Boxwood Dieback: Molecular Detection, In-Vitro Fungicide Efficacy, and Host Susceptibility for Managing a New Emerging Disease Caused by Colletotrichum Theobromicola

Harleen Kaur
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BOXWOOD DIEBACK: MOLECULAR DETECTION, IN-VITRO FUNGICIDE EFFICACY, AND HOST SUSCEPTIBILITY FOR MANAGING A NEW EMERGING DISEASE CAUSED BY COLLETOTRICHUM THEOBROMICOLA

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The Department of Plant Pathology and Crop Physiology

by

Harleen Kaur
B.S., Punjab Agricultural University, India, 2018
December 2020
ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my major advisor, Dr. Raghuwinder “Raj” Singh for being an amazing support and source of inspiration throughout my graduate program. I am thankful to him for providing me with the opportunity of pursuing my master’s degree in his lab and for making me a better researcher.

I would like to thank my co-advisor, Dr. Vinson Doyle and my committee member, Dr. Rodrigo Valverde for motivating me and for always being available when I needed guidance. I am grateful for all the valuable suggestions, comments and advises that I received during the course of my degree. Their support has truly been helpful without which I wouldn’t be able to accomplish my research objectives.

I would also like to thank my lab members, Dr. Monique De Souza and Tim Burks for encouraging me to do my best. I would like to thank them for helping me out whenever I faced any obstacles while working in the lab.

I am thankful to all the professors and graduate students in the Department of Plant Pathology and Crop Physiology for the constant support and for teaching me so much related to plant pathology and beyond.

Lastly, I would like to thank my parents, Harwant Kaur and Davinder Singh and my brother, Parampreet Singh for their unconditional love and support. I would like to thank them for always pushing me to go beyond my comfort zone and for encouraging me to pursue my higher education from outside my home country. I am blessed to have been a part of Louisiana State University Agricultural and Mechanical College.
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ABSTRACT

Boxwood (*Buxus* spp. L) is one of the most common and widely planted perennial ornamentals in both home gardens and commercial landscapes. Grown for its evergreen, dark green foliage, boxwood cultivation dates back to 4000 BC in Egypt. Although considered hardy, boxwood is susceptible to several plant pathogens. Recently reported boxwood dieback, a fungal disease caused by *Colletotrichum theobromicola*, has been spreading at an alarming rate within the United States. Boxwood dieback consists of symptoms that resemble to those caused by Phytophthora root rot, Volutella blight, and some abiotic disorders and can be easily misdiagnosed in nurseries and landscape plantings and may lead to ineffective management recommendations and practices. Additionally, previous studies have shown that it may take up to two to three months for the symptoms to appear under greenhouse conditions. Boxwood breeders and growers have shown great concerns regarding the disease due to its delayed onset of symptoms, non-availability of early, rapid, and accurate diagnostic tools, and lack of effective management practices. Therefore, the primary objectives of this study were to develop an accurate diagnostic method to detect the disease in boxwoods at early disease development stages and to devise effective disease management strategies including host range and screening of fungicide for their efficacy. A diagnostic *TaqMan* real-time PCR assay for *in planta* detection and quantification of *C. theobromicola* was developed. This species-specific assay targets calmodulin (CAL) gene and successfully detected the pathogen from symptomatic boxwood tissue at early stages of the disease development. Host range studies were conducted by screening a wide variety of boxwood cultivars under greenhouse conditions. Out of the 11 cultivars screened, ‘Little Missy’ was found to show latent infection to boxwood dieback. Finally, fungicide efficacy studies were conducted by screening nine chemical compounds to...
determine their effect on mycelial growth as well as spore germination inhibition of eight isolates of *C. theobromicola* collected from eight different states in the United States. Of the nine fungicides, difenoconazole+pydiflumetofen showed maximum mycelial growth and spore germination inhibition at 1 ppm active ingredient followed by fluxapyroxad+pyraclostrobin, and pyraclostrobin+boscalid at 5 ppm active ingredient. Azoxystrobin+benzovindiflupyr significantly inhibited mycelial growth at 1 ppm but reduced spore germination at 10 ppm active ingredient. This study provides the boxwood industry with much needed important and applied information regarding rapid and accurate detection, host susceptibility, and fungicide efficacy to effectively manage boxwood dieback and to reduce its further spread.
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. Boxwood

Boxwood (Buxus spp. L.) plants are a part of the Buxaceae family. Boxwood has been considered an important source of wood used for various purposes especially in building ornamental boxes and thus is named after the word “box” (Buxus means box in latin) (Batdorf 2004). Historically, boxwood has also been used to build tools, instruments, sculptures, and is considered an essential part of mankind’s practical and religious needs (Mitchell et al. 2018). Currently, it is used as a popular perennial shrub or small tree in the landscape and ornamental industry in the United States and other parts of the world. Boxwood is grown for its vibrant green color and evergreen growth and can tolerate heavy pruning without being stressed out. For these characteristics, boxwoods are also commonly grown as topiary and bonsai for decorative purposes (Lehtijärvi et al. 2017).

Boxwoods usually require low maintenance and are resilient compared to other landscape plants. Their hardy nature makes them suitable for growing in diverse environmental conditions. Boxwood stood as the most popular shrub holding the top sales amongst woody ornamentals in the United States surpassing the sales of arborvitae, azalea, holly, or hydrangea according to a report stated in 2014 (Calabro 2018). According to the USDA National Agricultural Statistics Service report in 2014, the annual wholesale value of boxwood was estimated to be more than $126 million.

Buxaceae is a small family consisting of several closely related genera. The largest genus in the Buxaceae is Buxus, consisting of about 70 species (Petruzello et al. 2013). The most commonly grown boxwood is Buxus sempervirens L., also known as “common boxwood”.

1
Additionally, different varieties of *B. microphylla* Siebold & Zucc. “Baby Gem”, Japanese or little leaf boxwood and *B. sinica* (Rehder & E.H.Wilson) M.Cheng “Wintergreen” or “Korean boxwoods” are also grown in the United States. Niemiera A. (2012) estimated about 400 varieties of *B. sempervirens* to be known by the boxwood industry. Various hybrids are also known to do well in terms of growth and resilience and the commonly used hybrids are crosses between *B. sempervirens* and *B. microphylla* including *B. microphylla* var. japonica, the Japanese boxwood, *B. sinica* var. insularis (syn. *B. microphylla* var. korean) which is also known as the “Wintergreen” boxwood as it can tolerate low temperature conditions (Daughtrey 2019).

### 1.2. *Colletotrichum* spp.

*Colletotrichum* is one of the most common plant pathogenic fungi infecting economically important crops and is distributed worldwide. It belongs to the Phylum: Ascomycota, Subphylum: Pezizomycotina, Class: Sordariomycetes, Subclass: Sordariomycetidae and Family: Glomerellaceae. The fungus has a wide host range and is responsible for the occurrence of anthracnose diseases. Anthracnose occurs on a large variety of important agricultural crops, affecting both quality and quantity of the produce leading to high yield losses worldwide. According to one study, anthracnose causes drastic yield losses in soybean with an estimation of about 16-26% and 30-50% in the United States and Thailand, respectively and up to 100% losses in Brazil and India (Hartman et al. 1999). It is also considered a huge problem in the horticultural industry. Miles and Schilder (2008) reported that preharvest anthracnose fruit losses in blueberry ranged from 10-20%, whereas postharvest losses can reach up to 100%. This indicates the significance and prevalence of phytopathogenic *Colletotrichum* spp. in agriculture and in the area of plant pathology.
The genus *Colletotrichum* is complex and needs in-depth exploration of molecular and phylogenetic studies to answer questions about its species complex and its genetic variability. Reports have shown association of a *Colletotrichum* species with several plants implying to its association with wide host range. On the other hand, more than one *Colletotrichum* species are known to infect a single host (Da Silva and Michereff 2013; Lima et al. 2013; Schena et al. 2014; Talhinhas et al. 2015). This association may vary from an endophytic relationship to phytopathogenic colonization. This unpredictable and fluctuating behavior and lifestyle of *Colletotrichum* spp. make it a significantly important fungal organism that holds high economic importance in agriculture (Dean et al. 2012).

*Colletotrichum* spp. have been categorized into several species complexes based on the analysis of several markers and phylogenetic studies (Taylor et al. 2000). A number of species complexes have been identified within the *Colletotrichum* genus, including three newly identified complexes i.e. *dracaenophilum*, *magnum*, and *orchidearum* (Marin-Felix et al. 2017; Damm et al. 2019). These species complexes are very difficult to distinguish from each other only on the basis of their morphology. *Colletotrichum theobromicola* Delacr., the causal agent of boxwood dieback belongs to the *Colletotrichum gloeosporioides* species complex which consists of approximately 40 or more closely related species (Jayawardena et al. 2016; Khodadadi et al. 2020; Sharma et al. 2017, Weir et al. 2012).

*Colletotrichum theobromicola* has been categorized as a distinct species in the *Colletotrichum gloeosporioides* species complex based on phylogenetic analyses using multiple loci (Rojas et al. 2010; Weir et al. 2012). *C. theobromicola* was considered as a phytopathogen based on its association with cocoa plants (*Theobroma cacao* L.) (Rojas et al. 2010). Since then,
it has been known to be associated with several other host plants. *C. theobromicola* infection was also reported to cause severe symptoms on eucalyptus in Brazil (Rodrigues et al. 2014).

Accurate identification and differentiation of *C. theobromicola* from other closely related species is challenging. Three commonly used markers to amplify this species are actin (ACT), chitin synthase (CHS-1), and the nuclear ribosomal internal transcribed spacers (nrITS). Additionally, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), calmodulin, manganese-superoxide dismutase (SOD2), β-tubulin 2 (TUB2), glutamine synthetase (GS) (Weir et al. 2012), DNA lyase (APN2), and the intergenic region between DNA lyase and the mating type locus (MAT1-2) (Rojas et al. 2010; Doyle et al. 2013) are some of the other loci that can amplify *C. theobromicola* but differentiation from other closely related species within the *Colletotrichum gloeosporioides* species complex is not assured.

1.3. Boxwood dieback

1.3.1. Disease discovery

Boxwood dieback caused by *C. theobromicola* was first discovered in 2011 in Louisiana from a commercial landscape on *B. microphylla* var. *japonica* (Mull. Arg.) Rehder & E.H. Wilson (R. Singh, *personal communication*). During subsequent years, the disease was also reported from several U. S. states including North Carolina, New York, Virginia, South Carolina (Singh et. al 2015), and Texas (Hawk et. al 2017). During the fall of 2011, symptomatic boxwood plants were collected from a commercial landscape in East Baton Rouge parish and processed at the LSU AgCenter’s Plant Diagnostic Center. Tissue from symptomatic plants consistently produced a fungus and identified as *Colletotrichum* sp. based on fungal
morphological characters. To further identify the fungus, DNA from single spore isolates (PDC11290, PDC11305, and PDC14313) was extracted and then amplified using three genetic markers i.e. actin (ACT), chitin-synthase (CHS-1), and internal transcribed spacer region (ITS). Multiple loci amplification and sequencing resulted into species identification as *C. theobromicola*. Pathogenicity tests were conducted by inoculating healthy boxwoods with isolate PDC11290 of *C. theobromicola*. A seven-day-old culture of isolate PDC11290 was grown on ¼-strength PDA at 28°C. Ten Japanese boxwoods were inoculated by forming a rectangular wound of 25 × 5 mm on the stem. A same sized culture agar strip with the fungus was placed on the wound such that the fungal mycelium gets in direct contact with the wood tissue underneath the bark. It was secured with parafilm to cover the entire wound. All inoculated plants were maintained in the greenhouse at 28 ± 2°C along with ten control plants inoculated with agar strips without the fungus. Plants inoculated with the fungus successfully showed dieback symptoms after about three months of inoculation. The fungus was re-isolated to prove the Koch’s postulate. Control plants remained symptomless (this work was completed by Dr. Singh from 2011 to 2014).

Previous literature showed the association of *Colletotrichum* spp. with boxwood (Holcomb 1967). Farr et al. (1989) also proved the pathogenic behavior of *Colletotrichum* sp. in boxwoods, causing a disease known as limb blight.

1.3.2. Disease symptoms

Infected boxwood plants initially exhibit light green colored foliage on the affected twigs (Fig. 1.1.A). As the disease progresses, leaves turn tan-colored and tend to remain attached to the affected twigs. Infection causes bright dark black discoloration of the affected twigs beneath the
bark, which extends along the affected stems and branches (Fig. 1.1.B). Diseased plants exhibit random dieback in the canopy (Fig. 1.1.C). Symptomatic plant parts produce salmon colored conidia and black fungal acervuli when kept in a moist chamber for a week (Singh and Doyle 2017).

The aboveground symptoms produced by \textit{C. theobromicola} in boxwoods are very similar to those produced by Phytophthora root rot. This resemblance can easily lead to misdiagnosis of the disease, which in turn results in implementation of ineffective management recommendations and mismanagement of the disease (Singh and Doyle 2017). Similar response or symptoms could be produced as a result of other abiotic stresses.

Figure 1.1. \textit{B. microphylla} “Baby Gem” infected with boxwood dieback caused by \textit{C. theobromicola} exhibiting (A) early symptoms of light green foliage in the middle of the canopy, (B) bright black discoloration of internal tissue and (C) random dieback with tan colored foliage.

1.3.3. Disease management

Management strategies to tackle boxwood dieback are limited as it is a recently discovered disease. Moreover, there is a high possibility of misdiagnosing the disease as its
symptoms resemble other diseases, such as Phytophthora root rot as well as abiotic stresses including cold or herbicide injury. At present growers are recommended to follow good agricultural practices to prevent or mitigate the disease development and spread in nurseries and landscapes.

Affected or symptomatic plants should be completely removed and destroyed. Removing only the affected parts of the plant such as symptomatic twigs and branches does not help as the pathogen is systemic in the affected plants. Furthermore, clean and disinfested tools in nurseries and landscapes are recommended for pruning and cutting boxwoods. Growers must be careful and avoid any injury to the plants, as injuries could cause stress to the plants making them more prone to the disease. Conducting regular diagnosis of boxwood plants for early and accurate detection of the disease is another approach to manage the disease.

1.4. Problem and approach

Boxwood is an important perennial landscape shrub that is used worldwide for its evergreen growth and resilient nature. Since boxwood dieback was first reported in the United States in 2015, it has been spreading at an alarming rate throughout the country. Lack of knowledge of disease epidemiology and effective management strategies helps the pathogen to spread rapidly in the boxwood industry. Discussions among plant pathologists and boxwood producers have shown that boxwood is at greater risk in both nurseries and landscapes to boxwood dieback. Boxwood dieback threatens the multimillion-dollar boxwood industry in the United States.

Moreover, detection of boxwood dieback through conventional methods takes longer time, which can lead to delayed management of the disease. Therefore, new diagnostic tools
need to be developed to detect the disease accurately and rapidly at early growth stages to mitigate disease losses. Previous studies have shown that it may take up to two to three months for the symptoms to develop under greenhouse conditions which raises great concerns regarding the disease due to its delayed onset of symptoms. Additionally, boxwood dieback can easily be misdiagnosed with Phytophthora root rot due to similar aboveground symptoms in plants. Developing rapid and accurate diagnostic tools for the identification of boxwood dieback at early growth stages is necessary to prevent the increased cost of production resulting from misdiagnosis until effective disease control measures can be developed. Other aspects of disease management including host range studies, and information pertaining to fungicide efficacy to manage boxwood dieback disease is lacking.

The primary objectives of this research project were to develop a reliable detection method for boxwood dieback and devise an effective disease management strategy, including studying host susceptibility and fungicide efficacy. This study provides critically needed information on main aspects of the disease necessary to reduce economic losses for commercial wholesale, retail trade, and private stakeholders in Louisiana and all over the United States. The aim of this study is to save the economically important boxwood industry.
CHAPTER 2. A DIAGNOSTIC TAQMAN REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF COLLETOTRICHUM THEOBROMICOLA, CAUSAL AGENT OF BOXWOOD DIEBACK

2.1. Introduction

Boxwood (Buxus spp. L.) is a common and popular landscape plant in the United States and other parts of the world. Although it’s considered to be hardy, boxwood is susceptible to several diseases and disorders. Several foliar blights and root rots are known to affect boxwood, including an emerging disease in the United States, boxwood dieback, caused by Colletotrichum theobromicola Delacr. It has now been reported in the United States from Louisiana, New York, North Carolina, South Carolina, Virginia (Singh et al. 2015; Singh and Ratcliff 2016), and Texas (Hawk et al. 2018). Symptoms produced by boxwood dieback has been discussed in chapter 1 section 1.3.2 (pages 6-7) (Figs. 1.1 A, B and C). Because boxwood dieback produces aboveground symptoms similar to those reported for Phytophthora root rot caused by Phytophthora spp. (Singh and Doyle 2017), visual disease diagnosis may be inaccurate and result in costly and ineffective management. Although the pathogen can be easily isolated from affected twigs, previous studies have shown that it may take two or more months for symptoms to appear after inoculation under greenhouse conditions (Hawk et al. 2018; Singh et al. 2015). This delay in the onset of disease symptoms may lead to an unwitting dissemination of the causal agent. It also precludes the development of a monitoring system that would be useful for quarantine purposes. Therefore, an accurate and rapid diagnostic tool to detect C. theobromicola in suspected boxwood plant material at early growth stages is required so that boxwood producers can implement effective disease management strategies and prevent the spread of the disease.
The pathogen causing boxwood dieback, *C. theobromicola* belongs to the *C. gloeosporioides* (Penz.) Penz. & Sacc. species complex, which consists of about 40 or more closely related species (Jayawardena et al. 2016; Khodadadi et al. 2020; Sharma et al. 2017; Weir et al. 2012). Differentiating among species within the species complex is challenging due to a lack of distinctive morphological features. Studies have been conducted to identify specific markers to differentiate *Colletotrichum* species and suggested that three loci, APN2/MAT-IGS, GAP2-IGS, and APN2 are most suitable for reliably assigning isolates to a species within the *Colletotrichum gloeosporioides* species complex (Vieira et al. 2019). However, no particular marker has been found to target *C. theobromicola* specifically. The main objective of this study was to develop a diagnostic *TaqMan* real-time PCR assay for early in planta detection and quantification of *C. theobromicola* in boxwood as sequencing multiple loci for accurate diagnosis of the disease is more expensive and more effort than a single qPCR reaction. Moreover, this will eliminate an additional step of isolation of the pathogen, which is time consuming and delays effective management measures.

2.2. Materials and Methods

2.2.1. Fungal isolates

The *C. theobromicola* isolates used in this study were obtained from infected boxwood plants collected from eight states in the United States, including AL, LA, MO, NC, OK, SC, TX, and VA (Table 2.1). The pathogen was isolated from symptomatic tissue as described previously (Singh et al. 2015; Singh and Doyle 2017). Ten-day old, single-spore cultures grown on quarter-strength potato dextrose agar media (¼ PDA) at 28°C were used for DNA extractions followed by PCR testing. *Colletotrichum theobromicola* isolated from hosts other than boxwood (i.e. *Anacardium occidentale* L. and *Theobroma cacao* L. from Brazil and Panama, respectively),
were also included in this study to confirm that the designed primers and probes are robust to genetic variation within \textit{C. theobromicola} and not just to the known haplotypes from boxwood (Table 2.1). Other \textit{Colletotrichum} species within the \textit{C. gloeosporioides} species complex including \textit{C. chrysophilum} (PN3) W.A.S. Vieira, W.G. Lima, M.P.S. Câmara & V.P. Doyle; \textit{C. fructivorum} (21ss) V. Doyle, P.V. Oudem & S.A. Rehner; \textit{C. gloeosporioides} (Coll20); \textit{C. nupharicola} (NJ2B) D.A. Johnson, Carris & J.D. Rogers; \textit{C. rhexiae} (CH6-2) Ellis & Everh., and \textit{C. siamense} (Coll126) Prihastuti, L. Cai & K.D. Hyde, were also tested in this study to determine primer and probe specificity. In addition to culture isolates, DNAs extracted from symptomatic plant (bark) tissues were also tested to detect \textit{C. theobromicola} from boxwoods. Bark tissue DNA were extracted from healthy or asymptomatic boxwood plants, greenhouse plants inoculated with \textit{C. theobromicola}, and nursery and landscape plants affected by boxwood dieback.

Table 2.1. \textit{C. theobromicola} isolates tested from different locations and hosts using \textit{TaqMan} qPCR assay

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Location</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDCColl I</td>
<td>Alabama (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>Isolate 136</td>
<td>Brazil</td>
<td>Cashew</td>
</tr>
<tr>
<td>PDC18454</td>
<td>Louisiana (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>PDC18029</td>
<td>Missouri (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>PDC14485</td>
<td>North Carolina (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>PDC19024A</td>
<td>Oklahoma (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>GJS08-43E2</td>
<td>Panama</td>
<td>Cocoa</td>
</tr>
<tr>
<td>GJS08-48E2</td>
<td>Panama</td>
<td>Cocoa</td>
</tr>
<tr>
<td>PDC19042</td>
<td>South Carolina (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>PDC17484</td>
<td>Texas (USA)</td>
<td>Boxwood</td>
</tr>
<tr>
<td>PDCColl D</td>
<td>Virginia (USA)</td>
<td>Boxwood</td>
</tr>
</tbody>
</table>
2.2.2. DNA extraction from single-spore isolates and infected plant material

DNA extractions from single-spore cultures of eight United States isolates were performed using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer guidelines with following modifications. Single-spore isolates were grown on ¼ PDA at 28°C for 10 days. Mycelium from each isolate was scraped and added individually to 2 ml fast-prep lysing matrix A tubes (MP Biomedicals, LLC., Irvine, CA) for DNA extraction. DNA samples were stored at -17°C. The extracted DNA quality and quantity were determined using a NanoDrop™ 2000c spectrophotometer using 2 μl of DNA sample (Thermo Fisher Scientific Inc., Wilmington, DE) at the ratio of absorbance at 260 and 280 nm. DNA from known *C. theobromicola* infected boxwood collected from landscape, nursery, and greenhouse and asymptomatic plants was extracted from 100 mg of bark tissue (not the woody tissue) using the DNeasy Plant Mini Kit. DNA was stored and quantified as described above. DNAs of *C. theobromicola* from cashew (Brazil) and cocoa (Panama) along with the DNAs of other *Colletotrichum* species from the *C. gloeosporioides* complex were obtained from a DNA collection in our laboratory.

2.2.3. Primers and probe design

Known sequences of *Colletotrichum* species within the gloeosporioides complex were retrieved from the GenBank database (NCBI, Bethesda, MD) using accession numbers obtained from Jayawardena et al. 2016, Vieira et al. 2017, and Weir et al. 2012. Previous studies reported 11 different markers suitable for differentiating *Colletotrichum* species (Doyle et al. 2013; Rojas et al. 2010; Vieira et al. 2017; Weir et al. 2012). Previously published sequences representing 11 different markers, including nuclear ribosomal internal transcribed spacers (nrITS), β-tubulin (TUB2), actin (ACT), intergenic spacer between GAPDH and a hypothetical protein (GAP2-
IGS), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), manganese-superoxide dismutase (SOD2), DNA lyase (APN2), calmodulin (CAL), glutamine synthetase (GS), chitin synthase (CHS-1), and the intergenic spacer between DNA lyase and the mating type locus MAT1-2-1 (APN2/MAT and APN2), were used to design species-specific primers for detection of C. theobromicola. The sequences were aligned and compared using MAFFT Sequence Alignment online version 7 (https://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2013; Katoh et al. 2019; Kuraku et al. 2013) with advanced iterative refinement settings of G-INS-i. The parameters chosen for aligning the target sequences were as follows: Scoring matrix for nucleotide sequences = 200PAM/K = 2; Gap opening penalty = 1.53; Offset value = 0.0. Aligned sequences were visualized using AliView (Larsson 2014). The number of sequences across the multiple sequence alignments of the 11 different markers ranged from 43 to 260 sequences per alignment (mean = 155) representing a range of 7 to 40 species (mean = 30).

Comparative analysis of all the loci was carried out where data across multiple species were available. The alignments were analyzed visually as well as using a software toolset for deciphering and managing biological sequences using the R programming language (DECIPHER R package) (Wright 2015). The DECIPHER R package designed various primer sets from 11 selected markers based on several variables using the DesignPrimers function. Markers that produced an amplicon size less than 100 bp or greater than 300 bp, and markers that amplified species other than C. theobromicola were excluded. Three loci (CAL, APN2/MAT and APN2) that resulted in species-specific amplification were shortlisted based the appropriate amplicon size and target specificity. Out of these three loci, CAL was selected based on empirical amplification of DNA from a range of isolates of C. theobromicola that were isolated from boxwoods as well as other hosts.
The target CAL fragments between the newly designed primers were analyzed with the PrimerQuest tool (Integrated DNA Technologies) to develop a TaqMan fluorescent probe. Based on the fragment length (up to 30 bp i.e. 23), GC content (30-80% i.e. 56.5%) and melting temperature ~10°C higher than the primer Tm (i.e. 61.3°C), a probe was designed. This TaqMan probe was labeled with a fluorescent reporter dye (6 FAM) at the 5′-end and a non-fluorescent quencher dye (3 BHQ) at the 3′-end.

2.2.4. Primer specificity

Conventional PCR

The polymerase chain reactions were carried out using a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mix for PCR consisted of the following reagents for a single reaction forming a total reaction volume of 25 μl: 12.5 μl Go Taq Green Master Mix (Promega, Madison, WI), 8.5 μl nuclease-free water (Qiagen Inc.), 1 μl of each forward (10 μM) and reverse (10 μM) primers i.e. CAL\textsubscript{Ct}F and CAL\textsubscript{Ct}R (Integrated DNA Technologies, Inc.), and 1 μl DNA template in 0.2 ml Eppendorf PCR Tubes (Eppendorf, Inc., Hamburg, Germany). Nuclease-free water was used as negative control. The following PCR parameters were used to amplify target DNA; 4 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50°C and 45 s at 72°C, and a final extension at 72°C for 7 min. The PCR products were visualized on a 2% agarose gel run at 110 V for 30-40 min.

Real-time TaqMan PCR assay

A Bio-Rad CFX real-time PCR detection system (Bio-Rad Laboratories) was used to perform quantitative PCR in 96 well PCR plates (Bio-Rad Laboratories). Each reaction was performed with a total volume of 25 μl, containing Omnimix™ HS lyophilized PCR master mix (Takara Bio Inc., Shiga, Japan) (one bead for two reactions), 1.25 μl (10 μM) of each forward
and reverse primers (CALCtF and CALCtR, respectively), 1 μl (10 μM) TaqMan probe (CALCtP) (Integrated DNA Technologies), 19 μl nuclease-free H2O, and 2.5 μl of template DNA using the following qPCR protocol: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 63.5°C for 30 s. Standard curves were generated by amplifying 10-fold serial dilutions of C. theobromicola isolate PDCColl I (DNA from culture) and PDC20027-P5 (DNA from C. theobromicola infected boxwood plant tissue).

2.3. Results

2.3.1. Primers and probe

The species-specific forward CALCtF (5’AAG GTC AGC TAC GAG AAT GTT3’) and reverse CALCtR (5’GAC GCA TAC CAA TTG TAA TCG ATA CT3’) primers were designed and the target region was analyzed with the IDT PrimerQuest tool to design a species specific TaqMan probe CALCtP (5’CTG CTG CGG TCG ATG TTG ACT CT3’) (Fig. 2.1). The TaqMan probe was labeled with a fluorescent reporter dye (6 FAM) at 5’-end and a non-fluorescent quencher dye (3 BHQ) at the 3’-end.
Figure 2.1. Screenshot of calmodulin alignments of *C. theobromicola* and other closely related *Colletotrichum* species in gloeosporioides species complex highlighting newly designed species-specific probe CALCtP sequence that will bind selectively to *C. theobromicola* due to differences among species in this region.

### 2.3.2. Primers specificity

The newly designed primers CALCtF and CALCtR were tested for their specificity against the NCBI database using the primer blast global alignment algorithm. Top searches that aligned with the primers were *C. theobromicola* with 100% identity and 100% query coverage. This gave a confirmation that the primers do not bind to any distantly related species and validates primer specificity. The primers CALCtF and CALCtR amplified a 226 bp PCR product with DNA extracted from *C. theobromicola* cultures as well as DNA extracted from symptomatic boxwood plant tissue (Figs. 2.2 and 2.3). No amplification was observed for the
non-template control. To further check the specificity of primers CAL\textit{Ct}F and CAL\textit{Ct}R, closely related \textit{Colletotrichum} species from the \textit{C. gloeosporioides} complex including, \textit{C. chrysophilum}, \textit{C. fructivorum}, \textit{C. gloeosporioides}, \textit{C. nupharicola}, \textit{C. rhexiae}, and \textit{C. siamense} were tested using conventional PCR and did not result in amplification (Fig. 2.2). The results from conventional PCR showed that the primers CAL\textit{Ct}F and CAL\textit{Ct}R are species-specific and only amplified DNA of \textit{C. theobromicola}, including those isolated from cashew, cocoa, boxwoods, and symptomatic boxwood plant tissue. No amplification was observed with DNAs extracted from healthy or asymptomatic boxwood bark tissues (Fig 2.3).

Figure 2.2. A 2\% agarose gel showing amplification of \textit{C. theobromicola} DNA (L2 to L12) isolated from 10 d old cultures and non-amplification of other closely related \textit{Colletotrichum} species in the \textit{C.gloeosporioides} species complex (L13 to L18) using species-specific primers CAL\textit{Ct}F/CAL\textit{Ct}R. M: marker (50-1000 bp); L1: non template control; L2: PDC Coll I (\textit{Buxus} sp.: Alabama); L3: Isolate 136 (\textit{Anacardium occidentale}: Brazil); L4: PDC18451 (\textit{Buxus} sp.: Louisiana); L5: PDC18029 (\textit{Buxus} sp.: Missouri); L6: PDC14485 (\textit{Buxus} sp.: North Carolina); L7: PDC19024A (\textit{Buxus} sp.: Oklahoma); L8: GJS08-43 (\textit{Theobroma cacao}: Panama); L9: GJS08-48 (\textit{Theobroma cacao}: Panama); L10: PDC19042 (\textit{Buxus} sp.: South Carolina); L11: PDC17484 (\textit{Buxus} sp.: Texas); L12: PDCColl D (\textit{Buxus} sp.: Virginia); L13: PN3 (\textit{C. chrysophilum}); L14: CH6-2 (\textit{C. rhexiae}); L15: NJ2B (\textit{C. nupharicola}); L16: Coll 126 (\textit{C. siamense}); L17: 21ss (\textit{C. fructivorum}); L18: Coll20 (\textit{C. gloeosporioides}). *L stands for lane.
Figure 2.3. A 2% agarose gel showing amplification of *C. theobromicola* DNA (L4 to L9) from symptomatic plant tissue and non-amplification of healthy or asymptomatic plant tissue (L2 and L3) using species-specific primers CALCtF/CALCtR. M: marker (50-1000 bp); L1: non-template control; L2: PDC20026-P1 (asymptomatic boxwood from nursery); L3: PDC20026-P2 (asymptomatic boxwood from greenhouse); L4: PDC20027-P1 (symptomatic boxwood from nursery); L5: PDC20027-P2 (symptomatic boxwood from nursery); L6: PDC20027-P3 (symptomatic boxwood from landscape); L7: PDC20027-P4 (symptomatic boxwood from landscape); L8: PDC20027-P5 (symptomatic boxwood from greenhouse); L9: PDC20027-P6 (symptomatic boxwood from greenhouse).
* L stands for lane.

2.3.3. Real-time *TaqMan* PCR assay

The real-time PCR conducted with primers CALCtF, CALCtR and probe CALCtP amplified culture DNA from *C. theobromicola* isolates from ten different locations listed in Table 2.1 (Fig. 2.4). The no template control and other *Colletotrichum* species from the *Colletotrichum gloeosporioides* species complex did not amplify and no fluorescence was observed. The threshold cycle (Ct) value for *C. theobromicola* culture DNA ranged from 16.13 to 25.57 with an average of 21.35 cycles (Appendix 1).

A standard curve was generated by serially diluting PDCColl I. isolate of *C. theobromicola* DNA with concentrations ranging from 14000 to 1.4 pg μl⁻¹ (Fig. 2.5). A linear
relationship between DNA quantity and the Ct value was observed with $y = 14.820$, $R^2 = 0.999$, a slope of -3.488 with a 93.5% PCR assay amplification efficiency (Fig 2.6). The lowest DNA concentration that the designed primers and probe were able to detect was 1.4 pg µl$^{-1}$.

Additionally, the *TagMan* real-time PCR amplified *C. theobromicola* from DNA extracted from bark collected from the transition zone between healthy and symptomatic tissue from known boxwood dieback affected plants collected from nursery (PDC20027-P1, PDC20027-P2), landscape (PDC20027-P3, PDC20027-P4) and from artificially inoculated boxwoods in the greenhouse (PDC20027-P5, PDC20027-P6) (Fig. 2.7). No amplification was observed from DNA extracted from asymptomatic boxwoods (PDC20026-P1, PDC20026-P2). The Ct value for plant tissue ranged from 22.32 to 27.16 with an average of 24.27 cycles.

A standard curve for symptomatic boxwood plant tissue was generated by serial dilution of plant PDC20027-P5 DNA concentrations ranging from 2790 to 2.7 pg µl$^{-1}$ and resulted in a slope of -3.501 and $R^2$ value of 0.999 with a 93% PCR efficiency (Figs. 2.8 and 2.9). The lowest detection level with symptomatic plant DNA tissue was determined to be 2.7 pg µl$^{-1}$. 
Figure 2.4. Amplification curves of *C. theobromicola* DNAs from 11 fungal isolates listed in Table 1 using species-specific primers CALCtF/CALCtR and TaqMan probe CALCtP. No amplification was observed from DNA samples isolated from *C. chrysophilum*, *C. fructivorum*, *C. gloeosporioides*, *C. nupharicola*, *C. rhexiae*, and *C. siamense*.

Figure 2.5. Amplification of a 10-fold serial dilution of *C. theobromicola* fungal isolate PDCColl I with DNA concentrations ranging from 14000 pg to 1.4pg.
Figure 2.6. A standard curve generated by amplification of 10-fold serial dilution of *C. theobromicola* fungal isolate PDCColl I with \( E = 93.5\% \), \( R^2 = 0.999 \), and Slope = -3.488.

Figure 2.7. Amplification curves of *C. theobromicola* DNAs tested from plant tissue (PDC20027-P1, PDC20027-P2, PDC20027-P3, PDC20027-P4, PDC20027-P5 and PDC20027-P6) using species-specific primers CALCtF/CALCtR and TaqMan probe CALCtP. No amplification was observed from DNA samples isolated from asymptomatic plant tissue (PDC20026-P1 and PDC20026-P2).
Figure 2.8. Amplification of a 10-fold serial dilution of *C. theobromicola* DNA PDC20027-P5 (boxwood plant inoculated with *C. theobromicola* in the greenhouse) extracted from plant tissue (bark) with DNA concentrations ranging from 2790 pg to 2.79 pg.

Figure 2.9. A standard curve generated by amplification of ten-fold serial dilution of *C. theobromicola* isolate PDC20027-P5 (boxwood plant inoculated with *C. theobromicola* in the greenhouse) with E = 93%, R² = 0.999, and Slope = -3.501.
2.4. Discussion

Boxwood dieback caused by *C. theobromicola* is an emerging disease in the nursery and landscape industry in the United States. The pathogen is spreading at alarming rates and affecting the boxwood industry negatively. The exact impact of the disease is not known due to its recent discovery, but a number of boxwood growers and breeders have presented their concerns at various regional and national horticulture industry meetings. Detection of boxwood dieback in the early stages of disease development is an important step towards implementing the disease management strategies and to prevent further spread in the surrounding plantations or in boxwood liners in the nurseries. Delayed onset of symptoms produced by boxwood dieback is a challenge for early and accurate detection. Under greenhouse conditions, artificial inoculation of healthy boxwoods resulted in initial symptoms of light-green or chlorotic foliage in as long as six weeks after inoculation followed by dieback symptoms after 12 weeks of inoculation (Singh et al. 2015). Previously, De Silva et al. (2017) summarized the quiescence or latent behavior of various species in the *C. gloeosporioides* species complex. The same phenomenon may explain the delayed symptom development by *C. theobromicola* infections in boxwoods.

Current diagnosis of boxwood dieback relies on isolation of the pathogen followed by amplification and sequencing of multiple loci, including ACT (actin), CHS-1 (chitin synthase), and ITS (internal transcribed spacer region) (Singh et al. 2015). Morphological characteristics to identify *C. theobromicola* are not considered reliable as these features can change with successive sub-culturing, variable growing conditions and time (Weir et al. 2012). These methods are expensive and time consuming and may take up to 3-4 weeks for accurate identification. Additionally, delayed diagnosis may lead to accidental spread of the pathogen through pruning with contaminated tools, overhead irrigation practices, and other poor cultural
practices. In order to avoid delayed diagnosis and further transmission of the disease in nurseries and landscapes, a rapid and reliable disease detection method is critically warranted.

Therefore, the main goal of our study was to develop a diagnostic assay for rapid, accurate, and reliable detection and quantification of *C. theobromicola* in the early stages of disease development. The diagnostic *TaqMan* real-time PCR assay developed in this study successfully detected *C. theobromicola* from DNA isolated from both culture isolates as well as symptomatic boxwood bark tissue. This assay is species-specific as it only amplified *C. theobromicola* from boxwood, cashew and cocoa. The primers CALCtF/CALCtR and the probe CALCtP did not amplify *C. chrysophilum, C. fructivorun, C. gloeosporioides, C. nupharicola, C. rhexiae, and C. siamense* (Fig. 2.2). The assay is highly sensitive and detected *C. theobromicola* at early infection stages from boxwood liners and from fully grown boxwood plants with a quantification limit of 1.4 and 2.79 pg of fungal DNA from culture and plant tissue, respectively. The newly designed calmodulin primers CALCtF and CALCtR amplified a 226 bp fragment of *C. theobromicola* both from culture isolates and plant bark tissue. Calmodulin, along with other markers, have been tested in previous studies for identification of *Colletotrichum* species within the *Colletotrichum gloeosporioides* species complex (Vieira et al. 2019) and was not considered as the best marker for species differentiation. However, the calmodulin gene does contain unique sequence motifs that allowed us to successfully distinguish *C. theobromicola* from other closely related species in this study.

The efficiency of the newly developed *TaqMan* real-time PCR is within acceptable limits between 90–100% (−3.6 ≥ slope ≥ −3.3) (qPCR Efficiency Calculator; Thermoscientific: https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-
web-tools/qpcr-efficiency-calculator.html). These parameters indicate that the primers and *TaqMan* probe we have designed enable the accurate detection as well as quantification of *C. theobromicola* in boxwoods, thereby leading to a newly designed approach for boxwood dieback detection. The specificity, sensitivity, and PCR efficiency discussed above validate the new *TaqMan* real-time PCR to detect *C. theobromicola*, causal agent of boxwood dieback. The conventional and real-time diagnostic assays can serve as an essential tool in Plant Diagnostic Clinics for an easy and rapid detection of boxwood dieback as well as in Research Labs to conduct advanced studies on this pathogen using the species-specific primers and probe. The results of this study will not only help in the diagnosis of affected boxwoods with boxwood dieback but will also help in identifying *C. theobromicola* in/from other host plants.

Early and accurate detection is critical in managing this pathogen known to cause delayed visible symptoms. Management of boxwood liners infected with *C. theobromicola* at early stages of the disease development may stop the disease in nurseries and prevent it from being introduced into landscapes. Accurate detection will help landscapers and nursery growers implement effective disease management strategies and reduce the costs associated with boxwood cultivation. Currently, boxwood dieback has been officially and unofficially reported from several states in the United States and has led to significant losses. This newly developed assay provides the boxwood industry with a rapid and reliable diagnostic tool to detect boxwood dieback at early stages of the disease development.
CHAPTER 3. SCREENING OF BOXWOOD CULTIVARS TO DETERMINE HOST RANGE OF BOXWOOD DIEBACK

3.1. Introduction

Recently discovered boxwood dieback has created a great concern in the boxwood nursery and landscape industry due to its rapid spread in several parts of the United States. Effective management strategies to prevent further disease spread are currently lacking. Due to limited knowledge of disease management strategies, growers are recommended to adopt good cultural practices to create unfavorable conditions for the survival of the pathogen and to reduce its spread in nurseries and landscapes. Since, no control methods have previously been described in the literature to manage *C. theobromicola* in boxwoods, this study focuses on determining host plant resistance as an alternate method for disease prevention. Host resistance has been proven to be a major strategy to fight against *Colletotrichum* species in agriculture (Goswami et al. 2011). A study conducted by Mahto et al. in 2020, shows development of transgenic chili and tomato lines to incorporate resistance against anthracnose caused by *Colletotrichum* sp. Results of their studies revealed successful identification and development of host resistance that can be used to prevent spread of the pathogen.

Although, an association of *Colletotrichum* species with *Buxus* species as an endophyte has previously been established (Liu et al. 2007), but *C. theobromicola* as a pathogen has never been reported in the Buxaceae prior to 2015 (Singh et al. 2015). Boxwood dieback caused by *C. theobromicola* is the first report of this fungus causing disease and killing infected boxwoods (Singh et al. 2015). So far, all the commonly grown boxwood cultivars including English, Japanese, and Korean boxwoods are found to be susceptible to boxwood dieback (Singh et al. 2017). Previously, studies have been conducted where boxwood cultivars were compared for
their susceptibility to another important boxwood disease called boxwood blight caused by *Calonectria pseudonaviculata* (Ganci et al. 2013). The literature also shows that some cultivars are resistant to boxwood blight and can be used as a possible strategy to protect boxwoods against boxwood blight (Ruhl et al. 2018). In the case of boxwood dieback, the information on susceptibility of boxwood cultivars to this disease is lacking and presents great challenges for boxwood growers to select more resilient cultivars. The main objective of this host range study was to determine the susceptibility of available boxwood cultivars against the pathogen *C. theobromicola*, and to identify naturally resistant/tolerant cultivars available in the boxwood industry.

3.2. Materials and Methods

3.2.1. Colletotrichum theobromicola isolate

The *C. theobromicola* fungal isolate PDC11305 used in this study was isolated from symptomatic boxwood plants obtained from a commercial landscape in East Baton Rouge parish in Louisiana in 2011. The fungus was isolated from symptomatic plant tissue and grown on quarter strength potato dextrose agar medium (¼ PDA) as described by Singh et al. in 2015. The isolate PDC11305 was revived from long term storage and plates were incubated at 28°C for seven to ten days to produce fungal inoculum.

3.2.2. Boxwood cultivars

Eleven boxwood cultivars were used in this experiment. Out of these 11, four cultivars including, *B. sempervirens* ‘English boxwood’, *B. microphylla var. japonica* ‘Japanese boxwood’, *B. microphylla var. korean* ‘Korean boxwood’ and *B. microphylla var. japonica* ‘Gregem’ also known as ‘Baby Gem’ are commonly available in the Louisiana boxwood industry.
Other cultivars screened in the host range study were *Buxus* x 'Conrowe ‘Gordo’ PP#19924, *B. sempervirens* ‘Dee Runk’, *B. microphylla* ‘Winter Gem’, *B. sempervirens* × *B. microphylla* var. korean ‘Green Velvet’, *B. sinica* ‘Sunburst’, *B. sempervirens* ‘Green Ice’, and *B. microphylla* ‘Little Missy’. These boxwood genotypes were maintained in a greenhouse at 27-30°C and 85% relative humidity. Plants were observed for six months for boxwood dieback symptom development. The experimental plants were a combination of liners (‘Dee Runk’, ‘Green Velvet’ and ‘Sunburst’ – 3.78 liter pots) and fully matured plants (‘Baby Gem’, ‘English’, ‘Gordo’, ‘Green Ice’, ‘Japanese’, ‘Korean’, ‘Little Missy’, and ‘Winter Gem’ – 11.35 liter pots).

### 3.2.3. Inoculation methods

Boxwood cultivars were inoculated with a 7-10-day old single spore culture of *C. theobromicola* isolate PDC11305 as described by Singh and Doyle (2017). The cultures were grown on ¼ PDA and incubated at 28°C to prepare the inoculum. A rectangular wound (approximately 2.5 cm in length) was made on the stem and the bark was pulled back to expose the woody tissue. Agar strips of the same size as the wound were taken from culture of isolate PDC11305 and placed on the wounds. The culture strips were placed on the wound so that the fungal mycelia face the woody tissue of the plant and the strip was sandwiched between the woody tissue and the bark. The wound was sealed by wrapping Parafilm around the plant stem. Controls plants were inoculated as described above with agar strips without fungal mycelia.

### 3.2.4. Greenhouse experiment setup

Three plants each of the 11 boxwood cultivars were inoculated with *C. theobromicola* agar strips in addition to two control plants that were inoculated with agar strips only (without fungus) in December of 2019. The inoculated and control plants were maintained at 27-30°C and
85% relative humidity in a greenhouse. Plants were examined regularly for any symptom development. The entire experiment was repeated again in March 2020.

3.3. Results

Boxwood cultivars ‘Sunburst’ (Fig. 3.1) and ‘Dee Runk’ were the first to show symptoms with 30 days after inoculation. Inoculated branches turned light green and gradually became tan colored within 60 days, with extended black discoloration beneath the bark above and below the area of inoculation. In case of cultivar ‘Green Velvet’ all inoculated and control plants died within 30 days after inoculation. Inoculated plants revealed bright black discoloration and *C. theobromicola* was re-isolated. Neither *C. theobromicola* nor *Phytophthora* spp. was isolated from control plants of ‘Green Velvet’.

Four boxwood cultivars, including English, Japanese, Wintergreen and ‘Baby Gem’, developed the initial disease symptoms, consisting of the foliage turning light green within 40-45 days after inoculation. Light green foliage turned to tan colored after 90 days of inoculation and the bright black discoloration was observed beneath the bark on inoculated plants, which extended downward and upward from the point of inoculation into healthy stems resulting in dieback symptoms. Control plants of these cultivars remained healthy and did not develop any boxwood dieback symptoms including tan colored foliage and bright black discoloration of the woody tissue.

Similarly, ‘Winter Gem’ showed prominent dying back of the plants within 60 days of inoculation. Cultivars ‘Green Ice’ and ‘Gordo’ exhibited symptoms after 90 days of inoculation. Inoculated plants of cultivar ‘Little Missy’ remained symptomless even after 90 days of inoculation. The bright black discoloration stayed confined to the inoculated area and did not
extend below or above this area on inoculated plants. There was no random dying back of stems and the plants looked healthy or asymptomatic until the end of the experiment (Fig 3.2).

To confirm if cultivar ‘Little Missy’ was truly resistant/tolerant to boxwood dieback, the plants were re-inoculated using branches that were previously not inoculated and were incubated as described above. After three months of re-inoculation, foliage on both previously as well as newly inoculated branches on ‘Little Missy’ plants began to turn light green and eventually led to the development of tan colored leaves. Bright black discoloration was observed beneath the bark which extended into the healthy plant tissue (Fig 3.3). Control plants remained asymptomatic or healthy.

Irrespective of the time taken for these cultivars to become symptomatic, all the inoculated plants were positive for boxwood dieback. Based on these results, it was concluded that none of the cultivars screened in this host range study are resistant/tolerant to boxwood dieback caused by *C. theobromicola* (Fig. 3.4)
Figure 3.1. Boxwood cultivar ‘Sunburst’ exhibiting symptoms of boxwood dieback caused by *C. theobromicola* in the host range study (A) Control plant showing no extended discoloration from the area on inoculation on the stem (B) Inoculated plant showing dark black discoloration underneath the bark extending towards the healthy woody tissue (C) Random dieback present on the inoculated plant (left) and control plant with no symptoms of dieback (right).
Figure 3.2. Boxwood cultivar *Buxus* ‘Little Missy’ showing no signs of susceptibility to boxwood dieback in host range screening through pathogenicity test (A) Control plant showing no extended discoloration from the area on inoculation on the branch (B) Black discoloration underneath the bark remains restricted to the inoculated area and does not extend towards the healthy wood tissue (C) Comparison of asymptomatic control plant (left) as well asymptomatic pathogen inoculated plant (right) showing no symptoms of boxwood dieback after three months of inoculation.
Figure 3.3. Boxwood cultivar ‘Little Missy’ exhibiting symptoms of boxwood dieback caused by *C. theobromicola* in the host range study (A) Inoculated plant showing dark black discoloration underneath the bark extending towards the healthy woody tissue (B) Random dieback present on the inoculated plants.
3.4. Discussion

Boxwood dieback is an important disease that is difficult to manage due to a lack of effective management practices. Boxwood growers and breeders are advised to adopt good cultural practices including, disinfecting tools, removing and disposing symptomatic plants, and avoiding unnecessary plant injuries while handling and planting to avoid spread of the pathogen in the nursery and landscape planting. These recommended management strategies are general for any plant disease problem and disease specific management practices to manage boxwood dieback are crucial in mitigating disease development and spread in boxwood industry. One of the disease management practice is to breed and incorporate resistant or tolerant varieties or cultivars into the planting systems. Host resistance plays a critical role in disease prevention,
maintain healthy plants, and to prevent further spread of the pathogen. Currently, no information on host resistance/tolerance is available on the association of *Colletotrichum* species and plants in the Buxaceae family.

The genus *Colletotrichum* is very diverse with several different species complexes. *Colletotrichum gloeosporioides* has been observed to affect major nursery plants including several landscape and ornamental plants (Coates et al. 2015). Moreover, previous studies have shown an endophytic relationship between *Colletotrichum* species and members of the Buxaceae family (Liu et al. 2007), but no parasitic behavior has been reported before. Exploring the host range of *C. theobromicola* on boxwoods may lead to the identification of a resistant/tolerant cultivar that can replace other commonly grown highly susceptible cultivars. The results from host range study showed that all the boxwood cultivars screened were susceptible to boxwood dieback. All the inoculated plants exhibited tan colored foliage with dying back of branches. Typical black discoloration was seen extending underneath the bark from the inoculated area. Out of all the boxwood cultivars, ‘Little Missy’ remained asymptomatic for extended periods but eventually produced symptoms 90-135 days after inoculation. This fact suggests that cultivar ‘Little Missy’ may possess tolerance to boxwood dieback disease and may require heavy disease inoculum to produce symptoms. Another possible explanation of delayed symptom production could be attributed to latent infection, where the pathogen is present in or on the host but does not cause symptoms, also known as the latent phase of the pathogen. This suggests that ‘Little Missy’ could have potential to be used as a tolerant cultivar. But, needless to mention that this cultivar should be studied further at molecular level to understand its phylogenetic traits and to have a comprehensive theory on how this cultivar manages to escape *C. theobromicola* infection.
Future pathogenicity screening studies should be carried out to determine if the tolerance shown by ‘Little Missy’ in the greenhouse is true under field conditions.
CHAPTER 4. IN VITRO SCREENING OF FUNGICIDES AGAINST BOXWOOD DIEBACK PATHOGEN, COLLETOTRICHUM THEOBROMICOLA

4.1. Introduction

Boxwood (Buxus spp. L) is among one of the most widely used ornamental plants in the landscape industry. Its hardy nature and evergreen growth makes it a valuable plant in home gardens and commercial landscapes. Despite its hardiness, boxwood is susceptible to various diseases and disorders. One of the recently discovered diseases is boxwood dieback that has affected boxwood industry all over the United States. Boxwood dieback is caused by the fungal pathogen, Colletotrichum theobromicola. It was first reported in the United States from LA, NC, NY, SC, and VA in 2015 (Singh et al. 2015), and from TX in 2018 (Hawk et al. 2018). Since it is a newly discovered disease, effective management strategies to mitigate disease development and spread are lacking. Moreover, the disease can easily be confused with other boxwood diseases especially Phytophthora root rot, which adds up to the difficulty in managing this disease.

Although current management practices include removing symptomatic plants and disinfecting pruning tools in nurseries, information on fungicides for managing boxwood dieback is lacking. Integrating cultural and preventative chemical management practices will help boxwood growers and landscapers combat this disease effectively and profitably. Currently, cultural practices, including planting of disease-free healthy boxwoods, removal of symptomatic plants, and disinfestation of pruning or cutting tools are recommended. At present, knowledge of fungicides in managing boxwood dieback effectively is lacking. Therefore, the objective of this study was to conduct in vitro screening of fungicides to determine their effectiveness in controlling C. theobromicola, causal agent of boxwood dieback.
4.2. Materials and Methods

4.2.1. Fungal isolates for mycelium and spore germination inhibition studies

*Colletotrichum theobromicola* isolate PDC14313 was used for mycelial and spore germination inhibition studies. In addition, screening of eight *C. theobromicola* isolates collected from different states in the United States (Table 4.1) against six fungicides with the greatest inhibition rate was carried out at fungicide concentrations of 1, 5, 10, 25, and 50 ppm of active ingredient as described in the methods used in the mycelial growth inhibition study in section 4.2.2.

Table 4.1. Boxwood dieback *C. theobromicola* isolates collected from eight U. S. states used in isolate screening study

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Location</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDCColl I</td>
<td>Alabama</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC18454</td>
<td>Louisiana</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC18029</td>
<td>Missouri</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC14485</td>
<td>North Carolina</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC19024A</td>
<td>Oklahoma</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC19042</td>
<td>South Carolina</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDC17484</td>
<td>Texas</td>
<td><em>Buxus</em> sp.</td>
</tr>
<tr>
<td>PDCCollD</td>
<td>Virginia</td>
<td><em>Buxus</em> sp.</td>
</tr>
</tbody>
</table>
4.2.2. *In vitro* fungicides screening procedure

Nine fungicides (Table 4.2) that previously reported to have good to excellent efficacy against *Colletotrichum* spp. were tested based on reports from the 2018 Guide to Ornamental Diseases (Syngenta and Chase 2018). For this assay, desired concentrations were prepared from stock solutions (1000 ppm) of each fungicide in autoclaved full-strength potato dextrose agar (PDA) medium. Initially, eight concentrations (0.1, 1.0, 5.0, 10, 25, 50, 75, and 100 ppm) were used for preliminary testing of nine fungicides. Media amended with different fungicides was poured in to 15 X 100 mm petri dishes (VWR International, LLC., Radnor, PA), and left undisturbed to solidify. A two mm diameter mycelial plug was taken from a 7-10-day old culture and placed in the middle of individual fungicide amended PDA media plates. The PDA plates with no fungicides were included as control treatments. Plates were then incubated at 26±2°C under a 12 h photoperiod for seven days. Mycelial growth diameter was measured using a Vernier caliper (General Tools & Instruments, LLC, Secaucus, NJ) at two perpendicular points from the center of the colony. Mean mycelial growth inhibition rates in percentage were calculated. Fungicides that did not inhibit any mycelial growth compared to control (0 ppm fungicides) were taken out of the study and further screening was performed with seven fungicides at concentrations ranging from 0, 0.1, 1, 5, 10, 25, and 50 ppm to determine the LD₅₀ values. Each fungicide concentration treatment was replicated three times and the entire experiment was repeated five times. Mycelial growth was measured after seven days as described above. Spore germination inhibition was performed by preparing a spore suspension from a 7 -10 days old culture of PDC14313. Four fungicides (rated as highly effective from mycelial growth inhibition rate study) at four concentrations ranging from 0, 1, 5, 10, and 25 ppm were used for spore germination inhibition study. Spore suspension of *C. theobromicola*
was serially diluted and a final concentration of 150 spores per 100 µL was poured and spread on fungicide amended media plates. Plates were incubated at 28°C in an incubator. Spore germination readings were taken for seven consecutive days by counting the number of germinated spores on fungicide amended plates as compared to the controls (0 ppm fungicide).

Each fungicide and concentration treatments in this study were repeated five times and the entire experiment was repeated three times.

In addition, the effect of fungicides on mycelial growth of eight *C. theobromicola* isolates collected from various states in the United States (Table 4.1) was carried out with four highly effective fungicides obtained from the fungicide screening assay using the same procedure of mycelial inhibition experiment.

Table 4.2. List of fungicides screened for *in vitro* fungicide efficacy study

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Active Ingredient</th>
<th>Established Efficacy</th>
<th>FRAC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broadform</td>
<td>trifloxystrobin+fluopyram</td>
<td>Unknown</td>
<td>7</td>
</tr>
<tr>
<td>Camelot O</td>
<td>copper octanoate</td>
<td>Good</td>
<td>M1</td>
</tr>
<tr>
<td>Concert II</td>
<td>chlorothalonil+propiconazole</td>
<td>Very good/ Excellent</td>
<td>3 + M5</td>
</tr>
<tr>
<td>Dithane Rainshield 75DF</td>
<td>mancozeb</td>
<td>Very Good</td>
<td>M3</td>
</tr>
<tr>
<td>Mural</td>
<td>azoxystrobin+benzovindiflupyr</td>
<td>Excellent</td>
<td>11 + 7</td>
</tr>
<tr>
<td>Orkestra Intrinsic</td>
<td>fluxapyroxad+pyraclostrobin</td>
<td>Excellent</td>
<td>7 + 11</td>
</tr>
<tr>
<td>Pageant Intrinsic</td>
<td>pyraclostrobin+boscalid</td>
<td>Very good/ Excellent</td>
<td>7 + 11</td>
</tr>
<tr>
<td>Postiva</td>
<td>difenoconazole+pydiflumetofen</td>
<td>Unknown</td>
<td>3 + 7</td>
</tr>
<tr>
<td>26/36 fungicide</td>
<td>thiophanate-methyl+iprodione</td>
<td>Good</td>
<td>1 + 2</td>
</tr>
</tbody>
</table>

### 4.2.3. Assessment of mycelial growth inhibition rate

The fungal mycelial growth inhibition rate ($I_c$) at different fungicide concentrations was calculated as previously described by Nakpalo et al. (2017) using the following formula.

$$I_c = \frac{D_0 - D_c}{D_0} \times 100$$
Where, $D_0$ is the diameter of control (0 ppm) and $D_c$ is the diameter of the fungal growth at a given concentration of the fungicide.

### 4.2.4. Assessment of spore germination inhibition

The number of germinated spores were counted and $I_g$ (spore germination inhibition rate) was calculated using the following equation as described by Nakpalo et al. (2017):

$$I_g = \frac{N_o - N_c}{N_o} \times 100$$

Where, $N_o$ is the number of germinated spores on control i.e. only PDA medium and $N_c$ is the number of germinated spores on a medium containing a given concentration of the fungicide.

### 4.2.5. Statistical analysis

Differences between and within treatment groups and controls were analyzed using analysis of variance (ANOVA) with Tukey-HSD test at 5% for the fungicide efficacy study. The isolate screening was analyzed using ANOVA for factorial in PROC GLM (SAS 9.4, Institute Inc., Cary, NC, 2013) Tukey-HSD at 5% of significance to differentiate means. PROC MIXED (SAS 2013) procedures were used for spore germination screening with fungicides as fixed effects. Tukey-HSD analysis at 5% of significance allowed us to compare the least square means and determine whether fungicides effects were significant for spore germination inhibition.

### 4.3. Results

#### 4.3.1. Fungicide screening

None of the fungicides applied at 0.1 ppm active ingredients were effective. Azoxystrobin+benzovindiflupy, difenoconazole+pydiflumetofen, fluoxapyroxad+pyraclostrobin, and pyraclostrobin+boscalid showed 78%, 78%, 56%, and 54%
mycelial growth inhibition at 1 ppm, respectively. Thiophanate-methyl+iprodione showed 79% mycelial reduction at 5 ppm, whereas propiconazole+chlorothalonil inhibited mycelial growth by 56% at 10 ppm. Fluopyram+trifloxystrobin was the least effective with mycelial inhibition rate of 53% at 50 ppm active ingredient. There was a significant difference ($F = 367.571, p > 0.0001$) in the mycelial growth inhibition rate between the fungicides screened with a coefficient of variation of 1.94% at 5% Tukey-HSD test (Fig 4.1) (Appendix 2).

Difenconazole+pydiflumetofen was the most effective fungicide at 1 ppm active ingredient, whereas broadform was rated the least effective even at higher concentrations of 25 and 50 ppm active ingredient (Fig 4.2).

A significant interaction between fungicides and their concentrations was observed, which implies that the mycelial growth inhibition rate significantly varied at different concentrations of the same fungicide screened. Individual performance in terms of effectiveness in mycelial growth inhibition of each fungicide is shown in figure 4.1.

All fungicides showed a significant higher mycelial growth inhibition when fungicide concentration was increased from 0.1 to 5 to 10 ppm except difenoconazole+pydiflumetofen and trifloxystrobin+fluopyram. These two fungicides show significant mycelial growth inhibition rate when active ingredient concentration was increased from 5 to 10 ppm (Fig 4.1). When the concentration increased from 10 to 25 ppm, all fungicides but fluxapyroxad+pyraclostrobin showed significant difference in mycelial growth inhibition rate. Additionally, no significant difference in mycelial inhibition rate was observed in all fungicides when the concentration was increased from 25 to 50 ppm except chlorothalonil+propiconazole. This fungicide not only showed a significant increase in mycelial inhibition rate when the active ingredient concentration
was increased from 25 to 50 ppm, but every time the concentration was increased the mycelial inhibition rate also increased in a linear pattern.

Based on the mycelial growth inhibition rate percentage, the LD$_{50}$ values of all fungicides were calculated and fungicides were categorized into four groups as highly effective (+++++++), medium effective (++++), least effective (++), and ineffective (-) (Table 4.2).

![Mean mycelial growth inhibition rate (%)](image)

**Figure 4.1.** Mean mycelial growth inhibition rate (%) of *C. theobromicola* at different concentration of seven fungicides screened. Means followed by different letters within each fungicide category are statistically different at Tukey HSD 5% level.
Figure 4.2. Visual comparison of mycelial growth inhibition rate of *C. theobromicola* at different active ingredient concentrations as compared to non-fungicide control; (A): difenoconazole+pydiflumetofen (highly effective); (B): trifloxystrobin+fluopyram (least effective).

Table 4.2. Classification of nine screened fungicides based on their effectiveness to inhibit mycelial growth of *C. theobromicola*.

<table>
<thead>
<tr>
<th>Efficacy range</th>
<th>Effectiveness*</th>
<th>Fungicide (active ingredient)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &lt; 1 ppm</td>
<td>+++++</td>
<td>azoxystrobin+benzovindiflupyr difenoconazole+pydiflumetofen fluxapyroxad+pyraclostrobin pyraclostrobin+boscalid</td>
</tr>
<tr>
<td>1 ppm &lt; IC&lt;sub&gt;50&lt;/sub&gt; &lt; 10 ppm</td>
<td>+++</td>
<td>chlorothalonil+propiconazole thiophanate-methyl+iprodion</td>
</tr>
<tr>
<td>10 ppm &lt; IC&lt;sub&gt;50&lt;/sub&gt; &lt; 25 ppm</td>
<td>++</td>
<td>trifloxystrobin+fluopyram</td>
</tr>
<tr>
<td>25 ppm &lt; IC&lt;sub&gt;50&lt;/sub&gt; &lt; 100 ppm</td>
<td>-</td>
<td>copper octanoate mancozeb</td>
</tr>
</tbody>
</table>

*++++++ Highly effective +++ Medium effective ++ Least effective – Ineffective
4.3.2. Isolate screening

The effects of six (medium to highly effective, Table 4.2) fungicides were determined on eight isolates of *C. theobromicola* isolated from *Buxus* spp. collected from different locations in the United States. These eight isolates were compared to identify any differences or similarities on mycelial growth inhibition rate (%) through ANOVA. No significant difference was observed in the mycelial growth inhibition rate among eight isolates screened at different concentration of six fungicides ($F=0.437$, $p=0.878$). The results were further confirmed by Tukey’s Post Hoc test which showed that there were no significant differences among the isolates collected from eight states within the United States (Fig. 4.3) (Appendix 3). However, within each isolate at different fungicide mean concentrations resulted in significantly different mycelial growth inhibition rates ($F=44.982$, $p\leq0.0001$) (Fig 4.3). These results concur our findings from the mycelial growth inhibition rate studies.

Overall, the results showed that all eight isolates perform in a similar manner against different fungicides with minor differences which were statistically not detected. Azoxystrobin+benzovindiflupyr and difenoconazole+pydiflumetofen showed the highest mycelial inhibition rate consistently against all the isolates and therefore, performed the best. Whereas, chlorothalonil+propiconazole showed the lowest rate of mycelial inhibition rate consistently of the six fungicides tested against the eight isolates. The remaining fungicides showed mixed results ranging from high to medium effectiveness.
Figure 4.3. A bar graph representing mycelial growth inhibition rate of eight isolates of *C. theobromicola* isolated from *Buxus* spp. from eight locations in the United States to six fungicides. *Means followed by same letters within each isolate are statistically not different by Tukey-HSD test at 5% level.

### 4.3.3. Spore germination

All fungicides exhibited significant differences in their effectiveness on spore germination of *C. theobromicola* (*F*=363.01, *p*≤0.001) (Appendix 4). The overall spore germination inhibition rate (%) ranged from 48% (azoxystrobin+benzovindiflupyr) to 96% (difenoconazole+pydiflumetofen), respectively, at lowest effective concentration for all fungicides.

Maximum spore germination inhibition was shown by difenoconazole+pydiflumetofen (96%) followed by pyraclostrobin+boscalid (68%) at 5 ppm (Fig 4.4).

Fluxapyroxad+pyraclostrobin inhibited spore germination by 58% and azoxystrobin+benzovindiflupyr exhibited the least spore inhibition rate of 48% at 5 ppm. Based
on these results, difenoconazole+pydiflumetofen showed maximum spore inhibition, therefore proved to be the best fungicide to inhibit spore germination of *C. theobromicola* followed by pyraclostrobin+boscalid and fluxapyroxad+pyraclostrobin which showed intermediate spore inhibition rate. Azoxytrobin+benzovindiflupyr performed greatly in the mycelial growth inhibition study but poorly in inhibiting spore germination of *C. theobromicola*.

Furthermore, performance of each fungicide with respect to each concentration was determined individually through fungicide-concentration interaction using mean spore germination inhibition rate (%). Performance of fungicides within each concentration showed significant difference (*F*=11.87, *p*≤ 0.001) and with increasing concentrations, fungicides showed increase in spore germination inhibition rate.

Overall, the results showed that all four fungicides are significantly different (*F*=363.03, *p*≤ 0.001) from each other in terms of spore germination inhibition. Additionally, effectiveness of fungicide was directly proportional to the active ingredient concentration.
Figure 4.4. Mean spore germination inhibition exhibited by four shortlisted fungicides against *C. theobromicola*. Means followed by different letters for each fungicide are statistically different by Tukey-HDS test at 5%.

### 4.4. Discussion

Boxwood dieback is a newly discovered disease and information on fungicides effective in managing this disease is lacking. Currently, cultural practices to reduce disease spread are recommended for both commercial and home landscapes. The disease has been successfully detected from boxwood liners in the propagative and wholesale nurseries. The main goal of this study was to screen fungicides that have previously been tested to manage *Colletotrichum* spp. in different hosts.
Several studies have been conducted to observe the effect of different fungicides on *Colletotrichum* species effecting different hosts. One of the studies showed azoxystrobin giving 100% control against *Colletotrichum* species in mango (Sundravadana et al. 2007). Another study showed benzovindiflupyr being the most effective active ingredient in inhibiting mycelial growth of *C. gloeosporioides* and *C. acutatum* (Ishii et al. 2016). Although, several studies have been conducted against other *Colletotrichum* spp., but this is the novel study conducted to screen fungicides against *C. theobromicola* isolated from *Buxus* spp. Combinations of active ingredients were tested, and it was concluded that these active ingredients provide satisfactory results to control *C. theobromicola* under *in vitro* conditions. Results from this study showed great potential for azoxystrobin in combination with benzovindiflupyr, where effectiveness of chemicals in mycelial growth inhibition is as high as 90%.

In general, usage of strobilurins are highly effective in managing anthracnose diseases caused by *Colletotrichum* spp. (Conner et al. 2004; Rava 2002). In this study, strobilurins gave a range of effectiveness starting from medium effective to highly effective with different combinations. Similarly, several other fungicide combinations gave satisfactory results in this experiment.

The preliminary *in vitro* screening revealed that copper octanoate and mancozeb were not effective at any concentrations. Further screening of seven fungicides showed that azoxystrobin+benzovindiflupyr, difenoconazole+pydilflumetofen, fluxapyroxad+pyraclostrobin and pyraclostrobin+boscalid were very highly effective with an IC$_{50}$ < 1 ppm followed by chlorothalonil+propiconazole, and thiophanate-methyl+49prodione as medium effective IC$_{50}$ < 10 ppm. Trifloxystrobin+fluopyram was categorized as least effective at IC$_{50}$ < 25 ppm active ingredient.
Interestingly, azoxystrobin+benzovindiflupyr was considered one of the best fungicides inhibiting mycelial growth, however, it gave significantly poor results for spore germination inhibition. Although, the number of spores germinated did not reduce much when compared to the controls, but the mycelial growth remained significantly smaller until seven days into the experiment. Overall, the active ingredient combination of azoxystrobin+benzovindiflupyr, difenoconazole+pydilumefoten, fluxapyroxad+pyraclostrobin and pyraclostrobin+boscalid gave best results in inhibiting mycelial growth as well as spore germination inhibition of \textit{C. theobromicola}. These fungicides are potential candidates that should be investigated in depth to understand the inhibitory mode of actions and mechanisms under greenhouse and field research trials.
CHAPTER 5. CONCLUSIONS AND FUTURE IMPLICATIONS

The study conducted in this research successfully led to the development of a real-time TaqMan PCR assay that detected *C. theobromicola*, causal agent of boxwood dieback. This assay is species specific and will lead to rapid as well as accurate detection of the pathogen not only from boxwoods but from any other host species. The assay does not detect any other closely related species within the *Colletotrichum gloeosporioides* species complex, making it a reliable method for diagnostic purposes. Boxwood dieback was initially reported from five states in the United States, spreading to approximately ten states at present. This indicates that the pathogen is spreading at an alarming rate and strict actions need to be taken in order to mitigate the disease. The first step to control the disease is to accurately and rapidly identify it and locate the source of inoculum. The TaqMan assay developed in this study has proven to be an excellent tool for detecting it in early disease developmental stages. Furthermore, the conventional PCR species specific primers CALCtF and CALCtR can be used in the Plant Clinics that lack real time PCR capabilities to accurately identify the disease. The real-time TaqMan assay with great specificity and sensitivity will provide excellent tool for rapid and accurate boxwood dieback detection. Growers will have an opportunity to get their boxwood material tested at any growth stage to prevent the introduction or spread of *C. theobromicola* in their production systems.

Furthermore, the host range study proved that in addition to the most commonly available cultivars in Louisiana, the boxwood hybrids that were assumed to perform better in the nursery and landscape are also susceptible to boxwood dieback. Although, one cultivar ‘Little Missy’ showed the occurrence of delayed symptoms, but eventually the pathogen caused plant death as the time progressed. This study will help the growers, breeders as well as landscapers understand that the boxwood hybrids which are not even introduced in the industry are most likely to be
ruled out in case of boxwood dieback resistance. But field trials are yet to be conducted which will confirm the results that are obtained through the greenhouse studies.

Finally, the fungicide efficacy study led to the shortlisting of four potential fungicides that were highly effective in inhibiting mycelial growth of the fungus that causes boxwood dieback. Moreover, it was also proved that the shortlisted fungicides were not only effective in inhibiting mycelial growth but also inhibited the spore germination in case of *C. theobromicola* at different concentrations. Since, boxwood dieback is a newly discovered and an emerging disease that holds great potential to become a threat in nearby future, it is extremely important to come up with effective management strategies to combat the disease. Chemical control is one of the most widely used strategies against plant diseases. No fungicide studies have been conducted for this particular disease so far and therefore, this study was performed to see if there are any potential fungicides available in the market that can be further used for *in-planta* testing. The study successfully provided us with a few potential fungicides that might in future become effective chemical control techniques through further field studies.

Overall, the results obtained from the studies conducted in this research will provide diagnosticians, growers, landscapers, breeders and fungicide industries with valuable information that can lead to efficient detection and effective management practices to tackle boxwood dieback and to prevent further disease spread. Having said that, follow up studies are necessary to confirm the results and to imply these findings in the ornamental and landscape industries. Further studies will include *in-planta* or field studies to follow up with the host range as well as fungicide study. Future research will also consist of studying the disease epidemiology and spread of *C. theobromicola* through root grafting or through soil transmission. *C. theobromicola* is also suspected to be infecting boxwood foliage therefore, further studies will provide
information whether the infection is only on stems or if the pathogen can also enter the plants by infecting leaves.
APPENDIX A. The Ct values of *C. theobromicola* isolates tested from different locations and hosts using qPCR assay

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Location</th>
<th>Host</th>
<th>Ct value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDC Coll I</td>
<td>Alabama (USA)</td>
<td>Boxwood</td>
<td>22.70</td>
</tr>
<tr>
<td>Isolate 136</td>
<td>Brazil</td>
<td>Cashew</td>
<td>18.64</td>
</tr>
<tr>
<td>PDC 18454</td>
<td>Louisiana (USA)</td>
<td>Boxwood</td>
<td>24.72</td>
</tr>
<tr>
<td>PDC 18029</td>
<td>Missouri (USA)</td>
<td>Boxwood</td>
<td>19.93</td>
</tr>
<tr>
<td>PDC 14485</td>
<td>North Carolina (USA)</td>
<td>Boxwood</td>
<td>25.51</td>
</tr>
<tr>
<td>PDC 19024 A</td>
<td>Oklahoma (USA)</td>
<td>Boxwood</td>
<td>20.88</td>
</tr>
<tr>
<td>GJS 08-43 E2</td>
<td>Panama</td>
<td>Cocoa</td>
<td>21.98</td>
</tr>
<tr>
<td>GJS 08-48 E2</td>
<td>Panama</td>
<td>Cocoa</td>
<td>21.34</td>
</tr>
<tr>
<td>PDC 19042</td>
<td>South Carolina (USA)</td>
<td>Boxwood</td>
<td>20.35</td>
</tr>
<tr>
<td>PDC 17484</td>
<td>Texas (USA)</td>
<td>Boxwood</td>
<td>22.70</td>
</tr>
<tr>
<td>PDC Coll D</td>
<td>Virginia (USA)</td>
<td>Boxwood</td>
<td>16.13</td>
</tr>
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</table>
APPENDIX B. *In vitro* mean mycelial inhibition of *C. theobromicola* isolate PDC14313 at different concentrations of seven fungicides tested

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>azoxystrobin+benzovindiflupyr</td>
<td>43.41 AB</td>
</tr>
<tr>
<td>chlorothalonil+propiconazole</td>
<td>4.52 E</td>
</tr>
<tr>
<td>difenoconazole+pydiflumetofen</td>
<td>45.14 A</td>
</tr>
<tr>
<td>fluxapyroxad+pyraclostrobin</td>
<td>37.64 C</td>
</tr>
<tr>
<td>pyraclostrobin+boscalid</td>
<td>40.81 B</td>
</tr>
<tr>
<td>thiophanate-methyl+iprodione</td>
<td>3.17 E</td>
</tr>
<tr>
<td>trifloxystrobin+fluopyram</td>
<td>31.49 D</td>
</tr>
</tbody>
</table>

*Means followed by different letters in the column are statistically different by Tukey-HSD test at 5%*
APPENDIX C. Effect of six shortlisted fungicides on mycelial growth inhibition in eight isolates collected from different locations

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>AL</th>
<th>LA</th>
<th>MO</th>
<th>NC</th>
<th>OK</th>
<th>SC</th>
<th>TX</th>
<th>VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>azoxystrobin+benzovindiflupyr</td>
<td>86.00</td>
<td>85.61</td>
<td>86.50</td>
<td>86.48</td>
<td>86.84</td>
<td>87.20</td>
<td>86.6</td>
<td>85.70</td>
</tr>
<tr>
<td>chlorothalonil+propiconazole</td>
<td>38.64</td>
<td>53.03</td>
<td>48.02</td>
<td>54.44</td>
<td>41.59</td>
<td>35.15</td>
<td>32.87</td>
<td>51.98</td>
</tr>
<tr>
<td>difenoconazole+pydiflumetofen</td>
<td>89.02</td>
<td>92.31</td>
<td>90.00</td>
<td>91.26</td>
<td>89.80</td>
<td>87.04</td>
<td>89.24</td>
<td>90.46</td>
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<tr>
<td>fluxapyroxad+pyraclostrobin</td>
<td>68.32</td>
<td>70.89</td>
<td>67.84</td>
<td>69.41</td>
<td>69.00</td>
<td>71.09</td>
<td>68.41</td>
<td>70.16</td>
</tr>
<tr>
<td>pyraclostrobin+boscalid</td>
<td>69.66</td>
<td>68.97</td>
<td>70.92</td>
<td>71.01</td>
<td>69.76</td>
<td>70.85</td>
<td>68.07</td>
<td>70.34</td>
</tr>
<tr>
<td>thiophanate-methyl+iprodione</td>
<td>76.05</td>
<td>73.31</td>
<td>72.11</td>
<td>73.73</td>
<td>73.59</td>
<td>72.96</td>
<td>73.31</td>
<td>73.46</td>
</tr>
</tbody>
</table>

*Means followed by different letters within each isolate (in the column) are statistically different by Tukey-HSD test at 5%
APPENDIX D. Effect of four shortlisted fungicides on spore germination in *C. theobromicola* at four concentrations

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>azoxystrobin+benzovindiflupyr</td>
<td>35.52 C c</td>
</tr>
<tr>
<td>difenoconazole+pydiflumetofen</td>
<td>87.28 B a</td>
</tr>
<tr>
<td>fluxapyroxad+pyraclostrobin</td>
<td>30.10 D c</td>
</tr>
<tr>
<td>pyraclostrobin+boscalid</td>
<td>43.54 B b</td>
</tr>
</tbody>
</table>

*Means followed by different UPPER-case letters in the rows are statistically different by Tukey-HSD test at 5%

*Means followed by different lower-case letters in the columns are statistically different by Tukey-HSD test at 5%
REFERENCES


VITA

Harleen Kaur was born and raised in Chandigarh, India. She completed her schooling from India and joined bachelor’s program in Agriculture Honors with ‘Horticulture’ as her major at Punjab Agricultural University, Ludhiana, Punjab, India in 2014. She obtained her bachelor’s degree in summer 2018. In fall 2018, She joined the Department of Plant Pathology and Crop Physiology at Louisiana State University to pursue her master’s degree in Plant Pathology under the supervision of Dr. Singh. She expects to graduate in fall 2020.