ABSTRACT

LLANOS MELO, ALEJANDRO KLEPER. Evaluation of SDHI Fungicides and Host Plant Defense Inducers for the Management of Plant Pathogens in Two Different Cropping Systems. (Under the Direction of Drs. Sara M. Villani and Barbara B. Shew).

Succinate dehydrogenase inhibitor (SDHI) fungicides and host plant defense inducers are important alternatives for the management of plant pathogens. SDHIs are single-site fungicides that block the production of energy in fungi by disrupting the electron transport chain in aerobic organisms. Another important fungicide group is the host plant defense inducers whose molecules inhibit or slow the development of pathogens by enhancing the defense system of plants. Several new SDHIs and host plant defense inducers are now available on the market, making them promising tools to be included in the integrated management of new pathosystems. However, an improved understanding of their efficacy and mechanisms need to be studied. Evaluation of these molecules in four pathosystems and two different cropping systems was conducted. In apple, Glomerella leaf spot and bitter rot caused by *Colletotrichum* spp. and fire blight caused by *Erwinia amylovora* were evaluated. In boxwood, boxwood blight caused by *Calonectria pseudonaviculata* was studied. These diseases are causing devastating economic losses in the southeastern US.

Nowadays, management of these diseases can be challenging due to resistance and restriction applications. It is necessary to look for more alternatives to be implemented in the integrated pest management of these cropping systems. Therefore, the objectives of this research were to 1) evaluate the effect of four SDHI fungicides for *C. fioriniae* and *C. fructicola* by mycelium-growth assay and characterization of *sdh* genes for *C. fructicola*, 2) evaluate the effect of four SDHI fungicides for *C. pseudonaviculata* by mycelium-growth assay, and 3) analyze the

effect of host plant defense inducers against boxwood blight and fire blight. Also, evaluate PR gene expression in apple tissue after application of host plant inducers.

The effective concentration at which mycelial growth was inhibited by 50% (EC_{50}) was calculated for *C. fructicola* and *C. fioriniae* for benzovindiflupyr and penthiopyrad. Furthermore, the sensitivity of isolates of *Colletotrichum* spp. from commercial orchards to benzovindiflupyr and penthiopyrad was also evaluated. Significant positive correlations between benzovindiflupyr and penthiopyrad were observed for *C. fructicola* and for *C. fioriniae*. In addition, the target genes for SDHI fungicides, *sdhB*, *sdh*C, and *sdhD* were identified and characterized for *C. fructicola*.

EC_{50} values of isolates of *C. pseudonaviculata* collected from North Carolina (NC) and outside NC to penthiopyrad, benzovindiflupyr, fluxapyroxad, and boscalid were calculated. Moreover, positive correlations were found between benzovindiflupyr and fluxapyroxad, benzovindiflupyr and penthiopyrad, and penthiopyrad and fluxapyroxad in isolates collected from NC.

The performance of host plant defense inducers in two different cropping systems was analyzed. Acibenzolar-S-methyl and *Bacillus mycoides* isolate J were studied in boxwood and apple, and the plant-derived extract from *Reynoutria sachalinensis* was included in trial on apples. In boxwood, drench applications of acibenzolar-S-methyl showed significantly less disease incidence than the untreated program. The frequency of applications did not increase the efficacy for boxwood blight control with either of the host plant defense inducers tested. In apple, the incidence of blossom blight was significantly reduced in comparison with the untreated with all the host plant defense inducer spray programs. The gene expression of PR-2 and PR-8 from apple-leaf tissue treated with acibenzolar-S-methyl and *Reynoutria sachalinensis* extract increased significantly compared to the untreated program at one and seven days after application. In addition, *Bacillus mycoides* isolate J increased the expression of PR-1 at one and seven days. These results contribute to having a better comprehension of these products and their use in an integrated pest management program for these pathosystems.

Evaluation of SDHI Fungicides and Host Plant Defense Inducers for the Management of Plant Pathogens in Two Different Cropping Systems.

by
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APPROVED BY:

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DEDICATION

I dedicate this accomplishment undoubtedly to my beloved parents, Alejandro Llanos and Gladys Melo and my siblings Diego and Vanessa, for their constant love and support during this time.

No estaria aquí si no fuese por ustedes, muchas gracias por permitirme ser parte de sus vidas ¡Los quiero mucho!

BIOGRAPHY

Alejandro K. Llanos was born and raised in Lima, Peru, and got his B.S. in Agronomy from The National Agrarian University - La Molina. Being an undergraduate student, Alejandro was very involved in academics and extracurricular activities. In his third year, he realized his love for plant pathology. It led him to participate in the undergraduate plant pathology group and he became the president of this group one year later. He organized several trials and events in coordination with the Department of Plant Pathology.

After he graduated, he received a job in the Research and Development Department of Bayer Crop Science. In that position, Alejandro conducted field experiments around Peru testing different fungicides, insecticides, and herbicides on various crops. It was a fruitful experience for him where he realized the critical situation of fungicide resistance in some pathosystems. This situation sparked his interest in the study of fungicide resistance. He then received a scholarship to study English in an intense program (Beca Ingles) and to study his master overseas (Beca Presidente de la Republica) sponsored by the Peruvian government.

In 2018, Alejandro was accepted at North Carolina State University and started his Master of Science in plant pathology under Dr. Sara Villani's direction. He worked on evaluating SDHI and host plant defense inducers in critical plant diseases in the southeastern US. During his experience working in Dr. Villani's lab, he enhanced his knowledge in this area by doing biomolecular experiments and field trials. Alejandro wants to become a reference in this field of study in the future and to spread what he has learned at NCSU to Peru and other countries in South America.

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CHAPTER I

Literature Review

Disease management

Diseases of agricultural plants, particularly those caused by fungal and fungal-like pathogens, have historically been considered one of the greatest threats to food security worldwide (Oliver & Hewitt 2014). Some of these plant diseases have generated several detrimental situations in our history such as starvation (potato late blight caused by *Phytophthora infestans* (Mont.) de Bary), loss of native species (chestnut blight caused by *Cryphonectria parasitica* (Murrill) Barr) and a direct impact to economic crops (leaf blight in corn caused by *Bipolaris maydis* (Y. Nisik & C. Miyake) Shoemaker) (Maloy 2005). Thus, efficient methods for management of these pests are necessary for the availability of food (Chandler et al. 2011).

Integrated Pest Management (IPM) is defined as '...a decision-based process involving coordinated use of multiple tactics for optimizing the control of different classes of pests (insects, pathogens, weeds, and vertebrates) in an ecologically and economically sound manner (Prokopy & Kogan 2003). The first use of this term appeared post World War II (Ehler 2006). IPM has developed into a sustainable approach to diminish the impact of plant diseases. Effective IPM combines several control measures including cultural, physical, biological, and chemical control to minimize the damage of different pests along with prevention, avoidance, suppression, monitoring, and forecasting (Campbell & Arthur 2007; Vetek et al. 2009). Nevertheless, in some pathosystems, the use of chemical control has become the primary tool for maintaining healthy crops and reliable yields (Brent & Hollomon 2007; McDougall 2018). Even though social pressure and regulations exist to reduce pesticide use, fungicides are still a fundamental tool in many agricultural ecosystems (Stukenbrock & McDonald 2008).

Glomerella leaf spot and bitter root in apple

Glomerella leaf spot and bitter rot are important diseases of apple in North Carolina. *Colletotrichum fructicola* (= *C. ignotum*) Prihastuti, L. Cai & K.D Hyde, cause of Glomerella leaf spot, produces mycelium with a cottony texture, initially white, becoming grey to greyish green in culture. The conidia are one-celled, smooth-walled, hyaline, and cylindrical (Fuentes-Aragón et al. 2018; Prihastuti et al. 2009). *Colletotrichum fioriniae* (formerly *C. acutatum* f. sp. fioriniae) Marcelino & Gouli ex R.G. Shivas & Y.P Tan, cause of bitter rot, produces hyaline, single-celled, smooth-wall conidia, that vary from fusiform to cylindrical and on branched conidiophores. In PDA, *C. fioriniae* mycelium is light grey and cottony in texture. The reverse side presents a pale pink coloration with flecking used to differentiate from other species in the *C. acutatum* species complex species (Damm et al. 2012; Kou et al. 2014; Shivas & Tan 2009).

Both diseases must be managed annually under North Carolina's environmental conditions. Glomerella leaf spot can cause up to 75% to 100% defoliation when not managed effectively (Sutton & Sanhueza 1998). Warm temperatures and high humidity are ideal for infection of the plant, triggering rapid symptom development (Anderson 1956; Villani 2017). Glomerella leaf spot starts as small red to purple colored specks that develop into asymmetrical, brown, and usually, concentric lesions five to seven days after infection, causing chlorosis and rapid defoliation (Leite et al. 1988; Taylor 1971; Villani 2017). Apple bitter rot disease is characterized by small, sunken black to brown colored spots from 1 to 5 mm diameter. These spots expand to larger concentric and rotted lesions on fruit (Wang et al. 2015). The predominant causal species of the disease varies depending on the region. Recently, it has been reported that *C. fioriniae* was the most prevalent species causing bitter rot, while *C. fructicola* is typically the primary causal agent of leaf spot symptoms in the southern region (Johnson 2019; Munir et al. 2016).

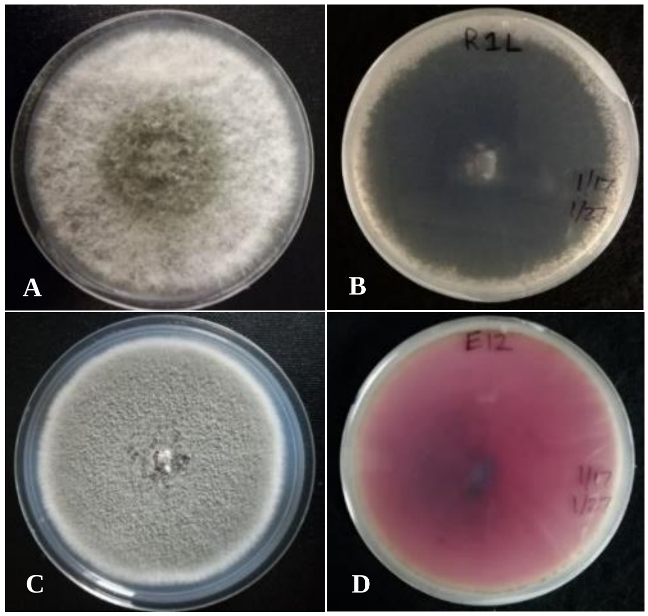

Figure 1.1 Cultural and morphological characteristics of *C. fructicola* **A,** front view; **B,** reverse view; and *C. fioriniae* **C,** front view; **D,** reverse view on Potato Dextrose Agar (PDA) medium.

Management of Glomerella leaf spot and bitter rot

The management of these apple diseases is based on cultural practices, resistant cultivars, and fungicide applications. Sanitation practices are vital to reduce initial inoculum in the orchard and include the pruning and removal of infected tissue (fruit mummies, cankers) where mycelium and conidia can overwinter, post-harvest application of urea to fallen leaves before green tip, and reducing leaves underneath the tree canopy (Villani 2017; Walgenbach et al. 2019). Cultivars that are descendants of 'Red Delicious' show moderate to complete resistance to Glomerella leaf spot whereas cultivars that have 'Golden Delicious' ancestry are susceptible to Glomerella leaf spot and fruit rot (Araujo & Stadnik 2013; Villani 2017).

Fungicide applications for control of Colletotrichum diseases in apple start at the beginning of petal fall and continue on 7 to 14-day intervals until harvest (Villani 2017). Species of *Colletotrichum* can be managed with multisite and single-site fungicides.

Multi-site inhibitor fungicides are elemental tools for disease control due to their broad-spectrum activity and lack of cross-resistance. Among these fungicides, mancozeb and captan are commonly used on apple. Mancozeb belongs to FRAC group M03 and captan to M04 (FRAC 2019). These fungicides show high efficacy against plant diseases caused by *Colletotrichum* spp. in some crops (Gao et al. 2017; Keinath 2018; MacKenzie et al. 2009). However, a drawback for this group is the lack of persistence and poor distribution on the plant surface (Smith & MacHardy 1984). Currently, these active ingredients are usually combined with single-site fungicides (Villani & Nance 2016).

Single-site fungicides provide high specificity to control for apple diseases, but the potential for fungicide resistance is a constant concern. For example, resistance to dodine, myclobutanil, thiophanate-methyl, kresoxim-methyl, and insensitivity to difenoconazole has been reported in apple scab pathogen (*Venturia ineaqualis*) populations, in several apple productions regions in the Eastern US. Furthermore, some isolates were resistant to more than one fungicide active ingredient (Chapman et al. 2011; Villani et al. 2015). Among the single-site fungicides, the respiratory inhibitors used for management of *Colletotrichum* include quinone-outside inhibitors (QoI) and succinate dehydrogenase inhibitors (SDHIs) (Ishii et al. 2016). However, inappropriate application practices with single-site fungicides, including an excessive number of applications and reduced rates, can lead to selection for fungicide resistant isolates in the population (Beckerman et al. 2014).

Like QoIs, succinate dehydrogenase inhibitors (SDHIs) fungicides are a class of fungicides that are at risk for developing resistance due to their single-site inhibiting activity (Sierotzki & Scalliet 2013). In fungicide efficacy studies, some species of *Colletotrichum* species (e.g., *C. gloeosporioides*, *C. acutatum*, *C. cereale*, and *C. orbiculare*) showed reduced sensitivity to fluopyram, fluxapyroxad, and boscalid. Another SDHI, penthiopyrad, was effective against three species, but not *C. orbiculare*. Benzovindiflupyr inhibited all species of *Colletotrichum* studied *in vitro* and also controlled Colletotrichum diseases in apple, peach, and cucumber (Ishii et al. 2016).

Fire blight in apple.

Erwinia amylovora (Burrill) Winslow et al. a gram-negative bacterium, is the causal agent of fire blight, a devastating disease on apple and other species in the family Rosaceae. Fire blight was first described in 1780 in an apple orchard in the present state of New York, US (Bonn & van der Zwet 2000). The bacterium infects pome fruit worldwide because of the susceptibility of popular commercial cultivars (Bonn & van der Zwet 2000; Peil et al. 2009).

The fire blight pathogen causes symptoms, including blossom, shoot, and rootstock blight (Norelli et al. 2003). Typical symptoms are curling and browning of young growing shoots (shepherd's crook), making infected tissue appear as being scorched by fire (Emeriewen 2015). When the bacterium colonizes intercellular spaces (parenchyma), their infection will trigger necrosis and the production of bacterial ooze (Thomson 2000).

Management of fire blight in apple

Management of *Erwinia amylovora* is a challenge because of the lack of synthetic compounds with systemic properties (Aćimović et al. 2015). Pruning infected twigs and chemical treatments such as preventive copper applications can manage the disease in early stages. However, once the pathogen infects the reproductive or vegetative tissue, these control measures are ineffective (Singh et al. 2019). Application of antibiotics was a standard strategy for managing this bacterium, but streptomycin-resistant strains are an increasing concern (Tancos et al. 2016; Chiou & Jones 1993). In addition, antibiotic application raises concerns about environmental impact and possible transfer of antibiotic resistance to human pathogens (McManus 2014; Sundin & Bender 1996). Due to the limitations of chemical control, host plant defense inducers and biological agents have been studied as alternatives for fire blight management (Johnson & Temple 2013; Maxson-Stein et al. 2002).

Boxwood blight

Boxwood is cultivated in the US and around the world as an ornamental plant (Maurer et al. 2017). These plants are popular because of their deer resistance, mostly evergreen foliage, low maintenance, and relatively long life span. However, boxwood is susceptible to plant pathogens such as the nematode *Meloidogyne incognita* (Kofoid & White), Chitwood, leaf infecting fungus *Pseudonectria buxi* (DC.) Seifert, Phytophthora root rot caused by *Phytophthora* spp., and rust caused by *Puccinia buxi* Sowerby (Strouts & Winter 2000; Blake et al. 2020). Currently, the most detrimental disease is boxwood blight caused by *Calonectria pseudonaviculata* (Crous, J.Z., Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous. The disease was first reported in 1998 in the United Kingdom (Henricot et al. 2000). The first reports of this pathogen in the US were made in 2012 in North Carolina and Connecticut (Ivors et al. 2012). Currently, the pathogen has been isolated and identified in more than 28 states in the USA and three Canadian provinces (Sakalidis et al. 2019; Yang & Hong 2017).

Boxwood blight symptoms occur on leaves and stems. Foliar symptoms include circular, dark brown spots that enlarge over time, and infected leaves became black-brown or necrotic.

Black streaks or cankers also form on the stem. These symptoms result in defoliation and death of severely infected plants (Henricot & Culham 2002; Ivors et al. 2012; Williams-Woodward 2015). Some species and cultivars are more susceptible than others. *Buxus sempervirens* 'Suffruticosa' (English boxwood) and *B. sempervirens* (American or common boxwood) are highly susceptible to this pathogen (Douglas 2011). There are no boxwood species immune to *C. pseudonaviculata*, but several cultivars have been identified with reduced susceptibility to the pathogen. The pathogen is also capable of infecting the genus *Pachysandra* and *Sarcococca* in the Buxaceae family (Henricot et al. 2008).

Management of boxwood blight

Growers are advised to use pathogen-free material and inspect plants before buying them. New plant materials should be isolated from other boxwoods and checked for symptoms for at least a couple of months before planting. Growers should provide adequate space between plants to enhance air circulation and decrease relative humidity and avoid overhead irrigation and handling plants when wet. Removing leaf debris may reduce inoculum. Plants should be inspected periodically, and infected plants bagged and removed to avoid spread (Douglas 2011). Finally, the use of cultivars with reduced susceptibility is an important alternative for the management of this disease. Asiatic boxwood cultivars exhibit an increased level of resistance compared to European species (Ganci 2014; Gehesquière 2014; Henricot et al. 2008; Shishkoff & Olsen 2015).

Fungicides are usually needed to manage boxwood blight (Douglas 2011). Fungicides can not eradicate the disease once plants have already been infected, and they have to be applied at 7 to 14-day intervals (Bush et al. 2016). However, fungicide selection, timing, and application strategies could reduce the incidence of boxwood blight. Several fungicides that appear to be useful in managing boxwood blight include propiconazole, myclobutanil, thiophanate-methyl, fludioxonil, pyraclostrobin, kresoxim-methyl, prochloraz, and chlorothalonil (Henricot & Wedgwood 2013; LaMondia 2015). Highly effective control has been reported with products containing chlorothalonil or fludioxonil (Ivors et al. 2012). The combination of systemic and protectant fungicides in a single application is an effective disease management strategy (LaMondia 2015; Maurer et al. 2017).

Fungicides for disease control

Fungicides have been used for over 200 years in crop protection (Brent & Hollomon 2007). Modern multi-site fungicides such as copper, mancozeb, and captan can provide excellent control of several foliar and fruit diseases including those caused by *Colletotrichum, Venturia*, *Alternaria,* and *Septoria*. These fungicides are unlikely to select for resistance in target fungi. However, they lack systemic activity and have to be applied before infection (Klittich 2008).

Important fungicide classes with single target sites include the dicarboximides, phenylamides, phenylpyrroles, anilinopyrimidines, carboxylic acid amides, defense activators, Quinone outside inhibitors (QoI), and succinate dehydrogenase inhibitors (SDHI) (Brent & Hollomon 2007; Morton & Staub 2008; Oliver & Hewitt 2014). Due to their high specificity, control failures due to fungicide resistance has been reported for all these classes of fungicides (FRAC 2019). The first fungicide resistance was reported in 1969 for a benzimidazole fungicide used to control powdery mildew in greenhouses (Morton & Staub 2008). Some fungicide groups such as dicaborximides, phenylamides, Qols, and SDHIs have a medium to high risk of selecting for fungicide resistance, while carboxylic acid amides and phenylpirroles have low to medium risk. An exception is host plant defense inducers, for which the MOA is unknown, and no resistance reported to date (FRAC 2019).

Respiration inhibitors

Interruption of the electron transport chain has been a key mechanism of inhibition of fungal pathogens (Kim et al. 2013). Five main complexes make electron transfer possible during aerobic respiration of living organisms: I, NADH dehydrogenase; II, succinate dehydrogenase; III, cytochrome bc1 complex; IV, cytochrome c oxidase; and proton Adenosine triphosphatase (ATPase) (Oliver & Hewitt 2014). Respiration inhibitors interact with complexes such as complex II and III, and ATPase (Sierotzki 2015). Respiration inhibitor fungicides play an essential role in the management of several diseases such as *Venturia inaequalis*, *Monilia fructicola, and Colletotrichum spp.* (Amiri et al. 2010; Cox 2015; Ishii et al. 2016; Piccirillo et al. 2018). Quinone outside inhibitor (QoI) and succinate dehydrogenase inhibitor (SDHI) fungicides are among the most significant group of fungicides inhibiting respiration (FRAC 2019).

Mode of action of SDHI fungicides

SDHIs are single-site fungicides that block the production of energy in fungi by disrupting the electron transport chain in aerobic organisms (Bartlett et al. 2002; Ruy et al. 2006; Semar et al. 2014). Succinate dehydrogenase (SDH) is a complex protein, sometimes named commonly as succinate: ubiquinone oxidoreductase (SQR) or complex II (Amiri et al. 2010; Hägerhäll 1997; Kuhn 1984). The SDH enzyme is ubiquitous in cells of all aerobic organisms and is located in the mitochondrial membrane (Oliver & Hewitt 2014). Four nuclear-encoded subunits form this complex protein: SDHA, SDHB, SDHC, and SDHD and two substrate-binding sites (Cecchini 2003; Lemire & Oyedotun 2002; Oliver & Hewitt 2014; Semar et al. 2014).

Two subunits of the SDH complex are located in the mitochondrial matrix. SDHA, a hydrophilic flavoprotein, catalyzes the oxidation of succinate to fumarate. This oxidation process is an essential step in the Krebs cycle, also called mitochondrial tricarboxylic acid (TCA) cycle or the citric acid cycle. SDHB, an iron-sulfur protein, has three iron-sulfur clusters that are necessary for the electron transfer from succinate to ubiquinone. These subunits are called soluble catalytic dimers. The remaining subunits of the SDH complex, SDHC and SDHD are lipophilic transmembrane subunits that help to anchor the protein complex to the mitochondrial membrane (Glattli et al. 2010; Lemire & Oyedotun 2002; Scallieti et al. 2010; Semar et al. 2014; Stammler et al. 2015). Ubiquinone binding site (Qp site) is a compartment formed by subunits B, C, and D and close to the (Fe-S) cluster (Horsefield et al. 2004). The subunits SDHC and SDHD trigger the reduction of ubiquinone (UQ) to ubiquinol (UQH2). The ubiquinone, a hydrophobic-membrane bound electron carrier, will help to transport the electron through the membrane in form of UQH2 during the electron transport chain mechanisms (Horsefield et al. 2006).

SDH inhibitors affect the succinate dehydrogenase enzyme by blocking complex II by overlapping in the ubiquinone-binding site (Qp site). They have a strong effect inside the fungal mitochondrion target, triggering a rapid decrease of cellular ATPs and accumulation of reactive oxygen species (Glattli et al. 2010; Oliver & Hewitt 2014; Scallieti et al. 2010; Semar et al. 2014; Stammler et al. 2015).

The first commercialized SDHI compound, carboxin, was registered in 1969 as a seed treatment fungicide. This active ingredient is active against bunt and smut fungi associated with cereals (Stammler et al. 2015; Walter 2010). Carboxins were used mainly to control species of *Rhizoctonia*, *Ustilago*, and *Tilletia caries* in cereals, maize, cotton, oilseed rape, and legumes (Oliver & Hewitt 2014).

In 1975, oxycarboxin was registered for foliar application, but similar to its predecessor did not provide broad-spectrum activity against diverse species of fungal pathogens (Oliver & Hewitt 2014; Stammler et al. 2015; Torriani et al. 2017). A milestone for the use of SDHI fungicides in disease management was reached when boscalid was introduced to the market in 2003 by BASF (Oliver & Hewitt 2014; Walter 2010). It showed a broad spectrum and foliar activity along with efficacy against *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, *Sclerotinia sclerotiorium*, *Septoria tritici*, and *Puccinia recondita* (Stammler et al. 2008, Oliver & Hewitt 2014; Walter 2010). Currently, boscalid is registered as a stand-alone active ingredient or pre-mixed with the fungicide, pyraclostrobin for canola, cereal, fruit, vegetable, ornamental crops, and turfgrass (Stammler et al. 2008; Walter 2010). The next generation of the SDHI fungicide class includes benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, isofetamid, isopyrazam, penflufen, penthiopyrad, and sedaxane. (Oliver & Hewitt 2014; Stammler et al. 2015).

SDHIs are among the most well-studied fungicide classes. As a result of rapid chemical innovation, the number of molecules in the SDHIs has risen to 23 distributed across 11 chemical groups (Sierotzki & Scalliet 2013; Stammler et al. 2015; FRAC 2019). Because of their high efficacy, they undoubtedly have become a group with significant importance in the last 30 years of agriculture (Walter 2010).

Resistance of target fungi to SDHI fungicides

The first report of resistance to SDHIs came in the mid-1970s, only five years after the introduction of carboxin. Resistance was reported in *Ustilago maydis* (cause of corn smut) and *Ustilago hordei* (causing covered smut of barley) (Ben-Yephet et al. 1975; Georgopoulos et al. 1975). This situation opened a new area of study in fungicide resistance (Stammler et al. 2015). Similar to carboxin, resistance to other SDHI fungicides such as flutolanil and boscalid was reported soon after they were released into the market (Avenot & Michailides 2010; Honda et al. 1996).

Modern molecular tools applied to fungicide research studies have helped to clarify the mechanism of development of resistance of plant pathogens in this fungicide class. Mutations in *sdhB, sdhC,* and *sdhD* have been characterized that confer different levels of insensitivity to SDHI fungicides. Among these mutations, the most commonly reported is found in the subunit SDHB. The presence of amino acid substitutions in SDHC and SDHD are associated with resistance that is variable across species. A mutation in the *sdhB* gene is found in the ubiquinone binding site (Qp), which results in the substitution of the highly conserved histidine (H) with tyrosine (Y), has been widely associated with a reduction in the sensitivity to this class (Avenot & Michailides 2010; Sierotzki & Scalliet, 2013; Walker et al. 2010). In addition to tyrosine, the additional substitution of histidine (H) with leucine (L) or arginine (R) has been reported (Walker et al. 2010).

The target site mutation in the *sdhB* gene depends on the fungal species because of the variability of this gene. In general, the most frequent target site mutation points, or single nucleotide polymorphisms (SNPs), reported so far are H/Y, L or R at 257, 267, 272, 277 or P225L/T/F (De Miccolis Angelini et al. 2010; Sierotzki & Scalliet 2013; Skinner et al. 1998). The effect of mutation in the same homologous site varies depending on the fungal species (Stammler et al. 2015).

For instance, point mutations reported in *Botrytis cinerea* and *Alternaria alternata* include H272Y/R/L and H277Y/R/L, respectively (Amiri et al. 2014; Avenot & Michailides 2007; Stammler et al. 2010). Other less frequent mutations characterized for *Botrytis cinerea* are P225F/L/T and N230I in the *sdhB* gene (Leroux et al. 2010; Veloukas et al. 2011). Also, the point mutations in *sdhB* gene have differing effects on the fitness of *Botrytis cinerea* (Ishii 2015;

Lalève et al. 2014; Veloukas et al. 2014).

Additional mutations in subunits C and D have also been reported in phytopathogenic fungi (Stammler et al. 2015). For instance, *Botrytis cinerea* shows substitutions in D-H132R, C-A85V, whereas substitutions in *Alternaria alternata* include C-H134R, D-D123E, and D-H133R. *Pyrenophora teres* presents another example, with C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H143R, D-D145G mutations (Stammler et al. 2015).

Fungi with SDHI resistance present partial or incomplete cross-resistance between other members of this class. The level of resistance to SDHIs varies depending on the fungal species, place of substitution, the substituting residue, and the chemical feature of the active ingredient (Avenot et al. 2014; Sierotzki & Scalliet 2013). For instance, positive cross-resistance between boscalid, penthiopyrad, and fluopyram was reported in *Alternaria alternata* (Avenot & Michailides 2010). Conversely, no cross-resistance between boscalid and fluopyram was reported for *Corynespora cassicola* and *Podosphaera xanthii* (Ishii et al. 2011). Furthermore, some studies in *Botrytis cinerea* reported that all mutations tested conferred resistance to boscalid and, in some cases to fluopyram and carboxin as well (Lalève et al. 2014). In addition, mutations in the Sdh genes can cause different patterns. For example, the mutation P225F in the SDHB confers resistance to all SDHIs in *Botrytis cinerea*. However, a mutation from H272 to Y272 in the same subunit reduces sensitivity to boscalid, isopyrazam and bixafen, but increases sensitivity to fluopyram (Sierotzki & Scalliet 2013).

Systemic acquired resistance and host plant defense inducers

An important mechanism involved in protection of the plant against plant pathogens is called systemic acquired resistance (SAR). It consists of an inducible defense that provides long-lasting protection in distant, pathogen-free tissue of the plant against a broad range of pathogens (Ádám et al. 2018). SAR is triggered in plants by beneficial microorganisms, earlier localized exposure to a pathogen, pathogen-derived elicitors such as lipids, carbohydrates, glycopeptides, and glycoproteins, and synthetic chemical application (Choi & Hwang 2011; Gozzo 2003; Raskin 1992; Ryals et al. 1994; Ryals et al. 1996; Walters et al. 2005).

Host plant defense inducers provide broader options for integrated pest management and are formulated and marketed for disease management in several cropping systems. Among the host plant defense inducers registered in the US are acibenzolar-S-methyl (ASM), *Bacillus mycoides* (Isolate J) and *Reynoutria sachalinensis* (giant knotweed extract). Acibenzolar-S-methyl acts as an analog of salicylic acid (SA), triggering the accumulation of pathogenesis-related (PR) protein such as PR-1 (anti-oomycete activity), PR-2 (β-1,3-glucanase), and PR-8 (class III chitinase) (Aćimović et al. 2015; Friedrich et al. 1996; Görlach et al. 1996; Maxson-Stein et al. 2002; Metraux et al. 1991). Application of *Bacillus mycoides* (Isolate J), a gram-positive saprophytic bacterium, was reported to induce oxidative burst and production of peroxide in sugar beet. This product provided 38% to 91% control against Cercospora leaf spot, caused by *Cercospora beticola* Sacc. in sugar beet (Bargabus 2003). The efficacy of *B. mycoides* (Isolate J) was reported for the management of *Glomerella cingulata* var. *orbiculare*, the causal agent of anthracnose of cucumber (Neher et al. 2008). In addition, *B. mycoides* (Isolate J) was effective against downy mildew in a highly susceptible of grapevine cultivar (Wilcox 2017). Another important defense inducer, *Reynoutria sachalinensis*, acts as a promoter of anthraquinone elicitor (FRAC 2019). This plant extract was reported to act against powdery mildew by activation of phytoalexins (Daayf et al. 1997). An increase in PR-2 gene expression was reported in courgette, a variety of summer squash (*Cucurbita pepo* var. *cylindrical*), when the tissue was treated with *R. sachalinensis* extract (Margaritopoulou et al. 2020).

SAR mechanisms and Protein related (PR) gene activation

The activation of SAR triggers several signaling cascade reactions inside the cell. The SAR activation in the plant is highly correlated to production of specific molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radicals ($^{\bullet}OH$) (Choi & Hwang 2011; Lamb & Dixon 1997). Several signal responses are triggered upon infection. One signal is the increase in SA concentration in plant tissue (Garcion & Métraux 2006). SA is found at high concentration near the infection site or at the site of the hypersensitive reaction (HR) (Enyedi et al. 1992). HR, a programmed cell death response by plants, is another visual symptom primarily of Effector-triggered immunity (ETI). It consists of the development of necrotic lesions at the pathogen-inoculation site by the accumulation of reactive oxygen species (ROS) and activation of defense-related genes such as PR genes (Vlot et al. 2009).

As part of the SAR mechanism, the production of pathogenesis-related (PR) proteins play an essential role in the protection of the plant from further infection. For activation of the PR genes, several essential chemical reactions occur, including the reduction of nonexpresser of PR genes 1 (NPR1) from the inactive state (oligomeric) to an active state (monomeric). NPR1 is a positive regulator of systemic acquired resistance (SAR), which is activated by SA via a mechanism not entirely understood. This process leads to nuclear localization of NPR1 and induces the expression of PR via transcription factors (Garcion & Métraux 2006; Kinkema et al. 2000; Mou et al. 2003). Most genes induced by SA are related to members of the PR gene family. Among this group, PR-1 is a significant marker for SA and SAR evaluation (Garcion & Métraux 2006; Van Loon & Van Strien 1999). As a consequence of these reactions, PR gene expression in infected plant tissue increases, developing long-lasting, and broad-spectrum protection against phytopathogens (Vlot et al. 2009).

Studies of PR gene expression in response to pathogen infection and host plant defense inducer application have been conducted in several pathosystems. A study in tomato reported a correlation in the resistance to *Peronospora tabacina* and *Phytophthora parasitica* var *nicotianae* with high expression of PR-1 (Alexander et al. 1993). In *Capsicum annum*, significant levels of induced expression of PR-1 and PR-10 were reported after inoculation of avirulent *Xanthomonas campestris* on leaves (Lee & Hwang 2005). In apple, the overexpression of PR-1, PR-2, PR-4, PR-5, PR-8, and PR-10 has been reported in several studies (Aćimović et al. 2015; Bai et al. 2013; Bonasera et al. 2006; Brisset et al. 2000; Maxson-Stein et al. 2002; Ziadi et al. 2001). However, evaluation of PR gene expression on apple after foliar application of induced resistance compounds under field conditions has not been investigated.

Research Justification and Objectives

Chemical pest control is one of the most helpful tools used in agriculture. Even though this component is part of integrated pest management (IPM) and shows the highest level of control for some pathogens, incorrect application of fungicides can be detrimental for agriculture, the environment, and society. Therefore, a strong understanding of the effects of these fungicide active ingredients on pathogens is required. Additionally, more sustainable tools are necessary and should be included and promoted as part of integrated pest management to preserve the effectiveness of the fungicide active ingredients. While there have been several studies regarding SDHI fungicide sensitivity evaluation and resistance surveillance in important pathogens of annual cropping systems, investigations of this chemical class in perennial fruit and ornamental systems have not been as prevalent. In addition, because of the frequency and duration of disease management programs on perennial crop systems in the southeastern US, alternative tools for protection against pathogens are imperative. Given the registration of several host defense inducers in recent years, it is important to develop application strategies to maximize their efficacy against bacterial and fungal diseases in perennial woody hosts. Therefore, this research investigates SDHI fungicide sensitivity and surveillance protocols for detecting fungicide resistance in three different pathosystems: Glomerella leaf spot and bitter rot of apple *(Malus domestica*) caused by *Colletotrichum* spp. and boxwood blight in *Buxus* spp. caused by *Calonectria pseudonaviculata* (*Cps*). In addition, strategies for the application of host plant defense inducers are also investigated: boxwood blight of *Buxus sempervirens* 'Justin Brouwers', and fire blight of *Malus domestica* 'Rome Beauty' caused by *Erwinia amylovora*.

This study aims to determine the baseline sensitivity (EC_{50}) of two species *Colletrotrichum fructicola* and *Colletotrichum fioriniae*, and evaluate the efficacy against *Calonectria pseudonaviculata* with four succinate dehydrogenase inhibitors (SDHIs) fungicides through evaluation of in vitro mycelium growth inhibition. Given the moderate to high resistance risk for SDHI fungicides, it is important to gain a better understanding of the efficacy of SDHI fungicides for Glomerella leaf spot and bitter rot management, determine the risk of cross-resistance between SDHIs, and to develop thresholds for resistance monitoring. Evaluation of potential fungicide sensitivity shifts was assessed in populations of *C. fructicola* and *C. fioriniae*, two predominant causal agents of Glomerella leaf spot and bitter rot in the southern US, in different commercial orchards. Characterization of *sdhB, sdhC* and *sdhD* genes and identification of a putative point mutation for SDHI resistance will be proposed for *C. fructicola*.

In addition, evaluation of the performance of host plant defense inducers for managing boxwood blight and fire blight was performed. Furthermore, a measurement of protein-related (PR) gene expression after SAR inducers application was made in apple to suppress fire blight disease.

The results obtained in this study will help to monitor sensitivity shifts of *C. fructicola* and *C. fioriniae* to SDHI fungicides determined by established baseline sensitivities. The characterization of the SDH genes of *C. fructicola* will provide a better understanding of the mechanisms of fungicide resistance in this species. Evaluation of SDHI against *C. pseudonaviculata* will help identify potential new single-site fungicides for boxwood blight management programs. Finally, the understanding of application methods and effectiveness of alternative products such as host plant defense inducers will expand the management toolbox for farmers and reduce the risk of developing fungicide resistance that could potentially be triggered by the use of the single-site fungicides.

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CHAPTER II

Characterization of the *CfsdhB*, *CfsdhC*, and *CfsdhD* genes in *Colletotrichum fructicola* and SDHI fungicide sensitivity of *Colletotrichum* spp. causing Glomerella leaf spot and bitter rot in North Carolina

ABSTRACT

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Glomerella leaf spot and bitter rot are two serious diseases of apple (*Malus domestica*) caused by *Colletotrichum* spp. in North Carolina. *C. fructicola* is the most prevalent species causing Glomerella leaf spot and *C. fioriniae* the most prevalent cause of bitter rot in major apple production regions of the Southeast. The utilization of multi-site and single-site fungicides is essential for the management of these diseases. Several new succinate dehydrogenase inhibitor (SDHI) fungicides that are registered for apple, formulated as either single-active ingredient products, or as pre-mixed products with another active ingredient, are increasingly being incorporated into management programs for Glomerella leaf spot and bitter rot. Given the moderate to high resistance risk for SDHI fungicides, it is important to gain a better understanding of the efficacy of SDHI fungicides for Glomerella leaf spot and bitter rot management, determine the risk of cross-resistance between SDHIs, and to develop thresholds for resistance monitoring. In this study, the baseline sensitivity of *C. fioriniae* and *C. fructicola* to benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid was investigated. Twenty-one isolates of *C. fructicola* and 11 isolates of *C. fioriniae* with no prior exposure to single-site fungicides were used to determine the effective concentration at which isolate mycelial growth was inhibited by 50% (EC_{50}). The mean EC_{50} for mycelium of *C. fructicola* was 0.169 µg/ml for benzovindiflupyr and 0.330 µg/ml for penthiopyrad, whereas EC_{50} for *C. fioriniae* was 0.442

μg/ml for benzovindiflupyr and 0.509 μg/ml for penthiopyrad. For both species, fluxapyroxad and boscalid were relatively ineffective at reducing mycelial growth with EC_{50} mean values exceeding 100 μg/ml in both cases. Sensitivity of isolates of *Colletotrichum* spp. from commercial orchards to benzovindiflupyr and penthiopyrad was also evaluated. Sixty-seven percent of orchard populations of *C. fructicola* had reduced sensitivity to benzovindiflupyr, as were 25% of *C. fioriniae* populations. Conversely, 100% of *C. fioriniae* populations had reduced sensitivity to penthiopyrad compared to the baseline population, but all populations of *C. fructicola* were sensitive. Significant positive correlations between percentage relative growth values for benzovindiflupyr and penthiopyrad were observed for *C. fructicola* (P < 0.0001, r = 0.55) and for *C. fioriniae* (P < 0.0001, r = 0.50). In addition, the target genes for SDHI fungicides, *sdhB*, *sdh*C, and *shD* were identified and characterized for *C. fructicola*. No putative mutation was found in *sdhB* among isolates characterized as having reduced sensitivity to any of the four SDHI fungicides evaluated. This study provides useful information for the management of Glomerella leaf spot and bitter rot, giving an understanding of the performance of these fungicide active ingredients and the potential fungicide resistance risk on these fungi.

INTRODUCTION

Glomerella leaf spot and bitter rot, caused by members of the *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* species complexes are considered the most devastating fungal diseases of apple (*Malus domestica*) in the United States. In the southeastern US, co-symptomatic apple foliage and fruit has predominantly been caused by *Colletotrichum fructicola* (*Colletotrichum gloeosporioides* species complex) whereas fruit infections in trees absent of Glomerella leaf spot symptoms were predominantly caused by *Colletotrichum fioriniae* (*Colletotrichum acutatum* species complex) (Damm et al. 2012; Johnson 2019; Johnston et al.

2005). However, the presence of other *Colletotrichum* species has varied among regions, cultivars, and tissue infected (González et al. 2006; González & Sutton 2004; Munir et al. 2016; Velho et al. 2016). On leaves, early symptoms of Glomerella leaf spot appear as small red to purple colored specks. As the infection progresses, the specks develop into asymmetrical, brown, and usually concentric lesions, causing chlorotic and rapid defoliation (Leite et al. 1988; Taylor 1971; Villani 2017). Early symptoms observed for bitter rot appear as small, sunken black to brown colored spots (1 to 5 mm diameter). As the fruit matures, these spots expand to larger concentric lesions, and maceration of fleshy tissue from the fruit surface to the apple core develops in a v-shaped pattern. Unfortunately, the presence of these symptoms affects the marketability of apple production.

The paucity of disease-resistant apple cultivars in the US and the marginal benefit gained from cultural management practices mandates multiple fungicide applications to manage these diseases. In North Carolina, multi-site protectant fungicides, including mancozeb, captan, and ziram serve as the backbone of management programs. Fungicides are applied beginning at petal fall on 7 to 10-day intervals. Quinone outside inhibitor (QoI) fungicides are strategically applied prior to predicted extended precipitation events and during the immediate pre-harvest period (Johnson et al. 2018). However, alternative modes of action are needed to manage these diseases to accommodate QoI resistance management practices and annual application limits. The succinate dehydrogenase inhibitor (SDHI) fungicides, applied either as a single-active-ingredient formulated product, or as a formulated pre-mixed fungicide, provide an alternative mode of action against *Colletotrichum* spp. SDHIs are single-site fungicides that block the production of energy in fungi by disrupting the electron transport chain in aerobic organisms (Bartlett et al. 2002; Ruy et al. 2006; Semar et al. 2014). Specifically, SDH inhibitors affect the succinate dehydrogenase enzyme by blocking complex II, a vital component involved in the electron transport chain (Glattli et al. 2010; Oliver & Hewitt 2014; Scallieti et al. 2010; Semar et al. 2014; Stammler et al. 2015).

Due to their highly specific mode of action, SDHI fungicides have been classified as medium to high risk for resistance development (FRAC 2019). In the past decade, there have been several reports of resistance in phytopathogens including *Alternaria alternata* (Avenot et al. 2014, 2019), *Botrytis cinerea* (Amiri et al. 2014; Fernández-Ortuño et al. 2017; Veloukas et al. 2011; Yin et al. 2011), *Corynespora cassiicola* (Miyamoto et al. 2009), *Alternaria solani* (Miles et al. 2014), *Didymella bryoniae* (Avenot et al. 2012; Stevenson et al. 2008), *Podosphaera xanthii* (Ishii et al. 2011; Miyamoto et al. 2010), and *Zymoseptoria tritici* (= *Mycosphaerella graminicola*) (Scalliet et al. 2012; Skinner et al. 1998). Resistance to SDHI fungicides has been associated with point mutations leading to amino acid substitutions in three of four subunits of the SDH complex (SDHB, SDHC, and SDHD). The most common mutation leading to a reduction in SDHI sensitivity is located in the subunit SDHB in which a highly conserved histidine (H) residue is replaced with tyrosine (Y), leucine (L) or arginine (R) (Avenot & Michailides 2010; Sierotzki & Scalliet 2013; Walker et al. 2010). Other common mutations are H134R in SDHB, and H133R in SDHD (De Miccolis Angelini et al. 2010; Sierotzki & Scalliet 2013; Skinner et al. 1998). Depending on the mutation location, substituting residue, number, and fungal species, variations in resistance degree and specific SDHI fungicides have been observed (Stammler et al. 2015).

Limitations in the number of applications for specific fungicides, the long growing season in the southeastern US, and environmental conditions in NC necessitate a diverse fungicide program to maintain adequate control of Glomerella leaf spot and bitter rot (Villani &

Nance 2016). In field trials, the next generation SDHI fungicides including benzonvidiflupyr, penthiopyrad, and fluxapyroxad have been demonstrated as possible new tools for Glomerella leaf spot and bitter rot management (Johnson et al. 2018; Villani & Nance 2016). Knowledge of the potential for SDHI resistance in these fungi should be integrated into disease management programs to minimize crop losses due to bitter rot. Therefore, the aims of this study were to 1) characterize the baseline sensitivity of *C. fructicola* and *C. fioriniae* to the SDHI fungicides benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid, 2) analyze population sensitivity shifts of *C. fructicola* and *C. fioriniae* to benzovindiflupyr and penthiopyrad, 3) evaluate the potential for cross-resistance between SDHI fungicides, and 4) identify and characterize the *sdhB*, *sdhC*, *sdhD* genes in *C. fructicola* by comparing homologous regions of *sdh* genes from other phytopathogenic fungi with a reference genome of *C. fructicola.*

MATERIALS AND METHODS

Collection of isolates of *Colletotrichum fructicola* and *Colletotrichum fioriniae* from apple leaves and fruit.

In July and August 2016, isolates of *Colletotrichum* spp. were collected from apple leaves and fruit symptomatic of Glomerella leaf spot and bitter rot. Leaves and fruits were collected from apple orchards located in North Carolina (eight orchards), and Georgia (four orchards). These isolates were collected from orchards in the southeastern US that represented a range in fungicide application practices, including nine conventional, one research, and two baseline orchards. Sample isolates that were obtained to calculate baseline fungicide sensitivity were obtained from trees that to our or the grower's knowledge had never been exposed to applications of single-site conventional fungicides, including SDHI and QoI fungicides.

Colletotrichum species and the number of isolates collected for each orchard are listed in Table 2.1.

Isolates were obtained by arbitrarily collecting a minimum of 20 symptomatic leaves or fruit from each orchard. No more than three samples were obtained from each tree. In total, 161 isolates of *C. fructicola* were collected from symptomatic leaves or fruit from seven orchards in Western NC. A second collection of *C. fioriniae* isolates was obtained from four orchards in GA and one in NC where a total of 80 bitter rot samples had been collected in a previous study (Johnson 2019; Table 2.1). In accordance with Johnson et al. (2019), isolation from leaf tissue was performed by immersing it in 0.6% NaOCl for three minutes. Leaves were rinsed twice and dried. Small pieces of the margin of the lesion were placed onto Lima Bean Agar (LBA) amended with streptomycin and chloramphenicol (LBA++). Fruit tissue was surface sterilized with a sterile paper towel saturated with 70% ethanol. Small pieces from a single lesion were placed on LBA++. For both tissues, Petri dishes were incubated for seven days at 25°C under constant fluorescent light. A region of fungal growth was selected and transferred to a LBA medium to obtain a pure culture. Conidia from pure culture were harvested after ten days of growth. Monoconidial cultures were obtained by the suspension of harvested conidia in 1 ml of sterile distilled water and, 100 μl aliquot was evenly distributing it onto the surface of PDA. A single conidium was selected after 24 hours and placed on a new plate of PDA. Once pure monoconodial cultures were obtained, isolate mycelium was stored in potato dextrose broth (PDB)/glycerol storage medium (50% PDB Difco Laboratories, Maryland, USA; 50% sterile glycerol) at -80°C until fungicide sensitivity assays were conducted.

Table 2.1 List of *Colletotrichum fructicola* and *Colletotrichum fioriniae* isolates from apple tissue collected in NC and GA in 2016.

Orchard	Species	Cultivar (state)	#Isolates[a]	Tissue	Disease[d]
A[b]	*C. fioriniae*	unknown (GA)	11	fruit	BR
B	*C. fioriniae*	Honeycrisp (NC)	15	fruit	BR
D	*C. fioriniae*	Honeycrisp (GA)	25	fruit	BR
F	*C. fioriniae*	Rome Beauty (GA)	14	fruit	BR
G	*C. fioriniae*	Jonagold (GA)	15	fruit	BR
n=5			n=80		
H	*C. fructicola*	Golden Delicious (NC)	28	fruit-leaf	GLS
I	*C. fructicola*	Golden Delicious (NC)	16	fruit-leaf	GLS-BR
M[b]	*C. fructicola*	Gala (NC)	21	fruit-leaf	GLS-BR
N	*C. fructicola*	Gala (NC)	27	fruit-leaf	GLS-BR
O	*C. fructicola*	Golden Delicious (NC)	27	fruit-leaf	GLS-BR
P	*C. fructicola*	Gala (NC)	25	fruit-leaf	GLS-BR
W[c]	*C. fructicola*	Gala (NC)	17	fruit-leaf	GLS-BR
n=7			n=161		

[a] Number of isolates tested from each orchard
[b] Baseline orchard
[c] Research orchard
[d] Disease diagnosed at the time of collection BR = Bitter rot, GLS = Glomerella leaf spot

Fungicides tested

The effect of four commercially formulated SDHI fungicides was evaluated on mycelium of *C. fructicola* and *C. fioriniae.* Baseline sensitivities were determined for benzovindiflupyr (Aprovia, 10% a.i., EC, Syngenta), penthiopyrad (Fontelis, 20.4% a.i, SC, Corteva), fluxapyroxad (Sercadis, 26.55% EC, BASF), and boscalid (Endura, 70% WDG, BASF)

Evaluation of minimal and complete media for the evaluation of SDHI fungicides in isolates of *C. fructicola* and *C. fioriniae*

Based on previous reports, a preliminary experiment was conducted to assess the effects of minimal and complete media on sensitivity to SDHI fungicides in culture (Amiri et al. 2010; Hu et al. 2011). The mycelial growth habit of ten baseline isolates of *C. fructicola* was evaluated at five concentrations of benzovindiflupyr and penthiopyrad on standard potato dextrose agar (PDA, 39.5 g in 1 liter H_2O), ¼ th strength potato dextrose agar (¼ PDA, 9.75 g in 1 liter H_2O), glycerol yeast extract agar (GLYE; 10 g glycerol, 10 g yeast extract, 15 g agar, 6 g NaNO3, 1.5 g KH2PO4, 0.5 g MgSO4, and 0.5 g KCl in 1 liter H_2O) (Villani et al. 2016), and yeast bacto acetate agar (YBA; 20 g yeast extract, 20 g bacto peptone, 40 g sodium acetate, 15 g agar in 1 liter H_2O) (Miyamoto et al. 2010; Stammler & Speakman 2006). Final concentrations of active ingredient of formulated fungicides included in the different media were 0 (control), 0.00001, 0.0001, 0.001, 0.01 and 0.1 μg a.i./ml. Four mycelium plugs obtained from cultures actively growing on PDA were used per concentration (two plugs per petri dish). Culture plates were incubated at 25°C with constant light, and mycelium diameter was measured after six days.

Fungal cultures tested on PDA and ¼ th strength PDA grew uniformly while growth on GLYE was irregular. Mycelium on YBA produced upward protrusions from the medium. Cultures grown on YBA had the slowest and most inconsistent growing pattern in comparison with other media tested (Figure 2.1). The most consistent and lowest EC_{50} value (effective concentration at which growth was inhibited by 50%) was obtained on standard PDA. Similar results were obtained in previous studies with SDHI fungicides (Amiri et al. 2014; Avenot et al. 2012; Villani et al. 2016). Therefore, we concluded that PDA was an appropriate medium for evaluating sensitivity of *Colletotrichum* spp. to SDHI fungicides.

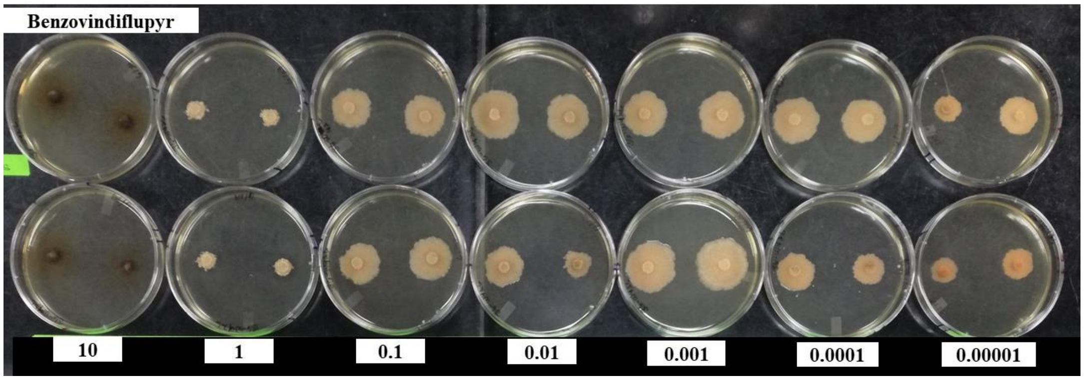

Figure 2.1 Mycelial growth of *Colletotrichum fructicola* on YBA amended with 0.00001, 0.0001, 0.001, 0.01 and 0.1 µg a.i/ml benzovindiflupyr.

Evaluation of sensitivity to SDHI fungicides in baseline populations of *Colletotrichum fructicola and C. fioriniae*

A total of 21 isolates of *C. fructicola* were obtained from Glomerella leaf spot and fruit lesions collected from an organic orchard (Orchard M). Eleven isolates of *C. fioriniae* were recovered from fruit with symptoms of bitter rot from a homeowner orchard in GA in which fungicides had never been applied (Orchard A). These populations were selected to develop a baseline sensitivity EC_{50} to four SDHI fungicides benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid.

A mycelium relative growth assay was used to calculate the EC_{50} value for each fungicide (Hou et al. 2018; Thomas et al. 2012; Villani et al. 2016). PDA was amended with a formulated product of the active ingredient for each fungicide. Final concentrations of active ingredient included in the amended media for benzovindiflupyr, penthiopyrad, and boscalid were 0.001, 0.01, 0.1, 1.0, and 10.0 µg a.i./ml. and for fluxapyroxad 0.01, 0.1, 1.0, and 10.0 µg a.i./ml. The control consisted of PDA without fungicides amendments. The amendments were diluted to a standard volume of 1 ml/liter medium and added to the autoclaved media (20 minutes – 156

KPa) when the temperature was approximately 50˚C before being poured into 100-mm diameter Petri dishes.

For the mycelium inhibition assay, each isolate was concurrently tested with the four SDHI fungicides mentioned above, and amended media was prepared no more than 24 hours prior to the experiment initiation. For each fungicide at each concentration, there were two replications (two Petri dishes) and two observations (two mycelial plugs) per replicate isolate. Therefore, a total of four 7-mm mycelium plugs were used per fungicide concentration by isolate combination. The 7-mm mycelium plugs were obtained from actively growing colonies on PDA. Two plugs were placed equidistantly with their mycelium in contact with the amended media. Once plugs were placed, they were incubated at room temperature (approximately 25˚C) under continuous fluorescence light. Proximity of the plate to the light source was determined to not influence isolate growth (data not shown), so plates for each isolate were stacked in ascending order of fungicide concentration to facilitate data collection. Following 3 to 4 days of incubation for isolates of *C. fructicola* or 5 to 8 days for isolates of *C. fioriniae,* radial colony diameter was determined by measuring with a 0 to150 mm electronic digital caliper (Westward, Lake Forest, Illinois).

Relative percent inhibition was calculated comparing the mycelial growth in the amended media at each isolate and fungicide concentration with the unamended control as described by Yoshimura et al. 2004. To determine the EC_{50} for each isolate, the relative percent inhibition of colony growth at each $\log_{10}$-transformed concentration was used to create a dose-response curve in Microsoft Excel (Redmond, WA). In addition, variation factors (V_f) for each fungicide were determined by dividing the highest EC_{50} value among isolates with the lowest EC_{50} value among isolates (Villani et al. 2016). Finally, t-tests were conducted in the PROC TTEST procedure in

SAS (Statistical Analysis System version 9.4, Cary, NC) to compare the distribution of benzovindiflupyr and penthiopyrad EC_{50} values in *C. fructicola* and *C. fioriniae*.

Sensitivity of commercial orchard populations of *C. fructicola* and *C. fioriniae* to benzovindiflupyr and penthiopyrad

Single conidial isolates of *C. fructicola* and *C. fioriniae* with previous exposure to single-site fungicides, including the SDHI fungicide boscalid, were evaluated for shifts in sensitivity to benzovindiflupyr and penthiopyrad. For each fungicide, PDA was amended at the baseline EC_{50} concentration previously determined for each *Colletotrichum* spp.

For each isolate the experiment was designed and evaluated as described above with two replicates and with two observations per replicate for each isolate. The level of sensitivity of each isolate was expressed as mean percent relative growth. Relative growth was calculated by comparing the longitudinal growth of the isolate on the fungicide-amended medium to that unamended medium for each isolate (Frederick et al. 2014).

To analyze shifts in sensitivity between orchard populations, the nonparametric Kolmogorov-Smirnov (K-S) one-sample test was performed with the PROC NPAR1WAY procedure in SAS (Statistical Analysis System version 9.4, Cary, NC). In this analysis, the distribution of relative mycelial growth values obtained per each isolate for the baseline population was compared with the distribution of the relative mycelial growth values obtained for the exposed orchard ("test orchard"). The test was performed for *C. fructicola* and *C fioriniae* with benzovindiflupyr and penthiopyrad.

K-S one-sample test results were used to classify the sensitivity distribution for both *Colletotrichum* species and for each orchard population of interest (Frederick et al. 2014; Köller et al. 1997). The probability of showing a statistical difference between the mean baseline orchard, designated as the "sensitive-standard" orchard, distribution and the orchard populations tested, designated as the test orchard, was determined ($P_{T=S}$). From this analysis, three outcomes regarding population sensitivity phenotype to benzovindiflupyr and penthiopyrad were determined. First, if $P_{T=S} \geq 0.05$, the tested orchard would be classified as "sensitive" since no statistical difference was observed between the distribution of the tested orchard and the "sensitive-standard" orchard. Second, if $P_{T=S} < 0.05$, and the mean relative growth of the tested orchard was greater than that of the "sensitive standard" population, the tested orchard population was classified as "reduced sensitive." Finally, a third scenario was possible if $P_{T=S} \geq 0.05$ and the mean relative growth of the tested orchard was less than that of the "sensitive-standard" population. In this case, tested orchard was also classified as sensitive. Correlation coefficients (*r*) were calculated with the PROC CORR procedure in SAS (Statistical Analysis System version 9.4, Cary, NC) by analyzing the relative percent growth to determine the level of cross-sensitivity between benzovindiflupyr and penthiopyrad for both species.

Characterization of the *C. fructicola sdhB, sdhC, and sdhD* genes

Using the BLAST function of CLC Main Workbench (Version 7.8.1, Qiagen, Aarhus, Denmark) assembled contigs of a *C. fructicola* isolate, strain 15060, were searched for homologs to the *sdhB (CfsdhB)*, *sdhC (CfsdhC)*, and the *sdhD (CfsdhD)* genes. Species and accession numbers were as follows: *sdhB* gene, *Alternaria alternata* (EU178856.1), *Venturia inaequalis* (KR139837.1), *Zymoseptoria tritici* (XM_003850705), and *Botrytis cinerea* (AUG44772.1); the *sdhC* gene of *A. alternata* (KJ426267), *V. inaequalis* (MH484042), *Z. tritici* (JX869229), *and B. cinerea* (CQ253443); and the *sdhD* gene of *A. alternata* (AHW57950.1), *V. inaequalis* (MH484042), and *B. cinerea* (ACT83439.1).

Based on single regions of homology for each *sdh* gene, primers were designed to amplify genomic DNA (gDNA) and complementary DNA (cDNA) for each *sdh* gene in *C. fructicola*. For each *sdh* gene, four primers were used to amplify the entire gene including regions upstream and downstream of the respective gene (Table 2.2). Amplification of the *sdhB, sdhC,* and *sdhD* genes of *C. fructicola*, was accomplished for six baseline isolates: M17F, M11F, M38F, M2F, M36L, and M28F. In order to extract total gDNA, the mycelium of each isolate was grown on PDA at room temperature (~25°C). After ten days, when mycelium covered approximately 90% of the Petri dish, the mycelium of each isolate was scraped gently from the surface. Approximately 100 mg were harvested with a sterile scalpel and placed in a 2 ml round bottom microcentrifuge tube. Frozen tissue was homogenized with a sterile 5-mm stainless steel grinding bead (Qiagen, Valencia, CA) using a TissueLyser II (Qiagen) at 30 cycles per second for 30 s. DNA extraction was performed the Omega E.Z.N.A. Plant DNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. A Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, DE) was used to determine the quantity and quality of the DNA extracted.

For each primer set (two sets per gene; see Table 2.2), polymerase chain reaction (PCR) was performed in 25 µl reaction volumes and contained the following: 12.5 µl of 2x EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Mountain View, CA), 9.5 µl dH2O, 1.0 µl of DNA (20 to 40 ng), and 1.0 µl of each primer at a final concentration of 0.4 µM. All PCR reactions were conducted in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA). The PCR conditions for amplification were similar for all reactions with the exception of the annealing temperature: 3 min at 94°C; 30 cycles of 30 s at 94 °C, 30 s at optimal annealing temperature (see Table 2.2) and elongation 45 s at 72°C; followed by a final elongation at 72°C for 7 min.

All PCR reaction products were separated on a 2% agarose gel (Agarose BP160-100, Fisher Bioreagents, NJ) with 1x GelRed Nucleic Acid stain (Biotium, Inc., Fremont, CA) at 80 volts for 60 min. Photographs of the gel were taken with a Gel Doc EZ Imager (Bio-Rad) through Image Lab software (version 5.2.1; Bio-Rad).

The PCR products were purified following the instructions provided with the Wizard SV Gel and PCR clean up system (Promega Corp., Madison, WI). Using the same primers as for amplification, sequencing reactions were performed in the forward and reverse directions using Sanger Cycle Sequencing Reaction set-up through capillary run by the Genomic Sciences Laboratory, North Carolina State University, on an ABI 3730 genetic analyzer. Consensus sequences for each isolate were assembled and edited with CLC Main Workbench (version 8.1.1; Qiagen) software for each *sdh* gene.

RNA extraction, cDNA synthesis, PCR and sequence region coding

To elucidate the coding region of *sdhB, sdhC*, and *sdhD* of *C. fructicola* (*CfsdhB, CfsdhC,* and *CfsdhD*), RNA was extracted from the six isolates (M17F, M11F, M38F, M2F, M36L, and M28F) used for gDNA extraction. Isolates recovered from -80°C storage were transferred to PDA. Following ten days of incubation under light and temperature conditions described above, mycelium was gently harvested (50 to100 mg) from the surface of the media with a sterile scalpel. The mycelium was ground in liquid nitrogen with a sterile mortar and pestle. For each isolate, RNA extraction was accomplished following the instructions of the RNeasy® Plant Mini Kit (50) (Qiagen Lab, Hilden, Germany).

Following RNA extraction, contaminating DNA was removed with the Invitrogen Turbo DNA-*free* ™ Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from the extracted RNA by using the kit protocol of Invitrogen SuperScript ™ IV VILO ™ Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania).

A single set of primers were designed and used for amplification of the coding regions for each *sdh* gene and for amplification of the three coding regions (Table 2.3). PCR reactions were carried out in 25 µl volumes and contained 12.5 µl of 2x EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Mountain View, CA), 9.5 µl dH2O, 1.0 µl of cDNA, and 1.0 µl of each primer (0.4 µM final concentration). Amplifications were performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA) with the following parameters: 3 min at 94°C (initial denaturation); 30 cycles of 30 s at 94°C (denaturation), 30 s at optimal annealing temperature (see Table 2.3), and 45 s at 72°C; followed by a final elongation of 7 min at 72°C. Finally, PCR products were separated, photographed, sequenced, and assembled as described above for gDNA.

Table 2.2 Primers used to amplify gDNA of *sdhB, sdhC* and *sdhD* genes of *C. fructicola*.

Primer set	Target gene	Part of the gene	Primer	Direction	Sequence (5'-3')	Annealing temperature
1	*sdhB*	front	gDNA sdhB_Cfruct F1	Forward	CTT GCC ACT CCG AAT ACC A	54°C
			gDNA sdhB_Cfruct R1	Reverse	GAA GGG GGT GAT GGT GAT G	
2	*sdhB*	End	gDNA sdhB_Cfruct F2	Forward	TGG AGG TAC GAC AAA GAC	54°C
			gDNA sdhB_Cfruct R2	Reverse	ACA TCA ACT CTC ACA CTC A	
3	*sdhC*	front	gDNA sdhC_Cfruct F1	Forward	GCC ATT GAA TCC TTG GGT GTG T	55°C
			gDNA sdhC_Cfruct R1	Reverse	CGA ACC ACG TCT GCT CCA TCT T	
4	*sdhC*	End	gDNA sdhC_Cfruct F2	Forward	CAG AGC CTG GAA CCT TCA AAC C	64°C
			gDNA sdhC_Cfruct R2	Reverse	AAC TCC AAT CGC CGT CTC CC	
5	*sdhD*	front	gDNA sdhD_Cfruct F1	Forward	TAA TAC TAC GGT GAT CCG GCC A	64°C
			gDNA sdhD_Cfruct R1	Reverse	CAG GAG TGT CTC TCT GCA GGT A	
6	*sdhD*	End	gDNA sdhD_Cfruct F2	Forward	GCA TGG CTA ACC ATA GTC CAG G	62°C
			gDNA sdhD_Cfruct R2	Reverse	CCA AGG AGT TCA AAC TCC AGC C	

Table 2.3 Primers used to amplify the coding region of *sdhB*, *sdhC* and *sdhD* of *C. fructicola*.

Primer set	Target gene	Primer	Direction	Sequence (5'-3')	Annealing
1	*sdhB_cDNA*	cDNA sdhB_Cfruct F	Forward	ATG GCT GCC CGG ATG GC	52°C
		cDNA sdhB_Cfruct R	Reverse	TCA AGC CTT CCA GAT GCG AGC G	
2	*sdhC_cDNA*	cDNA sdhC_Cfruct F1	Forward	ATG ATT GCG CAG CGG GTG G	62°C
		cDNA sdhC_Cfruct R1	Reverse	TTA CCA CGC AAA AGC CAG ACC G	
3	*sdhD_cDNA*	cDNA sdhD_Cfruct F2	Forward	ATG GCA GCT CTC CGC T	64°C
		cDNA sdhD_Cfruct R2	Reverse	TTA AAA AGC CAT AGC CTT CTT G	

Characterization of putative point mutations in *sdhB* conferring resistance to SDHI fungicides

Following characterization of the three *sdh* genes, ten isolates with different sensitivity phenotypes for benzovindiflupyr and penthiopyrad were selected (Table 2.4). PCR amplification and sequencing of the *sdhB* coding region for those isolates was performed. Each isolate was aligned and edited with CLC Main Workbench (version 7.9.1; Qiagen) software for the studied region. The sequence analysis was accomplished for each isolate in order to identify putative mutations in *sdhB* conferring resistance to *C. fructicola*.

Table 2.4 Isolates used for characterization of putative mutations of *sdhB* in *C. fructicola*.

Number	**Isolate**	**Benzovindiflupyr RGI (%)[a]**	**Penthiopyrad RG(%)[a]**
		Sensitive	**Sensitive**
1	I1L	56.0	46.3
2	I7L	60.6	49.8
		Reduced sensitivity	**Sensitive**
3	W4F	38.7	60.8
		Sensitive	**Reduced Sensitivity**
4	T92L	52.1	38.1
5	M28F	62.8	44.3
6	H10L	49.5	36.3
		Reduced sensitivity	**Reduced sensitivity**
7	H7L	32.9	37.0
8	I5L	30.5	29.3
9	M43L	41.3	34.5
10	R7L	37.2	25.6

[a] RGI%= Relative growth inhibition of the isolates cultured in PDA with the EC_{50} value calculated for benzovindiflupyr and penthiopyrad. Isolates presenting less than 45% of RGI were classified as sensitive, while isolates presenting more than 45% of RGI were classified as reduced sensitivity.

RESULTS

Sensitivity of baseline population of *C. fructicola* and *C. fioriniae* to benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid.

Mycelial growth inhibition assays were used to determine baseline sensitivities to the SDHI fungicides benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid. The baseline populations of *C. fructicola* and *C. fioriniae* exhibited greater sensitivity to benzovindiflupyr and penthiopyrad than to fluxapyroxad and boscalid (Figure 2.2A, 2.2B).

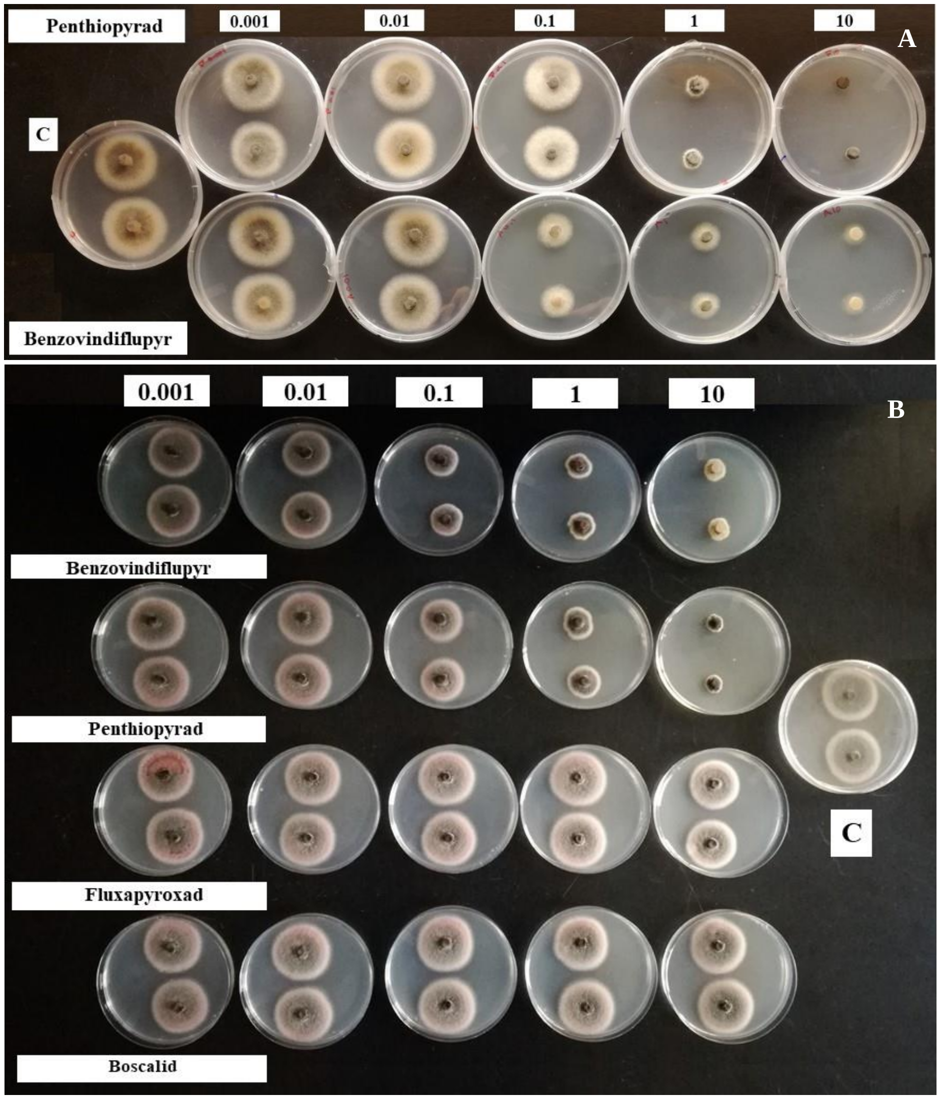

Figure 2.2 A, Mycelial growth of *Colletotrichum fructicola* on PDA amended with 0.001, 0.01, 0.1, 1, 10 µg a.i /ml benzovindiflupyr (bottom) and penthiopyrad (top), C = Control. **B,** Mycelial growth of *Colletotrichum fioriniae* on PDA amended with 0.001, 0.01, 0.1, 1, 10 µg a.i /ml benzovindiflupyr (top), penthiopyrad (middle-top), fluxapyroxad (middle-bottom), and boscalid (bottom), C = Control.

For *C. fructicola,* benzovindiflupyr EC_{50} values ranged from 0.070 to 0.306 µg/ml with a mean of EC_{50} = 0.169 ± 0.070 µg/ml (Figure 2.3A). The majority of isolates (12 of 21) tested for baseline sensitivity had EC_{50} values between 0.100 to 0.200 µg/ml, with the minimum and maximum EC_{50} values separated by a variation factor V_f of 4.4. For penthiopyrad, EC_{50} values were slightly greater ranging from 0.186 to 0.530 µg/ml with a mean of EC_{50} = 0.330 ± 0.090

μg/ml (Figure 2.3B). The majority of isolates evaluated (11 of 21) ranged between 0.300-0.400 μg/ml, with a V_f of 2.9. The range of EC_{50} value calculated for fluxapyroxad was from 17.250 μg/ml to 579.634 μg/ml with a V_f of 33.6. Boscalid did not inhibit growth of *C. fructicola* at the concentrations tested, and EC_{50} value was reported as >100 μg/ml.

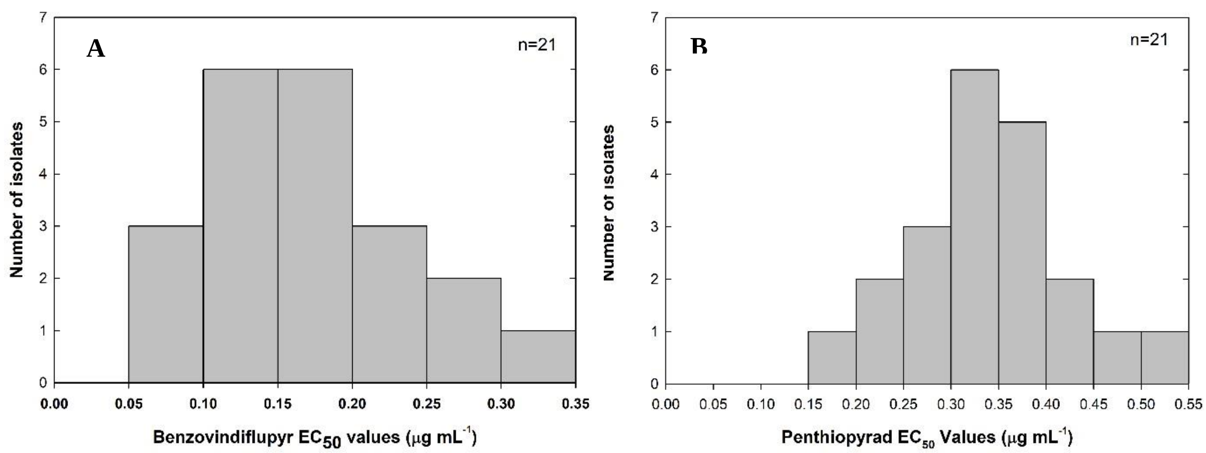

Figure 2.3 Distribution of effective concentration at which mycelial growth on agar was inhibited by 50% (EC_{50}) in baseline isolates of *Colletotrichum fructicola* for **A,** benzovindiflupyr (n = 21) and **B,** penthiopyrad (n = 21). The baseline population was collected from apple orchards in North Carolina that have never been exposed to single-site fungicides.

A baseline population of *Colletotrichum fioriniae* was also evaluated for sensitivity to the four SDHI fungicides. Similar to *C. fructicola*, *C. fioriniae* isolates showed high levels of sensitivity to benzovindiflupyr and penthiopyrad. EC_{50} values for benzovindiflupyr ranged from 0.174 to 0.865 μg/ml with a mean $EC_{50} = 0.442 \pm 0.175$ μg/ml with a V_f of 5.0 (Figure 2.4A). Similar to *C. fructicola*, EC_{50} values for penthiopyrad was slightly higher compared to benzovindiflupyr, with EC_{50} values ranging from 0.163 to 0.629 μg/ml with mean $EC_{50} = 0.509$ $\pm$ 0.130 μg/ml (Figure 2.4B). The minimum and maximum EC_{50} values were separated by a variation factor V_f of 3.9. Fluxapyroxad and boscalid failed to inhibit *C. fioriniae*, with mean EC_{50} values >100 μg/ml.

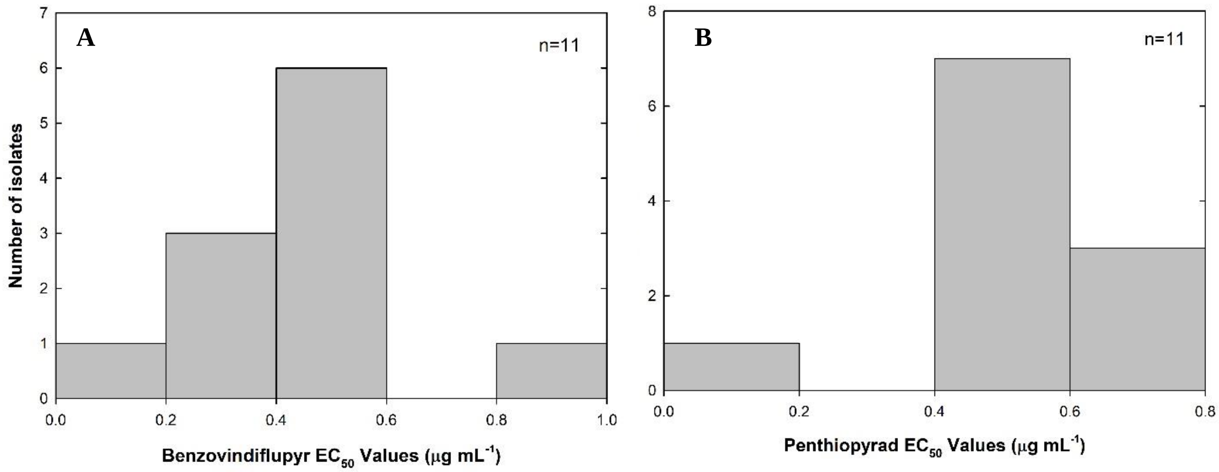

Figure 2.4 Distribution of effective concentration at which mycelial growth was inhibited by 50% (EC_{50}) values for baseline isolates of *Colletotrichum fioriniae* for **A,** Benzovindiflupyr (n=11) and **B,** Penthiopyrad (n=11). The baseline population was collected from apple orchards in Georgia that have never been exposed to single-site fungicides.

A t-test was used to compare the distribution of benzovindiflupyr and penthiopyrad EC_{50} values for *C. fructicola* and *C. fioriniae*. Significant differences were found between the *Colletotrichum* species for benzovindiflupyr ($P = 0.0002$) and penthiopyrad ($P < 0.0001$; figure 2.5).

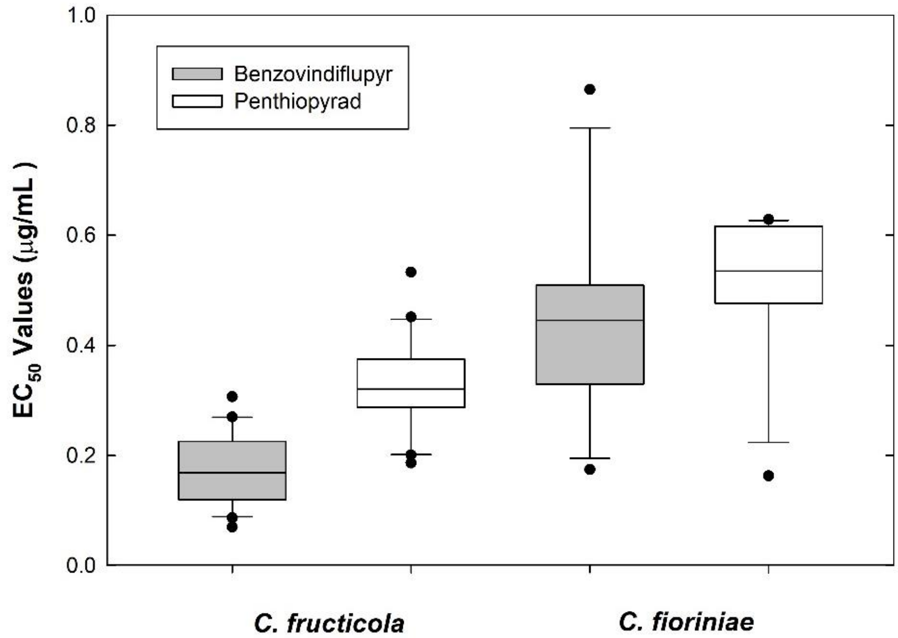

Figure 2.5 Boxplot of EC_{50} values of *C. fructicola* and *C. fioriniae* with benzovindiflupyr and penthiopyrad. Every dot represents an outlier value.

Evaluation of mycelial growth sensitivity in populations of *C. fructicola* and *C. fioriniae* exposed to benzovindiflupyr and penthiopyrad

The percentage of orchard populations with reduced sensitivity to SDHIs depended on the species of *Colletotrichum* and the fungicide. Four out of 6 orchards (66.7%) with populations of *C. fructicola* demonstrated reduced sensitivity to benzovindiflupyr while populations of *C. fioriniae* with reduced sensitivity to this fungicide were found in only 1 out of 4 (25%) orchards. Populations of *C. fructicola* from commercial orchards were all sensitive to penthiopyrad, but 4 of 4 (100%) of *C. fioriniae* populations had reduced sensitivity. The mean percent relative growth for each population (orchard) was obtained. For *C. fructicola*, the means of percent relative growth for benzovindiflupyr ranged from 40.7 ± 1.7% (Orchard W) to 50.5 ± 1.4% (Orchard H) and from 42.8 ± 1.3% (Orchard N) to 52.9 ± 1.6% (Orchard I) for penthiopyrad (Table 2.5). For *C. fioriniae*, the range of mean relative growth values observed for benzovindiflupyr ranged from 47.5 ± 1.0% (Orchard D) to 60.2 ± 3.3% (Orchard F), while the mean percent relative growth values for penthiopyrad ranged from 71.6 ± 1.4% (Orchard G) to 83.4 ± 3.0% (Orchard E) (Table 2.6).

Table 2.5 Mean mycelial percent relative growth of *Colletotrichum fructicola* populations and K-S one sample test values to determine sensitivity to benzovindiflupyr and penthiopyrad.

Orchard Designation	Benzovindiflupyr			Penthiopyrad		
	Mean % RG[a]	**Pr>Ksa[b]**	**Phenotype**	**Mean % RG[a]**	**Pr>Ksa[b]**	**Phenotype**
W	40.7 ± 1.7	0.9335	Sensitive	46.6 ± 2.0	0.7666	Sensitive
N	46.0 ± 2.1	0.0189[c]	Reduced sensitivity	42.8 ± 1.3	0.0215[c]	Sensitive
H	50.5 ± 1.4	0.0001[c]	Reduced sensitivity	51.6 ± 1.7	0.8814	Sensitive
I	47.4 ± 1.9	0.0113[c]	Reduced sensitivity	52.9 ± 1.6	0.1709	Sensitive
P	41.4 ± 0.6	0.1545	Sensitive	44.5 ± 0.6	0.0063[c]	Sensitive
O	45.8 ± 0.9	0.0005[c]	Reduced sensitivity	49.8 ± 0.9	0.2607	Sensitive

[a] Mean of the percentage of relative growth (%RG) in the Orchard. Mean relative growth of the orchard population of *Colletotrichum fructicola* on medium amended with EC_{50} value of benzovindiflupyr (0.169 µg/ml) and penthiopyrad (0.330 µg/ml) with that non-fungicide medium. Values are means and standard errors of *n* isolates for each population.

[b] The distribution of benzovindiflupyr and penthiopyrad sensitivity response for *Colletotrichum fructicola* population was determined using the K-S one-sample test (EC_{50}) in SAS (version 9.4, Cary, NC).

[c] Orchard population differed from the baseline Orchard M

Table 2.6 Mean mycelial percent relative growth of *Colletotrichum fioriniae* populations and K-S one sample test values to determine sensitivity to benzovindiflupyr and penthiopyrad.

Orchard Designation	Benzovindiflupyr			Penthiopyrad		
	Mean % RG[a]	**Pr>Ksa[b]**	**Phenotype**	**Mean % RG[a]**	**Pr>Ksa[b]**	**Phenotype**
B	51.7 ± 2.2	0.7302	Sensitive	71.8 ± 1.8	0.0072[c]	Reduced sensitivity
D	47.5 ± 1.0	0.3775	Sensitive	72.6 ± 0.9	<.0001[c]	Reduced sensitivity
F	60.2 ± 3.3	0.0290[c]	Reduced sensitivity	73.4 ± 3.5	0.0034[c]	Reduced sensitivity
G	49.7 ± 1.4	0.7990	Sensitive	71.6 ± 1.4	0.0126[c]	Reduced sensitivity

[a] Mean of the percentage of relative growth (%RG) in the Orchard. Mean relative growth of the orchard population of *Colletotrichum fioriniae* on medium amended with EC_{50} value of benzovindiflupyr (0.442 µg/ml) and penthiopyrad (0.509 µg/ml) with that non-fungicide medium. Values are means and standard errors of *n* isolates for each population.
[b] The distribution of benzovindiflupyr and penthiopyrad sensitivity response for *Colletotrichum fructicola* population was determined using the K-S one-sample test (EC_{50}) in SAS (version 9.4, Cary, NC).
[c] Orchard population differed from the baseline Orchard M

The percentage relative growth (%RG) of *Colletotrichum fructicola* on benzovindiflupyr and penthiopyrad was moderately correlated ($P < 0.001$, $r = 0.55$, n = 187) (Fig.2.6A). A similar result was obtained with *Colletotrichum fioriniae* ($P < 0.001$, $r = 0.503$, n = 104), where the sample correlation was significantly positive although weak (Fig 2.6B).

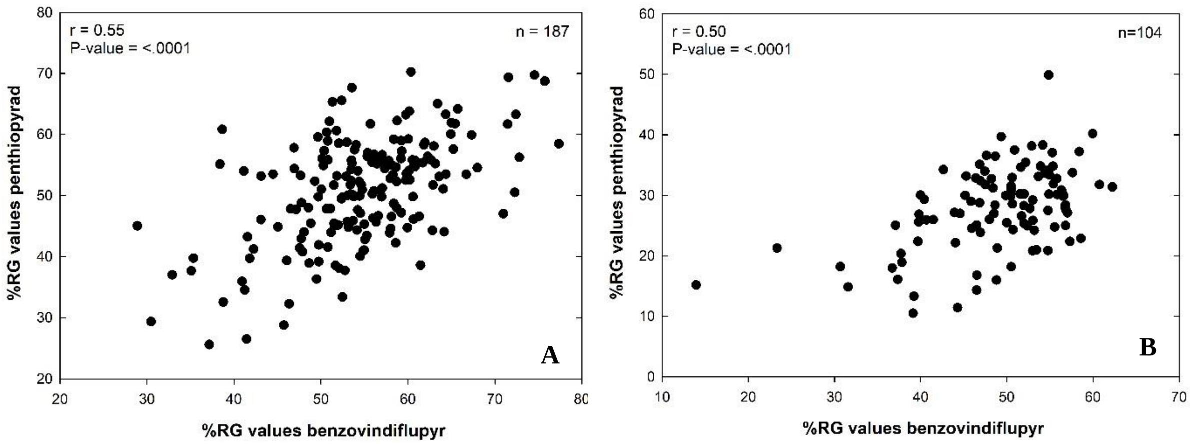

Figure 2.6 Correlation between percentage relative growth (%RG) of mycelium on media amended with benzovindiflupyr and penthiopyrad for **A,** *Colletotrichum fructicola* with 187 isolates and **B,** *Colletotrichum fioriniae* with 104 isolates. The sample correlation coefficient (*r*) was calculated for each *Colletotrichum* spp.

Identification and characterization of *sdhB, sdhC, and sdhD* genes of *C. fructicola*

The *CfsdhB, CfsdhC, and CfsdhD* genes were cloned from six baseline isolates in order to identify regions of previously characterized mutations that may confer future resistance to SDHI fungicides. Amplification of the *CfsdhB, CfsdhC, and CfsdhD* with primers targeting gDNA revealed consensus sequences of 1247 bp, 1070 bp, and 816 bp, respectively. For *CfsdhB*, the amplification of cDNA revealed an 831 bp fragment. Alignment of cDNA and gDNA of the *C. fructicola sdhB* gene revealed a 92 bp intron, and a 53 bp intron at nucleotide positions 360 and 604, respectively. Including the introns, the total length of the *CfsdhB* gene was 976 bp. The translated protein of CfSDHB shared 92.8% identity with *Colletotrichum graminicola*, 84.8% identity with *Verticillium dahliae,* and 77.5% identity with *Penicillium expansum* SDHB proteins. Residues commonly associated with reduced sensitivity or resistance to SDHI fungicides in other phytopathogens were noted at positions P201F/L/T, N206I, and H248Y/R/L. None of these mutations was identified in the sequenced baseline isolates. For *CfsdhC*, amplification of cDNA revealed a 567 bp fragment. Alignment of cDNA and gDNA of the *C. fructicola sdhC* gene revealed a 79 bp intron at nucleotide position 41 and a 131 bp intron at position 203. Including introns, the total length of the *CfsdhC* gene was 778 bp. The translated protein of CfSDHC shared 91.0% identity with *C. graminicola* and 63.4% identity with *V. dahliae.* A residue commonly associated with resistance to SDHI fungicides in other phytopathogens was noted at amino acid position 145 in which a histidine residue is replaced with arginine. This mutation was not identified in the sequenced baseline isolates.

Lastly, for *CfsdhD*, amplification of cDNA revealed a 513 bp fragment. Alignment of cDNA and gDNA of the *C. fructicola sdhD* gene revealed a 64 bp intron at nucleotide position 143 and a 65 bp intron at nucleotide position 225. Including introns, the total length of the *CfsdhD* gene was 642 bp. The translated protein of CfSDHD shared 65.3% identity with *C. graminicola*, 59.3% identity with *V. dahliae,* and 47.4% with *A. alternata.* Residues commonly associated with resistance to SDHI fungicides in other phytopathogens were noted at amino acid position 100 in which an aspartic acid residue is replaced with glutamic acid and at position 110 in which histidine is commonly replaced by arginine. These mutations were not identified in the sequenced baseline isolates.

Evaluation of putative point mutations of sdhB in isolates of *C. fructicola* with reduced SDHI sensitivity

Amplification of gDNA of 10 isolates with different phenotypes (sensitive and reduced sensitive) of benzovindiflupyr and penthiopyrad using cDNA primers to flanked *sdhB* were performed. The *sdhB* gen of these isolates was analyzed to identify single-nucleotide polymorphisms at position 200, 205, and 247. None of these mutations was found in the sequenced isolates selected.

DISCUSSION

A better understanding of single-site fungicide performance and the development of fungicide resistance is essential for the management of Glomerella leaf Spot and bitter rot, two devastating diseases of apple in the southeastern US. SDHI fungicides registered for apple include benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid. The development of baseline sensitivities and identifying putative molecular mechanisms of resistance to these fungicides is essential for improved comprehension of the risk of these fungicides and future resistance monitoring studies in *Colletotrichum fructicola* and *C. fioriniae*. During this study, baseline populations of *C. fructicola* and *C. fioriniae* were evaluated for their sensitivity to four SDHI fungicides. EC_{50} values revealed that both species were highly sensitive to both benzovindiflupyr and penthiopyrad, but not fluxapyroxad or boscalid. Linear correlation analyses suggested a moderate level of cross-sensitivity between the benzovindiflupyr and penthiopyrad. Finally, the characterization of *sdhB, sdhC, and sdhD* was completed for *C. fructicola,* and analysis of putative point mutations was conducted in isolates of *C.fructicola* with different SDHI-sensitivity phenotypes.

Baseline sensitivities showed a unimodal distribution for both *Colletotrichum* spp. for both benzovindiflupyr and penthiopyrad. Benzovindiflupyr and penthiopyrad had efficacy against mycelial growth of both *Colletotrichum* spp. For both species, benzovindiflupyr demonstrated greater intrinsic activity against mycelial growth compared to penthiopyrad.

C. fioriniae showed a broader range in sensitivity to SDHIs in comparison with *C. fructicola*. This difference could be because isolates of *C. fioriniae* were collected from a larger geographical area (NC and GA) than *C. fructicola* (NC). A previous study reported that the low range of EC_{50} could have been influenced by a small collection geographical area (Thomas et al. 2012; Villani et al. 2015). Another factor that could influence the range of the EC_{50} values would be the standard number of applications typically used in a specific production area. The number of applications for single-site fungicides increases selection for resistance in some pathosystems such as *Botrytis cinerea* and *Venturia inaequalis* (Bardas et al. 2010; Frederick et al. 2014; Samuel et al. 2011). In addition, the range of the baseline sensitivities for *C. fructicola* and *C. fioriniae* was narrow for both SDHI fungicides as evidenced by low V_f values. A smaller range in the sensitivity of isolates in a baseline population to penthiopyrad was found in *Didymella bryoniae* and some species of *Colletotrichum* spp. (except *C. orbiculare*) compared to both *Colletotrichum* spp. studied in our experiment (Ishii et al. 2016; Thomas et al. 2012). The range in EC_{50} values for benzovindiflupyr for both *Colletotrichum* spp. was similar to baseline sensitivity found in previous studies with *Bipolaris maydis* and other species of *Colletotrichum* spp. (Hou et al. 2018; Ishii et al. 2016). In our study, the range in the population's sensitivity population provided a V_f for *C. fructicola* of 4.4 and for *C. fioriniae* of 5.0 with benzovindiflupyr. For penthiopyrad, a V_f of 2.9 for *C. fructicola* and V_f of 3.9 for *C. fioriniae* was calculated. This information gives us a better understanding of the resistance development process in *Colletotrichum* spp. for these fungicides. Having a small V_f could indicate that resistance could evolve more slowly in this species or population in comparison with pathogens with high V_f (Villani et al. 2015, 2016).

The level of sensitivity to SDHI fungicides observed for *C. fructicola* and *C. fioriniae* coincides with a previous report where some species in the *C. acutatum* complex infecting apple (*C. fioriniae* and *C. nymphaeae)* were less sensitive to several fungicides evaluated *in vitro* (thiophanate-methyl, myclobutanil, and captan) compared with species of *C. gloeosporioides* complex species (*C. siamense* and *C. fructicola*) that infect apple at the mycelium level (Munir et al. 2016). Conversely, evaluation of sensitivity to trifloxystrobin and pyraclostrobin with many of the same isolates utilized in this study found that orchard populations of *C. fioriniae* more sensitive to the QoI fungicides compared to populations of *C. fructicola* (Johnson 2019). To the extent of our knowledge, this is the first characterization of the baseline sensitivity of *C. fructicola* and *C. fioriniae* for these fungicides.

Different levels of cross-resistance to other active ingredients with members of the same SDHI class were reported depending on the fungal pathogen (Ishii et al. 2011; Sierotzki & Scalliet, 2013; Stammler et al. 2015; Veloukas et al. 2013). In our study, a correlation indicating possible cross-sensitivity between benzovindiflupyr and penthiopyrad in both *C. fructicola* (r = 0.55) *and C fioriniae* (*r* = 0.50) was identified. The variety of phenotypes in each isolate may explain the weak correlation between these fungicides. Four different phenotypes were observed among the isolates tested: 1) sensitive to benzovindiflupyr and penthiopyrad, 2) reduced sensitivity to benzovindiflupyr, and sensitive to penthiopyrad 3) sensitive to benzovindiflupyr and reduced sensitivity to penthiopyrad and 4) reduced sensitivity to benzovindiflupyr and penthiopyrad. In a similar study in *Venturia inaequalis*, a high level of correlation between benzovindiflupyr and penthiopyrad was reported (Villani et al. 2016). The greater intrinsic activity of benzovindiflupyr in comparison with penthiopyrad has been reported in previous studies with other *Colletotrichum* spp. (*C. gloesporoides*, *C. acutatum*, *C. cereal*, and *C. orbiculare*) (Ishii et al. 2016).

SDHI fungicides have a unique mode and site of action (Complex II). Due to their features, the absence of cross-resistance has previously been reported with other chemical classes (QoIs, benzimidazoles, or anilinopyririmidines (Amiri et al. 2010; Avenot et al. 2008; Stammler et al. 2007). It was expected that a moderate to a high degree of correlation between benzovindiflupyr and penthiopyrad would be observed; however, it was unexpected that fluxapyroxad did not show high efficacy in both *Colletotrichum* spp. since all three active ingredients are members of the same SDHI chemical class (pyrazole-4-carboxamides) (FRAC, 2019). In contrast with our study, a strong cross-resistance for boscalid and fluxapyroxad and penthiopyrad was reported in *Botrytis cinerea* on strawberry (Amiri et al. 2014).

Fluxapyroxad showed poor activity against the *Colletotrichum* spp. in our study (>100 µg/ml). The high efficacy of benzovindiflupyr and the low efficacy of fluxapyroxad obtained in our study coincides with a previous field trial where fluxapyroxad showed lower efficacy than benzovindiflupyr for the control of Glomerella leaf spot and bitter rot was reported in NC (Johnson 2019). It is important to report that EC_{50} values obtained from the baseline sensitivity in *C. fructicola* for fluxapyroxad showed a surprisingly high variation with a range from 17.3 µg/ml to 579.6µg/ml. The high variation of *C. fructicola* in response to fluxapyroxad should be studied to understand its causes.

Differences observed in the inhibitory effects among different SDHI fungicides is likely due to different levels of intrinsic activity observed in the binding site of the complex II enzyme

(Avenot & Michailides 2010; Scalliet et al. 2012). In our study, benzovindiflupyr and penthiopyrad showed higher activity against *Colletotrichum* spp compared to fluxapyroxad and boscalid. It may indicate that their affinity to the target binding site is different even though they have the same mode of action. Variation in non-conserved regions could trigger structural conformation changes in the binding site (SDHB, -C, and –D), affecting the affinity and efficacy of some SDHI fungicides against different pathogens (Cecchini 2003; Sierotzki et al. 2010).

There are several reports about differences in the effectiveness of SDHIs at different fungal growth stages. Boscalid strongly inhibits conidial germination, germ tube development, appressoria formation, and mycelial growth (Stammler et al. 2007; Stammler & Speakman 2006). Furthermore, several studies reported that this molecule has a stronger effect on spore germination in comparison with mycelium in some species such as *Monilia fructicola*, *Alternaria alternata*, and *Botrytis cinerea* (Amiri et al. 2010; Myresiotis et al. 2008; Zhang et al. 2007). Despite the observation that benzovindiflupyr demonstrated strong activity against conidial germination and mycelial growth for *Bipolaris maydis*, the EC_{50} values obtained were only slightly different at 0.5060 µg/ml and 0.3446 µg/ml respectively (Hou et al. 2018). *Venturia inaequalis*, was more sensitive to benzovindiflupyr in conidial germination (EC_{50} = 0.0016) in comparison with mycelium growth (EC_{50}=0.043 µg/ml) (Villani et al. 2016). This situation could give us an idea that the measured baseline EC_{50} value for some SDHI fungicides depends on species and the fungal stage evaluated. Therefore, further studies have to be conducted to evaluate the performance of these active ingredients against conidial germination and germ tube development, a fungal stage that requires a high level of energy (Allen 1965; Taubitz et al. 2007).

Modern molecular studies in fungicide resistance have helped to clarify the mechanism of development of resistance of plant pathogens in the SDHI fungicide class. Nucleotide mutations in *sdhB, sdhC,* and *sdhD* have been reported to confer different levels of insensitivity to SDHI fungicides (Avenot & Michailides 2010; Sierotzki & Scalliet 2013; Walker et al. 2010). Additional studies in *Mycosphaerella graminicola* reported 27 amino acid substitutions occurring in the three mentioned subunits, which indicate that resistance in this fungicide class may be conditioned by several mutations across the subunits (Scalliet et al. 2012). Therefore analyzing these SDH genes is needed to gain a better understanding of cross-resistance in the SDHI group (Sierotzki & Scalliet 2013). In our study, the *sdhB*, *sdhC* and *sdhD* gene were identified and characterized for isolates of *C. fructicola* obtained from the baseline population. Among these genes, the most common mutation reported in several phytopathogens is found in the subunit SDHB (Sierotzki & Scalliet 2013; Walker et al. 2010). \For the identification of potential point mutation in *sdhB,* different isolates that presented different phenotypes (sensitive and reduced sensitive described above) were chosen among the orchards. The target site mutation, single nucleotide polymorphisms (SNPs), in the *sdhB* gene is going to depend on the fungal species due to the variability of this gene. In general, the most frequent target site mutation points reported so far are H/Y, L or R at 257, 267, 272, 277 or P225L/T/F (De Miccolis Angelini et al. 2010; Sierotzki & Scalliet 2013; Skinner et al. 1998). According to the evaluation in the *sdhB* putative gene, some tentative point mutations were identified as P200, N205, H247. No point mutations in the *sdhB* of *C. fructicola* were found in any of the isolates sequenced. Similar results were reported for *Colletotrichum* spp. isolates insensitive to boscalid (Ishii et al. 2016). Additional mutations in subunits C and D were reported from some plant pathogen isolates (Stammler et al. 2015). Therefore, further analysis in *sdhC* and *sdhD* should be performed to have a whole panorama of point mutations in the subunits that affect SDHI sensitivity in *C. fructicola.*

Overall, benzovindiflupyr and penthiopyrad inhibit mycelium of *C. fructicola* and *C. fioriniae*, showing a mean EC_{50} value below 1.0 µg/ml. These results could suggest that application programs that include these active ingredients could provide control following infection by the pathogen. Constant monitoring of pathogen populations should be conducted in the future to track their sensitivity over time. The correlation found between benzovindiflupyr and penthiopyrad raises awareness about the importance of rotation strategies with SDHI fungicides during the apple growing season. Identification and characterization of *sdhB*, *sdhC*, and *sdhD* will contribute to a better understanding the mechanism of *C. fructicola* for resistance development to this fungicide class. No mutation point was found in the *sdhB.* Therefore, further studies investigating the *sdhC* and *sdhD* genes would help us to understand resistance development better. The understanding of the effect in the phenotypic and molecular levels is helpful in developing management strategies to decrease the risk of fungicides.

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CHAPTER III:

Evaluation of in vitro efficacy of Succinate Dehydrogenase Inhibitor (SDHI) fungicides against the boxwood blight pathogen, *Calonectria pseudonaviculata*

ABSTRACT

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Boxwood blight, caused by the fungal pathogen *Calonectria pseudonaviculata* (*Cps*), has rapidly emerged as a detrimental plant disease in the ornamental industry since it was reported in the United States in 2012. One of the strategies to manage this fungal disease is the use of preventive fungicides. The recent registration of novel single-site fungicides, such as the succinate dehydrogenase inhibitors (SDHIs) for the control of boxwood blight, requires an assessment of sensitivity and propensity for cross-resistance in the pathogen. Thirty-four isolates of *Cps* were classified into two groups based on the area sampled, with 23 isolates from North Carolina (NC), and 11 isolates from outside NC (US states and countries). The effective fungicide concentration that inhibits mycelial growth by 50% relative to the control (EC_{50}) was calculated for all isolates. The EC_{50} mean values for mycelial growth of NC *Cps* isolates of benzovindiflupyr, boscalid, fluxapyroxad, and penthiopyrad were 0.67, 19.41, 8.89, and 0.58 µg/ml. Within this group three significant positive correlations for EC_{50} values were found for benzovindiflupyr and fluxapyroxad ($P = 0.028$, $r = 0.46$), benzovindiflupyr and penthiopyrad ($P < 0.0042$, $r = 0.57$), and penthiopyrad and fluxapyroxad ($P < 0.0001$, $r = 0.78$). The results of this study demonstrate the efficacy of SDHI fungicides in reducing mycelial growth of these fungicides and provide information on fungicide cross-sensitivity.

INTRODUCTION

Boxwood *(Buxus* spp.*)* has been cultivated since its use as clipped hedges in an Egyptian garden dating in ~4,000 B.C (Niemiera 2012). The genus *Buxus* has 91 species dispersed in four continents: 10 species in Africa, 45 in America, 34 in Asia, and 2 in Europe (Batdorf 2004). Boxwood is susceptible to attack by several pathogens, among them *Meloidogyne incognita* (Kofoid & White) Chitwood, twig and leaf blight caused by *Pseudonectria buxi* (DC.) Seifert, Phytophthora root rot caused by *Phytophthora parasitica* Dastur, and rust caused by *Puccinia buxi* Sowerby (Strouts & Winter 2000; Blake et al. 2020). However, a recently emerging fungal pathogen, *Calonectria pseudonaviculata* (Crous, J.Z., Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous is causing damage to boxwoods worldwide.

C. pseudonaviculata (*Cp*s), was first reported in the United States (US) in 2012 and was first isolated and identified from containerized and field-grown American and English boxwood (*Buxus sempervirens* and *Buxus sempervirens* 'Suffruticosa') in nurseries in Connecticut, North Carolina and Oregon (Ivors et al. 2012; Iriarte et al. 2016; Daughtrey 2019). To date, the pathogen has been identified in more than 28 states in the US and three Canadian provinces (Sakalidis et al. 2019; Yang & Hong 2017).

Symptoms of boxwood blight first appear on lower canopy leaves and as stem lesions. Foliar symptoms include dark brown lesions with a yellow halo that enlarge over time. As the infection progresses, infected leaves become chlorotic to brown in color. Severe leaf infection results in rapid defoliation reducing the photosynthetic area, which ultimately may cause death of the plant via repeated defoliation events that reduce plant health. Infected stems present symptoms as dark purple to black colored lesions (Henricot & Culham 2002; Ivors et al. 2012).

Since its introduction into the US, boxwood blight management has required an integrated strategy including sanitation, cultural management, planting of less susceptible species and cultivars, and fungicide applications. Indeed, incorporating more tolerant species such as *B. microphylla* var. japonica ‘Green Beauty’, and *B. sinica* var. insularis ‘Nana’ into landscapes has lessened the impact of boxwood blight in newly established plantings (Daughtrey 2019; Ganci 2014). Historical plantings of highly susceptible boxwoods including *Buxus sempervirens* ‘Suffruticosa’ and *B. sempervirens* have greater susceptibility to the pathogen (Douglas 2011).

The application of fungicides is used to manage and mitigate boxwood blight (Douglas 2011). However, there are limitations to this approach because fungicides are not able to eradicate the disease once plants have been infected by the fungus. Currently, in North Carolina, the multisite protectant fungicide, chlorothalonil, is applied on a 7 to 14-day interval between the months of April and October to suppress boxwood blight. Seasonal applications and the protective mode of action of this fungicide limit its utility for stand-alone management of boxwood blight (Bush et al. 2016). Fungicides for managing boxwood blight remains relatively limited to a few FRAC groups, including chloronitriles, dithiocarbamates, methyl benzimidazole carbamates, Quinone outside Inhibitors (Qol), Demethylation Inhibitors (DMI), and Succinate Dehydrogenase Inhibitors (SDHI) (Daughtrey 2019; La Mondia 2019; Palmer & Shishkoff 2014). In addition to boscalid, which is formulated with pyraclostrobin for boxwood blight, a new generation of SDHI fungicides, fluxapyroxad, and benzovindiflupyr, have recently been registered for disease management in ornamental crops. These fungicides, formulated in premixtures with QoI fungicides, may provide an efficacious alternative mode of action for management of boxwood blight.

SDHI fungicides represent a diverse assemblage of active ingredients divided into 11 chemical groups (FRAC 2019; Sierotzki & Scalliet 2013). These fungicides interfere with energy production in fungi by disrupting the electron transport chain, ultimately reducing target organism development (Bartlett et al. 2002; Ruy et al. 2006; Semar et al. 2014). These compounds trigger a rapid decrease of cellular adenosine triphosphate (ATP) (Glattli et al. 2010; Oliver & Hewitt, 2014; Scallieti et al. 2010; Semar et al. 2014; Stammler et al. 2015). The target site of this group of fungicides is succinate dehydrogenase (SDH), a complex protein, more commonly known as succinate: ubiquinone oxidoreductase (SQR) or complex II (Amiri et al. 2010; Hägerhäll 1997; Kuhn 1984). This SDH enzyme is ubiquitous in the mitochondrial membrane of aerobic microorganisms. SDHI fungicides inhibit activity of the succinate dehydrogenase enzyme by blocking complex II, which is a vital component involved in the electron transport chain by overlapping in the ubiquinone-binding site (Qp site). The SDHI fungicides have a broad spectrum and affect germination of asexual spores (conidia) and hyphal growth and extension (Avenot & Michailides 2010; Veloukas & Karaoglanidis 2012; Oliver & Hewitt 2014).

Given the limited information about the efficacy of SDHI fungicides against *Cps*, an improved understanding of the response of this pathogen to SDHI fungicides can contribute to deployment of these fungicides for managing boxwood blight disease. The objective of this study was to determine in vitro efficacy of *Cps* to four SDHI fungicides. We hypothesize that there would be different inherent efficacy and cross-sensitivity among the SDHI fungicides tested in the isolates of *Cps*. Therefore cross-resistance would be more prevalent in those SDHI that share the same chemical class.

MATERIALS AND METHODS

Isolates utilized in this study.

Thirty-four isolates of *C. pseudonaviculata* that were sampled from symptomatic boxwood leaves and stems from within and outside NC were evaluated (Table 3.1). The NC sample consisted in part of nine isolates obtained during 2017 and 2018 from diseased leaves and stems of English boxwood *Buxus sempervirens* 'Suffruticosa' obtained from four counties (Mitchell, Yancey, Buncombe and Avery Co).

Two different isolation methods were followed, depending on the type of plant tissue. Leaves with visible lesions were placed in a moist chamber for 2 to 3 days at room temperature (~25˚C) under constant fluorescent light to encourage sporulation. Sporulation was then observed under the dissecting scope. Single conidia observed on infected leaf lesions using a dissecting microscope (100X) were picked with a sterile dissecting needle. Conidia were transferred to a 9-cm diameter plastic Petri plate containing potato dextrose agar (PDA) amended with 50 µg/ml streptomycin and 50 µg/ml chloramphenicol (PDA ++) (Sigma-Aldrich, St Louis, MO). Mycelium was transferred to a new plate of PDA after 1 or 2 days to maintain purity and avoid contamination. Stem lesions were surface sterilized in 0.6% NaOCl solution and rinsed with sterile distilled water. Small pieces of symptomatic stem tissue were placed equidistantly on PDA++, and after 2 or 3 days actively growing mycelium was transferred to PDA to obtain a pure culture. Monoconidial cultures were obtained by culturing isolates on PDA and maintained at 25°C at constant fluorescent light and sealed with parafilm. Once mycelium covered the 9-cm diameter petri dish, from 8 to 10 mL of sterile distilled H_2O was added, and plates were allowed to stand for 2 to 3 h. Mycelium was then scraped with a plastic rod cell spreader, and the water was discarded. Petri dishes were rinsed with sterile distilled water to remove any remnant mycelium debris and were stored upside-down at 25°C (Dart et al. 2015; Marine et al. 2018). Sporulation was observed after 4 or 5 days. Fifteen milliliters of sterile distilled water were added to the Petri dish, and conidia were dislodged with a plastic-rod cell spreader. Conidia were collected in a sterile 50 ml Falcon tube. Fifteen µl of the suspension was evenly spread onto PDA++. Under a dissecting microscope, conidial germination was observed in the medium after 24 h. and an approximately a 5-mm agar plug containing one germinating conidium was cut with a scalpel and placed in a new plate of PDA to obtain a monoconidial culture.

In addition, the NC sample contained 14 isolates sampled from diseased boxwood leaves and stems collected between 2015 and 2018, were kindly provided by Dr. Marc Cubeta (Table 3.1). Also, 11 monoconidial cultures originating from diseased boxwood samples collected between 2001 and 2016 in CT, NY, France, Germany, Iran, New Zealand, Slovenia, Turkey, and the United Kingdom and obtained from Dr. JoAnne Crouch were included in this study.

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Table 3.1 List of *Calonectria pseudonaviculata* isolates from diseased boxwood in the study.

Isolate	Location[a]	Species	Source	Variety	Year
BXB 17A -1	Mitchell	*B. sempervirens*	Leaf	English (Suffruticosa)	2017
BXB 17B -1	Mitchell	*B. sempervirens*	Leaf	English (Suffruticosa)	2017
BXB 17E -2	Yancey	*B. sempervirens*	Leaf	English (Suffruticosa)	2017
BXB 17D -1	Yancey	*B. sempervirens*	Steam	English (Suffruticosa)	2017
BXB 17E -1	Yancey	*B. sempervirens*	Leaf	English (Suffruticosa)	2017
BXB 18B - 24L	Buncombe	*B. sempervirens*	Leaf	English (Suffruticosa)	2018
BXB 18B - 25L	Buncombe	*B. sempervirens*	Leaf	English (Suffruticosa)	2018
BXB 18C - 8S	Buncombe	*B. sempervirens*	Steam	English (Suffruticosa)	2018
BXB 18F -1	Avery	*B. sempervirens*	Leaf	English (Suffruticosa)	2018
BD2-55T	Surry	*B. sempervirens*	Leaf	English (Suffruticosa)	2015
BB22C-2-55T-1	Surry	*B. sempervirens*	Leaf	Varder Valley'	2017
BD7C-55	Surry	*B. sempervirens*	Leaf	Dee Runk	2015
BB21C-55T	Surry	*B. sinica var. insularis*	Leaf	Nana	2016
30405GV	Surry	*B. sempervirens ‘Suffruticosa’* × *B. sinica var. insularis*	Leaf	Green Velvet	2017
BB22-55I	Surry	*B. sempervirens*	Leaf	Justin Brouwers	2016
BBGG-1-55T	Surry	*B. sempervirens ‘Suffruticosa’* × *B. sinica var. insularis*	Steam	Green Velvet	2017
30405GM	Surry	*B. sempervirens ‘Suffruticosa’* × *B. sinica var. insularis*	Leaf	Green Mound	2017
BD7C-55 -2	Surry	*B. sempervirens*	Leaf	Dee Runk	2015

[a] NC county or country

Table 3.1 (continued).

Isolate	Location[a]	Species	Source	Variety	Year
BB20C - 55I	Surry	*B. microphylla var. japonica*	Leaf	NewGen Independence	2017
BB10b-1-55t-1	Surry	*B. microphylla*	Stem	John Baldwin	2015
BB22-55I	Surry	*B. harlandii*	Leaf	Richard	2016
BXB TM7	Surry	*B. sempervirens*	Leaf	Justin Brouwers	2016
JAC 17-36	USA: NC	*Buxus* spp.	Unknown	Unknown	2016
JAC 13-218	France	*B. sempervirens*	Unknown	Unknown	2006
JAC 13-200	Germany	*Buxus* spp.	Unknown	Unknown	2005
JAC 13-237	Iran	*B. sempervirens*	Unknown	Unknown	2013
JAC 13-182	New Zealand	*B. sempervirens*	Unknown	Unknown	2002
JAC 13-127	Slovenia	*B. sempervirens*	Unknown	Unknown	2011
JAC 13-183	Turkey	*B. sempervirens*	Unknown	Unknown	2012
JAC 13-129	Turkey	*B. sempervirens*	Unknown	Unknown	2012
CBS 114417	United Kingdom	*B. sempervirens*	Unknown	Unknown	2001
JAC 14-15	United Kingdom	*B. sempervirens*	Unknown	Unknown	2007
JAC 13-09	USA: CT	*Buxus* spp.	Unknown	Unknown	2011
JAC 14-108	USA: NY	*Buxus* spp.	Unknown	Unknown	2013

[a] NC county or country

Fungicides experiments.

Commercial formulations of four succinate dehydrogenase inhibitors (SDHIs) fungicides were tested in this study to determine the sensitivity of *C. pseudonaviculata* mycelial growth (Amiri et al. 2010; Hou et al. 2018; Villani et al. 2016). These included benzovindiflupyr (Aprovia, 10% a.i., EC, Syngenta), penthiopyrad (Fontelis, 20.4% a.i, SC, Corteva), fluxapyroxad (Sercadis, 26.55% EC, BASF), and boscalid (Endura, 70% WDG, BASF)

Fungicide stock solutions were prepared and stored at 4°C. Fungicides were added to autoclaved (20 minutes at 156 Kpa) potato dextrose agar (PDA) medium after the medium reached approximately 50˚C. Final concentrations of active ingredient included in the amended medium for benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid were 0.001, 0.01, 0.1, 1.0, and 10.0 µg a.i./ml. The control consisted of PDA without fungicide.

To evaluate mycelial growth sensitivity, the in vitro efficacy of the four SDHI fungicides were tested with each of the 34 isolates of *Cps* simultaneously. The amended media with the SDHI fungicides was prepared not greater than 24 h from the initiation of the experiment. In our study, two plastic 9-cm diameter Petri dishes were used as replicates with two observations (two mycelial plugs) per replicate for each fungicide at each concentration. Therefore, a total of four 7-mm mycelium plugs were used per fungicide concentration and isolate. The four 7-mm diameter mycelium plugs were taken from the active growth of 7-day-old cultures on PDA. The mycelium plugs were placed upside-down (mycelium in contact with the amended media) equidistant from each other on PDA. Each plate was sealed with parafilm and incubated at room temperature (25˚C) under constant fluorescent light and radial mycelial growth was measured between 7 to 9 days of incubation. The diameter of each colony was measured in two directions per concentration of each fungicide with a 0 to 150 mm electronic digital caliper (Westward, Lake Forest, Illinois). Each isolate was tested once during the experiment.

Data analysis

For each isolate from NC (n = 23) and outside of NC (n = 11), the effective concentration that inhibited isolate growth by 50% (EC_{50}) values was calculated in Microsoft Excel (Redmond, WA) using relative percent inhibition by comparing the mycelial growth in the fungicide amended medium at each isolate and fungicide concentration with the non-fungicide amended control (Yoshimura et al. 2004). Percent inhibition data were subjected to a log-transformation (log_{10}) prior to analysis and use to develop a dose-response curve in Excel program (Villani et al. 2016). A variation factor (V_f) for each fungicide was determined using the following equation (isolate with the highest EC_{50} value/ isolate with the lowest EC_{50} value). Pearson correlation analysis was performed with the PROC CORR procedure in SAS (Statistical Analysis System version 9.4) for the NC isolates group by analyzing the EC_{50} to determine the level of cross-resistance between the SDHI fungicides tested.

RESULTS

Sensitivity of *C. pseudonaviculata* to benzovindiflupyr, penthiopyrad, fluxapyroxad, and boscalid

All fungicides tested inhibited mycelial growth of *Cps* compared to the unamended control treatment. *Cps* exhibited the greatest sensitivity to penthiopyrad and benzovindiflupyr, followed by fluxapyroxad and boscalid (Figure 3.1).

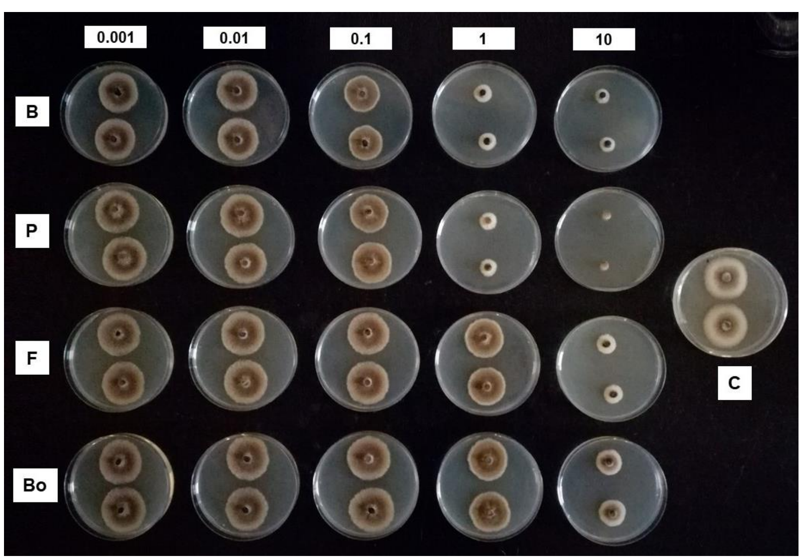

Figure 3.1 Mycelial growth of *C. pseudonaviculata* on PDA medium amended with benzovindiflupyr (B), penthiopyrad (P), fluxapyroxad (F) or, boscalid (Bo) at concentrations of 0.001, 0.01, 0.1, 1, 10 µg/ml compared to the non-fungicide amended control (C)

EC_{50} values were calculated for each isolate with the four SDHI mentioned previously. For benzovindiflupyr EC_{50} values ranged from 0.27 to 1.93 µg/ml, for penthiopyrad EC_{50} values ranged from 0.37 to 1.08 µg/ml, for fluxapyroxad EC_{50} values, the range obtained was from 3.63 to 42.42 µg/ml, and boscalid EC_{50} values ranged from 4.59 to 55.36 µg/ml (Table 3.2).

Table 3.2 EC_{50} values of *Calonectria pseudonaviculata* isolates from diseased boxwood in the study.

Isolate	Benzovindiflupyr (µg/ml)	Penthiopyrad (µg/ml)	Fluxapyroxad (µg/ml)	Boscalid (µg/ml)
BXB TM7	0.563	0.477	7.744	16.553
BXB 17A -1	0.548	0.514	6.783	8.397
BXB 17E -2	0.591	0.456	6.168	30.537
BXB 17D -1	0.803	0.544	10.809	30.490
BXB 17B -1	0.644	0.511	9.070	22.868
BXB 17E -1	1.045	0.713	9.171	11.169
BXB 18B - 24L	0.460	0.406	5.047	5.805
BXB 18B - 25L	0.395	0.374	3.626	4.587
BXB 18C - 8S	0.540	0.580	5.687	6.615
BXB 18F -1	0.503	0.480	6.123	9.264
BD2-55T	0.268	0.450	7.044	13.631
BB22C-2-55T-1	1.235	0.525	9.349	18.297
BD7C-55	0.361	0.629	11.568	17.532
BB21C-55T	0.814	0.795	9.041	32.273
30405GV	0.664	0.552	7.950	55.355
BB22-55I	0.916	0.805	10.225	6.636
BBGG-1-55T	0.693	0.624	11.450	14.782
30405GM	0.532	0.528	6.703	14.487
BD7C-55	0.625	0.560	7.335	28.127
BB20C - 55I	0.580	0.587	8.928	12.531
BB10b-1-55t-1	0.775	0.580	11.091	18.153
BB22-55I	0.894	0.567	7.243	40.333
JAC 17-36	0.974	0.957	26.301	28.012
JAC 13-218	0.609	0.622	25.856	26.112
JAC 13-200	0.780	1.030	12.840	17.104
JAC 13-237	0.926	0.974	42.416	21.193
JAC 13-182	0.852	0.589	14.337	7.477
JAC 13-127	1.931	1.072	15.709	30.089

Table 3.2 (Continued)

Isolate	Benzovindiflupyr (μg/ml)	Penthiopyrad (μg/ml)	Fluxapyroxad (μg/ml)	Boscalid (μg/ml)
JAC 13-183	1.005	0.992	4.509	21.831
JAC 13-129	0.952	1.078	6.814	15.475
CBS 114417	1.021	0.885	41.026	34.919
JAC 14-15	1.035	0.835	6.265	10.169
JAC 13-09	1.072	0.723	3.764	7.201
JAC 14-108	1.166	0.911	16.645	15.163

Isolates from North Carolina

EC_{50} values for benzovindiflupyr ranged from 0.27 to 1.24 μg/ml with a mean EC_{50} of 0.67 ± 0.23 μg/ml and a V_f of 4.6. Fifty-seven percent of the isolates (13 of 23) had EC_{50} values between 0.40 to 0.80 μg/ml (Figure 3.2A). For penthiopyrad, EC_{50} values ranged from 0.37 to 0.96 μg/ml with a mean EC_{50} = 0.58 ± 0.14 μg/ml with a V_f of 5.0. Seventy percent of the isolates (16 of 23) had EC_{50} values were between 0.40 to 0.60 μg/ml (Figure 3.2B). Mean EC_{50} values for fluxapyroxad and boscalid were higher than benzovindiflupyr and penthiopyrad. For fluxapyroxad, EC_{50} values ranged from 3.63 to 26.30 μg/ml, with a mean EC_{50} = 8.89 ± 4.35 μg/ml, and a V_f of 7.3. Forty-three percent of the isolates (10 of 23) had EC_{50} values ranged from 7.00 to 10.00 μg/ml (Figure 3.2C). Mean EC_{50} values for boscalid were 19.41 ± 12.56 μg/ml with a V_f of 12.1 and ranged from 4.59 to 55.35 μg/ml (Figure 3.2D).

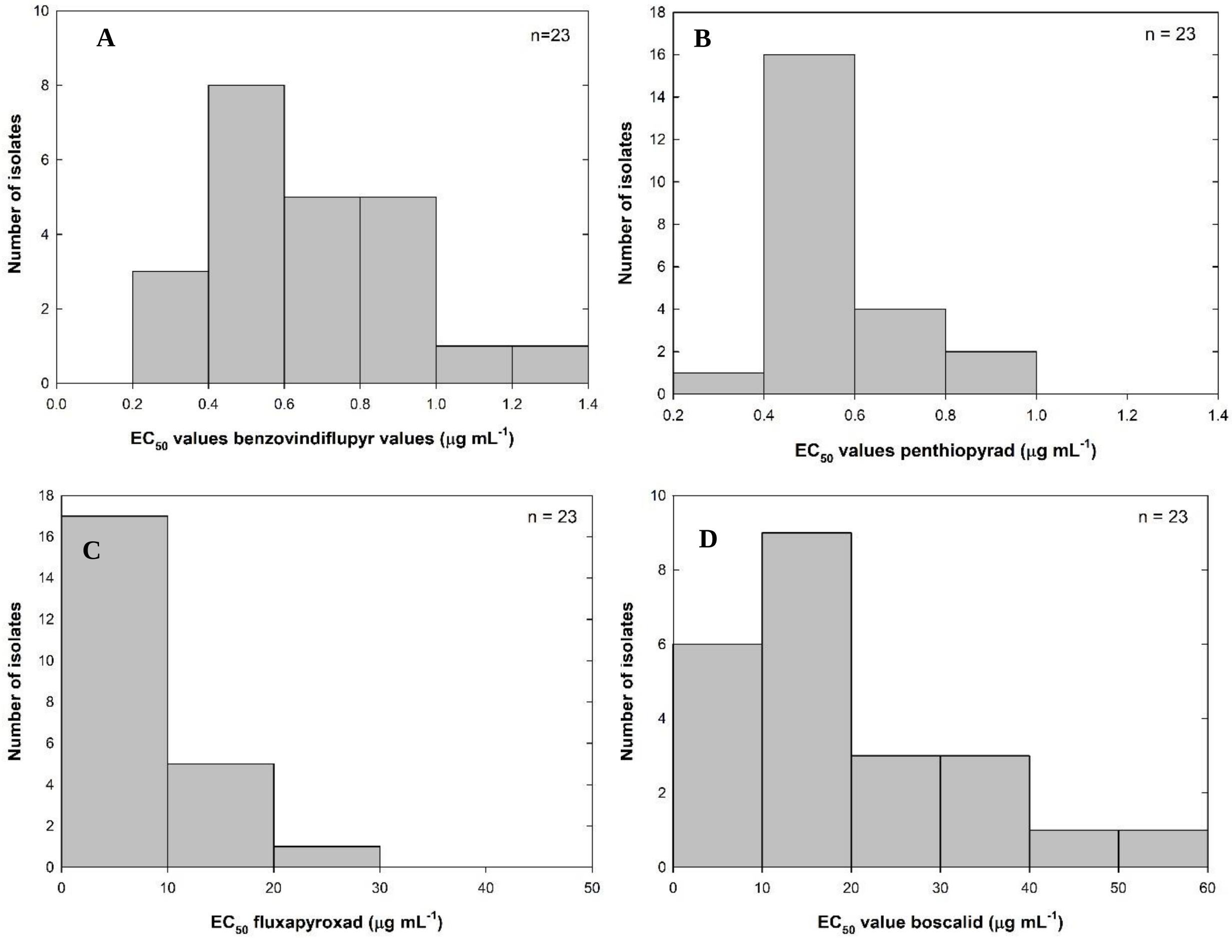

Figure 3.2 Distribution of effective concentration that inhibited radial mycelial growth by 50% (EC_{50}) for isolates of *C. pseudonaviculata* collected from boxwood in North Carolina for **A,** benzovindiflupyr (n = 23), **B,** penthiopyrad (n = 23), **C,** fluxapyroxad (n = 23), and **D,** Boscalid (n = 23).

Isolates collected outside of North Carolina

EC_{50} values for benzovindiflupyr ranged from 0.61 to 1.93 µg/ml and a V_f of 3.2. Approximately 72% of the isolates (8 of 11) tested had EC_{50} values between 0.80 to 1.20 µg/ml (Figure 3.3A). For penthiopyrad, a range from 0.59 to 1.08 µg/ml and a V_f of 1.8. Seventy-two percent of the isolates (8 of 11) had EC_{50} values between 0.80 to 1.20 µg/ml (Figure 3.3B). The EC_{50} values for fluxapyroxad ranged from 3.76 to 42.42 µg/ml and a V_f of 11.3. Sixty-four percent of the isolates (7 of 11) tested with fluxapyroxad showed values over 10 µg/ml (Figure

3.3C). Boscalid EC_{50} values ranged from 7.20 to 34.92 µg/ml with a V_f of 4.8. Forty-five percent of the isolates (5 of 11) had EC_{50} values of > 20 µg/ml (Figure 3.3D).

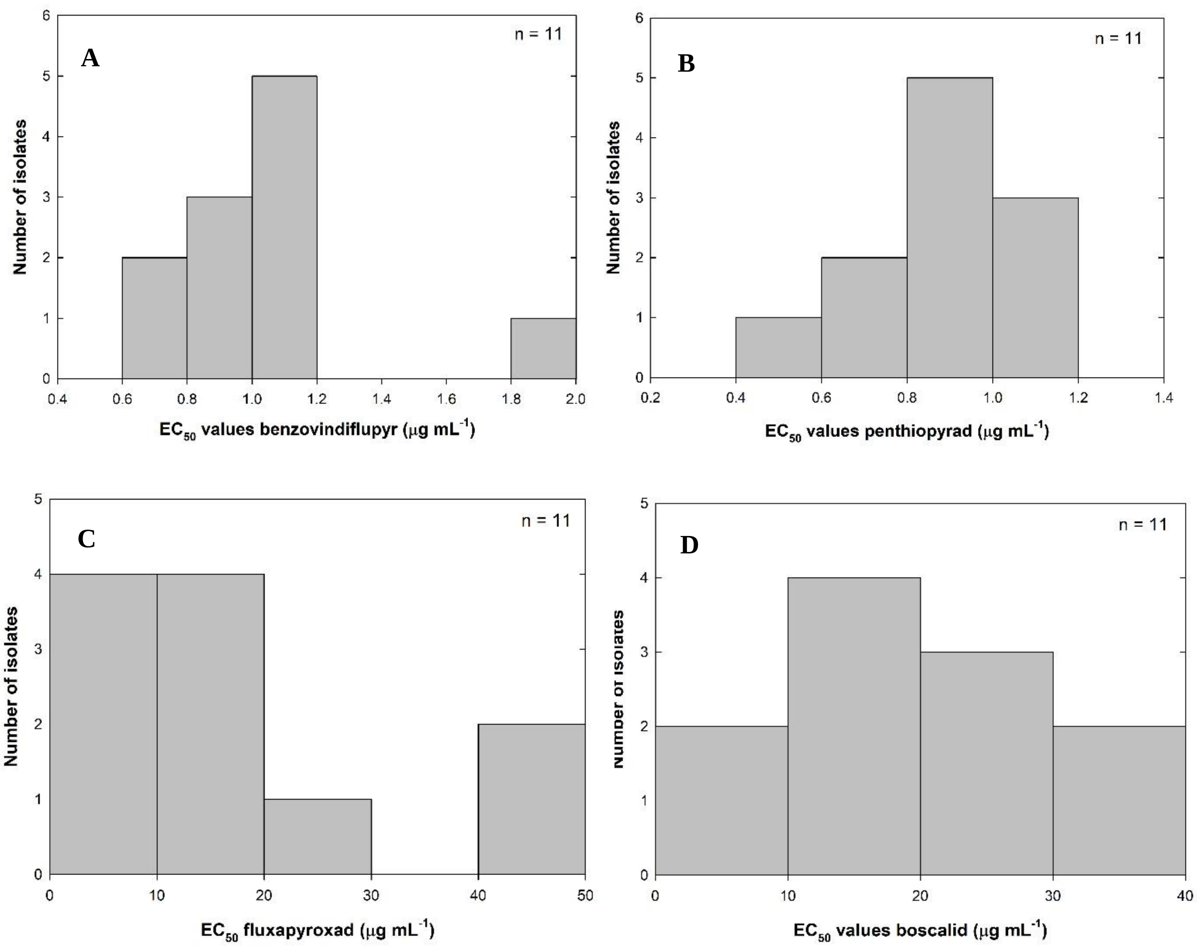

Figure 3.3 Distribution of effective concentration that inhibited radial mycelial growth by 50% (EC_{50}) for isolates of *C. pseudonaviculata* sampled from boxwood outside of North Carolina for **A,** benzovindiflupyr (n = 11), **B,** penthiopyrad (n = 11), **C,** fluxapyroxad (n = 11), and **D,** boscalid (n = 11).

Positive correlations were found in three pairs of fungicide EC_{50} values for the isolates sampled from North Carolina as follows: benzovindiflupyr and fluxapyroxad ($P = 0.028$, $r = 0.46$), benzovindiflupyr and penthiopyrad ($P = 0.0042$, $r = 0.57$) and penthiopyrad and fluxapyroxad ($P < 0.0001$, $r = 0.78$) (Figure 3.4). EC_{50} values for boscalid were not correlated with those of other fungicides.

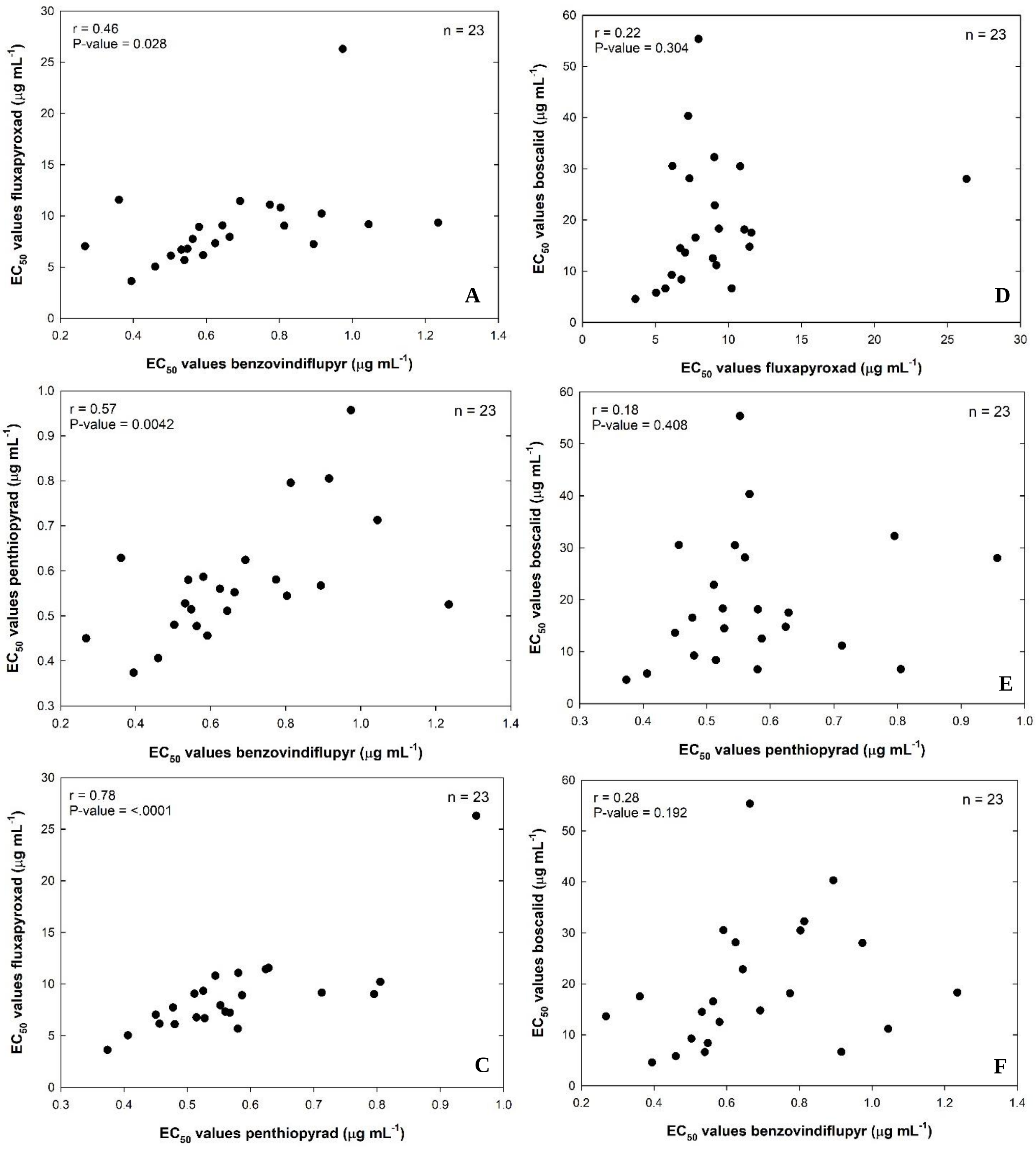

Figure 3.4 Correlation between effective concentration at which mycelial growth was inhibited by 50% (EC_{50}) values of isolates of *Calonectria pseudonaviculata* from North Carolina between **A,** benzovindiflupyr and penthiopyrad, **B,** benzovindiflupyr and fluxapyroxad, **C,** penthiopyrad and fluxapyroxad, **D,** fluxapyroxad and boscalid, **E,** penthiopyrad and boscalid, and **F,** benzovindiflupyr and boscalid. The sample correlation coefficient (*r*) was calculated for each isolate that was collected from the boxwood plant, presenting typical symptoms of boxwood blight.

DISCUSSION

The registration of new single-site fungicides, such as the SDHIs, provides additional chemical alternatives for managing plant diseases of economically important crops. SDHI fungicides are becoming an essential tool to manage several plant pathogens due to their broad-spectrum activity and absence of cross-resistance to other fungicides groups (Avenot & Michailides 2010; Stammler et al. 2007; Yanase et al. 2007). However, a significant limitation is that this fungicide class has a medium to high risk of selection for resistance (FRAC 2019). Thus, for a better understanding of these fungicides, evaluation of *Cps* isolates to SDHI fungicides is an essential initial approach.

Among the SDHI fungicides evaluated, all had an inhibitory effect on the mycelial growth of *Cps*. The highest efficacy was found with penthiopyrad and benzovindiflupyr for all the isolates tested. In our study, a high level of mycelial growth inhibition was found with benzovindiflupyr. This fungicide also is highly active against *Bipolaris maydis, Didymella bryoniae*, and *Venturia inaequalis* (Hou et al. 2018; Thomas et al. 2012; Villani et al. 2016b). In addition, previous studies found a high level of inherent efficacy of this fungicide against several *Colletotrichum* spp. (Ishii et al. 2016). As with benzovindiflupyr, penthiopyrad strongly inhibited mycelial growth of the *Cps* isolates tested. In contrast to our results, penthiopyrad did not inhibit mycelial growth of all *Colletotrichum* species evaluated despite their relatively high levels of sensitivity to benzovindiflupyr (Ishii et al. 2016). Compared to benzovindiflupyr and penthiopyrad, fluxapyroxad and boscalid were less effective inhibitors against of mycelial growth in all *Cps* isolates tested. These results coincide with Palmer and Shishkoff (2014), who found an EC_{50} value of 6.07 µg/ml in a mycelial inhibition assay of boscalid plus pyraclostrobin.

Despite benzovindiflupyr, penthiopyrad, and fluxapyroxad belonging to the same SDHI chemical group (FRAC 2019), these fungicides showed a different efficacy for inhibiting mycelial growth of *Cps*. Modern molecular tools have helped to clarify the mechanism of development of resistance of plant pathogens in this fungicide class. Mutations in *sdhB, sdhC,* and *sdhD* have been characterized that confer different levels of insensitivity to SDHI fungicides. Among these mutations, the most commonly reported is found in the subunit SDHB. Also, the presence of mutations in SDHC and SDHD is variable across species. An explanation for the different levels in the intrinsic activity in the SDHI fungicide class may be due to the diversity of amino acid residues in the binding site (SDHB, SDHC, and SDHD) (Avenot & Michailides 2010; Sierotzki & Scalliet 2013; Walker et al. 2010). Furthermore, this could explain why fungal populations exhibit partial or incomplete cross-resistance to different SDHIs (Avenot et al. 2014; Sierotzki & Scalliet 2013). In other studies, cross-resistance between boscalid and fluopyram was not observed for *Corynespora cassicola* and *Podosphaera xanthii* (Ishii et al. 2011). Conversely, a positive cross-resistance between boscalid, penthiopyrad, and fluopyram was reported in *Alternaria alternata* (Avenot & Michailides 2010).Therefore, further study in the characterization and the structural composition of the SDH genes in *Calonectria pseudonaviculata* is needed to test the hypothesis that these factors provide different binding strength among SDHI fungicides.

The efficacy of fungicides against conidial germination and mycelium growth varies depending on the fungicide class. For example, quinone-outside inhibitors (Qol) fungicides are commonly characterized by their low efficacy against mycelium growth, but high efficacy against conidial germination, whereas DMI fungicides are highly inhibitory to mycelium growth (Henricot et al. 2008; Palmer & Shishkoff 2014). The inhibitory effects of benzovindiflupyr and penthiopyrad on mycelium growth of *Cps* suggests they have potential curative activity against early infection. Curative activity was reported for benzovindiflupyr and fluxapyroxad, where effect against *Cps* was observed within 48 hours after infection (La Mondia 2019). Evaluation of inhibition at conidial germination of these SDHI fungicides is needed to give a complete assessment of their performance.

In other phytopathogens, cross-resistance between several SDHI active ingredients and chemical classes have been observed. However, the degree of cross-resistance has been associated with the target pathogen and particular SDHI fungicide (Ishii et al. 2011; Sierotzki & Scalliet 2013; Stammler et al. 2015; Veloukas et al. 2013). In our study, a correlation analysis was conducted to study the potential risk of fungicide resistance development. Positive correlations between benzovindiflupyr and fluxapyroxad, benzovindiflupyr and penthiopyrad, and penthiopyrad and fluxapyroxad suggest that selection of resistance for one of these fungicides could trigger an indirect selection for the second fungicide. These results are similar to a previous study where it was found that isolates resistant to fluxapyroxad were resistant to boscalid in *Botrytis cinerea* (Amiri et al. 2014). These correlations attributed to their common target and chemical class, pyrazole-4-carboxamides (FRAC 2019). Boscalid did not show a correlation with any of the SDHI fungicides tested in the NC *Cps* isolates. This result differs from a baseline sensitivity study where a positive correlation between penthiopyrad and boscalid was found ($P < 0.0001$, r = 0.75) in *Didymella bryoniae* (Thomas et al. 2012). Also, a significant positive sensitivity-resistance correlation was found ($P < 0.0001$, r = 0.53) between boscalid and penthiopyrad in *Alternaria alternata* (Avenot et al. 2014). A high correlation between boscalid and fluxapyroxad and penthiopyrad was reported in *Botrytis cinerea* on strawberry (Amiri et al. 2014). This behavior suggests partial resistance in SDHI and shows that cross-resistance in this class of fungicide depends on the fungal pathogen. Due to the correlation between some SDH fungicides, the application of these fungicides has to be limited during the season to avoid resistance development risk of *Calonetria pseudonaviculata*.

In conclusion, benzovindiflupyr and penthiopyrad showed the highest level of mycelial growth inhibition of *Cps*. The potential curative effect of these fungicides could be an important tool to be integrated into the application programs for the management of boxwood blight in rotation or combination with other fungicides classes such as DMIs and Qols. Finally, the correlation found provides us with important information for rotating application programs in the boxwood industry.

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CHAPTER IV:

Evaluation of host plant defense inducers for the management of boxwood blight (caused by *Calonectria pseudonaviculata*) and fire blight (caused by *Erwinia amylovora*)

ABSTRACT

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Host plant defense inducers, molecules that inhibit or slow pathogen development by enhancing the defense system of plants, are promising tools for integrated management of plant disease. An improved understanding of application strategies and the molecular mode of action of defense elicitors in fruit and ornamental pathosystems is imperative to their utilization for disease control. Application strategies for managing boxwood blight in boxwood and fire blight in apple were evaluated for two consecutive years. Acibenzolar-S-methyl (formulated as Actigard) and *Bacillus mycoides* isolate J (formulated as Lifegard) were studied in boxwood and apple, and the plant-derived extract of *Reynoutria sachalinensis* (formulated as Regalia) was evaluated in apple. The relative expression of genes encoding pathogenesis-related proteins PR-1, PR-2, and PR-8 was analyzed for leaf and flower tissue of apple. For boxwood, different application methods and timings for Actigard and Lifegard were evaluated in two different systems, container and field production. Drench applications of Actigard showed significantly less disease incidence than the untreated control plants ($P < 0.001$) under container production. High efficacy of Actigard was also observed under field production despite highly favorable conditions for development of boxwood blight. The frequency of application did not reduce disease with either of the host plant defense inducers tested in both production systems. In apple, application of host plant defense inducers in rotation with streptomycin at different bloom stages was conducted. The incidence of blossom blight was significantly reduced compared with the

untreated program with all host plant defense inducers. PR gene expression increased ~1.6 fold for PR-2 and ~1.5 fold for PR-8 genes in apple leaves one day following application with Actigard and Regalia. After seven days, an increase of ~2 fold for PR-2 and ~1.3 for PR-8 was observed with both programs as well. Also, an increase of 1.7 fold at one day and 2.0 fold at seven days following application was observed with Lifegard. PR-2 gene expression in apple blossoms increased, 1.9 fold with Actigard at one day following application. After seven days, an increase of 1.5 fold for PR-8 was observed with Lifegard.

INTRODUCTION

The use of synthetic chemicals has been an essential component for managing plant diseases for over 200 years (Brent & Hollomon 2007). Historically, protective and curative applications of multi-and single-target site fungicides and antibiotics have been the cornerstone for managing bacterial and fungal diseases of perennial specialty crops. However, because of their specific mode of action and intense use, they are prone to resistance development in fungal and bacterial plant pathogen populations. This resistance in these plant pathogens raises concerns in the agricultural community due to excessive fungicide applications in cropping systems (Fernández-Ortuño et al. 2008; FRAC 2019; Jones 1981; Sierotzki & Scalliet 2013). Several multi-site bactericides and fungicides have come under scrutiny in recent years because of their potential adverse effects on non-target organisms and the environment (McManus 2014; Sundin & Bender 1996). Furthermore, the lack of synthetic bactericides and fungicides with fully systemic activities makes therapeutic management of systemic pathogens particularly challenging (Aćimović et al. 2015). Therefore, efficient alternatives or complementary methods for managing bacterial and fungal plant pathogens are essential (Chandler et al. 2011).

Plants can protect themselves against abiotic and biotic stresses by different mechanisms (Raskin 1992). A well-known mechanism involved in this protection is systemic acquired resistance (SAR), which consists of an inducible defense within the plant with long-lasting protection in distant pathogen-free plant tissue from diverse pathogens (Ádám et al. 2018). SAR is triggered in plants by beneficial microorganisms, earlier localized exposure to a pathogen, pathogen-derived elicitors (carbohydrates, glycopeptides, glycoproteins, lipids), and synthetic chemicals (Choi & Hwang 2011; Gozzo 2003; Raskin 1992; Ryals et al. 1994; Ryals et al. 1996; Walters et al. 2005).

Early studies to better define and understand the mechanism(s) of host plant defense inducers began in the 1960s when an enhanced level of resistance in tobacco leaves was observed in response to the inoculation of tobacco mosaic virus (TMV). This process was named system acquired resistance (SAR) (Israel & Ross 1967; Ross 1961). Later, the involvement of salicylic acid (SA) in the host resistance response was identified (Israel & Ross 1967; White 1979). The activation of SAR triggers several signaling cascade reactions inside plant cells and has been reported in several species including *Arabidopsis thaliana* (L.) (Dempsey et al. 1993), *Cucumis Sativus* L. (Dalisay & Kuc 1995), *Capsicum annuum* L. (Lee & Hwang 2005), and *Solanum lycopersicum* L. (Christ & Mösinger 1989).

For activation of pathogenesis-related (PR) genes, several essential chemical reactions happen including reduction of the cotranslational activator Non-Expresser of Pathogenesis Related gene 1 (NPR1) from an inactive state (oligomeric) to an active state (monomeric). NPR1 is a positive regulator of SAR, which is activated by SA with a mechanism that is not entirely understood. This process will lead to nuclear localization of NPR1 that allows inducing the expression of PR genes via transcription factors (Garcion & Métraux 2006; Kinkema et al. 2000;

Mou et al. 2003). Most genes induced by SA are related to members of the PR gene family. Among this group, PR-1 is a significant marker for SA and SAR evaluation (Garcion & Métraux 2006; Van Loon & Van Strien 1999). As a consequence of all these reactions, PR gene expression in infected plant tissue increases, developing long-lasting and broad-spectrum protection against phytopathogens (Vlot et al. 2009).

Studies of PR gene expression have been conducted in several plant pathosystems. The expression of PR genes varies among plant species. In tobacco, expression of PR genes (PR-1. PR-2, PR-3, and PR-5) were analyzed by infecting tobacco with the tobacco mosaic virus (TMV) (Brederode et al. 1991). A study in tomato reported a correlation in the resistance of this plant against *Peronospora hyoscyami* f.sp *tabacina* Skalický and *Phytophthora parasitica* var *nicotianae* (Breda de Haan) Tucker with high expression of PR-1 (Alexander et al. 1993). In pepper (*Capsicum annum* L.), significant levels of expression of PR-1 and PR-10 were reported after leaf inoculation with avirulent *Xanthomonas campestris* (Lee & Hwang 2005). Recently studies by Ramezani et al. (2017) demonstrated high PR gene expression (i.e., thaumatin-like (TLP) and ribosome-inactivating proteins (RIP), and defensin genes) triggered by potassium phosphate against *Pseudoperonospora cubensis* in cucumber (*Cucumis sativus* L.).

Host plant defense inducers provide broader options for integrated pest management (IPM), with the objective of combining several control approaches to minimize damage by pathogenic microbes and insects (Campbell & Arthur 2007; Vetek et al. 2009). Host plant defense inducers are formulated and marketed primarily for managing plant diseases in several cropping systems and can activate local and systemic resistance in the distal part of the plant tissue due to mobile plant signaling molecules involved in systemic acquired resistance response (Gozzo & Faoro 2013). However, a high level of plant disease suppression is not always reached under field conditions compared to more controlled conditions such as in greenhouses (Walters & Fountaine 2009). An important question in the use of these products is the duration of the induced defense response. Also, how do application methods (e.g. targeted plant tissue) influence product performance and response under different diseases or insect pressure? Therefore, an understanding of application timing in relation to plant growth and mechanism(s) are essential for managing plant pathogens under field conditions.

An evaluation of soil application of Actigard (acibenzolar-S-methyl) at different rates and frequencies found a reduction in the foliar citrus canker incidence caused by *Xanthomonas citri* subsp. *citri* (Graham & Myers 2011). The frequency and rate of application of acibenzolar-S-methyl influenced management of bacterial spot caused by *Xanthomonas* spp. in tomato by reducing disease on a weekly rather than a bi-weekly schedule (Huang et al. 2012). In apple, reduction in the incidence of fire blight, caused by *Erwinia amylovora*, was reported by foliar application and trunk injection of Actigard (Aćimović et al. 2015; Thomson et al. 1998; Zeller & Zeller 1999).

Several formulated host plant defense inducers, including Actigard, Lifegard (*Bacillus mycoides* Isolate J), and Regalia (plant-derived extract of *Reynoutria sachalinensis*) are currently registered and commercially available for use on some vegetables and fruits crops in the US. Acibenzolar-S-methyl acts as an analog of SA, triggering accumulation of PR-proteins, such as PR-1 (anti-Oomycete activity), PR-2 (β-1,3-glucanase) and PR-8 (class III chitinase) (Aćimović et al. 2015; Friedrich et al. 1996; Görlach et al. 1996; Maxson-Stein et al. 2002; Metraux et al. 1991). *Bacillus mycoides* (Isolate J), a gram-positive saprobic soil associated bacterium, induces an oxidative burst and peroxide production in sugar beet and was found to suppress the disease causing activity of *Cercospora beticola* Sacc. 38-91% (Bargabus 2003). *Bacillus mycoides*

(Isolate J) also reduced the incidence and severity of cucumber anthracnose caused by *Glomerella cingulata* var. *orbiculare* (Neher et al. 2008). In addition, the biological plant activator Lifegard reduced downy mildew in a highly susceptible grape cultivar (Wilcox 2017). Another important host plant defense inducer, the plant-derived extract of *Reynoutria sachalinensis*, acts as a promoter of anthraquinone elicitor (FRAC 2019). The plant-derived extract of *Reynoutria sachalinensis* reduces the disease of powdery mildew fungi (*Sphaerotheca fuliginea*) by activating phytoalexins, reducing pathogen growth and development (Daayf et al. 1997). An increase in PR-2 gene expression was reported in courgette, a variety of summer squash (*Cucurbita pepo* var. *cylindrical*), when the tissue was treated with Regalia (Margaritopoulou et al. 2020).

In this study, we evaluated host plant defense inducers for managing boxwood blight caused by the fungus *Calonectria pseudonaviculata* and fire blight disease of apple caused by the bacterium *Erwinia amylovora*. *C. pseudonaviculata* is primarily a leaf pathogen that results in repeated defoliation and a reduction in boxwood health, while *E. amylovora* infects flowers, leaves, woody tissue, and fruits of apple and other species in the Rosacea. This study aimed to assess different application strategies with two host plant defense inducers (Actigard and Lifegard) in boxwood for managing boxwood blight in container and field production. In apple, our objective was to evaluate different rotation applications with a host plant defense inducer (Actigard, Lifegard, and Regalia) and streptomycin. In addition, the expression of PR genes (PR-1, PR-2, and PR-8) was evaluated in leaf and blossom tissue after treatment. We hypothesize that different application strategies of host plant defense inducers would provide different levels in the reduction of the infection of boxwood blight. In apple, we hypothesize that host plant defense inducer applications would reduce the incidence of fireblight compared to no treatment and would enhance the level of PR expression in leaf and blossom tissue.

MATERIALS AND METHODS

Evaluation of plant defense inducers for the management of boxwood blight

Site selection

Experiments were conducted at two geographic locations in 2018 and 2019 to evaluate boxwood blight management for both container and field production. Two experiments were conducted at the Mountain Horticultural Crops Research and Extension Center in Mills River, NC (container production experiment), and four experiments at a boxwood production field in Surry County, NC, with a history of boxwood blight disease (field production experiment).

Container production experiments, artificial inoculation

One and a half-year-old boxwood plants (cv. 'Justin Brouwers' *Buxus sempervirens*) were obtained from Saunders Brother Nursery (Piney River, VA) and used for each container experiment. Boxwood plants grown in 11.4 liter containers were acquired in July of 2017 and 2018. These plants were immediately transplanted to an 18.9 liter container with pine bark potting media (Sungro, Agawam, MA) with the addition of Micromax and lime. The plants were placed on a gravel container pad approximately 35-cm from each other underneath shade cloth. Boxwood plants were fertilized by adding 18-5-12 fertilizer plus 5 to 6 month Osmocote to each container one month prior to the initiation of the experiment.

Application rates, methods, and frequency for the host plant defense inducers utilized in this study are listed in Table 4.1. For foliar applications, fungicides were applied to runoff (i.e. until the fungicide solution began to drip off of the leaves) using a 15-liter hand pump backpack sprayer. Approximately 150 ml of the fungicide was deposited onto each plant at a pressure of

275 kPa. For drench applications, 1 liter of the fungicide solution was distributed evenly onto the pine bark medium. No application was made in the controls throughout the experiment.

Application dates for the first, second, and third applications were 13 July (seven days prior to inoculation), 27 July (seven days post-inoculation), and 10 August (21 days post-inoculation) in 2018, and 12 July, 26 July, and 09 August in 2019. Treatments were arranged in a randomized complete block design consisting of four single-plant replications per treatment.

Table 4.1 Treatments evaluated for the evaluation of host plant defense inducers for the management of boxwood blight in container production.

Treatment	Trade name	Active ingredient	Appl. method	Freq. of appl.[a]	Dose
1	Untreated	-	-	-	-
2	Actigard	acibenzolar-S-methyl	Drench	1	0.6 g/liter
3	Lifegard	*Bacillus mycoides* isolate J	Drench	1	3.4 g/liter
4	Actigard	acibenzolar-S-methyl	Drench	3	0.6 g/liter
5	Lifegard	*Bacillus mycoides* isolate J	Drench	3	3.4 g/liter
6	Actigard	acibenzolar-S-methyl	Foliar	1	0.04 g/liter
7	Lifegard	*Bacillus mycoides* isolate J	Foliar	1	0.34 g/liter
8	Actigard	acibenzolar-S-methyl	Foliar	3	0.04 g/liter
9	Lifegard	*Bacillus mycoides* isolate J	Foliar	3	0.34 g/liter

[a]1 app **=** Only one application before inoculation; 3 app = one application before inoculation and two applications after inoculation.

Inoculum preparation

The in vitro conidia concentration from isolates of *Calonectria pseudonaviculata* collected previously in North Carolina was assessed to select isolates for the experiment (data not shown). Three isolates collected in 2017 from different counties in NC from *B. semipervirens* (English boxwood) were selected, 17A-1 (Mitchell Co), 17D-1 (Yancey Co), and 17E-1 (Yancey Co). A previous inoculation of these isolates on *Buxus sempervirens* cv. ‘Justin Brouwers’ under container production conditions was performed to confirm pathogenicity before the experiment. To produce inoculum for experiments in each year, conidia were produced using the protocols of

Dart et al. (2015) and Marine et al. (2018) by culturing each isolate on potato dextrose agar (PDA) incubated at 25°C under constant fluorescent light. Once the mycelium covered the entire surface of the PDA, the Petri dish was flooded with 8 to 10 ml of sterile water for 2 h. The mycelium was gently dislodged from the agar surface with a plastic-rod cell spreader, and the water was discarded. Petri dishes were rinsed to discard remnant mycelium. Plates were stored at 25°C and placed upside-down under constant fluorescent light. After four to five days of incubation, conidia were observed with a microscope. A total of 10 ml of sterile water was added to each Petri dish, and conidia were liberated using a sterile plastic-rod cell spreader and sterile distilled water.

The conidia-water suspension was decanted in a 1 liter glass bottle and then combined with 5 liters of water for application. Conidia from all three isolates were combined in equal volumes (1:1:1 v/v). The concentration of conidia was quantified using a hemocytometer. A conidial suspension (1.0 x 10^4 conidia/ml) was applied to each plant with a 15-liter hand pump sprayer onto the top surface of the leaves until runoff. Inoculation was conducted on 30 July 2018 and 19 July 2019. The inoculation was performed seven days after the first application of a host plant defense inducer. Boxwoods were covered by a plastic sheet (Plastic Drop Cloth, Poly-America, TX) for 24 h after inoculation. Once the plastic sheet was removed, overhead irrigation was applied to encourage disease development. Boxwoods were watered for 1.5 hours, three times per day (4.5 hours/day) throughout the entirety of the experiment (Ganci 2014; Ivors et al. 2012). The second and third applications of the host plant defense inducer were conducted 7 and 21 days after inoculation.

Disease assessment

The incidence of boxwood blight disease was assessed on 20 August in 2018 (10 days after 3rd application) and 21 August in 2019 (12 days after 3rd application). For the 2018 experiment, disease incidence was determined by evaluating the number of symptomatic leaves with boxwood blight symptoms from 10 leaves per shoot and expressed as a percentage. A total of 10 shoots was evaluated per treatment replicate. Unfavorable weather conditions for the development of boxwood blight disease in 2019 resulted in little disease development on inoculated boxwood plants. However, necrotic stem lesions developed in all of the untreated plants. Therefore, the severity of the stem lesions was considered for evaluating the performance of host plant defense inducers in 2019. The severity of stem lesion was calculated based on the percentage of area in a standardized stem length (8 cm). A total of 10 stems were evaluated per treatment replicate.

Disease incidence of boxwood blight per shoot and severity of boxwood blight on stem data were subjected to analysis of variance (ANOVA) for a randomized block design using the Generalized Linear Mixed Models (GLIMMIX) procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Also, linear contrast analysis was performed for the experiment.

Field experiments, natural inoculum

For these experiments, boxwoods (*Buxus sempervirens* cv. 'Justin Brouwers') were planted on a research plot in 2017 in Surry County, NC. Plants were spaced in 2.5- m between plants and 2.5- m between rows adjacent to a field with a history of severe boxwood blight. To promote disease development, 400 cm^3 of diseased leaves were distributed on the soil surface at the base of each plant. A total of four experiments, two experiments per year were conducted in the same field. An experiment was conducted in 2018 to evaluate the frequency of drench applications for managing boxwood blight disease. Host plant defense inducers were applied two, four, or six times during the experiment (Table 4.2). A second experiment evaluating the effects of drench and foliar applications was conducted. Six applications were made for each application method and product (Table 4.3). The experiments were repeated in 2019.

For both experiments, the interval between applications was 14 days, and the final evaluation was approximately 15 days after the last application. The application dates in 2018 were 24 July, 7 August, 21 August, 4 September, 18 September, and 2 October. During the 2019 experiment applications were made on 25 July, 6 August, 20 August, 3 September, 17 September, and 1 October.

A total of 180 to 220 ml of fungicide solution were used per plant for foliar applications. Applications were made using a 15-liter hand pump backpack sprayer to run-off. For drench applications, one liter of the fungicide solution was poured evenly around the plant using a one-liter-plastic container. The treatments were arranged in a randomized complete block design consisting of four single-plant replications. Similar to container production, Actigard at 0.04 g/liter and 0.6 g/liter and Lifegard at 3.4 g/liter and 0.34 g/liter were sprayed and poured at the base of each plant, respectively.

Table 4.2 Experiment 1: Evaluation of the frequency of drench applications of host plant defense inducers for the management of boxwood blight.

Treatment	Trade name	a.i	Timing [a]
1	Untreated	-	-
2	Actigard	acibenzolar-S-methyl	A,D
3	Lifegard	*Bacillus mycoides* isolate J	A,D
4	Actigard	acibenzolar-S-methyl	A,B,D,E
5	Lifegard	*Bacillus mycoides* isolate J	A,B,D,E
6	Actigard	acibenzolar-S-methyl	A,B,C,D,E,F
7	Lifegard	*Bacillus mycoides* isolate J	A,B,C,D,E,F

[a]Application timings: 2018: A = 24 Jul; B = 7 Aug; C = 21 Aug; D = 4 Sep; E = 18 Sep; F = 2 Oct. 2019: A = 25 Jul; B = 6 Aug; C = 20 Aug; D = 3 Sep; E = 17 Sep; F = 1 Oct.

Table 4.3 Experiment 2**:** Evaluation of efficacy of host plant defense inducers under drench and foliar application after six applications.

Treatment	Trade name	a.i	Method	Timing [a]
1	Untreated		N/A	-
2	Lifegard	*Bacillus mycoides* isolate J	Drench	A,B,C,D,E,F
3	Actigard	acibenzolar-S-methyl	Drench	A,B,C,D,E,F
4	Actigard	acibenzolar-S-methyl	Foliar	A,B,C,D,E,F
5	Lifegard	*Bacillus mycoides* isolate J	Foliar	A,B,C,D,E,F

[a]Application timings: 2018: A = 24 Jul; B = 7 Aug; C = 21 Aug; D = 4 Sep; E = 18 Sep; F = 2 Oct. 2019: A = 25 Jul; B = 6 Aug; C = 20 Aug; D = 3 Sep; E = 17 Sep; F = 1 Oct.

The incidence of boxwood blight was obtained by evaluating the number of shoots infected on 10 arbitrarily selected shoots per evaluation. Five evaluations were made for each plant. Therefore, a total of 50 shoots evaluated per plant. This evaluation was made 15 days after the last application.

Disease incidence on shoots and severity on stems data were subjected to analysis of variance (ANOVA) using Generalized Linear Mixed Models (GLIMMIX) procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC).

Evaluation of plant defense inducers for managing fire blight of apple

The experiment was conducted in 2017 and 2018 at the Mountain Horticultural Crops Research and Extension Center (MHCREC) in Mills River, NC on 17-year-old 'Rome Beauty' apple trees (*Malus* x *domestica* Norkh) on semi-dwarfing rootstocks. Twenty trees in 2017 and 24 trees in 2018 were used with four single-tree replicates per treatment.

Four applications for the management of fire blight were made during the experiment at 20%, 40%, 80% bloom, and 50% petal fall using gas-powered backpack sprayers (Solo Mist Blower, 200 PSI) and were applied to runoff. Harbour (streptomycin) was tested as a standard antibiotic in the experiment. Three plant host defense inducers Actigard, LifeGard, and Regalia (*Reynoutria sachalinensis* extract) were applied in rotation with streptomycin. The description of the treatments is in Table 4.4. The experimental design followed a randomized complete block design with four single-tree replicates per treatment.

Trees were inoculated with a suspension of *Erwinia amylovora* strain Ea 273 (Wodzinski et al. 1994) approximately four hours after the 80% bloom treatment each year. Isolate Ea 273 was grown in Luria Bertani broth on a shaker at 160 rpm at 27 °C overnight. A 15-liter hand pump backpack sprayer was used to apply a final bacterial suspension of 1 x 10^6 UFC/ml plus phosphate buffer to blossoms until run-off. The inoculation was made four hours after the streptomycin application.

Table 4.4 Host plant defense inducers strategy applications for the control of blossom and shoot blight for the management of fire blight on apple cv. 'Rome Beauty' in 2017 and 2018.

Treatment	Trade name	i.a.	Dose/ha.	Timing[a]	Year
1	Untreated	-	-	-	2017/2018
2	Harbour	Streptomycin	1.68 kg	3	2017/2018
3	Lifegard	*Bacillus mycoides* isolate J	315 g	1,3	2017/2018
	Harbour	Streptomycin	1. 68 kg	2,4	
4	Actigard	Acibenzolar-S-methyl	140 g	1,3	2017/2018
	Harbour	Streptomycin	1.68 kg	2,4	
5	Regalia	*Reynoutria sachalinensis* extract	2.34 l	1,3	2017/2018
	Harbour	Streptomycin	1.68 kg	2,4	
6	Lifegard	*Bacillus mycoides* isolate J	315 g	1-4	2018

[a]Application timing in 2017: 1 = 20% bloom (April 21); 2 = 40% bloom (April 23) : 3 = 80% bloom (April 26) and 4 = 50% petal fall (May 3) Application timing in 2018; 1 = 20% bloom (April 17); 2 = 40% bloom (April 18) : 3 = 80% bloom (April 20 and May 1) and 4 = 50% petal fall (May 9)

Evaluation

The incidence of blossom blight was evaluated by counting the number of blighted blossoms out of 5 blossom clusters selected arbitrarily, and the incidence was expressed in a percentage value (percent blossom blight). A total of 20 such collections was taken for each treatment replicate. Shoot blight incidence was also evaluated in this experiment. The assessment was made by evaluating the number of blighted shoots in the total shoots on a scaffold branch expressed in a percentage value. A total of 5 such random collections were evaluated for each treatment replication. The evaluation of blossom blight and shoot blight symptoms were made after bloom on 18 May and 20 June in 2017 and on 24 May and 13 June in 2018, respectively.

Analysis of Pathogenesis-related (PR) gene expression in flower and leaf tissue following exogenous applications of host plant defense inducers

Sample Collection

During 2017, leaves and blossoms that were sprayed with the host plant defense inducers tested in the above field trial were collected at one and seven days after the initial 20% bloom spray. An additional collection was made at 35 days after the initial application for apple leaf tissue only. For each replicate, five separate blossoms and leaves were collected assayed at each time point. After collection, the tissue was immediately placed in 50 ml polyethylene-Falcon tubes and flash frozen with liquid nitrogen. Samples were stored at -80◦C until they were processed.

Extraction of RNA, cDNA synthesis and reverse transcription PCR

Samples of frozen leaves and blossoms were ground with a mortar and pestle in liquid nitrogen. RNA was extracted from frozen ground tissue using the Qiagen RNeasy® Plant Mini Kit (Qiagen Lab, Hilden, Germany), and DNA was removed using the Invitrogen Turbo DNA-

free Kit TM (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. RNA concentration and quality were measured with a Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, DE). All samples were diluted to a final concentration of 50 ng/µl prior to RT-qPCR analysis.

RT-qPCR using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA) was used to determine gene expression. A standard curve was obtained by using RNA from untreated apple leaf tissue with an initial concentration of 50 ng/µl followed by serial dilutions of 5.0, 0.5, 0.05, and 0.005 ng/µl. Four sets of previously developed primers were used for the amplification of Actin, PR-1, PR-2, and PR-8 genes (Aćimović et al. 2015; Maxson-Stein et al. 2002) (Table 4.5). Amplification of the PR genes was carried out in a 20 µl reactions, containing 10 µl of SYBR Green Rxn Mix, 0.25 of iScript Reverse Transcriptase, 6.65 µl dH2O, 2.5 µl of RNA (5 ng/µl) and 0.3 µl of each primer (0.5 µM final concentration). The amplification reactions were performed in a Bio-Rad CFX Connect TM Real-Time System (Bio-Rad, Hercules, CA) with the following parameters: 10 min at 50°C; 40 cycles of 1 min at 95°C, 10 s at 95°C, and 30 sec at 59°C. A total of three technical replicates per sample in the RT-qPCR reaction was performed at the same time.

Table 4.5 Primers used to amplify coding region of Actin, PR-1, PR-2, and PR-8 in the study.

Target gene	Primer ID	Direction	Sequence (5'-3')	Reference
Actin	aj748	Forward	AACTTCGTGTTGCTCCTGA AGAG	Maxson-Stein et. al., 2002
	aj749	Reverse	CAGTAGTACGACCACTGGC ATAGAG	
PR1	aj708	Forward	GTAGGCGTTGGTCCCTTGA C	Maxson-Stein et. al., 2002
	aj709	Reverse	GATTGCAGTCGCCAACATG T	
PR2	aj778	Forward	TCCGATGCCATTGCTTTTG	Maxson-Stein et. al., 2002
	aj779	Reverse	TTATGGACGAAACGGCAA CA	
PR8	aj780	Forward	CTCTTTTGAGCAGTTGGAA CCA	Maxson-Stein et. al., 2002
	aj781	Reverse	TGCCGGTAACCCCATGAA	

Evaluation of PR gene expression in apple blossom and leaves

The actin housekeeping gene was used to normalize expression levels of pathogenesis-related genes (PR-1, PR-2, and PR-8). Relative gene expression in apple tissue was determined by the mean of three technical replicates of the PR gene of interest normalized by the mean of three technical replicates of the actin housekeeping gene for the same treatment, time point, and type of plant tissue. A standard curve was used to determine the mean of starting quantity of gene of interest. The means of the normalized PR gene expression values were subjected to analysis of variance (ANOVA) for comparing treatment means of one gene at one time point and one type of plant tissue (Aćimović et al. 2015).

RESULTS

Evaluation of application strategies of host plant defense inducers for the management of boxwood blight in container production.

The incidence of boxwood blight was high across all programs in the 2018 container experiment. The disease incidence on leaves ranged from 59.5 ± 18.3% to 93.0 ± 8.1%, with the highest incidence occurring in the untreated program. Among all of the programs evaluated, a single drench application of Actigard prior to inoculation provided the greatest level of control against boxwood blight, and this program did not significantly differ from Actigard programs in which three applications were made, regardless of application method. Among the Lifegard treatments, only the three application program reduced the incidence of boxwood blight compared to the untreated program. The three foliar-application program of Lifegard provided a level of control that was equivalent to a single Actigard drench application (Figure 4.1A). Contrast analysis showed a difference only in the product factor ($P < 0.0001$) (Table 4.6).

In 2019, severity on stems ranged from 3.9 ± 4.5% to 14.7 ± 1.3%, with the highest incidence occurring in the untreated program. Among the Actigard treatments, the three-application drench program obtained the highest level of control compared to the untreated program. Actigard treatments with one application (drench or foliar) did not differ compared to those in which three applications were made of the same application method. Among Lifegard treatments, only three application programs of this host defense inducer provided a level of control that differed from the untreated program regardless of application method (Figure 4.1B). Contrast analysis showed a difference in all three factors tested; product ($P < 0.0001$), timing ($P = 0.0008$), and method of application ($P = 0.0018$) (Table 4.6).

Table 4.6 Linear contrasts comparing effects of host plant defense inducers, timing of application, and application method on boxwood blight incidence (2018) or severity (2019) in the susceptible *Buxus sempervirens* cultivar ‘Justin Brouwers.’

2018					
Product	**Mean**	**Timing**	**Mean**	**Met. appl.**	**Mean**
Acibenzolar-S-Methyl	64.7	After	68.7	Drench	71.2
Bacillus mycoides isolate J	78.6	Before	74.6	Foliar	72.1
Estimate	13.9		5.9		0.9
Pr>t	<0.0001		0.1071		0.864
2019					
Product	**Mean**	**Timing**	**Mean**	**Met. appl.**	**Mean**
Acibenzolar-S-Methyl	7.1	After	7.9	Drench	8.00
Bacillus mycoides isolate J	11.4	Before	10.728	Foliar	10.6
Estimate	4.3		2.9		2.7
Pr>t	<0.0001		0.0008		0.0018

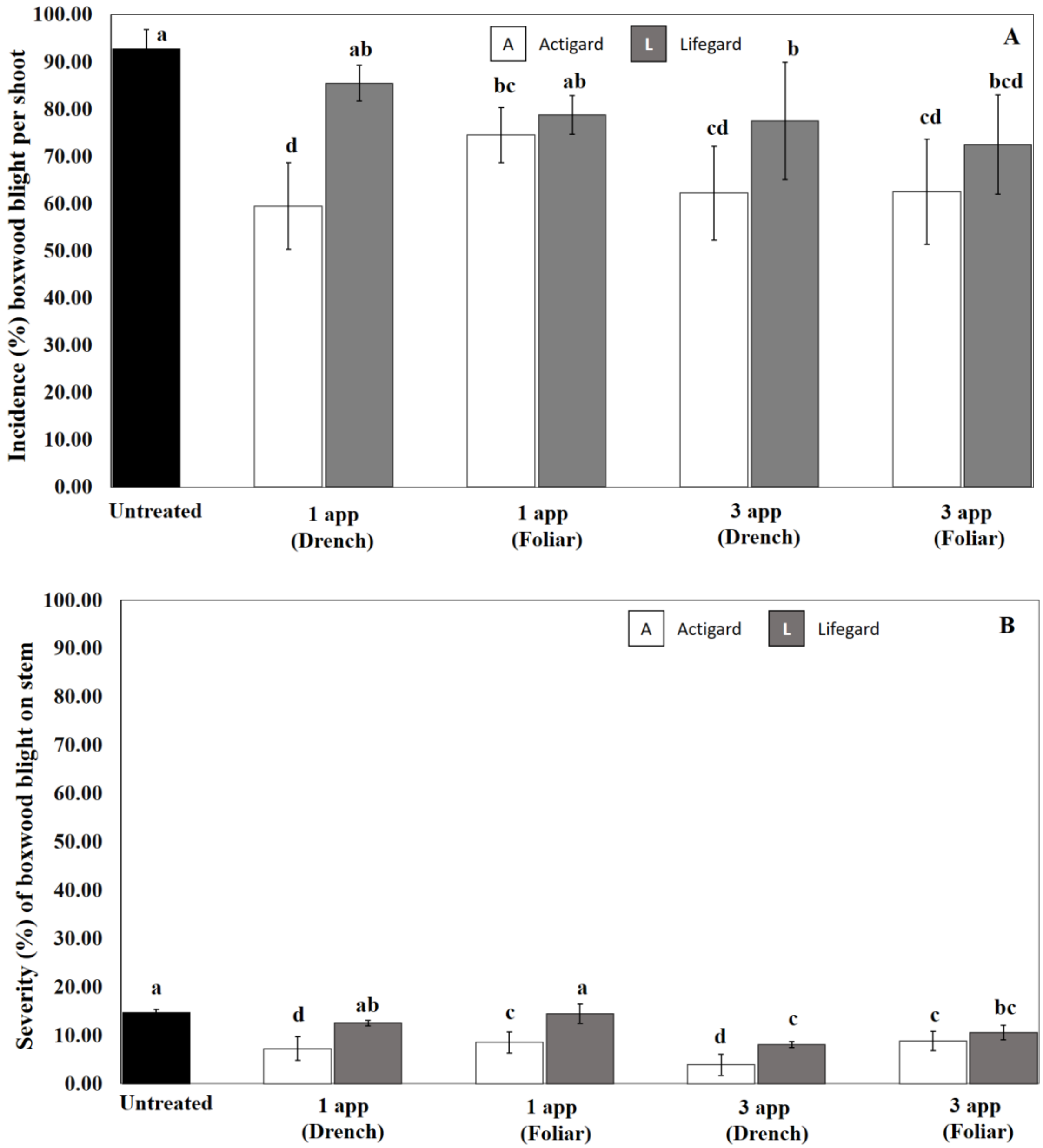

Figure 4.1 Effect of host plant defense inducers, application method, and application timing on boxwood blight of container-grown plants of the susceptible *Buxus sempervirens* cultivar 'Justin Brouwers.' **A,** Incidence on shoots in 2018; **B,** Severity on the stems in 2019.

Evaluation of frequency of drench application of host plant defense inducers for the management of boxwood blight in field production.

As with the container production experiment in 2018, a high incidence of boxwood blight was observed across all programs in the field experiment. The disease incidence on leaves ranged from 24.0% ± 17.9% to 92.0% ± 11.3%, with the highest incidence occurring when Lifegard was applied four times as a drench. Among all of the programs evaluated, six drench applications of Actigard provided the greatest level of control against boxwood blight; however, this program did not differ from Actigard programs in which two or four applications were made. Actigard programs provided better control than Lifegard programs with two, four, or six drench applications. Only Lifegard programs in which six applications were made reduced disease relative to the untreated control (Figure 4.2A).

The results obtained during the experiment in 2019 showed a different trend in comparison with 2018. Due to unfavorable weather conditions for the development of boxwood blight, incidence of foliar boxwood blight was low, ranging from 4.5 ± 3.0% to 18.0 ± 13.0%, with the highest incidence occurring in the untreated program. In contrast to results from 2018, among all of the programs evaluated, four drench applications of Lifegard showed the greatest level of control against boxwood blight. This program did not differ from the Lifegard drench program in which two applications were made. In contrast to the results obtained in 2018, programs with Lifegard and Actigard did not differ from each other at two, four, or six drench application (Figure 4.2B).

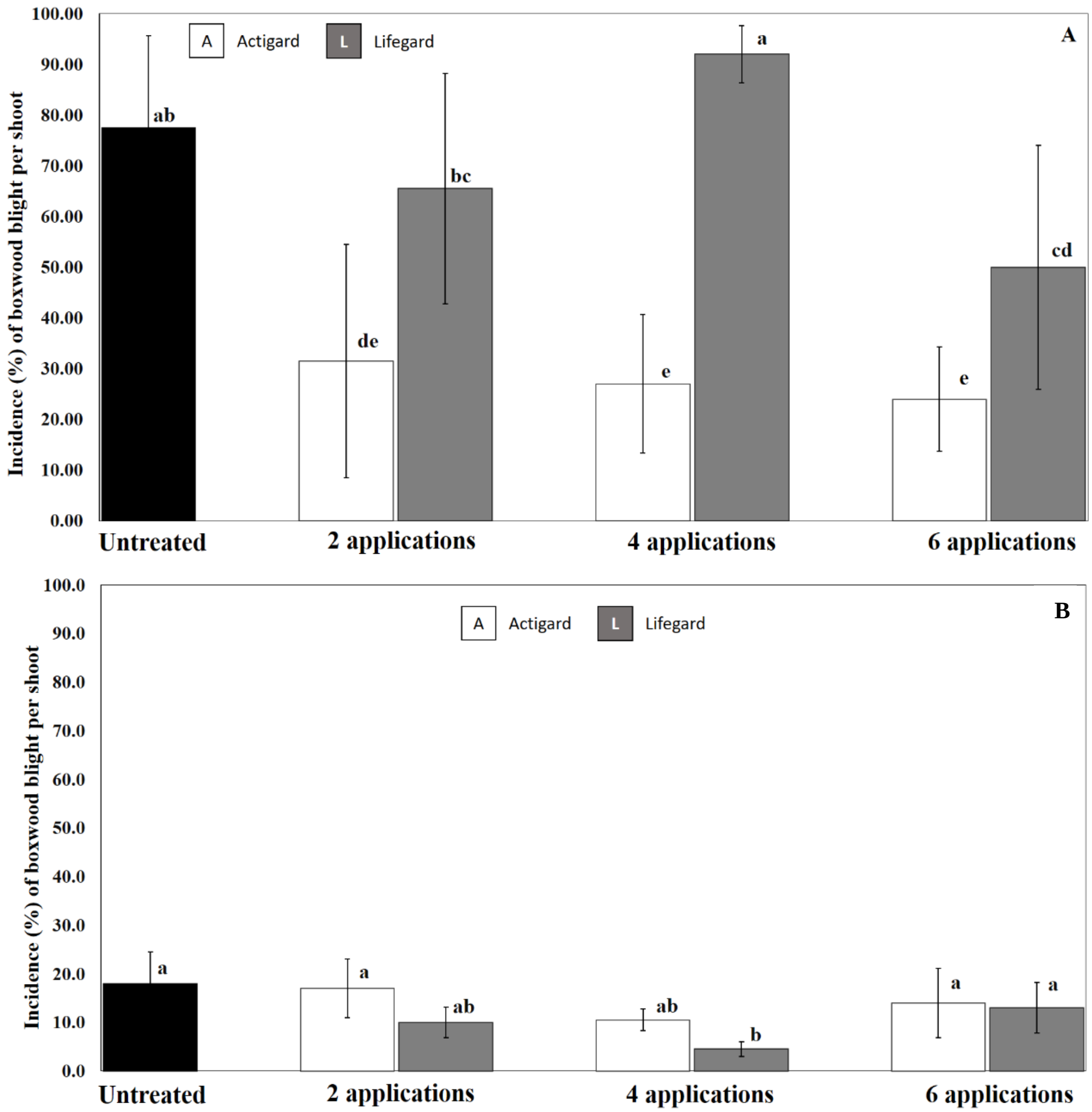

Figure 4.2 Effect of drench-application frequency of host plant defense inducers on boxwood blight under field production of *Buxus semipervirens,* susceptible cultivar 'Justin Brouwers.' Incidence on shoot in **A,** 2018 and **B,** 2019.

Evaluation of application method of Actigard and Lifegard for the management of boxwood blight

In 2018, the disease incidence on leaves ranged from 24.0 ± 17.8% to 88.7 ± 16.3%, with the highest incidence occurring in the six foliar applications program of Actigard. Actigard was effective against boxwood blight when applied in six drench applications program, but was not effective when applied to foliage. In contrast, the six foliar applications program of Lifegard showed lower disease incidence than six drench applications. However, these programs did not differ from each other (Figure 4.3A).

As in the previous field experiment, incidence of foliar boxwood blight was low in 2019, ranging from 10.5 ± 3.0% to 18.0 ± 12.9%, with the highest incidence occurring in the untreated program. No differences were found among the programs tested (Figure 4.3B).

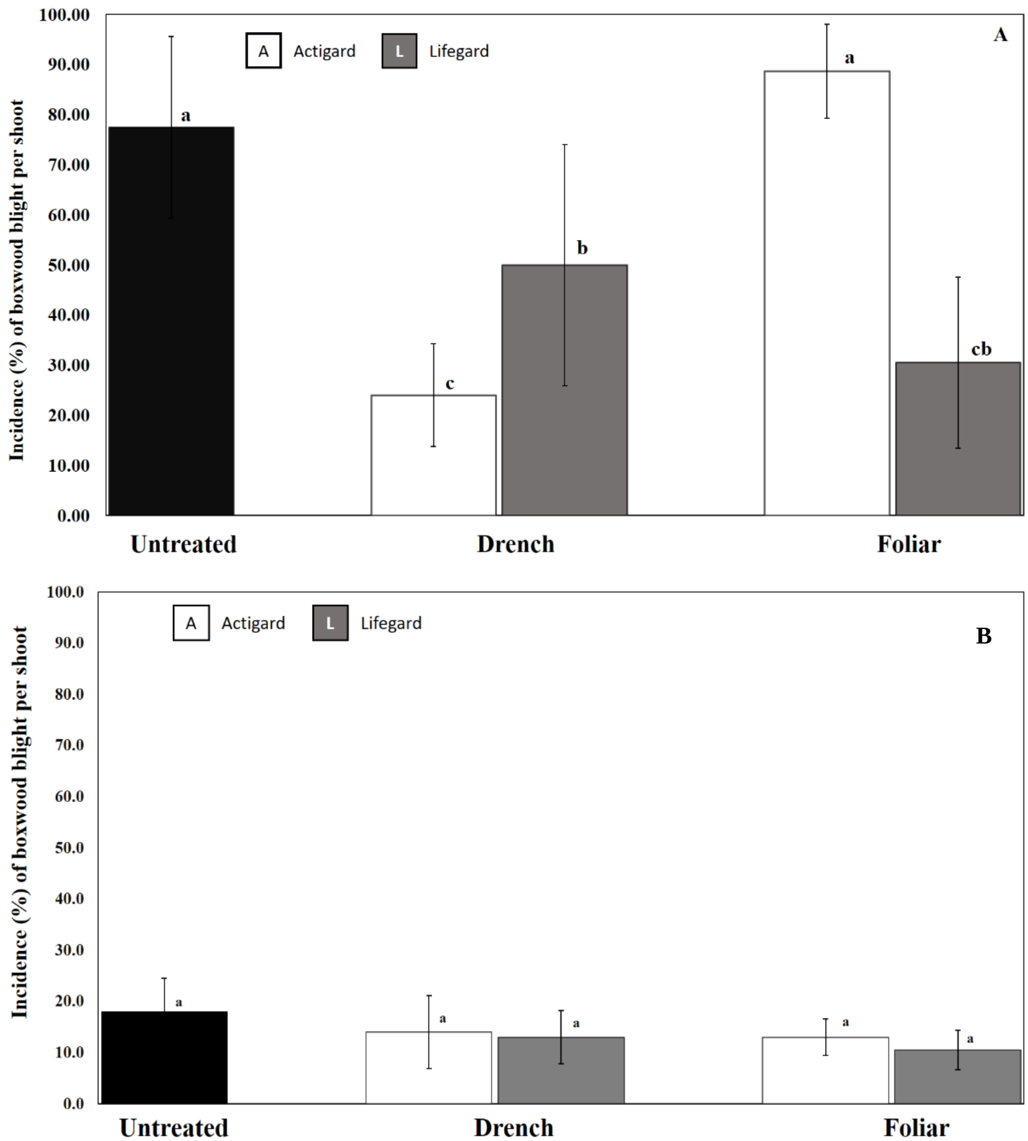

Figure 4.3 Effect of foliar and drench application of host plant defense inducers on boxwood blight under field production of *Buxus semipervirens*, susceptible cultivar 'Justin Brouwers.' Incidence on shoots in **A,** 2018 and **B,** 2019.

Effect of plant defense inducers on blossom and shoot blight for the management of fire blight on Apple.

The incidence of blossom blight ranged from 9.4 ± 2.0% to 58.3 ± 2.4%, with the highest incidence occurring in the untreated program in 2017. All host plant defense inducers, when rotated with streptomycin, significantly reduced the incidence of blossom blight compared to the untreated program. Among the programs in which host defense inducers were included, Lifegard demonstrated the greatest efficacy against blossom blight with a mean incidence of 26.8% ± 2.8; however, the incidence in this program did not differ significantly from the Actigard or Regalia programs. Conversely, a single application of streptomycin at 80% bloom provided a level of blossom blight control that was significantly greater than all other programs in the experiment (Figure 4.4A).

In 2018, the range of the mean incidence of blossom blight ranged from 14.2% ± 1.8 to 80.3% ± 2.4, with the highest incidence occurring in the untreated program. As with the 2017 results, all host plant defense inducers differed from the untreated program. Among the programs in which host defense inducers were included, Actigard and Regalia demonstrated the greatest efficacy against blossom blight with a mean incidence of 34.1% ± 6.9 and 32.2% ± 10.7; however, these programs did not differ from each other. The Lifegard program without streptomycin rotation had a higher incidence of blossom blight than the programs that rotated any of the host plant defense inducers with streptomycin. Finally, a single application of streptomycin at 80% bloom, provided a level of blossom blight control that was significantly greater than all other treatment programs in the experiment, as in 2017 (Figure 4.4B).

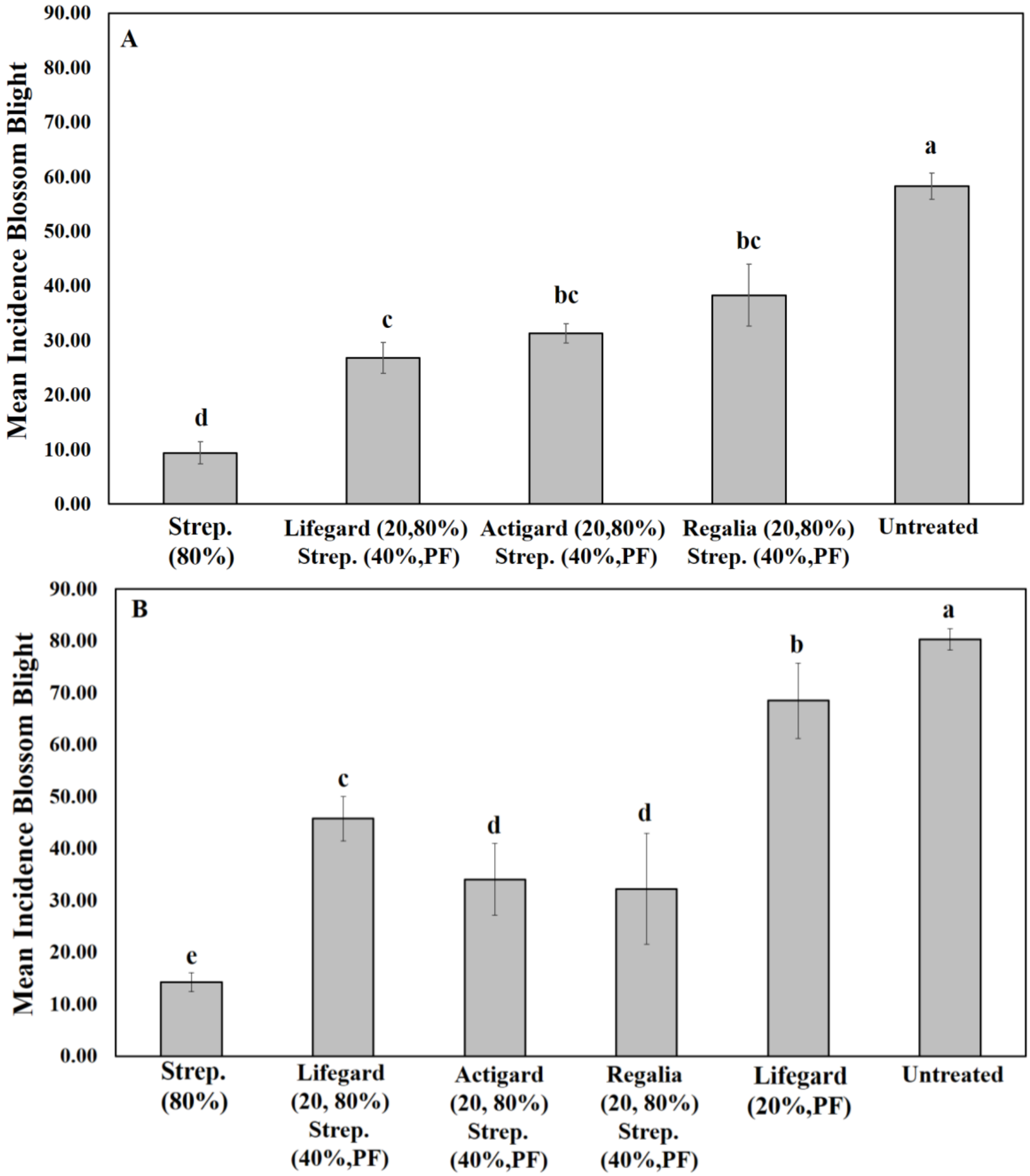

Figure 4.4 Mean incidence of blossom blight infection by different host plant defense inducers and streptomycin applications at different apple phenological stage (20%, 40%, 80% bloom, and 50% petal fall = PF) **A,** 2017 and **B,** 2018.

In the 2017 experiment, the mean incidence of shoot blight ranged from 21.2% ± 2.2 to 54.7% ± 1.4, with the highest incidence in the untreated program. The only host plant defense inducer treatment that differed with the untreated was Lifegard program. The Actigard and Regalia programs did not differ from each other. The highest control was obtained with streptomycin sprayed at 80% bloom (Figure 4.5A).

During the 2018 experiment, the mean incidence of shoot blight ranged from 24.1% ± 4.5 to 63.2% ± 8.1. The highest incidence was observed in Lifegard program without streptomycin rotation. The Lifegard program without streptomycin had the lowest efficacy in comparison with all treatments in which host plant defense inducers were rotated with streptomycin. The performance of the host plant defense inducers in rotation with streptomycin were not different from each other. As all the experiments conducted previously, streptomycin sprayed at 80% bloom showed the highest efficacy among all the programs (Figure 4.5B).

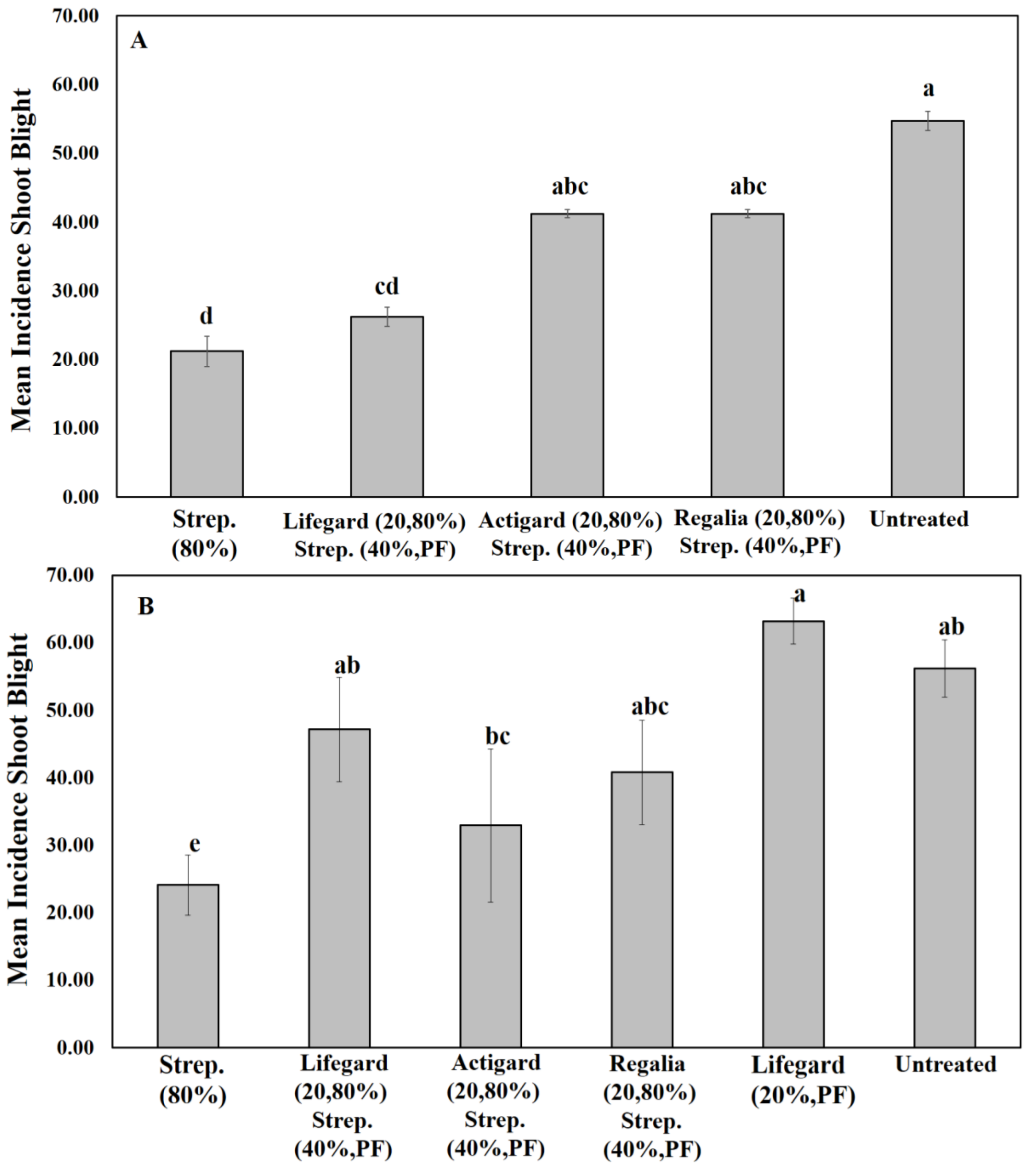

Figure 4.5 Mean incidence of shoot blight infection by different host plant defense inducers and streptomycin applications at different apple phenological stage (20%, 40%, 80% bloom, and 50% petal fall = PF) **A,** 2017 **B,** 2018.

Expression of PR-1, PR-2 and PR-8 in leaves and blossoms following application of host plant defense inducers.

One day after the application of host plant defense inducers, the highest relative gene expression of PR-1 was observed in leaves treated with Lifegard. Applications of Actigard and Regalia resulted in higher PR-2 and PR-8 expression in comparison with the untreated program. At seven days, the highest relative PR gene expression was observed in leaves treated with Lifegard. Actigard and Regalia showed a significantly higher value in the PR-2 and PR-8 gene expression than with the untreated program; however, they did not differ from each other. At 35 days after the first application, only Lifegard showed a higher value in the PR-1 gene expression in comparison with all the programs tested. No difference was found in the PR-2 and PR-8 gene expression with any of the treatments (Table 4.7).

One day after the application of host plant defense inducers, the only differences in blossoms were found in the PR-2 gene expression, with Actigard being the only program to cause a difference in PR-2 expression compared with the untreated program. Finally, at seven days, Lifegard was the only treatment that differed from the untreated program in the expression of PR-8. No differences were found in PR-1 and PR-2 gene expression (Table 4.8).

Table 4.7 Relative gene expression values for PR-1, PR-2, and PR-8 in apple leave tissue after the initial host plant defense inducer application in 2017.

1 day						
	PR-1		PR-2		PR-8	
Untreated	1.00 ± 0.00	b	1.00 ± 0.00	b	1.00 ± 0.00	c
Acibenzolar-S-methyl	1.38 ± 0.16	ab	1.63 ± 0.31	a	1.51 ± 0.20	ab
Reynoutria sachalinensis	1.25 ± 0.20	b	1.68 ± 0.34	a	1.57 ± 0.18	a
Bacillus mycoides isolate J	1.73 ± 0.22	a	1.35 ± 0.17	ab	1.20 ± 0.11	bc
7 day						
	PR-1		PR-2		PR-8	
Untreated	1.00 ± 0.00	b	1.00 ± 0.00	b	1.00 ± 0.00	c
Acibenzolar-S-methyl	1.30 ± 0.40	b	2.00 ± 0.21	a	1.38 ± 0.15	a
Reynoutria sachalinensis	1.05 ± 0.03	b	2.12 ± 0.29	a	1.31 ± 0.18	ab
Bacillus mycoides isolate J	2.00 ± 0.31	a	0.85 ± 0.04	b	1.06 ± 0.03	bc
35 day						
	PR-1		PR-2		PR-8	
Untreated	1.00 ± 0.00	a	1.00 ± 0.00	a	1.00 ± 0.00	a
Acibenzolar-S-methyl	1.20 ± 0.08	a	1.25 ± 0.18	a	1.04 ± 0.20	a
Reynoutria sachalinensis	1.20 ± 0.33	a	1.11 ± 0.10	a	1.00 ± 0.44	a
Bacillus mycoides isolate J	0.41 ± 0.26	b	1.17 ± 0.45	a	1.11 ± 0.46	a

Table 4.8 Relative gene expression values for PR-1, PR-2, and PR-8 in apple blossom tissue after the initial host plant defense inducer application in 2017.

1 day						
	PR-1		PR-2		PR-8	
Untreated	1.00 ± 0.00	a	1.00 ± 0.00	b	1.00 ± 0.00	a
Acibenzolar-S-methyl	1.04 ± 0.20	a	1.93 ± 0.52	a	1.41 ± 0.39	a
Reynoutria sachalinensis	1.00 ± 0.43	a	1.76 ± 0.39	ab	1.30 ± 0.31	a
Bacillus mycoides isolate J	1.11 ± 0.46	a	1.04 ± 0.25	b	1.15 ± 0.16	a
7 day						
	PR-1		PR-2		PR-8	
Untreated	1.00 ± 0.00	ab	1.00 ± 0.00	a	1.00 ± 0.00	b
Acibenzolar-S-methyl	0.78 ± 0.20	b	1.16 ± 0.12	a	1.19 ± 0.23	ab
Reynoutria sachalinensis	0.71 ± 0.25	b	1.14 ± 0.23	a	1.16 ± 0.09	ab
Bacillus mycoides isolate J	1.60 ± 0.40	a	0.96 ± 0.22	a	1.48 ± 0.22	a

DISCUSSION

Host plant defense inducers have become an alternative tool for the management of plant pathogens. Their applications could help to reduce the risk of development of fungicide resistance and the dependency and risk of antibiotics in agriculture. Therefore, an improved understanding of application timing, frequency, and method is essential for their efficient use and improved efficacy in perennial specialty cropping systems. This study assessed application strategies for two host plant defense inducers in the management of boxwood blight caused by *Calonectria pseudonaviculata*. Experiments were conducted in two different production systems, container and field, during two consecutive years, 2018 and 2019, in North Carolina. Additionally, the efficacy of three host defense inducers was evaluated for the management of fire blight in apple in 2017 and 2018. Host plant defense inducers trigger induction of plant pathogenesis-related (PR) as a response to stop or reduce the infection of a pathogen or other events (Van Loon et al. 1994; Van Loon & Van Strien 1999). Therefore, an evaluation of three PR genes after the application of host plant defense inducer was considered. PR induction has been demonstrated in several experiments in apple, among them PR-1, PR-2, PR-5, PR-8, and PR-10 have been previously studied (Bonasera et al. 2006; Brisset et al. 2000; Ziadi et al. 2001). PR-1 (anti-oomycete activity), PR-2 (β-1,3-glucanase), and PR-8 (class III chitinase) were analyzed and demonstrated enhanced expression in previous PR induction studies in apple (Aćimović et al. 2015; Maxson-Stein et al. 2002). Therefore, these three PR genes were selected to measure their induction after the application of host plant defense inducers to blossoms and leaves.

Evaluation of application strategies of host plant defense inducers under container production of boxwood showed that Actigard applied as a drench had the highest control among the treatments tested. Actigard triggers systemic acquired resistance in the plant as a mimic of salicylic acid, which acts as an endogenous plant stress hormone (Oliver & Hewitt 2014; Tietjen 2017). However, Actigard is recommended to be applied in a foliar spray (Toquin et al. 2012). The poor performance of the foliar application on boxwood could be attributed to the characteristics of boxwood leaves. Boxwood has a thick waxy cuticle as a natural protection for moisture loss (Batdorf 1995). The constitution of the leaves could have an influence on the poor efficacy of foliar application of Actigard. This active ingredient may not have been able to penetrate the leaf surface; as a consequence, SAR could not be triggered. A study encountered this problem when the control of boxwood leaf miner was lower when the leaves were hardening off because of a lack of insecticide penetration (D'eustachio & Raupp 2001). The same pattern was observed under natural inoculum conditions in the field in the 2018 experiment, where Actigard applied by drench had better performance than Lifegard when they were applied at different frequencies (2, 4, or 6 applications). An additional study under field conditions showed that the action of Actigard to control *Calonectra pseudonaviculata* was significant when they were applied under drench comparing with foliar application. Overall, these results suggest that Actigard could be taken up by the roots, triggering SAR, while Lifegard showed several limitations under both conditions. In the case of Lifegard several foliar and drench applications did not show a statistical difference between them; but overall, foliar applications tended to have greater control. In our experiment, low control was observed in comparison with studies in different pathosystems. A reduction of 60-80% was reported against *Cercospora beticola*, causal agent of Cercospora leaf spot in sugar beet, and a delay of the disease onset and reduction of spore production was observed against *Glomerella cingulata* var. *orbiculare*, causal agent of anthracnose of cucumber (Bargabus 2003; Neher et al. 2009). Lifegard induces system acquired resistance by induced a biphasic oxidative burst (Bargabus 2003). One possible answer for the low performance in the foliar application could be the thick waxy cuticle and the small surface area in boxwood in comparison with the two species above mentioned. This cuticle could decrease the interaction between the product and the plant. It is also possible that the isolate of *Bacillus mycoides* was not effective against *C. pseudonaviculata*. *Bacillus mycoides* is considered a ubiquitous organism; however, the Isolate J was isolated from sugar beet leaves (Bargabus 2003). A previous study observed that two different isolates of *Bacillus mycoides* isolated from two different sites of the plant, endosphere of potato and the soil of the same geographical region, showed different levels of colonization and competition (Yi 2018). Some future experiments have to be done in order to understand the impact of waxy cuticle in the performance of host plant defense inducers, such as evaluating the effect of surfactants and adjuvants. In addition, activation of the growth by application of nitrogen before or after the application of host plant defense inducers in order to stimulate new shoots with poorly developed cuticles that will allow to penetrate the product easily should be evaluated.

In all the experiments conducted, an increase in the frequency of application of the host plant defense inducers did not enhance the efficacy for the control of boxwood blight. This result was similarly reported by (Aćimović et al. 2015), who stated that an increase in efficiency was not observed with one or two applications of Actigard when it was applied by trunk injection in apple for the control of fire blight. One explanation for this might be that system acquired resistance provides in the plant a long-lasting systemic immunity to other secondary infections (Choi & Hwang 2011). It could suggest that our interval of application could have been too short in comparison with this immunity response process.

It is important to mention that unfavorable conditions such as high temperature with average of 22.7°C and lower relative humidity with average of 75% were presented in 2019, triggering adversity for the development of the disease in the container and field conditions. Optimal temperature conditions for the infection of *C. pseudonaviculata* have been reported in vitro at 25°C, stopping its growth at 30°C and a lethal temperature with 33°C (Henricot & Culham 2002). Additional studies reported that the optimum temperature in the range from 24 to 28°C (Hunter & Barnett 1978). Species in the genus *Calonectria* are found predominantly under humid conditions being prevalent in tropical and subtropical areas (Crous et al. 1991; Crous & Wingfield 1994). The negative impact of the weather on the development of the disease in boxwood was observed in both systems production. Even after a second inoculation to the same experiment in container production, just a few symptoms were observed. However, more consistent necrotic lesions were observed on the stem in under these conditions; therefore, efficacy was measured as disease severity on stems. An explanation of the presence of lesions on the stem could rely on the morphological structure of boxwoods, as stems are not as much exposed to the environment as leaves are. The stems were covered by dense leaves, which creates a different microclimate, decreasing the temperature and maintaining humidity, two important variables for the infection and colonization of *C. pseudonaviculata.* Besides, hot and dry weather stimulates the close of guard cells to prevent the plant from losing water (Hofstra & Hesketh 1969; Schulze et al. 1972). Therefore the weather conditions could have negatively influenced the penetration and efficacy of host plant defense inducer to control this pathogen.

The performance of host plant defense inducers declined when the pressure of *C. pseudonaviculata* was high. In our study, better control was observed under field production than with container production, where artificial inoculation and controlled conditions triggered high level of disease. Similar observations were also reported by Aćimović et al. (2015), who stated that control of blossom blight with Actigard was low under high disease pressure in the field. There are some possible explanations for these results in our experiment. First, in order to provide favorable conditions for disease development, overhead irrigation was applied for 1.5 hrs three times during the day (Ganci 2014; Ivors et al. 2012). This factor could have been counterproductive for the efficacy of the products, which could have been leached or rinsed from treated areas in the foliar application method. Besides, roots were constrained within the containers, whereas the root system was better developed under field conditions, perhaps providing greater potential for absorption of the inducers. Future experiments to evaluate root architecture and absorption surface should be conducted to determine the effect of the surface with the performance of host plan defense inducer.

A second pathosystem was evaluated in 2017 and 2018 to gain insight into application strategies for newly registered host plant defense inducers for the control of fire blight in apple. Two different stages of infection, blossom blight and shoot blight, were examined to better understand the immediate, mid-range, and relatively longer-term effects of host defense inducers for the management of fire blight. Application of a host plant defense inducer in rotation with streptomycin suppressed blossom and shoot blight compared to the untreated programs. However, in the 2018 experiments, even four applications of Lifegard without streptomycin rotation did not provide good control on blossom blight and shoot blight. In previous studies, the effect of Actigard for the control of blossom blight ranged from 50 to 60% on 'golden delicious' and 'James Grieve' (Brisset et al. 2000; Zeller & Zeller 1999). An experiment demonstrated the effect of *Bacillus mycoides* for the control of Cercospora leaf spot in sugar beet, showing 60-80% of control under controlled conditions (Bargabus 2003). Similar experiments with

Reynoutria sachalinensis were reported to have an effect on powdery mildew in giant knotweed and in tomato (Herger & Klingauf 1990; Konstantinidou-Doltsinis et al. 2006).

In both 2017 and 2018, a single application of streptomycin applied at 80% bloom provided a greater level of control against both blossom and shoot blight compared to programs in which the antibiotic was applied in rotation with host defense inducers at 40% bloom and 50% petal fall. Application of host plant defense inducers in rotation with streptomycin at the different bloom stages controlled blossom blight and shoot blight compared to the untreated program. Similar results were reported in a previous study where two Actigard applications and one application of streptomycin reduced the incidence of blossom blight in comparison with the non-treated program (Cox et al. 2011). A higher incidence in shoot blight was reported in comparison with the incidence of blossom blight in both years. This could be explained because small infections of blossom blight can trigger an increase in the infection of shoot blights. That is why it is suggested that earlier application of host plant defense inducer on flower buds and growing shoots would protect open flowers and young shoot tissue, reducing the blossom blight and shoot blight (Brisset et al. 2000).

PR induction is a consequence of systemic acquired resistance (SAR). This is a process that involves a signal pathway inside the plant against disease infection, which could be triggered after interaction with a pathogen or by certain synthetic or natural compounds (Hammerschmidt 2007; Van Loon et al. 1994). A consistent result was obtained with Actigard and Regalia on apple leaves, where enhanced expression of PR-2 and PR-8 was induced within 24 hours of product application and was sustained seven days following their application. The level of expression increased for PR-2 and decreased for PR-8 at seven days, but not significantly, even though two applications of host plant defense inducers were made in this interval. These results may suggest that there was no additive effect from the second application of the host plant defense inducers. This data could explain the results obtained for boxwood blight, where the frequency of application likewise did not affect control. A rapid expression in treated apple leaves was also reported three days after the treatment for PR-2 with Actigard (Brisset et al. 2000). Our results coincided with this previous study where expression of PR-2 and PR-8 increased one day after apple leaves were treated with Actigard and Regalia. However, another study found an increase in the level of expression of PR-1 (Aćimović et al. 2015). Application of Actigard in our experiment showed a higher value in the expression of PR-1 in comparison with the untreated program; however, no difference was found at 1, 7, and 35 days. The same PR genes (PR-2 and PR-8) were found overexpressed with the application of Actigard in 'Jonathan' but not for PR-1 (Maxson-Stein et al. 2002). *Reynoutria sachalinensis* used as leaf extract enhances natural defense against powdery mildew in the treated plants by activation of phytoalexins (Daayf et al. 1997). Some molecules are reported to be induced after application of this host plant defense inducer such as peroxidase, polyphenoloxidase, and chitinase activities in cucumber leave analyzed (Herger & Klingauf 1990). These previous studies coincided with the results obtained in the apple treated tissue with Regalia where the induction of PR-2 and PR-8 were reported. However, as with Actigard, the level of the PR-1 was not increased by treatment with Regalia. A recent study found similar patter where induction of PR-2 was observed but not in PR-1 in summer squash (*Cucurbita pepo* var. *cylindrical*) in tissue sprayed with this active ingredient (Margaritopoulou et al. 2020). However, an increase in the expression of PR-1 was observed on foliage at one and seven days after the initial application with Lifegard compared with the untreated program. Evaluation of PR genes expression in apple blossoms showed that only Actigard at one day with PR-2 and Lifegard at seven days with PR-8 had a significant increase compared to the untreated program. The density of stomata in leaves is higher than in fruit of *Malus* spp. and decreases along with the development of the fruit (Blanke & Lenz 1989). Therefore, probably the low density of stomata in fruit could affect the penetration of the products in the apple tissue and as a consequence system acquire resistance would be induced or would not be as efficient as it was in leaves. The interval of PR inductions in a previous study for PR-2 showed a sustained induction locally and systematically for 17 days (Brisset et al. 2000). That could explain why the level of the PR genes on treated apple-leaf tissue did not differ at 35 days after application. Therefore an in-depth study testing interval of applications for host plant defense inducers is necessary to understand this mechanism and to determine adequate intervals between application.

This study provides useful information for developing strategies for application of host plant defense inducers on specific perennial crops such as boxwood blight caused by *Calonectria pseudonaviculata* and firebligh caused by *Erwinia amylovora* and their potential use in an Integrated Pest Management (IPM) program. Besides, the analysis of PR genes in the apple tissue could help us to understand the mechanism that is involved in System Acquire Resistance inside the plant triggering PR genes.

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