ABSTRACT

MILLER, MEGAN ELIZABETH. Investigations into the Use of Thermotherapy to Inactivate the Boxwood Blight Pathogen *Calonectria pseudonaviculata* (Under the direction of Dr. Marc A. Cubeta).

Boxwood are popular ornamental shrubs because of their evergreen and deer-repelling properties. In 2011, the fungal, foliar boxwood blight pathogen, *Calonectria pseudonaviculata* was identified in the United States. A second species of the boxwood blight pathogen, *Calonectria henricotiae* occurs in Europe and is a currently a quarantined pathogen in the United States. These pathogens cause blighting of boxwood leaves and twigs, eventually leading to defoliation. Current control strategies for boxwood blight are limited to the use of preventative fungicides and the planting of partially resistant cultivars. The inconspicuous leaf symptoms on boxwood cultivars with high levels of partial resistance can make disease scouting challenging, especially during propagation. Growers require a method of control to ensure that boxwood cuttings are not diseased before they are moved into propagation houses were environmental conditions are highly conducive for disease development. We proposed the utilization of hot water thermotherapy during the propagation process as a method of eliminating the pathogen from diseased tissue prior to propagation. For thermotherapy to become a viable disease management option, the boxwood must have a higher thermal inactivation point than the pathogens. The objectives of this research were to (1) determine the thermal death kinetics of *C. pseudonaviculata*'s and *C. henricotiae*'s thermal death kinetics at 45°C, 47.5°C, 50°C, 52.5°C, and 55°C, (2) determine the ability of heat treated *C. pseudonaviculata* conidia to cause infection, and (3) determine the rooting response of different boxwood cultivars after exposure to heat.

To determine the thermal death kinetics of *C. pseudonaviculata* conidia, three field isolates were heat treated in 45°C to 55°C water for up to 20 min. As time of exposure increased at each temperature, the percentage of conidial germination decreased. The predicted time required to inactivate 90% of *Calonectria pseudonaviculata* conidia ranged from 3 to 26 min. When the thermal death kinetics of *C. pseudonaviculata* were compared with *C. henricotiae*, it was discovered that *C. henricotiae* conidia were inactivated faster that *C. pseudonaviculata* conidia at 45°C. At 47.5°C, the rate of inactivation for each species was dependent upon time. Detached 'Justin Brouwers' (*B. sinica* var, *insularis*) were inoculated with conidia heat treated at 45°C for up to 32 min and at 47.5°C for up to 12 min. Conidia of *Calonectria pseudonaviculata* treated for 28 min at 45°C were viable, but did not cause disease on artificially inoculated boxwood leaves. Disease was not observed after leaves were inoculated with conidia treated at 47.5°C for 9 min, but was observed again after leaves were inoculated with conidia treated at 47.5°C for 12 min.

The rooting response of the cultivars 'Green Beauty' (*Buxus microphylla* var. *japonica*), 'Green Velvet' (*B. sinica* var. *insularis* x *B. sempervirens*), 'Justin Brouwers' (*B. sinica* var. *insularis*), and 'Nana' (*B. sinica* var. *insularis*) was assessed after heat treatment in 45°C and 47.5°C water for 0 to 60 min in 5 min intervals. Root production was assessed after 3 months. The number of roots produced by boxwood cuttings from all cultivars treated in 45°C water for up to 60 min was not significantly lower than the non-treated control. A similar response was observed for cuttings of all cultivars treated in 47.5°C water for up to 60 min, expect for the cv. 'Nana' which produced fewer roots than the non-treated control after treatment for 35 min at 47.5°C.

Investigations into the Use of Thermotherapy to Inactivate the Boxwood Blight Pathogen *Calonectria pseudonaviculata*

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Plant Pathology

Raleigh, North Carolina

2017

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DEDICATION

To the Miller Girls: Mary, Marilyn, and Missi

BIOGRAPHY

Megan Elizabeth Miller was born on October 14, 1991 in Peoria, Illinois, where she lived until she was 18 years old. As a student at Limestone Community High School, Megan passed her time by participating in marching band and theater or studying. She also worked as both a professional popcorn popper and a Master Bear Builder while in high school.

In August 2010, Megan began attending Eastern Illinois University studying environmental biology with a minor in chemistry. As an undergraduate, she first performed research in a Dr. Andrew Methven's mycology lab fruiting different species of *Coprinus*. Seeking to apply her knowledge of mycology to plant protection, Megan participated in an internship with Dr. JoAnne Crouch at the USDA-ARS-Systematic Mycology and Microbiology Lab in Beltsville, MD in 2012. Her time in Beltsville was spent characterizing the fungal endophytes in the turf grass *Danthonia spicata* and exploring Washington D.C. The following summer, Megan continued her undergraduate research working with Dr. Marc Cubeta as a Kelman Scholar in the Department of Plant Pathology at NC State University. While at State, she utilized microsatellite markers to determine the genetic diversity in the reproductive biology of the blueberry pathogen *Monilinia- vacinii-corymbosi*.

After graduating from Eastern in 2014, Megan returned to Dr. Marc Cubeta's lab to work with the boxwood blight pathogen, *Calonectria pseudonaviculata* for her Master's degree. Her time spent in Dr. Cubeta's lab allowed her to develop as lab scientist and extension plant pathologist. Megan hopes to continue her career in agriculture in extension.

ACKNOWLEDGMENTS

I would like to thank my committee members, Drs. Marc A. Cubeta, Barbara Shew, and David Shew for their continual feedback and understanding. I would like to give special thanks to Dr. Marc Cubeta for pushing me to take opportunities interacting with boxwood growers, attending professional and extension meetings, and participating in outreach events. These experiences allowed me to develop my ability to speak to a wide variety of audiences and provided me with a sweeping view of the different aspects of plant pathology. I would like to acknowledge the funding sources for this work, The North Carolina Department of Agriculture and Consumer Services, IR-4, and the Horticultural Research Institute. Also, thank you to the Saunders Brothers and Farm Market Inc. for donating plants.

Working with the all of the current and past members of the Cubeta lab, including Kathleen Burchhardt, Bri Hoge, Elizabeth Thomas, Lindsey Becker, Marie Meckman, and Rachel Norris has been a wonderful experience, and I am thankful to have gained both friends and colleagues from this experience. My thanks is further extended to Dr. Nina Shishkoff for counting countless conidia and to Dr. Peter Ojiambo for helping with data analysis. I would further like to recognize Ted Mays for his great advice and hospitality, and Bennett Saunders and his brothers for teaching me the value of a family farm and answering my questions about boxwood cutting propagation. Also, thank you to Dr. Methven for first introducing me to the world of fungi.

Finally, I would like to thank my family and friends for supporting me along the way. To my Mom, thank you for supporting me from kindergarten to 18th grade. I would like to thank my grandma for teaching me the importance of independent thinking, and my aunt for instilling a love of science in me from a very young age. Thank you to Patrick Snyder for

continually encouraging me to follow my dreams, and also thank you to my cat Toby for providing endless hours of entertainment. I would especially like to thank Emma Wallace for being a wonderful roommate and friend during this experience, and my friends Nathan Miller, Michal Cannon, Bri Hoge, and Katie Neufeld for always providing great company.

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CHAPTER I. Literature Review

Boxwood

Boxwood are woody-ornamental shrubs that are popular because they are deer resistant, mostly evergreen, low maintenance, and have relatively long life spans. Whole plants are commonly used in the landscape as hedges, specimen plants and topiary, and cuttings are sold as holiday greenery for boxwood wreaths and trees. The first boxwood plants in the United States were imported from Amsterdam and planted in Long Island, New York in 1653 (The American Boxwood Society 2016). Today, boxwood sales generate approximately $126 million in revenue annually in the United States (USDA. National Agricultural Statistics Service 2014). Whole plants sell for an average of $50.00 per square foot and cuttings used for holiday decorations are sold for between $0.30 and $0.35 per pound (Kays and Drohan 2003).

Boxwood are in the genus *Buxus* and family Buxaceae. Species from this genus can occur worldwide, but are commercially cultivated from Europe and Asia. Cultivars of the boxwood species *Buxus sempervirens* originated in Europe, while cultivars of *Buxus harlandii, Buxus microphylla*, and *Buxus sinica* are Asiatic in origin (Vane Laere et al. 2011). There are 217 registered boxwood cultivars of which 148 are in commercial production (The American Boxwood Society 2016).

Boxwood Blight

The fungus *Calonectria pseudonaviculata* (Crous J.Z. Groenewald & C.F. Hill) L.Lombard, M.J. Wingf, & Crous. (syn=*Cylindrocladium psedonaviculatum* Crous, J.Z. Groenewald & C.F. Hill =*Cylindrocladium buxicola* Henricot) is a pathogen of boxwood

leaves and stems (Henricot and Culham 2002). Foliar symptoms are characterized by brown to black circular leaf lesions, surrounded by a yellow halo when fully mature. Eventually, the infected leaves become necrotic, resulting in blighting and defoliation of the plant (Henricot and Culham 2002). The fungus can also infect and cause black cankers on stems (Weeda and Dart 2012). While root and crown infections have been demonstrated from direct application of inoculum, disease symptoms from these plant parts have never been reported in a natural setting, suggesting that crown and root infections do not contribute to the boxwood blight disease cycle (Dart et al. 2015) The pathogen produces similar symptoms on naturally infected *Pachysandra terminalis* (Japanese spurge) (Douglas et al. 2012) and species of *Sarcococca* (sweet box) under laboratory and field conditions (Henricot et al. 2008; Malapi-Wight et al. 2016).

Boxwood blight is a relatively new disease to the US and was initially described in 2011 from Connecticut and North Carolina, (Ivors et al. 2012). Since this discovery, the pathogen has been isolated and identified from infected boxwood in 18 additional states (AL, DE, FL, GA, KS, KY, MA, MD, NJ, NY, OH, OR, PA, RI, TN, VA, VT, WV, and three Canadian provinces (British Columbia, Quebec and Ontario). The first report of the occurrence of boxwood blight was in a nursery in Hampshire, United Kingdom (UK) in 1994. However, the causal agent was not determined. The disease was found in again 1997 and a species of *Cylindrocladium* was identified as the cause (Henricot, Perez Sierra, and Prior 2000). A species of *Cylindrocladium* was also isolated from boxwood in New Zealand showing leaf lesion and stem canker symptoms and misidentified as *Cylindrocladium spathulatum* (Ridley 1998). In New Zealand in 2002, the pathogen was identified as *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenewald & C.F. Hill (Crous,

Groenewalk, and Hill 2002) and later that year in the UK as *Cylindrocladium buxicola* Henricot (Henricot and Culham 2002). Henricot and Culham (2002) sequenced the ribosomal 5.8S, internal transcribed spacer (ITS) ribosomal DNA, beta-tubulin and high mobility group (HMG) of the *MAT2* mating type regions of 17 isolates collected in the UK and one from New Zealand from symptomatic species of *Buxus sempervirens*. They determined that the genomic regions of the isolates collected from the UK and the US were identical, indicating that *Cylindrocladium buxicola* and *Cylindrocladium pseudonaviculatum* were the same organism (Henricot and Culham 2002).

Boxwood blight was more devastating in the UK than in New Zealand, and all research from the region subsequently referred to the pathogen as *Cylindrocladium buxicola*. When the pathogen was isolated from infected boxwood in the US in 2011, the pathogen was identified as *Cylindrocladium pseudonaviculatum* (Ivors et al. 2012). Prior to the publication of the first report of boxwood blight in the US, the 'One Fungus One Name' ruling of The International Code of Nomenclature for algae, fungi, and plants established rules of priority for naming fungi, including the rule that if multiple names for the same organism occurred in the literature, the oldest given name should be given priority. This ruling led the authors of the 2012 report to name the organism *Cylindrocladium pseudonaviculatum*, as this was the first reported name in the literature, even though *Cylindrocladium buxicola* was more commonly used (McNeill et al. 2012; Ivors et al. 2012). However, the 'One Fungus One Name' ruling also stipulated that the teleomorph name of a species will be given priority over the anamorph name, causing Lombard et al. (2010), to propose that *Cylindrocladium pseudonaviculatum* should be called *Calonectria pseudonaviculata* because *Cylindrocladium* is the anamorphic genus while *Calonectria* is the teleomorphic genus (Lombard et al. 2010).

Currently, the pathogen is referred to as *Calonectria pseudonaviculata*. Due to popular and widespread use in previously published literature and the sexual stage of the fungus has not been observed, a proposal was published in 2012 to conserve the name *Cylindrocladium buxicola* (Hencricot et al. 2012) A ruling on the official name is still undecided.

In 2015, a second species of the boxwood blight pathogen, *Calonectria henricotiae* sp. nov. was discovered in a population study using genealogical concordance phylogenetic species recognition criteria on four independent nuclear loci. This species, found in five countries in Europe is considered a quarantine pathogen in the US (Gehesquière et al. 2015). While this pathogen was hypothesized to be more fungicide resistant and thermal tolerant, Shishkoff (2016) showed that microsclerotia produced by *C. henricotiae* are more sensitive to sanitizers than those of *C. pseudonaviculata* (Shishkoff,2016)

Pathogen Biology

Unlike many species of *Calonectria* which produce sexual fruiting structures (perithecia and ascospores), *C. pseudonaviculata* does not appear to produce these structures on infected plants or in sexual compatibility experiments conducted *in vitro*. Sequence analysis of the mating type loci from field populations of the fungus suggest that this species is heterothallic (Henricot and Culham 2002; Gehesquière et al. 2015). Because ascospores are not produced, conidia serve as the source of primary and secondary inoculum. The conidia form on penicillately branches and conidiophores bearing phialides and the conidiophores contain a stipe extension ending in a vesicle with a pointed tip. The conidia are covered in an adhesive mucilage causing them to aggregate during conidiogenesis, forming the characteristic cylindrical shaped cluster of conidia. Conidia are splash dispersed onto

surrounding leaves and adhere to the leaf surface. A hyphal germination tube is produced which directly penetrates the plant or indirectly penetrates the stomata within three hours (Henricot et al. 2008). After penetrating the leaf, the hyphae colonizes the mesophyll layer and condiophores bearing conidia are produced on the abaxial surface of the leaf within seven days (Henricot et al. 2008). Spores can also be produced on infected stem tissue (Weeda and Dart 2012). Microsclerotia are formed within 1-2 weeks of the pathogen infecting leaves (Dart et al. 2015). Blighted and necrotic boxwood leaves containing microsclerotia detach from the plant and fall to the soil, where the microsclerotia serve as initial inoculum in the spring. These survival structures can persist and produce conidia in soil for up to 5 years after the initial infection (Henricot et al. 2008).

Current Control Measures

Upon the discovery of boxwood blight in the US and abroad, fungicides have been investigated for boxwood blight pathogen control. Preventative applications of propiconazole, myclobutanil, thiophanate-methyl, fludioxonil, pyraclostrobin, kresoxim-methyl, chlorothalonil, or mixes of these chemistries such as Concert II (propiconazole + chlorothalonil), Danconil WeatherStick (chlorothalonil), Opponent (epoxiconazole + kresoxim-methyl + pyraclostrobin), Spectro 90 WDG (thiophanate-methyl + chlorothalonil), and Strike Plus 50 WDG (triazole + strobilurin) are effective if applied before the pathogen incubation period and symptom development (Ivors, Lacey, and Ganci 2012; Henricot and Wedgwood 2013; LaMondia 2015). While systemic fungicides have been shown to reduce the mycelial growth or conidial germination of *Calonectria pseudonaviculata in vitro*, these

fungicides have little curative effect or have not been treated as post-infection treatments (LaMondia 2015; Ivors, Lacey, and Ganci 2012; Henricot and Wedgwood 2013).

Due to the ineffectiveness fungicides used alone and the cost associated with multiple preventative fungicide applications, the management of boxwood blight relies heavily on the use of resistant cultivars. More than 90 species of *Buxus* are known and all commercial cultivars tested to date are susceptible (Ehsen 2011; Henricot et al. 2008; Ganci 2014). Ganci (2014) showed that cultivars of Asiatic boxwood species (e.g., *Buxus harlandii, B. microphylla*, and *B. sinica*) are more resistant to boxwood blight than cultivars of the European species of *B. sempervirens*. Shishkoff (2015), showed similar patterns when assessing host resistance in cuttings from the National Boxwood Collection. Asiatic cultivars were often considered to have higher levels of partial resistance than the European species (Shishkoff and Olsen 2015; Miller, Norris, and Cubeta 2016).

Cultural control measures also reduce the spread of *Calonectria pseudonaviculata*. The movement of boxwood plants between fields has contributed to the spread of the pathogen into new nurseries. The adhesive mucilage that aids the conidia in sticking to the surface of leaves was hypothesized to allow them to adhere to equipment, clothing and pruning shears. An experiment was performed in which boots, gloves, and pruning shears were exposed to a spore suspension, and the contaminated materials were subsequently rubbed on leaves of *Buxus sempervirens* 'Suffruticosa'. Conidia adhered to boots, gloves and pruning shears, but no infection was observed on leaves exposed to inoculum on these materials (Gehesquiere 2014). These results suggest that human mediated spread of *Calonectria pseudonaviculata* on boots, gloves and pruning shears may not play a

significant role in the spread of the pathogen, human mediated pathogen dispersal is still possible (Gehesquiere 2014).

The production of healthy boxwood cuttings during propagation is critical in preventing the spread of the pathogen from diseased plants to clean nursery stock. To stimulate boxwood rooting, woody cuttings are placed in a humid propagation house, with the floor heated to 21.1°C and misted frequently. This warm, moist environment is conducive for the infection by *Calonectria pseudonaviculata* and development of boxwood blight, so rooting disease free cuttings is critical. Furthermore, lesion development on resistant cultivars is slower with symptoms observed as "pin-point" lesions (Ganci 2014). These pin-point lesions are often challenging for propagators to detect, since they are very much small and lack the characteristic yellow halo around the necrotic center associated with fully developed lesions of boxwood blight. Because fungicides cannot be effectively used curatively, a cultural control measure such as heat treatment of cuttings needs to be investigated as a possible way to minimize the introduction and spread of pathogen into propagation houses.

Concepts and Principles of Heat Treatments

Thermotherapy, or heat treatment, has long been used to rid soil and plants of viruses, bacteria, nematodes, and fungi. Heat treatments of planting materials, historically called, plant thermotherapy gained popularity in the early 1960s (Baker 1962). The objectives of plant thermotherapy have not changed since they were first established. Heat treatment has and continue to be used to establish or maintain an uninfected mother plant to supply uninfected seeds and seedlings for field or greenhouse propagation. However, if heat

treatment cannot completely eliminate a pathogen, it may be used to reduce the level of a pathogen, or to discover if a pathogen is present by increasing the moisture level which can lead to the appearance of pathogen signs For heat treatment to successfully kill a pathogen, there must be a differential response in heat sensitivity between the plant host and pathogen (Baker 1962). The same concept and principle of a differential response has been applied to soil heat treatments in which a balance between killing the plant pathogen and protecting the beneficial soil biota needs to be established. Experiments have shown that the majority of plant pathogenic fungi and bacteria in soil are killed between 46.1°C and 62.8°C after the soil is exposed to heat for 30 min (Baker, K.F., and Roistacher 1957). . Plant pathogenic viruses are more resistant to heat treatment and require exposure to 71.1°C water for 30 min. These inactivation temperatures led researchers at the University of California Division of Agricultural Sciences to recommend soil heat treatments at 82°C for 30 min to eradicate soil-borne pests such as nematodes, insects, weed seeds, and pathogenic bacteria and fungi (Baker, K.F., and Roistacher 1957). However, using a lower temperature of 60°C for 30 min has been found to eliminate most pathogenic bacteria and fungi, while still retaining the populations of beneficial microorganisms in the soil (Hartmann and Kester 1975).

The differential between the plant host and pathogen thermal inactivation points is influenced by the moisture content of the host. The moisture content in the planting material is directly proportional to susceptibility of the plant to thermal damage or death (Baker, 1962). Initial research on the heat treatment of wheat seeds found that seeds with a moisture level of 3% could survive dry air treatments at 114.4°C for 60 min. Seeds with 35% moisture died after exposure to 58.9°C for 30 min (Hutchinson 1944). As early as 1962, Baker hypothesized that using woody cuttings, which have a lower moisture content, for the heat

treatment of ornamentals would be significantly more successful than heat treating green stems. Green stems of woody plants did not survive hot-water treatment at 51.7°C for 30 min, but hardened off cuttings survived (Baker 1962). The boxwood industry uses woody cuttings to propagate plants, indicating that these hardened off propagative tissues may survive hot-water treatment.

The next factor influencing the ability of a plant to survive heat treatment is the condition of the planting material. Seeds with a damaged coat, or wounded vegetative material increases sensitivity to heat. Finally, because of the slow penetration of heat into plant tissues, treating the smallest plant part relevant to propagation such as cuttings instead of a whole plant is imperative to the success of heat treatment (Baker 1962).

Inactivation of pathogens

Because the success of thermotherapy relies on a temperature differential between the host and pathogen, understanding the thermal death kinetics of the pathogen is of paramount importance for predicting when the pathogen will be inactivated *in planta*. For example, conidia of *Botrytis cinerea* Pers. 1794 and *Monilinia fructigena* Honey 1945, two postharvest pathogens of strawberry and cherry, are thermally inactivated at 45°C, with spores of *B. cinerea* and *M. fructigena* being killed after 15 and 3 min, respectively (Marquenie et al. 2002). Conidial germination of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. 1884, the causal agent of mango anthracnose, is halted after heat treatment at 55°C for 5 min (Soppee and Sangchote 2005).

The thermal death kinetics of soil pathogens have also been investigated to determine if soil naturally infested with certain pathogens can be heat treated to reduce pathogenic

microbes. A spore suspension of the soil fungus *Verticillium dahlia* Kleb 1913, was heat treated at 45°C. and spore viability was reduced to 0.01%, but the pathogen was never completely inactivated (Castejon-Munoz and Bollen 1993). The fungus *Fusarium oxysporum* f. sp. *dianthi* (Prill. & Delacr.) W.C. Snyder & H.N. Hansen 1940 was more thermal tolerant than *V. dahlia* and treatment of conidia for 30 min at 55°C was required to reduce survival to less than 0.001%. It was also that the sublethal heating at of the spores at 45°C induced thermotolerance allowing the pathogen to survive subsequent heat treatment at 55°C (Castejon-Munoz and Bollen 1993). The propagules of the common soil pathogens *Pythium ultimum* Trow 1901, *Thielaviopsis basicola* (Berk. & Broome) Ferraris 1912 and *Rhizoctonia solani* J.G. Kuhn 1858 have also been heat treated to test the temperatures at which these pathogens would need to be heated to be inactivated in soil during the solarization process. At 50°C, 33 min was needed to inactivate 90% of the *Pythium ultimum* oospores and 68 min to inactivate 90% of the *Thielaviopsis basicola* endospores (or aleuriospores) treated. Actively growing cultures of *Rhizoctonia solani* were inactivated after treatment at 50°C for 10 min (Pullman, DeVay, and Garber 1981).

Heat treatment of seeds

Seeds are especially good candidates for heat treatment due to their small size and low moisture content. Therefore, they are frequently treated using different forms of thermotherapy such as hot water, vapor, hot air, and/or hot oil treatments to eradicate of wide range of plant pathogens. Two fungal pathogens of soybean seeds, *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner 1927 and *Phomopsis sojae*, Lehman 1922, have been successfully inactivated by submerging infected seed in soybean oil heated to 90°C for 5 min.

Soybean oil was used instead of water to prevent the seeds from being rehydrated, therefore protecting them from the heat (Pyndji, Sinclair, and Singh 1987; Zinnen and Sinclair 1982). The age of the seeds affected their ability to survive heat treatment, and soybean seeds that had been in storage for one year prior to heat treatment were unable to germinate. However, soybean seeds less than one month old were unaffected by the heat treatment (Pyndji, Sinclair, and Singh 1987). *Xanthomonas* and *Pseudomonas* bacteria have been successfully eliminated from the seeds of many different plant species such as tomato, tobacco, rice, barley, cucumber, crucifers, pumpkin, guar, and cotton using hot water, aerated steam, and dry heat treatments (Grondeau, Samson, and Sands 2011). In addition to eliminating bacteria, dry heat (70°C) for 24 h also eliminated *Tomato Mosaic Virus* from tomato seeds (Silva, Freitas, and Nascimento 2010). Heat treatment can also be used to eliminate nematode seed infection. Both moist heat (40°C for 15-30 min and 60°C for 5-10 min) and dry heat (60°C for 6 h and 95°C for 4-12 h) either eliminated or reduced the plant parasitic nematodes *Aphlenchoides besseyi*, *Ditylenchus* sp., and *Ditylenchus dipaci* from infested maize, oat and rice seeds (Tenete et al. 1999).

Heat treatment of corms

Corms are underground, vegetative, plant stems that allow plants to overwinter or survive conditions such as drought and heat. Their known thermal tolerance makes them especially good candidates for successful thermotherapy. In 1959, Dutch growers began heat treating corms of Gladilous to eradicate the fungal pathogens *Fusarium oxysporum* f. sp. *gladioli* (Massey) W.C. Snyder & H.N. Hansen 1940, *Botrytis gladiolorum* Timmerm. 1941, *Stromatinia gladioli* (Drayton) Whetzel 1945, and *Rhizoctonia solani* (Schenk et al 1959).

Further research showed that the climate in which the corm and cormel (new growth at the base of the corm) is produced affect the ability of the cormel to survive treatment at higher temperatures. For example, cormels grown during the summer in subtropical climates can survive heat treatment at 57°C for 30 min, the temperature required to eliminate *Fusarium oxysporum* f. sp. *gladioli*. However, cormels produced in the winter in subtropical climates or in moderate climates cannot survive hot water treatment at 57°C for 30 minutes. These differences in heat tolerance due to plant physiology suggest that a universal procedure for heat treatment of gladiolus corms and cormels will be challenging (Bald 1956; Roistacher, Baker K, and Bald 1957; Vigodsky 1967; Schenk 1961).

Heat Treatments of Woody Propagative Materials

Woody propagative materials such as scions from fruit trees or vegetative cuttings from ornamentals are also amenable for heat treatments to eliminate pathogens due to their low moisture content. Thermotherapy of scions reduced the levels of bacterial infection of pecan, pear and apple. Scions from pear and apple were wrapped in wet cloth and incubated for 5 h at 45°C. After this treatment, the bacterium *Erwinia amyylovora* could not be isolated from the scions. However, heat treating the scions at 50°C for either 1 or 2 h did not kill the pathogen completely (Keck et al. 1995). Heat treatments of pecan scions have been successful at reducing *Xylella fastidiosa*, the causal agent of bacterial leaf scorch disease. Treatment of scions at 46°C for 30 min significantly reduced transmission of *X. fastidiosa* during grafting (Sanderlin and Melanson 2008).

The use of thermotherapy to remove pathogens from grape vine cuttings was first reported in 1959. Heat treatment at 50°C for 30 min inactivated *Agrobacterium tumefaciens*

biovar 3 from dormant grape vine cuttings without causing harm to the cuttings (Burr et al. 1989). *Xylella fastidiosa* was also inactivated via heat treatment at 50°C for 20 min (Goheen, Nyland, and Lowe 1973). Fungal pathogens have also been successfully managed in grapevines through the use of hot water treatment. The fungi *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf & Mugnai) Crous & W. Gams 2000 and a species of *Phaeoacremonium* W. Gams, Crous & M.J. Wingf., 1996, the causal agents of Petri disease, were eliminated from grapevines treated in hot water at 50°C for 30 min (Fourie and Halleen 2004).

Thermotherapy has also been used to manage web blight of azalea caused by binucleate *Rhizoctonia* anastomosis group U (AG-U). The pathogen was eliminated twelve different azalea cultivars after at treatment in hot water at 50°C for 21 min. However, heat treatment of infected cuttings at 55°C for 5 min killed the pathogen, but prevented the azalea cuttings from rooting (Copes and Blythe 2009, 2011).

Project Objectives

Because of the limited availability and utility of systemic fungicides, boxwood growers are relying on the use of partially resistant cultivars and focusing their efforts on preventing the spread of the pathogen between fields through integrated cultural practices to manage boxwood blight caused by *C. pseudonaviculata*. While partially resistant cultivars can reduce the level of disease in a field, the challenge of scouting for the pinpoint lesion symptoms on infected plants could provide a potential opportunity for the pathogen to enter propagation houses, and spread to clean propagation stock. Heat treatment of boxwood cuttings taken from the field before they enter the propagation house could potentially

provide an economical method to prevent spread of the pathogen. To determine if boxwood is a candidate for heat treatment, the following research objectives were developed and investigated;

1. Examine the susceptibility of boxwood cultivars to boxwood blight.
2. Determine the ability of boxwood cuttings sampled from cultivars with varying levels of resistance to survive exposure to hot water and form roots after treatment.
3. Determine the thermal death kinetics of *Calonectria pseudnaviculata* and *C. henricotiae* conidia.

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Chapter II.

Title: Thermal inactivation of the boxwood blight pathogens *Calonectria pseudonaviculata and Calonectria henricotiae*

Abstract

The fungi *Calonectria pseudonaviculata* and *Calonectria henricotiae* cause disease on boxwood, ornamental plants in the genus *Buxus*. Current strategies for managing boxwood blight disease are restricted to the use of preventative fungicides and partially resistant cultivars. In this study, we investigated the potential of thermotherapy for managing boxwood blight to reduce fungicide dependence and eliminate *C. henricotiae* and *C. pseudonaviculata* in boxwood propagation stock. We initially examined the thermal death kinetics of conidia produced from three field isolate of *C. pseudonaviculata* treated in water at 45°C, 47.5°C, 50°C, 52.5°C and 55°C. As time of exposure increased at each temperature the percentage of conidial germination decreased. The predicted times of 3 to 26 min required to inactivate 90% of *C. pseudonaviculata* conidia decreased as water temperature increased from 45°C to 55°C. A comparison of the thermal death kinetics of *C. pseudonaviculata* with *C. henricotiae* conidia treated in water at 45°C and 47.5°C suggest that conidia of *C. henricotiae* were inactivated faster ($P < 0.0001$) than *C. pseudonaviculata* conidia at 45°C. At 47.5°C, the rate of inactivation of each species also was dependent on the time of exposure to heat. Some conidia of *C. pseudonaviculata* treated for 28 min at 45°C were viable as indicated by the 8.33% germination on culture media but did not cause blight on artificially inoculated boxwood leaves. Disease was not observed after leaves were artificially inoculated with conidia treated at 47.5°C for 9 min (3.2% germination).

INTRODUCTION

Boxwood, in the genus *Buxus,* are woody, ornamental shrubs frequently used in the landscape as hedges, specimen plants and topiary. The fungus *Calonectria pseudonaviculata* (Crous J.Z. Groenewald & C.F. Hill) L.Lombard, M.J. Wingf, & Crous. (syn=*Cylindrocladium psedonaviculatum* Crous, J.Z. Groenewald & C.F. Hill =*Cylindrocladium buxicola* Henricot) was identified in the United States in 2011 and now occurs in at least 21 states and 3 Canadian provinces (Ivors et al. 2012). Foliar symptoms are characterized by brown to black circular leaf lesions, surrounded by a yellow halo when fully mature. Conidia are produced on mature lesions and splash dispersed to surrounding leaves and plants. Microsclerotia are formed within 1 to 2 weeks of the pathogen infecting the leaves (Dart et al. 2015). Eventually, the entire leaf becomes necrotic resulting in blighting and defoliation of the plant (Henricot and Culham 2002). The fungus can also infect stems causing the formation of black cankers (Weeda and Dart 2012).

In 2015, a second boxwood blight pathogen was discovered in a population study of isolates from diseased boxwoods in Belgium, Canada, Croatia, France, Germany, Italy, New Zealand, Slovenia, Spain, Switzerland, Netherlands, the United Kingdom, and the United States. Based on phylogenetic analysis of four independent nuclear loci this species was identified as *Calonectria henricotiae* sp. nov. (Gehesquière et al. 2015). *C. henricotiae* only occurs in Belgium, Germany, Slovenia, the Netherlands, and the United Kingdom and is a quarantine pathogen in the US (Gehesquière et al. 2015). Gehesquiere et al (2015), hypothesized that *C. henricotiae* was more fungicide resistant and thermal tolerant than *C. pseudonaviculata,* but Shishkoff (2016) showed that microsclerotia produced by *C.*

henricotiae are more sensitive to sanitizers than *C. pseudonaviculata*. (Shishkoff, 2016; Gehesquière et al., 2015).

Currently, *C. pseudonaviculata* is managed with frequent, preventative fungicide applications and by planting cultivars with high levels of partial resistance (Ivors, Lacey, and Ganci 2012; Henricot and Wedgwood 2013; Lamondia 2014; Shishkoff and Olsen 2015; Ganci 2014; Miller, Norris, and Cubeta 2016). Disease symptoms develop more slowly on cultivars with higher relative levels of resistance, making symptom identification challenging for plant propagators (Ganci 2014). Nurserymen must propagate clean boxwood cuttings to prevent introduction of the pathogen and disease into propagation houses.

Thermotherapy can be used to eliminate plant pathogens from propagative material. For thermotherapy to be successful, the pathogen must have a lower thermal inactivation point than the plant host (Baker 1962). Understanding thermal death kinetics of the pathogen and host is critical for developing applied protocols for the thermal inactivation of *Calonectria pseudonaviculata in planta*. Chan et al (1996) compared the thermal death kinetics of bacterial and fungal postharvest pathogens of papaya to determine whether treatment in hot water would prevent loss in storage. Conidia of *Botrytis cinerea* Pers. 1794 and *Monilinia fructigena* Honey 1945, postharvest pathogens of strawberry and cherry, were killed after 15 and 3 min, respectively, when treated at inactivated at 45°C (Marquenie et al. 2002). The determination of the thermal death kinetics (inactivation) of conidia of these fungi was critical for developing postharvest heat treatments for cherry and strawberry (Marquenie et al. 2002).

While thermotherapy is commonly used to rid fruits of postharvest pathogens, few woody, ornamental systems have been investigated to determine whether heat treatments

could eliminate fungal pathogens from dormant cuttings. Heating *Rhizoctonia solani* Kühn to 50°C for 10 min inhibits mycelial growth (Pullman, DeVay, and Garber 1981). Subsequent experiments showed that heat treating azalea cuttings infected with binucleate *Rhizoctonia* AG-U (teleomorph=*Ceratobasidium*) to 50°C for 20 min inactivated the pathogen within the plant without damaging the ability of the azalea cuttings to produce roots (Copes and Blythe 2009, 2011). The time required to inactivate the pathogen *in planta* was approximately double the time required to kill the pathogen *in vitro* (Copes and Blythe 2011, 2009; Pullman, DeVay, and Garber 1981). Because the thermal inactivation point of the binucleate *Rhizoctonia* AG-U was lower than the thermal inactivation points of azaleas, growers have used this practice to heat treat cuttings before placing them in a propagation house.

The objective of our study was to determine the thermal death kinetics of the boxwood blight pathogens *Calonectria pseudonaviculata* and *Calonectria henricotiae*. We tested the hypothesis that the number of germinated *Calonectria henricotiae* and *Calonectria pseudonaviculata* conidia will decrease as temperature and time of exposure increase. Our second hypothesis was that the time required to thermally inactivate conidia of *Calonectria henricotiae* will be less than conidia of *Calonectria pseudonaviculata*. We also tested the hypothesis that inactivating 90% of *Calonectria pseudonaviculata* conidia will reduce infectivity of the fungus on boxwood leaves.

MATERIALS AND METHODS

Isolates and conidia production

Conidia from three isolates of *Calonectria pseudonaviculata* and three isolates of *Calonectria henricotiae* were used for heat treatment experiments (Table 2.1). Three methods were used to produce conidia for the experiments. For experiments with *C.*

pseudonaviculata isolates TM7 and BD6c conducted at North Carolina State University (NCSU), six mycelial plugs from the edge of an actively growing plate were placed in 10 mL of sterilized deionized H_20 in a 15 mL Falcon tube (Corning, Inc., Tewksbury, MA). The tube was shaken vigorously for 30 sec, and 500 µl aliquots were taken from the tube, placed onto Potato Dextrose Agar (PDA) (BD Difco™, Sparks, MD) in 9-cm diameter plastic petri dishes, and spread with a sterile glass rod (flood inoculation method). Plates were incubated at 23°C with an 18-h photoperiod and conidia were harvested 7-16 days after incubation. The flood inoculation method was not useful for producing conidia for isolate BB5b. Instead, five mycelial plugs taken from the edge of an actively growing culture of *Calonectria pseudonaviculata* isolate BB5b were placed on the surface of a PDA plate plates and were incubated at 23°C until mycelium covered the entire surface of the plate. The aerial mycelium in each plate was scraped with a sterile metal spatula and plates were incubated at 23°C with an 18-h photoperiod, for 7-16 d to induce sporulation.

Since *Calonectria henricotiae* is a quarantined pathogen in the US, all experiments with this fungus were conducted at the Foreign Disease-Weed Science Research Quarantine Facility in Ft. Detrick, MD. To produce conidia for these experiments, one plug was taken from the actively growing edge of either three *Calonectria pseudonaviculata* or three *Calonectria henricotiae* isolates (described above, Table 2.1) and placed in the center of a glucose yeast-extract tyrosine agar (GYET) (Hunter 1992). The plates were incubated for up to 30 days at 20°C. Cubes of colonized agar where then transferred to Synthetic Nutrient-Poor Agar (SNA) and incubated for 4 to 8 days at 20°C with continuous illumination. Spores were harvested 3 to 4 days after the induction of sporulation (Leslie and Summerell 2006).

Harvesting and Quantification of Conidia

Conidia were harvested by adding 15 mL of 0.01% Tween 20 (Bio-Rad Laboratories Inc., Richmond, CA) and agitating the surface of the colony with a bacterial loop to release the conidia from the condiophores. The spore suspension from each plate was filtered through either four layers of sterile cheese cloth or poured directly into a 50 mL Falcon Tube. Conidial suspensions were quantified using a hemacytometer (Reichert Scientific Instruments, Buffalo, NY) and diluted in sterile distilled H_2O to 10,000 conidia/mL.

Heat treatment and thermal death kinetics of Calonectria pseudonaviculata conidia

A suspension of 10,000 conidia/mL produced separately for each isolate was aliquoted into 300 µl PCR tubes (BioExpress, Kaysville, UT) for heat treatment in a Mastercycler® ep thermal cycler (Eppendorf, Hamburg, Germany). The conidial suspensions from each isolate were treated individually at 45°C in 2 min intervals for 0-20 min. At 47.5°C, 50°C, 52.5°C, and 55°C, the conidia were treated in 1 min intervals for 0-10 min. Non-treated conidia for each temperature served as the (0) control. Tubes of each isolate and time treatment combination were arranged in the thermal cycler in a randomized complete block design with 3 blocks and 1 tube per block. Conidia in the non-treated (0) control were kept on ice for the duration of the heat treatment. As tubes were removed from the thermal cycler, they were placed on ice. When all tubes were removed from the thermal cycler, the suspension in each tube was mixed on a vortexer for 10 sec, and 300 µl of the suspension inside each tube was pipetted on PDA. Plates were incubated for 24 h at 23°C in a dark incubator. Germination was assessed by arbitrarily evaulating 50 conidia for the presence or absence of a germ tube. A conidium was considered germinated if the germ tube was twice the width of the conidium. This experiment was conducted twice.

Heat treatment of Calonectria pseudonaviculata and Calonectria henricotiae conidia

A separate solution containing 10,000 conidia/ml was produced for each of the three isolates of *Calonectria henricotiae* and three isolates of *Calonectria pseudonaviculata*, aliquoted into PCR tubes (LabSource, Northlake, IL), and placed into a PTC-100 Peltier thermal cycler (BioRad Laboratories, Inc., Richmond, CA) as described above. For this experiment, conidia of each isolate were treated at 45°C in 4 min intervals for 0-20 min and at 47.5°C in 2 min intervals for 0-10 min. Tubes for each treatment were arranged in a completely randomized design, with three replications of each isolate at each time for each temperature. A non-treated (0) control from each isolate was kept on ice for the duration of the experiment. When all tubes were removed from the thermal cycler, each tube was mixed with a vortex and 300 µl of the 10,000 conidia/ml solution inside each tube was placed on a PDA plate. After 24 h incubation in the dark at 20°C, the percent germination of 50-100 conidia arbitrarily chosen from the plate was evaluated. A conidium was considered germinated if the germ tube was twice the width of the conidium. The experiment was conducted twice.

Infectivity of heat-treated Calonectria pseudonaviculata conidia

Conidia from isolate BD6c were produced using the flood inoculation method, then harvested quantified, and diluted as described above. Conidia (10,000 spore/ml) were placed in 300 µl PCR tubes (BioExpress, Kaysville, UT) and heat treated in a Mastercycle® ep thermal cycler (Eppendorf, Hamburg, Germany) at 45°C in 4 minute intervals for 0-32 min and at 47.5°C in 3 min intervals for 0-12 min. Tubes were arranged in a randomized complete block design containing four blocks and one replication of each time in each block. A non-treated (0) control was kept on ice for the duration of the experiment. When all tubes

were removed from the thermal cycler, each tube was mixed with a vortex and 250 µl of the 10,000 conidia/ml solution inside each tube was placed on a PDA plate and the percent conidial germination was assessed as described previously. The remaining 50 µl of conidia in each PCR tube was aliquoted onto four individual boxwood leaves, so that each of the four leaves was inoculated with 50 µl of the heat treated spore suspension. The boxwood leaves (cv. 'Justin Brouwers') were taken from two-year old boxwood liners provided by Saunders Brothers Nurseries and Farm Market. Two control treatments were included in this experiment, leaves inoculated with the non-treated (0) control and leaves that were not inoculated with conidia. Leaves were incubated in moist chambers and assessed for disease incidence (percentage or number of leaves with disease symptoms) and the presence or absence of conidia each day for 13 days.

Data Analysis

To determine the thermal death kinetics of *C. pseudonaviculata* conidia, percent conidial germination data were analyzed using Proc Reg in SAS 9.4 (SAS 9.4, Cary, NC), to fit the equation Log(standard germination)=-kt/2.303 where standard germination = germ per time/germ of control for each replication, k=rate constant, and t=time (Chan et al. 1996). The decimal reduction value (1-D) or the time required to kill 90% of conidia was calculated from the equation D=2.303/k (Chan et al. 1996). Before analysis, any time points with a standard germination greater than 100% were designated as 100%. This method developed by Chan et al. (2009) was also used to analyze the percent conidial germination of heat treated spores that were used to infect boxwood leaves. The presence or absence of disease was subsequently assessed on each of the four inoculated leaves and converted to a percentage of infected leaves after the 13 d incubation period.

To determine and compare the thermal inactivation rates of *Calonectria henricotiae* and *Calonectria pseudonaviculata* conidia, percent conidial germination data was standardized by dividing the number of germinated conidia at each time point by the mean number of germinated conidia in the non-treated 0-control, for each isolate. Data from two experimental runs were combined and the standardized conidial germination data were analyzed using PROC GLM (SAS 9.4 Cary, N.C.) to test for the effects of *Calonectria* species and time. Treatments were compared using Fisher's Least Significant Difference (LSD).

RESULTS

Heat treatment and thermal death kinetics of Calonectria pseudonaviculata conidia

The time required to inactivate conidia at each temperature was used to generate the thermal death curves and to calculate the decimal reduction values for each isolate of *C. pseudonaviculata* (Figure 2.1) At all temperatures tested, the percentage of conidial germination decreased as time of exposure increased (Figure 2.1). At 50°C, 52.5°C, and at 55°C all isolates died within the 10 min treatment, with TM7 dying the fastest at 52.5°C and 55°C. However, this isolate was the least insensitive to heat treatment at 45°C. The thermal death curves provided k-values necessary to calculate the decimal reduction values (1-D) or the time required to inactivate 90% of the conidia (Table 2.1). The modified Chan et al (1996) model predicted that it would require a maximum time of 16.4 min for conidial inactivation to reach 90% when isolate BD6c was heat treated at 45°C for 20 min and a minimum time of 3.1 min when treated at 52.5°C. (Figure 2.1, Table 2.2). The maximum predicted time required to inactivate 90% of BB5b conidia at treated 45°C was 15.2 min, and the minimum predicted time was 3.5 min after treatment at 52.5°C. (Figure 2.1, Table 2.2).

Isolates BB5b and BD6c had the lowest decimal reduction values of 3.1 min and 3.5 min respectively at 52.5°C. (Table 2.2). Isolate TM7 was predicted to survive longer than isolates BD6c and BB5b at 45°C, but survived for less time than these isolates at 52.5°C and 55°C (Table 2.2). The rapid inactivation of conidia from TM7 at 52.5°C and 55°C prevented further analysis of our data for this isolate. Differences in the predicted time to inactivate 90% of the conidia treated at 45°C and 47.5°C were observed within individual isolates. For example, in run 1 the predicted time required to kill 90% of the conidia of isolate BD6c at 45°C was 10.2 minutes, whereas in run 2, 16.4 min were needed to inactivate 90% of the conidia. A similar pattern was observed for this isolate at 47.5°C, where in run 1 the predicted time required to kill 90% of the conidia was 8.2 min and 15.3 min in run 2 (Table 2.2).

Heat treatment of C. pseudonaviculata and C. henricotiae conidia

As time of exposure increased at 45°C and 47.5°C, the percentage of conidial germination decreased (Figure 2.2). At 45°C ($p < 0.0001$) *C. henricotiae* conidia were inactivated faster than *C. pseudonaviculata* conidia. At 47.5°C, the rate of inactivation of each species depended upon the length they were exposed to the heat ($p<0.0001$) (Table 2.3). After exposure to 45°C for 20 min, the percentage of conidial germination of *C. henricotiae* and *C. pseudonaviculata* was 0.58% and 5.3%, respectively (Figure 2.2. After exposure to 47.5°C for 10 min, the percentage of conidial germination of *C. henricotiae* and *C. pseudonaviculata* was 0.572% and 2.794%, respectively (Figure 2.3).

Infectivity of Heat Treated Calonectria pseudonaviculata conidia

Analysis of BD6c conidia treated at 45°C for 32 min and 47.5°C for 12 min resulted in R^2 values of 0.9289 and 0.90492, respectively. The predicted time required to inactivate

90% of the conidia for 45°C was 19.6 minutes, and treating conidia at 47.5°C resulted in a decimal reduction value of 8.4 min (Figure 2.3). Conidia treated for 28 min at 45°C were viable (8.33% germination) but did not cause disease on artificially inoculated boxwood leaves. A disease incidence of 100% was observed on the leaves inoculated with non-heat treated conidia. Disease was not observed after leaves were artificially inoculated with conidia treated at 47.5°C for 9 min (3.2%). Disease incidence was 50% on boxwood leaves artificially inoculated with conidia treated at 47.5°C for 12 min, when 2.4% of the conidia germinated after the imposed heat treatment.

DISCUSSION

The primary objective of our research was to determine the thermal death kinetics of the boxwood blight pathogens *C. pseudonaviculata* and *C. henricotiae*, which was necessary to predict whether these fungi could be eliminated from cuttings of boxwood during hot water treatment. Furthermore, we determined whether conidia of *C. pseudonaviculata* treated with hot water for specified times could infect detached boxwood leaves. In our study, we determined the thermal death kinetics of *Calonectria pseudonaviculata* conidia treated at 45°C, 47.5°C, 50°C, 52.5°C, and 55°C, because plant pathogenic organisms are frequently inactivated at temperatures in this range (Chan et al. 1996).

For all three *Calonectria pseudonaviculata* isolates examined in this study, the percentage of conidial germination decreased as time of exposure to heat increased. This finding supports hypothesis 1 that increased exposure to heat will decrease the percentage of conidial germination or more completely kill pathogen propagules. Heat treatment of *C. pseudonaviculata* at 45°C and 47.5°C for 20 min greatly reduced conidial germination. Similarly, exposure to these two temperatures for 15-30 min has also been shown to reduce

the mycelial growth and conidial germination of *Colletotricum musae* and *Fusarium proliferatum* (Lopez-Caberra and Marrero-Dominguez 1998). *C. pseudonaviculata* was often killed in 5 minutes or less when treated at 52.5°C and 55°C, which may allow for quick and efficient *in planta* pathogen inactivation at these temperatures. However, hot water treatments of azalea cuttings at temperatures above 50°C has been shown to result in decreased root production (Copes and Blythe 2009; Copes et al. 2011). Even though pathogen inactivation occurs quickly at 52.5°C and 55°C, treatment of infected boxwood cutting should be limited to temperatures between 45°C and 50°C to reduce negative rooting effects.

The three *C. pseudonaviculata* isolates behaved differently depending upon the treatment temperature. Even though isolate TM7 survived the longest at the minimum treatment temperature of 45°C, it was the most sensitive to the highest temperatures of 52.5°C and 55°C, indicating that this isolate was the least thermal tolerant. Isolate BD6c was least tolerant of treatment at 45°C, but was most tolerant of treatment at the highest temperatures of 52.5°C and 55°C indicating that this isolate was most thermal tolerant. After treatment at 52.5°C and 55°C, isolate BB5b survived longer than isolate TM7 but was more sensitive than isolate BD6c, indicating that this isolate has a relatively moderate thermotolerance compared to the other two isolates. Isolate BD6c's reduced sensitivity to higher temperature treatments could be influenced by the warmer season in which it was isolated. Isolate BD6c was collected in Surry Co., N.C. in June, while BB5b and TM7 were isolated in December and January. These results may further indicate that *in planta* pathogen sensitivity to heat treatment may vary depending upon the season in which a boxwood cutting is heat treated.

The data collected from the heat treatment of the *Calonectria pseudonaviculata* conidia was analyzed using the methodology of Chan et al (1996). This method used a transformed regression to calculate the rate constant (k) which allowed for the calculation of the decimal reduction value (1-D) or the time (in minutes) required to inactivate (kill) 90% of using the equation D=2.303/k. While, the calculated decimal reduction values were useful for comparing thermal tolerance (sensitivity) of three isolates of *Calonectria pseudonaviculata*, the inability of the Chan et al (1996) model to account for multiple isolates increased the amount of variation between isolates in the dataset. Furthermore, the Chan et al (1996) model failed to account for the variation in sensitivity to the imposed heat treatment that occurred between experimental runs of the same isolate, preventing the data from separate experimental runs from being combined in our study. For example, in run 1 of heat treatment of isolate BD6c at 45°C, it was predicted that 10.2 minutes would be required to inactivate 90% of the conidia. However, the second time isolate BD6c was treated at this temperature, the model predicted that 16.4 minutes are required to inactivate 90% of the conidia. Variation was also observed between the three replications that were treated at each time at each temperature. This variation is reflected by the relatively low r^2 values in Table 2.2. A similar method of data analysis was used to predict the thermal death kinetics of *Colletotrichum gloesporiodes*, resulting in r^2 values greater that 0.9 (Chan et al. 1996). However, the Chan et al (1996) analysis was performed on the mean of three conidial germination studies at each time point. Analyzing the mean, rather than the replications of the *C. gloesporiodes* germination rates decreased the variation in their study and highly increased the r^2 values. In contrast, our analysis included three conidial germination replications, allowing the model to account for variation in germination between replications and more realistically predict the

time required to inactivate *Calonectria pseudonaviculata*. Understanding the variation between replications of an isolate within a heat treatment and between different heat treatments of the same isolates at the same temperature is necessary for predicting how long it will take to inactivate *C. pseudonaviculata in planta*.

The hypothesis that *Calonectria pseudonaviculata* is more thermal tolerant than *Calonectria henricotiae* was tested by treating conidia of three *C. pseudonaviculata* isolates and three *C. henricotiae* isolates at 45°C and 47.5°C. Previous research provided evidence that *C. henricotiae* has a greater rate of mycelial growth and colony expansion than *C. pseudonaviculata* at 28°C and 31°C, indicating that *C. henricotiae* may be slightly more thermal tolerant than *C. pseudonaviculata* (Gehesquière et al. 2015). In our study, after conidia from isolates of *C. henricotiae* and *C. pseudonaviculata* were treated at 45°C, the *C. henricotiae* conidia died faster. The rate of inactivation for each species changed as they were exposed to 47.5°C over time, but the germination rates of *C. henricotiae* were lower than the germination rates of *C. pseudonaviculata* after exposure to this temperature for 10 min. Because *C. henricotiae* was shown to die faster than *C. pseudonaviculata* at 45°C and had a lower final conidial germination rate at 47.5°C, using hot water to treat plants infected with *C. henricotiae* may be more effective than heat treating plants infected with *C. pseudonaviculata*. Because *C. henricotiae* is only found in Belgium, Germany, Slovenia, The Netherlands, the United Kingdom, and is quarantined in the United States, heat treating plants infected with *C. henricotiae* could allow for the trade of these plants into areas where this species has not yet been introduced (Gehesquière et al. 2015).

After investigating the thermal death kinetics of *C. pseudonaviculata* and *C. henricotiae*, the infectivity of BD6C conidia treated at 45°C for up to 32 min and at 47.5°C

for up to 12 min was examined. The modified Chan et al. (1996) model predicted it would require 19.6 min to inactivate 90% of conidia at 45°C and 8.4 min at 47.5°C. BD6C conidia still caused infection in 25% of leaves after the conidial suspension was treated at 45°C for 24 min, at which time only 7.8% of the conidia were germinating. An infection did not occur when conidia were treated for 28 min or longer even though the germination rate had increased to 8.3%. BD6c conidia treated at 47.5°C caused infection in 50% of leaves when the germination rate was only 2.4%. The results from the infectivity experiment were contrary to our hypothesis (#3) that infection and disease symptoms would not occur after 90% of the conidia were inactivated. Conidial germination was reduced faster when the conidia were exposed to 47.5°C then when conidia were exposed to 45°C. However, the few conidia that were still germinating after exposure to 47.5°C were still able to cause infection, indicating that a shorter exposure time to the higher temperature of 47.5°C does not seem to affect the physiological processes that moderate infection in the surviving conidia as much as an increased duration of exposure to the lower temperature of 45°C. Conidia that survive heat treatment to sub-lethal temperatures may acquire thermotolerance, potentially allowing for increased survival at previously lethal temperatures (Castejon-Munoz and Bollen 1993). Heat shock proteins that develop after the exposure to sub-lethal heat are often responsible for conveying this newly acquired thermotolerance (Maheshwari, Bharadwaj, and Bhat 2000).

Infecting boxwood leaves with heat-treated conidia provided a more specific *in planta* pathogen inactivation window than what was provided by the thermal death kinetics of *Calonectria pseudonaviculata* conidia alone. We now hypothesize that it will take at least 28 min to inactivate *C. pseudonaviculata in planta* at 45°C. We also hypothesize that it will take a minimum of 12 minutes to inactivate *C. pseudonaviculata in planta* at 47.5°C. Further

testing should be performed to ensure that any pathogen propagules surviving at this temperature have not acquired increased thermal tolerance. Because the amount of time required to inactivate the conidia varies by isolate, the amount of time infected cuttings are exposed to hot water should be maximized. Heat treatments are only effective if the plant host and pathogen have differential thermal death points. Therefore, treatment of boxwood cuttings in water at 45°C and 47.5°C should be performed to ensure that these temperatures do not damage boxwood cuttings. Because the conidia of *Calonectria pseudonaviculata* conidia were more thermotolerant than *Calonectria henricotiae* conidia after treatment at 45°C, heat treatment at that temperature may have greater utility for boxwood cuttings infected with *C. henricotiae*.

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Table 2.1: Isolate designation and source (*Buxus* sp.), location, and date isolated for conidial thermal inactivation experiments.

Isolate	Species	Source	Date Isolated	Location
BD6c	*Calonectria pseudonaviculata*	*B. sempervirens* 'Suffruticosa'	6/18/14	Surry Co., NC
BB5b	*Calonectria pseudonaviculata*	*B. sempervirens* 'Suffruticosa'	12/7/11	Surry Co., NC
TM7	*Calonectria pseudonaviculata*	*B. sempervirens*	1/26/15	Surry Co., NC
T	*Calonectria pseudonaviculata*	*B. sempervirens 'Suffruticosa'*	2001	Belgium
182	*Calonectria henricotiae*	*B. sempervirens*	2009	Belgium
78	*Calonectria henricotiae*	*B. sempervirens*	2011	Netherlands
55	*Calonectria henricotiae*	*Buxus*, sp.	2007	Germany

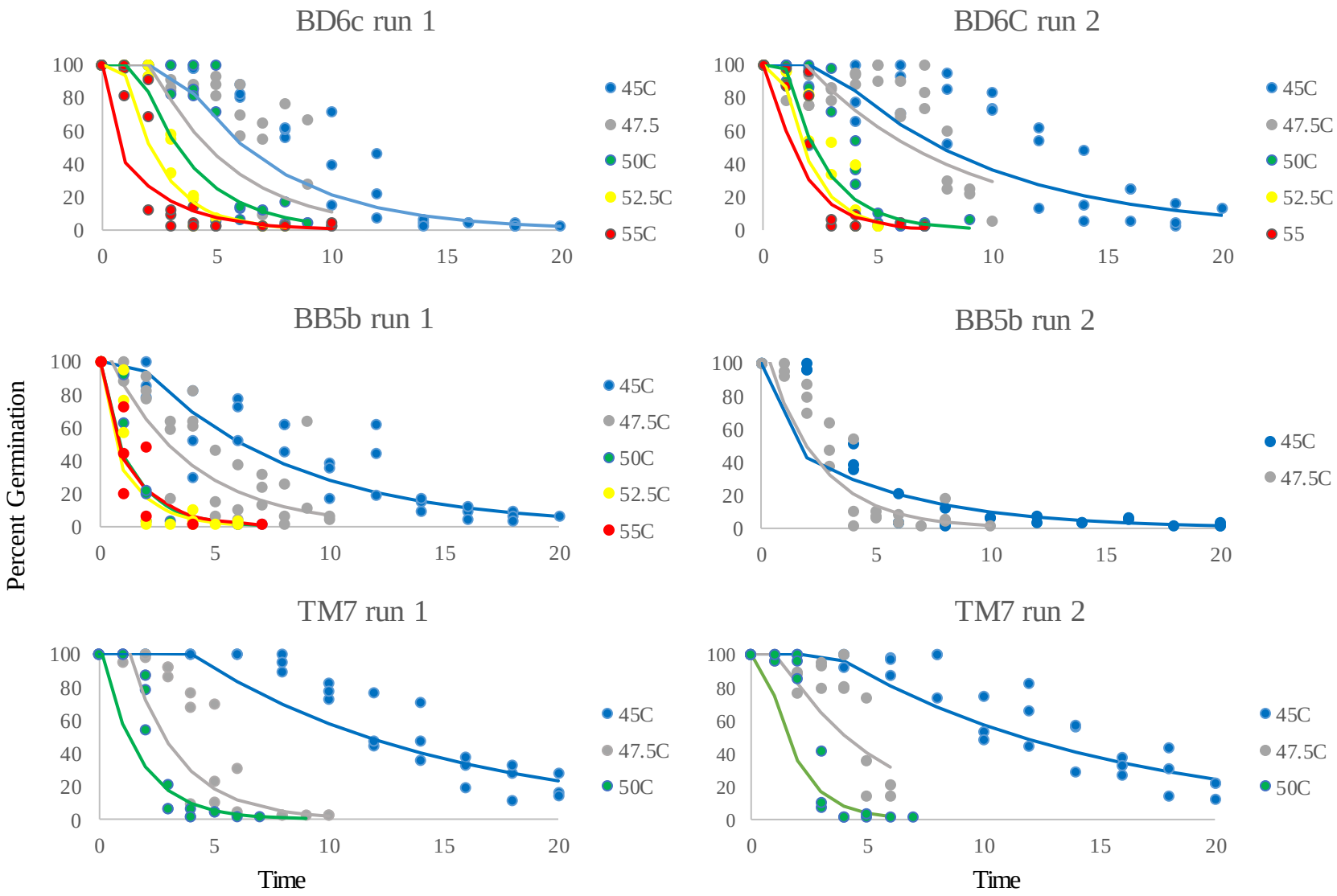

Figure 2.1. Thermal death kinetics using the equation log(std. germ) = -kt/2.303 based on percentage of conidial germination of three isolates of *Calonectria pseudonaviculata* after exposure to 45°C, 47.5°C, 50°C, 52.5°C, and 55°C for two independent runs.

Table 2.2:The thermal death rate constant (k) and 1-D values (min) derived from the equations Log(std. germ)=-kt/2.303, where std.germ=(germ. per time)/(germ. per control), k=rate constant, and t=time of conidia from three isolates of *Calonectria pseudonaviculata* treated in water at 45°C, 47.5°C, 50°C, 52.5°C, and 55°C.

Run	Temp (°C)	Isolate	[z]K (min^{-1})	[y]R^2	[x]1-D (min)
1	45	Bd6c	0.22536	0.8366	10.2192
		BB5b	0.15107	0.815	15.24459
		TM7	0.09137	0.731	25.20521
	47.5	Bd6c	0.28139	0.5237	8.18437
		BB5b	0.28076	0.572	8.202735
		TM7	0.45593	0.7892	5.051214
	50	Bd6c	0.4025	0.6496	5.721739
		BB5b	0.65043	0.8087	3.540735
		TM7	0.60213	0.7729	3.824755
	52.5	BD6c	0.57921	0.8348	3.976105
		BB5b	0.66453	0.6599	3.465607
	55	BD6c	0.42481	0.6245	5.421247
		BB5b	0.61384	0.7671	3.751792
2	45	BD6c	0.14034	0.621	16.51015
		BB5b	0.18369	0.6997	12.53743
		TM7	0.08572	0.7814	26.86654
	47.5	BD6c	0.15095	0.4745	15.25671
		BB5b	0.42892	0.7195	5.3693
		TM7	0.23873	0.5456	9.646881
	50	BD6c	0.55212	0.7584	4.171195
		TM7	0.8069	0.74514	2.854133
	52.5	BD6c	0.73246	0.727	3.144199
	55	BD6c	0.68155	0.7183	3.20618

[z]K=Rate constant in the equation:
Log(std. germ)=-kt/2.303
[y]R^2=Variation explained by the model
[x]1-D is the decimal reduction value or the time required to kill 90% of the conidia, calculated from the equation D=2.303/k

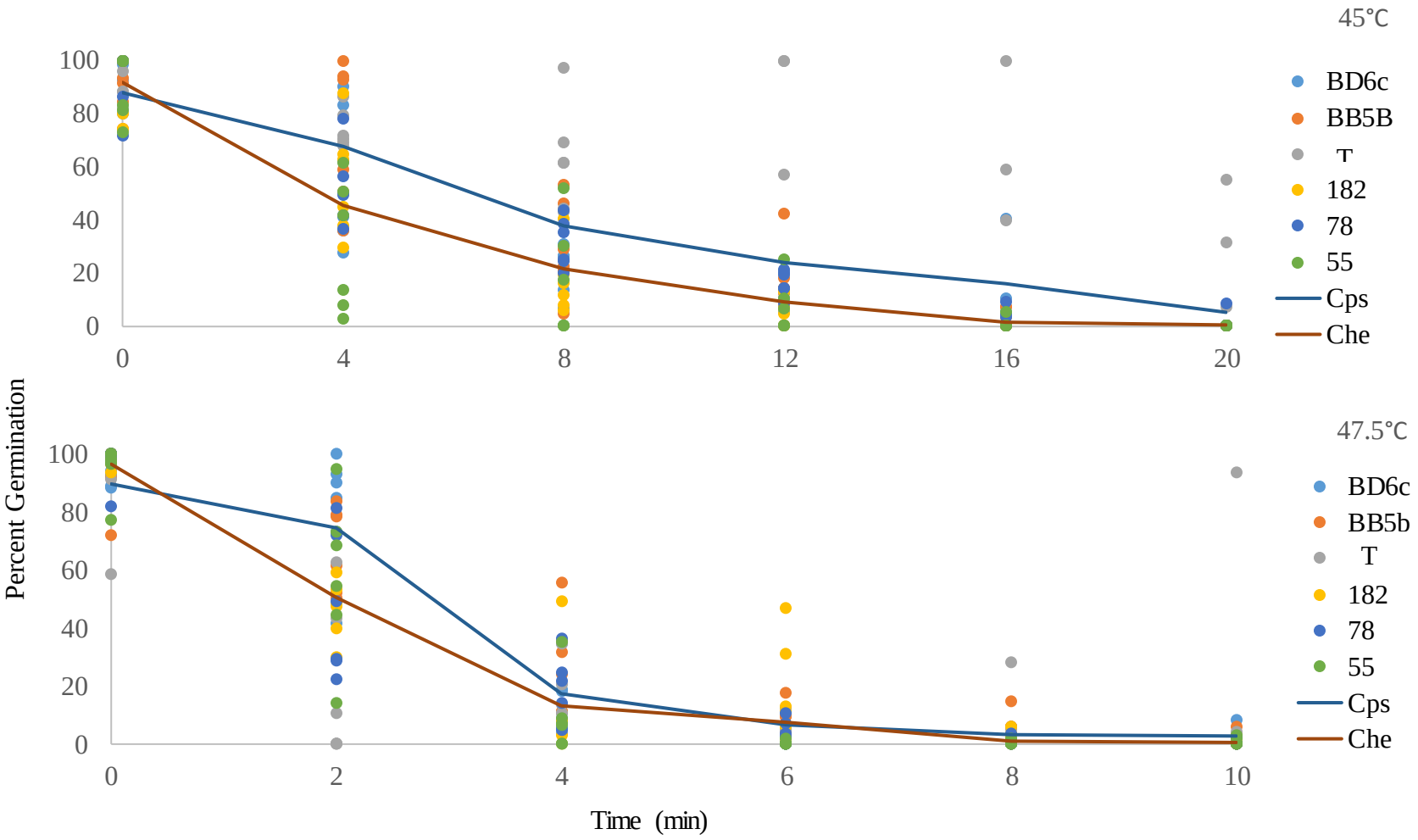

Figure 2.2: Thermal death kinetics of three isolates of *Calonectria pseudonaviculata* and three isolates of *C. henricotiae* and the percentage of conidial germination for each species after treatment in water at 45°C and 47.5°C.

Table 2.3: Analysis of variance table and sources of variation for determining the effect of species and time after conidia of *Calonectria henricotiae* and *Calonectria pseudonaviculata* were treated in water at 45°C and 47.5°C.

Temp	Source	DF	Mean Square	F	Pr>F
45°C	Model	11	19339.87	51.55	<0.0001
	Time	5	40314.54	107.47	<0.0001
	Species	1	7021.959	18.72	<0.0001
	Time*Species	5	797.0917	2.12	0.0639
	Error	204	151.85		
	R^2	0.735			
	Coefficient of Variation (%)	56.29			
47.5°C	Model	11	24684.16	162.55	<0.0001
	Time	5	52132.55	349.9	<0.0001
	Species	1	930.8452	6.13	0.0141
	Time*Species	5	986.43	6.5	<0.0001
	Error	204	151.85		
	R^2	0.898			
	Coefficient of Variation (%)	40.61			

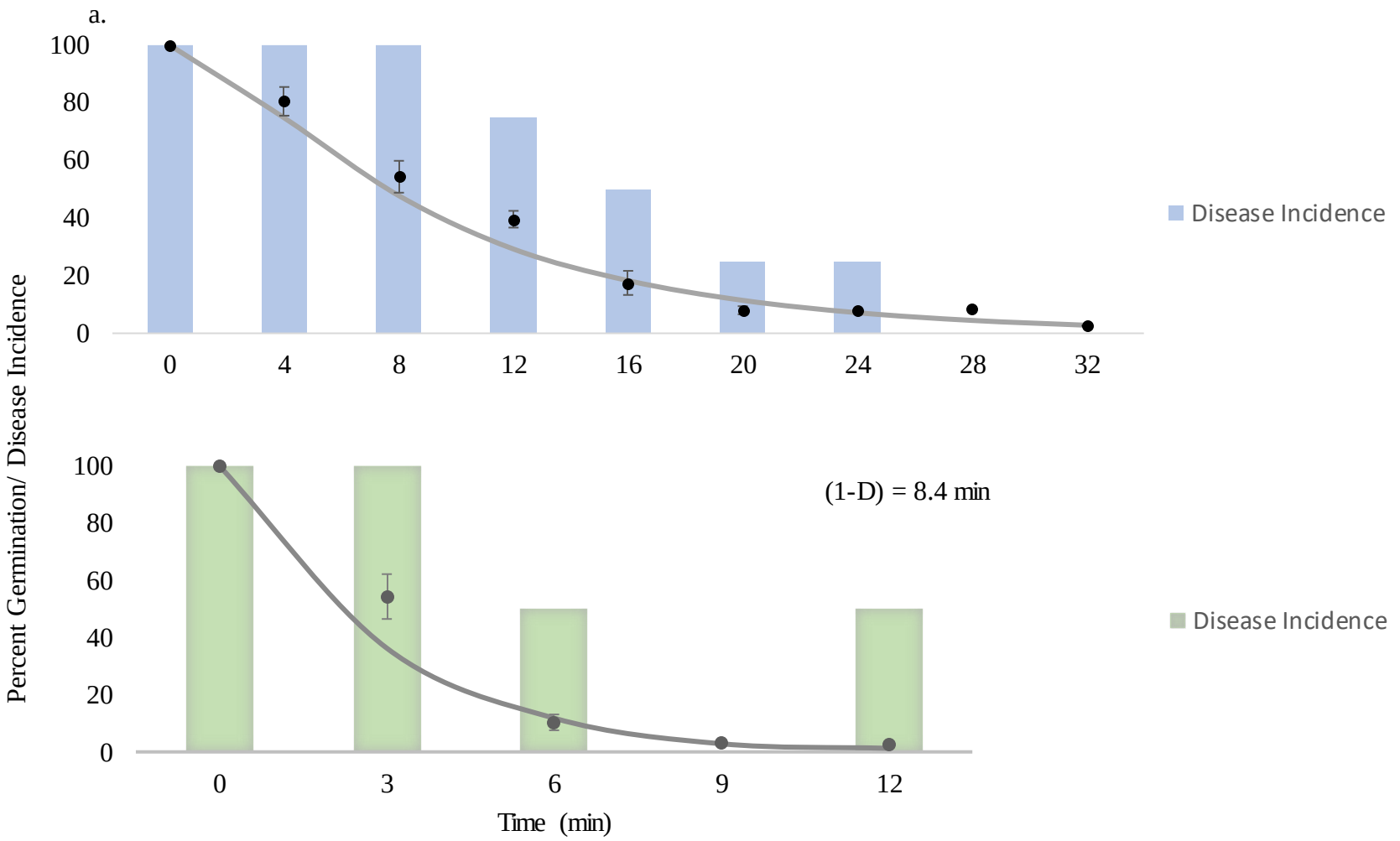

Figure 2.3: Thermal Death Curve of BD6C conidia heat treated at (a) 45°C and (b) 47.5°C and the percentage of leaves infected out of four on detatached 'Justin Brouwers' leaves inoculated with heat treated conidia

Chapter III.

Title: Rooting response of boxwood cultivars to hot water treatment

ABSTRACT - Boxwood blight caused by the fungal pathogen *Calonectria pseudonaviculata* is a newly described and emerging disease of boxwood. Current disease management strategies include the use of partially resistant cultivars and preventative fungicides. The inconspicuous leaf symptoms on boxwood cultivars with partial resistance make disease scouting challenging, especially during propagation. Growers need to ensure that cuttings of boxwood are not diseased before moving them into propagation houses where environmental conditions are conducive for disease development. Recently, we have been investigating the potential utility of hot water to eliminate *Calonectria pseudonaviculata* from boxwood cuttings during propagation. For this practice to become a viable disease management option, an understanding of the response of boxwood cuttings to treatment with hot water is needed. The objective of this research was to assess the rooting response of boxwood cuttings treated with hot water. We tested the hypotheses that 1) treatment of boxwood cuttings in 45°C or 47.5°C water for 30 min will not reduce root production and 2) root production will vary among boxwood cultivars when treated for more than 30 min in 45°C or 47.5°C water. For our experiments, boxwood cuttings from cultivars 'Green Beauty' (*Buxus microphylla* var. *japonica*), 'Green Velvet' (*B. sinica* var. *insularis* x *B. sempervirens*), 'Justin Brouwers' (*B. sinica* var. *insularis*), and 'Nana' (*B. sinica* var. *insularis*) were treated in 45°C or 47.5°C water for 0 to 60 min at 5 min intervals. Boxwood cuttings were incubated for 3 months and assessed for root production. The number of roots produced by boxwood cuttings from all cultivars treated in 45°C water for up to 60 min was not significantly lower than the non-treated control. A similar response was observed for cuttings of all cultivars treated in

47.5°C water for up to 60 min, except for cv. 'Nana' which produced fewer roots than the non-treated control after treatment for 35 min at 47.5°C.

INTRODUCTION

Boxwood (*Buxus* sp.) are woody ornamentals that are popular because they are deer resistant, mostly evergreen, low maintenance, and have relatively long life spans. Boxwood are commonly used in the landscape as hedges, specimen plants and topiaries, while boxwood cuttings are sold as holiday greenery in boxwood wreaths and trees. Annual sales of boxwood in the United States in 2014 was approximately $126 million (USDA National Agricultural Statistics Service, 2014). Boxwood blight, caused by the *Calonectria pseudonaviculata* (Crous J.Z. Groenewald & C.F. Hill) L. Lombard, M.J. Wingf & Crous. (syn=*Cylindrocladium pseudonaviculatum* Crous J.Z. Groenewald & C.F. Hill = *Cylindrocladium buxicoloa* Hencricot) is a recently described and emerging disease of boxwood leaves and stems that negatively impacts the health of boxwood throughout the world. The disease was first reported in the United States in Connecticut and North Carolina in 2011 (Ivors et al. 2012) and has been subsequently found in 16 additional states and three Canadian provinces. Leaf symptoms are typically characterized by brown to black, circular lesions with a necrotic center surrounded by a yellow halo. Eventually, the entire leaf becomes necrotic resulting in blighting and defoliation of the plant (Henricot and Culham, 2002). The pathogen can also cause black streaking stem cankers (Weeda and Dart, 2012).

Management of boxwood blight requires integrated use of cultural control, preventative fungicide and host plant resistance strategies. Recent investigations suggest that all commercial cultivars of boxwood are susceptible to blight (Ganci 2014; Shishkoff and Olsen 2015; Miller, Norris, and Cubeta 2016). *Buxus sempervirens* 'Suffruticosa', the most

commonly grown boxwood is highly susceptible and severely affected by the disease, but cultivars with partial resistance have been identified (Ganci 2014; Shishkoff and Olsen 2015; Miller, Norris, and Cubeta 2016). Asiatic species of boxwood (e.g., *Buxus harlandii, B. microphylla,* and *B. sinica*) are usually less susceptible to boxwood blight than cultivars within the European species *Buxus sempervirens*. However, the Asiatic cultivar 'Justin Brouwers' (*Buxus sinica var. insularis*) is one of the few Asiatic cultivars tested that is highly susceptible to the disease, while the cultivars 'Green Beauty' (*Buxus microphylla* var. *japonica*) 'Green Velvet' (*B. sinica* var. *insularis* x *B. sempervirens*) and 'Nana' (*B. sinica* var. *insularis*), are relatively resistant (Ganci 2014).

Increased times for symptom development (incubation period) and sporulation (latent period) slows the progression of disease development in partially resistant cultivars (Ganci 2014). Under controlled environmental conditions, asexual spore production on the abaxial leaf surface of partially resistant cultivars is delayed by up to 7 days compared to susceptible cultivars (Ganci 2014). Disease symptoms on cultivars with partial resistance are often manifested as small inconspicuous dark necrotic spots that subsequently produce characteristic boxwood blight lesions. The inconspicuous nature of disease symptoms on partially resistant cultivars creates challenges for disease scouting, especially during propagation with boxwood cuttings. Preventing the introduction of boxwood blight into a propagation house is imperative, as the environment is hot and humid which would increase disease development. Because the frequent application of preventative fungicides is often cost prohibitive, growers are interested in an alternative strategy for managing boxwood blight disease during the propagation stage.

Hot water treatment, a type of thermotherapy, has been used to mitigate diseases caused by bacteria, fungi, nematodes, and viruses in corms, fruits, seeds, tubers, and vegetables (Baker, 1962). Treatment of woody propagative material with hot water has also been previously shown to eliminate or reduce plant pathogenic bacteria species from pecan, pear, and apple scions, and grape vine cuttings (Sanderlin and Melanson 2008; Burr et al. 1989; Goheen, Nyland, and Lowe 1973). However, studies investigating the response of woody plants infected with fungal pathogens to hot water treatment are more limited. The fungal pathogens that cause Petri disease of grape, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf & Mugnai) Crous & W. Gams 2000 and a species of *Phaeoacremonium* W. Gams, Crous & M.J. Wingf., 1996, have successfully been eliminated from grapevines treated in hot water at 50°C for 30 minutes (Fourie and Halleen 2004). More recently, azalea cuttings artificially inoculated with the fungal pathogen, binucleate *Rhizoctonia* anastomosis group U (AG-U) were treated in 50°C water for 21 minutes, which prevented development and spread of azalea web blight disease without reducing rooting of the cuttings (Copes and Blythe 2009, 2011). Woody ornamentals may be amenable to hot water treatment due to their relatively low moisture content (Baker 1962). Before hot water treatments of boxwood cuttings infected with *Calonectria pseudonaviculata* can be deployed, comprehensive investigations into the rooting response of the boxwood cuttings after a heat treatment must be performed.

The objective of this study was to assess the rooting response of boxwood cuttings treated with hot water. We tested the hypotheses that 1) treatment of boxwood cuttings in 45°C or 47.5°C water for 30 min will not reduce root production and 2) root production will vary among boxwood cultivars when treated for more than 30 min in 45°C or 47.5°C water.

MATERIALS AND METHODS

Source and maintenance of boxwood cultivars

Four boxwood cultivars 'Green Beauty' (*Buxus microphylla* var. *japonica*), 'Green Velvet' (*B. sinica* var. *insularis* x *B. sempervirens*), 'Justin Brouwers' (*B. sinica* var. *insularis*), and 'Nana' (*B. sinica* var. *insularis*), ranging in age from 3-to 5-years old were obtained from Saunders Brothers Nursery in Piney River, VA. Prior to experimentation, plants were dug from the field and placed in a greenhouse maintained at 18°C at North Carolina State University (NCSU) for 6 to10 d.

Hot water treatment experiments

To examine the sensitivity of boxwood cultivars to hot water treatment, cuttings were removed from the top 15 cm of the mother plant and the bottom 7.5 cm of leaves were removed. Two cuttings of each individual cultivar were placed in 50 mL Falcon tubes (Corning, Inc., Tewksbury, M.A.) with six 1-cm diameter holes to facilitate water movement through the tube. Cuttings from each cultivar were individually treated at both 45°C and 47.5°C in a completely randomized design in a 19-L circulating water bath (Model 289; Precision-Fisher Scientific, Hampton, NH). Due to limited space in the water bath, cuttings of each cultivar was heat treated in two separate rounds, a 0-30 min round and a 35-60 min round, with tubes removed in 5 min intervals during each round. A non-treated control (0) and a room temperature control (RT) submerged in 22.5°C water for the 30 min in the 0-30 min round and 60 min in the 35-60 min round. For each time point in the two rounds, there were three replicate tubes containing two cuttings (subsamples). After treatment, cuttings were dipped in C- Grow-K rooting hormone (Coor Farm Supply, Smithfield, N.C.) for 3 s, and planted in soilless potting media in a 32-cell tray in a randomized complete block design.

Experiment 1 was conducted 3 -10 March 2016, and Experiment 2 was conducted from 14-17 March 2016.

Growth conditions and root assessment

Treated boxwood cuttings were placed in a PVC and plastic propagation house at the Horticulture Field Laboratory at NCSU, with floor heated to 21°C and cuttings were fog misted for 6 s every 8 min for the duration of the experiment. The ambient air temperature outside the propagation house was 20°C in March, 22°C in April, and 25°C in May. Beginning on 7 June 2016 for Experiment 1 and 13 June 2016 for Experiment 2, cuttings were removed from the trays and the number of roots greater than 1-cm in length was counted.

Statistical analysis

Prior to analysis, the two subsamples in each replicate were averaged to provide the mean number of roots at each time point. An analysis of variance was performed for each temperature, cultivar, and round separately by fitting a generalized linear mixed model using the PROC GLIMMIX procedure in SAS (Version 9.3, Cary, N.C.), where the duration of exposure to each temperature was a fixed effect and block was a random effect, denoted by the RANDOM statement in SAS. Dunnett's Multiple Comparison Analysis was performed to determine significant differences between the 0 and RT controls and cuttings submerged in hot water.

RESULTS

Experiment 1

Due to differences in root production between Experiments 1 and 2, data from these two experiments were not combined. With the exception of the 'Green Beauty' RT control in

the 35-60 min round of treatment at 45°C, the number of roots greater than 1-cm in length produced by the RT and 0 controls were similar (Table 3.1). Heat treatment of boxwood cuttings at 45°C for up to 30 min did not significantly reduce the number of roots produced for any of the cultivars heat treated. Treating cuttings of the boxwood cultivars 'Green Beauty', 'Green Velvet' and 'Justin Brouwers' in 45°C water for up 60 min did not significantly reduce root production compared to the 0 control. However, the number of roots produced by the cultivar Nana after treatment at 45°C for 55 and 60 min was significantly reduced from 37.8 roots to a mean of 20.3 and 17.0 roots, respectively (Table 3.1).

Treatment of cuttings from all cultivars at 47.5°C for 30 min did not reduce the number of roots produced when compared to the 0 control. Treating cuttings from 'Green Beauty', 'Green Velvet' and 'Justin Brouwers' for 60 minutes at 47.5°C also did not reduce root production. Submerging cuttings from the cultivar 'Nana' in 47.5°C water reduced root production at all sample times in the 35-60 minute round compared to the 0 control (Table 3.1).

Experiment 2

All of the RT controls in Experiment 2 produced a similar number of roots as the 0 control (Table 3.2). Treatment of cuttings from all cultivars in 45°C water for up to 30 min did not significantly reduce root production compared to the 0 control. The cultivars 'Green Beauty', 'Justin Brouwers' and 'Nana' were successfuly submerged in 45°C for up to 60 min without significantly reducing the number of roots produced. However, the cultivar 'Justin Brouwers' produced no roots after submersion in 45°C water for 55 min.

The mean number of roots produced by the cultivar 'Nana' was reduced to 4.2 after 30 min of exposure to 47.5°C water from an average of 28.4 roots produced by the 0 control for this cultivar. All other cultivars produced similar amounts of roots to their respective 0 controls when exposed to 47.5°C for 30 min. Root production was reduced in the cultivars 'Green Beauty' and 'Green Velvet' after exposure to 47.5°C for 60 min. The mean number of roots produced by 'Justin Brouwers' for the 0 control treatment was low at 3.8, and no roots were produced when cuttings were exposed to 47.5°C water for 35-60 min. Exposure of cultivar 'Nana' to 47.5°C for 35-40 min reduced root production compared to the 0 control, with no root produced after 45 min of exposure (Table 3.2).

DISCUSSION

Heat treatment at 45°C and 47.5°C has been successful in other plant pathosystems and inactivation of pathogenic microbes has occurred both *in vitro* and *in planta*. Treating asexual spores of the soilborne fungal pathogen *Verticillium dahlia* Kleb at 45°C reduced viability to 0.01% (Castejon-Munoz and Bollen 1993). Both mycelial growth and conidial germination of *Colletotrichum musae* and *Fusarium proliferatum*, two fungal crown rot pathogens of banana (*Musa* spp.), were inhibited by exposure to 45°C and 47.5°C. Furthermore, these temperatures where shown to inactivate these pathogens *in planta* when infected banana fruits were heat treated for 15-30 min (Lopez-Caberra and Marrero-Dominguez 1998). However, except for the studies of (Copes and Blythe 2009, 2011) and Fourie and Halleen (2004), information about the response of woody plant propagative materials to hot water treatment is limited.

In our study, heat treating cuttings from all four boxwood cultivars at 45°C for 30 minutes did not significantly lower root production compared to the non-treated control. At 47.5°C, cuttings from all cultivars except 'Nana' were able to successfully withstand treatment for 30 min without the treated cuttings producing less roots than the non-treated cuttings. Unlike treatment at 45°C and 47.5°C for up to 30 min, heat treating boxwood cuttings at these two temperatures for 35-60 min resulted in a differential rooting response between the cultivars tested. This result supported the hypothesis that root production will vary among cultivars after treatment at 45°C and 47.5°C for at least 35 min. For example, the number of roots produced by the cultivars 'Green Beauty' and 'Green Velvet' was not reduced by treatment in 45°C water for 60 minute in Experiments 1 or 2. However, treating cuttings from the cultivar 'Nana' at 45°C for 55 and 60 minutes significantly reduced the number of roots produced compared to the 0 control in Experiment 1. This same differential cultivar response was seen when heat treating 'Nana' cuttings for 35-60 min at 47.5°C, as root production was consistently reduced compared to the number of roots produced by the untreated control. Root production in 'Green Velvet' and 'Green Beauty' was also reduced compared to the non-treated control after exposure 47.5°C for 60 min in Experiment 2.

The cuttings heat treated in Experiment 2 produced less roots than cuttings from the same mother plants in Experiment 1, which was likely influenced by production of new growth on the mother plants at the beginning of Experiment 2. During the production of new foliar growth, the water content of the plant increases, and plants with higher water content are less amenable to heat treatment. Even non-heat treated plants produce less roots during the production of new growth, so it is recommended that dormant plants be used for cutting

production, especially if hot water treatments will be used (Baker 1962). While we did not measure the water content of the cuttings before treatment, boxwood growers typically discontinue cutting production by mid-March, the time at which our second experiment was performed, to deter losses caused by propagation of actively growing plant material. The Asiatic species and cultivars of boxwood used in this study were chosen based on preliminary experiments suggesting that commercial cultivars produced from species of *Buxus sempervirens,* which originated in Europe, were not able to withstand heat treatment (data not shown). Furthermore, the number of roots produced by the 0 controls was rarely different from the number of roots produced by the RT controls, indicating that the reductions in rooting that occurred in the heat treated cuttings were caused by exposure to hot water over time, and not just exposure to water for long periods of time.

An understanding of differences in thermal tolerance between cultivars and species of a plant is essential for deploying hot water treatment as a measure for disease control (Grondeau, Samson, and Sands 2011). The primary objective of our research was to determine whether boxwood cuttings from cultivars with partial resistance to boxwood blight could tolerate exposure to hot water. Future heat treatments will only be successful if there is a differential response between the pathogen and the host to heat treatment. Upon the completion of the rooting period following the heat treatment, cuttings must also meet the grower standard of having 5.0 roots over 1.0 cm in length. Because we showed that the dormancy of the cuttings did affect their rooting ability, we did not use the results from Experiment 2 when determining our pathogen inactivation windows. The longest time period in which each individual cultivar produced an acceptable number of roots after treatment at 45°C and 47.5°C represents the maximum time that particular cultivar can be heat treated. To

prevent a negative rooting response during future experiments that heat treat *Calonectria pseudonaviculata in planta,* the pathogen will need to be inactivated within 60 minutes at 45°C for all cultivars tested, within 35 minutes at 47.5°C for 'Nana', and within 60 minutes at 47.5°C for 'Green Beauty', 'Green Velvet' and 'Justin Brouwers.' Experiments have shown that *Calonectria pseudonaviculata* conidia were able to infect detached 'Justin Brouwers' boxwood leaves after they were treated for 24 minutes at 45°C, when germination was 7.7%, and after 12 min at 47.5°C, when germination was 2.4% (Chapter 2). Infection did not occur past 24 or 12 minutes, so these represent the minimum time hypothesized to inactivate *C. pseudonaviculata* in planta, giving a minimum inactivation window of 24 min at 45°C and a minimum of 12 minutes at 47.5°C.

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Table 3.1: Mean root production of four different boxwood cultivars after treatment in 45°C and 47.5°C for 0-60 minutes for Rooting Experiment 1.

			Duration of Submission (min)								
Temp	Round	CV[z]	0/0	5/35	10/40	15/45	20/50	25/55	30/60	RT/RT	Prob.[y]
45	0-30	GB	28.33[x]	13.5	16.83	16.67	24.17	17.5	18	13.67	0.0665
		GV	35	40	31.83	28.3	23.5	19.5	23.83	22.3	0.5646
		JB	21.5	26.67	24.17	24	26.67	23.5	22.17	15.5	0.5816
		Nana	29.17	27	35	17.5	23.5	23.17	21.67	33.33	0.5959
	35-60	GB	20.83	19.5	11.3	21.0	11.4	21.67	12.5	0*	0.0005
		GV	36	21.17	15.5	24.33	23.83	23.33	24.83	20.3	0.2685
		JB	28.83	21.67	26.17	27.67	19.17	30.5	26.83	27.83	0.7544
		Nana	37.83	18.33	25.5	25.17	28.0	20.3*	17.0*	28.0	0.0176
47.5	0-30	GB	28.83	13.5	16.83	16.7	24.17	17.5	19	13.7	0.1520
		GV	24.67	34.83	35.5	28.5	22.3	22.17	20.3	13.83	0.2840
		JB	21.3	26.5	25.8	31.3	21.67	27.67	16.5	30.17	0.0669
		Nana	20.67	35.5*	31	17.3	21.3	19.17	14	31.83	0.0011
	35-60	GB	13.67	11.67	7.5	12.67	8.5	10.83	8.5	13.67	0.4342
		GV	26.33	28.67	32.67	17.67	22.17	13.17	14.67	24.33	0.6498
		JB	25.5	16.8	20.17	11.8	17.8	6.17	12.67	21.83	0.1440
		Nana	24.83	14.3*	12.5*	6.0*	4.67*	2*	1.33*	28.17	<0.0001

[z] (GB)-*B. mirocphylla* var. *japonica* cv. 'Green Beauty, (GV)- *B. sinica* var. *insularis* x *B. sempervirens* cv. 'Green Velvet', (JB)-*B. sinica* var. *insularis* cv. 'Justin Brouwers', (Nana)-*B. sinica* var. *insularis* cv.'Nana'

[y]Probability (based on Type III sum of squares from the GLIMMIX procedure of SAS 9.3) of duration of exposure at each temperature affecting root growth

[x]Mean number of roots greater than 1.0 cm in length after the 3 month growing period.

*Root development is significantly different (α=0.05) from the non-treated control based on the Dunnett's Multiple Comparisons test, which compares all treatments to the control.

Table 3.2: Mean root production of four different boxwood cultivars after treatment in 45°C and 47.5°C for 0-60 minutes for Rooting Experiment 2.

			Duration of Submission (min)								
Temp	Round	CV[z]	0/0	5/35	10/40	15/45	20/50	25/55	30/60	RT/RT	Prob.[y]
45	0-30	GB	19.67[x]	16.83	26	21.5	13.67	15.83	16.0	11.67	0.3674
		GV	19.0	24.17	17.17	16.7	19.17	17.7	13.17	18.0	0.6071
		JB	3.17	18.33	11.0	5.5	17.3	10.17	9.0	7.67	0.5312
		Nana	32.67	35.67	31.67	25.5	16.5	26.0	21.33	26.0	0.6071
	35-60	GB	17.17	18.3	13.67	13.5	9.17	17.67	9.3	15.17	0.2905
		GV	19.17	14.0	10.0	13.17	14.67	5.0*	10.3	19.83	0.0374
		JB	5.0	12.0	4.5	4.17	4.67	0	1.17	11.83	0.3230
		Nana	21.17	17.5	17.3	17.83	11.33	17	8.3	27.17	0.0736
47.5	0-30	GB	20.3	9.17	16.17	11.67	12.83	11.67	11.33	19.5	0.1327
		GV	14.83	21.33	14.0	22.5	28.17	14.0	9.83	19.5	0.0643
		JB	26.5	24	17.4	18.3	16.3	13.3	13.3	20.17	0.3419
		Nana	24.83	21.0	19.17	10.67	14.50	12.0	4.17*	20.67	0.0104
	35-60	GB	14.5	5.83	13.67	6.3	5.17	4.5	3.7*	13.5	0.0227
		GV	18	14	13.5	14.17	8.83	4.17	0*	23.3	0.0059
		JB	3.83	0	0	0	0	0	0	10.83*	<0.0001
		Nana	25.17	8.3*	3.83*	0*	0*	0*	0*	29.83	<0.0001

[z] (GB)-*B. mirocphylla* var. *japonica* cv. 'Green Beauty, (GV)- *B. sinica* var *insularis* x *B. sempervirens* cv. 'Green Velvet', (JB)-*B. sinica* var. *insularis* cv. 'Justin Brouwers', (Nana)-*B. sinica* var. *insularis* cv. 'Nana'

[y]Probability (based on Type III sum of squares from the GLIMMIX procedure of SAS 9.3) of duration of exposure at each temperature affecting root growth

[y]Mean number of roots greater than 1.0 cm in length after the 3 month growing period.

*Root development is significantly different (α=0.05) from the non-treated control based on the Dunnett's Multiple Comparisons test, which compares all treatments to the control.

APPENDIX

Appendix A: Evaluation of boxwood cultivars for resistance to boxwood blight, 2015.

Miller, M. E., Norris, R. S., & Cubeta, M. A. (2016). Evaluation of boxwood cultivars for resistance to boxwood blight, 2015. *Plant Disease Management Reports*, 10: OT009

This trial evaluated the relative level of resistance in 23 boxwood cultivars provided by The United States National Arboretum (Washington, DC) to Calonectria pseudonaviculata (Cps) the causal agent of boxwood blight. Two-year-old boxwood liners in 1-gal pots were placed on a container pad at the Tidewater Research Station in Plymouth, NC and arranged in a randomized complete block design with four replications of one plant per replicate. The plants were fertilized with 20-20-20 fertilizer at the labeled rate in March 2016. Inoculum was prepared by flooding potato dextrose agar plates containing a sporulating field isolate of *Calonectria pseudonaviculata* (TM7) with a 0.01% solution Tween 20 and distilled water. The suspension was diluted to 3.0x105 spores/fl oz and applied to plants on 10 Jun by spraying with a pressurized sprayer until runoff. White, plastic 10-gal garbage bags were placed over plants overnight to increase relative humidity. The following morning, the bags were removed and the plants were overhead irrigated four times a day for 10 min to promote environmental conditions conducive for disease development. The percentage of the entire plant showing symptoms such as leaf lesions or stem streaking was estimated for each plant on 24 Jun, 22 Jul, 5 Aug, 9 Sep, and 4 Dec. Defoliation was also rated for each plant using a semi-qualitative scale in which no defoliation received a 1, 1% received a 2, 1 to 10% received 3, 10 to 30% received 4, and greater than 30% defoliation received a rating of 5. Average maximum temperatures for Jun, July, Aug, Sep, Oct, Nov, and Dec were 88, 89, 87, 83, 73, 67, and 67°F; average minimum temperatures were 69, 70, 67, 66, 53, 48, and 49°F; and total rainfall amounts were 5.91, 3.45, 2.06, 6.46, 4.83, 5.41, and 5.46-in, respectively.

The area under the disease progress curve (AUDPC) for the percent diseased plant area was calculated 177 days post inoculation (dpi). Analysis of variance was performed using PROC GLM in SAS (SAS Institute Inc., Cary, NC) and the means were separated using the Fisher's Least Significant Difference Post Hoc test.

Varder Valley had the highest level of disease and highest AUDPC value at 177 dpi. This cultivar was significantly more susceptible than the cultivar Suffruticosa, which was included as a susceptible control. Suffruticosa and Denmark had similar levels of disease 177 dpi and had similar AUDPC values, indicating these cultivars have moderately low levels of resistance to boxwood blight relative to the most susceptible cultivar Varder Valley. John Baldwin, Arborescens 31793, and Green Mound all had the lowest AUDPC values. Therefore, these cultivars are more resistant than Varder Valley, Suffruticosa, and Denmark, the most susceptible cultivars in the study. However, the two-year-old plants provided for use in this study had underdeveloped plant architecture resulting in lower humidity within the canopy and causing less disease development. For example, the cultivar Sempervirens was rated resistant in this trial, but mature plants often are severely affected by the pathogen.

	Boxwood Blight		Leaf Defoliation
Boxwood Cultivar	Final Disease Severity (4 Dec)	AUDPC	**Average Defoliation (±Standard Deviation) (4 Dec)
Varder Valley	44.50 a*	3057.9 a*	1.5 (±0.50)
Suffruticosa	28.75 b	2722.9 b	1.0 (±0)
Denmark	19.25 bc	1309.4 b	2.0 (±1.0)
Scupi	9.00 cd	1121.3 bc	1.0 (±0)
Marginata	8.75 cd	493.9 bcd	1.5 (±0.5)
National	6.25 cd	718.8 bcd	1.5 (±0.5)
Graham Blandy	6.00 cd	694.0 bcd	1.0 (±0)
Wintergem	4.25 d	270.8 cd	1.0 (±0)
Handsworthienesis	3.25 d	226.4 cd	1.5 (±0.5)
Sempervirens	2.75 d	357.1 bcd	1.5 (±0.5)
Northland	2.75 d	370.3 bcd	1.5 (±0.5)
Northern New York	1.50 d	90.6 cd	1.0 (±0.5)
Buxus sp.	1.50 d	94.5 cd	1.0 (±0)
Pincushion	1.00 d	126.0 cd	1.0 (±0)
Harlandii	1.00 d	172.6 cd	1.0 (±0)
Myrtifolia	1.00 d	322.6 bcd	1.0 (±0)
Arborescens 31793	0.75 d	51.9 d	1.0 (±0)
Edgar Anderson	0.75 d	167.6 cd	1.0 (±0)
John Baldwin	0.75 d	79.5 d	1.0 (±0)
Baby Gem	0.67 d	112.5 cd	1.0 (±0)
Buxus bodineiri	0.50 d	88.3 cd	1.5 (±0.5)
Arborescens 57953	0.50 d	119.9 cd	1.0 (±0)
Green Mound	0.50 d	37.0 d	1.0 (±0)
LSD	14.1	1037.3	ns

*Means within a column followed by the same letter are not significantly different (P=0.05) based on the Fisher's Protected LSD test.

**Average defoliation of each cultivar assessed at 177 dpi. F-test for overall defoliation model was not significant (ns).