*Vaccinium corymbosum* interspecific hybrids) blueberries is a significant barrier to production (Barrau et al., 2006), and major losses from blueberry fruit rots can occur both in the field and after harvest during postharvest handling and storage (Neugebauer et al., 2024). The primary postharvest fruit rots of blueberries are caused by fungi, with *Botrytis cinerea* (gray mold), *Alternaria* spp. (Alternaria fruit rot), and *Colletotrichum* spp. (anthracnose fruit rot) as the major contributors (Bell et al., 2021; Neugebauer et al., 2024). Though there are many different species of *Alternaria* that cause postharvest diseases in different fruits, *A. alternata, A. tenuissima,* and *A. arborescens* are the most common species that cause Alternaria rot in blueberries (Neugebauer et al., 2024). A survey conducted in California on Alternaria rot on blueberries showed that 62% of the isolates were *A. alternata,* 33% were *A. arborescens* and 5% were *A. tenuissima* (Zhu and Xiao, 2015). These pathogens are very important because they cause

rots not only in blueberries but in many other diverse fruits and vegetables including apple, pepper, mandarin, and pomegranates (Cabral et al., 2016; Luo et al., 2017; Elfar et al., 2018; Wang et al., 2021).

Infection by *Alternaria* spp. can occur as early as bloom, but infections typically remain latent and become apparent when fruit ripens (Neugebauer et al., 2024). Initially the ripe fruit shrivels or flattens. The damaged part later gets covered with a greenish mass of mycelium and spores. The berries may look dry in the field but become soft and watery when stored after harvesting. Fruit are exposed to the pathogen from plant debris in the field or from leaf spots caused by the same pathogen (Troncoso-Rojas and Tiznado-Hernández, 2014). In conventional blueberry production in the U.S., the primary way to reduce *Alternaria* spp. infections is to apply different classes of fungicides starting from bloom through harvest (Neugebauer et al., 2024). Several site-specific fungicides including quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), demethylation inhibitors (DMIs), phenylpyrroles, and anilinopyrimidines (APs) are utilized in Georgia and elsewhere for Alternaria fruit rot control (Sial et al., 2023; Neugebauer et al., 2024).

Researchers recommend these fungicides for use in blueberries because of their efficacy against *Alternaria* spp. However, because these fungicides are used widely in a variety of crops, there is considerable selection pressure that can lead to the development of resistance to these fungicides, and resistance development can be a common issue (Deising et al., 2008). Frequent use of relatively few specific fungicides results in a high selection pressure. The pathogens that cause blueberry fruit rots have already been found to be resistant to some fungicides in different parts of the U.S. In a recent publication (Wang et al., 2022), sensitivity profiles of *A. alternata* isolates from blueberry fields to quinone outside inhibitors (QoIs), boscalid, fluopyram,

fludioxonil, cyprodinil, and polyoxin D in California were examined. Out of 143 isolates, all were considered resistant to boscalid and sensitive to fludioxonil and cyprodinil while 32, 69, and 42 isolates were sensitive, low resistant, and resistant to fluopyram, respectively. In addition, 60 of the 143 isolates were QoI-resistant. Fungicide resistance in *Colletotrichum gloeosporioides*, the pathogen responsible for anthracnose rot, has already been documented in blueberry in Georgia (Ali et al., 2019), with resistance to pyraclostrobin, boscalid, and thiophanate-methyl identified. To maximize efficacy of the fungicide spray program and minimize further resistance development, it is important to know the current fungicide resistance status of the *Alternaria* spp. in blueberries and to monitor any early shifts in the pathogen's sensitivity. An assessment of the resistance status against currently utilized fungicides may provide an opportunity to make changes to the fungicide recommendations to improve management of Alternaria rot. There is no data available on the sensitivity status of the *Alternaria* spp. on blueberries in Georgia. While $EC_{50}$ values are usually the determinant of the sensitivity status of a particular fungicide against a specific pathogen, there are no $EC_{50}$ values from Georgia available for any fungicides against the *Alternaria* spp. causing disease on blueberries. Therefore, in our study, *Alternaria* species associated with blueberry fruit rot in Georgia were isolated and identified with morphological and molecular methods, verified as pathogens via pathogenicity testing, and utilized in fungicide sensitivity assays to determine $EC_{50}$ values for fludioxonil, fluazinam, metconazole, cyprodinil, pydiflumetofen, boscalid, and pyraclostrobin.

## Materials and methods

### Pathogen isolation

For isolation of *Alternaria* spp., blueberries were collected from multiple locations within major blueberry-producing counties in Georgia including Appling, Bacon, Brantley, and Pierce (**Supplementary Figure 2.1**). Fungal isolates were cultured from symptomatic (rotting) berries on acidified ¼ strength potato dextrose agar (AqPDA). Agar was acidified using 184 µl lactic acid (85% w/w) per liter. AqPDA plates were incubated for 2-4 days at room temperature (~23°C) to allow for fungal growth. Once fungal growth was observed, pure culture isolates were obtained by hyphal tip cutting and maintained on AqPDA for 7 additional days. To store isolates, mycelial plugs (4 mm) were cut from the leading edges of fungal colonies and placed in 20% glycerol at 4°C.

### Morphological identification

For morphological identification, isolates were first cultured on potato dextrose agar (PDA) for 3-5 days. Mycelial plugs (4 mm) taken from the edge of each colony were transferred to two 9 cm plastic Petri dishes, one containing V8 agar and the other one containing PDA. Plates were sealed with parafilm and incubated in the dark at 25°C for 7 days. After this, Petri dishes with V8 agar were unsealed and kept at 25°C in 12hr-12hr light-darkness conditions for 2 to 3 additional days. Conidial characteristics were observed from the V8 agar plates under a light microscope at 400X magnification. Photographs of the plates were taken, and conidial lengths and widths were measured.

**Molecular identification**

DNA was extracted from each of the 46 isolates from 7-day-old PDA cultures. The mycelium was scraped off using a sterile loop and placed into a 2-mL microcentrifuge tube containing approximately twenty 2-mm zirconia/silica ceramic beads (Research Products International, Mount Prospect, IL). After grinding the sample by shaking for 30 seconds in a Biospec Mini Beadbeater-8 (BioSpec Products, Bartlesville, OK), DNA was extracted using a CTAB (cetyltrimethylammonium bromide) extraction method (Doyle and Doyle, 1987). The ITS1 and ITS4 primer set (**Table 2.1**) was used to amplify the internal transcribed spacer (ITS) region containing ITS1-5.8S-ITS2 of nuclear ribosomal DNA (rDNA) (White et al., 1990). For further identification of *Alternaria* spp., primer pair ATPDF1 and ATPDR1 (**Table 2.1**) was used to amplify the gene encoding the plasma membrane ATPase (Lawrence et al., 2013). For a subset of isolates, additional primer pairs (**Table 2.1**) were used to amplify sequences of the *Alternaria* major allergen (*Alt a1*), calmodulin (CAL), and the second largest subunit of RNA polymerase II (RPB2) (Hong et al., 2005; Lawrence et al., 2013). For PCR, a total reaction volume of 30 µl was used, and each reaction contained 15 µl 2X PCR Master Mix (Promega, Madison, WI), approximately 200 ng of DNA, and 10 mM of each primer (1 µl each). PCR was performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to the published reaction conditions for each primer set (references in **Table 2.1**). PCR products were visualized in a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium, Fremont, CA) using a Bio-Rad Molecular Image Gel Doc XR+ with Image Lab Software (Bio-Rad Laboratories, Hercules, CA). Amplified PCR products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tec, Inc., Norcross, GA) and Sanger sequenced in both directions by Eurofins Genomics (Louisville, KY). Isolates were initially confirmed as belonging to *Alternaria* spp. by comparison

of obtained ITS sequences with publicly available *Alternaria* spp. sequences in the GenBank database using the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST/).

**Phylogenetic analysis**

For identification of isolates to the species level, a phylogenetic analysis was performed using the plasma membrane ATPase gene sequence for each of these isolates and 56 reference isolates (**Supplementary Table 2.1**) previously classified as belonging to 49 different *Alternaria* spp. (Lawrence et al., 2013; Woudenberg et al., 2015; Zhu and Xiao, 2015; Luo et al., 2018; Elfar et al., 2019; Qian et al., 2022; Elfar et al., 2023; Yan et al., 2024). ATPase sequences were initially aligned with the CLUSTAL X program (Thompson et al., 1997) and further edited in MEGA7 (Kumar et al., 2016). Evolutionary analyses were conducted in MEGA7. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3447)). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

**Pathogenicity testing**

In total, 22 isolates were selected representing all the locations and isolated species of *Alternaria* for pathogenicity confirmation based on Zhu and Xiao (2015) with slight

modifications. Store-bought, firm, ripe organic blueberries were selected for inoculation experiments. Berries were surface sterilized by briefly dipping twice in 70% ethanol, once in 0.5% sodium hypochlorite, and twice in sterile distilled water. Air dried berries were fixed to the bottom of clamshells with double-sided tape with the stem-scar facing up. Spore suspensions were prepared as described in the "Morphological identification" section and standardized to a concentration of $10^5$ spores per milliliter of water after counting the number of spores with a hemocytometer. Each berry was inoculated with 20 µl of spore suspension on the stem scar site. For each isolate, three clamshells which each contained 9 berries (27 berries total) were inoculated. The clamshells were placed in a sealed plastic box at room temperature, and two sterile paper towels soaked with sterile distilled water were placed at the bottom of each box to ensure humid conditions. After 7 days, the berries were visually rated for disease incidence (as the presence of any spores or mycelium) and severity on a scale of 0 to 5 based on Saito et al. (2016) with a slight modification (**Supplemental Figure 2.2**). The Disease Index (DI) was calculated according to Fu et al. (2020). The DI was computed using the formula:

$$\mathbf{DI} = \frac{\sum(\boldsymbol{n} \times \mathbf{corresponding\ DS})}{\boldsymbol{N} \times \mathbf{5}} \times \mathbf{100}$$

where, DI = Disease Index, DS = Disease Severity, n = the number of berries corresponding to each disease rating, N = the total number of berries inoculated. Re-isolation from diseased berries and identification of the obtained isolates was carried out to fulfill Koch's postulates.

**Fungicide sensitivity assessment**

For sensitivity testing, seven technical grade fungicides were used including cyprodinil (purity 99.9%), fludioxonil (99.5%), fluazinam (98.4%), metconazole (98.9%), pydiflumetofen (99.2%), boscalid (97.1%), and pyraclostrobin (98.5%) from Sigma-Aldrich Corp. (St. Louis, MO,

USA) and dissolved in acetone for the preparation of stock solutions (1,000 µg/mL). PDA was amended with each fungicide to final concentrations ranging from 0.01 to 100 µg/mL (**Table 2.2**) alongside non-amended control plates. These concentrations were used to ensure a fungal growth inhibition range from only slightly to almost complete inhibition. Fungicide sensitivity tests were repeated two times for each of the 46 isolates and each test consisted of two Petri plates with each concentration of each fungicide. Mycelial growth inhibition assays were carried out for each of the fungicides, except pyraclostrobin, for the determination of the $EC_{50}$ values (50% mycelial growth inhibition).

For pyraclostrobin, spore germination inhibition assays were performed. For mycelial growth inhibition assays, mycelial plugs (4 mm in diameter) were removed from the margins of colonies grown on PDA and placed upside-down on the fungicide-amended and fungicide-free PDA media which were incubated at 25±1°C. After 4-5 days, the colony growth of each isolate was measured (the 4 mm diameter of the inoculation plug was subtracted from the colony diameter) and the percent inhibition (PI) values for each fungicide rate was calculated using the formula:

$$\boldsymbol{PI = \frac{a - b}{a} \times 100}$$

where a = colony growth of the control plate, and b = colony growth of the fungicide-amended plate.

The $EC_{50}$ for each isolate was determined based on the percent inhibition on each of the different fungicide concentrations used. Relative growth inhibition was regressed against the $\log_{10}$ fungicide concentration using Statistical Analysis System (SAS Institute Inc., Cary, NC) for calculation of the $EC_{50}$ values. For the pyraclostrobin spore germination inhibition assay, spores from each isolate of *Alternaria* spp. were produced, scraped off with sterile plastic loops,

suspended in 10% tween 20, and adjusted to $10^5$ spores per milliliter using a hematocytometer. Water agar plates were prepared for the four tested concentrations (0.01, 0.1, 1, and 10 μg/μL) of pyraclostrobin. In the control plate, no pyraclostrobin was added. Then, 100 μL of the spore suspension was added and dispersed onto each of these plates. After incubation of these plates at 28°C for 24 hours, germination of 100 randomly selected spores from each plate was observed, counting those germinated and those not germinated. Based on these observations, percent inhibition (PI) values for each of the fungicide rates were calculated using the previous formula where a = number of spores germinated in the control plate, and b = number of spores germinated in the fungicide-amended plate. The $EC_{50}$ values were calculated in the same way as for the mycelial growth inhibition assay using Statistical Analysis System (SAS Institute Inc., Cary, NC).

To examine correlations between $EC_{50}$ values for the two SDHI fungicides examined (boscalid and pydiflumetofen), the Pearson correlation coefficient (R) and the associated p-value were computed using SigmaPlot 16 (Systat Software Inc., San Jose, CA). For pydiflumetofen, since baseline information establishing the thresholds for resistance and reduced sensitivity were not available, the frequency distribution of the $EC_{50}$ values was further subjected to a Shapiro-Wilk test for normality (JMP®, Version 17.2.0. SAS Institute Inc., Cary, NC, 1989–2023) to evaluate for the presence of values that may indicate reduced sensitivity to this fungicide.

**Mutation identification in fungicide-resistant *Alternaria* spp. isolates**

To determine if the fungicide-resistant *Alternaria* spp. isolates possess mutations known to be associated with fungicide resistance, sequencing the fungal *sdhB, sdhC, sdhD,* and *cytB* genes was carried out via PCR with specific primers (**Table 2.1**). A total reaction volume of 20 μl was used, and each reaction contained 10 μl 2X PCR Master Mix (Promega, Madison, WI),

approximately 200 ng of genomic DNA, and 10 mM of each primer (1 µl each). PCR was performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to the previously published reaction conditions for each primer set (Avenot et al., 2008a; Avenot et al., 2009; Vega and Dewdney, 2014). Amplified PCR products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tec, Inc., Norcross, GA) and Sanger sequenced in both directions by Eurofins Genomics (Louisville, KY).

## Results

### Morphological characteristics

A total of 46 *Alternaria* spp. were isolated from rotting berries from 16 commercial blueberry farms in southeastern Georgia (**Supplementary Table 2.2**). Growth characteristics and conidial morphology of these isolates were consistent with those of *A. alternata, A. tenuissima*, *A. dumosa* and *A. limoniasperae* as described by Simmons (1967; 2007). Among these, 43 of 46 isolates were identified as *A. alternata*. These isolates were initially greyish green to olive brown in color on the PDA plates (45–47 mm in 5 days) (MB21-397; **Figure 2.1A**) and whitish green on V8 agar (35–40 mm in 5 days) (data not shown). The conidia were generally ovoid to ellipsoid and ranged from 8.7–21.2 × 7.2–11.3 µm in size (n=30) with one to four transverse and zero to two longitudinal septa per conidium (**Figure 2.1B**). The conidiophores of these isolates were singular, short, and measured 17.9–60.5 × 2.8–6.6 µm in size (n=15) (**Figure 2.1C**). One isolate (MB21-456) was identified as *A. tenuissima* and was characterized by grayish color on PDA (50–52 mm in 5 days) (**Figure 2.1E**) and whitish gray on V8 (45–50 mm in 5 days) (data not shown). The conidia were ovoid with a tapering apical beak and a size of 11.5–31.5 × 5.1–12.7 µm (n=30), with one to five transverse and zero to one longitudinal septa per conidium (**Figure 2.1F**).

Conidiophores 15.6–57.4 × 3.1–6.8 µm (n=15) were arising singly and short (**Figure 2.1G**). Another isolate (MB21-363) was identified as *A. dumosa* and was characterized by brown color on PDA (35–40 mm in 5 days) (**Figure 1I**) and whitish cottony gray on V8 (43–47 mm in 5 days) (data not shown). Conidia were ovoid size of 17.5–41.5 × 4.5–8.4 µm (N=30), with one to seven transverse and zero to one longitudinal septa per conidium (**Figure 2.1J**). The conidiophores of this isolate were singular, long, and measured 37.5–115.3 × 3.0–4.3 µm (n=15) (**Figure 2.1K**). The final isolate (MB21-475) was identified as *A. limoniasperae* and was light brown-green on PDA (43-45 mm in 5 days) (**Figure 2.1M**) and whitish grey on V8 (45–50 mm in 5 days) (data not shown). Conidia were narrow-ellipsoid to ovoid 25.3–45.3× 6.5–8.9 µm (n=30) with one to five transverse and one to two longitudinal septa per conidium (**Figure 2.1N**). The primary conidiophores were large 65–110 × 3–5 µm (n=10), but the secondary conidiophores were short 3–21×2–4 µm (n=10) (**Figure 2.1O**).

**Molecular identification and phylogenetic analysis**

The results of ITS sequencing (Genbank accession numbers OR041698-OR041743) confirmed all 46 isolates as belonging to the genus *Alternaria*. Sequences obtained from other gene regions further confirmed this assessment (Genbank accession numbers OR091105-OR091150 [ATPase], PP662487-PP662508 [*Alt a1*], PP662470-PP662475 [CAL], and PP662476-PP662481 [RPB2]). Phylogenetic analysis of *Alternaria* spp. isolates using the ATPase gene enabled the identification of all isolates to the species level (**Figure 2.2**). Based upon this analysis, all isolates from this study segregated with isolates from section *Alternaria* within the genus *Alternaria*, with the vast majority of isolates from this study forming a single clade with reference isolates of *A. alternata*. In total, 43 isolates from this study were identified as *A. alternata* and the remaining

isolates were classified as *A. tenuissima* (n=1), *A. dumosa* (n=1), and *A. limoniasperae* (n=1) (**Figure 2.2**).

**Pathogenicity testing**

Inoculation of detached blueberry fruit with *Alternaria* isolates yielded lesions of dark brown mycelium growth and rotten berries during the seven days following initial inoculation (**Figure 2.1D, 2.1H, 2.1L, 2.1P**). To fulfill Koch's postulates, re-isolation from diseased berries and identification of the obtained isolates was performed to confirm the presence of *Alternaria* spp. in the rotting berries. Isolates of *A. alternata* and *A. tenuissima* had higher disease indexes and equal or higher disease incidences on blueberry fruits as compared to the isolates of *A. dumosa* and *A. limoniasperae*. The disease indexes and disease incidences (%) ranged from 40.0–82.2 and 88.9-100%, respectively, for the 19 *A. alternata* isolates tested, and were 54.1 and 100%, 19.3 and 85.2%, and 39.3 and 88.9% for the isolates of *A. tenuissima*, *A. dumosa*, and *A. limoniasperae*, respectively (**Table 2.3**).

**Fungicide sensitivity of *Alternaria* spp. isolates**

For fludioxonil, the $EC_{50}$ values for the 43 *A. alternata* isolates ranged from 0.037 to 0.234 µg/mL (**Figure 2.3A**). The mean $EC_{50}$ value for these isolates was 0.124 µg/mL with a standard deviation of 0.043 µg/mL. The $EC_{50}$ values for the other three isolates were 0.199 µg/mL for MB21-363 (*A. dumosa*), 0.158 µg/mL for MB21-456 (*A. tenuissima*), and 0.080 µg/mL for MB21-475 (*A. limoniasperae*). As a whole, the fludioxonil $EC_{50}$ values showed a near-unimodal distribution pattern skewed toward the left (low values) with a few isolates being less sensitive than most other isolates (**Figure 2.4A**).

For fluazinam, the $EC_{50}$ values for the 43 *A. alternata* isolates ranged from 0.025 to 0.125 µg/mL (**Figure 2.3B**). The mean $EC_{50}$ value for this fungicide was 0.065 µg/mL with a standard deviation of 0.025 µg/mL. The $EC_{50}$ values for the other three isolates were 0.053 µg/mL for MB21-363 (*A. dumosa*), 0.038 µg/mL for MB21-456 (*A. tenuissima*), and 0.057 µg/mL for MB21-475 (*A. limoniasperae*). The frequency distribution for this fungicide showed a near-unimodal pattern where most isolates had $EC_{50}$ values less than 0.01 µg/mL (**Figure 2.4B**).

For metconazole, the $EC_{50}$ values of the 43 *A. alternata* isolates ranged from 0.125 to 5.729 µg/mL (**Figure 2.3C**). The mean $EC_{50}$ value of these isolates for this fungicide was 1.153 µg/mL with a standard deviation of 1.194 µg/mL. The $EC_{50}$ values for the other three isolates were 1.914 µg/mL for MB21-363 (*A. dumosa*), 0.945 µg/mL for MB21-456 (*A. tenuissima*), and 0.300 µg/mL for MB21-475 (*A. limoniasperae*). For this fungicide, the frequency distribution of $EC_{50}$ values had a near-unimodal pattern (**Figure 2.4C**).

For cyprodinil, the $EC_{50}$ values of the 43 *A. alternata* isolates ranged from 0.015 to 0.404 µg/mL (**Figure 2.3D**). The mean $EC_{50}$ value was 0.124 µg/mL with a standard deviation of 0.086 µg/mL. $EC_{50}$ values for the other three were 0.404 µg/mL for MB21-363 (*A. dumosa*), 0.033 µg/mL for MB21-456 (*A. tenuissima*), and 0.023 µg/mL for MB21-475 (*A. limoniasperae*). The frequency distribution for this fungicide was unimodal and skewed slightly towards the higher values having a small number of isolates with higher $EC_{50}$ values (**Figure 2.4D**).

For pydiflumetofen, the 43 *A. alternata* isolates had $EC_{50}$ values ranging from 0.008 to 1.114 µg/mL (**Figure 2.5A**). The mean value was 0.131 µg/mL with a standard deviation of 0.238 µg/mL. The $EC_{50}$ values for the other three isolates were 0.026 µg/mL for MB21-363 (*A. dumosa*), 0.023 µg/mL for MB21-456 (*A. tenuissima*), and 0.463 µg/mL for MB21-475 (*A.*

*limoniasperae*). The frequency distribution of the $EC_{50}$ values for this fungicide did not fit a normal distribution. Since only data ranging from 0.008 to 0.067 µg/mL passes the Shapiro-Wilk goodness of fit test where the Shapiro–Wilk test statistic (W) is 0.93 ($\alpha = 0.05$), the $EC_{50}$ values higher than 0.067 were considered to have reduced sensitivity to pydiflumetofen. Based on this parameter, there were 12 isolates (11 *A. alternata* and 1 *A. limoniasperae*) with reduced sensitivity to pydiflumetofen (**Figure 2.6A**).

For boscalid, $EC_{50}$ values were found to vary greatly among the 43 *A. alternata* isolates, ranging from 0.551 to greater than 100 µg/mL. On the basis that $EC_{50}$ values above 5 µg/mL represent resistant isolates (Wang et al., 2022), there were 19 resistant and 24 sensitive isolates among the 43 collected *A. alternata* (**Figure 2.5B**). The $EC_{50}$ values of the sensitive isolates ranged from 0.551 to 4.157 µg/mL, with a mean of 1.805 µg/mL and standard deviation of 0.941 µg/mL. For the resistant isolates, $EC_{50}$ values ranged from 7.861 to greater than 100 µg/mL. Among these, there were 12 isolates that were extremely resistant, having $EC_{50}$ values greater than 100 µg/mL. Isolate MB21-363 (*A. dumosa*) was sensitive to boscalid, with an $EC_{50}$ value of 1.657 µg/mL. By contrast, isolates MB21-456 (*A. tenuissima*) and MB21-475 (*A. limoniasperae*) were resistant to boscalid with $EC_{50}$ values of greater than 100 µg/mL. The frequency distribution of the $EC_{50}$ values for boscalid showed a clear bimodal pattern with a shift towards higher $EC_{50}$ values (**Figure 2.6B)**.

The $EC_{50}$ values for pyraclostrobin were found to vary considerably among the 43 *A. alternata* isolates (**Figure 2.5C**) ranging from 0.040 to greater than 100 µg/mL. Assuming that $EC_{50}$ values above 10 µg/mL represent resistance (Avenot and Michailides, 2015), there were ten isolates determined to be resistant and 33 isolates determined to be sensitive. Among the sensitive isolates, the $EC_{50}$ values ranged from 0.040 to 8.762 µg/mL, with an average of 1.035 µg/mL and standard deviation of 1.691 µg/mL. By contrast, for the ten resistant isolates, the $EC_{50}$ values

ranged from 14.35 to greater than 100 µg/mL. Among these, three isolates were extremely resistant, with $EC_{50}$ values greater than 100 µg/mL. Isolates MB21-363 (*A. dumosa*), MB21-456 (*A. tenuissima*), and MB21-475 (*A. limoniasperae*) were sensitive to pyraclostrobin with $EC_{50}$ values of 0.81 µg/mL, 0.13 µg/mL, and 0.88 µg/mL, respectively. For the 46 *Alternaria* spp. isolates, the frequency distribution of the $EC_{50}$ values for pyraclostrobin showed a bimodal pattern suggesting a shift towards higher $EC_{50}$ values (**Figure 2.6C**). Of the ten *Alternaria* isolates found to be resistant to pyraclostrobin, six isolates (MB21-068, MB21-433, MB21-495, MB21-500, MB21-545, MB21-777) from three locations (sites 5 and 7 in Bacon County, and site 14 in Pierce County), were also resistant to boscalid (**Table 2.4**). Furthermore, five of these six isolates (all isolates except MB21-068) also demonstrated reduced sensitivity to pydiflumetofen (**Figures 2.5 and 2.6**).

**Mutation identification in fungicide resistant isolates**

**Mutations within sdhB, sdhC, and sdhD in isolates resistant to SDHI fungicides**

Portions of *sdhB*, *sdhC*, and *sdhD* were sequenced from 16 selected *Alternaria* spp. isolates, including 12 boscalid-resistant isolates from four locations and four boscalid-sensitive isolates from three locations. Obtained sequences (Genbank accession numbers OR091065-OR091072 and PP620128-PP620135 [*sdhB*], OR091073-OR091080 and PP620136-PP620143 [*sdhC*], OR091081-OR091091 and PP620144-PP620148 [*sdhD*]; **Supplemental Table 2.2**) did not indicate any nucleotide changes within the sensitive isolates that would result in amino acid changes versus the previously-published *sdhB* (EU178851), *sdhC* (FJ437067), or *sdhD* (FJ437068) sequences of isolate AaY16, a known SDHI-sensitive *A. alternata* isolate (Avenot et al., 2008a; Avenot et al., 2009). However, sequences from 9 of 12 boscalid-resistant isolates

indicated nucleotide changes that would result in amino acid changes. Among these, all five boscalid-resistant isolates from site 14 (isolates MB21-495, MB21-500, MB21-543, MB21-544, and MB21-545) were found to possess a guanine at nucleotide position 120 within the obtained sequence of *sdhD*, which would result in an amino acid change at amino acid position 133 from histidine to arginine (H133R) (**Figure 2.7, Supplementary Table 2.2**). Three of five boscalid resistant isolates from site 5 (MB21-068, MB21-405, MB21-433) were found to possess an adenine at nucleotide position 228 within the obtained sequence of *sdhC*, which would result in an amino acid change at amino acid position 134 from histidine to glutamine (H134Q) (**Figure 2.7, Supplemental Table 2.1**). In addition, the sequence of *sdhC* from boscalid-resistant isolate MB21-777 from site 7 had a cytosine at nucleotide 61 resulting in an amino acid change at position 79 from glycine to arginine (G79R) (**Figure 2.7, Supplemental Table 2.1**). Mutations in either *sdhC* or *sdhD* were noted in all six isolates previously determined to be double-resistant to both boscalid and pyraclostrobin, with the H133R mutation found in isolates MB21-495, MB21-500, and MB21-545 (from site 14), the H134Q mutation found in isolates MB21-068 and MB21-433 (from site 5), and the G79R mutation found in isolate MB21-777 (from site 7).

**Mutation in *cytB* in pyraclostrobin resistant isolates**

All ten pyraclostrobin-resistant isolates had a cytosine at nucleotide position 123 of the sequenced product (Genbank accession numbers OR091092-OR091104; **Supplemental Table 2.2**), whereas three sensitive isolates had a guanine at this position. This mutation results in a change from glycine to alanine (G143A) at amino acid position 143 (**Figure 2.7**).

**Relationships between boscalid and pydiflumetofen isolate fungicide-sensitivities**

Pearson correlation analysis (**Figure 2.8**) indicated that there was a statistically significant positive correlation between $EC_{50}$ values of boscalid and pydiflumetofen ($r=0.52$, $p<0.05$). Of the 12 isolates identified as having a reduced sensitivity to pydiflumetofen in this study, 11(92%) were also identified as being resistant to boscalid (**Figure 2.5A&B**). These included all nine isolates determined to have $EC_{50}$ values greater than 0.150 µg/mL for pydiflumetofen.

Of the 16 isolates for which succinate dehydrogenase gene sequences were obtained in this study, all seven isolates with an $EC_{50}$ of greater than 0.092 µg/mL for pydiflumetofen had detectable mutations in at least one succinate dehydrogenase gene (**Table 2.5**). These seven isolates were all resistant to boscalid. For isolates with $EC_{50}$ values for pydiflumetofen less than 0.092 µg/mL, only 2 of 9 isolates had a detectable mutation in a succinate dehydrogenase gene, and both of those isolates were resistant to boscalid (**Table 2.5**).

**Discussion**

Georgia routinely ranks amongst the top producers of blueberries in the U.S. (NASS, 2022), but fruit rot diseases cause significant yield losses in the state each year. Fungicides are routinely applied in Georgia to manage blueberry fruit rot pathogens, including *Alternaria* spp. (Sial et al., 2023; Neugebauer et al., 2024). However, relatively little work had been previously done with *Alternaria* spp. from Georgia (Kaur and Dutta, 2024), and prior to the work described here there was no comprehensive data available regarding either the identities of or fungicide resistance status of *Alternaria* spp. isolates causing fruit rot in Georgia blueberries. As such, we identified the species of *Alternaria* isolates associated with fruit rot in Georgia blueberries and evaluated the resistance status of the obtained fungal isolates versus commonly used fungicides. Specifically,

we determined the $EC_{50}$ values of *Alternaria* spp. isolates for seven important fungicides that are currently used to manage pre- and post-harvest fruit rot diseases of blueberry in Georgia. These $EC_{50}$ values give us the first detailed picture of the current fungicide sensitivity of *Alternaria* spp. isolates from blueberry in Georgia.

There are many different species of *Alternaria* that cause postharvest diseases in fruit crops; however, it is generally recognized that *A. alternata, A. tenuissima*, and *A. arborescens* are the primary species that cause Alternaria rot in blueberries (Neugebauer et al., 2024). In our study, we identified *A. alternata* to be the most abundant (93.5%) amongst the *Alternaria* spp. isolates cultured from blueberry fruit in Georgia. This finding is in agreement with previous work with *Alternaria* from blueberries in California, which found that the majority of isolates (61.5%) belonged to *A. alternata* (Zhu and Xiao, 2015). The remainder of isolates in our study were found to belong to other species including *A. tenuissima, A. limoniasperae*, and *A. dumosa* (1 isolate of each). Though the older literature describes *A. tenuissima* as the cause of Alternaria fruit rot (Milholland and Jones, 1972; Cline, 1996; Milholland and Cline, 2017), finding this species in low abundance is in agreement with the aforementioned study of blueberries in California which found that only 5% of isolates were *A. tenuissima*. Though *A. limoniasperae* and *A. dumosa* were not found by Zhu and Xiao (2015), based on the results of our pathogenicity testing of a selection of our *Alternaria* isolates, these two isolates were capable of rotting detached blueberry fruit, albeit with lower severities than all of the 19 *A. alternata* isolates and one *A. tenuissima* isolate we assayed. *A. dumosa* was recently reported to cause blueberry fruit rot in China (Wang et al., 2024a), but to our knowledge *A. limoniasporae* has not been previously reported as a cause of fruit rot on blueberries. Given these facts, and their low abundance among our collected isolates, these two

species seem likely to be of less importance than *A. alternata* in causing Alternaria fruit rot on blueberry in Georgia.

*Alternaria* isolates with resistance to pyraclostrobin and boscalid, as well as isolates with reduced sensitivity to pydiflumetofen, were identified in our study, and all tested isolates were determined to be sensitive to the other four fungicides examined: fluazinam, metconazole, fludioxonil, and cyprodinil. For these fungicides, $EC_{50}$ values were low or very low for all isolates and generally fell within ranges observed for fungicide-sensitive *Alternaria* isolates from blueberries or other crop systems (Mitani et al., 1996; Avenot and Michailides, 2015; Fonseka and Gudmestad, 2016; Gama et al., 2021; Haque and Parvin, 2022; Wang et al., 2022).

Fluazinam inhibits the development of appressoria and penetrating hyphae. For fluazinam, 39 of the isolates examined in our study had $EC_{50}$ values less than 0.1 µg/mL, with the values for the remaining seven isolates falling between 0.1 to 0.2 µg/mL. This range is somewhat higher than, but comparable to, the values observed from prior studies of *A. alternata* isolates from sugar beet in the U.S. (0.0004 to 0.0021 µg/mL) and pear in Japan (less than 0.1 µg/mL) (Mitani et al., 1996; Haque and Parvin, 2022). Resistance to fluazinam is not frequently reported; however, resistance has been found in *Phytophthora infestans* on potato where it had been extensively used (Schepers et al., 2018). In recent years, fluazinam has been found to be very effective against *Colletotrichum* spp. that cause anthracnose fruit rot of blueberry, and a study of 201 *C. gloeosporioides* isolates collected from the blueberries in Florida indicated no resistance to fluazinam when isolates were screened at a discriminatory dose of 1 µg/mL (Gama et al., 2021). Though fluazinam has not been widely used in Georgia blueberry production previously, given the recent identification of pyraclostrobin and boscalid resistant *C. gloeosporioides* in Georgia blueberry (Ali et al., 2019), this effective fungicide has been recently recommended as part of a rotation to control QoI

fungicide-resistant *Colletotrichum* on blueberry. Accordingly, the assessment of *Alternaria* isolate sensitivity in our study is particularly timely as fungal exposure to fluazinam is likely to increase in the coming years.

Metconazole is a DMI fungicide used for its efficacy against multiple fungal diseases including those caused by *Alternaria* spp. (Kumazawa et al., 2000; Fonseka and Gudmestad, 2016; Lee et al., 2021). Metconazole inhibits fungal cell membrane development by preventing ergosterol biosynthesis leading to disruption of cell membrane function, leakage of cytoplasmic contents, and hyphal inhibition (Wang et al., 2024b). Previous reports of $EC_{50}$ values for *A. alternata* in other crops are rare, and resistance among *Alternaria* species to metconazole does not appear to have been reported previously. There is no baseline sensitivity information for metconazole and *A. alternata* in Georgia blueberries, and in comparison to a previous baseline sensitivity study of *A. alternata* from potato (range 0.05 to 0.46 µg/mL; mean 0.26 µg/mL) (Fonseka and Gudmestad, 2016), the $EC_{50}$ values observed in our study (range 0.125 to 5.729 µg/mL; mean 1.146 µg/mL) were relatively higher. Despite this, the isolates in our study were still concluded to be sensitive based upon their unimodal frequency distribution and the fact that the minimum inhibitory concentration (MIC) was less than 10 µg/mL for all tested isolates. Furthermore, the isolate with the highest $EC_{50}$ in our study (5.729 µg/mL) had a resistance factor of less than 5 when compared to the mean $EC_{50}$. Reduced sensitivity to metconazole has been reported, in *Colletotrichum truncatum* from peach, with a mean $EC_{50}$ value of 16.6 µg/mL (Chen et al., 2016). The data collected in our study on metconazole sensitivity will be a valuable basis for comparison if shifts in sensitivity occur in *Alternaria* spp. from Georgia blueberries.

Cyprodinil, a broad-spectrum pyrimidinamine fungicide that is used to protect fruit plants, vines, cereals, and vegetables from a wide range of fungal pathogens (Ma and Ye, 1997), works

through inhibition of the biosynthesis of methionine and other thionic amino acids of fungi (Masner et al., 1994). Resistance to cyprodinil has not been frequently reported among *Alternaria* spp. but has been reported from other fungal species including *Botrytis cinerea*, where 30% of the isolates from strawberries were found to be resistant (Fernández-Ortuño et al., 2013). For cyprodinil, 22 of the isolates in our study had $EC_{50}$ values less than 0.1 µg/mL, 15 isolates were between 0.1 and 0.2 µg/mL, and the remaining nine isolates were between 0.2 and 0.4 µg/mL. These values, which ranged from 0.02 to 0.40 µg/mL with a mean value of 0.13 µg/mL were relatively lower than prior reports from fungicide-sensitive *A. alternata* from blueberries in California (mean 0.465 µg/mL) and baseline sensitivities established for *A. alternata* isolates from pistachio in California (range 0.001 to 1.184 µg/mL; mean 0.214 µg/mL) (Avenot and Michailides, 2015; Wang et al., 2022). This suggests that *A. alternata* isolates from Georgia blueberry remain sensitive to cyprodinil at this time, with mean $EC_{50}$ values from Georgia being only one-third and one-half the means from fungicide-sensitive isolates from California blueberries and California pistachios, respectively.

With respect to fludioxonil, the $EC_{50}$ values for our isolates ranged from 0.037 to 0.234 µg/mL, and as such were relatively lower than the baseline sensitivities (range 0.010 to 4.875 µg/mL) established for isolates from pistachio in California between 1998 and 2003 (Avenot and Michailides, 2015). Likewise, the mean $EC_{50}$ for our isolates (0.124 µg/mL) was relatively similar to the mean (0.078 µg/mL) from a recent study of *A. alternata* isolates from blueberry in California which found, as we did, that all tested isolates were sensitive to fludioxonil (Wang et al., 2022). While there is no baseline sensitivity information available for fludioxonil and *Alternaria* spp. from blueberry or any other crops in Georgia, we anticipate that the $EC_{50}$ values determined in our study will be valuable for future resistance monitoring efforts in Georgia, as fludioxonil is widely

used in conventional blueberry production in Georgia, typically as one component of combination products with cyprodinil in the commercial product Switch® (Syngenta Crop Protection, 2022) or with pydiflumetofen in the commercial product Miravis Prime® (Syngenta Crop Protection, 2023). While resistance to fludioxonil has been reported in *Alternaria* spp. from pistachio and crucifers (Iacomi-Vasilescu et al., 2004; Avenot and Michailides, 2015), significant fitness costs associated with fludioxonil resistance that have been observed in the laboratory with isolates of other fludioxonil-resistant fungal species (Li and Xiao, 2008) may tend to slow the development of widespread fludioxonil resistance in some cases despite repeated applications.

The fungicide boscalid has been utilized in blueberry production in the U.S. since 2003, typically as one component of a two-component mixture with pyraclostrobin in the commercial product Pristine® (BASF, 2003). Since that time, it has been widely used by Georgia blueberry growers to manage several diseases including fruit rots such as Alternaria leaf spot and fruit rot (*Alternaria* spp.), anthracnose ripe rot (*Colletotrichum* spp.), and Botrytis gray mold (*Botrytis cinerea*) (Sial et al., 2023). Perhaps not unexpectedly, given its long history of widespread use, we identified resistance to boscalid in 21 of 46 (46%) *Alternaria* isolates in our study. Moreover, among these isolates, 14 of 46 (30%) were extremely resistant to boscalid with $EC_{50}$ values greater than 100 µg/mL. These findings are similar to findings from blueberry *A. alternata* from California, where 33% of isolates were reported to have $EC_{50}$ values of greater than 100 µg/mL (Wang et al., 2022). By contrast, in comparison to Wang et al. (2022) where only 23% of isolates had $EC_{50}$ values less than 10 µg/mL, our study indicated a larger proportion of isolates remaining sensitive to boscalid with 25 isolates (54%) having $EC_{50}$ values less than 5 µg/mL. While there is no prior data regarding boscalid-resistant *Alternaria* spp. in Georgia in any crops, nor any baseline $EC_{50}$ values for *Alternaria* spp. in Georgia to compare with, our data overall suggest that a shift

has likely taken place (or is in progress) among *Alternaria* isolates from blueberry towards boscalid-resistance due to selection pressure from boscalid applications over the past two decades. This is not surprising based on observations from other crop systems. For example, prior to the introduction and use of boscalid, the baseline sensitivity to boscalid for 43 *A. alternata* isolates collected during 1999 and 2000 from pistachio in California showed that no resistant isolates were present and that $EC_{50}$ values ranged from 0.011 to 0.650 µg/mL (Avenot et al., 2014). However, just a decade later, 69 of 117 (59%) *A. alternata* isolates collected from pistachio orchards in the Central Valley of California were found to be extremely resistant to boscalid with $EC_{50}$ values greater than 100 µg/mL (Avenot and Michailides, 2015). In Georgia, while 21 isolates with boscalid resistance were identified in our study, these isolates originated from only 6 of 16 unique locations (with 16 out of 21 resistant isolates originating from only two of these locations) suggesting that resistance may not yet be widespread at this time. Continued resistance monitoring will be necessary, and the $EC_{50}$ values for boscalid determined in our study will be valuable for this effort going forward.

Resistance to the SDHI fungicide boscalid has been previously associated with mutations within the genes encoding subunits *sdhB*, *sdhC,* or *sdhD* of the succinate-dehydrogenase complex (Sierotzki et al., 2011; Avenot et al., 2014). One of the most common mutations for *sdhB* in *A. alternata* is H277Y/R (Avenot et al., 2008a), however, in our analysis of boscalid-resistant isolates this mutation was not found in any tested *Alternaria* isolates from Georgia blueberry. Nonetheless, two other common mutations previously reported in *Alternaria* spp. (Avenot et al., 2009; Metz et al., 2019), corresponding to H134Q in *sdhC* and H133R in *sdhD*, were found in some of our boscalid-resistant isolates. Furthermore, G79R, a less-frequently reported mutation in *Alternaria* spp. (Förster et al., 2022), was also identified within *sdhC* of a single boscalid-resistant isolate in

our study. Of note, all five isolates identified with the H133R mutation originated from a common location (site 14) and all three isolates identified with the H134Q mutation originated from a different common location (site 5) while the isolate with the G79R mutation was identified from a different site from the others, suggesting that resistance to boscalid in these locations likely developed independently. In addition, at least four isolates identified as having resistance to boscalid in our study (including some boscalid-resistant isolates from site 5 where the H134Q mutation was identified) did not have any detectable mutations within *sdhB*, *sdhC*, or *sdhD*. This is in agreement with prior reports suggesting that other determinants besides identifiable mutations in the succinate dehydrogenase subunit genes may play a role in resistance expression (Avenot et al., 2014; Förster et al., 2022).

Given the large number of isolates identified in this study with resistance to boscalid, and reports of cross-resistance among SDHI fungicides in some fungal pathogens (Avenot et al., 2014; Fernández-Ortuño et al., 2017; Alzohairy et al., 2023), we also investigated the sensitivity of isolates in our study to the SDHI fungicide pydiflumetofen. Pydiflumetofen has recently begun to be utilized for managing blueberry fruit rots in Georgia and is most commonly applied in combination with fludioxonil in the commercial product Miravis Prime® (Sial et al., 2023). For *Alternaria* isolates in our study, $EC_{50}$ values for pydiflumetofen ranged from 0.008 to 1.114 µg/mL, which were somewhat higher than were found in a prior study conducted on *A. alternata* isolates causing Alternaria leaf spot of almond in California (range 0.001 to 0.215 µg/mL) (Förster et al., 2022), but more similar to results from *A. alternata* isolates causing black spot disease on cherry in China (range 0.027 to 1.175 µg/mL) (Siling et al., 2023). However, in contrast to the conclusions of Siling et al. (2023), where isolates were characterized as being sensitive to pydiflumetofen based on a unimodal distribution of $EC_{50}$ values and the fact that they possessed

no identifiable mutations within the succinate dehydrogenase subunit genes, the frequency distribution of our isolates' $EC_{50}$ values was bimodal and several isolates did possess mutations in *sdhC* or *sdhD*. From our analysis, 12 of our isolates showed reduced sensitivity to pydiflumetofen. Among these, 11 were resistant to boscalid, and there was a statistically significant positive correlation between the sensitivity of isolates in our study to these two SDHI fungicides. Of note, despite the fact that cross-resistance between different SDHI fungicides is assumed (FRAC, 2024a) and has been identified in varying degrees in pathogens such as *A. alternata* for some SDHI fungicide combinations (Avenot et al., 2014), our results stand in contrast to recent prior work with *A. alternata* which did not find strong evidence for cross-resistance among several SDHI fungicide combinations including boscalid and pydiflumetofen (Förster et al., 2022). Nonetheless, the observed correlation in our study, along with the fact that significant numbers of isolates showed reduced sensitivity to pydiflumetofen, may have significant implications for the long-term efficacy of products containing pydiflumetofen for Alternaria fruit rot control in Georgia, and these results suggest that additional fungicide resistance monitoring will be necessary going forward to stay abreast of potential shifts of isolates toward resistance to pydiflumetofen.

In addition to finding resistance to boscalid, 10 of 46 (22%) *Alternaria* isolates from this study were identified with resistance to the QoI fungicide pyraclostrobin. Pyraclostrobin is typically applied to blueberries in Georgia in the form of the commercial product Pristine® (Sial et al., 2023), which includes boscalid as its other component. However, the singular use of other QoI fungicides, such as azoxystrobin, in blueberry production has a longer history. Given the numerous reports worldwide of resistance to QoI in multiple pathogen species (Fisher and Meunier, 2008), these fungicides are generally considered high risk for resistance development (FRAC, 2024b), and our identification of pyraclostrobin-resistant *Alternaria* isolates is, perhaps,

not surprising. As with boscalid, there are no baseline pyraclostrobin $EC_{50}$ values for *Alternaria* spp. from blueberry in Georgia; however, a previous baseline for *A. alternata* causing late blight of pistachios in California was developed using isolates collected from orchards without a previous history of Pristine® applications (Avenot et al., 2008b). In Avenot et al. (2008b) most isolates (77%) were sensitive to pyraclostrobin with $EC_{50}$ values less than 0.01 µg/mL, 17% had low resistance (mean $EC_{50}$ value = 4.71 µg/mL), and a single isolate was resistant with an $EC_{50}$ value greater than 100 µg/mL. That study, which like ours used a spore germination assay to assess *Alternaria* sensitivity to the strong spore germination inhibitory ability of QoI fungicides (Barilli et al., 2016), set a cutoff between sensitive and resistant isolates of 10 µg/mL (Avenot et al., 2008b). Based on this cutoff, out of 46 *Alternaria* spp. isolates in our study, 10 were found to be resistant and 36 sensitive to pyraclostrobin, and all 10 resistant isolates were confirmed to possess the G143A mutation frequently identified in QoI-resistant fungal pathogens (Fisher and Meunier, 2008). Among the 36 sensitive isolates, 28 (77%) had $EC_{50}$ values less than 1 µg/mL. By contrast, 3 of 10 resistant isolates in our study were extremely resistant ($EC_{50}$ values less than 100 µg/mL), with the remaining seven resistant isolates having a mean $EC_{50}$ value of 38.98 µg/mL. While a significant number of resistant isolates were found in our study, the proportion of resistant isolates is low in comparison to previous reports from pistachio and blueberry fields in California with a history of Pristine® applications, where 95% and 42%, respectively, were determined to be resistant to pyraclostrobin (Avenot et al., 2008b; Wang et al., 2022)

In our study, 6 of 46 (13%) isolates were resistant to both boscalid and pyraclostrobin, and five of these isolates showed reduced sensitivity to pydiflumetofen. This indicates that multiple fungicide resistance (including resistance to both components of Pristine®) is present among *Alternaria* isolates from Georgia blueberry. Multiple resistance to both components of Pristine®

has been reported before in *Alternaria* species, including in pistachio orchards where 7 of 59 isolates (12%) were found to be resistant to boscalid and pyraclostrobin (Avenot et al., 2008b), and in Georgia blueberries, *Colletotrichum gloeosporioides* isolates causing anthracnose ripe rot were recently found to be resistant to both of these fungicides as well (Ali et al., 2019). The presence of multiple fungicide resistance in Georgia blueberries has the potential to significantly reduce the efficacy of spray programs currently being utilized to control fruit rots. The extent and prevalence of multiple fungicide resistance should be monitored in the future, and growers should be encouraged to use tank mixes with other modes of action and multisite fungicides before control failures occur.

Taken together, the identification of the primary species associated with Alternaria fruit rot on Georgia blueberries, the characterization of fungicide sensitivity of *Alternaria* isolates, and the identification of fungicide resistance and fungicide resistance-associated mutations will aid in the management of this fruit rot disease in Georgia. Informed decisions regarding spray selection as well as more accurate identification and diagnosis of this issue are expected to result from this work.

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## Tables

**Table 2.1**. Primers used in this study.

| Primer Name | Sequence (5'-3') | Reference |
|---|---|---|
| ITS1 | TCCGTAGGTGAA CCTGCGG | White et al., 1990 |
| ITS4 | TCCTCCGCTTA TTGATATGC | |
| ATPDF1 | ATCGTCTCCATGACCGAGTTCG | Lawrence et al., 2013 |
| ATPDR1 | TCCGATGGAGTTCATGATAGCC | |
| Alt-for | ATGCAGTTCACCACCATCGC | Hong et al., 2005 |
| Alt-rev | ACGAGGGTGAYGTAGGCGTC | |
| CALDF1 | AGCAAGTCTCCGAGTTCAAGG | Lawrence et al., 2013 |
| CALDR1 | CTTCTGCATCATCAYCTGGACG | |
| RPB2DF | ACCGACACACAAATGCTGGAGC | Lawrence et al., 2013 |
| RPB2DR | CAAGACCCCAATGAGAGTTGTG | |
| SdhBF6 | AAGGAAGATCGCAAGAAGCTC | Avenot et al., 2008a |
| SdhBR6 | AATGGCTAGCGCAGGGTTCA | |
| SdhC-(A-G) F1 | CACCTGGCCATCTACAAGC | Avenot et al., 2009 |
| SdhC-(A-G) R1 | TGGTTCTTGAAACCAATACCG | |
| SdhD(C-A) S1 | CCACTGGAGCTTCGAGAGGA | |
| SdhD(C-A) R1 | GCTGTTCGAGTCTTGGGAAC | |
| cytb2f | CTATGGATCTTACAGAGCAC | Vega and Dewdney 2012 |
| DTRcytb2-INTr | GTATGTAACCGTCTCCGTC | |

**Table 2.2.** Fungicidal product commonly utilized for Alternaria fruit rot and leaf spot control in blueberry production, active ingredient, FRAC mode of action, and concentrations of active ingredient used in the mycelial growth inhibition assays conducted as part of this study.

| Trade Name | Active Ingredient | Group (FRAC MoA) | Concentration Used (µg/µl) |
|---|---|---|---|
| Quash | Metconazole | DMI (FRAC 3) | 0.01, 0.05, 0.1, 0.5, 1.0 |
| Omega 500F | Fluazinam | 2,6-dinitroanilines (FRAC 29) | 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 |
| Pristine | Pyraclostrobin | QoI (FRAC 11) | 0.01, 0.1, 1.0, 10 |
| | Boscalid | SDHI (FRAC 7) | 0.5, 1.0, 5.0, 10, 50, 100 |
| Switch 62.5WG | Cyprodinil | Anilopyrimidines (FRAC 9) | 0.1, 0.5, 1.0, 5.0, 10 |
| | Fludioxonil | Phenylpyrroles (FRAC 12) | 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 |
| Miravis Prime | Pydiflumetofen | SDHI (FRAC 7) | 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 |
| | Fludioxonil | Phenylpyrroles (FRAC 12) | 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 |

**Table 2.3**. Pathogenicity test results including disease severity index and incidence (%) of selected *Alternaria* spp. isolates.

| Species Identity | Isolate Name | Disease incidence (%) | Disease Severity Index |
|---|---|---|---|
| *A. alternata* | MB21-013 | 92.6 | 45.9 |
| | MB21-068 | 100 | 57.8 |
| | MB21-099 | 100 | 79.3 |
| | MB21-348 | 100 | 48.9 |
| | MB21-362 | 100 | 70.4 |
| | MB21-397* | 100 | 65.2 |
| | MB21-402 | 96.3 | 58.5 |
| | MB21-410 | 100 | 66.7 |
| | MB21-417 | 100 | 66.7 |
| | MB21-421 | 88.9 | 46.7 |
| | MB21-449 | 100 | 68.9 |
| | MB21-454 | 100 | 58.5 |
| | MB21-495 | 96.3 | 54.8 |
| | MB21-500 | 100 | 48.9 |
| | MB21-543 | 88.9 | 40.0 |
| | MB21-546 | 100 | 82.2 |
| | MB21-561 | 96.3 | 51.1 |
| | MB21-736 | 96.3 | 68.1 |
| | MB21-777 | 100 | 55.6 |
| *A. tenuissima* | MB21-456* | 100 | 54.1 |
| *A. dumosa* | MB21-363* | 85.2 | 19.3 |
| *A. limoniasperae* | MB21-475* | 88.9 | 39.3 |

*isolates depicted in Figure 2

**Table 2.4.** Frequency of boscalid and/or pyraclostrobin sensitivities of *Alternaria* spp. isolates collected from blueberry sites in Georgia in this study.

| County | Site Number | No. of Isolates | Boscalid Sensitivity (%) | | Pyraclostrobin Sensitivity (%) | | Resistance to both |
|---|---|---|---|---|---|---|---|
| | | | Sensitive | Resistant | Sensitive | Resistant | |
| Appling | 1 | 3 | 3 | 0 | 2 | 1 | 0 |
| | 2 | 2 | 2 | 0 | 2 | 0 | 0 |
| Bacon | 3 | 5 | 5 | 0 | 5 | 0 | 0 |
| | 4 | 2 | 2 | 0 | 2 | 0 | 0 |
| | 5 | 14 | 3 | 11 | 12 | 2 | 2 |
| | 6 | 1 | 1 | 0 | 1 | 0 | 0 |
| | 7 | 4 | 2 | 2 | 3 | 1 | 1 |
| | 8 | 1 | 1 | 0 | 1 | 0 | 0 |
| Brantley | 9 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 10 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 11 | 2 | 2 | 0 | 0 | 2 | 0 |
| Pierce | 12 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 13 | 2 | 2 | 0 | 2 | 0 | 0 |
| | 14 | 5 | 0 | 5 | 2 | 3 | 3 |
| | 15 | 1 | 1 | 0 | 1 | 0 | 0 |
| | 16 | 1 | 0 | 1 | 1 | 0 | 0 |
| Totals | 16 | 46 | 25 (54.3%) | 21 (45.7%) | 36 (78.3%) | 10 (21.7%) | 6 (13.0%) |

**Table 2.5.** SDHI fungicide sensitivity and mutation status of select *Alternaria* spp. isolates.

| Isolate Name | Site Number | Boscalid Resistance Status | Mutations (sdh genes) | $EC_{50}$ (µg/mL) | |
|---|---|---|---|---|---|
| | | | | Boscalid | Pydiflumetofen |
| MB21-347 | 5 | Sensitive | no mutations | 1.053 | 0.018 |
| MB21-358 | 6 | Sensitive | no mutations | 1.094 | 0.057 |
| MB21-362 | 2 | Sensitive | no mutations | 0.784 | 0.029 |
| MB21-363 | 5 | Sensitive | no mutations | 1.657 | 0.026 |
| MB21-449 | 5 | Resistant | no mutations | 8.154 | 0.020 |
| MB21-456 | 12 | Resistant | no mutations | >100 | 0.023 |
| MB21-479 | 5 | Resistant | no mutations | >100 | 0.008 |
| *MB21-068 | 5 | Resistant | H134Q (sdhC) | >100 | 0.054 |
| MB21-405 | 5 | Resistant | H134Q (sdhC) | >100 | 0.377 |
| *MB21-433 | 5 | Resistant | H134Q (sdhC) | >100 | 0.093 |
| *MB21-495 | 14 | Resistant | H133R (sdhD) | >100 | 0.822 |
| *MB21-500 | 14 | Resistant | H133R (sdhD) | 36.180 | 0.253 |
| MB21-543 | 14 | Resistant | H133R (sdhD) | 13.703 | 0.026 |
| MB21-544 | 14 | Resistant | H133R (sdhD) | >100 | 1.114 |
| *MB21-545 | 14 | Resistant | H133R (sdhD) | 26.720 | 0.359 |
| *MB21-777 | 7 | Resistant | G79R (sdhC) | >100 | 0.260 |

*indicates isolate with resistance to both pyraclostrobin and boscalid

**Supplementary Table 2.1.** Accession numbers of additional sequences utilized in phylogenetic analysis.

| | | | Accession Numbers | | | |
|---|---|---|---|---|---|---|
| **Isolate Name** | **Species** | **Reference** | **ATPase** | **Alt a1** | **CAL** | **RPB2** |
| CBS 577.94 | *Alternaria agripestis* | Lawrence et al. 2013 | JQ671932 | JQ646440 | JQ646266 | JQ646468 |
| CBS 118809 | *Alternaria alstroemeriae* | Elfar et al. 2023 | MH101803 | MH084526 | MH175185 | n/a |
| EGS 52-039 | *Alternaria alternantherae* | Lawrence et al. 2013 | JQ671892 | JN383511 | JQ646226 | n/a |
| EGS 34-016 | *Alternaria alternata* | Lawrence et al. 2013 | JQ671874 | AY563301 | JQ646208 | JQ646490 |
| X1122 | *Alternaria alternata* | Zhu and Xiao 2015 | KJ908253 | KJ920989 | KJ920970 | n/a |
| X1265 | *Alternaria alternata* | Zhu and Xiao 2015 | KJ908237 | KJ921004 | KJ920960 | n/a |
| CBS 594.93 | *Alternaria aragakii* | Lawrence et al. 2013 | JQ671935 | JQ646443 | JQ646269 | JQ646521 |
| EGS 39-128 | *Alternaria arborescens* | Lawrence et al. 2013 | JQ671880 | AY563303 | JQ646214 | JQ646487 |
| X1033 | *Alternaria arborescens* | Zhu and Xiao 2015 | KJ908248 | KJ921009 | KJ920941 | n/a |
| X1263 | *Alternaria arborescens* | Zhu and Xiao 2015 | KJ908226 | KJ921003 | KJ920947 | n/a |
| EGS 35-122 | *Alternaria argyroxiphii* | Lawrence et al. 2013 | JQ671926 | JQ646434 | JQ646260 | JQ646464 |
| CBS 118810 | *Alternaria betae-kenyensis* | Elfar et al. 2023 | MH101805 | JQ905104 | MH175189 | n/a |
| DAOM 231361 | *Alternaria bornmuelleri* | Lawrence et al. 2013 | JQ671791 | JN383516 | JQ646125 | JQ646520 |
| ATCC 18043 | *Alternaria botrytis* | Lawrence et al. 2013 | JQ671834 | AY563317 | JQ646168 | n/a |
| EEB 2232 | *Alternaria brassicicola* | Lawrence et al. 2013 | JQ671843 | AY563311 | JQ646177 | n/a |
| CBS 107.38 | *Alternaria burnsii* | Lawrence et al. 2013 | JQ671860 | JQ646388 | JQ646194 | JQ646457 |
| EGS 26-010 | *Alternaria carotiincultae* | Lawrence et al. 2013 | JQ671850 | AY563287 | JQ646184 | JQ646477 |
| ATCC 18044 | *Alternaria chartarum* | Lawrence et al. 2013 | JQ671828 | AY563319 | JQ646162 | n/a |
| EGS 41-188 | *Alternaria cheiranthi* | Lawrence et al. 2013 | JQ671830 | AY563290 | JQ646164 | n/a |
| CBS 109164 | *Alternaria cretica* | Lawrence et al. 2013 | JQ671916 | JQ646426 | JQ646250 | JQ646480 |
| EGS 31-021 | *Alternaria cucurbitae* | Lawrence et al. 2013 | JQ671836 | AY563315 | JQ646170 | JQ646452 |
| CBS 915.96 | *Alternaria dianthicola* | Lawrence et al. 2013 | JQ671810 | n/a | JQ646144 | JQ646465 |
| EGS 45-007 | *Alternaria dumosa* | Lawrence et al. 2013 | JQ671877 | AY563305 | JQ646211 | n/a |
| CBS 489.92 | *Alternaria eichhorniae* | Woudenberg et al. 2015 | MH101806 | KP123973 | MH175190 | n/a |
| EGS 38-073 | *Alternaria embellisia* | Lawrence et al. 2013 | JQ671793 | AY563322 | JQ646127 | n/a |
| EGS 37-143 | *Alternaria ethzedia* | Lawrence et al. 2013 | JQ671805 | AY563284 | JQ646139 | JQ646458 |
| EGS 42-049 | *Alternaria euphorbiicola* | Lawrence et al. 2013 | JQ671911 | AY563314 | JQ646245 | n/a |
| EGS 36-103 | *Alternaria eureka* | Lawrence et al. 2013 | JQ671771 | JN383507 | JQ646105 | JQ646473 |
| EGS 44-001 | *Alternaria frumenti* | Lawrence et al. 2013 | JQ671823 | JQ646378 | JQ646157 | JQ646509 |
| 0407-5-2 | *Alternaria gaisen* | Yan et al. 2024 | MW541812 | MW541814 | n/a | n/a |
| CBS 104.32 | *Alternaria gossypina* | Lawrence et al. 2013 | JQ671868 | JQ646395 | JQ646202 | n/a |
| CBS 107.41 | *Alternaria gypsophilae* | Lawrence et al. 2013 | JQ671859 | JQ646387 | JQ646193 | JQ646475 |
| EGS 50-184 | *Alternaria hordeicola* | Lawrence et al. 2013 | JQ671812 | JQ646372 | JQ646146 | n/a |
| EGS 49-062 | *Alternaria hyacinthi* | Lawrence et al. 2013 | JQ671778 | FJ266506 | JQ646112 | n/a |
| EGS 27-193 | *Alternaria infectoria* | Lawrence et al. 2013 | JQ671804 | FJ266502 | JQ646138 | n/a |
| CBS 118486 | *Alternaria iridiaustralis* | Woudenberg et al. 2015 | MH101807 | KP123981 | MH175191 | n/a |
| YZU 171616 | *Alternaria jacinthicola* | Luo et al. 2018 | MG781017 | MG781013 | MG781016 | n/a |
| EGS 41-158 | *Alternaria japonica* | Lawrence et al. 2013 | JQ671840 | AY563312 | JQ646174 | n/a |
| EGS 40-187 | *Alternaria leptinellae* | Lawrence et al. 2013 | JQ671773 | JQ646366 | JQ646107 | JQ646461 |
| C.FR13 | *Alternaria limoniasperae* | Elfar et al. 2019 | MH492694 | n/a | n/a | n/a |
| EGS 45-100 | *Alternaria limoniasperae* | Lawrence et al. 2013 | JQ671879 | AY563306 | JQ646213 | n/a |
| EGS 30-033 | *Alternaria longipes* | Lawrence et al. 2013 | JQ671864 | AY563304 | JQ646198 | JQ646515 |
| CBS 135.31 | *Alternaria malorum* | Lawrence et al. 2013 | JQ671800 | JQ646369 | JQ646134 | JQ646481 |
| EGS 31-061 | *Alternaria mouchaccae* | Lawrence et al. 2013 | JQ671799 | AY563279 | JQ646133 | JQ646459 |
| RC O1B9 | *Alternaria oxytropis* | Lawrence et al. 2013 | JQ671792 | JN383517 | JQ646126 | JQ646479 |
| EGS 29-180 | *Alternaria panax* | Lawrence et al. 2013 | JQ671846 | JQ646382 | JQ646180 | n/a |
| P354.8 | *Alternaria penicillata* | Lawrence et al. 2013 | JQ671788 | JN383502 | JQ646122 | JQ646469 |
| EGS 09-159 | *Alternaria petroselini* | Lawrence et al. 2013 | JQ671854 | AY563288 | JQ646188 | JQ646474 |
| EGS 41-130 | *Alternaria rosae* | Lawrence et al. 2013 | JQ671803 | JQ646370 | JQ646137 | n/a |
| EGS 19-016 | *Alternaria scirpicola* | Lawrence et al. 2013 | JQ671781 | AY563320 | JQ646115 | JQ646455 |
| ATCC 58177 | *Alternaria solani* | Lawrence et al. 2013 | JQ671898 | AY563299 | JQ646232 | JQ646507 |
| EGS 46-051 | *Alternaria sonchi* | Lawrence et al. 2013 | JQ671849 | AY563307 | JQ646183 | n/a |
| EGS 34-015 | *Alternaria tenuissima* | Lawrence et al. 2013 | JQ671875 | AY563302 | JQ646209 | n/a |
| SY-4 | *Alternaria tenuissima* | Qian et al. 2022 | MT416124 | MK593137 | MT416125 | n/a |

| | | | | | | |
|---|---|---|---|---|---|---|
| CBS 114.35 | *Alternaria tomato* | Lawrence et al. 2013 | JQ671861 | JQ646389 | JQ646195 | JQ646494 |
| CBS 116533 | *Alternaria vaccariae* | Lawrence et al. 2013 | JQ671858 | JQ646386 | JQ646192 | JQ646463 |

**Supplementary Table 2.2.** Accession numbers for sequences generated from isolates utilized in this study.

| Isolate Name | Collection Date | Isolation Location (Georgia, USA) | Site ID | Blueberry Type (Cultivar) | Accession Number | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ITS | ATPase | Alt a1 | CAL | RPB2 | cytB | sdhB | sdhC | sdhD |
| MB21-007 | 22-Apr-21 | Brantley County | Site 11 | SHB (Optimus) | OR041698 | OR091105 | n/a | n/a | n/a | OR091092 | n/a | n/a | n/a |
| MB21-008 | 22-Apr-21 | Brantley County | Site 11 | SHB (Optimus) | OR041699 | OR091106 | n/a | n/a | n/a | OR091093 | n/a | n/a | n/a |
| MB21-013 | 22-Apr-21 | Brantley County | Site 9 | SHB (V1) | OR041700 | OR091107 | n/a | n/a | n/a | OR091094 | n/a | n/a | n/a |
| MB21-068 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041701 | OR091108 | PP662487 | PP662470 | PP662476 | OR091095 | PP620128 | PP620136 | PP620144 |
| MB21-099 | 4-May-21 | Appling County | Site 1 | SHB (Farthing) | OR041702 | OR091109 | n/a | n/a | n/a | OR091096 | n/a | n/a | n/a |
| MB21-131 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041703 | OR091110 | n/a | n/a | n/a | OR091097 | n/a | n/a | n/a |
| MB21-280 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041704 | OR091111 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-341 | 17-May-21 | Bacon County | Site 3 | SHB (Farthing) | OR041705 | OR091112 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-347 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041706 | OR091113 | PP662488 | n/a | n/a | n/a | PP620129 | PP620137 | OR091081 |
| MB21-348 | 4-May-21 | Appling County | Site 1 | SHB (Farthing) | OR041707 | OR091114 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-358 | 17-May-21 | Bacon County | Site 6 | SHB (Indigocrisp) | OR041708 | OR091115 | PP662489 | n/a | n/a | n/a | OR091065 | OR091073 | OR091082 |
| MB21-362 | 4-May-21 | Appling County | Site 2 | SHB (Farthing) | OR041709 | OR091116 | PP662490 | PP662471 | PP662477 | OR091098 | OR091066 | OR091074 | OR091083 |
| MB21-363 | 7-May-21 | Bacon County | Site 5 | SHB (Star) | OR041710 | OR091117 | PP662491 | PP662472 | PP662478 | n/a | PP620130 | PP620138 | OR091084 |
| MB21-375 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041711 | OR091118 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-376 | 17-May-21 | Bacon County | Site 3 | SHB (Farthing) | OR041712 | OR091119 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-397 | 17-May-21 | Bacon County | Site 3 | SHB (Farthing) | OR041713 | OR091120 | PP662492 | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-400 | 4-May-21 | Appling County | Site 1 | SHB (Farthing) | OR041714 | OR091121 | PP662493 | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-402 | 4-May-21 | Brantley County | Site 10 | SHB (Patrecia) | OR041715 | OR091122 | n/a | n/a | n/a | OR091099 | n/a | n/a | n/a |
| MB21-405 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041716 | OR091123 | PP662494 | n/a | n/a | n/a | PP620131 | PP620139 | OR091085 |
| MB21-410 | 7-May-21 | Pierce County | Site 15 | SHB (Rebel) | OR041717 | OR091124 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-416 | 17-May-21 | Bacon County | Site 3 | SHB (Farthing) | OR041718 | OR091125 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-417 | 7-May-21 | Pierce County | Site 13 | SHB (Farthing) | OR041719 | OR091126 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-420 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041720 | OR091127 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-421 | 17-May-21 | Bacon County | Site 3 | SHB (Farthing) | OR041721 | OR091128 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-427 | 4-May-21 | Appling County | Site 2 | SHB (Farthing) | OR041722 | OR091129 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-428 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041723 | OR091130 | PP662495 | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-433 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041724 | OR091131 | PP662496 | n/a | n/a | OR091100 | PP620132 | PP620140 | PP620145 |
| MB21-449 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041725 | OR091132 | PP662497 | n/a | n/a | n/a | OR091067 | OR091075 | OR091086 |
| MB21-454 | 7-May-21 | Pierce County | Site 16 | SHB (Star) | OR041726 | OR091133 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-456 | 1-May-21 | Pierce County | Site 12 | SHB (Suziblue) | OR041727 | OR091134 | PP662498 | PP662473 | PP662479 | n/a | OR091068 | OR091076 | OR091087 |
| MB21-474 | 7-May-21 | Pierce County | Site 13 | SHB (Farthing) | OR041728 | OR091135 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-475 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041729 | OR091136 | PP662499 | PP662474 | PP662480 | n/a | n/a | n/a | n/a |
| MB21-479 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041730 | OR091137 | PP662500 | n/a | n/a | n/a | OR091069 | OR091077 | OR091088 |
| MB21-483 | 17-May-21 | Bacon County | Site 5 | SHB (Star) | OR041731 | OR091138 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-495 | 7-May-21 | Pierce County | Site 14 | SHB (Meadowlark) | OR041732 | OR091139 | PP662501 | PP662475 | PP662481 | OR091101 | OR091070 | OR091078 | OR091089 |
| MB21-500 | 7-May-21 | Pierce County | Site 14 | SHB (Meadowlark) | OR041733 | OR091140 | PP662502 | n/a | n/a | OR091102 | PP620133 | PP620141 | PP620146 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MB21-543 | 7-May-21 | Pierce County | Site 14 | SHB (Meadowlark) | OR041734 | OR091141 | PP662503 | n/a | n/a | n/a | OR091071 | OR091079 | OR091090 |
| MB21-544 | 7-May-21 | Pierce County | Site 14 | SHB (Meadowlark) | OR041735 | OR091142 | PP662504 | n/a | n/a | n/a | OR091072 | OR091080 | OR091091 |
| MB21-545 | 7-May-21 | Pierce County | Site 14 | SHB (Meadowlark) | OR041736 | OR091143 | PP662505 | n/a | n/a | OR091103 | PP620134 | PP620142 | PP620147 |
| MB21-546 | 25-May-21 | Bacon County | Site 7 | SHB (Farthing) | OR041737 | OR091144 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-561 | 17-May-21 | Bacon County | Site 7 | SHB (Farthing) | OR041738 | OR091145 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-736 | 14-Jun-21 | Bacon County | Site 4 | RE (Brightwell) | OR041739 | OR091146 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-754 | 14-Jun-21 | Bacon County | Site 8 | RE (Brightwell) | OR041740 | OR091147 | PP662506 | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-777 | 17-May-21 | Bacon County | Site 7 | SHB (Farthing) | OR041741 | OR091148 | PP662507 | n/a | n/a | OR091104 | PP620135 | PP620143 | PP620148 |
| MB21-778 | 17-May-21 | Bacon County | Site 7 | SHB (Farthing) | OR041742 | OR091149 | PP662508 | n/a | n/a | n/a | n/a | n/a | n/a |
| MB21-779 | 14-Jun-21 | Bacon County | Site 4 | RE (Brightwell) | OR041743 | OR091150 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |

**Figures**

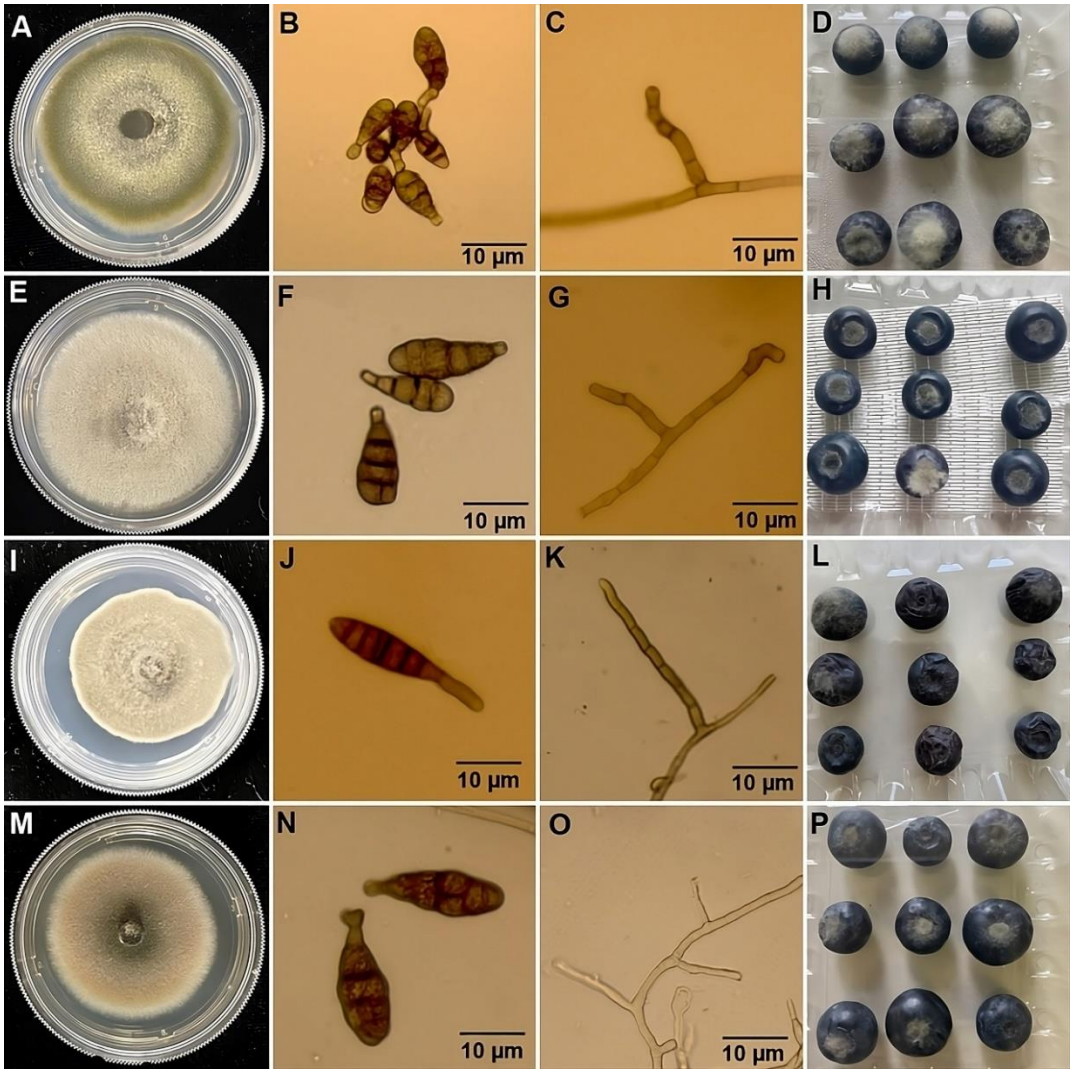


**Figure 2.1.** Morphological features & pathogenicity testing of representative isolates from blueberry of each *Alternaria* species. (A) Colony morphology on potato dextrose agar (PDA) after 5 days incubation at 22°C; (B) conidia; (C) conidiophore; (D) symptoms on blueberry fruits following inoculation with representative isolates after 7 days of incubation at 22°C. Based on morphological characteristics and phylogenetic analysis, the isolate depicted in panels A to D was identified as *A. alternata* (MB21-397); E to H were identified as *A. tenuissima* (MB21-456); I to L were identified as *A. dumosa* (MB21-363); and M to P were identified as *A. limoniasperae* (MB21-475).

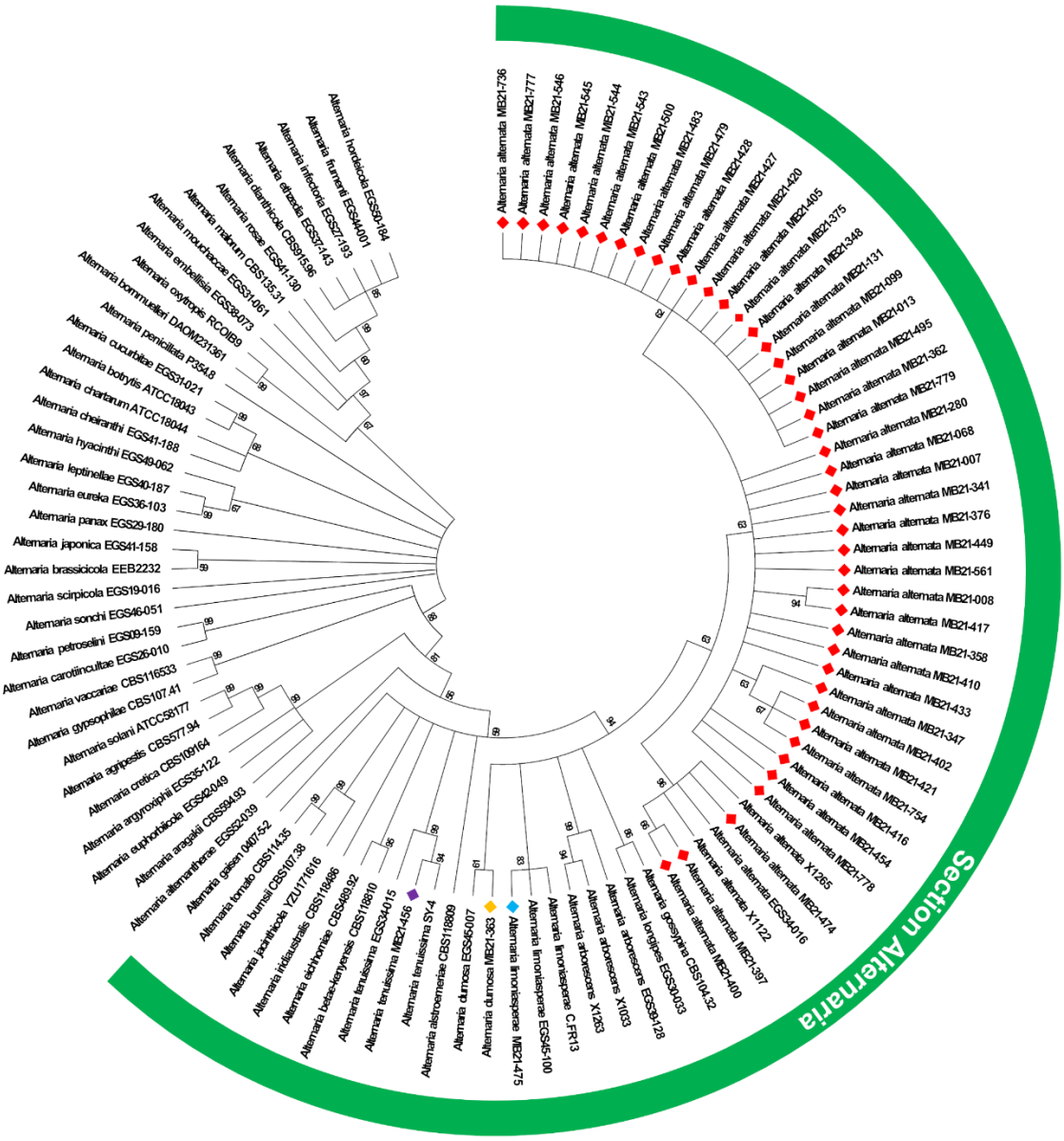


**Figure 2.2.** Maximum likelihood phylogenetic tree based on ATPase gene sequences of isolates from this study alongside other isolates from the genus *Alternaria*. Isolates from this study are indicated by a colored diamond. A red diamond indicates isolates identified as *A. alternata*, and the light blue, orange, and violet diamonds indicate isolates of *A. limoniasperae*, *A dumosa*, and *A. tenuissima*, respectively. Isolates from species within the section *Alternaria* (which is within the genus *Alternaria*) are indicated by the green semi-circle.

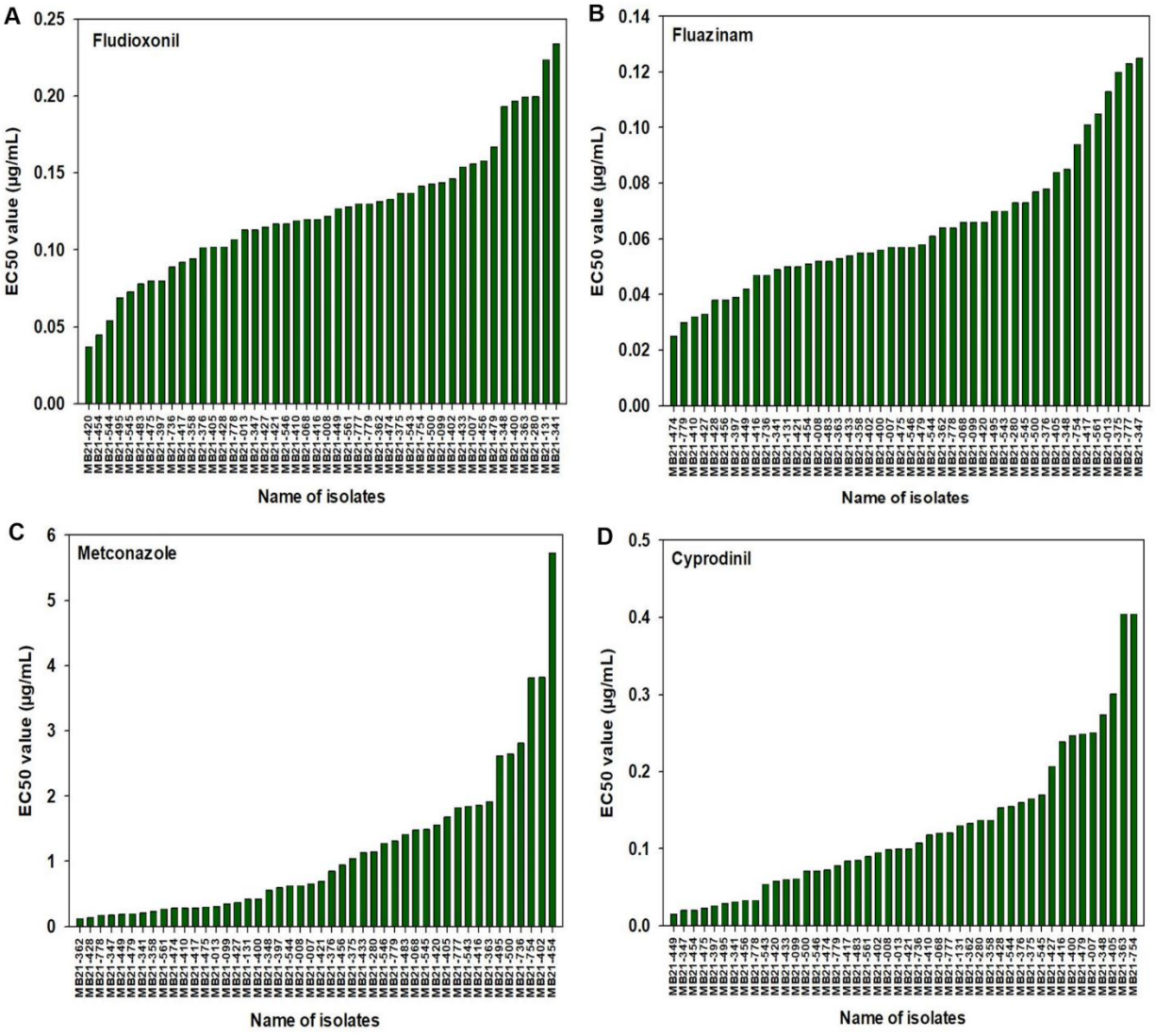


**Figure 2.3**. $EC_{50}$ values determined based on a mycelial growth inhibition assay from all 46 isolates used in this study for: (A) fludioxonil, (B) fluazinam, (C) metconazole, and (D) cyprodinil. Results are depicted for 43 *A. alternata*, one *A. tenuissima* (MB21-456), one *A. dumosa* (MB21-363), and one *A. limoniasperae* (MB21-475).

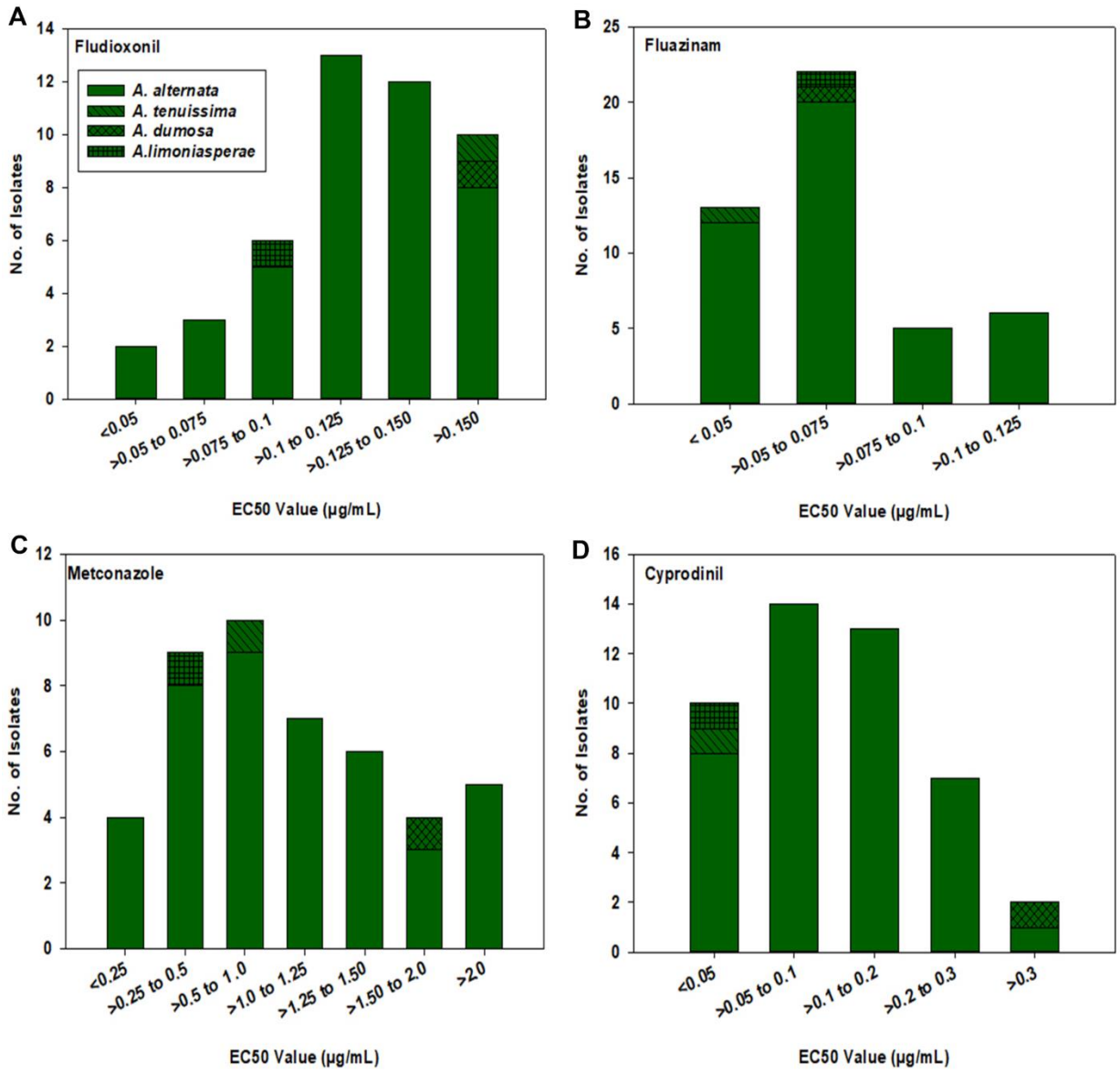


**Figure 2.4**. Frequency distribution of $EC_{50}$ values (based on a mycelial growth inhibition assay) for all 46 *Alternaria* spp. isolates from this study for: (A) fludioxonil, (B) fluazinam, (C) metconazole, and (D) cyprodinil. Results are depicted for 43 *A. alternata*, one *A. tenuissima* (MB21-456), one *A. dumosa* (MB21-363), and one *A. limoniasperae* (MB21-475) as indicated by the figure legend.

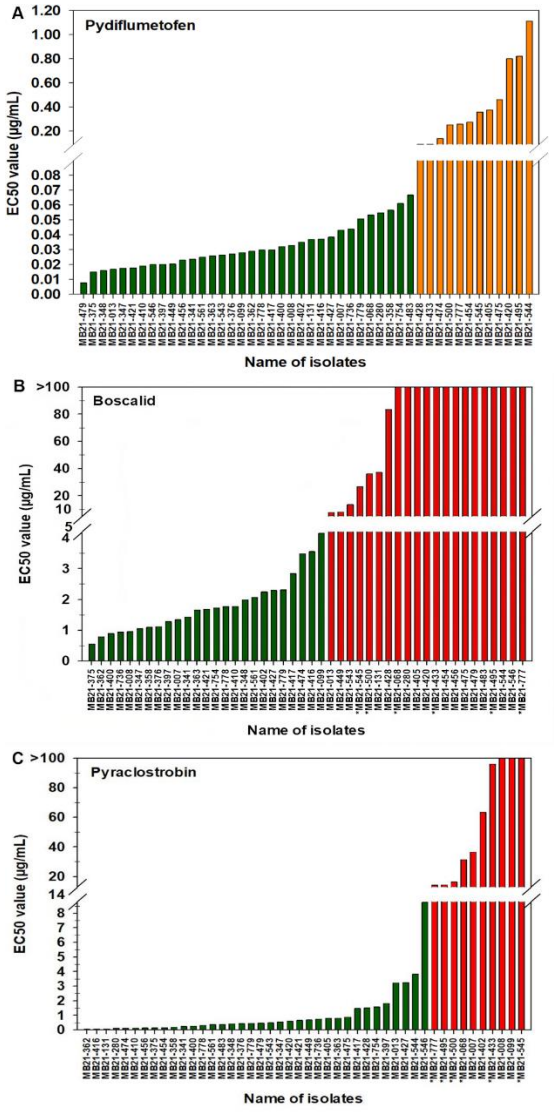


**Figure 2.5**. $EC_{50}$ values from all 46 isolates from this study determined for: (A) pydiflumetofen, (B) boscalid, and (C) pyraclostrobin from all 46 isolates from this study. Values were determined based on a mycelial growth inhibition assay for pydiflumetofen and boscalid and via a spore germination for pyraclostrobin. Results are depicted for 43 *A. alternata*, one *A. tenuissima* (MB21-456), one *A. dumosa* (MB21-363), and one *A. limoniasperae* (MB21-475). An asterisk (*) indicates those isolates that were double resistant to boscalid and pyraclostrobin. Green color indicates sensitive isolates, orange color indicates isolates with reduced sensitivity, and red color indicates resistant isolates.

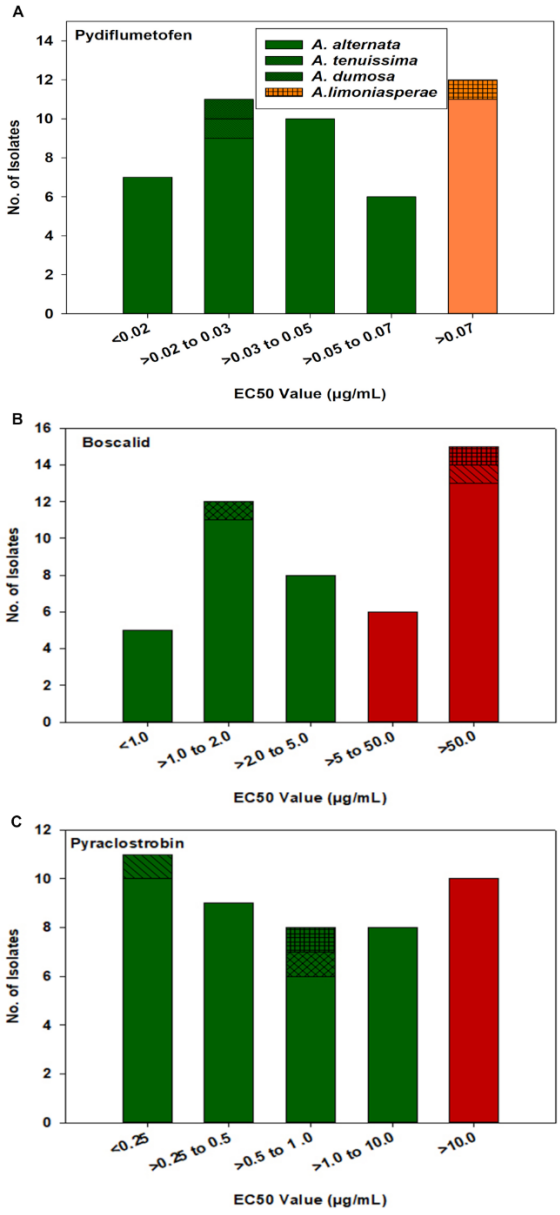


**Figure 2.6**. Frequency distribution of $EC_{50}$ values for all 46 *Alternaria* spp. isolates from this study for (A) pydiflumetofen, (B) boscalid, and (C) pyraclostrobin. Values were determined based on a mycelial growth inhibition assay for pydiflumetofen and boscalid and via a spore germination for pyraclostrobin. Results are depicted for 43 *A. alternata*, one *A. tenuissima* (MB21-456), one *A. dumosa* (MB21-363), and one *A. limoniasperae* (MB21-475) as indicated by the figure legend. Green color indicates sensitive isolates, orange color indicates isolates with reduced sensitivity, and red color indicates resistant isolates.

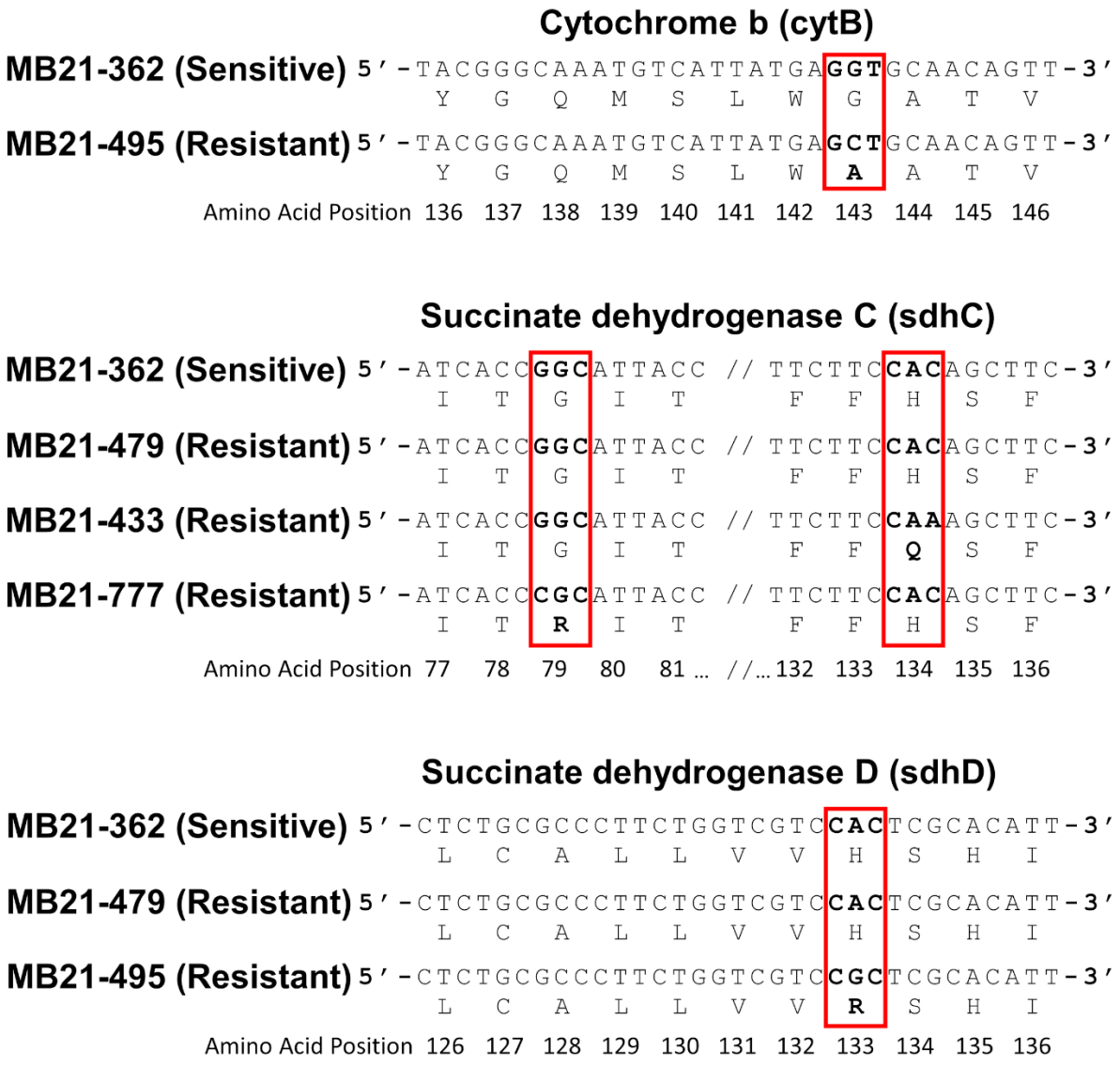


**Figure 2.7**. Partial nucleotide sequences of *cytB*, *sdhC*, and *sdhD* from selected *Alternaria* spp. isolates sequenced in this study alongside the corresponding amino acid within the predicted proteins. The sequences from pyraclostrobin-resistant isolates (including MB21-495; top panel) included a guanine (G) to cytosine (C) change at nucleotide position 123 within the sequenced portion of *cytB* which corresponds with an G143A amino acid change within the predicted protein sequence. Some boscalid-resistant isolates (including isolate MB21-433; middle panel), included a cytosine (C) to adenine (A) change at nucleotide position 228 within the sequenced portion of

*sdhC* which corresponds with an H134Q amino acid change within the predicted protein sequence, while other boscalid-resistant isolates (including MB21-477; middle panel) included a guanine (G) to cytosine (C) change at nucleotide position 61 which corresponds to a G79R amino acid change. Within the sequenced portion of *sdhD*, additional boscalid-resistant isolates (including MB21-495; bottom panel) included an adenine (A) to guanine (G) change at nucleotide position 120 which corresponds to an H133R amino acid change with the predicted protein sequence. No other nucleotide differences expected to result in amino acid changes within cytB, sdhB, sdhC, or sdhD were noted between resistant and susceptible isolates.

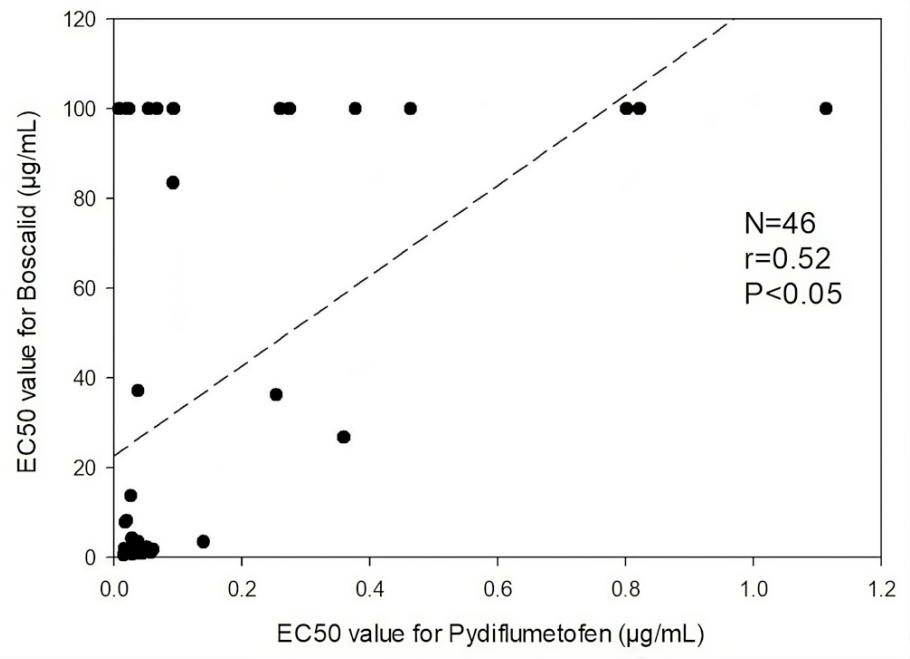


**Figure 2.8**. Graph depicting $EC_{50}$ values for boscalid (y-axis) versus $EC_{50}$ values for pydiflumetofen (x-axis) for each of the 46 *Alternaria* spp. isolates from blueberry tested in this study. Correlation trend line and statistics are indicated.


**Supplementary Figure 2.1.** Collection locations for *Alternaria* spp. isolates utilized in this study. Counties within Georgia (U.S.A.) where isolates were collected are shown in red and numbers of isolates collected in each respective county are indicated in parentheses.


**Supplementary Figure 2.2.** Disease index scale utilized in pathogenicity tests. On the scale: 0 = no fungal growth; 1 = growth restricted within the stem scar site (less than 4 mm growth); 2 = growth covering the entire stem scar site (~4 mm); 3 = growth beyond the stem scar site (>4 mm to 7 mm); 4 = growth covering most of the berry surface, and 5 = mushy berry with growth across the entire berry surface.

# CHAPTER 3

## Investigating the sensitivity of *Botrytis cinerea* isolates to fungicides commonly used for management of gray mold on Georgia blueberries[1]

[1]Beg, M.A., Aktaruzzaman, M., Lewis, K.J., & Oliver, J.E. To be submitted to: *Plant Disease*.

**Abstract**

Georgia is one of the largest producers of blueberries in the U.S., and postharvest fungal diseases negatively impact production. A recent survey found *Botrytis cinerea* to be the most abundant fruit rot pathogen on blueberry in Georgia, and fungicide resistance development is a major concern with this pathogen. The objective of this study was to investigate the sensitivity of the *B. cinerea* isolates to fungicides frequently used for management. A mycelial growth inhibition assay was used to assess the fungicide sensitivity of 60 *B. cinerea* isolates previously collected from rotting blueberries in Georgia. For fludioxonil, cyprodinil, pyraclostrobin, boscalid, and fenhexamid, $EC_{50}$ values were determined to be 0.001-0.991 µg/mL (mean 0.036 µg/mL), 0.001-17.845 µg/mL (mean 1.253 µg/mL), 0.002-7.074 µg/mL (mean 0.852 µg/mL), 0.009-5.163 µg/mL (0.976 µg/mL), and 0.001-3.991 µg/mL (mean 0.33 µg/mL), respectively. Based on these $EC_{50}$ values, all the 60 isolates were sensitive to fludioxonil, boscalid, and pyraclostrobin. Fludioxonil and pyraclostrobin sensitivity was further supported by the absence of resistance-associated mutations the in *bos1* and *cytB* genes, respectively, in selected *B. cinerea* isolates. Though $EC_{50}$ values and mutation analysis did not indicate resistance to boscalid and pyraclostrobin, 49 and 58 isolates, respectively, had minimum inhibitory concentrations (MIC) of >100 µg/mL to these fungicides. For cyprodinil, 50 sensitive, 6 low resistance, 1 weakly resistant, and 5 moderately resistant isolates were identified. Also, there were 48 sensitive, 12 moderately resistant, and 0 highly resistant isolates identified for fenhexamid. The A378T mutation in *erg27*, previously associated with moderate resistance to fenhexamid, was detected in three moderately resistant isolates, but the high-resistance-associated F412S mutation was absent from tested isolates. Gene expression analysis revealed increased expression of *atrB* and the mixed-function oxidase gene BC1G_16062 when *B. cinerea* isolates were induced with fludioxonil and cyprodinil, respectively.

No *atrB* overexpression was observed in fludioxonil-sensitive isolates, whereas BC1G_16062 showed higher expression in an isolate moderately resistant to cyprodinil compared to a sensitive isolate. Information provided by this study reiterates the importance of managing fungicide resistance in blueberry fruit rot pathogens and provides important baseline information regarding the fungicide sensitivity of *B. cinerea* isolates from blueberry going forward.

Keywords: *Botrytis cinerea*, blueberry, fruit rot, fludioxonil, cyprodinil, pyraclostrobin, boscalid, fenhexamid, fungicide resistance

## Introduction

Blueberries are an economically significant commodity in Georgia. According to the 2025 Farmgate Report, blueberries generated a farmgate value of $526 million on over 27,000 cultivated acres, a substantial increase over the $300 million reported in 2018 (UGA 2025). This growth has elevated blueberries to the eighth position in the agricultural commodity rankings in the state, and blueberries hold the highest share in farmgate value among all fruits and nuts produced in Georgia at 47.5% (UGA 2025). Nonetheless, this high-value crop faces several challenges from various diseases, including Phytophthora root rot, mummy berry, bacterial leaf scorch, and multiple postharvest diseases (Sial et al. 2025). Among the fungal diseases that affect blueberries before and after harvest, Botrytis blossom blight and fruit rot, also known as gray mold, is particularly important due to the causal pathogen's polycyclic nature and high conidial production (Harmon 2004; Williamson et al. 2007). The risk of blossom blight is greatest when flowers are in full bloom or early senescence, especially under hot and humid conditions. In some cases, the pathogen may spread from flowers to stems, leading to twig blight and dieback as well. Notably, the fungus can remain latent in developing fruit and may only express symptoms as the fruit ripen or after harvest (Dewey and Grant-Downton 2016).

The most effective management strategies for Botrytis blossom blight and gray mold involve timely fungicide applications and the removal of dead twigs or shoots that can serve as primary inoculum for the following season. According to the Southeastern Blueberry Integrated Management Guide, fungicides such as Switch® (cyprodinil + fludioxonil), Pristine® (pyraclostrobin + boscalid), and Elevate® (fenhexamid) have excellent efficacy against *Botrytis cinerea* (Sial et al. 2025). The active ingredients in these products represent various classes of fungicides, including quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors

(SDHIs), anilinopyrimidines, phenylpyrroles, and hydroxyanilides. However, a concerning issue is that *B. cinerea* has been reported in other regions to have developed resistance, to varying degrees, to all of these fungicide classes (Abbey et al. 2024a; Saito et al. 2016b). In the United States, fungicide resistance in *B. cinerea* isolates from blueberry has been reported in Michigan, where more than 50% of tested isolates were resistant to boscalid and pyraclostrobin, while approximately 20% showed resistance to fludioxonil and fenhexamid (Abbey et al. 2024a). Similarly, in California and Washington, a high proportion of *B. cinerea* isolates from blueberry were determined to be resistant to boscalid and pyraclostrobin (up to 66%), while moderate resistance was found to cyprodinil and fenhexamid. Isolates from California were sensitive to fludioxonil, but reduced sensitivity to this chemical was seen in 70% of Washington isolates (Saito et al. 2016b). Although Georgia is a major blueberry-producing state with the highest cultivated acreage among U.S. states (NASS 2022), no data is currently available on the fungicide resistance status of *B. cinerea* populations from blueberry. Moreover, there is no established baseline information with which to compare $EC_{50}$ values for the fungicides that are currently in use. As such, in order to assess the current sensitivity status of the *B. cinerea* population, the primary objective of this study was to determine the $EC_{50}$ values for *B. cinerea* isolates from Georgia blueberry versus several commonly used fungicides. The determined $EC_{50}$ values are expected to not only provide insight into the present level of *B. cinerea* fungicide sensitivity but also serve as a reference point for future comparisons to enable monitoring and detection of potential resistance development. In addition, a secondary aim of this study was to investigate the molecular mechanisms underlying resistance to some of these fungicides.

## Materials and Methods

### Pathogen isolation

For isolation of *Botrytis cinerea*, blueberries were collected from multiple locations within major blueberry-producing counties in Georgia including Appling, Bacon, and Pierce. Fungal isolates were cultured from symptomatic (rotting) berries on acidified ¼ strength potato dextrose agar (AqPDA). Agar was acidified using 184 microliter lactic acid (85% w/w) per liter. AqPDA plates were incubated for 4 to 5 days at room temperature (~23˚C) to allow for fungal growth. Once fungal growth was observed, pure culture isolates were obtained by hyphal tip cutting and maintained on AqPDA for an additional 3 to 4 days. To store isolates, mycelial plugs (4 mm) were cut from the leading edges of pure fungal colonies and placed in 20% glycerol at 4˚C.

### Morphological identification

For morphological identification, isolates were first cultured on 9 cm Petri plates of potato dextrose agar (PDA) which were sealed with Parafilm and placed at room temperature (~23˚C) for 4 to 5 days to allow for fungal growth. After this, plates were unsealed and kept at the same temperature in 12hr-12hr light-darkness conditions for 7 to 10 additional days to allow for sporulation. Conidial characteristics were observed under a light microscope (Vanguard IS-300, Vanguard Scientific Instruments, Inc.) at 400X magnification.

### Molecular identification

DNA was extracted from 7-day-old PDA cultures of each isolate. Mycelium was scrapped off using a sterile loop and placed into 2-mL microcentrifuge tubes containing approximately twenty 2-mm zirconia/silica ceramic beads (Research Products International, Mount Prospect, IL).

After grinding the sample by shaking for 30 seconds in a Biospec Mini Beadbeater-8 (BioSpec Products, Bartlesville, OK), DNA was extracted using the CTAB (cetyltrimethylammonium bromide) extraction method (Doyle and Doyle 1987). The ITS1 and ITS4 primer set (**Table 3.1**) was used to amplify the internal transcribed spacer (ITS) region containing ITS1-5.8S-ITS2 of nuclear ribosomal DNA (rDNA) (White et al. 1990), and primer pair 262/520L (**Table 3.1**) was used to amplify a portion of the *Bc-hch* gene (Fournier et al. 2003). For PCR, a total reaction volume of 30 µL was used, and each reaction contained 15 µL 2X PCR Master Mix (Promega, Madison, WI), approximately 200 ng of DNA, and 10 mM of each primer (1 µL each). PCR was performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to the previously published reaction conditions for each primer set (Fournier et al. 2003; White et al. 1990). PCR products were visualized in a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium, Fremont, CA) using a Bio-Rad Molecular Image Gel Doc XR+ with Image Lab Software (Bio-Rad Laboratories, Hercules, CA). Products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tec, Inc., Norcross, GA) and Sanger sequenced in both directions by Eurofins Genomics (Louisville, KY). Isolates were initially confirmed as belonging to *Botrytis* spp. by comparison of obtained ITS sequences with publicly available *Botrytis* spp. sequences in the GenBank database using the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST/).

**RFLP (Restriction fragment length polymorphism) characterization**

To differentiate between *Botrytis* spp. group I (comprised of *Botrytis cinerea*) or group II (comprised of *Botrytis pseudocinerea* and *Botrytis californica*), the *Bc-hch* gene was amplified using primer pair 262/520L and restriction digested according to previously published methods (Saito et al. 2016a). This marker distinguishes between two groups of *Botrytis* species based on

fragment patterns: Group I and Group II, both producing six fragments, five of which are identical. The key difference lies in the largest fragment. Group I produces a 517 bp fragment, while Group II yields a 601 bp fragment (Saito et al. 2016a). Briefly, 5 µL of the PCR amplified *Bc-hch* fragment were subjected to restriction digestion using the *HhaI* enzyme (R0139S, New England Biolabs, MA, USA). The 20 µL digestion reaction consisted of 0.1 µL of *HhaI*, 2 µL of the rCutSmart reaction buffer (B6004S, New England Biolabs, MA, USA), and 13 µL of nuclease-free water. The reaction was incubated at 37°C for 90 minutes in a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and visualized in a 1% agarose gel as described previously.

**Pathogenicity test**

Eight *Botrytis cinerea* isolates from five unique locations were selected for pathogenicity testing. For each isolate, store-bought, firm, ripe organic blueberries were selected for inoculation experiments. Berries were surface sterilized by briefly dipping berries twice in 70% ethanol, once in 0.5% sodium hypochlorite, and twice in sterile distilled water. Air dried berries were fixed to the bottom of clamshells with double-sided tape with the stem-scar facing up. Spore suspensions, prepared from the sporulating cultures described in the morphological identification section, were standardized to a concentration of $10^5$ spores per milliliter of water using a hemocytometer. Each berry was inoculated with 20 µL of spore suspension on the stem scar site. For each isolate, three clamshells, which each contained 9 berries (27 berries total) were inoculated. The clamshells were placed in a sealed plastic box at room temperature and two sterile paper towels soaked with sterile distilled water were placed at the bottom of each box to ensure humid conditions. After ~5 days,

the numbers of infected berries with visible fungal growth were counted and the disease incidence for each isolate was recorded.

**Fungicide sensitivity assessment**

Fungicide sensitivity was assessed for 60 selected *B. cinerea* isolates against six commonly used fungicides. These isolates originated from three major blueberry-producing counties in Georgia: Appling (n = 14), Bacon (n = 11), and Pierce (n = 35), representing a total of 19 unique field locations (**Table 3.5**). For sensitivity testing, six technical grade fungicides from Sigma-Aldrich Corp. (St. Louis, MO, USA) were used, including cyprodinil (purity 99.9%), fludioxonil (99.5%), fluazinam (98.4%), boscalid (97.1%), and pyraclostrobin (98.5%). Stock solutions of each (1,000 µg/mL) were first dissolved in acetone and then serially diluted to prepare different fungicide concentrations (**Table 3.2**) in amended PDA plates, alongside non-amended control plates. Concentrations were carefully selected and adjusted based upon reports in the prior literature (Avenot et al. 2018; Kim and Xiao 2010; Ma and Michailides 2005) to range from expected minor growth inhibition to complete growth inhibition for each respective fungicide. Moreover, salicylhydroxamic acid (SHAM) was added at 100 µg/mL (0.01%) to each tested concentration in the pyraclostrobin assay to inhibit the alternative oxidase pathway. Fungicide sensitivity tests were conducted individually for each fungicide and repeated twice for each of the 60 isolates. Mycelial growth inhibition assays were conducted individually for each fungicide for the determination of the $EC_{50}$ values (50% mycelial growth inhibition). For mycelial growth inhibition assays, 4-mm-diameter mycelial plugs were excised from the margins of actively growing pure colonies on PDA and placed upside-down onto fungicide-amended and non-amended (control) PDA media. Plates were incubated at 25 ± 1 °C. After 4 to 5 days of incubation, colony diameters were measured for

each isolate (excluding the original 4 mm plug), and percent inhibition (PI) was calculated for each fungicide concentration using the following formula:

$$PI = \frac{(a - b)}{a} \times 100$$

where a is the colony diameter on the control plate, and b is the diameter on the fungicide-amended plate.

The $EC_{50}$ value for each isolate against each fungicide was determined based on the percent inhibition observed at each fungicide concentration. Relative growth inhibition was regressed against the $log_{10}$-transformed fungicide concentrations, and $EC_{50}$ values for each isolate-fungicide combination were determined using a probit regression approach based on Finney's method (Finney 1952), with the aid of a spreadsheet calculator (Mekapogu 2021).

**Mutation identification (fludioxonil-bos1, cytB- pyraclostrobin, erg 27-fenhexamid)**

To determine whether *B. cinerea* isolates harbor mutations associated with resistance to fludioxonil, fenhexamid, and pyraclostrobin, partial sequences were obtained from the *bos1*, *erg27*, and *cytB* genes of selected isolates. PCR amplification and sequencing was performed according to the methodology described above with the respective primers listed in **Table 3.1**, using the previously published reaction conditions for each primer pair (Cosseboom and Hu 2021; Fillinger et al. 2008; Grabke et al. 2013; Leroux et al. 2010; Ma et al. 2007). The portions of *bos1* and *erg27* targeted for amplification corresponded to the portions of these genes encoding amino acids 288 to 569 for *bos1* and amino acids 281 to 474 for *erg27*. The portion of *cytB* targeted for amplification corresponded to the portions of the gene encoding amino acids 133 to 163 and, if present, would include the *Bcbi*-143/144 intron (Leroux et al. 2010).

**Gene Expression Analysis by qRT-PCR**

**atrB Expression in Response to Fludioxonil**

To assess the expression of the *atrB* gene in response to fludioxonil, four *B. cinerea* isolates were selected based on their determined $EC_{50}$ values (**Table 3.3)**. Although all were phenotypically categorized as sensitive, they represented the highest (MB21-184, and MB21-668) and lowest (MB21-226, MB21-486) $EC_{50}$ values to fludioxonil among our tested isolates (**Table 3.3**). Isolates were grown from transferred mycelium on potato dextrose agar (PDA) amended with either 0 ppm (control) or 0.05 ppm fludioxonil. Following incubation, mycelial tissue was harvested for RNA extraction on day 5.

Total RNA was extracted using the E.Z.N.A.® Plant RNA Kit (R6827, Omega Bio-tek, Inc., Norcross, GA, USA), following the manufacturer's protocol. RNA concentration and purity were assessed using a Thermo Scientific NanoDrop One$^{C}$ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA) on a CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). The reactions were set up and run using the CFX Maestro software (Bio-Rad Laboratories, Hercules, CA, USA). Gene-specific primers were used to amplify *atrB* (**Table 3.1**), and expression levels were normalized versus the housekeeping gene β-tubulin (Li et al. 2014). Three technical replications of each reaction were conducted.

The thermal cycling conditions for *atrB* expression analysis included an initial reverse transcription at 50°C for 10 minutes, followed by polymerase activation at 95°C for 1 minute. Amplification was carried out for 39 cycles of 95°C for 10 seconds and 60°C for 30 seconds. A melting curve analysis was performed from 65°C to 95°C with temperature increments of 0.5°C

every 0.05 seconds. A paired Student's t-test was performed using R (version 4.4.3) to determine if there were any statistically significant differences between the uninduced and induced expression of the gene across all isolates.

**BC1G_16062 (a mixed functional oxidase gene) and BC1G_12366 (a transmembrane gene) Expression in Response to Cyprodinil**

For cyprodinil-associated gene expression analysis, two *B. cinerea* isolates were selected: MB21-159 (sensitive; $EC_{50}$ = 0.001 ppm) and MB21-252 (moderately resistant; $EC_{50}$ = 17.845 ppm). MB21-159 was cultured from mycelium on PDA without cyprodinil and on PDA with 0.01 ppm cyprodinil, while MB21-252 was cultured from mycelium on PDA without cyprodinil and on PDA with 17.845 ppm cyprodinil. Mycelial tissue was harvested after incubation for RNA extraction.

RNA extraction and cDNA synthesis were performed as described above, using the same reagents, equipment, and software. Gene-specific primers (**Table 3.1**) were used to amplify BC1G_16062 (a mixed functional oxidase gene) and BC1G_12366 (a transmembrane gene), with β-tubulin serving as the internal reference (Li et al. 2014; Wang et al. 2018). Three technical replications for each reaction were performed. The thermal cycling conditions for BC1G_16062 and BC1G_12366 expression included an initial reverse transcription at 50°C for 10 minutes, followed by polymerase activation at 94°C for 30 seconds. Amplification consisted of 39 cycles of 94°C for 30 seconds and 60°C for 30 seconds. Melting curve analysis was carried out from 68°C to 95 °C, with 0.5°C increments every 5 seconds.

To determine whether there were significant differences in gene expression, pairwise Student's t-tests were conducted separately for BC1G_16062 and BC1G_12366 using R (version

4.4.3). For each gene, comparisons were made between (i) sensitive vs. moderate-resistant isolates under non-induced (without fungicide) conditions, (ii) sensitive vs. sensitive-induced (with fungicide) conditions, and (iii) moderate-resistant vs. moderate-resistant-induced conditions.

## Results

### Pathogen isolation, morphological identification and pathogenicity testing

In 2021, a total of 155 *B. cinerea* isolates were recovered from symptomatic blueberry fruit collected across three major blueberry-producing counties in Georgia: Pierce (n = 77), Appling (n = 39), and Bacon (n = 39). In terms of host background, 125 isolates were from southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) cultivars, while 30 were collected from rabbiteye blueberry (*V. virgatum*) cultivars. Isolates were obtained from 19 unique locations within these counties. Of these, 36 isolates originated from sites managed organically, while the remaining 119 isolates were collected from blueberries managed conventionally. The fungal isolates were fast-growing, typically producing creamy, soft-textured mycelium that filled the PDA plate within 5 to 7 days. Initially, the cultures appeared white, but upon maturation, they developed whitish, brownish, or dark gray coloration. Of the original 155 isolates, 117 isolates were characterized for colony type. Predominantly, three distinct colony types were observed (**Figure 3.1**). These included:

1. Conidial type – characterized by rapid and abundant spore production, often resulting in dark gray colonies
2. Sclerotial type – producing characteristic sclerotia with noticeable concentric zonation
3. Mycelial type – displaying a creamy texture and lacking both spores and sclerotia

Among all isolates, mycelial type colonies were the most common (n = 63), followed by conidial type colonies (n = 32) and sclerotial type colonies (n = 22). Pathogenicity testing of 8 isolates which included representatives of each of these morphotype indicated 100% disease incidence. Fungal growth was observed at 2 dpi on the stem scar site inoculated with fungal spores. This growth eventually overtook over the whole berry by 7 dpi. No fungal growth was observed in the uninoculated berries.

**Molecular identification**

All isolates were initially identified as *Botrytis* species based on ITS sequencing and obtained sequences (**Table 3.5**) indicated 100% similarity to *Botrytis* species in the NCBI database. Further confirmation was performed using amplification of the *Bc-hch* gene, which yielded an expected amplicon of 1171 bp (Fournier et al. 2003). In our study, all 60 tested isolates produced the 517 bp fragment, indicating that all isolates belonged to Group I and were *B. cinerea* (**Figure 3.2**).

**Fungicide sensitivity of *B. cinerea* isolates**

Of the 60 *B. cinerea* isolates tested for fungicide sensitivity, all were found to be sensitive to fludioxonil, with $EC_{50}$ values ranging from 0.001 to 0.991 µg/mL (**Figure 3.3A**). The mean $EC_{50}$ value was 0.04 µg/mL, with a standard deviation of 0.13 µg/mL. The frequency distribution exhibited a unimodal pattern, with 55 out of 60 isolates showing $EC_{50}$ values below 0.05 µg/mL (**Figure 3.4A**).

For pyraclostrobin, the $EC_{50}$ values among the 60 *Botrytis cinerea* isolates ranged from 0.002 to 7.074 µg/mL (**Figures 3.3B and 3.4B**). Since none of the $EC_{50}$ values exceeded 10 µg/mL

(Yue et al. 2024), all isolates were considered sensitive to this QoI fungicide. The mean $EC_{50}$ was 0.85 µg/mL, with a standard deviation of 1.23 µg/mL. Although $EC_{50}$ values were low, indicating sensitivity, the minimum inhibitory concentration (MIC, the lowest concentration required to completely inhibit fungal growth) was relatively high (**Figure 3.5A**). Even at the maximum tested concentration of 100 µg/mL, 58 out of 60 isolates were not fully inhibited, resulting in MIC > 100 µg/mL for the majority of isolates (**Figure 3.6**). The dose–response curve further confirmed this observation: while approximately 50% growth inhibition was achieved at 0.1 µg/mL, even the highest concentration tested (100 µg/mL) failed to suppress fungal growth beyond 80% (**Figures 3.5A and 3.6**).

For boscalid, the $EC_{50}$ values ranged from 0.009 to 5.163 µg/mL, with a mean $EC_{50}$ of 0.85 µg/mL and a standard deviation of 0.97 µg/mL (**Figures 3.3C and 3.4C**). All isolates were considered sensitive based on their low $EC_{50}$ values. However, similar to the result for pyraclostrobin, despite the apparent sensitivity based on $EC_{50}$ values, 49 isolates exhibited MIC values exceeding 100 µg/mL. The growth response curve and heatmap visualization revealed that although 0.5 µg/mL achieved approximately 50% growth inhibition, even the highest tested concentration of 100 µg/mL failed to suppress growth beyond 90% for most isolates (**Figures 3.5B and 3.6)**.

For cyprodinil, the $EC_{50}$ values of the 60 *B. cinerea* isolates ranged from 0.001 to 17.845 µg/mL, with a mean of 1.25 µg/mL and a standard deviation of 3.55 µg/mL (**Figure 3.3D**). Based on the classification proposed by Avenot et al. (2018), where $EC_{50}$ values below 1 µg/mL were considered sensitive, 1–5 µg/mL as low resistant, 5–10 µg/mL as weakly resistant, and 10–50 µg/mL as moderately resistant, 50 isolates in our study were found to be sensitive, 6 were low resistant, 1 was weakly resistant, and 3 were moderately resistant. The frequency distribution

showed a nearly unimodal pattern (right-skewed), with only a few isolates exhibiting higher $EC_{50}$ values (**Figure 3.4D**).

For fenhexamid, $EC_{50}$ values among the 60 *B. cinerea* isolates ranged from 0.001 to 3.991 µg/mL, with a mean of 0.33 µg/mL and a standard deviation of 0.71 µg/mL (**Figure 3.3E**). According to the classification scale proposed by Yin et al. (2016), which defines $EC_{50}$ values below 0.4 µg/mL as sensitive and values between 0.4 and 4 µg/mL as moderately resistant, 48 isolates were found to be sensitive, while 12 were moderately resistant. The frequency distribution of $EC_{50}$ values had a unimodal pattern (**Figure 3.4E**).

**Mutation Identification**

A total of thirteen *B. cinerea* isolates were sequenced to identify mutations in the *erg27* gene that might be associated with resistance to fenhexamid (**Tables 3.4 and 3.5**). Among these, the five sensitive isolates had $EC_{50}$ values ranging from 0.064 to 0.157 µg/mL. None of these possessed detectable mutations within *erg27*. Sequences were also obtained from eight isolates showing moderate resistance to fenhexamid, with $EC_{50}$ values ranging from 0.451 to 3.991 µg/mL. These isolates came from seven unique locations. Among this group of moderately resistant isolates, three isolates (MB21-668, MB21-746, and MB21-748) carried the A378T mutation, which has been previously reported to be associated with partial resistance to fenhexamid in *B. cinerea* (Yin et al. 2016). In addition, MB21-748 also contained an E375K substitution. The F412S mutation, a known marker of high-level resistance to fenhexamid, was not detected in any of the isolates. None of the nine pyraclostrobin-sensitive isolates tested carried the G143A mutation within *cytB* (**Table 3.5**). Similarly, no mutation was detected within *bos1* among the sequences obtained from eleven *B. cinerea* isolates with varying $EC_{50}$ values (**Table 3.5**).

**Gene expression of *atrB* with or without fludioxonil**

Among all tested isolates, MB21-226, MB21-486, and MB21-184 showed significantly higher levels of *atrB* gene expression when induced by fludioxonil, while no significant difference in expression was observed for isolate MB21-668 in the presence of fludioxonil (**Figure 3.7**). Specifically, isolate MB21-226 showed approximately a 3-fold increase in expression, MB21-486 exhibited about a 2-fold increase in expression, and MB21-184 demonstrated nearly an 8-fold increase in *atrB* expression following induction with 0.05 µg/mL of fludioxonil. Notably, in the absence of fludioxonil, no differences in *atrB* expression were observed between the isolates, regardless of whether they were previously observed to have relatively higher or lower $EC_{50}$ values versus fludioxonil.

**Gene expression of BC1G_16062 and BC1G_12366 with or without cyprodinil**

In the absence of fungicide, the moderately resistant isolate MB21-252 showed slightly higher expression of a mixed functional oxidase (MFO) gene BC1G_16062, which is more than twice that of the sensitive isolate MB21-159 (**Figure 3.8**). However, upon induction with 0.01 µg/mL cyprodinil, MB21-159 exhibited more than a 5-fold increase in gene expression. In contrast, induction with 17.845 µg/mL cyprodinil resulted in about a 3-fold increase in BC1G_16062 expression in the moderately resistant isolate MB21-252.

In the absence of fungicide, the sensitive isolate MB21-159 and the moderately resistant isolate MB21-252 showed no difference in the expression of the transmembrane gene BC1G_12366 (**Figure 3.8**). However, upon induction with 0.01 µg/mL cyprodinil, MB21-159 exhibited more than a 5-fold increase in gene expression. In contrast, no significant change in

BC1G_12366 expression was observed in the moderately resistant isolate MB21-252 following induction.

**Discussion**

*Botrytis cinerea* is a major fungal pathogen responsible for postharvest diseases in blueberries. It causes Botrytis blossom blight and gray mold in blueberries, posing significant challenges during both production and postharvest storage and distribution. Managing this pathogen is particularly difficult due to its ubiquitous presence. *B. cinerea* acts as both a pre- and postharvest pathogen in many fruit and vegetable cropping systems. It produces a large amount of spores which gives it the advantage to spread quickly and infect easily. In fact, a clamshell containing just one infected berry can release enough spores to contaminate the entire clamshell, rendering it unconsumable. However, although more than 30 different *Botrytis* species have been identified (Walker 2016), only a few have been reported in association with blueberries. Among them, *Botrytis cinerea* is most commonly linked to gray mold on blueberries, with occasional reports of *B. pseudocinerea* and *B. californica* (Saito et al. 2014; Saito et al. 2016a). For example, a 2012–2013 survey in Washington and California found that 98.3% (n = 656) of the isolates were *B. cinerea* causing disease on blueberries (Saito et al. 2016b). In our study, all 60 isolates tested showed the 517 bp RFLP band following digestion of the *Bc-hch* amplicon, characteristic of group II isolates, and as such we identified them as *B. cinerea*.

Despite the fact that all fungal isolates in our study were identified as *B. cinerea*, three different isolate types (mycelial-type, conidial-type, and sclerotial type) were identified based on their pattern of culture growth on PDA. A similar classification of three distinct morphological types in *B. cinerea* populations has been reported from kiwifruit in China, where isolates were also

grouped into three categories based on colony characteristics (Pei et al. 2019). Likewise, *Botrytis* isolates collected from blueberries in Michigan displayed the same three morphotypes. Interestingly, in that study, some isolates lacking conidia were categorized as part of an undescribed novel group of *Botrytis* species (Abbey et al. 2024a) and were recovered exclusively from blossoms and not from fruit. While the mycelial-type isolates described by Abbey et al. (2024a) were found in low numbers, which the authors suggested could indicate a fitness cost related to a possible inability to survive, reproduce through the growing season, and ultimately infect fruit due to their lack of conidial production, in our study, isolates belonging to the mycelial type accounted for 54% of isolates. These isolates lacked both conidia and sclerotia, at least under the conditions they were subjected to in the laboratory. This may indicate an adaptive advantage for colonizing fruit surfaces or thriving in postharvest environments where spore or sclerotial formation is less essential. By contrast, a substantial number of isolates exhibited either the conidial type (n = 32) or sclerotial type (n = 22) morphology in our study, both of which may confer advantages related to reproduction, dispersal, or long-term survival. These three morphological types may also differ in their virulence and epidemiological potential; however, further investigation is needed to determine whether they are associated with specific genetic lineages or levels of aggressiveness on blueberry.

Although cultural practices such as sanitation, avoiding machine harvesting, and careful postharvest handling can reduce the risk of postharvest diseases, the primary strategy to prevent both pre- and postharvest fruit rot remains the utilization of an effective fungicide spray program. As such, and given that no comprehensive in vitro sensitivity testing has been reported for *B. cinerea* populations from any crop in Georgia, we determined the $EC_{50}$ values for several commonly used fungicides against *B. cinerea*. This not only provides a more comprehensive

understanding of the current sensitivity status but also serves as a reference for monitoring potential shifts toward resistance in the future.

Fludioxonil is a highly effective fungicide for managing gray mold in various cropping systems, including blueberries (Kim et al. 2016). Although resistance to fludioxonil has been documented in *B. cinerea*, it remains relatively uncommon. A survey in Shanghai reported resistance in over one-third of the field isolates, including both moderately and highly resistant strains (Wang et al. 2021). Resistance to fludioxonil has also been reported in *B. cinerea* isolates from blackberry in Georgia (Fernández-Ortuño et al. 2014b) and from strawberries in Virginia, Maryland, and South Carolina (Fernández-Ortuño et al. 2013; Fernández-Ortuño et al. 2014a). In contrast, *B. cinerea* isolates from Michigan were consistently found to be sensitive to fludioxonil from 2019 to 2022 (Abbey et al. 2024a). Additionally, 100% of the *B. cinerea* populations from grape, pistachio, and pomegranate were reported to be sensitive to fludioxonil (Avenot et al. 2018). In our study, all 60 isolates were also found to be sensitive to this fungicide based on both $EC_{50}$ and MIC values. The highest $EC_{50}$ value observed was 0.991 µg/mL, which is considerably lower than the values reported for resistant isolates in previous studies. For example, Zhou et al. (2020) used 5 µg/mL as a discriminatory dose to identify resistant isolates, and their reported $EC_{50}$ values for resistant strains ranged from 18 µg/mL to over 100 µg/mL. In contrast, none of the isolates in our study exhibited growth at 1 µg/mL, confirming their sensitivity. Furthermore, mutations such as I365N/S, Q369P, and N373S in the *bos1* gene, previously linked to fludioxonil resistance (Wang et al. 2021), were not detected in any of the isolates we examined. The *atrB* gene, which encodes a membrane-bound drug efflux pump known as an ATP-binding cassette (ABC) transporter, has also been associated with resistance to fludioxonil (Li et al. 2014), with reports of 200-fold increases in expression of *atrB* in moderately resistant isolates and 30- to 100- fold overexpression

in low-resistant isolates compared to a sensitive isolate. In our study, although there was a trend of increased expression upon fungicide induction with fludioxonil, none of the tested isolates exhibited more than a 10-fold overexpression, which again confirms their sensitivity to fludioxonil.

Pyraclostrobin, a QoI fungicide widely used for managing gray mold in blueberries, has proven to be highly effective (Abbey et al. 2024b); however, due to its single-site mode of action, it carries a high risk for resistance development. Resistance in various pathogens, including *B. cinerea*, has been reported, most commonly through the G143A point mutation in the *cytB* gene. A study on *B. cinerea* isolates from blueberries reported a low frequency of sensitivity to pyraclostrobin (Abbey et al. 2024a). In 2019, 35% of the isolates were sensitive, but this number dropped drastically in 2020 and 2022, with only 8% and 11% of collected isolates being sensitive. Similarly, Cosseboom and Hu (2021) found that 92% of *B. cinerea* isolates collected from small fruits across the mid-Atlantic U.S. (2014–2019) were resistant to pyraclostrobin based on a discriminatory dose of 10 µg/mL. Among the *B. cinerea* isolates in our study, we found lower $EC_{50}$ values for pyraclostrobin, ranging from 0.002 to 7.074 µg/mL, suggesting sensitivity. Based on the scale proposed by Yue et al. (2024), which considers isolates with $EC_{50}$ values greater than 10 µg/mL as moderately resistant and greater than 100 µg/mL as highly resistant, all our isolates would be classified as sensitive. However, it should be noted that this interpretation would change if we had determined resistance using a 10 µg/mL discriminatory dose, as 58 out of 60 isolates grew at this concentration and the MIC exceeded 100 µg/mL for these isolates. Although the growth inhibition curve showed a sharp decline up to 1 µg/mL, there was little additional inhibition between 1, 10, and 100 µg/mL. While this response is atypical, it is not unprecedented, and a similar plateau in inhibition was observed in *Colletotrichum gloeosporioides* against fludioxonil,

where no significant difference in growth was found from 1 to 100 µg/mL (Schnabel et al. 2021). As stated, resistance to QoI fungicides is most commonly associated with the G143A substitution (Grasso et al. 2006), and it is well established that the *Bcbi*-143/144 intron hinders mutation at this codon in some *B. cinerea* isolates. In *B. cinerea*, three *cytB* genotypes have been described: isolates lacking both the G143A mutation and the *Bcbi*-143/144 intron ($S^-/I^-$), isolates with the intron but no mutation ($S^-/I^+$), and isolates with the mutation but no intron ($S^+/I^-$) (Leroux et al. 2010). In our study, we did not detect the G143A mutation or the *Bcbi*-143/144 intron in any of the isolates. Therefore, all isolates were of the $S^-/I^-$ phenotype. Although we classified our isolates as sensitive to pyraclostrobin based on the absence of the G143A mutation and their overall growth inhibition response, the relatively high MICs we observed for a large number of isolates suggests some type of partial insensitivity, and this complex or emerging resistance pattern should be investigated further.

Boscalid is an SDHI fungicide which is considered to have medium to high risk of development of resistance by fungal pathogens (FRAC 2024). In our study, the highest $EC_{50}$ value recorded for *B. cinerea* versus boscalid was around 5 µg/mL. Based on the scaling used by Liu et al. (2021), where $EC_{50}$ values less than 5 µg/mL were considered sensitive, all of our isolates were classified as sensitive. The growth response curve also showed that fungal growth was inhibited by more than 50%, even at a concentration of 1 µg/mL. However, similar to what was observed for pyraclostrobin, there was a sharp increase in growth inhibition up to 5 µg/mL, followed by a plateau from 5 to 100 µg/mL. Additionally, 100 µg/mL could reduce growth by up to only ~90% in 48 isolates, meaning the MIC for all these isolates was greater than 100 µg/mL. While this does represent somewhat better sensitivity for boscalid than for pyraclostrobin (where at the highest concentration only up to 80% of fungal growth was inhibited), it still suggests that a considerable

portion of our isolates were somewhat insensitive to this fungicide as well. Both pyraclostrobin and boscalid are components of the fungicide Pristine®, which is widely used in commercial blueberry production. Although neither active ingredient alone could completely inhibit fungal growth in our study, their combination may remain somewhat effective, as they both did significantly reduce fungal growth in our study, as reflected by their low $EC_{50}$ values. There have been no reports of gray mold control failure in blueberry fields in Georgia, and it is unclear at this time if the insensitivity we observed in vitro will impact control in the field. Nonetheless, since fungal growth is not completely inhibited by these fungicides, the portion of the population that survives could be repeatedly exposed to these fungicides and face high selection pressure, potentially leading to reduced sensitivity over time. In Michigan, *B. cinerea* isolates from blueberries showed the highest frequencies of resistance (>50%) to both components of Pristine® among the eight fungicides tested (Abbey et al. 2024a). This highlights the importance of using tank mixes, applying proper dosages, and rotating or combining with other fungicides to minimize the risk of resistance development with these fungicides.

Cyprodinil, an anilinopyrimidine fungicide, acts by inhibiting methionine biosynthesis. Due to its single-site mode of action, it is effective in managing gray mold in blueberries but also carries a high risk of resistance development. Resistance to cyprodinil has already been reported in *B. cinerea* populations from Michigan blueberries (Abbey et al. 2024a). In our study, $EC_{50}$ values ranged from as low as 0.001 µg/mL to as high as 17.845 µg/mL. Among the 60 isolates tested, 50 were classified as sensitive, while six showed low resistance, one was weakly resistant, and three were moderately resistant. A similar resistance trend was observed in *B. cinerea* isolates from California grape, pistachio, and pomegranate, where 83% of isolates were sensitive to cyprodinil, and 13%, 3%, and 1% showed low, weak, and high levels of resistance, respectively

(Avenot et al. 2018). Target gene mutation analysis associated with cyprodinil resistance has not yet been reported, but limited gene expression studies are available (Wang et al. 2018). Gene expression analysis of BC1G_16062, a mixed functional oxidase gene previously linked to cyprodinil resistance (Wang et al. 2018), further supports these findings. In our study, both the sensitive and moderately resistant isolates showed increased expression of this gene when induced with cyprodinil. However, in the absence of the fungicide, the moderately resistant isolate exhibited more than a 2-fold higher expression compared to the sensitive isolate. In contrast, no such trend was observed for another gene that had been previously associated with resistance to cyprodinil (Wang et al. 2018), the transmembrane gene BC1G_12366. Although we did not detect any highly resistant isolates, the presence of 10 isolates with low to moderate levels of resistance indicates a potential shift in the population toward reduced sensitivity. If proper fungicide rotations and resistance management strategies are not followed, the proportion of resistant isolates may increase in the coming years, and it will be important to use the reference point developed through this study to monitor continued changes in sensitivity to this fungicide.

Fenhexamid is commonly used for gray mold control due to its specific action on the ergosterol biosynthesis pathway. It belongs to the hydroxyanilide class of fungicides and works by inhibiting the 3-ketoreductase enzyme encoded by the *erg27* gene. In our study, 48 isolates were found to be sensitive to this fungicide, while 12 were classified as moderately resistant; however, no highly resistant isolates were detected. A similar trend was reported in *B. cinerea* isolates from Michigan blueberries, where 99% and 100% of the isolates were sensitive to fenhexamid in 2019 and 2020, respectively, but the sensitivity dropped slightly to 92% in 2022. In another study from China (Yin et al. 2016), $EC_{50}$ values ranged from 0.038 to 4.842 µg/mL, which is comparable to our range of 0.001 to 3.991 µg/mL. Based on $EC_{50}$ values, they identified 74 moderately resistant

isolates across nine different locations, with resistance frequencies ranging from 0% to 37%. Among these, 62 moderately resistant isolates (83.8%) carried both the P57A and A378T mutations in the *erg27* gene. Transformation assays further confirmed that inserting a single copy of *erg27* carrying these mutations into a wild-type strain led to partial resistance. In our study, three isolates were found to carry the A378T mutation. The presence of the A378T mutation in some isolates suggests the occurrence of moderate resistance to fenhexamid in the population, and the absence of F412S indicates that isolates with high resistance levels to fenhexamid are yet to emerge in *B. cinerea* populations infecting blueberries in Georgia.

In summary, all 60 *B. cinerea* isolates tested in this study were sensitive to fludioxonil, confirming its continued effectiveness. Although the $EC_{50}$ values for boscalid and pyraclostrobin were generally low, a high number of isolates (49 and 58, respectively) exhibited minimum inhibitory concentrations above 100 ppm, raising concerns about potential insensitivity and growing resistance development. For cyprodinil, a range of resistance levels was detected, including some low-resistant, weakly resistant, and moderately resistant isolates, and in the case of fenhexamid, some isolates displayed moderate resistance and the A378T mutation was identified. Overall, since the majority of isolates remain sensitive to the tested fungicides, they should continue to be effective components in blueberry spray programs when used in combination with other fungicides. However, if a high enough proportion of resistant isolates become present in a given location, control failures could occur. Since control failures against *B. cinerea* in Georgia have not been reported to date, the use of fungicide mixtures in spray programs, where the limited portion of the population exhibiting reduced sensitivity is suppressed by the complementary action of other fungicides, is continuing to provide effective control. Nonetheless, the detection of emerging resistance reported here, although currently limited, underscores the

importance of resistance management strategies including tank mixes, rotating fungicides with different modes of action, and avoiding overreliance on single-site fungicides. In addition, the $EC_{50}$ values generated in this study provide a valuable reference point for future monitoring and will be critical for detecting potential shifts in *B. cinerea* sensitivity in Georgia blueberry production systems going forward.

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## Tables

**Table 3.1.** Primers used in this study, including the amplified region, primer name, sequence, and reference.

| Sequenced region | Primer Name | Sequence (5'-3') | Reference |
|---|---|---|---|
| ITS region | ITS1 | TCCGTAGGTGAA CCTGCGG | White et al. 1990 |
| | ITS4 | TCCTCCGCTTA TTGATATGC | |
| *Bc-hch* | 262 | AAGCCCTTCGATGTCTTGGA | Fournier et al. 2003 |
| | 520 L | ACGGATTCCGAACTAAGTAA | |
| *bos1* | BF2 | CAACGTTATGGCACAAAATCTCA | Ma et al. 2007 |
| | BR2 | AAGTTTCTGGCCATGGTGTTCA | |
| *erg27* | Erg27beg | TGGGATTACCACCATGGGAGACAAGTG | Fillinger et al. 2008 |
| | Erg27end2 | CAATGGTTCCGCATTTCTTTGCCTCC | Cosseboom and Hu 2021 |
| | F412_F | GACATTACGTTCTCGCACACG | Grabke et al. 2013 |
| | F412_R | CAACCAGGAACTTCGGTTCG | |
| *cytB* | Qo13ext | GGTATAACCCGACGGGGTTATAGAATAG | Leroux et al. 2010 |
| | Qo14ext | AACCATCTCCATCCACCATACCTACAAA | |
| β-tubulin | Tubulin-F | GAGCTGTTTTCCCTTCCATTGTC | Li et al. 2014 |
| | Tubulin-R | GACGACACCGTGCTCGATTGG | |
| *atrB* | atrBfor | GCACTTGTGGCGAGTATCTATC | Li et al. 2014 |
| | atrBrev | TGCATCCCTCCATCCATAGC | |
| BC1G_16062 | BC1G_16062-F | CGGCCTCAAGTAAGATTCCCT | Wang et al. 2018 |
| | BC1G_16062-R | GGTAGGTCTCCATCCAAGCG | |
| BC1G_12366 | BC1G_12366-F | TACTTACGGGCAGCAACCAT | Wang et al. 2018 |
| | BC1G_12366-R | ACTGACTGATTGCGTGCTTC | |

**Table 3.2.** Fungicide product commonly utilized for Botrytis blossom blight and fruit rot control in blueberry production, active ingredient, FRAC mode of action and concentrations of active ingredient used in the mycelial growth inhibition assays conducted as a part of this study.

| Trade Name | Active Ingredient | Group (FRAC MoA) | Concentrations Used in Mycelial Growth Inhibition Assay (µg/µl) |
|---|---|---|---|
| Elevate | Fenhexamid | Hydroxyanilides (17) | 0.05, 0.1, 0.5, 1, 5, 10, 50 |
| Omega 500F | Fluazinam | 2,6-dinitroanilines (29) | 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 |
| Pristine | Pyraclostrobin | QoI (11) | 0.1, 1, 10, 100 |
| | Boscalid | SDHI (7) | 0.1, 0.5, 1, 5, 10, 50, 100 |
| Switch 62.5WG | Cyprodinil | Anilopyrimidines (9) | 0.1, 0.5, 1, 5, 10 |
| | Fludioxonil | Phenylpyrroles (12) | 0.01, 0.05, 0.5, 1, 5, 10 |

**Table 3.3.** Isolate ID, $EC_{50}$ values, resistance category, and treatment concentrations used for gene expression analysis of *atrB*, BC1G_16062, and BC1G_12366.

| Isolate ID | $EC_{50}$ (ppm) | Fungicide | Resistance Category | Concentrations (ppm) for Gene Expression Analysis |
|---|---|---|---|---|
| MB21-226 | 0.001 | Fludioxonil | Sensitive | 0, 0.05 |
| MB21-486 | 0.001 | Fludioxonil | Sensitive | 0, 0.05 |
| MB21-184 | 0.785 | Fludioxonil | Sensitive | 0, 0.05 |
| MB21-668 | 1.298 | Fludioxonil | Sensitive | 0, 0.05 |
| MB21-159 | 0.001 | Cyprodinil | Sensitive | 0, 0.01 |
| MB21-252 | 17.845 | Cyprodinil | Moderately Resistant | 0, 17.845 |

**Table 3.4.** Sensitivity of *B. cinerea* isolates to fenhexamid, along with detected mutations within *erg27*.

| Isolate ID | Site Number | Fenhexamid Sensitivity | Mutations in *erg27* |
|---|---|---|---|
| MB21-224 | 2 | Sensitive | None Detected |
| MB21-324 | 19 | Sensitive | None Detected |
| MB21-502 | 23 | Sensitive | None Detected |
| MB21-512 | 21 | Sensitive | None Detected |
| MB21-188 | 4 | Sensitive | None Detected |
| MB21-260 | 4 | Moderately Resistant | None Detected |
| MB21-720 | 4 | Moderately Resistant | None Detected |
| MB21-184 | 22 | Moderately Resistant | None Detected |
| MB21-187 | 2 | Moderately Resistant | None Detected |
| MB21-487 | 11 | Moderately Resistant | None Detected |
| MB21-668 | 5 | Moderately Resistant | A378T |
| MB21-746 | 3 | Moderately Resistant | A378T |
| MB21-748 | 27 | Moderately Resistant | A378T, E375K |
| MB21-511 | 11 | Moderately Resistant | n.s. |
| MB21-116 | 1 | Moderately Resistant | n.s. |
| MB21-266 | 6 | Moderately Resistant | n.s. |
| MB21-270 | 2 | Moderately Resistant | n.s. |

n.s. = not sequenced

**Table 3.5**. Isolate name, county of isolate, site number, and accession numbers for all isolates and sequences obtained in this study.

| Isolate Name | County | Site Number | ITS | bos1 | erg27 | cytB |
|---|---|---|---|---|---|---|
| MB21-108 | Appling | 1 | OR803133 | n.s. | n.s. | n.s. |
| MB21-116 | Appling | 1 | OR803140 | n.s. | n.s. | n.s. |
| MB21-129 | Appling | 1 | OR803152 | n.s. | n.s. | n.s. |
| MB21-086 | Appling | 2 | OR803116 | n.s. | n.s. | n.s. |
| MB21-087 | Appling | 2 | OR803117 | n.s. | n.s. | n.s. |
| MB21-088 | Appling | 2 | OR803118 | n.s. | n.s. | n.s. |
| MB21-091 | Appling | 2 | OR803120 | n.s. | n.s. | n.s. |
| MB21-166 | Appling | 2 | OR803182 | n.s. | n.s. | n.s. |
| MB21-177 | Appling | 2 | OR803192 | n.s. | n.s. | n.s. |
| MB21-187 | Appling | 2 | OR803200 | n.s. | PV817830 | PV686203 |
| MB21-224 | Appling | 2 | OR803234 | PV686194 | PV817832 | PV686207 |
| MB21-229 | Appling | 2 | OR803238 | n.s. | n.s. | n.s. |
| MB21-230 | Appling | 2 | OR803239 | n.s. | n.s. | n.s. |
| MB21-231 | Appling | 2 | OR803240 | n.s. | n.s. | n.s. |
| MB21-233 | Appling | 2 | OR803242 | n.s. | n.s. | n.s. |
| MB21-234 | Appling | 2 | OR803243 | n.s. | n.s. | n.s. |
| MB21-237 | Appling | 2 | OR803246 | n.s. | n.s. | n.s. |
| MB21-240 | Appling | 2 | OR803249 | n.s. | n.s. | n.s. |
| MB21-241 | Appling | 2 | OR803250 | n.s. | n.s. | n.s. |
| MB21-244 | Appling | 2 | OR803253 | n.s. | n.s. | n.s. |
| MB21-247 | Appling | 2 | OR803256 | n.s. | n.s. | n.s. |
| MB21-248 | Appling | 2 | OR803257 | n.s. | n.s. | n.s. |
| MB21-252 | Appling | 2 | OR803261 | n.s. | n.s. | n.s. |
| MB21-270 | Appling | 2 | OR803279 | n.s. | n.s. | n.s. |
| MB21-271 | Appling | 2 | OR803280 | n.s. | n.s. | n.s. |
| MB21-279 | Appling | 2 | OR803288 | n.s. | n.s. | n.s. |
| MB21-287 | Appling | 2 | OR803295 | n.s. | n.s. | n.s. |
| MB21-318 | Appling | 2 | OR803317 | n.s. | n.s. | n.s. |
| MB21-382 | Appling | 2 | OR803368 | n.s. | n.s. | n.s. |
| MB21-645 | Appling | 3 | OR803592 | n.s. | n.s. | n.s. |
| MB21-653 | Appling | 3 | OR803600 | n.s. | n.s. | PV686211 |
| MB21-674 | Appling | 3 | OR803620 | n.s. | n.s. | n.s. |
| MB21-695 | Appling | 3 | OR803641 | n.s. | n.s. | n.s. |
| MB21-696 | Appling | 3 | OR803642 | n.s. | n.s. | n.s. |
| MB21-703 | Appling | 3 | OR803648 | n.s. | n.s. | n.s. |
| MB21-718 | Appling | 3 | OR803661 | n.s. | n.s. | n.s. |
| MB21-719 | Appling | 3 | OR803662 | n.s. | n.s. | n.s. |
| MB21-746 | Appling | 3 | OR803686 | PV686202 | PV817840 | n.s. |
| MB21-747 | Appling | 3 | OR803687 | n.s. | n.s. | n.s. |
| MB21-138 | Bacon | 4 | OR803159 | n.s. | n.s. | n.s. |

| | | | | | | |
|---|---|---|---|---|---|---|
| MB21-188 | Bacon | 4 | OR803201 | PV686193 | PV817831 | PV686204 |
| MB21-196 | Bacon | 4 | OR803209 | n.s. | n.s. | n.s. |
| MB21-259 | Bacon | 4 | OR803268 | n.s. | n.s. | n.s. |
| MB21-260 | Bacon | 4 | OR803269 | PV686198 | PV817833 | n.s. |
| MB21-371 | Bacon | 4 | OR803359 | n.s. | n.s. | n.s. |
| MB21-657 | Bacon | 4 | OR803604 | n.s. | n.s. | n.s. |
| MB21-664 | Bacon | 4 | OR803610 | n.s. | n.s. | n.s. |
| MB21-665 | Bacon | 4 | OR803611 | n.s. | n.s. | n.s. |
| MB21-666 | Bacon | 4 | OR803612 | n.s. | n.s. | n.s. |
| MB21-720 | Bacon | 4 | OR803663 | n.s. | PV817839 | n.s. |
| MB21-741 | Bacon | 4 | OR803682 | n.s. | n.s. | n.s. |
| MB21-745 | Bacon | 4 | OR803685 | n.s. | n.s. | n.s. |
| MB21-490 | Bacon | 5 | OR803453 | n.s. | n.s. | n.s. |
| MB21-532 | Bacon | 5 | OR803490 | n.s. | n.s. | n.s. |
| MB21-551 | Bacon | 5 | OR803505 | n.s. | n.s. | n.s. |
| MB21-661 | Bacon | 5 | OR803607 | n.s. | n.s. | n.s. |
| MB21-663 | Bacon | 5 | OR803609 | n.s. | n.s. | n.s. |
| MB21-668 | Bacon | 5 | OR803614 | n.s. | PV817838 | n.s. |
| MB21-693 | Bacon | 5 | OR803639 | n.s. | n.s. | n.s. |
| MB21-694 | Bacon | 5 | OR803640 | n.s. | n.s. | n.s. |
| MB21-063 | Bacon | 6 | OR803094 | n.s. | n.s. | n.s. |
| MB21-163 | Bacon | 6 | OR803179 | n.s. | n.s. | n.s. |
| MB21-179 | Bacon | 6 | OR803194 | n.s. | n.s. | n.s. |
| MB21-232 | Bacon | 6 | OR803241 | n.s. | n.s. | n.s. |
| MB21-242 | Bacon | 6 | OR803251 | n.s. | n.s. | n.s. |
| MB21-262 | Bacon | 6 | OR803271 | n.s. | n.s. | n.s. |
| MB21-266 | Bacon | 6 | OR803275 | n.s. | n.s. | n.s. |
| MB21-267 | Bacon | 6 | OR803276 | n.s. | n.s. | n.s. |
| MB21-268 | Bacon | 6 | OR803277 | n.s. | n.s. | n.s. |
| MB21-269 | Bacon | 6 | OR803278 | n.s. | n.s. | n.s. |
| MB21-514 | Bacon | 9 | OR803473 | n.s. | n.s. | n.s. |
| MB21-542 | Bacon | 9 | OR803500 | n.s. | n.s. | n.s. |
| MB21-487 | Bacon | 11 | OR803450 | PV686200 | PV817835 | n.s. |
| MB21-503 | Bacon | 11 | OR803462 | PV686201 | n.s. | n.s. |
| MB21-506 | Bacon | 11 | OR803465 | n.s. | n.s. | n.s. |
| MB21-511 | Bacon | 11 | OR803470 | n.s. | n.s. | n.s. |
| MB21-520 | Bacon | 11 | OR803479 | n.s. | n.s. | n.s. |
| MB21-564 | Bacon | 11 | OR803516 | n.s. | n.s. | n.s. |
| MB21-089 | Pierce | 19 | OR803119 | n.s. | n.s. | n.s. |
| MB21-243 | Pierce | 19 | OR803252 | n.s. | n.s. | n.s. |
| MB21-290 | Pierce | 19 | OR803297 | n.s. | n.s. | n.s. |
| MB21-309 | Pierce | 19 | OR803310 | n.s. | n.s. | n.s. |
| MB21-310 | Pierce | 19 | OR803311 | n.s. | n.s. | n.s. |
| MB21-322 | Pierce | 19 | OR803321 | n.s. | n.s. | n.s. |
| MB21-324 | Pierce | 19 | OR803323 | n.s. | PV817834 | n.s. |

| | | | | | | |
|---|---|---|---|---|---|---|
| MB21-134 | Pierce | 20 | OR803156 | n.s. | n.s. | n.s. |
| MB21-136 | Pierce | 21 | OR803158 | n.s. | n.s. | n.s. |
| MB21-253 | Pierce | 21 | OR803262 | n.s. | n.s. | n.s. |
| MB21-264 | Pierce | 21 | OR803273 | n.s. | n.s. | n.s. |
| MB21-265 | Pierce | 21 | OR803274 | n.s. | n.s. | n.s. |
| MB21-486 | Pierce | 21 | OR803449 | n.s. | n.s. | n.s. |
| MB21-493 | Pierce | 21 | OR803456 | n.s. | n.s. | n.s. |
| MB21-512 | Pierce | 21 | OR803471 | n.s. | PV817837 | n.s. |
| MB21-135 | Pierce | 22 | OR803157 | n.s. | n.s. | n.s. |
| MB21-150 | Pierce | 22 | OR803168 | n.s. | n.s. | n.s. |
| MB21-151 | Pierce | 22 | OR803169 | n.s. | n.s. | n.s. |
| MB21-171 | Pierce | 22 | OR803186 | n.s. | n.s. | n.s. |
| MB21-176 | Pierce | 22 | OR803191 | n.s. | n.s. | n.s. |
| MB21-180 | Pierce | 22 | OR803195 | n.s. | n.s. | n.s. |
| MB21-181 | Pierce | 22 | OR803196 | n.s. | n.s. | n.s. |
| MB21-184 | Pierce | 22 | OR803198 | PV686192 | PV817829 | n.s. |
| MB21-193 | Pierce | 22 | OR803206 | n.s. | n.s. | n.s. |
| MB21-195 | Pierce | 22 | OR803208 | n.s. | n.s. | n.s. |
| MB21-199 | Pierce | 22 | OR803212 | n.s. | n.s. | n.s. |
| MB21-210 | Pierce | 22 | OR803222 | n.s. | n.s. | PV686205 |
| MB21-226 | Pierce | 22 | OR803235 | n.s. | n.s. | n.s. |
| MB21-235 | Pierce | 22 | OR803244 | n.s. | n.s. | n.s. |
| MB21-236 | Pierce | 22 | OR803245 | n.s. | n.s. | n.s. |
| MB21-246 | Pierce | 22 | OR803255 | n.s. | n.s. | n.s. |
| MB21-250 | Pierce | 22 | OR803259 | n.s. | n.s. | n.s. |
| MB21-281 | Pierce | 22 | OR803289 | n.s. | n.s. | n.s. |
| MB21-298 | Pierce | 22 | OR803303 | n.s. | n.s. | n.s. |
| MB21-227 | Pierce | 23 | OR803236 | n.s. | n.s. | n.s. |
| MB21-501 | Pierce | 23 | OR803460 | n.s. | n.s. | n.s. |
| MB21-502 | Pierce | 23 | OR803461 | n.s. | PV817836 | n.s. |
| MB21-513 | Pierce | 23 | OR803472 | n.s. | n.s. | n.s. |
| MB21-522 | Pierce | 23 | OR803481 | n.s. | n.s. | n.s. |
| MB21-528 | Pierce | 23 | OR803486 | n.s. | n.s. | n.s. |
| MB21-144 | Pierce | 24 | OR803163 | n.s. | n.s. | n.s. |
| MB21-249 | Pierce | 24 | OR803258 | n.s. | n.s. | n.s. |
| MB21-256 | Pierce | 24 | OR803265 | PV686197 | n.s. | n.s. |
| MB21-302 | Pierce | 24 | OR803305 | PV686199 | n.s. | PV686209 |
| MB21-154 | Pierce | 25 | OR803172 | n.s. | n.s. | n.s. |
| MB21-155 | Pierce | 25 | OR803173 | n.s. | n.s. | n.s. |
| MB21-159 | Pierce | 25 | OR803176 | n.s. | n.s. | n.s. |
| MB21-161 | Pierce | 25 | OR803177 | n.s. | n.s. | n.s. |
| MB21-170 | Pierce | 25 | OR803185 | n.s. | n.s. | n.s. |
| MB21-222 | Pierce | 25 | OR803232 | n.s. | n.s. | PV686206 |
| MB21-223 | Pierce | 25 | OR803233 | n.s. | n.s. | n.s. |
| MB21-228 | Pierce | 25 | OR803237 | n.s. | n.s. | n.s. |

| | | | | | | |
|---|---|---|---|---|---|---|
| MB21-238 | Pierce | 25 | OR803247 | PV686195 | n.s. | n.s. |
| MB21-239 | Pierce | 25 | OR803248 | PV686196 | n.s. | PV686208 |
| MB21-245 | Pierce | 25 | OR803254 | n.s. | n.s. | n.s. |
| MB21-261 | Pierce | 25 | OR803270 | n.s. | n.s. | n.s. |
| MB21-263 | Pierce | 25 | OR803272 | n.s. | n.s. | n.s. |
| MB21-342 | Pierce | 25 | OR803338 | n.s. | n.s. | n.s. |
| MB21-369 | Pierce | 25 | OR803357 | n.s. | n.s. | PV686210 |
| MB21-484 | Pierce | 25 | OR803447 | n.s. | n.s. | n.s. |
| MB21-489 | Pierce | 25 | OR803452 | n.s. | n.s. | n.s. |
| MB21-492 | Pierce | 25 | OR803455 | n.s. | n.s. | n.s. |
| MB21-507 | Pierce | 25 | OR803466 | n.s. | n.s. | n.s. |
| MB21-251 | Pierce | 26 | OR803260 | n.s. | n.s. | n.s. |
| MB21-504 | Pierce | 26 | OR803463 | n.s. | n.s. | n.s. |
| MB21-652 | Pierce | 27 | OR803599 | n.s. | n.s. | n.s. |
| MB21-655 | Pierce | 27 | OR803602 | n.s. | n.s. | n.s. |
| MB21-658 | Pierce | 27 | OR803605 | n.s. | n.s. | n.s. |
| MB21-697 | Pierce | 27 | OR803643 | n.s. | n.s. | n.s. |
| MB21-748 | Pierce | 27 | OR803688 | n.s. | PV817841 | n.s. |
| MB21-654 | Pierce | 28 | OR803601 | n.s. | n.s. | n.s. |
| MB21-656 | Pierce | 28 | OR803603 | n.s. | n.s. | n.s. |
| MB21-662 | Pierce | 28 | OR803608 | n.s. | n.s. | n.s. |
| MB21-667 | Pierce | 28 | OR803613 | n.s. | n.s. | n.s. |
| MB21-671 | Pierce | 30 | OR803617 | n.s. | n.s. | n.s. |
| MB21-672 | Pierce | 30 | OR803618 | n.s. | n.s. | n.s. |
| MB21-683 | Pierce | 30 | OR803629 | n.s. | n.s. | n.s. |

n.s. = not sequenced

**Figures**

**Figure 3.1.** Three distinct colony morphologies of *B. cinerea* after growth on potato dextrose agar (PDA) for 10–14 days: conidial type (left), sclerotial type (middle), and mycelial type (right).

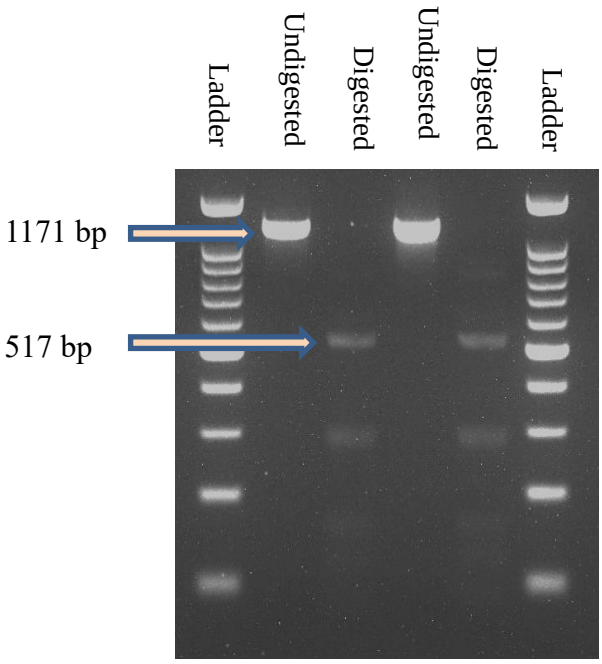
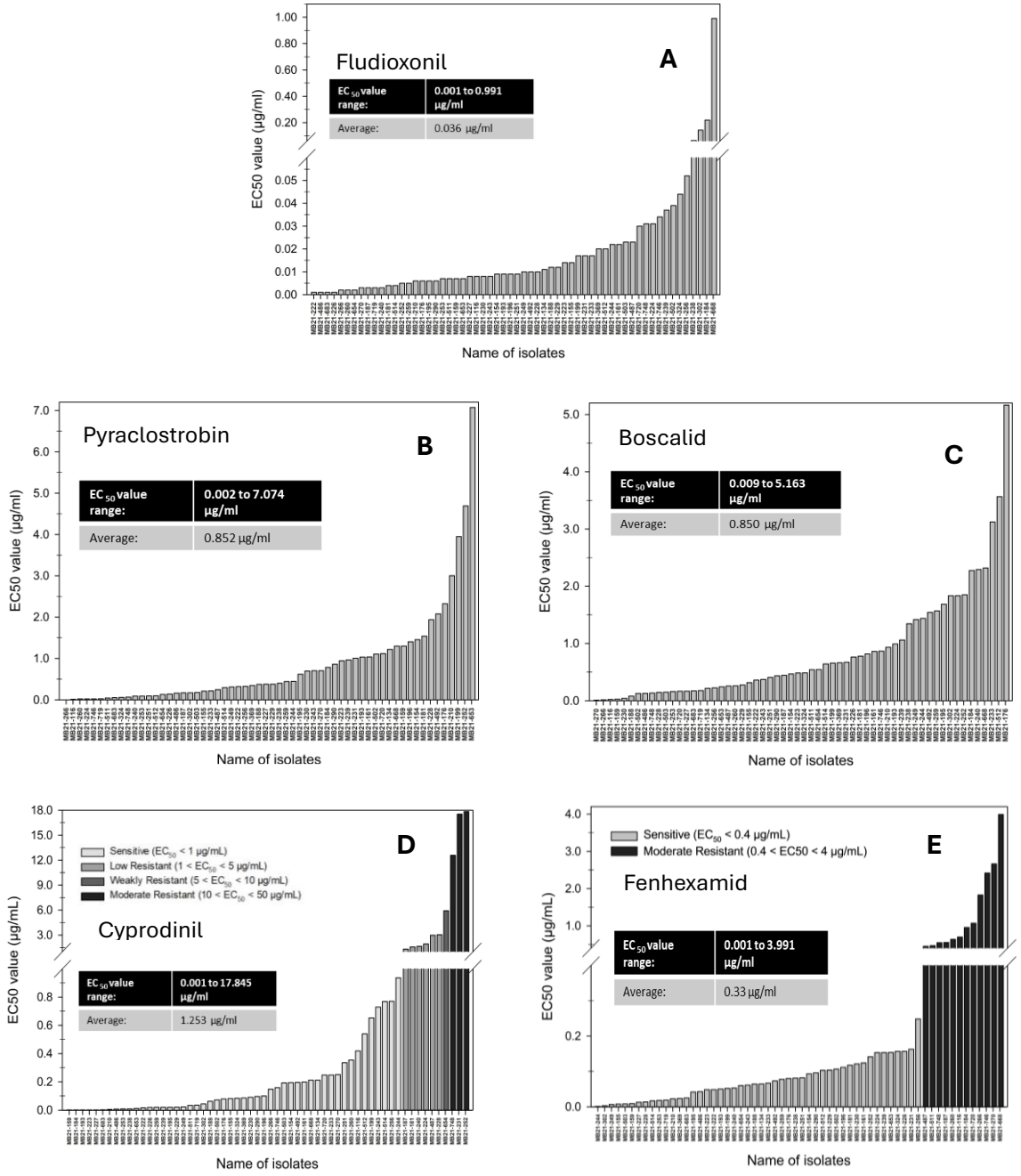



**Figure 3.2.** Restriction digestion of *Bc-hch* gene fragment (1171 bp) amplified using primer pair 260/520L and digested with the *HhaI*. All isolates showed the distinguishing 517 bp fragment characteristic of *B. cinerea* Group I. No 601 bp fragment indicative of Group II was observed for any isolate.




| | |
|---|---|
| EC50 value range: | 0.001 to 0.991 µg/ml |
| Average: | 0.036 µg/ml |

| | |
|---|---|
| EC50 value range: | 0.002 to 7.074 µg/ml |
| Average: | 0.852 µg/ml |

| | |
|---|---|
| EC50 value range: | 0.009 to 5.163 µg/ml |
| Average: | 0.850 µg/ml |

| | |
|---|---|
| EC50 value range: | 0.001 to 17.845 µg/ml |
| Average: | 1.253 µg/ml |

| | |
|---|---|
| EC50 value range: | 0.001 to 3.991 µg/ml |
| Average: | 0.33 µg/ml |

**Figure 3.3.** $EC_{50}$ values determined from mycelial growth inhibition assays for all 60 *B. cinerea* isolates screened against five fungicides: (A) fludioxonil, (B) pyraclostrobin, (C) boscalid, (D) cyprodinil, and (E) fenhexamid.

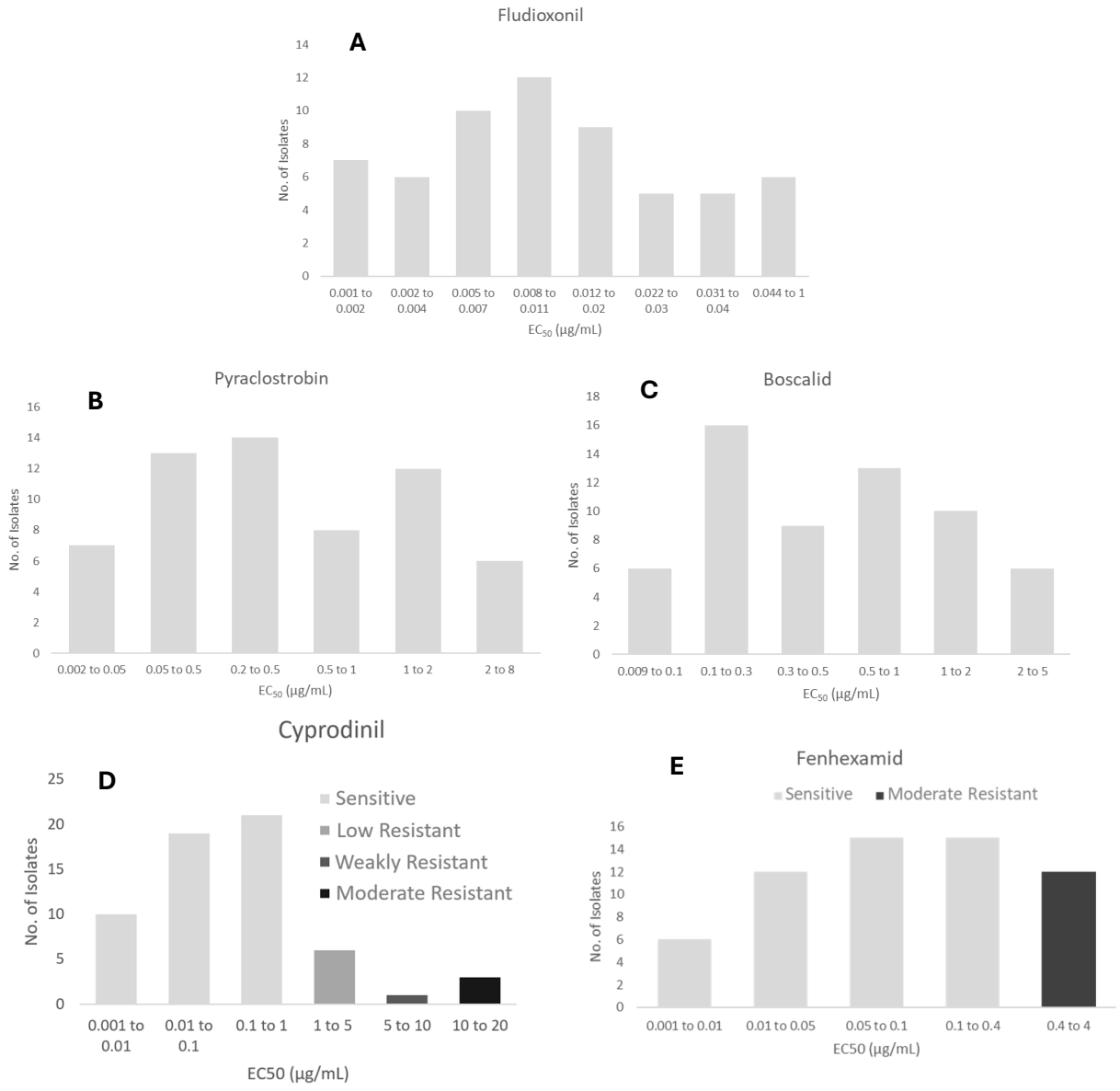



**Figure 3.4.** Frequency distribution of $EC_{50}$ values determined from mycelial growth inhibition assays for all 60 *B. cinerea* isolates tested against five fungicides: (A) fludioxonil, (B) pyraclostrobin, (C) boscalid, (D) cyprodinil, and (E) fenhexamid.

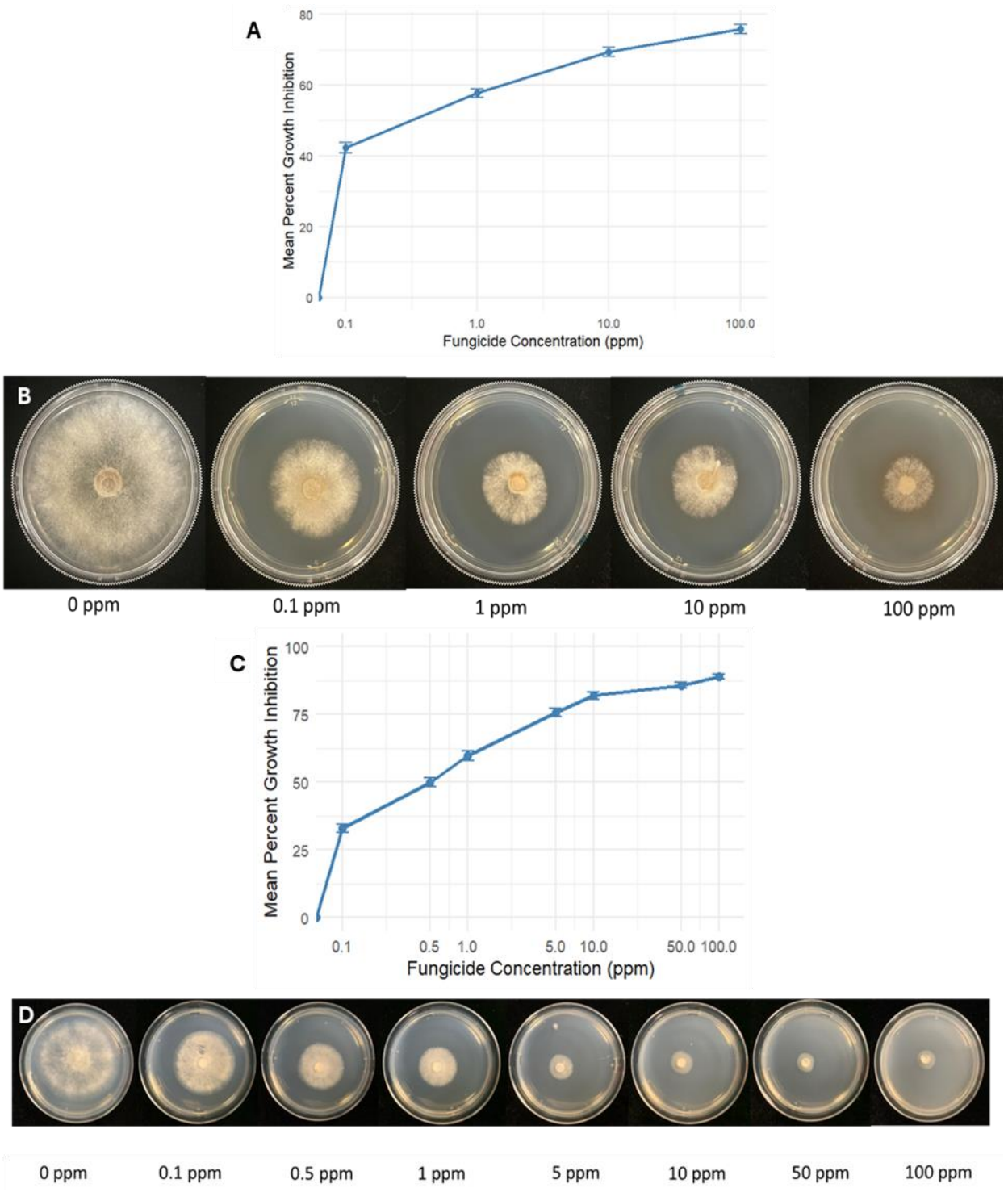



**Figure 3.5.** Dose-response curves and radial growth inhibition of *B. cinerea* isolates exposed to pyraclostrobin (A and B) and boscalid (C and D) at increasing fungicide concentrations. Growth inhibition was measured on PDA after 4 days of incubation. Each set includes the mean percent

growth inhibition curve with standard error bars (A and C) and corresponding mycelial growth inhibition across the fungicide gradient (B and D), demonstrating dose-dependent inhibition.

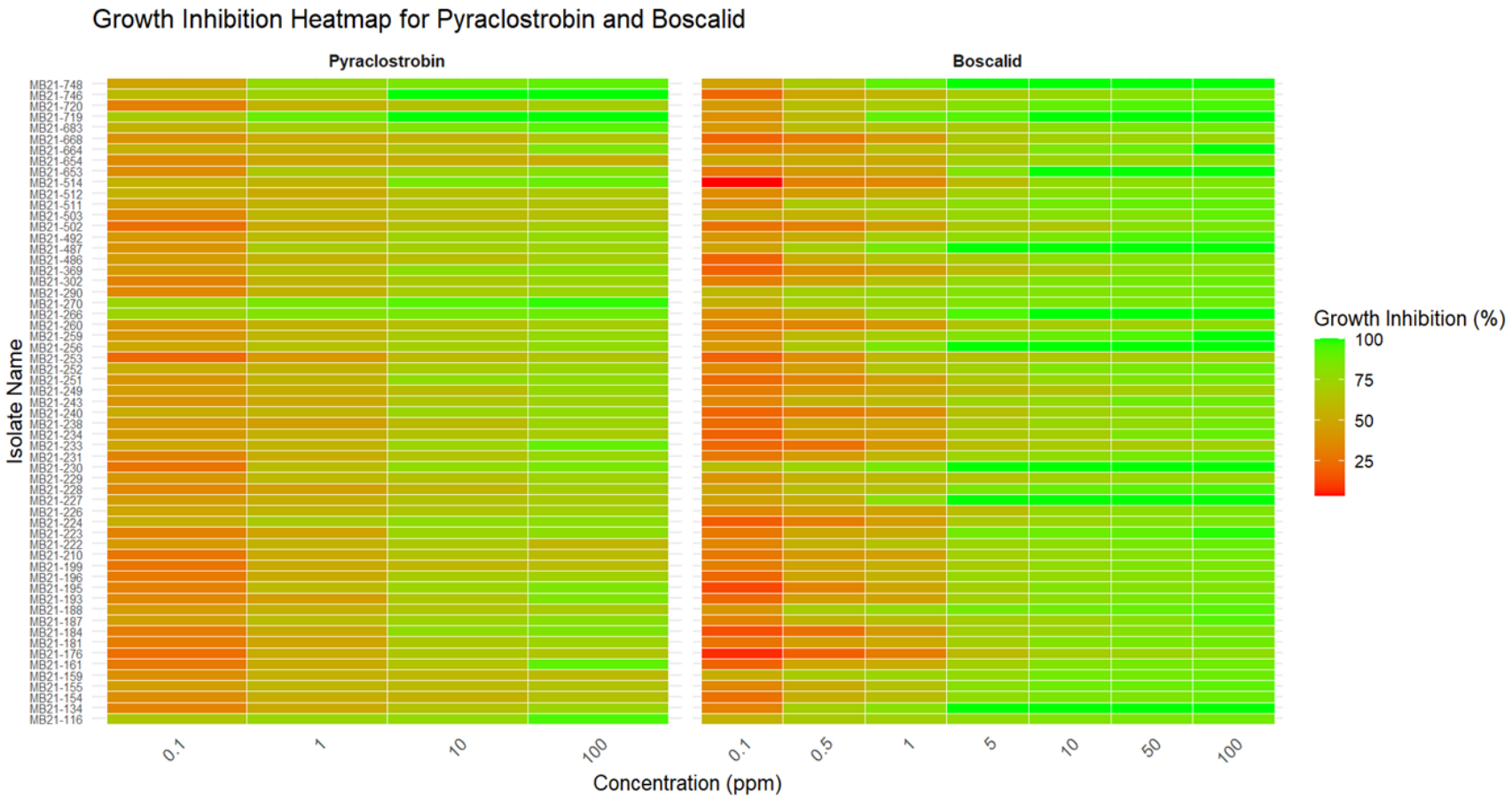



**Figure 3.6.** Growth inhibition heatmap for 60 *B. cinerea* isolates exposed to increasing concentrations of boscalid (left) and pyraclostrobin (right). Shades represent the percentage of mycelial growth inhibition at each fungicide concentration, with darker, red shades indicating greater inhibition than lighter, green shades. Growth was assessed on PDA after 4 days of incubation.

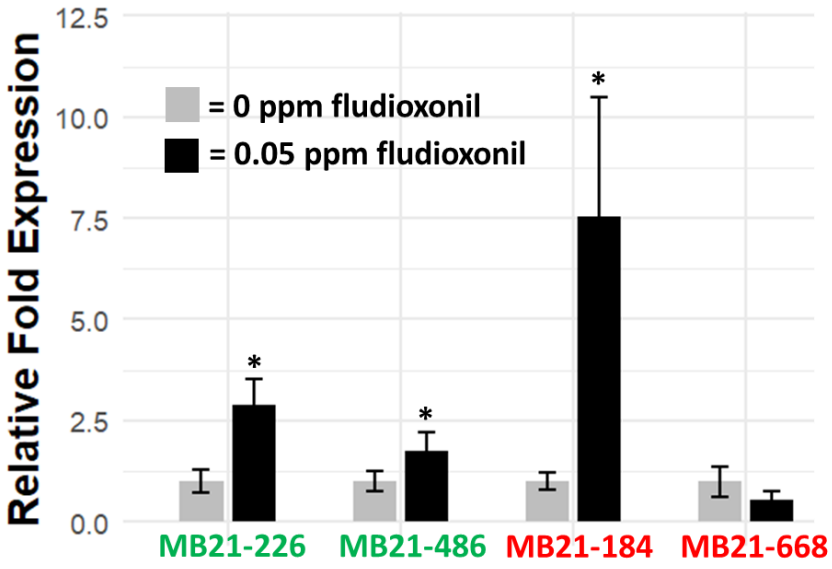



**Figure 3.7.** Relative fold expression of the *atrB* gene. **Green** indicates isolates with the lowest $EC_{50}$ values for fludioxonil, while **Red** represents those with the highest. The asterisk (*) indicates significantly increased expression (relative to the expression without fungicide exposure for each respective isolate) based on student's t-test ($\alpha = 0.05$) and error bars indicate ± standard deviation.

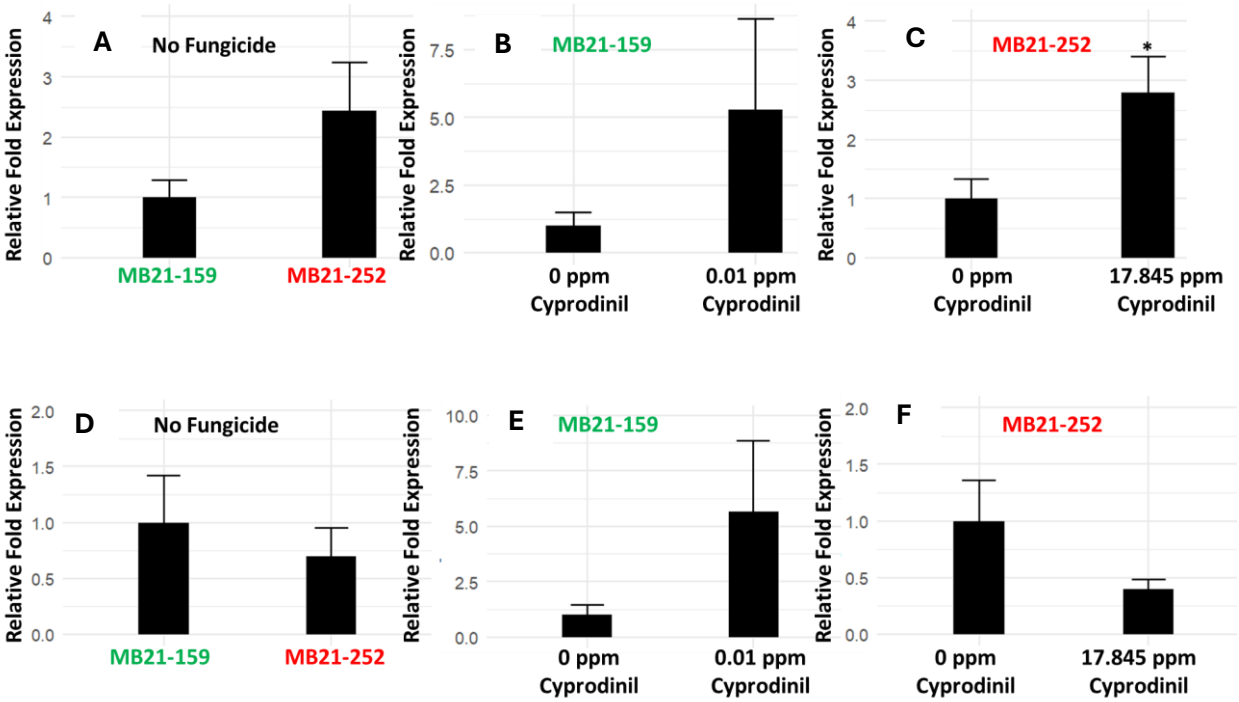



**Figure 3.8.** Relative expression of BC1G_16062 (A–C) and BC1G_12366 (D–F) in a *B. cinerea* sensitive (MB21-159) and a moderately resistant (MB21-252) isolate induced at different concentration of cyprodinil. **Green** indicates the sensitive isolate, while **Red** indicates the moderately resistant isolate. The asterisk (*) indicates significantly increased expression (relative to the other value) based on student's t-test ($\alpha = 0.05$) and error bars indicate ± standard deviation.

# CHAPTER 4

## Georgia blueberry *Neopestalotiopsis* isolates, which are phylogenetically indistinguishable from the emerging novel strawberry *Neopestalotiopsis* sp., are pathogenic to both blueberry and strawberry[1]

[1]Beg, M. A. & Oliver, J. E. (2025). Georgia blueberry *Neopestalotiopsis* isolates, which are phylogenetically indistinguishable from the emerging novel strawberry *Neopestalotiopsis* sp., are pathogenic to both blueberry and strawberry. Short communication article to be submitted to: *Plant Disease*.

**ABSTRACT**

A novel *Neopestalotiopsis* sp. causing an emerging crown, leaf, and fruit disease on strawberry has been associated with significant losses in recent years in the Eastern U.S and Canada. Particularly aggressive on strawberry relative to other *Neopestalotiopsis* spp., isolates of this novel species are morphologically similar to the relatively less aggressive species *Neopestalotiopsis rosae* and have been genetically differentiated from *N. rosae* based on multilocus sequence analysis as well as restriction fragment length polymorphisms (RFLP) within the fungal beta-tubulin gene. Here, from blueberry fruit collected from commercial blueberry production sites in southern Georgia (U.S.A.), we report the isolation, identification, and characterization of *Neopestalotiopsis* isolates which are phylogenetically indistinguishable from the novel *Neopestalotiopsis* sp. from strawberry. Isolates collected from blueberry across multiple growing seasons were identified as belonging to the novel *Neopestalotiopsis* sp. based on RFLP and multilocus sequence analysis. Moreover, these isolates were found to be capable of causing disease on both blueberry and strawberry plants in greenhouse experiments. These findings expand our understanding of this novel *Neopestalotiopsis* sp. and have implications for the diagnosis and management of *Neopestalotiopsis* diseases on small fruit crops.

**KEYWORDS:** *Neopestalotiopsis* sp., blueberry, strawberry, *Neopestalotiopsis rosae*, fruit rot, leaf spot

**MANUSCRIPT**

*Pestalotiopsis* sensu lato, which is comprised of three genera *Neopestalotiopsis, Pseudopestalotiopsis,* and *Pestalotiopsis* sensu stricto (Maharachchikumbura et al. 2014), are often considered as secondary pathogens in phytopathology, perhaps due to the relatively small number of studies verifying the pathogenicity of these genera relative to their frequent isolation from plant material (Darapanit et al. 2021). Nonetheless, the genus *Neopestalotiopsis* has been reported to cause diseases on a diverse range of hosts including mango, rubber tree, eucalyptus, macadamia, grapevine trunk, guava, and strawberry (Baggio et al. 2021; Diogo et al. 2021; Gerardo-Lugo et al. 2020; Maharachchikumbura et al. 2016; Pornsuriya et al. 2020; Prasannath et al. 2021). On strawberry, *Neopestalotiopsis rosae* has been reported to cause disease in multiple locations including China, Egypt, Germany, India, Italy, Mexico, Taiwan, and the United States (Baggio et al. 2021; Chandana et al. 2024; Dardani et al. 2025; Essa et al. 2018; Rebollar-Alviter et al. 2020; Schierling et al. 2024; Sun et al. 2021; Wu et al. 2020), and a newly identified novel *Neopestalotiopsis* sp. phylogenetically very close to *N. rosae* has been recently reported as causing an epidemic on strawberry in Florida (Baggio et al. 2021), other parts of the Eastern U.S. (Guan et al. 2023; Jimenez Madrid et al. 2024), and Canada (McNally et al. 2023). Isolates of this novel *Neopestalotioposis* sp. from strawberries were found to be more virulent than strawberry isolates of *N. rosae* (Baggio et al. 2021). In addition to being within a separate clade from *N. rosae* in a phylogenetic tree constructed using portions of the internal transcribed spacer (ITS), *β*-tubulin (*B-tub*), and *tef1* regions (Baggio et al. 2021), *Neopestalotiopsis* sp. isolates possess a reportedly unique point mutation in the *B-tub* gene, which has been used, in part, to distinguish isolates of this aggressive novel species from other *Neopestalotiopsis* spp. using high resolution melting analysis (Rebello et al. 2023). In addition, an RFLP technique has also been developed based on

this mutation to help differentiate this novel *Neopestalotiopsis* sp. from other morphologically similar species (Kaur et al. 2023), and this method is currently being used for routine diagnosis of this aggressive novel *Neopestalotiopsis* sp. from strawberry (Jimenez Madrid et al. 2024). Although *Neopestalotiopsis* spp. have generally not been regarded as significant pathogens in blueberry production, *Neopestalotiopsis clavispora* (Borrero et al. 2018; Jevremovic et al. 2022; Lee et al. 2019; Zheng et al. 2023) and additional *Neopestalotiopsis* spp. (Santos et al. 2022) have recently been reported to cause twig dieback, leaf spot, and/or canker on blueberries in multiple locations including China, Korea, Portugal, Serbia, and Spain. Likewise, while *Neopestalotiopsis* spp. are not widely recognized as postharvest pathogens, a few reports have identified some species as causing postharvest fruit rot in persimmon and red guava (Qin et al. 2023; Wang et al. 2024).

During the 2021 and 2022 growing seasons, as part of collection efforts largely focused on the identification of other fruit rot pathogens from rotting blueberries in Georgia (Beg et al. 2025; Bollenbacher 2023), a number of *Neopestalotiopsis* isolates were also recovered from blueberry fruit. Some of these isolates, which were morphologically similar to *N. rosae* (**Figure 4.1**), were chosen for further genetic characterization via sequencing of the ITS region. A subset of these isolates was found to have an ITS sequence identical (100%) to that of the previously identified aggressive *Neopestalotiopsis* strain 17-43L reported from Florida strawberry fields (Genbank accession number MK895144; **Supplementary Table 4.1**), and this finding prompted further investigation to determine whether the unique β-tubulin (*B-tub*) mutation previously described by Rebello et al. (2023) was present in these isolates. Sequencing of the *B-tub* gene (**Supplementary Table 4.1**) also revealed that some of these isolates possessed sequences that were 100% identical to those of the aggressive Florida strain from strawberries and included the characteristic mutation.

Following the RFLP protocol outlined by Kaur et al. (2023), the presence of this mutation was further validated (**Figure 4.2**). In total, four 2021 isolates from two unique locations in Bacon (3 isolates) and Pierce (1 isolate) counties and six 2022 isolates (all from the same location in Bacon County where the 2021 isolates originated) were identified as belonging to the novel *Neopestalotiopsis* sp. based on these methods. None of the blueberry production locations where these isolates were originally collected had any history of strawberry production. Subsequently, to allow for multilocus sequence analysis (MLSA), sequences of *tef1* (**Supplementary Table 4.1**) were obtained from a subset of these isolates, and subsequent phylogenetic analysis confirmed that these isolates belonged to the novel *Neopestalotiopsis* sp. (**Figure 4.3**).

For initial morphological identification of the pathogen, isolates of *Neopestalotiopsis* sp. were cultured on potato dextrose agar (PDA) and incubated in darkness at 25°C for five days. After this period, colonies exhibited a snowy white, cottony, and fluffy texture on the upper surface, while the underside appeared white to pale yellow (**Figure 4.1A & 4.1B**). Following initial incubation, the Petri dishes were unsealed and maintained under a 12-hour light/12-hour dark photoperiod at 25°C for an additional 7 days. Under these conditions, dark black acervuli developed, scattered across the plate (**Figure 4.1A**), with a prominent concentric ring of clustered acervuli forming at the center. Acervuli were carefully disrupted using a sterile scalpel, and a spore suspension was prepared in sterile distilled water. Spore measurements were conducted using a ZEISS Axioscope 5 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), where 20 randomly selected conidia were measured to determine their average dimensions. The mean spore length and width were determined to be 28.5 μm and 6.8 μm, respectively. Conidia were ellipsoid to fusiform and five-celled with from two to four flexuous appendages as described

in previous reports of this novel *Neopestalotiopsis* sp. (Baggio et al. 2021; Jimenez Madrid et al. 2024).

For molecular identification of the pathogen, MLSA was performed according to the methods reported by Baggio et al. (2021). Primers ITS1 and ITS4 (White et al. 1990), Bt2a and Bt2b (Glass and Donaldson 1995), and EF1-526F and EF1-1567R (Rehner 2001) were used to amplify portions of the ITS, *B-tub*, and *tef1* regions, respectively. For PCR, a total reaction volume of 30 µl was used, and each reaction contained 15 µl 2X PCR Master Mix, approximately 200 ng of genomic DNA, and 10 mM of each primer (1 µl each). PCR was performed using a BioRad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) according to the reaction conditions for each primer set in Baggio et al. (2021). After PCR, products were examined visually on 1% agarose gel alongside 100 bp DNA ladder (Biotium, Inc., Fremont, CA). The PCR amplicon was purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Inc. Norcross, GA) and then the concentration was assessed by using a Nanodrop One$^{C}$ (Thermo Scientific, Waltham, MA). Purified PCR products were sequenced via Sanger sequencing in both directions by Eurofins Genomics (Louisville, KY). For RFLP analysis, PCR products from *B-tub* were digested with *Bsa*WI (New England Biolabs, Ipswich, MA) according to the methods described previously by Kaur et al. (2023) and digested products were visualized on agarose gels as described above. Phylogenetic analysis (**Figure 4.3**) was conducted in Mega 7 (Kumar et al. 2016) with sequences (**Supplementary Table 4.1**) from the Georgia blueberry isolates as well as previously characterized isolates from *Neopestalotiopsis*, *Pseudopestalotiopsis*, and *Pestalotiopsis* (Baggio et al. 2021; Maharachchikumbura et al. 2014; Norphanphoun et al. 2019; Prasannath et al. 2021). The evolutionary history was inferred by using the Maximum Likelihood method based on the

Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). All positions with less than 95% site coverage were eliminated leaving a total of 1185 positions in the final dataset.

To determine if the isolates from blueberry identified as belonging to the novel *Neopestalotiopsis* sp. were capable of causing disease on blueberry and strawberry, pathogenicity tests were performed on plants of both hosts. For pathogenicity tests, *Neopestalotiopsis* sp. isolates 'MB21-284' and 'MB21-494' were both used to inoculate both hosts; however, no differences were noted between these isolates in terms of their pathogenicity in any of the tests. From cultures of these isolates, spores were harvested as described previously, suspended in sterile distilled water, and the concentration of the suspension was adjusted to approximately $10^5$ spores/ml using a hemacytometer. For blueberry, leaf and root pathogenicity tests were carried out using plants of the southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) cv. 'Rebel' (AgriStarts, Inc., Apopka, FL). Tests were conducted approximately 4-weeks after planting into plastic pots (27.9 cm) filled with pine bark and sand. Wounding and non-wounding methods were used in the leaf pathogenicity tests. Wounding of leaves was performed with sterilized needles, and both wounded and non-wounded leaves were sprayed with the prepared spore suspension. For comparison, sterile distilled water only was used as a negative control. After inoculation, leaf-inoculated plants were placed in plastic boxes (60.8 cm x 42.7 cm x 33.1 cm) with a water-soaked paper towel placed at the bottom to ensure humidity. Four days post-inoculation, symptoms began to be evident on both the wounded and non-wounded blueberry leaves (**Figure 4.4**). Dark brown circular lesions were observed with a yellow spot in the center (**Figure 4.4B**). Most of the lesions were observed on the edge of the leaves irrespective of the wounding. Black acervuli were also clearly visible with the naked eye within the lesions as had been previously reported on strawberries affected by *Neopestalotiopsis* (Baggio et al. 2021). To fulfill Koch's postulates, the

pathogen was reisolated from diseased leaf tissue (lesions) and re-identified as *Neopestalotiopsis* sp. according to the methods described previously.

For root inoculation of blueberry plants, the soil was first removed from the roots by rinsing in sterile distilled water, and then the root tips were trimmed to promote entry of the pathogen. Roots were inoculated by immersion in a suspension of ~$5 \times 10^5$ spores/ml or water (control) for 10 min. Root inoculated plants were potted into plastic pots (27.9 cm) filled with pinebark and sand and maintained in a greenhouse at 28°C (±2°C) with daily irrigation throughout the experiment. Root-inoculated plants were observed for 30 days post inoculation (dpi). By 7 dpi, the first visual symptoms (leaf yellowing) were observed. Most of the leaves of inoculated plants had turned yellow by 14 dpi (**Figure 4.4D**) and defoliation of ~80% of the foliage was observed by 28 dpi. By contrast, the leaves of the control plants that were root inoculated with sterile distilled water were green and no defoliation was observed at 28 dpi. At 30 dpi, to fulfill Koch's postulates, the pathogen was re-isolated from root tissue of inoculated plants on PDA and confirmed to be the novel *Neopestalotiopsis* sp. using previously described methods.

A blueberry fruit pathogenicity test was also performed using store-bought, firm, and ripe organic blueberries. These were initially surface sterilized by briefly rinsing (dipping) berries twice in 70% ethanol, once in 5% bleach (0.5% sodium hypochlorite) and then twice in sterile distilled water. Rinsed berries were then air dried and fixed to the bottom of clamshells with double-sided tape with the stem-scar facing up. Each individual berry was inoculated at the stem scar site with either 20 µl of spore suspension or with sterilized distilled water (for the control). For each single isolate, three clamshells which each contained 9 berries (27 berries total) were inoculated. Following inoculation, the clamshells were placed in a sealed plastic box for incubation, and two sterile paper towels soaked with sterile distilled water were placed at the bottom of each box to

ensure humid conditions. On inoculated berries, fungal signs (white mycelium) began to be evident at the inoculation site after three days post-inoculation and the upper portion of the berries became sunken. In 3 to 4 more days, the berry was slightly shrivelled and the white threadlike mycelium spread to a diameter of about 6 mm on the inoculation site. Fungal growth was sparse and scattered over the berry surface. Approximately 10 days post inoculation, black acervuli typical of *Neopestalotiopsis* were observed dotting the surface of the inoculated berries (**Figure 4.4C**). By contrast, no symptoms or fungal signs were observed on the uninoculated (sterile water only) control berries. To fulfill Koch's postulates, the fungus was reisolated from mycelium and acervuli on infected berries and confirmed to be the new *Neopestalotiopsis* sp. using the previously described methods.

For the leaf, fruit, and root inoculation of strawberry (*Fragaria x ananassa*), cut-top, bare-root transplants of cultivar 'Albion' were obtained (Indiana Berry & Plant Co., Plymouth, IN). For leaf inoculation, strawberry plants were potted into plastic pots (10 cm x 7.6 cm x 10.9 cm) and watered twice daily for 5 days until cut-top plants leafed-out. After that, plants were maintained in the greenhouse and inoculated using methods similar to those described previously for the blueberry leaf inoculation. On inoculated plants, the first symptoms were observed on the buds and blooms. The whole bud in the unopened leaves and petals and stigma in the opened leaves initially turned dark brown, and then dieback started from those sites causing the whole plants to collapse and die within 2-weeks post inoculation. Symptoms on the leaves of inoculated strawberry plants also started to appear within 3 to 4 days post inoculation, and leaves started to develop dark brown spots that eventually formed into larger lesions (**Figure 4.4E & 4.4F**). At 7-10 dpi, black acervuli were clearly visible with the naked eye on inoculated leaves and resembled observations reported by Baggio et al. (2021). By contrast, no signs or symptoms were observed on the

uninoculated control plants. Similarly, the fruit inoculation assay was carried out on attached fruit that developed on 'Albion' plants potted at the same time as those used for the leaf inoculation assay. For strawberry fruit inoculation, attached fruit were directly sprayed with a spore suspension prepared as described previously. Symptoms started to appear on the strawberry fruit after 3 days post inoculation. White mycelium was initially observed on the fruit (**Figure 4.4G**) and later the fruit appeared to shrivel and dry out. At 7-10 dpi, acervuli (appearing as black dots) were observed on the strawberry fruit as previously reported by Baggio et al. (2021). While strawberry plants were also root inoculated using methods similar to those utilized for the blueberry root inoculation assay described preivously, no visible symptoms were observed on strawberry plants root-inoculated with *Neopestalotiopsis* isolates.

Taken together, the results of this study indicate the pathogenicity on both blueberry and strawberry of *Neopestalotiopsis* sp. isolates recovered from blueberries in Georgia which belong to the same novel *Neopestalotiopsis* sp. responsible for the strawberry epidemic in Florida. Morphological identification, based on mycelial and conidial characteristics, along with molecular identification through a phylogenetic tree constructed using ITS, β-tubulin *(B-tub),* and *tef1* regions, confirmed that these isolates belong to the same aggressive *Neopestalotiopsis* sp. originally reported from Florida strawberry (Baggio et al. 2021). Pathogenicity testing conducted on the leaves, fruit, and roots of both blueberries and strawberries further confirmed that these isolates can cause disease on both hosts (**Figure 4.4**). *Neopestalotiopsis* sp. has not previously been considered as a major blueberry pathogen, but our findings from greenhouse experiments suggest that these isolates have the potential to cause disease on blueberry. To determine their capacity to trigger outbreaks in blueberries under field conditions, further epidemiological studies are needed; however, it should be noted that the identification of isolates of this novel species from

the same field site in both 2021 and 2022 suggests their ability to persist in field settings. Furthermore, the fact that these isolates recovered from blueberry can cause disease in strawberry is concerning because this novel *Neopestalotiopsis* sp. has become major pathogen in the strawberry production system in the Eastern U.S. and Canada (Baggio et al. 2021; Guan et al. 2023; Jimenez Madrid et al. 2024; McNally et al. 2023). Since blueberries and strawberries are often propagated in the same nurseries, these hosts may serve as pathogen reservoirs for each other, facilitating cross-infection and disease spread. Although the *Neopestalotiopsis* isolates identified from blueberries in this study were shown to have the potential to cause disease in both blueberry and strawberry, it should be noted that our findings did not determine whether their relative aggressiveness is comparable to that of isolates of this novel species from strawberry. A comparative pathogenicity assay, involving side-by-side testing of blueberry-derived novel *Neopestalotiopsis* sp. isolates alongside aggressive strawberry-derived novel *Neopestalotiopsis* sp. isolates from Florida and other regions would be necessary to determine this. If virulence of both the blueberry and strawberry isolates were found to be similar on strawberry, this would suggest the potential for movement of this novel species between blueberry and strawberry and have obvious implications for the management of the emerging crown, leaf, and fruit disease, especially in nursery and production environments where both of these small fruit hosts may be present. If, by contrast, the blueberry-derived novel *Neopestalotiopsis* sp. isolates were found to be relatively less virulent than the strawberry-derived isolates, this would suggest that identification of this novel species based solely on the presence or absence of mutations within the β-tubulin (*B-tub*) gene or on phylogenetic analysis using the three genes frequently utilized for MLSA of *Neopestalotiopsis* spp. (ITS, *B-tub*, and *tef1*) may be insufficient on their own to determine if isolates of this novel species belong to the "aggressive-type" on strawberry – with significant

implications for disease diagnostics. Regardless, given the demonstrated ability of the *Neopestalotiopsis* sp. isolates identified here from blueberries to cause significant symptoms on both blueberry and strawberry, it is crucial to further investigate their epidemiology, transmission mechanisms, and potential strategies for their management. This study emphasizes the need for continued surveillance of new *Neopestalotiopsis* spp. to understand potential interactions in diverse agricultural systems.

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## FIGURES

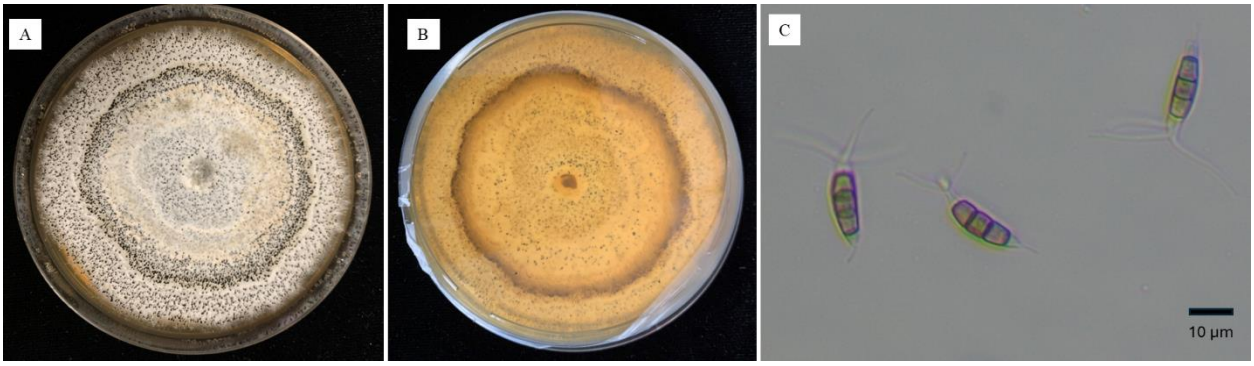


**Figure 4.1**. Morphological identification of *Neopestalotiopsis* sp. (A) Front view showing whitish mycelium with concentric rings of acervuli on potato dextrose agar. (B) Bottom view showing yellowish mycelium with concentric rings of acervuli on potato dextrose agar. (C) Five-celled conidia of *Neopestalotiopsis* sp. with three melanized internal cells, hyaline basal and apical cells, and three apical appendages.

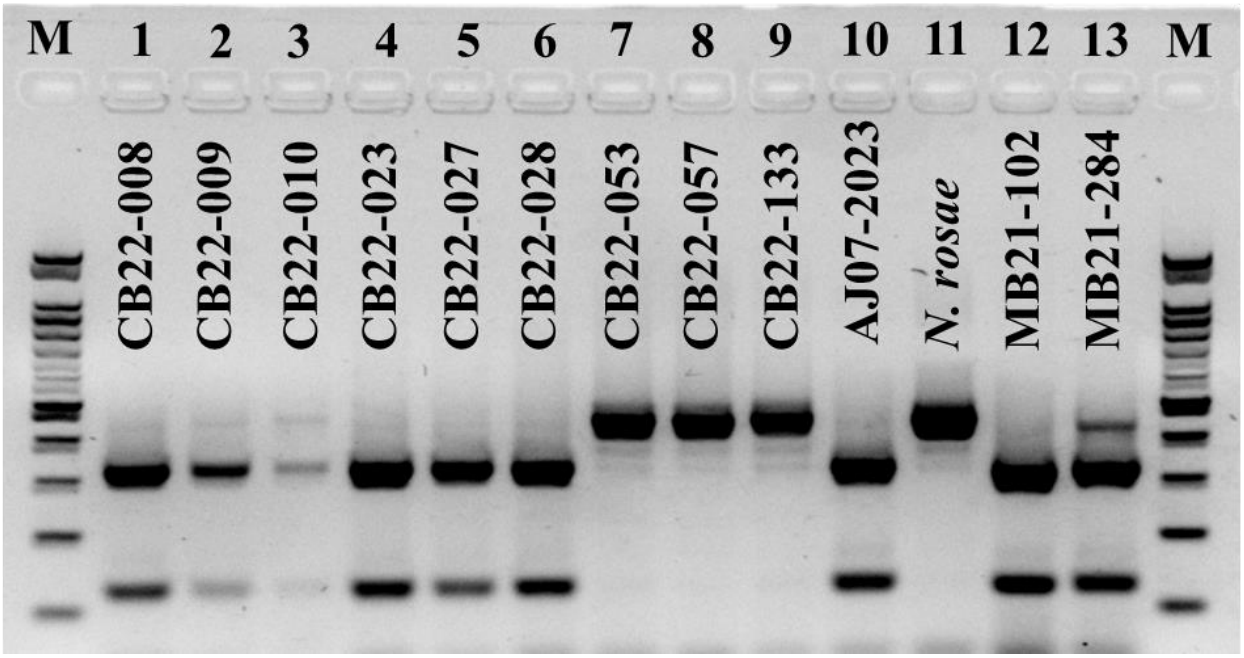


**Figure 4.2.** Restriction digestion with *Bsa*WI enzyme of the 420 bp *B-tub* gene region amplified with the Bt2a and Bt2b primer pair. Lanes 1-9 and 12-13 are isolates from Georgia blueberries and Lanes 10 and 11 are *Neopestalotiopsis* sp. isolate 'AJ07-2023' and a *Neopestalotiopsis rosae* isolate, respectively, from Jimenez Madrid et al. (2024). Products from *Neopestalotiopsis* sp. isolate 'AJ07-2023' (lane 10), 2022 Georgia blueberries isolates (lanes 1-6), 2021 Georgia blueberry isolates (lanes 12 and 13) show two fragments of 290 bp and 130 bp, indicating digestion due to the presence of a mutation within *B-tub*. In contrast, the *N. rosae* isolate (lane 11), and the 2022 Georgia blueberry isolates (lanes 7-9) display only the undigested 420 bp amplicon, indicating the absence of a restriction site at this location within *B-tub*. M = 100 bp marker.

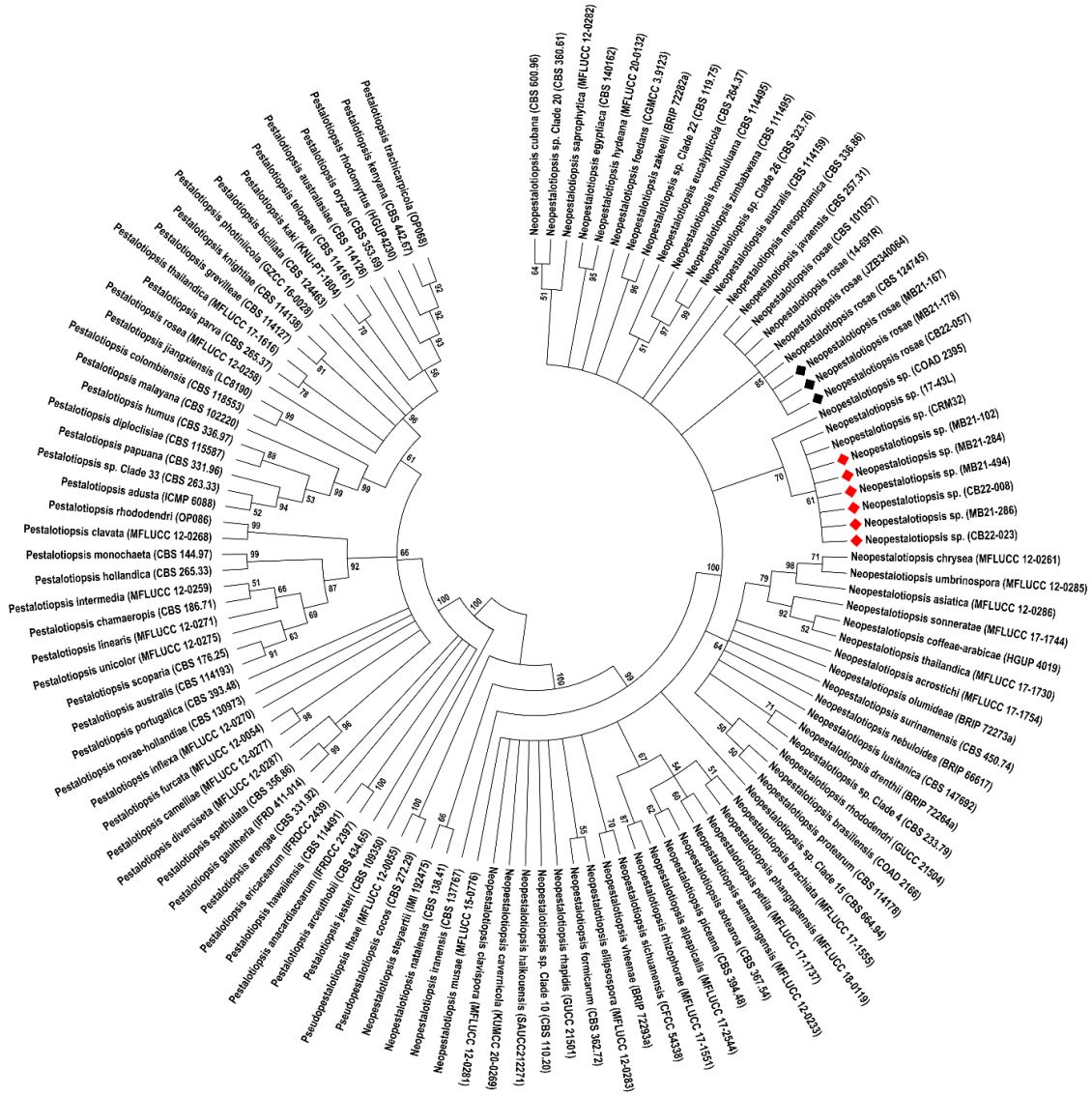


**Figure 4.3.** Maximum likelihood phylogenetic tree constructed using concatenated portions of the internal transcribed spacer (ITS), β-tubulin (*B-tub*), and *tef1* regions showing Georgia blueberry *Neopestalotiopsis* isolates from blueberry alongside other pestalotioid species. Georgia blueberry isolates identified as *Neopestalotiopsis rosae* are indicated with a black diamond and Georgia blueberry isolates identified as the novel *Neopestalotiopsis* sp. are indicated with a red diamond. Bootstrap support percentages (determined from 1000 replicates) are indicated on the individual branches. Branches were collapsed if bootstrap support was less than 50%.


**Figure 4.4.** Images from pathogenicity testing with *Neopestalotiopsis* sp. isolates on blueberry leaves (A & B), detached fruit (C), and roots (D) as well as strawberry leaves (E & F) and attached fruit (G). Panel A shows leaf spots on inoculated blueberry plants. Panels B and C show the typical signs of fungal fruiting bodies (acervuli) on leaves and fruit, respectively. Panel D shows yellowing and defoliation of the leaves following root inoculation. Panel E shows leaf spots and stem dieback on inoculated strawberry plants. Panels F and G show fungal signs including fruiting bodies (acervuli) on strawberry leaves and fruit, respectively.

## Supplementary information

**Supplemental Table 4.1.** List of Genbank accession numbers of sequences generated in this study as well as accession numbers and references for isolates used in phylogenetic analysis.

| Species (Isolate) | GenBank Accession Number | | | Reference[1] |
|---|---|---|---|---|
| | ITS | *B-tub* | *tef1* | |
| *Neopestalotiopsis acrostichi* (MFLUCC 17-1754) | MK764272 | MK764338 | MK764316 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis alpapicalis* (MFLUCC 17-2544) | MK357772 | MK463545 | MK463547 | Kumar et al. (2019) |
| *Neopestalotiopsis aotearoa* (CBS 367.54) | KM199369 | KM199454 | KM199526 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis asiatica* (MFLUCC 12-0286) | JX398983 | JX399018 | JX399049 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis australis* (CBS 114159) | KM199348 | KM199432 | KM199537 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis brachiata* (MFLUCC 17-1555) | MK764274 | MK764340 | MK764318 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis brasiliensis* (COAD 2166) | MG686469 | MG692400 | MG692402 | Bezerra et al. (2018) |
| *Neopestalotiopsis cavernicola* (KUMCC 20-0269) | MW545802 | MW557596 | MW550735 | Liu et al. (2021) |
| *Neopestalotiopsis chrysea* (MFLUCC 12-0261) | JX398985 | JX399020 | JX399051 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis clavispora* (MFLUCC 12-0281) | JX398979 | JX399014 | JX399045 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis coffeae-arabicae* (HGUP 4019) | KF412649 | KF412643 | KF412646 | Song et al. (2013) |
| *Neopestalotiopsis cubana* (CBS 600.96) | KM199347 | KM199438 | KM199521 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis drenthii* (BRIP 72264a) | MZ303787 | MZ312680 | MZ344172 | Prasannath et al. (2021) |
| *Neopestalotiopsis egyptiaca* (CBS 140162) | KP943747 | KP943746 | KP943748 | Crous et al. (2015) |
| *Neopestalotiopsis ellipsospora* (MFLUCC 12-0283) | JX398980 | JX399016 | JX399047 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis eucalypticola* (CBS 264.37) | KM199376 | KM199431 | KM199551 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis foedans* (CGMCC 3.9123) | JX398987 | JX399022 | JX399053 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis formicarum* (CBS 362.72) | KM199358 | KM199455 | KM199517 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis haikouensis* (SAUCC212271) | OK087294 | OK104870 | OK104877 | Zhang et al. (2022) |
| *Neopestalotiopsis honoluluana* (CBS 114495) | KM199364 | KM199457 | KM199548 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis hydeana* (MFLUCC 20-0132) | MW266069 | MW251119 | MW251129 | Huanaluek et al. (2021) |
| *Neopestalotiopsis iranensis* (CBS 137767) | KM074045 | KM074056 | KM074053 | Ayoubi and Soleimani (2016) |
| *Neopestalotiopsis javaensis* (CBS 257.31) | KM199357 | KM199437 | KM199543 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis lusitanica* (CBS 147692) | MW794110 | MW802843 | MW805406 | Diogo et al. (2021) |
| *Neopestalotiopsis mesopotamica* (CBS 336.86) | KM199362 | KM199441 | KM199555 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis musae* (MFLUCC 15-0776) | KX789683 | KX789686 | KX789685 | Hyde et al. (2016) |
| *Neopestalotiopsis natalensis* (CBS 138.41) | KM199377 | KM199466 | KM199552 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis nebuloides* (BRIP 66617) | MK966338 | MK977632 | MK977633 | Crous et al. (2020) |
| *Neopestalotiopsis olumideae* (BRIP 72273a) | MZ303790 | MZ312683 | MZ344175 | Prasannath et al. (2021) |
| *Neopestalotiopsis petila* (MFLUCC 17-1737) | MK764276 | MK764341 | MK764319 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis phangngaensis* (MFLUCC 18-0119) | MH388354 | MH412721 | MH388390 | Tibpromma et al. (2018) |
| *Neopestalotiopsis piceana* (CBS 394.48) | KM199368 | KM199453 | KM199527 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis protearum* (CBS 114178) | JN712498 | KM199463 | KM199542 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis rhapidis* (GUCC 21501) | MW931620 | MW980441 | MW980442 | Yang et al. (2021) |
| *Neopestalotiopsis rhizophorae* (MFLUCC 17-1551) | MK764278 | MK764343 | MK764321 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis rhododendri* (GUCC 21504) | MW979577 | MW980443 | MW980444 | Yang et al. (2021) |
| *Neopestalotiopsis rosae* (14-691R) | MK895142 | MK903338 | MK903334 | Baggio et al. (2021) |
| *Neopestalotiopsis rosae* (CB22-057) | OR800207 | PV492423 | PV492436 | This study |
| *Neopestalotiopsis rosae* (CBS 101057) | KM199359 | KM199429 | KM199523 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis rosae* (CBS 124745) | KM199360 | KM199430 | KM199524 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis rosae* (JZB340064) | MN495972 | MN968336 | MN968328 | Sun et al. (2021) |
| *Neopestalotiopsis rosae* (MB21-167) | OR803183 | PV492412 | PV492425 | This study |
| *Neopestalotiopsis rosae* (MB21-178) | OR803193 | PV492413 | PV492426 | This study |
| *Neopestalotiopsis samarangensis* (MFLUCC 12-0233) | JQ968609 | JQ968610 | JQ968611 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis saprophytica* (MFLUCC 12-0282) | JX398982 | JX399017 | JX399048 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis sichuanensis* (CFCC 54338) | MW166231 | MW218524 | MW199750 | Jiang et al. (2021) |
| *Neopestalotiopsis sonneratae* (MFLUCC 17-1744) | MK764280 | MK764345 | MK764323 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis* sp. (17-43L) | MK895144 | MK903340 | MK903336 | Baggio et al. (2021) |
| *Neopestalotiopsis* sp. (CB22-008) | OR800163 | PV492417 | PV492430 | This study |

| | | | | |
|---|---|---|---|---|
| *Neopestalotiopsis* sp. (CB22-023) | OR800175 | PV492420 | PV492433 | This study |
| *Neopestalotiopsis* sp. (COAD 2395) | MN238829 | MN689615 | MN704864 | Belisário et al. (2020) |
| *Neopestalotiopsis* sp. (CRM32) | OR999326 | OR997691 | PP003932 | Unpublished (Acosta-Gonzalez et al. 2023) |
| *Neopestalotiopsis* sp. (MB21-102) | OR803128 | PV492411 | PV492424 | This study |
| *Neopestalotiopsis* sp. (MB21-284) | OR803292 | PV492414 | PV492427 | This study |
| *Neopestalotiopsis* sp. (MB21-286) | OR803294 | PV492415 | PV492428 | This study |
| *Neopestalotiopsis* sp. (MB21-494) | OR803457 | PV492416 | PV492429 | This study |
| *Neopestalotiopsis* sp. Clade 10 (CBS 110.20) | KM199342 | KM199442 | KM199540 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis* sp. Clade 15 (CBS 664.94) | KM199354 | KM199449 | KM199525 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis* sp. Clade 20 (CBS 360.61) | KM199346 | KM199440 | KM199522 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis* sp. Clade 22 (CBS 119.75) | KM199356 | KM199439 | KM199531 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis* sp. Clade 4 (CBS 233.79) | KM199373 | KM199464 | KM199528 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis steyaertii* (IMI 192475) | KF582796 | KF582794 | KF582792 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis surinamensis* (CBS 450.74) | KM199351 | KM199465 | KM199518 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis thailandica* (MFLUCC 17-1730) | MK764281 | MK764347 | MK764325 | Norphanphoun et al. (2019) |
| *Neopestalotiopsis umbrinospora* (MFLUCC 12-0285) | JX398984 | JX399019 | JX399050 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis vheenae* (BRIP 72293a) | MZ303792 | MZ312685 | MZ344177 | Prasannath et al. (2021) |
| *Neopestalotiopsis zakeelii* (BRIP 72282a) | MZ303789 | MZ312682 | MZ344174 | Prasannath et al. (2021) |
| *Neopestalotiopsis zimbabwana* (CBS 111495) | JX556231 | KM199456 | KM199545 | Maharachchikumbura et al. (2014) |
| *Neopestalotiopsis* sp. Clade 26 (CBS 323.76) | KM199350 | KM199458 | KM199550 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis adusta* (ICMP 6088) | JX399006 | JX399037 | JX399070 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis anacardiacearum* (IFRDCC 2397) | KC247154 | KC247155 | KC247156 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis arceuthobii* (CBS 434.65) | KM199341 | KM199427 | KM199516 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis arengae* (CBS 331.92) | KM199340 | KM199426 | KM199515 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis australasiae* (CBS 114126) | KM199297 | KM199409 | KM199499 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis australis* (CBS 114193) | KM199332 | KM199383 | KM199475 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis biciliata* (CBS 124463) | KM199308 | KM199399 | KM199505 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis camelliae* (MFLUCC 12-0277) | JX399010 | JX399041 | JX399074 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis chamaeropis* (CBS 186.71) | KM199326 | KM199391 | KM199473 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis clavata* (MFLUCC 12-0268) | JX398990 | JX399025 | JX399056 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis colombiensis* (CBS 118553) | KM199307 | KM199421 | KM199488 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis diploclisiae* (CBS 115587) | KM199320 | KM199419 | KM199486 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis diversiseta* (MFLUCC 12-0287) | JX399009 | JX399040 | JX399073 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis ericacearum* (IFRDCC 2439) | KC537807 | KC537821 | KC537814 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis furcata* (MFLUCC 12-0054) | JQ683724 | JQ683708 | JQ683740 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis gaultheria* (IFRD 411-014) | KC537805 | KC537819 | KC537812 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis grevilleae* (CBS 114127) | KM199300 | KM199407 | KM199504 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis hawaiiensis* (CBS 114491) | KM199339 | KM199428 | KM199514 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis hollandica* (CBS 265.33) | KM199328 | KM199388 | KM199481 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis humus* (CBS 336.97) | KM199317 | KM199420 | KM199484 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis inflexa* (MFLUCC 12-0270) | JX399008 | JX399039 | JX399072 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis intermedia* (MFLUCC 12-0259) | JX398993 | JX399028 | JX399059 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis jesteri* (CBS 109350) | KM199380 | KM199468 | KM199554 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis jiangxiensis* (LC8190) | KY464144 | KY464164 | KY464154 | Liu et al. (2017) |
| *Pestalotiopsis kaki* (KNU-PT-1804) | LC552953 | LC552954 | LC553555 | Das et al. (2020) |
| *Pestalotiopsis kenyana* (CBS 442.67) | KM199302 | KM199395 | KM199502 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis knightiae* (CBS 114138) | KM199310 | KM199408 | KM199497 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis linearis* (MFLUCC 12-0271) | JX398992 | JX399027 | JX399058 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis malayana* (CBS 102220) | KM199306 | KM199411 | KM199482 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis monochaeta* (CBS 144.97) | KM199327 | KM199386 | KM199479 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis novae-hollandiae* (CBS 130973) | KM199337 | KM199425 | KM199511 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis oryzae* (CBS 353.69) | KM199299 | KM199398 | KM199496 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis papuana* (CBS 331.96) | KM199321 | KM199413 | KM199491 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis parva* (CBS 265.37) | KM199312 | KM199404 | KM199508 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis photiniicola* (GZCC 16-0028) | KY092404 | KY047663 | KY047662 | Chen et al. (2017) |
| *Pestalotiopsis portugalica* (CBS 393.48) | KM199335 | KM199422 | KM199510 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis rhododendri* (OP086) | KC537804 | KC537818 | KC537811 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis rhodomyrtus* (HGUP4230) | KF412648 | KF412642 | KF412645 | Song et al. (2013) |
| *Pestalotiopsis rosea* (MFLUCC 12-0258) | JX399005 | JX399036 | JX399069 | Maharachchikumbura et al. (2014) |

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|---|---|---|---|---|
| *Pestalotiopsis scoparia* (CBS 176.25) | KM199330 | KM199393 | KM199478 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis* sp. Clade 33 (CBS 263.33) | KM199316 | KM199414 | KM199489 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis spathulata* (CBS 356.86) | KM199338 | KM199423 | KM199513 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis telopeae* (CBS 114161) | KM199296 | KM199403 | KM199500 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis thailandica* (MFLUCC 17-1616) | MK764285 | MK764351 | MK764329 | Norphanphoun et al. (2019) |
| *Pestalotiopsis trachicarpicola* (OP068) | JQ845947 | JQ845945 | JQ845946 | Maharachchikumbura et al. (2014) |
| *Pestalotiopsis unicolor* (MFLUCC 12-0275) | JX398998 | JX399029 | JX399063 | Maharachchikumbura et al. (2014) |
| *Pseudopestalotiopsis cocos* (CBS 272.29) | KM199378 | KM199467 | KM199553 | Maharachchikumbura et al. (2014) |
| *Pseudopestalotiopsis theae* (MFLUCC 12-0055) | JQ683727 | JQ683711 | JQ683743 | Maharachchikumbura et al. (2014) |

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# CHAPTER 5

## Comparative Virulence of Blueberry Isolates of *Neopestalotiopsis rosae* and a novel *Neopestalotiopsis* sp.: Evaluating Their Relative Aggressiveness on Blueberry (*Vaccinium corymbosum* interspecific hybrids) and Strawberry (*Fragaria* × *ananassa*)[1]

[1]Beg, M.A. & Oliver, J.E. To be submitted to: *Plant Disease*.

**Abstract**

A novel *Neopestalotiopsis* sp., reported to be more aggressive than the closely related strawberry pathogen *Neopestalotiopsis rosae*, was recently identified as being associated with epidemics on strawberries in the Eastern United States and Canada. Concurrent work with *Neopestalotiopsis* isolates from Georgia blueberry fruit identified isolates from blueberry that are indistinguishable from strawberry isolates of the aggressive novel *Neopestalotiopsis* sp. based on currently recommended diagnostic methods including RFLP and multilocus sequence analysis. Initial pathogenicity assays with these blueberry isolates confirmed their ability to cause disease on both blueberry and strawberry; however, their relative virulence on these hosts compared to isolates of the novel *Neopestalotiopsis* sp. from strawberry was not investigated. To investigate this, we conducted greenhouse virulence assays on both blueberry and strawberry with novel *Neopestalotiopsis* sp. and *N. rosae* isolates collected from blueberry and an isolate of the novel *Neopestalotiopsis* sp. collected from strawberry. Results on blueberry cultivar 'Rebel' indicated that all isolates were able to cause disease; however, no consistent virulence differences were noted between isolates. On strawberry cultivar 'Albion', all isolates of the novel *Neopestalotiopsis* sp. from blueberry caused significant disease in all cases, whereas the *N. rosae* isolates did not, indicating that isolates of the novel *Neopestalotiopsis* sp. from blueberry are relatively more aggressive than the *N. rosae* isolates on this host. However, on strawberry cultivar 'Camino Real', disease severity was numerically less, and no consistent differences were noted between blueberry isolates of *N. rosae* and the novel *Neopestalotiopsis* sp. Interestingly, on both strawberry cultivars, the strawberry *Neopestalotiopsis* sp. isolate caused significantly more severe disease than any of the tested isolates from blueberry. Overall, while these results provide further confirmation that isolates of the novel *Neopestalotiopsis* sp. from blueberry are capable of causing disease on both

hosts, the blueberry isolates tested in these experiments were not as virulent on strawberry as the tested strawberry isolate of the novel *Neopestalotiopsis* sp. This suggests a need for further investigation regarding potential genetic factors present in strawberry isolates of this novel *Neopestalotiopsis* sp. that may explain their increased aggressiveness on strawberry relative to isolates of this novel species from blueberry.

KEYWORDS: Blueberry, Strawberry, *Neopestalotiopsis*, *Neopestalotiopsis rosae*, greenhouse assays, virulence, cross-host infection.

## Introduction

Pestalotioid fungi, frequently identified from plant material as endophytes or secondary pathogens (Darapanit et al. 2021), are increasingly being recognized as important pathogens in fruit crops such as blueberry and strawberry. While *Neopestalotiopsis* has not been considered to be a major pathogen in blueberry cultivation in the United States, there is growing international evidence of its pathogenic potential in this crop. A notable example comes from China, where a 2023 report documented *Pestalotiopsis* species causing blueberry leaf spots and stem cankers. Of 91 isolates recovered from symptomatic tissues, 57% were identified as *Neopestalotiopsis clavispora* (Zheng et al. 2023), suggesting the prevalence and potential impact of this species. *N. clavispora* is, in fact, one of the most frequently reported species in the genus and has also been associated with twig dieback and stem canker in blueberries in various countries, including Korea, Serbia, Spain, and Uruguay (Borrero et al. 2018; González et al. 2012; Jevremovic et al. 2022; Lee et al. 2019). Additional characterizations of *Neopestalotiopsis* populations from blueberries have been conducted in Portugal and South Africa. In South Africa, a recent study reported that 59% of the 48 isolates collected from symptomatic blueberry tissues were *N. rosae*, while 29% were identified as *N. hispanica*, both associated with leaf and twig blight (Van der Vyver et al. 2025). Similarly, in Portugal, a significant proportion of the *Neopestalotiopsis* isolates recovered from blueberry were identified as *N. rosae* and *N. vaccinii* (synonymous with *N. hispanica*) (Santos et al. 2022), further emphasizing the genus's emerging role in blueberry disease complexes.

In strawberry production in recent years, the genus *Neopestalotiopsis* has emerged as a significant threat. One species of *Neopestalotiopsis* that has been reported more frequently is *N. rosae*. This species has been reported to cause disease in strawberries in China, Egypt, Germany, India, Italy, Mexico, Taiwan, and the United States (Baggio et al. 2021; Chandana et al. 2024;

Dardani et al. 2025; Essa et al. 2018; Rebollar-Alviter et al. 2020; Schierling et al. 2024; Sun et al. 2021; Wu et al. 2020). This pathogen has the ability to cause leaf, root, crown, and fruit rot in strawberries, leading to significant economic losses. In addition to *N. rosae*, a distinct species, frequently referred to in the literature as a novel aggressive *Neopestalotiopsis* sp. was identified as the cause of a severe disease outbreak in Florida, USA, during the 2018–2019 growing season (Baggio et al. 2021). This novel species has been reported to be more aggressive than *N. rosae*, and was suggested to have been disseminated to strawberry farms on nursery-propagated plants (Baggio et al. 2021). Since it was first identified in Florida strawberries, this new species has subsequently been reported in other parts of the Eastern U.S. (Guan et al. 2023; Jimenez Madrid et al. 2024) and in Canada (McNally et al. 2023). This pathogen has been shown to be phylogenetically very close to, but distinct from, *N. rosae* based on sequenced portions of the internal transcribed spacer (ITS), β-tubulin (B-tub), and tef1 regions, but this novel species has not been assigned a formal species name in the published literature to date. Nonetheless, this new species has been diagnostically differentiated from other *Neopestalotiopsis* species based on a specific mutation within the β-tubulin (B-tub) gene (Kaur et al. 2023).

Recently, multiple *Neopestalotiopsis* sp. isolates carrying the same B-tub mutation and phylogenetically placed within the same clade as the novel aggressive *Neopestalotiopsis* sp. from Florida were identified from blueberry fruit in Georgia (Chapter 4), and initial pathogenicity testing confirmed that these isolate from Georgia blueberry were capable of causing disease on both blueberry and strawberry. These findings (that a pathogen known to cause epidemics in strawberry has been found in blueberry and is capable of causing disease on strawberry) immediately led to two hypotheses: First, that these blueberry isolates would be more aggressive (virulent) on strawberry compared to other *Neopestalotiopsis* spp. isolates collected from

blueberry, and second, that these isolates would also exhibit greater virulence on blueberry compared to other *Neopestalotiopsis* spp. isolates. To test these hypotheses, we conducted greenhouse pathogenicity tests with both blueberry and strawberry to compare the relative virulence of Georgia blueberry fruit isolates of *N. rosae* and the novel *Neopestalotiopsis* sp. as well as a recently characterized isolate of the novel *Neopestalotiopsis* sp. from Georgia strawberry (Jimenez Madrid et al. 2024).

## Materials and Methods

### Plant Materials

For all blueberry experiments (**Table 5.1**), southern highbush (*Vaccinium corymbosum* interspecific hybrids) cultivar 'Rebel' was used. Plants (in 72-cell trays) were purchased from AgriStarts, Inc. (Apopka, Florida) and transferred to 10 cm x 7.6 cm x 10.9 cm plastic pots filled with pine bark and sand. Plants were kept in the greenhouse for approximately four weeks prior to inoculation. For the strawberry experiments (**Table 5.1**), two strawberry (*Fragaria* × *ananassa*) cultivars were used: 'Albion' and 'Camino Real'. Two experiments were conducted with 'Albion' and two with 'Camino Real'. Bare root 'Albion' plants for strawberry experiments 1 and 2 were obtained from Indiana Berry & Plant Co. (Plymouth, Indiana) and Nourse Farms (Whately, Massachusetts), respectively. For cultivar 'Camino Real', plugs were acquired from Farm on Central (Carlisle, OH) and utilized for the third and fourth strawberry experiment. Strawberry plants were established in Jungle Growth™ Flower and Vegetable Professional Mix soil (Mulch and Soil Company, Fort Meyers, Florida) in 10 cm x 7.6 cm x 10.9 cm plastic pots and allowed to grow for at least 3 to 4 weeks in the greenhouse prior to inoculation.

**Fungal isolates and inoculum preparation**

For pathogenicity experiments with blueberry and strawberry, previously identified *Neopestalotiopsis* isolates were used (**Table 5.1**). These included *N. rosae* isolates 'MB21-167' and 'MB21-178' as well as novel *Neopestalotiopsis* sp. isolates 'MB21-494', 'MB21-102', 'MB21-284', and 'CB22-008' collected from Georgia blueberries in 2021 and 2022 (Chapter 4). In addition, novel *Neopestalotiopsis* sp. isolate 'AJ07-2023' collected from Georgia strawberries in 2023 (Jimenez Madrid et al. 2024) was also utilized in some experiments for comparison. Spore suspensions for use in the pathogenicity experiments were obtained from each respective isolate according to a method modified from that listed in Chapter 4. In brief, isolates of *Neopestalotiopsis* spp. were first cultured on potato dextrose agar (PDA) and incubated in darkness at 25°C for five days. After this period, Petri dishes were unsealed and maintained under a 12-hour light/12-hour dark photoperiod at 25°C for an additional seven days to allow for production of fungal acervuli. The dark, black acervuli that developed were carefully disrupted using a sterile scalpel, and a spore suspension was prepared in sterile distilled water. Using a hemocytometer, the spore concentration was adjusted to $10^5$ spores/ml to ensure inoculum uniformity among isolates across experiments.

**Inoculation methods and evaluation**

For pathogenicity experiments conducted on blueberry roots, soil was first removed from the roots by rinsing them repeatedly in sterile distilled water. Root tips were then trimmed to promote entry of the pathogen. Roots were inoculated by immersion for 10 min in a suspension of ~$5 \times 10^5$ spores/ml or water (control). For each respective isolate or the water (control), six plants were inoculated. After inoculation, root-inoculated plants were re-potted into plastic pots and

maintained in a greenhouse at 28°C (±2°C) with daily irrigation throughout the experiment. Root-inoculated plants were observed for 30 days post-inoculation (dpi).

For additional pathogenicity experiments with blueberry and strawberry, prepared spore suspensions were sprayed onto plants until runoff. Just as in the blueberry root inoculation experiment, for each isolate and the water (untreated) control, six individual plants were used as replicates, with each pot containing one plant considered as a single experimental unit throughout the experiment. After inoculation, potted plants were placed into plastic boxes (60.8 cm x 42.7 cm x 33.1 cm) containing water-soaked paper towels at the bottom to maintain high humidity (95-100%) throughout each experiment. Plants were assessed for disease over 21 dpi.

**Disease assessments**

In blueberry plants subjected to root inoculation, assessments were made based on the typical progression of symptoms which included the initial yellowing of leaves, subsequent leaf reddening, and eventual defoliation (**Figure 5.1A)**. Accordingly, to assess disease severity over time, for each plant, a disease severity index was calculated by numerically summarizing the symptoms across all leaves at each assessment time. Each yellow leaf was assigned a score of one point, each red leaf was assigned a score of two points, each defoliated leaf was assigned a score of three points, and the disease severity index was calculated using the formula:

$$X_i = \sum[(\mathrm{S1} \times 1) + (\mathrm{S2} \times 2) + (\mathrm{S3} \times 3)]$$

where S1 is the number of yellow leaves, S2 is the number red leaves, S3 is the number of defoliated leaves, and $X_i$ is the total score calculated for each individual plant at the $i^{th}$ observation.

In leaf-inoculated blueberry plants, a disease severity index was calculated by numerically summarizing the number of defoliated leaves and leaf spots across all leaves (**Figure 5.1B**). One point was assigned for each leaf spot and two points were assigned for each defoliated leaf. The total disease score for a plant was calculated as:

$$X_i = \sum[(S1 \times 1) + (S2 \times 2)]$$

where S1 is the number of leaf spots, S2 is the number of defoliated leaves, and $X_i$ is the total score calculated for each individual plant at $i^{th}$ observation.

For inoculated strawberry plants, a disease severity index was calculated based on the spots observed on the leaves or flower buds and the progression of disease into the stems (**Figure 5.2)**. One point was assigned for each spot that was less than ¼ of the leaf surface, two points were assigned for each leaf spot that was greater than ¼ of the leaf surface, and two points were assigned for each infected stem. Based on the symptoms observed on the plants, the following disease severity index was established for assessing the diseases on the strawberry plants:

$$X_i = \sum[(S1 \times 1) + (S2 \times 2) + (S3 \times 2)]$$

where S1 is the number of leaf spots that represent less than ¼ of the leaf surface, S2 is the number of leaf spots that represent more than ¼ of the leaf surface, S3 represents each infected stem, and $X_i$ is the total score calculated for each individual plant at $i^{th}$ observation.

**AUDPC Calculation and Statistical Analysis**

Area under disease progress curve (AUDPC) values for each plant were calculated based on the disease severity index calculated at each assessment timepoint using the following formula:

$$AUDPC = \sum_{i=1}^{N_{i-1}} [(X_i + X_{i+1})/2]\,(t_{i+1} - t_i)$$

where $X_i$ is the total score calculated for an individual plant at the $i^{th}$ evaluation, $X_{i+1}$ is the score of the $(i + 1)^{th}$ evaluation and $(t_{i+1} - t_i)$ is the number of days between two evaluations (Shaner and Finney 1977). AUDPC values calculated during pathogenicity experiments were analyzed using analysis of variance (ANOVA) to compare the disease severity among isolates in each experiment. Post hoc comparisons were made using Tukey's HSD test to identify significant differences between isolates ($\alpha = 0.05$). Statistical analyses were performed using the R software environment (v. 4.1.1).

## Results

### Pathogenicity of *Neopestalotiopsis* Isolates Following Root Inoculation of Blueberry

By 7 dpi, the first visual symptoms (leaf yellowing) were noted on root-inoculated blueberry plants. Between 7 and 14 dpi, leaves on the inoculated plants turned from yellow to red, and defoliation of many of the leaves had occurred by 14 dpi (**Figure 5.1A**). In contrast, the leaves of the control plants (root-inoculated with sterile distilled water) remained largely green throughout, and no defoliation had occurred on these plants by 21 dpi.

Among the tested isolates, *Neopestalotiopsis rosae* isolates ('MB21-167' and 'MB21-178') and the novel *Neopestalotiopsis* sp. isolate ('CB22-008') all induced similar root disease symptoms. The disease severity (AUDPC values) for 'MB21-167', 'MB21-178' (*N. rosae*), and 'CB22-008' (novel *Neopestalotiopsis* sp.) were significantly higher than those of the uninoculated control plants (**Figure 5.3A**). There were no statistically significant differences in terms of disease severity (AUDPC values) between the blueberry *N. rosae* isolates and the novel *Neopestalotiopsis* isolate.

**Blueberry leaf inoculation**

In the blueberry leaf-inoculation experiments (**Table 5.1**), symptoms began to appear on the leaves of the inoculated plants at 4 dpi. Dark brown circular lesions with a yellow center were initially observed (**Figure 5.1B**), predominantly developing along the leaf edges and at the nodes, eventually leading to leaf drop. Black fungal acervuli resembling those previously reported on strawberry affected by *Neopestalotiopsis* (Baggio et al. 2021) were clearly visible within the lesions. In the first blueberry leaf-inoculation experiment (**Figure 5.3B**), both *N. rosae* isolates ('MB21-167' and 'MB21-178') and the novel *Neopestalotiopsis* sp. isolate ('CB22-008') from blueberries induced similar types of symptoms; however, disease severity (as indicated by AUDPC values) varied significantly among the isolates (**Figure 5.3B**). While the disease severity for all isolates was numerically higher than for the untreated control plants, the disease severity observed for *N. rosae* isolate 'MB21-178' was relatively lower than that of *N. rosae* isolate 'MB21-167' and the novel *Neopestalotiopsis* sp. isolate ('CB22-008'). Similar leaf symptoms were observed in the second blueberry leaf-inoculation experiment. In this experiment, all isolates (including *N. rosae* 'MB21-167' and 'MB21-178', novel *Neopestalotiopsis* sp. isolates from blueberry 'MB21-494', 'CB22-008', and a novel isolate from strawberry 'AJ07-2023') caused numerically greater disease severity than the untreated control, however, the difference in disease severity between isolate 'MB21-167' (*N. rosae*) and the untreated control was not statistically significant (**Figure 5.3C**). The strawberry-derived *Neopestalotiopsis* sp. isolate 'AJ07-2023' caused significant disease in this experiment with relatively similar disease severity to the blueberry-derived *Neopestalotiopsis* sp. isolates tested.

**Strawberry Inoculation Experiments**

On inoculated strawberry plants, symptoms first appeared on the buds and blooms. Entire unopened buds, or the petals and stigmas of opened flowers, initially turned dark brown, followed by progressive dieback originating from these sites. This led to the collapse and death of the entire plant within 2 to 3 weeks post-inoculation. Leaf symptoms on inoculated plants also became evident by 3–4 dpi, beginning as dark brown spots that gradually expanded into larger lesions (**Figure 5.2**). By 7–10 dpi, black acervuli were clearly visible on the affected leaves with the naked eye, consistent with previous descriptions by Baggio et al. (2021). No fungal signs (acervuli) were observed on the uninoculated control plants.

In the first strawberry inoculation experiment (cv. 'Albion'), while disease severity after inoculation with *Neopestalotiopsis rosae* isolates ('MB21-167' and 'MB21-178') and novel *Neopestalotiopsis* isolates from blueberries ('CB22-008', 'MB21-102', and 'MB21-494') was numerically higher than in the uninoculated control (**Figure 5.4A**), only the three novel *Neopestalotiopsis* sp. isolates caused significantly more severe disease than the control treatment. In addition, two of the three tested novel *Neopestalotiopsis* sp. isolates from blueberry ('MB21-102' and 'MB21-494') caused statistically greater disease severity than the *N. rosae* isolates. In the second strawberry inoculation experiment (with cv. 'Camino Real'), overall disease severity values were relatively less than in the first experiment (**Figure 5.4B**). Though inoculation with all tested isolates resulted in numerically more disease than in the untreated control, only one isolate of the novel *Neopestalotiopsis* sp. ('MB21-494') resulted in disease severity that was significantly higher than the control. No statistically significant differences in disease severity were observed among the *N. rosae* and novel *Neopestalotiopsis* isolates from blueberry in this experiment.

In the third strawberry experiment (conducted with cv. 'Camino Real'), AUDPC values indicated that inoculation with the *N. rosae* isolate ('MB21-167'), the novel *Neopestalotiopsis* isolate from blueberry ('MB21-494'), and the novel isolate from strawberry ('AJ07-2023') each resulted in significantly higher disease severity compared to the control (**Figure 5.5A**). Although no statistically significant difference in disease severity was observed between the *N. rosae* isolate ('MB21-167') and the novel blueberry isolate ('MB21-494'), the strawberry-derived isolate ('AJ07-2023') caused significantly higher disease than any of the blueberry isolates. In fact, the average AUDPC value with this isolate in this experiment was nearly three times greater than the *Neopestalotiopsis* isolates from blueberry in this experiment.

In the fourth strawberry experiment (conducted with cv. 'Albion'), the tested *N. rosae* isolates ('MB21-167' and 'MB21-178'), novel *Neopestalotiopsis* isolates from blueberry ('MB21-494' and 'MB22-008'), and the strawberry-derived isolate ('AJ07-2023') all caused numerically higher disease severity compared to the control (**Figure 5.5B**). However, only the novel *Neopestalotiopsis* sp. isolates—whether from blueberry or strawberry—caused disease severity that was statistically greater than the control. In contrast, no statistically significant differences were observed between either of the *N. rosae* isolates or the control. No significant differences in disease severity were observed between the novel *Neopestalotiopsis* isolates from blueberry included; however, the strawberry-derived isolate ('AJ07-2023') caused statistically significantly higher disease than any of the blueberry isolates in this experiment. Notably, its AUDPC value was approximately three times greater than those of the *N. rosae* isolates.

**Discussion:**

While *Neopestalotiopsis* spp. isolates are frequently isolated from plant tissue (Rayanathoola et al. 2025), isolates from this genus are frequently assumed to be secondary pathogens. While *N. rosae* has been known to as the cause of Neopestalotiopsis leaf, crown, and fruit rot on strawberries for more than 50 years (Howard and Albregts 1973; Kenneth et al. 1968), this disease had generally not been considered as a major issue on strawberries until 2019-2020, when a novel aggressive *Neopestalotiopsis* sp. was first identified to be the culprit behind a devastating outbreak on strawberry in Florida (Baggio et al. 2021). Since then, increased attention has been focused on this genus, and it has become important to identify so-called "aggressive" *Neopestalotiopsis* isolates that may pose a threat to strawberry production. Since young plants infected with *Neopestalotiopsis* may not initially show symptoms of Neopestalotiopsis leaf, crown, and fruit rot until they are put out in the field and exposed to environmental conditions (wetness and humidity) that are ideal for disease development (Rebollar-Alviter et al. 2020), nursery providers are under increasing pressure to ensure that their planting stock is initially free from *Neopestalotiopsis* spp. that may cause this devastating disease (Avila-Hernández et al. 2025). This has, in turn, put pressure on diagnostic laboratories to identify the so called "aggressive" strain and differentiate it from less aggressive *Neopestalotiopsis* spp. that may also be present (Avila-Hernández et al. 2025). Since the relatively less aggressive *N. rosae*, and the "aggressive" novel *Neopestalotiopsis* sp. cannot be distinguished morphologically, laboratories have relied on genetic techniques including restriction fragment length polymorphisms (RFLP) to differentiate these two species based upon the presence of a single mutation within B-tub, or on multilocus sequence analysis (MLSA) based on the sequencing of the internal transcribed spacer (ITS), β-tubulin (B-tub), and tef1 regions. Based upon both of these methodologies, the isolates from Georgia

blueberry fruit utilized in this study were previously identified and classified as belonging to the same novel *Neopestalotiopsis* sp. as the "aggressive" isolates from strawberry (Chapter 4).

While the ability of these Georgia isolates to cause disease on blueberry and strawberry was previously confirmed (Chapter 4), the potential threat they pose to both strawberry and blueberry production largely depends on how relatively "aggressive" (virulent) they are on these hosts. If isolates of the novel *Neopestalotiopsis* sp. from blueberry are as aggressive on strawberry as the strawberry isolates of this species have been reported to be, this would suggest that they may belong to the same pathogenic population responsible for the strawberry disease outbreak in Florida or possess the potential to cause similar disease. This would have obvious implications for small fruit growers in the Southeast U.S. who may grow both crops and for nursery producers who may propagate and maintain blueberry and strawberry plants in the same facilities where the potential for cross infection or movement of aggressive strains from blueberry to strawberry may be high. Likewise, if these novel *Neopestalotiopsis* sp. isolates from blueberry are relatively more aggressive on blueberry than other *Neopestalotiopsis* isolates frequently isolated from blueberry (including *N. rosae*) this may suggest an emerging concern for Southeast blueberry growers. Accordingly, virulence assessments, such as the ones conducted in this study, are critical for understanding the situation with these isolates from blueberry.

In this study, greenhouse pathogenicity assays were conducted as part of this work on both blueberry and strawberry using *Neopestalotiopsis* spp. isolates originally cultured from both hosts. In these assays, the disease severity resulting from inoculations with isolates of the novel *Neopestalotiopsis* sp. from blueberry was compared with the disease severities caused by *N. rosae* isolates from blueberry and a novel *Neopestalotiopsis* sp. isolate from strawberry. On blueberry, results indicated the ability of the tested isolates to cause disease; however, significant differences

in disease severity were not noted between isolates of *N. rosae* and the novel *Neopestalotiopsis* sp. isolates from blueberry, nor between the isolates of the novel *Neopestalotiopsis* sp. from blueberry and the novel *Neopestalotiopsis* sp. from strawberry in these assays. While, to our knowledge, these results represent the first confirmation that isolates of this novel species from strawberry are capable of causing disease on blueberry, they do not indicate that it, nor the other novel *Neopestalotiopsis* sp. isolates from blueberry, are likely to be substantially more aggressive on blueberry than other *Neopestalotiopsis* spp. isolates that may be found on this host. Previous work comparing the aggressiveness of *Neopestalotiopsis* isolates on blueberry has been very limited and has utilized different inoculation and assessment methods than the ones utilized in our study to compare isolate aggressiveness (due to their having a different disease focus). In work primarily focused on blueberry twig blight and utilizing pathogenicity assays to compare the ability of *Pestalotiopsis* spp. and *Neopestalotiopsis* spp. to cause stem lesions, Santos et al. (2022) showed that isolates representing different species did differ in their aggressiveness on blueberry. Among these, based on a single isolate, *N. rosae* was noted as being the most aggressive species in terms of lesion length that resulted after wound inoculation of the stem.

On strawberry, disease severities resulting from our inoculations with isolates of the novel *Neopestalotiopsis* sp. from blueberry were frequently higher than disease severities resulting from inoculation with *N. rosae*, but results differed somewhat between cultivars. On cultivar ‘Albion’, all isolates of the novel *Neopestalotiopsis* sp. from blueberry caused significant disease in all cases, whereas the *N. rosae* isolates did not, suggesting that isolates of the novel *Neopestalotiopsis* sp. from blueberry are relatively more aggressive than the *N. rosae* isolates on this host. However, in our experiments with strawberry cultivar ‘Camino Real’, disease severity was numerically less,

and no consistent differences were noted between blueberry isolates of *N. rosae* and the novel *Neopestalotiopsis* sp. in these experiments.

Differences between strawberry cultivars in terms of their susceptibility to *Neopestalotiopsis* have been noted in previous studies. In particular, Guan et al. (2023) assessed the susceptibility of several strawberry varieties, including 'Albion' and 'Camino Real', to a single isolate of the aggressive novel *Neopestalotiopsis* sp. from Indiana in greenhouse trials on bare root and plug plants. In that study, both cultivars were determined to be very susceptible to the novel *Neopestalotiopsis* sp., with 'Albion' being found relatively less susceptible than 'Camino Real'. In addition, work by McNally (2024) with detached leaves, suggested that some numbered strawberry selections differed, relative to cultivars 'Albion' and 'Jewel', in their susceptibility versus an isolate of the novel *Neopestalotiopsis* sp. from Canada.

Of note, in our experiments on both strawberry cultivars, the strawberry *Neopestalotiopsis* sp. isolate we used caused significantly more severe disease than any of the tested isolates from blueberry. Prior work with strawberry isolates of the novel *Neopestalotiopsis* sp. has shown that these strains are relatively more aggressive than *N. rosae* isolates from strawberry (Baggio et al. 2021). Of note, in the work by Baggio et al. (2021), differences in virulence were noted even between isolates of the novel *Neopestalotiopsis* sp., with isolate 19-02L causing more severe disease than other isolates of this species. In our study, only a single isolate of the novel *Neopestalotiopsis* sp. from strawberry was utilized for comparison. It is unknown how the virulence of this isolate would compare to other *Neopestalotiopsis* sp. isolates from strawberry. As such, our results should be interpreted with caution. If, for example, our strawberry isolate ('AJ07-2023') has similar or greater virulence than isolate 19-02L from Baggio et al. (2021), then it may not be surprising to find that our other blueberry isolates of the novel *Neopestalotiopsis* sp. are

relatively less virulent than this isolate – given that Baggio even found isolates of the novel *Neopestalotiopsis* sp. from strawberry that were significantly less aggressive than 19-02L (but still more aggressive than *N. rosae* isolates from strawberry). As such, additional work with a more robust isolate pool may be necessary to fully understand the breadth of virulence variations within and between species.

Assuming for a moment that the results from our pathogenicity assays with a small number of isolates from blueberry and a single isolate from strawberry are representative of the situation – and that isolates of the novel *Neopestalotiopsis* sp. from blueberry truly are less aggressive than strawberry isolates of this species – this result calls into question the specificity of the diagnostic methods currently utilized to distinguish between *Neopestalotiopsis* spp. isolates that are potentially "aggressive" on strawberry and isolates that are less aggressive. Clearly, by all diagnostic methods currently employed to differentiate the novel *Neopestalotiopsis* sp. from other closely related species, the isolates from blueberry tested here and identified previously (Chapter 4) belong to this novel species (100% identical within all sequenced regions), and yet they did not appear to be as virulent on strawberry as the strawberry isolate of this species. This suggests that these diagnostic methods alone may not be enough to distinguish isolate aggressiveness, and more work is likely needed to assess the population of this novel species present in regions where strawberries are grown. Specifically, further investigation regarding potential genetic factors present in strawberry isolates of this novel *Neopestalotiopsis* sp. that may explain their increased aggressiveness on strawberry relative to isolates of this novel species from blueberry are merited with the goal of developing more precise diagnostic methods capable of identifying which strains of *Neopestalotiopsis* are more likely to threaten strawberry production.

Strictly focusing on our results on strawberry with our blueberry isolates only, it should be reiterated that our overall results indicated that isolates of the novel *Neopestalotiopsis* sp. from blueberry are more aggressive on strawberry than blueberry isolates of *N. rosae.* This does suggest the potential that isolates of this novel species from blueberry, to some degree, could pose a concern for strawberry production. The potential for these relatively more virulent isolates to move from blueberry to strawberry remains and should be considered in situations where both of these crops are being grown and propagated.

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## Tables

**Table 5.1**. Pathogenicity experiments conducted on different cultivars of blueberry and strawberry in this study.

| Experiment Name | Inoculation Type | Host (Cultivar) | Isolates Utilized (Species[1]) | Date |
|---|---|---|---|---|
| Blueberry Exp. 1 | Root inoculation | Blueberry (cv. 'Rebel') | MB21-167 & MB21-178 (*N. rosae*)<br>CB22-008 (*Neo.* sp. [blueberry]) | Mar. 2024 |
| Blueberry Exp. 2 | Leaf inoculation | Blueberry (cv. 'Rebel') | MB21-167 & MB21-178 (*N. rosae*)<br>CB22-008 (*Neo.* sp. [blueberry]) | Mar. 2024 |
| Blueberry Exp. 3 | Leaf inoculation | Blueberry (cv. 'Rebel') | MB21-167 & MB21-178 (*N. rosae*)<br>MB21-494 & CB22-008 (*Neo.* sp. [blueberry])<br>AJ07-2023 (*Neo.* sp. [strawberry]) | Mar. 2025 |
| Strawberry Exp. 1 | Leaf and bloom inoculation | Strawberry (cv. 'Albion') | MB21-167 & MB21-178 (*N. rosae*)<br>MB21-102, MB21-494 & CB22-008 (*Neo.* sp. [blueberry]) | Mar. 2024 |
| Strawberry Exp. 2 | Leaf and bloom inoculation | Strawberry (cv. 'Camino Real') | MB21-167 & MB21-178 (*N. rosae*)<br>MB21-102, MB21-494 & CB22-008 (*Neo.* sp. [blueberry]) | Nov. 2024 |
| Strawberry Exp. 3 | Leaf and bloom inoculation | Strawberry (cv. 'Camino Real') | MB21-167 (*N. rosae*)<br>MB21-494 (*Neo.* sp. [blueberry])<br>AJ07-2023 (*Neo.* sp. [strawberry]) | Nov. 2024 |
| Strawberry Exp. 4 | Leaf and bloom inoculation | Strawberry (cv. 'Albion') | MB21-167 & MB21-178 (*N. rosae*)<br>MB21-494 & CB22-008 (*Neo.* sp. [blueberry])<br>AJ07-2023 (*Neo.* sp. [strawberry]) | Mar. 2025 |

[1]Species names are abbreviated as follows: "*N. rosae*" refers to blueberry isolates identified as *Neopestalotiopsis rosae*; "*Neo* sp. [blueberry]" refers to blueberry isolates identified as the novel *Neopestalotiopsis* sp.; "*Neo* sp. [strawberry]" refers to strawberry isolates identified as the novel *Neopestalotiopsis* sp.

**Figures**

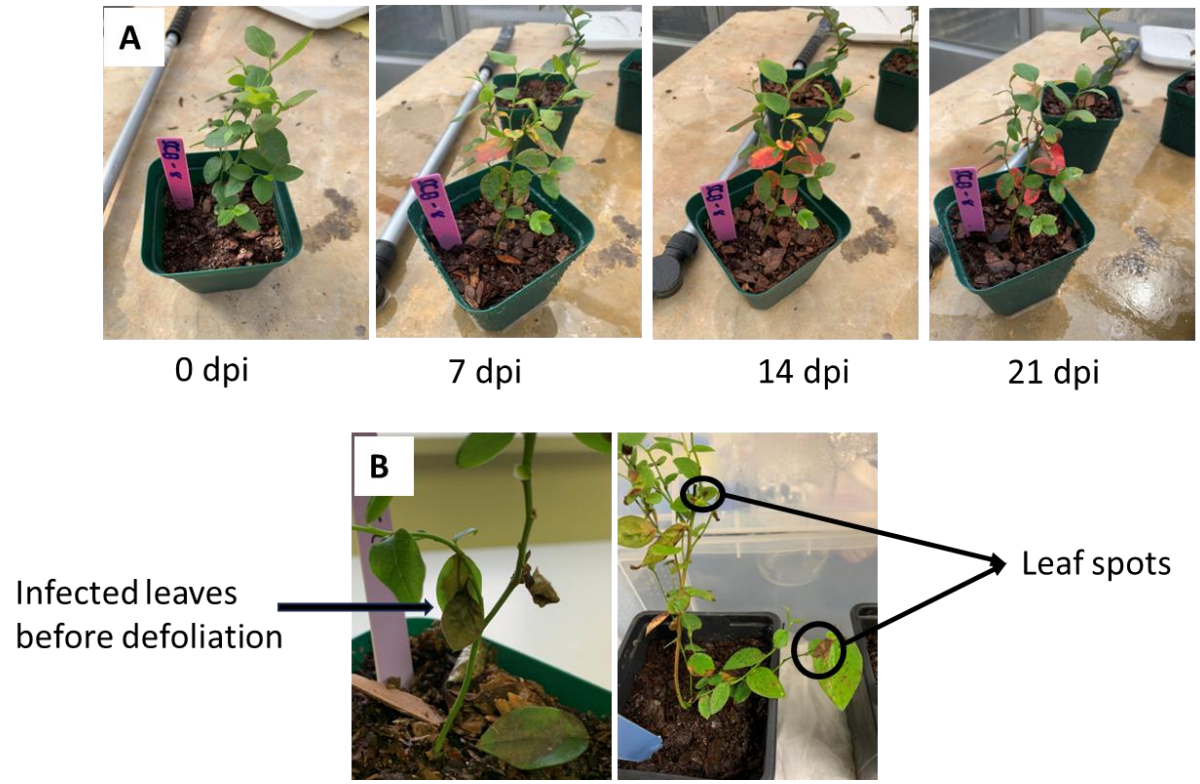


**Figure 5.1.** Disease symptoms observed on blueberry plants. (A) Yellowing, reddening, and defoliation of root-inoculated plants. (B) Defoliation and leaf spots of leaf-inoculated plants.

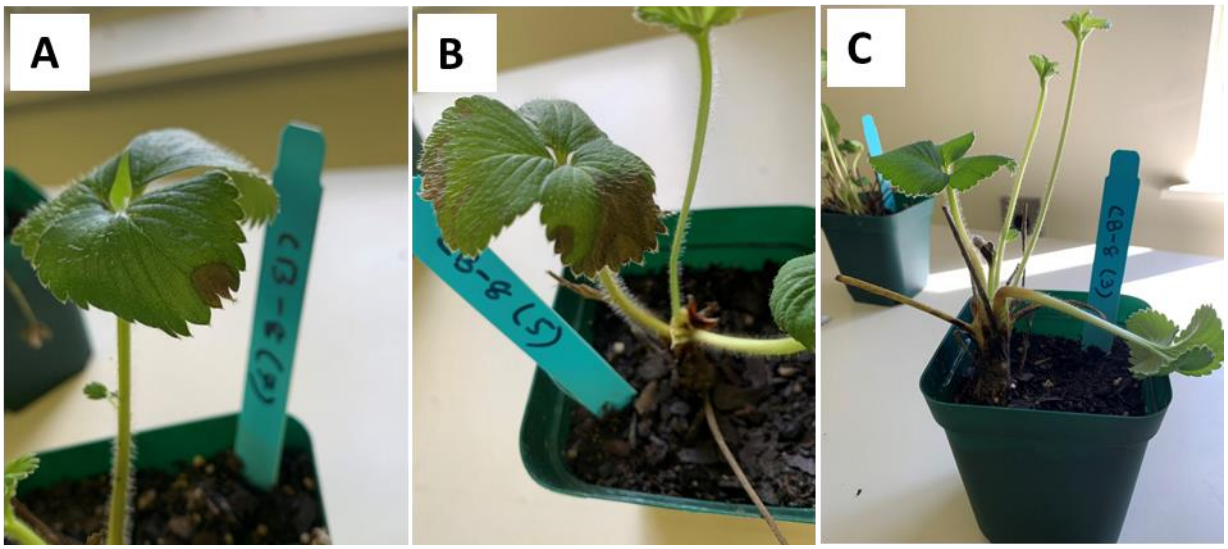


**Figure 5.2.** Disease symptoms observed on strawberry plants. (A) Newly developed leaf spots. (B) Expanded leaf spots covering more than one-quarter of the leaf surface. (C) Infected stem (lower left).

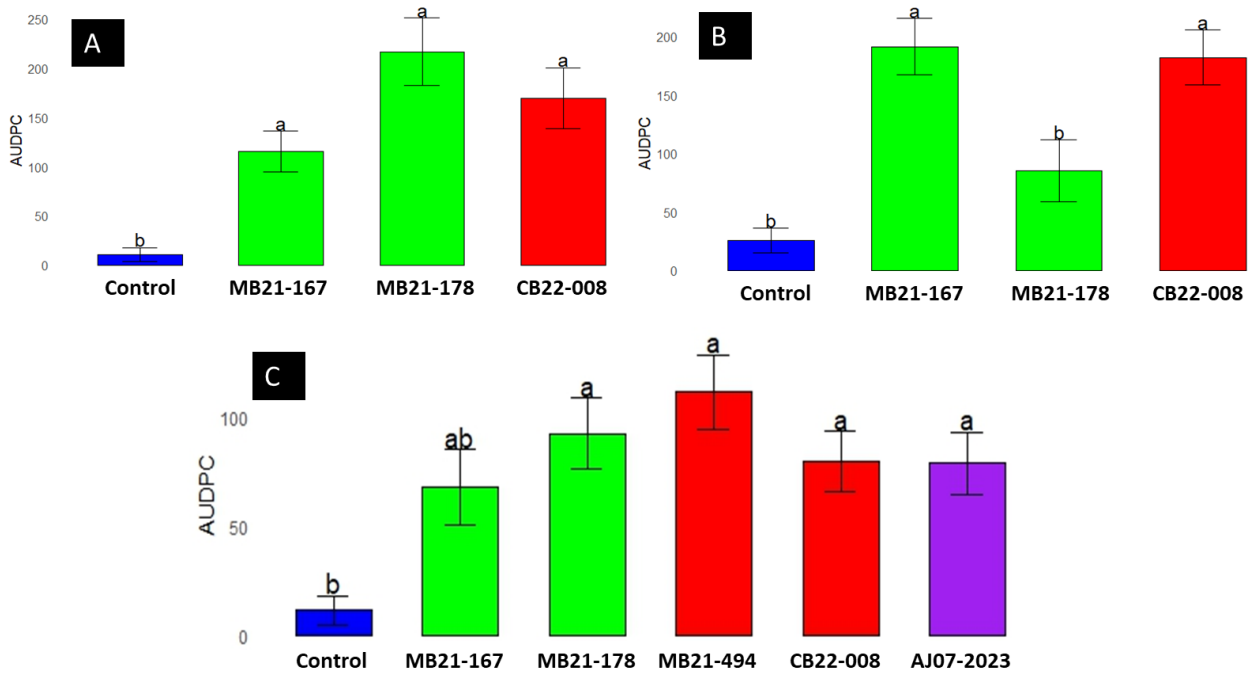


**Figure 5.3**. Disease severity (AUDPC; area under the disease progress curve) for *Neopestalotiopsis rosae* and novel *Neopestalotiopsis* sp. isolates collected from blueberry or strawberry based on leaf or root inoculation assays on blueberry (cultivar 'Rebel'). (A) Results from root inoculation assay. (B and C) Results from leaf inoculation assays. **Blue** bars represent the uninoculated control, **Green** bars represent *N. rosae* isolates, **Red** bars represent novel *Neopestalotiopsis* sp. isolates from blueberry, and **Purple** bars represents novel *Neopestalotiopsis* sp. isolates from strawberry. Significant differences within each panel (based on Tukey's HSD with $\alpha = 0.05$) are indicated by different letters above the bars.

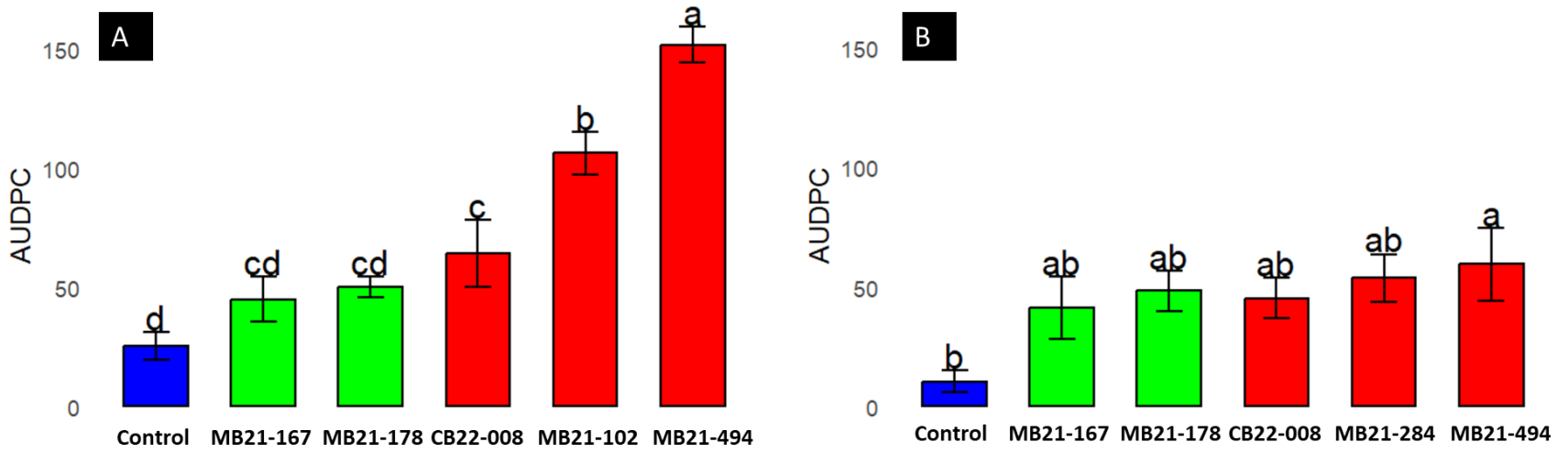


**Figure 5.4**. Disease severity (AUDPC; area under the disease progress curve) for *Neopestalotiopsis rosae* and novel *Neopestalotiopsis* sp. isolates collected from blueberry based on inoculation assays on strawberry. (A) Results from Strawberry Experiment 1 with cultivar 'Albion'. (B) Results from Strawberry Experiment 2 with cultivar 'Camino Real'. **Blue** bars represent the uninoculated control, **Green** bars represent *N. rosae* isolates, and **Red** bars represent novel *Neopestalotiopsis* sp. isolates from blueberry. An analysis of variance was performed to compare disease severity among isolates in each experiment. Significant differences within each panel (based on Tukey's HSD with $\alpha = 0.05$) are indicated by different letters above the bars.

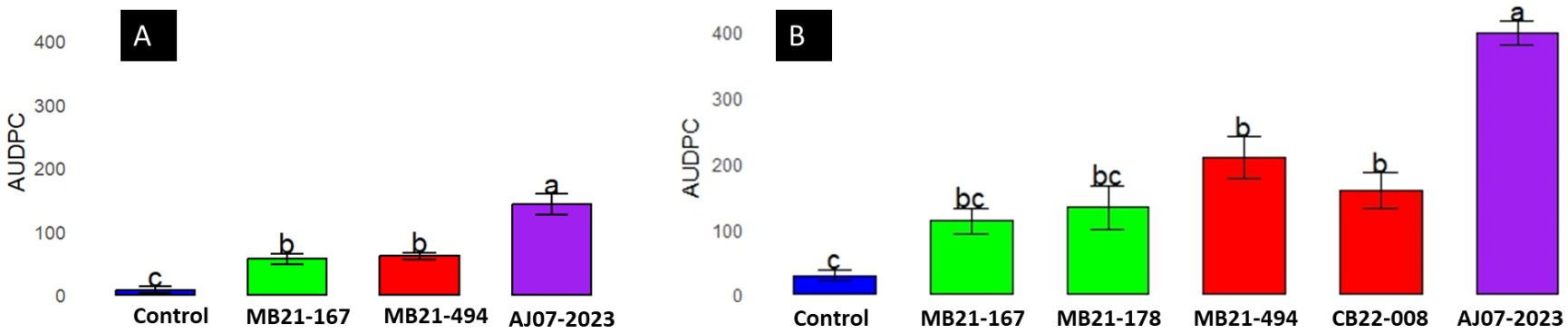


**Figure 5.5**. Disease severity (AUDPC; area under the disease progress curve) for *Neopestalotiopsis rosae* and novel *Neopestalotiopsis* sp. isolates collected from blueberry or strawberry based on inoculation assays on strawberry. (A) Results from Strawberry Experiment 3 with 'Camino Real' and (B) Results from Strawberry Experiment 4 with 'Albion'. **Blue** bars represent the uninoculated control, **Green** bars represent *N. rosae* isolates, **Red** bars represent novel *Neopestalotiopsis* sp. isolates from blueberry, and **Purple** bars represents novel *Neopestalotiopsis* sp. isolates from strawberry. An analysis of variance was performed to compare disease severity among isolates in each experiment. Significant differences within each panel (based on Tukey's HSD with $\alpha = 0.05$) are indicated by different letters above the bars.

# CHAPTER 6

# CONCLUSIONS

My dissertation research primarily focused on investigating the diversity and the characteristics of pathogens associated with postharvest fruit rots of blueberry in Georgia. Accordingly, part of this work involved fungicide sensitivity screening of two major fungal groups from Georgia blueberries: *Alternaria* spp. (Chapter 2) and *Botrytis cinerea* (Chapter 3). Additionally, a portion of this work (Chapters 4 and 5) identified and assessed the potential virulence of *Neopestalotiopsis* spp. isolates in two cropping systems: blueberry and strawberry.

To investigate pathogen diversity, fungal isolates were collected over two consecutive years from fields and storage facilities in major blueberry-producing counties across Georgia (summarized in Chapter 1). Pathogen identification was conducted using both morphological characteristics and molecular techniques and a total of 836 isolates were collected during the 2021 and 2022 seasons, representing over 15 fungal genera. While many were determined to be secondary pathogens with limited roles in fruit rots, a substantial number of isolates belonging to species of known fruit rot pathogens such as *Colletotrichum* spp., *Alternaria* spp., and *B. cinerea* were identified. These were associated with anthracnose rot, Alternaria fruit rot, and gray mold, respectively, and their pathogenicity was confirmed through in vitro assays. These three groups alone accounted for over 40% of the total isolates collected, confirming their importance as the major causal agents of postharvest diseases in Georgia. This comprehensive overview of pathogen diversity, previously unavailable, provides a valuable foundation for researchers to develop targeted management strategies focused on these predominant fungal groups.

The primary strategy for managing pre- and postharvest fruit rots relies on an effective fungicide spray program. However, the success of such programs depends on selecting appropriate fungicides that target specific pathogens at critical moments (optimal timings) in their life or disease cycles. One critical aspect affecting fungicide spray program efficacy is the potential for fungicide resistance to develop in the target fungal populations. With the repeated use of fungicides, resistance development has become increasingly common worldwide. Considering this, we conducted fungicide sensitivity assessments of fungicides commonly used by Georgia blueberry growers for control of the fruit rot pathogens *Alternaria* spp. (Chapter 2) and *Botrytis cinerea* (Chapter 3). Testing the fungicide sensitivity of 46 *Alternaria* isolates against 6 commonly used fungicides using mycelial growth inhibition assays allowed us to calculate the $EC_{50}$ values for individual isolates. Based on these values, resistance was detected to boscalid (46% of isolates) and pyraclostrobin (22% of isolates), and reduced sensitivity was detected to pydiflumetofen (26% of isolates). By contrast, isolates were sensitive to metconazole, fluazinam, fludioxonil, and cyprodinil. Resistant isolates were recovered from 6 of the 16 surveyed locations (37.5%), with six isolates (from three distinct locations) exhibiting dual resistance to both boscalid and pyraclostrobin. Known resistance mutations, including G143A in *cytB*, H134Q and G79R in *sdhC*, and H133R in *sdhD* were also identified within the resistant isolates. Interestingly, both fungicides against which resistance was identified in our study (pyraclostrobin and boscalid) are components of the commercial product "Pristine®" which is typically used by Georgia blueberry growers. These findings suggest that blueberry growers primarily relying on QoI fungicides, particularly Pristine® (pyraclostrobin + boscalid), for fruit rot management may encounter reduced efficacy in fields with resistant *Alternaria* populations. However, since resistance was not identified to other fruit rot fungicides, spray programs utilizing a rotation or mixture of fungicides should remain

effective, as the fungicides to which the *Alternaria* spp. are still sensitive should remain capable of suppressing resistant populations, providing complementary control and reducing selection pressure.

Using the same methods used for *Alternaria* spp., 60 selected *B. cinerea* isolates were assessed for fungicide sensitivity (Chapter 3). While all *B. cinerea* isolates were found to be sensitive to fludioxonil, boscalid, and pyraclostrobin based on the determined $EC_{50}$ values, 49 and 58 of the isolates, for boscalid and pyraclostrobin, respectively, exhibited minimum inhibitory concentrations exceeding 100 ppm. This suggests that increasing fungicide concentrations beyond a certain point may not proportionally enhance inhibition, highlighting the existence of an optimal dosage range. Understanding this optimal dose is critical for effective field-level applications, suggesting that future work should focus on further investigation into the field efficacy of these fungicides. For cyprodinil, resistance profiling identified six low-resistant, one weakly resistant, and three moderately resistant isolates, and in the case of fenhexamid, 12 *B. cinerea* isolates were found with moderate resistance, but no highly resistant isolates were detected. Accordingly, molecular screening revealed the presence of the A378T mutation in the *erg27* gene in three isolates moderately resistant to fenhexamid; however, the F412S mutation commonly associated with high-level resistance to fenhexamid was not observed in any of the tested isolates. Taken together, the overall fungicide sensitivity profiles of the *B. cinerea* isolates examined in this study indicate that resistance development (except to fludioxonil, to which all isolates remain fully sensitive) may be in the early stages with no high resistance observed but a number of weak or moderate resistances or tolerance identified. Although resistance appears to be currently limited, the situation may worsen in the coming years, and both researchers and growers should remain vigilant and prepared for potential shifts. Our findings suggest that continuous monitoring of

blueberry *B. cinerea* resistance going forward is merited and necessary. If resistance against certain fungicides rises significantly, rotations with other effective modes of actions, reliance on multisite modes of action, and temporarily suspending the use of the resistance-associated fungicides to allow for the re-establishment of a sensitive population may help improve the disease management scenario.

As previously stated, long-term surveillance should be continued to track emerging resistance patterns in major fungal groups such as *Colletotrichum*, *Botrytis*, and *Alternaria* spp. from blueberry. Findings from these efforts should be regularly revisited and spray programs updated based on the current fungicide sensitivity status of these fungal pathogens. Since the number of fungicides labelled for use on blueberry and effective for fruit rot control is limited, and since the development of new ones is relatively slow, it is not practical to simply remove a fungicide from the spray program. Hence, going forward, it is important to test and implement integrated disease management strategies that combine fungicide rotation, cultural practices, and biocontrol agents to reduce reliance on high-risk fungicides and to delay resistance development. It should be noted that one limitation of our fungicide resistance assessments is that they were based on in vitro assays. As the true efficacy of fungicides for disease management in the field can only be accurately assessed under real-world field conditions, it is essential that fungicide efficacy trials be conducted in locations where resistance has been identified.

Another important area to explore is the molecular profile of resistant isolates. We have identified several mutations, some of which were consistently associated with resistance and some which were not. Identifying the genes involved in resistance development, and their polymorphisms, is important not only for understanding resistance but also for aiding in the development of new fungicides or in the modification of existing ones. To identify these genes,

transcriptome analysis can be used to assess the upregulation or downregulation of gene expression. Polymorphic or differentially expressed genes can then be cloned and studied further to determine their potential role in resistance.

During the initial collection of fungi from blueberry fruit conducted to help improve our understanding of pathogen diversity (summarized in Chapter 1), a significant amount of *Neopestalotiopsis* spp. isolates were also identified alongside other fungal pathogen groups. While *Neopestalotiopsis* has not been considered a major pathogen in blueberry production in the United States, it is considered quite significant in strawberry production. Based on the currently utilized diagnostic technique (identifying a mutation in *β-tubulin* gene of *Neopestalotiopsis* spp. isolates), we confirmed the presence, in rotting blueberries, of the novel *Neopestalotiopsis* sp. currently causing ongoing disease epidemics in strawberry in Florida, other parts of Eastern U.S., and Canada (Chapter 4). Based on a more robust three gene phylogeny utilizing the portions of the internal transcribed spacer (ITS), *β-tubulin* (*B-tub*), and *tef1* regions widely used in the identification of species within the *Neopestalotiopsis* group, we further confirmed the presence of isolates of the novel *Neopestalotiopsis* sp. in a blueberry planting over two consecutive years – suggesting a persistent presence of the pathogen.

In the *Neopestalotiopsis* population collected from blueberry in our study, *Neopestalotiopsis rosae* isolates were also identified. This species has been frequently reported to cause disease on strawberry in different parts of the world (including in the U.S.) and less frequently on blueberry in a few countries (not including the U.S.). To understand the comparative virulence of the *N. rosae* and novel *Neopestalotiopsis* sp. isolates from Georgia blueberry, and a novel *Neopestalotiopsis* sp. isolate from strawberry, pathogenicity tests were conducted on both blueberry and strawberry. Our pathogenicity results on blueberry indicated that all of these isolates

caused diseases on the host, however, there were no differences in relative virulence between these species. On the contrary in strawberry cultivar 'Albion', blueberry isolates of the novel *Neopestalotiopsis* sp. were relatively more virulent than blueberry isolates of *N. rosae,* but differences were not evident in strawberry cultivar 'Camino Real'. Interestingly, in further pathogenicity testing of novel *Neopestalotiopsis* sp. isolates from blueberry alongside a single novel *Neopestalotiopsis* sp. isolate from strawberry, the strawberry isolate showed significantly higher disease severity than tested isolates from blueberry on both cultivars.

The ability of cross-host infection of these *Neopestalotiopsis* spp. in blueberry and strawberry can be troublesome in the locations (e.g. nurseries, commercial fields) where these hosts are grown concurrently. The *Neopestalotiopsis* group are not typically seen as primary pathogens, but based upon recent reports of this group causing disease on different hosts, their pathogenicity should be tested on other crops, especially on the other small fruits. Moreover, because of the heightened virulence of the emerging novel *Neopestalotiopsis* sp., a continued monitoring of the *Neopestalotiopsis* spp. population among the small fruits grown in Georgia is necessary in order to determine if there is any rise in the proportion of isolates belonging to the novel *Neopestalotiopsis* sp. going forward.

The observed differences in virulence between the strawberry and blueberry isolates of the novel *Neopestalotiopsis* sp. suggest that further investigations are needed to examine isolate pathogenicity differences and the potential genetic factors underlying these differences. If our results, which at this point are based on a single isolate from strawberry and a small number of isolates from blueberry, turned out to be consistent with further, more robust, examinations of isolates from blueberry and strawberry in general, this may raise several questions regarding whether there may be other consistent genetic differences between the strawberry and blueberry

isolates of this novel *Neopestalotiopsis* sp. (and/or whether all these isolates truly belong to the same species). To this end, genome sequencing and SNP-based analysis may be necessary to compare these isolates at the genomic level and may identify potential virulence factors that contribute to the pathogen's aggressiveness on strawberry. Moreover, the novel *Neopestalotiopsis* sp. has not been given any formal species name. Therefore, comparative genetic and phenotypic analysis is required to determine whether the novel *Neopestalotiopsis* sp. is a new species or a subpopulation of an existing species with heightened virulence. Finally, additional, identification and characterization of the *Neopestalotiopsis* spp. population from the small fruits grown in Georgia using pan-genome analysis may help to answer many of the questions regarding the virulence, evolution, host adaptation, and the genetic factors associated with this emerging pathogen and closely-related *Neopestalotiopsis* species.