ABSTRACT

GANCI, MIRANDA LYNN. Investigation of Host Resistance in *Buxus* Species to the Fungal Plant Pathogen *Calonectria pseudonaviculata* (=*Cylindrocladium buxicola*), the Causal Agent of Boxwood Blight and Determination of Overwinter Pathogen Survival. (Under the direction of Drs. K.L. Ivors and D.M. Benson).

Boxwood blight, caused by *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum*, *Cylindrocladium buxicola*) is an emerging disease on boxwood (*Buxus* spp.). Host resistance is a disease management strategy and in this study *Buxus* cultivars were screened for resistance to the pathogen. Natural pathogen spread was simulated on an outdoor shaded container pad to identify field resistance in cultivars. Disease resistance was also assessed on detached branches of several *Buxus* cultivars incubated in humidity chambers. In total, over 80 cultivars from four commercially grown *Buxus* species were screened and a wide range of resistance was observed, however, no complete resistance was found. In general, the Asiatic types expressed higher levels of partial resistance than others.

Components of partial resistance to boxwood blight in *Buxus* cultivars were evaluated on whole plants in a controlled environment chamber; variables measured included disease severity, incubation period, latent period, lesion area, and conidia production. The cultivars were chosen because they expressed a range of resistance reactions to the pathogen in the container pad experiments. The cultivars and species *B. harlandii*, *B. microphylla* 'John Baldwin', *B. microphylla* var. *japonica* 'Green Beauty', *B. sempervirens* 'Dee Runk', and *B. sinica* var. *insularis* 'Nana' expressed field resistance, whereas the cultivars *B. microphylla* var. *japonica* 'Morris Midget', *B. sempervirens* 'American' and 'Vardar Valley' were susceptible. Differences in components of partial resistance were found across the boxwood cultivars tested. Resistance in the cultivars *B. sinica* var. *insularis* 'Nana', *B. harlandii*, and

B. microphylla var. *japonica* ‘Green Beauty’ was attributed to their minimal disease severity, longer incubation period, and longer latent period. The Asiatic cultivar *B. microphylla* var. *japonica* ‘Morris Midget’ had higher disease severity, shorter latent period, and higher conidia production. The susceptibility of *B. sempervirens* ‘American’ was attributed to shorter incubation and latent periods, large lesion area, and high disease severity.

The pathogen *C. pseudonaviculata* forms microsclerotia in diseased leaves and stems. Abscised infected leaves harbor these survival structures, which can serve as inoculum for future disease epidemics. Under favorable conditions, microsclerotia germinate to form conidia, the primary inoculum for this disease. Survival of *C. pseudonaviculata* was investigated within infected leaves placed on the surface or subsurface (5 cm) of field soil or soilless potting media from September through May in 2012 to 2013 and again in 2013 to 2014. Throughout the duration of both the field soil experiment and soilless media experiments in 2012 to 2013 the proportion of leaves with sporulation was reduced for leaves on the surface compared to the subsurface. Additionally, in 2013 to 2014, results from both experiments indicated that the proportion of leaves with sporulation and the proportion of leaves with microsclerotia were greater for leaves from the subsurface treatments compared to the surface treatments. There was a statistically significant correlation between the proportion of leaves with sporulation and the proportion of leaves with microsclerotia. Results demonstrate that *C. pseudonaviculata* can survive over the winter in infected leaves in North Carolina.

Investigation of Host Resistance in *Buxus* Species to the Fungal Plant Pathogen *Calonectria pseudonaviculata* (=*Cylindrocladium buxicola*), the Causal Agent of Boxwood Blight and Determination of Overwinter Pathogen Survival

by
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DEDICATION

I dedicate these studies to the fungus which I have come to hate, yet respect and understand through thousands of hours of research. May we continue to always learn more about managing it!

I am also so grateful for the never ending support from my parents, Patti and Charlie Ganci, my loving sister, and patient friends.

BIOGRAPHY

Miranda Lynn Ganci was born and raised in South Florida. She moved to North Carolina to pursue an associate degree in Culinary Arts at Johnson and Wales University. Upon completing that degree she realized that her love of food extended beyond the kitchen door and she became interested in agriculture. After graduating with a Bachelor of Science in Plant and Soil Science from North Carolina State University she decided to combine her interest in fungi and agriculture and join the Plant Pathology Department at NC State in order to learn how to manage diseases caused by fungi and other organisms. After completing her Master of Science degree she plans on applying her skills towards helping growers sustainably manage plant diseases.

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LITERATURE REVIEW

Introduction

Boxwood (*Buxus* spp.) are ornamental plants commonly used in the landscape as hedges, specimen plants, and topiary. Boxwood plants generally are low maintenance plants and did not have any major devastating disease problems until the emergence of boxwood blight. Boxwood blight is a foliar disease that affects plants in the *Buxaceae* family. The causal agent is the fungal pathogen *Calonectria pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous (syn. = *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenew. & C.F. Hill = *Cylindrocladium buxicola* Henricot) (3, 12, 26). The fungus is classified in the phylum *Ascomycota*, class *Sordariomycetes*, order *Hypocreales* and family *Nectriaceae* (17, 25, 33). The pathogen was initially detected in the United States in 2011 in North Carolina and Connecticut (19) and at the time of this publication has been identified in 15 states (AL, CT, DE, GA, MA, MD, NC, NJ, NY, OH, OR, PA, RI, TN, and VA) and three Canadian provinces (British Columbia, Ontario, and Québec). Symptoms include brown to black circular lesions that often have a yellow halo, dark margin and necrotic center. The lesions expand in a zonate pattern resulting in necrosis and defoliation of the leaf. The pathogen can also cause black streaking stem cankers. The asexual stage (= anamorph) of the pathogen contributes to disease development and the sexual stage (= teleomorph) has not been observed.

The value of boxwood plants sold in the U.S. is over $100 million annually (37). Susceptible cultivars infected by the boxwood blight pathogen can become completely defoliated resulting in plants which are unsaleable. While boxwood blight can severely

affect the aesthetic quality of plants, infection does not always result in plant death. Young seedlings are especially likely to be killed by the pathogen. Death can also result when plants are infected by the opportunistic fungal pathogen *Pseudonectria buxi,* (DC) Seifert, Gräfenhan & Schroers causal agent of Volutella blight (34), after initial infection by *C. pseudonaviculata*.

History and nomenclature

In 1994, a new foliar blight disease of boxwood was detected in a Hampshire, United Kingdom (UK) nursery. At the time, the pathogen causing the disease was unknown. In 1997, an outbreak of boxwood blight occurred in multiple nurseries throughout the UK. It was determined that a *Cylindrocladium* (=*Calonectria*) species was the causal agent of boxwood blight, however, it was speculated that the pathogen was an unidentified species (15). Meanwhile, in the late 1990s, a new foliar blight disease on boxwood was also identified in New Zealand. In June 2002, the pathogen causing boxwood blight in New Zealand was formally published as *Cylindrocladium pseudonaviculatum* (3) and in December 2002, the same pathogen was formally published as *Cylindrocladium buxicola* in the UK (12). Researchers from the UK, Henricot and Culham (12), established the phylogenetic status of *C. buxicola* (= *C. pseudonaviculata*) by sequencing the ribosomal 5.8S RNA gene and the flanking internal transcribed spacers (ITS), the beta-tubulin gene, and the high mobility group (HMG) of the *MAT2* mating type gene. The analysis included isolates obtained from *Buxus sempervirens* displaying symptoms of boxwood blight in New Zealand and established that they were identical with those isolated from the UK.

In Europe, where boxwood blight has had serious economic impact and been the focus of management studies for over 15 years the causal agent has been referred to as *C. buxicola* (= *C. pseudonaviculata*) (13). However, upon the detection of the pathogen in North America (8, 19) the first assigned name has been re-introduced into the literature because *C. pseudonaviculatum* has priority over *C. buxicola*. In an effort to reduce confusion over the name of the boxwood blight pathogen a proposal to conserve the more commonly used name, *C. buxicola* has been submitted to the journal TAXON (13).

According to Article 59.1 (revised in 2012) of The International Code of Nomenclature for algae, fungi, and plants, only one scientific name should refer to each species of fungi (26), a concept commonly referred to as 'One Fungus One Name' (35, 41). The name for the teleomorph *Calonectria* De Not. 1867 (type: *C. pyrochroa* (Desm.) Sacc. 1878) has priority over the name for the anamorph *Cylindrocladium* Morgan 1892 (type: *C. scoparium* Morgan 1892) (31). When multiple generic names refer to the same organism, priority is assigned to the oldest generic name unless there is a proposal to make an exception to the principle of priority (26). The official name for the boxwood blight pathogen has undergone many changes and the names *C. pseudonaviculata, Cylindrocladium buxicola,* and *Cylindrocladium pseudonaviculatum* all appear in the literature. Throughout this publication the pathogen will be referred to as *Calonectria pseudonaviculata*.

Pathogen biology

Perithecia of *Calonectria pseudonaviculata* have not been observed during disease development or on infected plants. Additionally, sexual compatibility and mating experiments in the laboratory have not resulted in perithecia development (12). Both

homothallic and heterothallic mating systems are found within *Calonectria* (24). The absence of perithecia development by *C. pseudonaviculata* suggests that the species is heterothallic (12).

The infectious propagules of *C. pseudonaviculata* are asexual conidia. Conidia are borne from penicillate branched conidiophores bearing phialides; the structure also includes a stipe extension which terminates in a vesicle with a papillate (pointed) tip. Conidia are covered in a sticky mucilage. During conidiogenesis conidia stick to one another to form a characteristic cylindrical cluster (2). Conidia are splash dispersed by irrigation water and wind-driven rain. Not only does the mucilage help conidia adhere to plant tissue, the sticky covering also facilitates short and long distance conidia dispersal by people, equipment and animals. Once conidia land on host leaves they are capable of germinating in as few as three hours. Germination can occur on the abaxial or adaxial leaf surface. Hyphae penetrate through stomata on the abaxial leaf surface (9). The fungal mycelium proliferates intercellularly in the mesophyll layer. After re-emergence from stomata the fungus produces conidiophores bearing conidia on the abaxial surface. The disease cycle can be completed in less than seven days (14). Conidia can also develop on stem tissue (40). Many *Calonectria* species are capable of infecting root tissue, but *C. pseudonaviculata* has not been isolated from roots (11).

Pathogen survival

Many species of *Calonectria* (= *Cylindrocladium*) cause root, crown, and foliar diseases on field, forest, and ornamental crops (2). The ability of many of the pathogenic *Calonectria* species to form microsclerotia contributes to pathogen survival and subsequent

production of inoculum when conidiophores and conidia form on germinating microsclerotia. In boxwood, azalea (*Rhododendron* spp.), peanut (*Arachis hypogaea*), and spruce (*Picea* spp.) tissues, microsclerotia of *Calonectria* are formed by the aggregation of chains of chlamydospores. (1, 22, 32, 40). Microsclerotia formed by *C. pseudonaviculata* are similar to those formed by *Rhizoctonia solani* as they are not enclosed by a hard rind typical of some other fungal sclerotia such as those formed by *Sclerotium rolfsii*. Microsclerotia of *C. pseudonaviculata* have only been detected in leaves and stems, but other plant pathogenic species of *Calonectria* can form microsclerotia in flowers, leaves, stems, and roots (1, 22, 32, 40).

It has been shown that *C. floridanum*, *C. pseudonaviculata*, and *C. scoparium* are capable of producing over 1,000 microsclerotia per abscised infected leaf while *C. indusiata* (syn. *C. theae*) produces fewer than 200 per leaf (22, 40). Interestingly, microsclerotia of *C. floridanum* and *C. scoparium* were not specifically associated with stomata in azalea (22), although *C. pseudonaviculata* microsclerotia are apparently associated with stomata in boxwood (9).

Abscised infected boxwood leaves and stems can harbor microsclerotia. When soil and plant debris were collected from the top 5 cm of a boxwood blight infested field, pathogen inoculum density averaged 25.2 colony forming units per 10 gram of soil (4). *C. pseudonaviculata* microsclerotia were recovered at greater rates from the debris fraction of soil (debris > 850 µm in diameter) than from the soil fraction (particle size range 38 µm to 850 µm diameter): therefore it was determined that inocula are primarily present in defoliated infected leaves and stems (4). It is unknown if *C. pseudonaviculata* microsclerotia can

survive free of plant debris in naturally infested field soil. In peanut fields, *C. ilicicola* microsclerotia can be found in root tissue or free in soil (30). Additionally, *C. floridanum* and *C. scoparium* microsclerotia have also been isolated from infested soils (27, 29, 36).

Host range

The fungal pathogen *C. pseudonaviculata* is capable of infecting hosts in the *Buxaceae* family. Natural infection by *C. pseudonaviculata* and subsequent development of boxwood blight has been observed on species of *Buxus* (boxwood) (15) and *Pachysandra* (spurge) (5). Symptoms of boxwood blight developed when detached branches of *Sarcococca* (sweet box) were artificially inoculated (14). However, artificial inoculation on an outdoor container pad, of popular ornamental plants found in the *Ericaceae* family including azalea, *Pieris,* and *Rhododendron,* did not result in development of symptoms (Ganci, *unpublished*).

In nursery and field production of boxwood, the pathogen can infect several hundred thousand plants within a few weeks when environmental conditions are ideal for disease development (6). In addition to causing devastation in production settings, boxwood blight has also been identified in native stands of *Buxus* in Iran (28), the Republic of Georgia (10) and at the famed Box Hill in Surrey, United Kingdom (15). Boxwood are the most commercially popular hosts of *C. pseudonaviculata*. All known species and cultivars of *Buxus* that have been exposed to the pathogen are susceptible to some degree (7, 9, 14). The boxwood cultivars *Buxus sempervirens* ‘American’ and ‘Suffruticosa’ (English) are two of the most popular commercial cultivars but they also appear to be among the most susceptible; disease severity ratings can exceed 75% diseased leaves and defoliation. Over 95 species of

Buxus are known (38) and screening diverse host germplasm for pathogen resistance followed by planting resistant cultivars is an important disease management strategy.

Management strategies

As a new and emerging disease, boxwood blight is a threat to the economic vitality of boxwood producers and disease management strategies must be developed in order to reduce economic losses caused by the disease. In addition to host resistance, management strategies should incorporate cultural and chemical controls. Sanitation is an important tool to prevent human induced pathogen movement and disease introduction. The primary means of natural spread of *C. pseudonaviculata* is by splash dispersal of conidia onto new hosts. However, human activity can greatly increase the risk for pathogen spread. For example, the pathogen can reach new host plants and production areas via contaminated equipment, humans, and tolls such as pruning shears. Preventing movement from infested areas to clean areas and surface sterilizing equipment can reduce the risk of pathogen spread. After the pathogen is established it is very difficult to eradicate from production areas (22, 23).

The pathogen *C. pseudonaviculata* is not systemic and therefore it may be theoretically possible to prune out of infected parts of plants. However, this management strategy may be risky because any infected plant debris not removed from the site can serve as inoculum for future epidemics. In production areas, complete destruction or removal of infected plants is advised. A typical six year old *B. sempervirens* ‘American’ plant may easily have over 10,000 leaves; if each infected leaf can serve as an inoculum source it is clear that site sanitation and removal of debris are integral to disease management.

Flaming soils can greatly reduce the levels of *C. pseudonaviculata* inocula in infested field sites by eliminating infected debris from the soil surface (4). However, if flaming does not completely eliminate debris it may not be a completely effective strategy. Soil solarization has been shown to effectively eliminate *C. pauciramosa* and *C. polizzii* microsclerotia from the soil surface to a depth of 30 cm. The successful elimination of inocula in soil was achieved under solarization in tunnel greenhouses (39). The effectiveness of soil solarization is highly dependent on exposure time (20) and unfortunately frequent rainfall in boxwood field production areas on the east coast of the United States may prevent soils from attaining the consistently high temperatures required to reduce or eliminate pathogen viability.

The effect of fungicides on mycelial growth and germination of conidia was evaluated in-vitro by Henricot et al. 2008 (14) and LaMondia 2014 (21). The following active ingredients were found to reduce *C. pseudonaviculata* mycelial growth and conidia germination: boscalid + pyraclostrobin (14), chlorothalonil (21), epoxiconazole + kresoxim-methyl + pyraclostrobin (14), epoxiconazole + pyraclostrobin (14), fludioxonil (21), fludioxonil + cyprodinil (21), kresoxim-methyl (14, 21), mancozeb (21), pyraclostrobin (21), and trifloxystrobin (21).

Additionally, boxwood blight can be effectively managed in-vivo, when fungicides are applied to the plants preventatively. The most effective active ingredients included boscalid + pyraclostrobin (16), chlorothalonil (16, 18) (Ivors, *unpublished*), chlorothalonil + propiconazole (Ivors, *unpublished*), chlorothalonil + thiophanate-methyl (18) (Ivors, *unpublished*), epoxiconazole + kresoxim-methyl + pyraclostrobin (16), prochloraz (16),

trifloxystrobin + triadimefon (Ivors, *unpublished*). There are no registered uses for the active ingredient epoxiconazole in the US; experiments utilizing this active ingredient were conducted in the UK. Curative applications of fungicides did not provide adequate disease control. Effective disease management can only be achieved if plants are sprayed preventatively and not during pathogen latent period or after symptom development (16, 18) (Ivors, *unpublished*).

Research objectives

The overall goal of this research was to provide information that will lead to better management of boxwood blight. The objectives of this research were:

1) To investigate host resistance in *Buxus* species to the fungal pathogen *Calonectria pseudonaviculata*
    a. Screen commercial cultivars for resistance and evaluate disease severity
    b. Assess components of partial resistance across resistant and susceptible cultivars
2) To determine survival of *Calonectria pseudonaviculata* in abscised infected leaves exposed to the surface and subsurface of field soil and soilless potting media

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CHAPTER 1. Determination of survival of *Calonectria pseudonaviculata* in abscised infected leaves exposed to the surface and subsurface of field soil and soilless potting media

INTRODUCTION

Boxwood blight is caused by the fungal pathogen *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum*, *Cylindrocladium buxicola*). Boxwood (*Buxus* spp.) is the most economically important host. Infection on susceptible hosts leads to foliar and stem lesions, leaf blighting, and defoliation. Weeda and Dart (16) provided evidence that the pathogen can survive in soil as microsclerotia embedded in infested plant material such as abscised leaves and stems. Microsclerotia are survival structures that germinate to form conidia, which serve as inoculum for future disease epidemics (16). Microsclerotia of C. *scoparium* survived for at least seven years in a Wisconsin nursery soil (15). In an outdoor experiment in the United Kingdom, *C. pseudonaviculata* was capable of surviving for at least five years in infected leaf material on the soil surface and buried in the soil subsurface (5 cm) (4). Microsclerotia contribute to pathogen survival and are important in the boxwood blight disease cycle (16).

Boxwood blight disease development is most severe under humid and warm conditions (18 to 25°C). These conditions occur commonly in the spring and fall in boxwood production regions in western North Carolina. After plant infection and leaf defoliation the abscised infected leaves that remain in a production area can serve as inoculum for disease epidemics. North Carolina growers produce container grown and field grown boxwood; thus *C. pseudonaviculata* within abscised infected plant material may overwinter in a variety of production environments. Furthermore, abscised leaves not only remain on the surface they

may become buried due to heavy rain events, equipment use, and human activity in production areas. Location of the pathogen propagule in the soil profile can affect survival. Pataky and Beute (10) identified that *C. ilicicola* microsclerotia had a reduced survival rate on the soil surface compared to the soil subsurface (25 cm). The purpose of this study was to determine the overwinter survival of *C. pseudonaviculata* in leaves on the surface or buried in the subsurface of field soil or soilless potting media in western North Carolina. Overwinter pathogen survival should be investigated to understand the risk of inoculum buildup in production areas where infected plant debris is not removed.

MATERIALS AND METHODS

Overwinter survival of *C. pseudonaviculata* within boxwood leaves on the surface and in the subsurface of field soil and on the surface and in the subsurface of soilless potting media was evaluated from September until May during 2012 to 2013 and again in 2013 to 2014. Experiments were performed at the North Carolina State University Mountain Horticultural Crops Research Station in Mills River, NC.

2012 to 2013 Experiments

Symptomatic leaves were collected from various boxwood plants including cultivars of *B. sempervirens* and *B. microphylla*. Approximately 80% of collected leaves came from *B. sempervirens* plants. Plants were either exposed to the pathogen via direct inoculation with a water suspension of 10,000 conidia/ml or indirect inoculation via dispersal of conidia produced on artificially inoculated plants. Five symptomatic leaves per sample were placed into nylon mesh bags (7.62 cm x 7.62 cm) and were exposed to the surface of field soil

(Hayesville loam) or soilless potting mix (pine bark) or were buried 5 cm deep in field soil or in soilless potting mix. These conditions reflected the locations in which infected defoliated leaves might be found in a production area.

The field soil treatment area was exposed to full sun and natural precipitation and the soil was a Hayesville loam. This reflects typical conditions for field-produced boxwood. The soilless potting media was a pine bark mix and it was placed in 3.8 liter plastic containers. The soilless media treatment was exposed to partial shade (under 55% shade cloth) and overhead irrigation when the experiment was established on 7 Sep 2012. The shade cloth was removed and the irrigation was turned off from 8 Nov 2012 until 10 Apr 2013. This reflects typical conditions for container produced boxwood.

A split-plot experiment design was utilized for each substrate type: field soil and soilless media. The main unit of substrate was divided into subplots of depth: surface (0 cm) and subsurface (5 cm). In each experiment, the nylon mesh bag samples, which contained five infected leaf subsamples, were arranged in a completely randomized design (with respect to time) with five replicates per treatment combination.

The experiments were conducted for eight months beginning in September 2012 and survival of the pathogen within leaves was assessed at the beginning of the experiments and each month thereafter. At the beginning of the experiments, 20 symptomatic leaves were placed into a humid chamber in order to establish the survival of the pathogen at time zero. The humid chamber consisted of a clear plastic box (38.1 cm x 17.78 cm x 10.16 cm) on the lab bench which received ambient temperature and light. Survival of the pathogen was assessed by determining the proportion of infected leaves with spore production after

incubation in a humid chamber for four days. In each of eight months (Oct 2012 to May 2013), five replicate nylon mesh bags each containing five symptomatic leaf subsamples were removed from each of the two treatment conditions in the field soil experiment and each of the two treatment conditions in the soilless media experiment. Nylon mesh bags were collected at 0, 29, 63, 104, 130, 167, 194, 239, and 252 days after experiment set-up. After substrate debris was removed, leaves were placed onto potato carrot agar (PCA; carrot at 20 g/liter, potato at 20 g/liter, and Agar (Sigma) at 20 g/liter) amended with antibiotics (ampicillin at 30 mg/liter and streptomycin sulfate at 133 mg/liter) in 100 x 15 mm petri dishes; the adaxial surface of the leaf was in contact with the media. After four days of incubation (23°C, 24 h photoperiod), leaves were viewed with a stereoscope (Olympus SZX7) at 10X-40X and pathogen survival was assessed by recording the proportion of infected leaves retrieved from each nylon mesh bag with spore production.

Soil and soilless media surface and subsurface (5 cm) temperature data were collected with a WatchDog weather station (2000 series, Spectrum Technologies, Inc.). Air temperature (2 m) data was obtained from the Mountain Horticultural Crops Research Station -CRONOS Database (NC Climate Retrieval and Observations Network Of the Southeast Database; State Climate Office of North Carolina). The WatchDog weather station was located at the site of the experiments while the CRONOS weather station was located approximately 140 m from the site of the experiments.

2013 to 2014 Experiments

The 2013 to 2014 treatment conditions were the same as the 2012 to 2013 experiments except a shade treatment was added to the field soil experiment. A split-split-

plot experiment design was utilized for the field soil experiment. The main unit of substrate (field soil) was divided into subplots of depth: surface (0 cm) and subsurface (5 cm). The subplots of depth were further divided into subunits of sun exposure: shade or full sun. All of the treatments in the field soil experiment received natural precipitation and the shade treatment was under a 55% shade cloth for the duration of the experiment. A split-plot experiment design was utilized for the soilless potting media experiment as described for the 2012 to 2013 experiment. All of the treatments in the soilless potting media experiment received natural precipitation and were under a 55% shade cloth for the duration of the experiment. In each experiment, nylon mesh bag samples, which contained 10 infected leaf subsamples, were arranged in a completely randomized design (with respect to time) with seven replicates per treatment combination.

In addition, the role of microsclerotia in the overwinter survival of the boxwood blight pathogen (16) was investigated in the 2013 to 2014 experiments. In each experiment, each replicate nylon mesh bag sample included 10 infected leaf subsamples; five leaves were used to assess pathogen survival and five leaves were used to analyze the presence of microsclerotia. Throughout the experiments, survival of the pathogen was assessed by calculating the proportion of infected leaves, from each nylon mesh bag, with spore production. The role of microsclerotia in the survival of *C. pseudonaviculata* was investigated by calculating the proportion of infected leaves, from each nylon mesh bag, that contained microsclerotia.

Symptomatic leaves were collected from *B. sempervirens* ‘Suffruticosa’ plants, 12 days after they were inoculated with a spray suspension of 15,000 conidia/ml applied until

run-off. Ten symptomatic leaves were placed into nylon mesh bags and placed in the various treatments. At the beginning of the experiments, 35 symptomatic leaves were placed onto PCA: after incubation for four days the leaves were examined to establish the proportion of infected leaves with spore production at time zero. Additionally, 35 leaves were cleared and examined to establish the proportion of infected leaves that contained microsclerotia at time zero.

In each of eight months (Oct 2013 to May 2014), seven nylon mesh bags each containing ten symptomatic leaves were removed from each of the four treatment conditions in the field soil experiment and each of the two treatment conditions in the soilless media experiment. Five leaf subsamples were analyzed for pathogen survival while the other five were analyzed for the presence of microsclerotia. Nylon mesh bags were collected at 0, 26, 61, 99, 135, 164, 183, 222, and 253 days after experiment set-up. The survival analysis was performed as described above. To assess the presence of microsclerotia within infected leaves, the leaf pigment was cleared using a modification of the method described in Linderman (7). Leaves were freed from substrate debris by wiping with a dry paper towel and placed in plastic specimen cups. Four days after leaf collection, 15 to 30 ml of 5% sodium hydroxide (w/v) was added. At 8 to 10 days post collection, the leaves were drained and fresh 5% sodium hydroxide (w/v) was added. At 20 to 30 days post collection leaves were drained, flushed twice with distilled water, placed in 15 to 30 ml 95% ethanol, and analyzed for the presence of microsclerotia. Cleared leaves were mounted in lactophenol cotton blue and viewed with a compound scope (Olympus BX41) at 40-400X. Due to the destructive nature of the leaf clearing process, the same leaf which was assessed for the

presence of microsclerotia could not be assessed for the ability of the pathogen to produce conidia.

Weather data was collected as described previously, except that only subsurface field soil and soilless media temperature data were collected by the WatchDog weather station. This is in contrast to the 2012 to 2013 experiments in which both surface and subsurface temperatures were recorded for each substrate.

Statistical analysis

An analysis of variance for the significance of treatment effects on the proportion of leaves with spore production and on the proportion of leaves with microsclerotia was conducted for the soil and soilless media experiments. The MIXED procedure of SAS (version 9.4; SAS Institute) was used. In the split-plot soil substrate experiment in 2012 to 2013 and in the split-plot media substrate experiments in 2012 to 2013 and 2013 to 2014, the main unit of substrate, the subplot unit of depth, and time were considered fixed effects. In the split-split-plot soil substrate experiment in 2013 to 2014 the main unit of substrate, the subplot unit of depth, the subunit of sun exposure and time were considered fixed effects. In all experiments, replication, of the nylon mesh bags that contained the infected leaves, within time was considered a random effect.

The CORR procedure of SAS was used to assess the correlation between the proportion of leaves with sporulation and the proportion of leaves with microsclerotia. Data from the 2013 to 2014 soil and soilless media experiments was combined for the analysis.

RESULTS

2012 to 2013 Experiments

The proportion of infected leaves with sporulation was 1, after incubation in a humid chamber, in a subsample of leaves collected prior to placement in treatment conditions in the field soil and soilless media experiments. Throughout the duration of both the field soil experiment and soilless media experiment the proportion of leaves with sporulation was reduced for leaves on the surface compared to the subsurface (Fig. 1).

In the field soil experiment, the proportion of leaves with sporulation was 0 for the surface treatment by January 2013, after 130 days of treatment exposure. While the proportion of leaves with sporulation from the subsurface treatment was 0.44. By May 2013, after 252 days of treatment exposure, the proportion of leaves with sporulation remained at 0 for leaves from the surface and was reduced to 0.19 for leaves from the subsurface treatment (Fig. 1). Results from an ANOVA indicated that the time ($P < 0.0001$) and depth ($P < 0.0001$) treatments both had a significant effect on the proportion of infected leaves with sporulation while the interaction term was not significant ($P = 0.0592$).

In the soilless media experiment, after an overwintering period of 130 days the proportion of leaves with sporulation was 0.48 and 0.92 for leaves from the surface and subsurface treatments, respectively. By May 2013, after 252 days of exposure to the treatment conditions, the proportion of leaves with sporulation retrieved from the surface was 0.05 whereas the proportion of leaves with sporulation was 0.8 for leaves from the subsurface (Fig. 1). Results from an ANOVA indicated that the time ($P = 0.0002$) and depth ($P < 0.0001$) treatments both had a significant effect on the proportion of infected leaves with

sporulation. The interaction of depth x time was also significant ($P = 0.0221$) indicating that the response over time differed for each level of depth.

2013 to 2014 Experiments

Just prior to the start of the experiments, the proportion of infected leaves with sporulation was 0.94 in a subsample of leaves assayed as previously described. Throughout the duration of both the field soil experiment and soilless media experiment the proportion of leaves with sporulation was reduced for leaves on the surface compared to the subsurface (Fig. 2). Additionally, prior to the start of the experiments, a subsample of infected leaves were cleared and the proportion of leaves with microsclerotia was 0.03. However, after infected leaves were exposed to the treatment conditions the proportion of leaves with microsclerotia increased; the proportion of leaves with microsclerotia was higher for leaves from the subsurface compared to the surface for both the soil and soilless media experiments (Fig. 3&4).

In the field soil experiment, the proportion of leaves with sporulation was reduced to 0.49 (Fig. 2) for the leaves retrieved after 26 days (October, 2013) of exposure to the surface and full sun treatment combination: whereas the proportion of leaves with microsclerotia was 0.3 (Fig. 4). After the same time period, the proportion of leaves with sporulation was only reduced to 0.86 for the leaves retrieved from the surface treatment under shade (Fig. 2) and the proportion of leaves with microsclerotia was 0.68 (Fig. 4). Additionally, after 26 days, the leaves retrieved from the subsurface had a proportion of 0.89 and 0.94 leaves with sporulation after exposure to full sun and shade, respectively (Fig. 2). The proportion of leaves with microsclerotia was over 0.9 for both subsurface treatments (Fig. 4). By May

2014, after 253 days of exposure to the treatment conditions, the proportion of leaves with sporulation and the proportion of leaves with microsclerotia was above 0.5 for all treatment combinations with the exception of leaves retrieved from the surface with full sun exposure (Fig. 2). Results from an ANOVA indicated that the time ($P < 0.0001$), depth ($P < 0.0001$), and sun exposure ($P < 0.0001$) treatments all had a significant effect on the proportion of infected leaves with sporulation and on the proportion of leaves with microsclerotia. Additionally, all two way interactions and the time x depth x sun exposure interaction had a significant ($P < 0.0001$) effect on the proportion of leaves with sporulation. However, only the interaction terms time x depth ($P < 0.0001$) and sun exposure x depth ($P < 0.0001$) had a significant effect on the proportion of leaves with microsclerotia.

In the soilless media experiment, the proportion of leaves with sporulation was 0.97 for leaves retrieved after 26 days (October 2013) of exposure to both the surface and subsurface (Fig. 2). After the same time period, the proportion of leaves with microsclerotia was above 0.9 for leaves from the surface and subsurface (Fig. 4). By May 2014, after 253 days of exposure to the treatment conditions, the proportion of leaves with sporulation was 0.73 and 0.91 for leaves retrieved from the surface and subsurface, respectively (Fig. 2). While the proportion of leaves with microsclerotia was 0.57 and 0.9 for the leaves from the surface and subsurface, respectively (Fig. 4). An analysis of variance indicated that time ($P < 0.01$), depth ($P < 0.0001$), and the time x depth interaction ($P < 0.01$) all had a significant effect on the proportion of leaves with sporulation and on the proportion of leaves with microsclerotia.

Results from the CORR procedure in SAS indicated that there was a statistically significant relationship ($r = 0.92$, $P < 0.0001$) between the proportion of leaves with sporulation and the proportion of leaves with microsclerotia. The correlation analysis was conducted with the combination of data from the soil and soilless media experiments.

Lower subsurface soil and soilless media temperatures were recorded in the winter of 2013 to 2014 (Fig. 6) compared to 2012 to 2013 (Fig. 5). Additionally, lower minimum air temperatures were recorded in the winter of 2013 to 2014 compared to 2012 to 2013 (Fig. 7).

DISCUSSION

From September 2012 through May 2013, leaves infected with the fungal pathogen, *C. pseudonaviculata*, were exposed to either the surface or subsurface (5 cm) of field soil or soilless potting media and the proportion of leaves with sporulation was assessed monthly. Additionally, from September 2013 through May 2014 infected leaves were exposed to the following treatment combinations: the surface or subsurface (5 cm) of field soil with either full sun or shade exposure and the surface or subsurface (5 cm) of soilless potting media. The proportion of leaves with sporulation and the proportion of leaves with microsclerotia was assessed monthly.

Results of this experiment demonstrate that *C. pseudonaviculata* can form microsclerotia in infected boxwood leaves and survive over the winter in western North Carolina. After an overwintering period from September through May, the proportion of leaves with sporulation was greatest in leaves from the subsurface compared to the surface, in all of the soil and soilless media experiments. Pataky and Beute (10) also showed that

survival of microsclerotia of *C. ilicicola* was greater at 25 cm below the soil surface compared to the surface. Results from the 2013 to 2014 field soil experiment also indicate that the pathogen is less likely to survive in leaves on the surface exposed to full sun compared to shade exposure.

Results from 2013 to 2014 indicated that there is a statistically significant relationship between the proportion of leaves with sporulation and the proportion of leaves with microsclerotia; this suggests that the primary pathogen survival structure within leaves is most likely microsclerotia. Weeda and Dart (16) also provided evidence that microsclerotia are the survival propagules of the boxwood blight pathogen, as has been demonstrated for *C. ilicicola* in peanut and soybean (5) and *C. floridanum* in many ornamental crops (8, 9). In general, the proportion of leaves with sporulation was slightly higher than the proportion of leaves with microsclerotia. Perhaps the pathogen also survived as mycelium or conidia or some microsclerotia were not identified in this study due to their small size or physiological state of development.

In the 2013 to 2014 experiments, the proportion of leaves with sporulation were higher than for the 2012 to 2013 for all treatment combinations. Differences in survival could be attributed to differences in environment during the experiment, such as moisture or temperature, or differences in experimental methods, such as shade exposure and source of infected leaves.

Soil moisture content can affect pathogen survival. Dart, Hong and Bradley (2) identified that the optimum soil moisture content for recovery of *C. pseudonaviculata* with plant baits was 1000% of field capacity (flooded soil). Thies and Patton (15) reported that

microsclerotia viability in *C. scoparium* was greatly reduced after air drying (15). Griffin et al. (3) reported that microsclerotia of *C. ilicicola* were not able to survive after incubation in a soil that was air dried for 24 h, however, the loss of germination was reversed when the microsclerotia were incubated for seven days in air-dried soils that were moistened (3). Pataky and Beute (10) reported that at 4°C, microsclerotia viability of *C. ilicicola* was highest in moist soil. Soil moisture content was not investigated in the present study. In the 2012 to 2013 experiment the soilless media experiment received shade and irrigation from 9 Sep 2012 until 8 Nov 2012 and from 10 Apr 2013 until 27 May 2013. While in the 2013 to 2014 experiment the soilless media experiment received shade for the duration of the experiment. Perhaps there was greater moisture retention in the soilless media 2013 to 2014 experiment due to the consistent shade exposure and thus a higher proportion of leaves with sporulation was identified compared to the 2012 to 2013 experiment.

Temperature is an important environmental factor which affects microsclerotia survival of *Calonectria* spp. (6, 11, 14). Rowe (13) identified that winter temperatures in eastern North Carolina were not capable of killing microsclerotia of *C. ilicicola* within infected peanut roots. However, in western Virginia, viable microsclerotia of *C. ilicicola* was not recovered after naturally infested field soil was incubated for three months (October to December) at a depth of 14.2 cm under field conditions; soil temperatures in December were -4.7°C minimum, 4.4°C maximum, and -1.7°C mean (12). Minimum temperatures recorded in the subsurface (5 cm) for field soil and for soilless media were lower in 2013 to 2014 than in 2012 to 2013. Additionally, in general lower minimum air temperatures were recorded in 2013 to 2014 compared to 2012 to 2013. This suggests that low winter temperatures did not

negatively affect pathogen survival in leaves on the surface or in the subsurface considering that the proportion of leaves with sporulation was higher for leaves from the 2013 to 2014 experiment compared to the 2012 to 2013 experiment.

Kuruppu et al. (6) demonstrated that microsclerotia germination was decreased at incubation in field soil above 35°C. Weather data collected from the surface of field soil and soilless media in the 2012 to 2013 experiment indicated that maximum temperatures were over 35°C and 30°C on the surface of soilless media and field soil, respectively in both April and May 2013. These temperatures could have contributed to the decline in the proportion of leaves with sporulation from leaves on the surface of soilless media after March 2013. It is unknown if similar high temperatures were reached on the surface treatments in the 2013 to 2014 experiments, however maximum air temperature was higher March through May in the 2013 to 2014 experiment compared to the 2012 to 2013 experiment. Future studies should assess the survival of the pathogen over the entire year in order to understand if high temperatures during the spring and summer can reduce pathogen survival.

Previous studies that investigated survival of *Calonectria* spp., indicated that the adverse effects of extreme temperature on pathogen growth could be reversed after the pathogen was returned to ambient conditions (6, 12). Pataky and Beute (10) evaluated the microsclerotia viability of *C. ilicicola* on the surface and in the subsurface of field soil over the winter in North Carolina and they observed an increase in microsclerotia viability in the spring of both experimental years (10). In the 2012 to 2013 experiments, the proportion of leaves with sporulation appeared to increase in leaves from the subsurface of field soil and soilless media in April and May, respectively, although the change was not statistically

significant. In the 2013 to 2014 experiments, the proportion of leaves with sporulation was greater in February compared to January for leaves from the surface of soilless potting media and from the surface of field soil with sun and shade exposure. However, this was followed by a decrease in the proportion of leaves with sporulation in the following month. Similar to the 2012 to 2013 experiments, there was an increase in the proportion of leaves with sporulation from the subsurface of soilless media treatment in April 2014. Even though there were some cases where pathogen survival increased in the spring, overall warmer spring temperatures did not increase pathogen recovery under all treatment conditions.

Differences in experimental methods could have affected pathogen survival. In the 2012 to 2013 experiment, leaves were collected from boxwood blight susceptible and resistant boxwood cultivars. In the 2013 to 2014 experiment, infected leaves were only collected from the susceptible cultivar *B. sempervirens* 'Suffruticosa'. Perhaps microsclerotia development and pathogen survival were inhibited inside leaves from the more resistant cultivars leading to a reduction in the proportion of leaves with sporulation in 2012 to 2013 compared to 2013 to 2014 experiments. The majority of collected infected leaves in the 2012 to 2013 experiment were from susceptible cultivars so it does not seem likely that the differences in the proportion of leaves with sporulation are entirely attributable to this difference in experimental methods.

Results from the current study indicate that *C. pseudonaviculata* is capable of surviving over the winter (September through May) in leaf debris under conditions commonly encountered in a boxwood production environment. Leaf debris is likely to end up on the surface of field soil or soilless media after infected boxwood leaves defoliate and

during removal of infected boxwood plants. Furthermore, abscised leaves not only remain on the surface they may become buried due to heavy rain events, equipment use, and human activity in production areas. Debris should be removed from production areas as completely as possible so that microsclerotia in leaf material are not available to germinate and produce conidia, and initiate disease epidemics in the future. The proportion of leaves with sporulation was reduced for leaves retrieved from the surface compared to the subsurface. Push flamers have been used to disintegrate infected leaves on the soil surface (1). Anecdotal evidence has suggested that if this management method is used much care should be taken to avoid blowing leaves into shady conditions during flaming because any leaves that are not disintegrated can serve as inoculum. Future studies should investigate the rate of development of microsclerotia in attached and abscised tissue so that management strategies can be directed towards disturbing microsclerotia formation. The boxwood blight pathogen is capable of persisting in many environments due to its ability to form microsclerotia and the current study has provided some insight into which environments the pathogen is most likely to survive in infected leaves.

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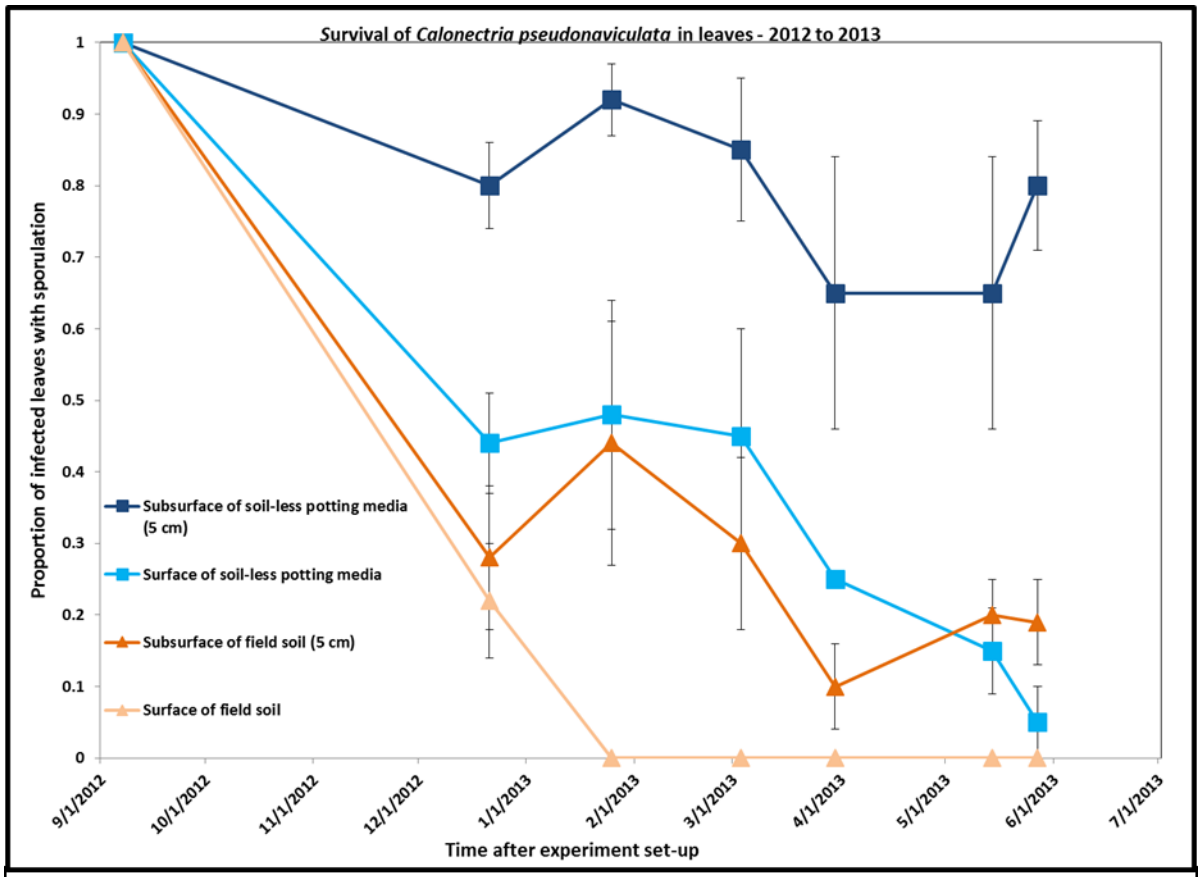

Fig. 1. Proportion of infected leaves with sporulation by *Calonectria pseudonaviculata* after exposure to different environmental treatment conditions (2012 to 2013). Error bars represent standard error of the mean.

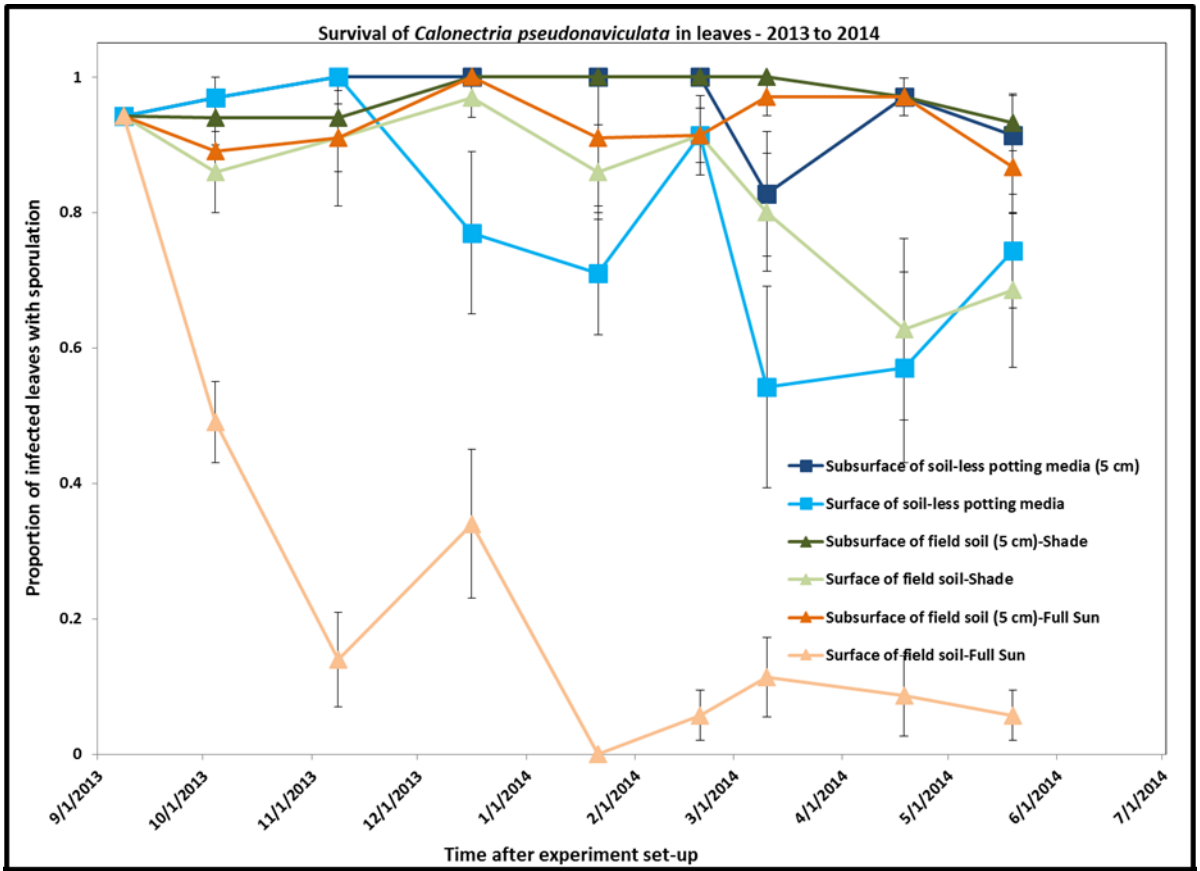

Fig. 2. Proportion of infected leaves with sporulation by *Calonectria pseudonaviculata* after exposure to different environmental treatment conditions (2013 to 2014). Error bars represent standard error of the mean.

Microsclerotia of *Calonectria pseudonaviculata* in cleared leaves of boxwood

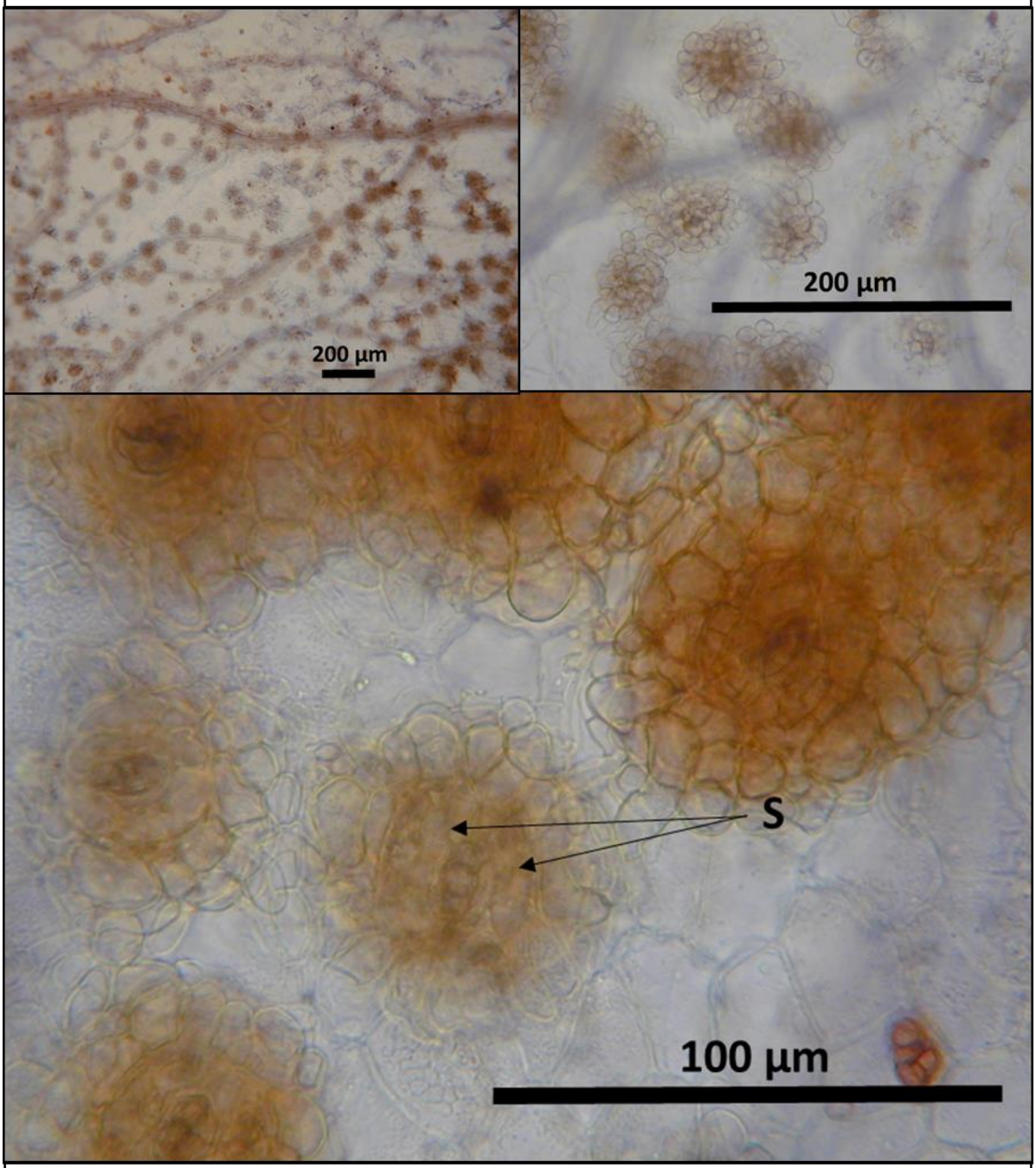

Fig. 3. Microsclerotia of *Calonectria pseudonaviculata* are pathogen survival structures that form within leaves. They are often found growing near stomata. **S**, Stomatal guard cells.

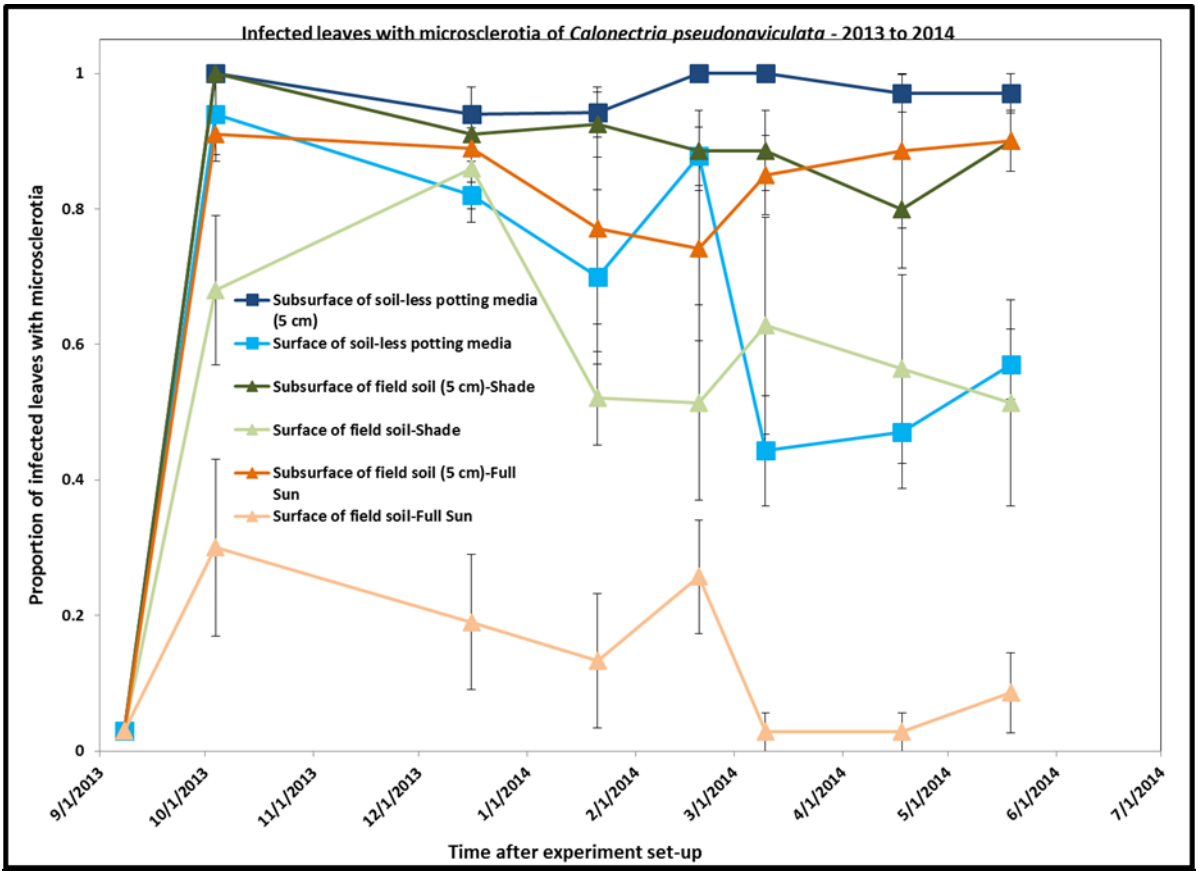

Fig. 4. Proportion of infected leaves with microsclerotia of *Calonectria pseudonaviculata* after exposure to different environmental treatment conditions (2013 to 2014). Error bars represent standard error of the mean.

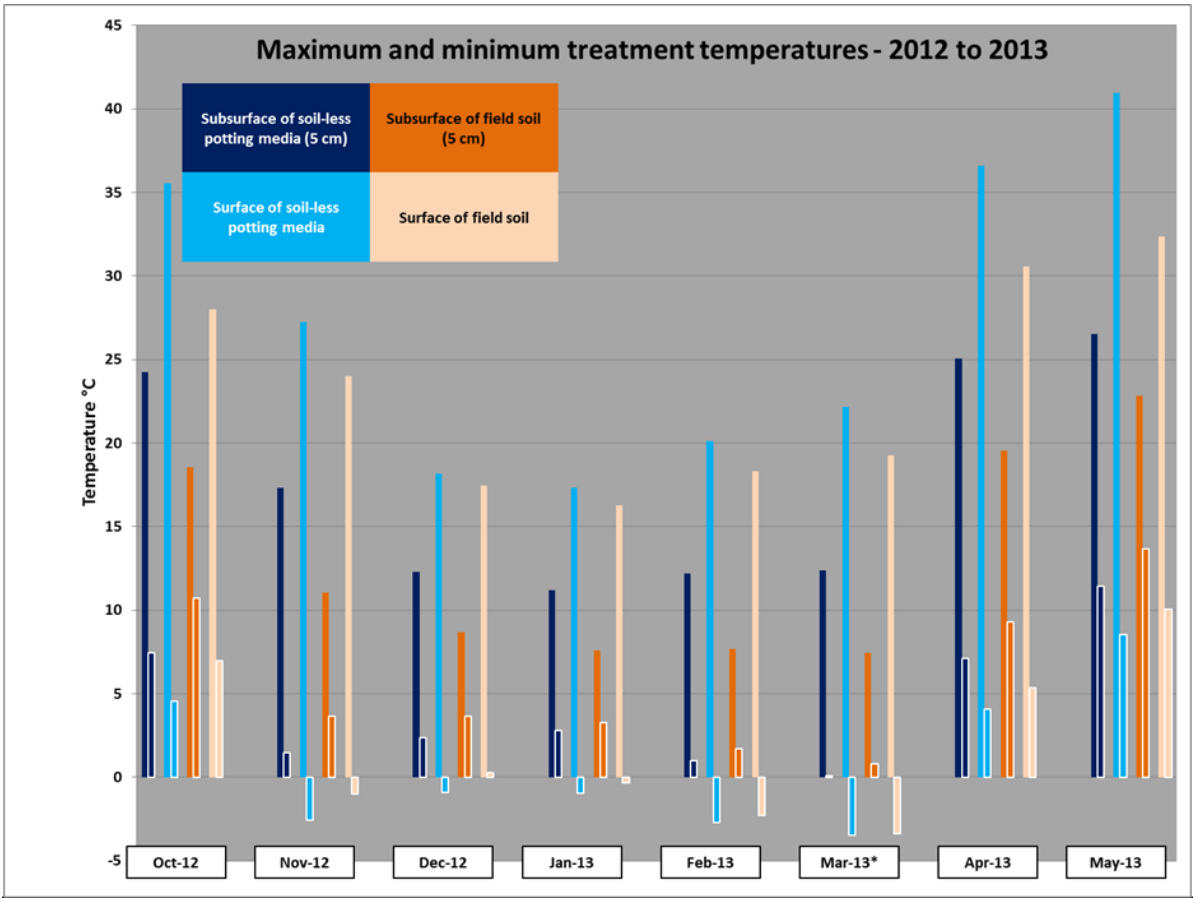

Fig. 5. Maximum and minimum surface and subsurface temperature for the different environmental treatments in the 2012 to 2013 experiment. Bars with white outline represent minimum temperatures. *Data only includes 1 Mar to 10 Mar.

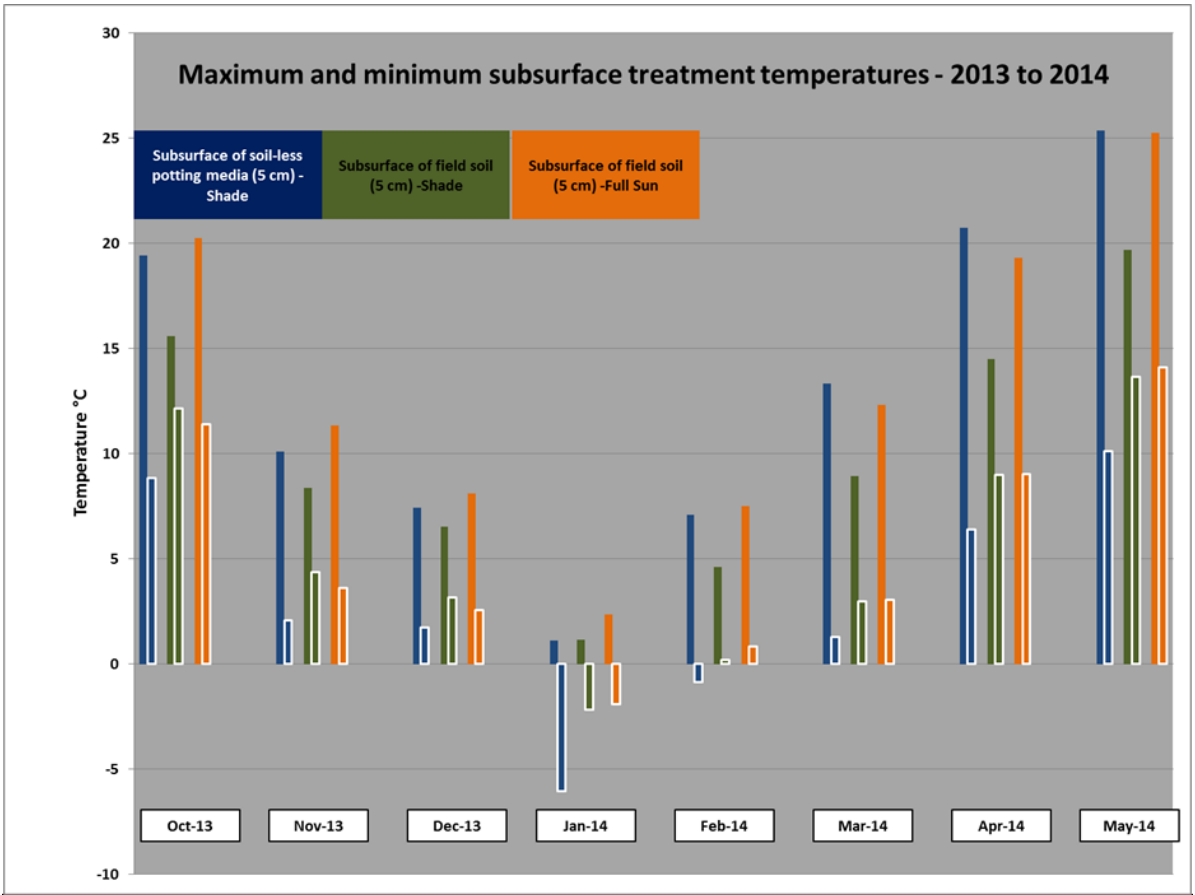

Fig. 6. Maximum and minimum soil subsurface temperature for the different environmental treatments in the 2013 to 2014 experiment. Bars with white outline represent minimum temperatures.

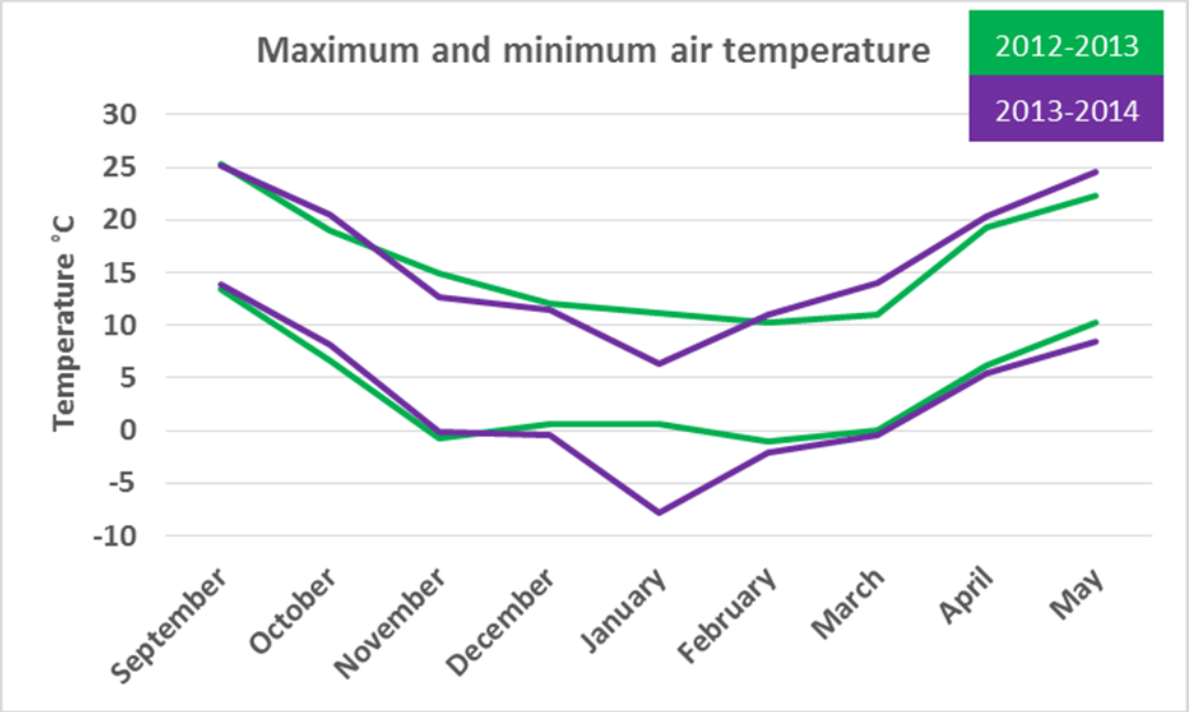

Fig. 7. Maximum and minimum air temperature at 2 m height at the Mountain Horticultural Crops Research and Extension Center, Mills River, NC

CHAPTER 2. Susceptibility of commercial *Buxus* species and cultivars to the boxwood blight pathogen, *Calonectria pseudonaviculata*

INTRODUCTION

Boxwood blight is a foliar disease caused by the fungal pathogen *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum = Cylindrocladium buxicola*). As infection progresses, leaf lesions often expand in a zonate pattern and leaves defoliate. This disease was first described in the United Kingdom in the mid to late 1990s (10). Epidemics of boxwood blight have resulted in economic and emotional loss due to the financial and historical value of boxwood. The use of host resistance is a sustainable and affordable disease management strategy. As such, researchers in Europe began conducting host susceptibility assays in hopes of identifying boxwood blight resistant *Buxus* cultivars in the mid to late 2000s (7-9).

In 2011, boxwood blight was detected in the United States (12). It was immediately apparent that this disease would be economically important in North Carolina and elsewhere in the U.S. because the pathogen destroyed millions of dollars of container and field boxwood in a relatively short time period (12). Boxwood cultivars most severely impacted were *B. sempervirens* ‘American’ and ‘Suffruticosa’; these cultivars are the most popular, most commonly grown, and apparently most susceptible to boxwood blight among boxwood cultivars grown in the U.S. After the U.S. detection of boxwood blight, host resistance screening of *Buxus* germplasm commonly found in the U.S. nursery trade was identified as an important objective.

In previous host resistance studies performed on *Buxus* cultivars the Asiatic types were identified as generally more resistant than the European types (7, 8). With ample genetic diversity in species of *Buxus* the use of host resistance could be a practical disease management strategy for future boxwood plantings. Over 95 species of *Buxus* have been identified with indigenous origins around the globe (19). The most common in the nursery trade are cultivars and hybrids of the European type, *B. sempervirens*. Other popular cultivated species are of Asiatic origin and these include *B. harlandii*, *B. microphylla* (littleleaf), and *B. sinica* (Korean). Cultivars of *B. sinica* var. *insularis* are commonly referred to as Korean boxwood and occasionally referred to as *B. microphylla* var. *koreana*. Throughout this publication the Korean boxwood will be referred to as *B. sinica* var. *insularis.* Van Laere et al. (19) performed genetic analysis of *Buxus* and found that the European species *B. sempervirens*, and *B. balearica* clustered together and the Asiatic cultivar *B. colchica* also grouped within this cluster (it has leaf morphology similar to *B. sempervirens*). Batdorf (2) provides evidence that *B. colchica* is a synonym for *B. sempervirens*. The Asiatic species, *B. microphylla* (the Korean types were categorized under this species name), *B. harlandii*, *B. hyrcana*, *B. myrica*, *B. henyri*, *B. bodinieri*, and *B. wallichiana* clustered together (19). Previously, the cultivar 'Justin Brouwers' had been classified with *B. sinica* var. *insularis*. However, Van Laere et al. (19) identified that 'Justin Brouwers' had been misclassified and it actually belongs with the *B. sempervirens* group.

Morphologically, the European species are characterized as having elliptical, darker green leaves with an acute tip (*B. colchica* and *B. wallichiana* also have this leaf morphology) (19) and the Asiatic species are described as having oblanceolate to obovate

medium green leaves with an obtuse tip (13). Flower morphology does not distinguish European from Asiatic species (21, 22).

Hybrid boxwood cultivars such as *Buxus* 'Green Gem', 'Green Mound', 'Green Mountain', and 'Green Velvet' have been developed from crosses between *B. sempervirens* and *B. microphylla* (reportedly Korean types) (20). These are commonly known as the Sheridan hybrids as they were introduced by Sheridan nurseries of Oakville, Ontario, Canada.

Disease resistance in plants is the ability to actively reduce pathogen development and reproduction and decrease disease development (1, 15, 16). Tolerance is commonly used to describe a host on which pathogen colonization and reproduction are not reduced relative to susceptible types even though there are less symptoms or yield loss compared to a susceptible host (6, 15). Resistance in plants can be identified by the expression of a major gene (complete resistance) or many genes (quantitative resistance) (16, 18). The basis for tolerance is less understood and it might be related to the concept of avoidance; wherein yield loss is not incurred or symptoms do not develop in abundance due to factors such as leaf shape or plant architecture. The terms 'resistance' and 'tolerance' are often used interchangeably, however, throughout this publication the term 'resistance' will be used to describe reduced disease development on the host.

Detached branch or leaf assays have been used to screen for host resistance. Detached branch assays are utilized to screen a large amount of germplasm in a relative small space within a controlled environment. When a strong correlation is found between the results of

detached and whole plant screening assays then the detached assay can be used for primary selection of germplasm; this is more rapid and economical than field evaluation (14, 17).

The objective of this study was to screen commercial *Buxus* cultivars for resistance to the boxwood blight pathogen, *Calonectria pseudonaviculata*.

MATERIALS AND METHODS

Susceptibility of commercial boxwood cultivars to the boxwood blight pathogen, *C. pseudonaviculata* was evaluated in 2012, 2013, and 2014 at the Mountain Horticultural Crops Research Station in Mills River, NC. Experiments were performed on plants on a shaded container pad with daily overhead irrigation. Additionally, susceptibility and resistance screening was evaluated on detached branches of boxwood in a humidity chamber; this was done for a subset of the cultivars screened in the outdoor trials.

2012 container pad trial

In the 2012 trial, 23 cultivars of boxwood were randomized in plots within each of four blocks. The experiment consisted of a randomized complete block design. Each plot consisted of six subsamples of a test cultivar and two inoculum reservoir plants (Fig. 1). This design was used to simulate natural plant to plant spread of the pathogen instead of exposing plants to extraordinarily high concentrations of the pathogen by artificial inoculation. Test cultivars were placed around inoculated *B. sempervirens* 'Suffruticosa' plants; these were the inoculum reservoir plants. The experiment also included positive and negative control plots, which consisted of six *B. sempervirens* 'Suffruticosa' plants surrounding either two inoculum reservoir plants or two non-inoculated *B. sempervirens* 'Suffruticosa' plants, respectively.

Test cultivars were donated from Saunders Brothers nursery. The nursery was free of boxwood blight disease. The test cultivars ranged in age from approximately two to five years old. Most test cultivars ranged from 10.16 cm to 60.96 cm height and were most commonly growing in 3.8 liter (1 gal) containers, although some plants were grown in 7.6 (2 gal) or 11.4 liter (3 gal) containers, which contained either field (clay loam) soil or pine bark soilless potting media. Test cultivars were sprayed with the protectant fungicide, chlorothalonil, on 14 May 2012 by the company providing the plants and were delivered to the Mountain Horticultural Crops Research Station on 24 May 2012. On 6 Jun 2012 dolomitic lime was applied at a rate of 30 g per 3.8 liter and on 18 Jun 2012 10-6-4 granular fertilizer was applied at a rate of 21 g per 3.8 liter.

The inoculum consisted of a combination of isolates collected from infected field-grown and container-grown boxwood leaves in North Carolina during boxwood blight disease epidemics in 2011 and 2012. Inoculum was prepared by growing *C. pseudonaviculata* on full strength potato dextrose agar for 30 days. A conidial suspension for inoculum was prepared by flattening aerial mycelium with a semi-micro spatula and cultures were placed inside of an incubator (constant 22 °C, 18 h photoperiod with fluorescent light). After an incubation period of five days, conidia were dislodged from the culture and into a collection beaker with a stream of water dispensed from a hand held spray bottle. The suspension was filtered through cheesecloth and the concentration of conidia was counted using a hemocytometer. The inoculum reservoir plants were direct spray inoculated with a 10,000 conidia/ml suspension until run-off with a hand held pump up sprayer on 12 Jul 2012. After inoculation the plants were covered with plastic bags in order to increase humidity in

the plant canopy; this is ideal for boxwood blight disease development. The bags were removed from the plants the following morning and irrigated daily every 1.5 to 2 h for a 10 to 15 min duration from 9:00 am until 8:00 pm to sustain leaf wetness. Irrigation output was approximately 0.5 cm/10 min duration. On 16 Jul 2012, 4 days post inoculation (dpi) of the inoculum reservoir plants, the test cultivars were placed around the inoculum reservoir plants. The test cultivar containers were touching the containers of the inoculum reservoir plants; however, due to the variation in test cultivar plant size the distance between the test cultivar leaves and inoculum reservoir leaves ranged from approximately 2.54 cm to 15.24 cm (Fig. 1). On 16 July 2012 irrigation was changed to four times daily for a 30 min duration. Disease did not develop as quickly as was expected on the positive control. On 31 Jul 2012, 19 dpi, the inoculum reservoir plants had over 75% diseased and defoliated leaves, but the susceptible positive control *B. sempervirens* 'Suffruticosa' had less than 10% diseased leaves and defoliation. To increase disease development, a direct inoculation of the test cultivars was performed on 8 Aug 2012 with a 1,000 conidia/ml suspension until run-off.

Disease evaluations were performed on 10 Aug 2012 (19 days of exposure to the inoculum reservoir plants), 24 Aug 2012 (36 days of exposure to the inoculum reservoir plants), and 9 Sep 2012 (50 days of exposure to the inoculum reservoir plants). Disease severity was evaluated as percent of diseased and defoliated leaves with a modified Horsfall-Barratt scale (11) (0 = 0, 1 = 1 lesion, 2 = 0.6 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, 8 = 75 to 87%, 9 = 87 to 94%, 10 = 94 to 97%, 11 = 97 to 100%, 12 = 100%). Leaf drop was evaluated with a qualitative scale; none, trace < 1%,

low = 1 to 10%, moderate = 10 to 30%, and high > 30%. The disease severity and leaf drop rating was assigned to the group of six subsamples for each test cultivar within each block.

2013 container pad trial

In the 2013 experiment, 29 commercial cultivars were arranged in a randomized complete block design. The experiment design this year was different from the year prior due to a limited number of plants provided by the supplier; each of six blocks included a single subsample plant of each test cultivar arranged randomly into plots. Each plot consisted of six different test cultivars and two inoculum reservoir plants (Fig. 1). This differed from the 2012 experiment because in that experiment each of four blocks consisted of a random arrangement of plots where each plot contained six subsample plants of the same test cultivar. The 2013 experiment included a plant of *B. sempervirens* 'Suffruticosa' in each plot as a positive control (Fig. 1) and *B. sinica* var. *insularis* 'Nana' and *B. microphylla* var. *japonica* 'Green Beauty' as negative controls. These cultivars expressed resistance in the 2012 experiment.

Test cultivars were donated from Longwood gardens, Saunders Brothers nursery, and Spring Meadow nursery. All nurseries were free of boxwood blight disease. The test cultivars ranged in age from approximately two to five years old. Most test cultivar plants ranged from 10.16 cm to 60.96 cm height and were most commonly growing in 3.8 liter (1 gal) containers, although some were in 7.6 (2 gal) or 11.4 liter (3 gal) containers, which contained either field (clay loam) soil or pine bark soilless potting media. Test cultivars were delivered to the Mountain Horticultural Crops Research Station on 17 Apr 2013 and they did not receive any fungicide application in the spring of 2013 prior to delivery. On 16 May 2013

10-6-4 granular fertilizer was applied at a rate of 21 g per 3.8 liter and on 24 May 2013 dolomitic lime was applied at a rate of 30 g per 3.8 liter. On 3 Jun 2013, the insecticide, dinotefuran was applied at a rate of 0.6 g per 3.8 liter of suspension for the control of fungus gnats.

The inoculum reservoir plants were inoculated with a 10,000 conidia/ml suspension to run-off, as described previously, on 24 May 2013. The following morning, the plastic bags were removed from the plants and daily irrigation was applied every two h for a 10 min duration from 6:00 am until 10:00 pm. Irrigation output was approximately 0.5 cm/10 min duration. On 29 May 2013, 5 days post inoculation (dpi) of the inoculum reservoir plants, the test cultivars were placed around the inoculum reservoir plants. On 3 Jun 2013 (5 days of exposure to the inoculum reservoir plants) irrigation was changed to four times daily for a 10 min duration. By 6 Jun 2013 (13 dpi) the inoculum reservoir plants had 3 to 6% diseased leaves and the positive control *B. sempervirens* 'Suffruticosa' had 1% diseased leaves. By 13 Jun 2013 (20 dpi) the inoculum reservoir plants were showing 50-75% diseased and defoliated leaves and the positive control *B. sempervirens* 'Suffruticosa' plants had over 20% diseased and defoliated leaves.

Disease evaluations were performed on 14 Jun 2013 (16 days of exposure to the inoculum reservoir plants) and 21 Jun 2013 (23 days of exposure to the inoculum reservoir plants). Disease severity was evaluated as percent of diseased leaves with a modified Horsfall-Barratt scale; this differed from the 2012 and 2014 experiments where disease severity was evaluated as percent of diseased and defoliated leaves. Leaf drop was evaluated with the same qualitative scale as described previously. After the rating performed on 21 Jun

2013, the test cultivars with an average Horsfall-Barratt rating of 5 or above (> 12%) were removed from the experiment because they were considered susceptible.

In order to verify field resistance, the 14 remaining cultivars plus the positive control, *B. sempervirens* 'Suffruticosa', were direct spray inoculated with a suspension of 10,000 conidia/ml on 21 Jun 2013 and placed in plastic bags overnight. Over the next 82 days disease development was minimal so the test cultivars were direct inoculated a second time with a suspension of 15,000 conidia/ml on 11 Sep 2013. A disease severity evaluation was performed on 10 Sep 2013 (81 days after the inoculation on 21 Jun 2013). Disease severity was evaluated as percent of diseased and defoliated leaves with a modified Horsfall-Barratt scale (similar to the 2012 and 2014 experiments). Leaf drop was evaluated with a qualitative scale: none, trace, low, moderate, and high. On 16 Oct 2013, the cultivars that remained in the 2013 experiment were transplanted from pots on the container pad to a 1 x 1 meter spacing in an outdoor field adjacent to the container pad; one plant per test cultivar was randomly arranged in each of 5 blocks. The field was in full sun and did not have overhead irrigation (drip irrigation lines were installed). A final disease severity evaluation was done for the field-planted cultivars on 15 Nov 2013 (147 days after the inoculation on 21 Jun 2013).

2014 container pad trial

In the 2014 experiment, 60 commercial cultivars were arranged in a randomized complete block design. Of the 60 cultivars, 32 had been previously tested in either 2012 or 2013. The experiment consisted of a randomized complete block design as described for the 2013 experiment (Fig. 1). As in 2013, the 2014 experiment included a plant of *B.*

sempervirens ‘Suffruticosa’ in each plot as a positive control and *B. sinica* var. *insularis* ‘Nana’ and *B. microphylla* var. *japonica* ‘Green Beauty’ as negative controls. Test cultivars were donated from Saunders Brothers nursery and Spring Meadow nursery. The test cultivars ranged in age from approximately two to five years old. Most test cultivar plants ranged from 10.16 cm to 60.96 cm height and were most commonly growing in 3.8 liter (1 gal) containers, although some were in 7.6 (2 gal) or 11.4 liter (3 gal) containers, which contained either field (clay loam) soil or pine bark soilless potting media.

Test cultivars were delivered to the Mountain Horticultural Crops Research Station on 4 Apr 2014 and they did not receive any fungicide application in the spring of 2014 prior to delivery. The inoculum reservoir plants were inoculated with a 10,000 conidia/ml suspension to run-off, as described previously, on 26 May 2014. The following morning, the plastic bags were removed from the plants and daily irrigation was applied every two h for a 10 min duration from 6:00 am until 10:00 pm. Irrigation output was approximately 0.5 cm/10 min duration. On 2 June 2014, 7 days post inoculation (dpi) of the inoculum reservoir plants, the test cultivars were placed around the inoculum reservoir plants. On 9 Jun 2014 (7 days of exposure to the inoculum reservoir plants) irrigation was changed to four times daily for a 10 min duration. By 9 Jun 2014 (14 dpi) the inoculum reservoir plants had 6 to 12% diseased leaves and the positive control *B. sempervirens* ‘Suffruticosa’ had 3 to 6% diseased leaves. By 17 Jun 2014 (22 dpi) the inoculum reservoir plants were showing 50-75% diseased and defoliated leaves and the positive control *B. sempervirens* ‘Suffruticosa’ plants had over 20% diseased and defoliated leaves.

Disease evaluations were performed on 10 Jun 2014 (8 days of exposure to the inoculum reservoir plants), 17 Jun 2014 (15 days of exposure to the inoculum reservoir plants) and 26 Jun 2014 (24 days of exposure to the inoculum reservoir plants). After the rating performed on 26 Jun 2014, the test cultivars with an average Horsfall-Barratt rating of 5 or above (> 12%) were removed from the experiment because they were considered susceptible. The 48 remaining cultivars plus the positive control, *B. sempervirens* 'Suffruticosa', were direct spray inoculated with a suspension of 10,000 conidia/ml on 27 Jun 2014 and placed in plastic bags overnight. Disease severity evaluation was performed on 3 July 2014 (6 dpi), 10 July 2014 (13 dpi), 17 July 2014 (20 dpi), and 25 Aug 2014 (59 dpi). Disease severity was evaluated as percent of diseased and defoliated leaves with a modified Horsfall-Barratt scale. Leaf drop was evaluated with a qualitative scale as previously described.

Weather data for the Mountain Horticultural Crops Research Station were obtained from the CRONOS Database ((NC Climate Retrieval and Observations Network Of the Southeast Database), State Climate Office of North Carolina).

Detached branch trial

Twenty one commercial cultivars were evaluated; these were evaluated in the 2014 container pad trial as well. Two separate experiments were performed on detached branches in humid chambers. A range of susceptible and resistant *Buxus* cultivars were selected for this trial based on the results of the 2012 and 2013 container pad trials. Cultivars were donated from Saunders Brothers nursery and Spring Meadow nursery. The cultivars ranged in age from approximately two to five years old. Most test cultivar plants ranged from 10.16

cm to 60.96 cm height and were most commonly growing in 3.8 liter (1 gal) containers, although some were in 7.6 (2 gal) or 11.4 liter (3 gal) containers, which contained either field (clay loam) soil or pine bark soilless potting media. Detached branches were cut from *Buxus* cultivars maintained in a greenhouse. Plants used as sources of detached branches were hand-watered daily and received liquid 20-20-20 fertilizer applications at a rate of 200 ppm until saturation one time per week. The systemic insecticide, imidacloprid (Marathon 1% Granular, OHP Inc.) was applied at a rate of 14 g per 3.8 liter on 3 Jan 2014 for the control of boxwood leaf miner *Monarthopalpus flavus*.

Each detached branch consisted of a main stem cutting of primarily mature leaves. The height of detached branches ranged from 10 cm to 41 cm. Depending on the size of the branch, the branches were placed in either a 250 or 125 ml Erlenmeyer flask containing either 150 or 75 ml of water, respectively. Humid chambers consisted of clear plastic boxes (Cambro Manufacturing Company, 45.72 cm x 66.04 cm x 38.1 cm). Saturated absorbent towels (Mr. Clean, Cham-it cloth) were placed in each box. Temperature and humidity data were obtained from a digital weather station (AcuRite). Humidity levels ranged from approximately 75 to 99% inside of plastic boxes when the lids were on and 40 to 70% when lids were off. The experiment consisted of a randomized complete block design. Each plastic box represented a block and there were six blocks in the experiment. In each block there was one detached branch of each test cultivar randomly arranged. Plastic boxes were maintained under ambient fluorescent light and temperature in the lab.

Test cultivars were direct spray inoculated with a 10,000 conidia/ml suspension until run-off; approximately 200 ml of inoculum was used for each block of 21 detached branches.

After inoculation, test cultivars were spritzed with water two times per day. In the first and second experiment, the lids were kept on the plastic boxes until symptom development, 3 dpi. In the first experiment the lids were removed from the plastic boxes 4 dpi. From 17 dpi until the conclusion of the experiment at 24 dpi the lids were placed on the boxes nightly. In the second experiment, the lids were placed on the plastic boxes nightly from 4dpi until the conclusion of the experiment at 23 dpi in order to increase humidity levels throughout the duration of the experiment.

Disease severity was evaluated as percent leaf area diseased and defoliated with the same modified Horsfall-Barratt scale mentioned earlier. Leaf drop was evaluated with the same qualitative scale mentioned earlier. In the first experiment, disease evaluations were performed at 4, 8, 13, 17, 21, and 24 dpi. In the second experiment, disease evaluations were performed at 3, 8, 14, 20, and 23 dpi.

Some of the cultivars included in the container pad and detached branch susceptibility experiments were variegated cultivars (such as *B. microphylla* 'Golden Dream' and 'Wedding Ring). On variegated leaves the lesion morphology caused by *C. pseudonaviculata* infection can resemble natural variegation within the leaf (Fig. 2). To better assess lesion characteristics, leaves were collected, photographed, incubated, and evaluated for *C. pseudonaviculata* sporulation so that lesions caused by the pathogen could be correctly identified.

Relationship between container pad and detached branch trials

The relationship between disease severity results in the detached branch trial and the 2014 container pad trial was evaluated for 19 commercial cultivars.

Effect of geographic origin on disease severity

The effect of the geographic origin of *Buxus* species on disease severity was evaluated for test cultivars in the 2012, 2013, and 2014 container pad trials and in the detached branch trial.

Statistical analysis

Container pad trials

The GLM procedure of SAS (version 9.4; SAS Institute) was used to perform an analysis of variance to evaluate the effect of *Buxus* test cultivars on disease severity response for the 2012 container pad trial at 50 days of exposure to the inoculum reservoir plants, for the 2013 container pad trial at 23 days of exposure to the inoculum reservoir plants, and for the 2014 container pad trial at 24 days of exposure to the inoculum reservoir plants. Additionally, an analysis of variance was performed to evaluate the effect of *Buxus* test cultivars, after direct inoculation, on disease severity response for the 2013 container pad trial at 81 and 147 dpi and for the 2014 container pad trial at 59 dpi. Disease severity ratings were back transformed to the midpoint of the corresponding disease severity range.

Detached branch trials

The effect of *Buxus* cultivars on disease severity response at 24 and 23 dpi was analyzed as described previously. Additionally, a Levene's test was used in the HOV test option in the GLM procedure to assess homogeneity of variance between the two experiments. There was not a significant effect ($P = 0.8509$) of experiment on disease severity ratings and thus the data from the two experiments were combined.

Relationship between container pad and detached branch trials

Disease severity results for 19 cultivars were used to explore the relationship between results obtained from the container pad and detached branch trials. The CORR procedure of SAS was used to generate Pearson correlation coefficients. The correlation was evaluated between disease severity results obtained from the detached branch trial (23 and 24 dpi combined) and 2014 container pad trial at 24 days of test cultivar exposure to the inoculum reservoir plants and between the detached branch trial and 2014 container pad trial at 59 dpi of the test cultivar plants.

Effect of geographic origin on disease severity

An analysis of variance was performed in order to evaluate the effect of the geographic origin of *Buxus* species on disease severity response for the 2012 container pad trial at 50 days of test cultivar exposure to the inoculum reservoir plants, for the 2013 container pad trial at 23 days of test cultivar exposure to the inoculum reservoir plants, for the 2014 container pad trial at 24 days of test cultivar exposure to the inoculum reservoir plants, and for the detached branch trial at 24 and 23 dpi (data sets combined). Prior to analysis species were separated into the following independent variable groups; Asiatic, European, and Hybrid.

RESULTS

2012 container pad trial

For the container pad experiment conducted in 2012 the analysis of variance indicated that cultivar significantly affected disease severity ($P < 0.0001$) after 50 days of test cultivar

exposure to the inoculum reservoir plants (7 Sep 2012). There was a wide range of disease severity ratings for the cultivars tested. Disease severity ranged from 0.025% for *B. microphylla* var. *japonica* 'Green Beauty' to 61% for *B. sempervirens* 'Suffruticosa' (positive control). High and moderate leaf drop occurred most commonly on the cultivars with the highest disease severity rating. The unexposed negative controls of *B. sempervirens* 'Suffruticosa' incurred 22% diseased and defoliated leaves indicating that inoculum spread to the plants from the inoculum reservoir plants in other plots (Fig. 3).

Significant disease development in the 2012 container pad experiment did not begin until the second week in August; approximately three and a half weeks after the test cultivars were moved next to the inoculum reservoir plants (Fig. 4).

2013 container pad trial

There was abundant rainfall during the 2013 experiment. Significant disease development began on the test cultivars about two weeks after the plants were placed next to the inoculum reservoir plants (Fig. 5).

Cultivar significantly affected disease severity ($P < 0.0001$) after 23 days of test cultivar exposure to the inoculum reservoir plants (21 Jun 2013). As in the 2012 experiment there was a wide range of disease severity ratings. The cultivars *B. sempervirens* 'Aurea Pendula', 'Latifolia Maculata', 'Arborescens', 'Denmark', and 'Handsworthii' had ratings above 40% diseased leaves which was higher than the rating of 35% for the positive control *B. sempervirens* 'Suffruticosa'. The cultivars included as negative controls had low disease severity ratings, with 1% diseased leaves for *B. microphylla* var. *japonica* 'Green Beauty' and 5% for *B. sinica* var. *insularis* 'Nana' (Fig. 6).

For the disease severity evaluations conducted after the test cultivars were direct inoculated, the analysis of variance indicated that cultivar significantly affected disease severity at 81 dpi (10 Sep 2013) ($P < 0.0001$) and 147 dpi (15 Nov 2013) ($P < 0.0001$). There was a slight increase in the disease severity ratings on 10 Sep 2013 for plants that were inoculated directly, including; *B. sempervirens* 'Suffruticosa', 'Rotundifolia', 'Hohman's Dwarf", *B. sp.* 'Franklin's Gem', *B. sinica* var. *insularis* 'Nana', *B. sp.* 'Wedding Ring', and *B. microphylla* var. *japonica* 'Green Beauty' compared to the disease severity ratings recorded after 23 days of test cultivar exposure to the inoculum reservoir plants (21 Jun 2013) (Fig. 6&7). However, there was a decrease in disease severity ratings for all cultivars between 81 and 147 dpi with the exception of *B. harlandii* 'Richard' (Fig. 7).

2014 container pad trial

There was abundant rainfall during the 2014 experiment. Significant disease development began on the test cultivars about 1.5 weeks after the plants were placed next to the inoculum reservoir plants (Fig. 8).

Cultivar significantly affected disease severity ($P < 0.0001$) after 24 days of test cultivar exposure to the inoculum reservoir plants (26 Jun 2014). As in the 2012 and 2013 experiments there was a wide range of disease severity ratings. The cultivars *B. microphylla* var. *japonica* 'Morris Midget' and 'Grace Hendricks Philips' had ratings above 50% diseased and defoliated leaves which was higher than the rating of 48% for the positive control *B. sempervirens* 'Suffruticosa'. The cultivars *B. microphylla* 'Winter Gem', 'John Baldwin', and 'Jim Stauffer', *B. harlandii* and *B. harlandii* 'Richard', and *B.* sp. 'Wee Willie' had low disease severity ratings, with less than 1% diseased and defoliated leaves. The cultivars

included as negative controls had low disease development as well, with less than 3% diseased and defoliated leaves for both *B. microphylla* 'Green Beauty' and *B. sinica* var. *insularis* 'Nana' (Fig. 9).

For the disease severity evaluations conducted after the test cultivars were direct inoculated, the analysis of variance indicated that cultivar significantly affected disease severity at 59 dpi (25 Aug 2014) ($P < 0.0001$). Disease severity ratings ranged from 0.7% for *B. harlandii* 'Richard' to 99% for *B. sempervirens* 'Jensen' and the positive control 'Suffruticosa' (Fig. 10).

Detached branch trial

Cultivars significantly affected disease severity ($P <0.0001$). The cultivar *B. sempervirens* 'Suffruticosa' had the second highest disease severity rating of 48% leaf area diseased and defoliated. While *B. sempervirens* 'Rotundifolia' had the highest disease severity rating of 55%. Out of 21 cultivars evaluated, 14 cultivars had less than 10% leaf area diseased and defoliated after direct inoculation. The following cultivars were exceptions; *B. sempervirens* 'Longwood' (16%), *B. microphylla* 'Golden Dream' (17%), *Buxus* 'Green Velvet' (22%), *B. microphylla* 'Wedding Ring' (25%), and *B. sempervirens* 'Justin Brouwers' (31%) (Fig. 11).

Relationship between container pad and detached branch trials

The CORR procedure of SAS indicated that there was a weak correlation between disease severity results obtained from the detached branch trial (23 and 24 dpi combined) and 2014 container pad trial at 24 days of test cultivar exposure to the inoculum reservoir plants

($r = 0.42$, $P < 0.0001$) and between the detached branch trial and 2014 container pad trial at 59 dpi of the test cultivar plants ($r = 0.36$, $P < 0.0001$).

Effect of geographic origin on disease severity

In the three years of container pad experiments and the detached branch trial, the geographic origin of *Buxus* species had a significant effect ($P < 0.0001$) on disease severity response as indicated by an analysis of variance. Overall, the European group (*B. sempervirens*) cultivars had the highest disease severity while the cultivars in the Asiatic group had the lowest disease severity ratings (Fig. 12).

DISCUSSION

The disease severity ratings recorded for all of the experiments indicated a wide range of susceptibility in *Buxus* cultivars to the boxwood blight pathogen, *C. pseudonaviculata*. In this study, the Asiatic types generally expressed more resistance than the European types; this is similar to the results found in previous host resistance studies (7, 8). In the 2012 container pad trial the cultivars were exposed to the pathogen by placing them adjacent to the inoculum reservoir plants rather than inoculating them directly with *C. pseudonaviculata*. In general, the *B. sempervirens* types had higher disease severity ratings than the Asiatic types (which include *B. harlandii*, *B. microphylla*, and *B. sinica*) in the 2012 experiment. However, there were exceptions; *B. sempervirens* 'Dee Runk' and 'Fastigiata' had the lowest disease severity ratings of the *B.* sempervirens types. They have an upright and moderately compact plant architecture and a columnar growth pattern. It is possible that there is less humidity (and therefore less disease development) in the canopy of 'Dee Runk' and 'Fastigiata'

compared to the dense canopies of the susceptible cultivars, *B. sempervirens* 'American' and 'Suffruticosa'. In contrast, *B. microphylla* 'Morris Midget' and 'Morris Dwarf' were more susceptible than most of the Asiatic types. They have a very dense and compact plant architecture, which suggests that high humidity within the plant canopy contributes to disease development in these cultivars.

In the 2012 container pad experiment there were multiple rain events between the time the test cultivars were exposed to the inoculum reservoir plants and the time of significant disease development within the experiment; yet it took three and a half weeks for significant disease to develop. This result was unexpected because under ideal weather conditions the pathogen can complete its life cycle in less than one week. Perhaps, in addition to daily irrigation on the container pad, heavier rain events such as those that occurred on 27 Jul (1.68 cm) and 31 Jul (1.9 cm) 2013 are required to trigger rapid significant disease development. Hypothetically, the heavy rain event served to disperse conidia from inoculum reservoir plants onto the test cultivars. Additionally, the heavy rain event on 31 Jul was followed by almost daily precipitation for a week which most likely provided extended periods of leaf wetness and higher humidity in the plant canopy which is ideal for pathogen infection and growth. We are unable to determine whether, the weather during this period, or the direct inoculation with a low concentration of 1,000 conidia/ml suspension conducted on 8 Aug 2012, or both, resulted in the increase in disease observed following the inoculation.

As in 2012, in the 2013 container pad trial the cultivars were exposed to the pathogen via the inoculum reservoir plants. In this experiment there were relatively more *B.*

sempervirens types (20) in comparison to Asiatic types (8). Nevertheless, putative resistance was most frequently found in the Asiatic types. The *B. sempervirens* types which had a disease severity rating of less than 20%, *B. sempervirens* 'Angustifolia', 'Rotundifolia', and 'Longwood', all have an upright growth pattern and moderately dense canopy.

The disease severity ratings recorded 81 days post the direct inoculation performed on 21 Jun 2013 indicated that only *B. sempervirens* 'Suffruticosa' and 'Rotundifolia' had a disease severity rating increase of more than 5% compared to the disease rating performed after 23 days of exposure to the inoculum reservoir plants. There was generally a decrease in disease severity from the rating performed at 81 dpi (10 Sep 2013) to the rating performed at 147 dpi (15 Nov 2013) despite the fact that the test cultivars were direct inoculated for a second time on 11 Sep 2013.

Even though there was abundant precipitation during the 2013 experiment, there was not any rain for 3 days following the direct inoculation of the test cultivars on 21 Jun 2013. Perhaps this contributed to minimal pathogen infection and growth after the direct inoculation despite the fact that the test cultivars were covered with plastic bags after inoculation and received overhead irrigation. Another consideration is that the plants could have been covered with plastic bags too early on the day of inoculation, effectively killing some of the inoculum if temperatures in the bag were too high.

In the 2014 container pad experiment the Asiatic cultivars tended to be more resistant than the European cultivars. Notable exceptions include *B. microphylla* 'Morris Midget' and 'Grace Hendrick Phillips'. Both of these cultivars have a dense canopy and compact plant architecture and were identified as susceptible in 2012. In contrast to the results from 2013,

B. sempervirens ‘Longwood’ was one of the most susceptible of the *B. sempervirens* types in 2014. Notably, the ‘Longwood’ plants in the 2014 experiment were smaller and putatively younger than the plants in the 2013 experiment. In the future it is important to conduct disease resistance screens on plants of the same age in each experiment. Considering that *C. pseudonaviculata* was identified in the U.S. in 2011 there was not time to cultivate plants of the same age for the initial resistance screens that were conducted for this study.

The disease severity rating performed 39 days after direct inoculation (8.25.14) indicated that there was an increase of more than 25% for *B. sempervirens* ‘Rotundifolia’ and ‘Angustifolia’ compared to the rating performed after 24 days of exposure to the inoculum reservoir plants (6.26.14). While the cultivars *B. microphylla* ‘Green Beauty’, ‘Northern Emerald’, *B. sinica* var. *insularis* “Nana’, and *B. harlandii* had a disease severity rating increase of less than 2% during the same time period.

In the 2014 experiment, the inoculation of the inoculum reservoir plants was conducted prior to multiple rain events and there was significant disease development 1.5 weeks after the cultivars were placed next to the inoculum reservoir plants. In addition, a conscious effort was made to perform a direct inoculation on the test cultivars immediately before a predicted rain event. It is possible that the rain events contributed to overall higher disease severity ratings in 2014 compared to 2013.

As in the whole plant field trials, the Asiatic cultivars tended to be the most resistant cultivars in the detached branch trial. However, there were some notable discrepancies between disease severity ratings incurred in the container pad trials compared to the detached branch trials. Surprisingly, *B. sempervirens* ‘Latifolia Maculata’ had the lowest disease

severity rating of the *B. sempervirens* cultivars in the detached branch trial but one of the highest ratings in the 2013 and 2014 container pad trials. Additionally, *B. sempervirens* 'American' only incurred 6% leaf area diseased and defoliated in the detached branch trial but had a disease severity rating of over 40% diseased and defoliated leaves in the 2012 and 2014 container pad trials. The Asiatic cultivars, *B. microphylla* 'Golden Dream' and 'Wedding Ring' both had higher disease severity ratings in the detached branch trial than in the container pad trials. Interestingly, these are both variegated cultivars, and even though considerable time was spent analyzing lesion morphology for different cultivars it is possible that incorrect severity ratings were assigned due to incorrect documentation of boxwood blight symptoms. It is not surprising that there was a weak correlation between the disease severity results from the detached branch and container pad trials. Future studies should investigate improved methods for conducting detached leaf or branch susceptibility and resistance assays.

This study utilized a modified Horsfall-Barratt scale to rate disease severity as either, percent of diseased and defoliated leaves (2012, 2013 after direct inoculation of the test cultivars, and 2014), percent of diseased leaves (2013 prior to the direct inoculation of test cultivars) and leaf area diseased and defoliated (detached branch trial). The cultivars in this study were of various size and shape; assessing disease severity on plants with different size leaves and number of leaves (some cultivars had 100 fold more leaves than other cultivars) is inherently challenging. The container pad trials were conducted on whole plants and considering the relative leaf shapes and plant sizes it was only realistic to assess percentage of diseased and defoliated leaves. While the detached branch trials were conducted on a less

amount of plant material and thus it was possible to assess percentage of leaf area diseased and defoliated. In any case, the disease severity ratings could have possibly been improved with the use of a different rating scale; the accuracy of disease severity ratings with the Horsfall-Barratt scale has been questioned in other pathosystems (4, 5). Perhaps the use of a nearest percent scale would have resulted in increased accuracy and decreased variance among the ratings.

The goal of this study was to screen *Buxus* cultivars for resistance to the boxwood blight pathogen, *C. pseudonaviculata*. Over 80 cultivars were screened and a wide range of resistance was observed, with the Asiatic types generally expressing more resistance. Because it would be difficult to continually spray preventative fungicides on a frequent basis in the landscape to manage this disease on susceptible cultivars, resistant cultivars should be planted into landscapes to effectively manage boxwood blight. Considering the high economic and historical value of some *B. sempervirens* plantings, caution should be used during site selection for resistant cultivars. They incur relatively low levels of pathogen development and presumably reproduction, but they are not immune. The resistant cultivars could essentially serve as inoculum reservoirs and transfer the pathogen and boxwood blight disease to locations which include susceptible cultivars. Additionally, site selection for resistant cultivars is limited to the hardiness zones for which they are adapted; Batdorf (3) provides extensive reference to hardiness zones for cultivars of *Buxus*. Future studies should investigate the nature of the resistance in *Buxus* cultivars so that they may be effectively used in boxwood breeding programs.

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Design of Experiment in 2012, 2013, and 2014

Fig. 1. Design of experiment in 2012, 2013, and 2014. The 2012 (left) trial included 4 blocks with 6 plants of each cultivar in the same plot. The 2013 and 2014 (right) trial included 6 blocks with 1 plant of each cultivar in each block distributed randomly among plots. The red arrows indicate the location of the inoculum reservoir plants. The blue arrow indicates the susceptible positive control which was in each plot in the 2013 and 2014 trial.

Lesion morphology on the variegated cultivar *B. microphylla* 'Golden Dream'

Fig. 2. On variegated cultivars such as *B. microphylla* 'Golden Dream' it can be hard to distinguish between boxwood blight symptoms and natural variegation. The photograph on the left illustrates a lesion caused by *Calonectria pseudonaviculata*. The center and right photographs most likely illustrate natural variegation in the leaf.

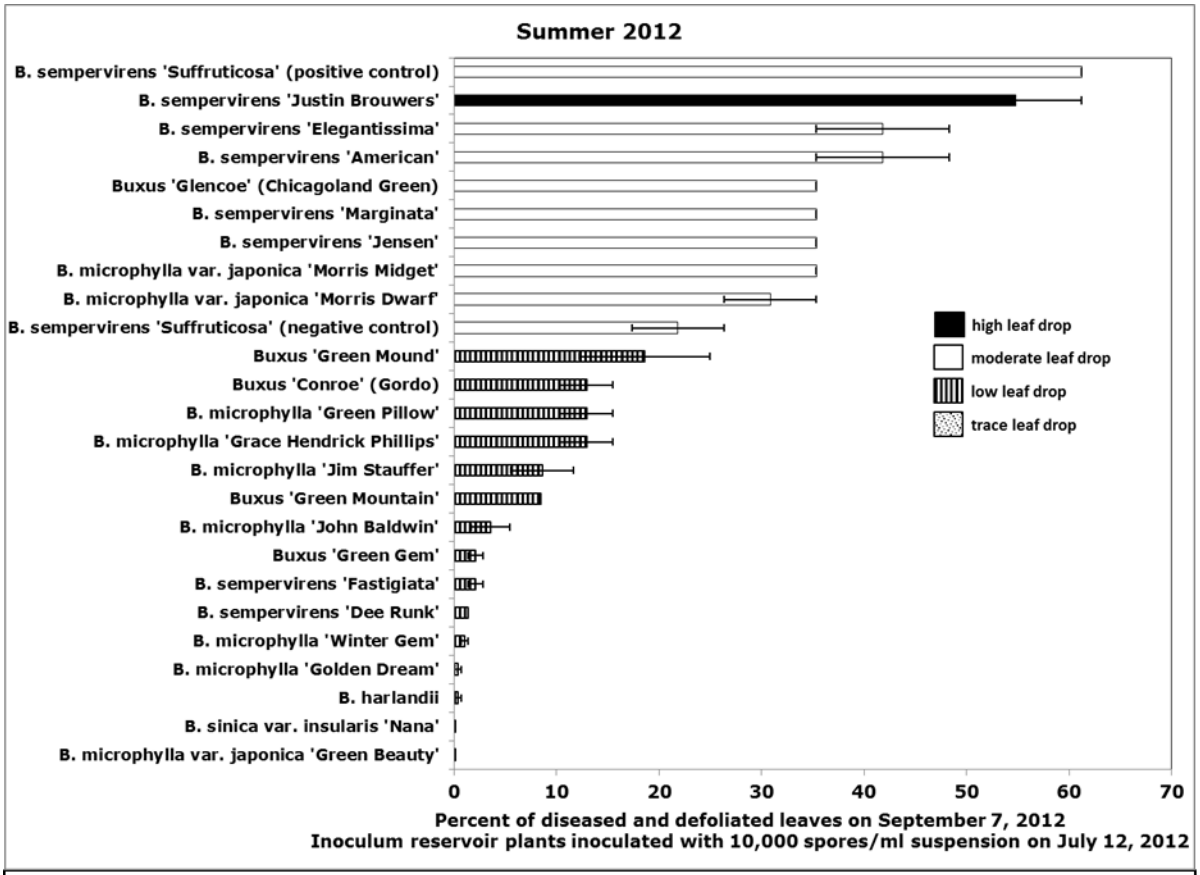

Fig. 3. Susceptibility of *Buxus* cultivars to boxwood blight in 2012. Cultivars were exposed to the pathogen by sitting next to inoculum reservoir plants for 50 days. Experiment conducted on an outdoor shaded container pad with overhead irrigation at the Mountain Horticultural Crops Research and Extension Center. Error bars represent standard error of the mean. Leaf drop ratings are indicated by the color of the bar.

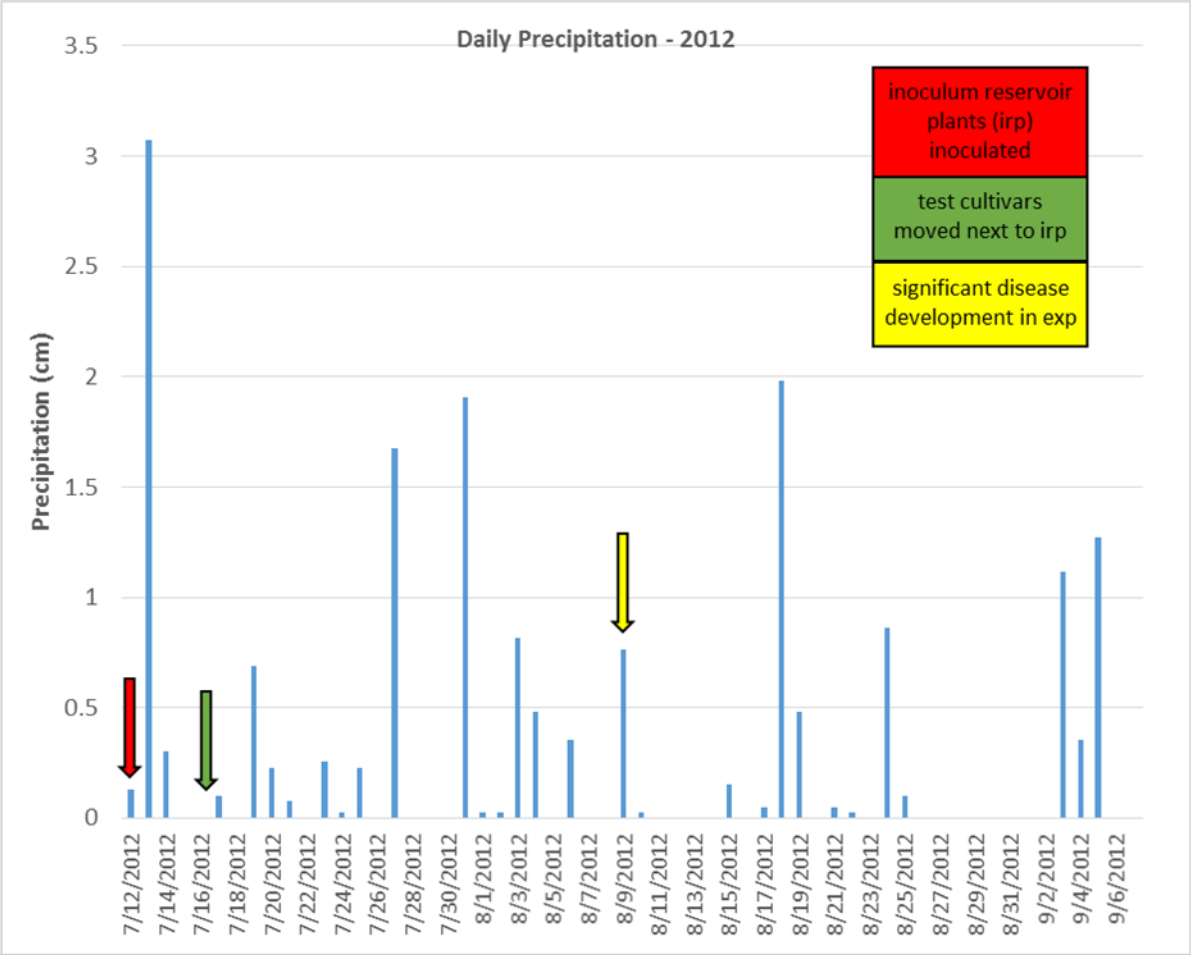

Fig. 4. Daily precipitation during the 2012 container pad susceptibility trial at the Mountain Horticultural Crops Research and Extension Center, Mills River, NC. Arrows indicate events during the experiment.

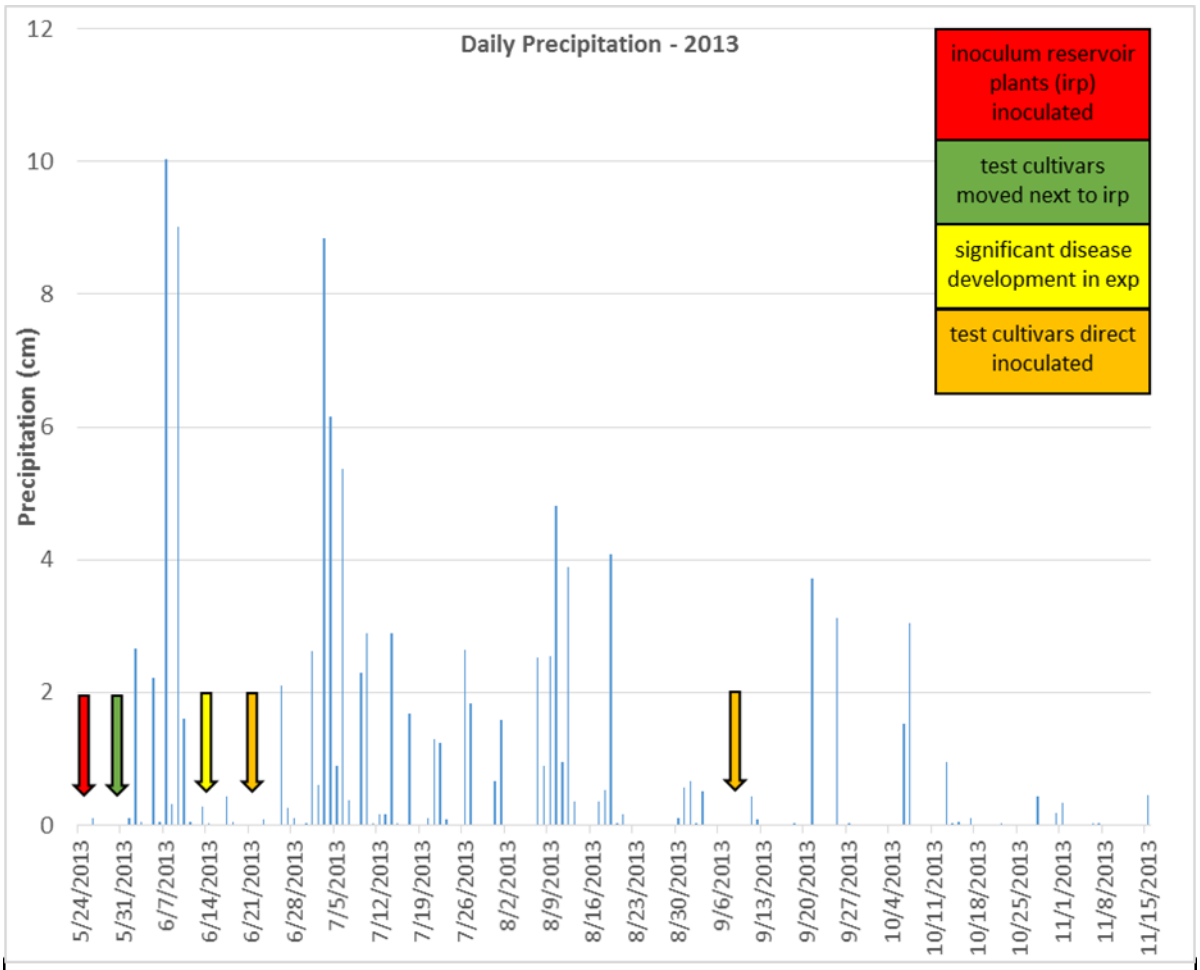

Fig. 5. Daily precipitation during the 2013 container pad susceptibility trial at the Mountain Horticultural Crops Research and Extension Center, Mills River, NC. Arrows indicate events during the experiment.

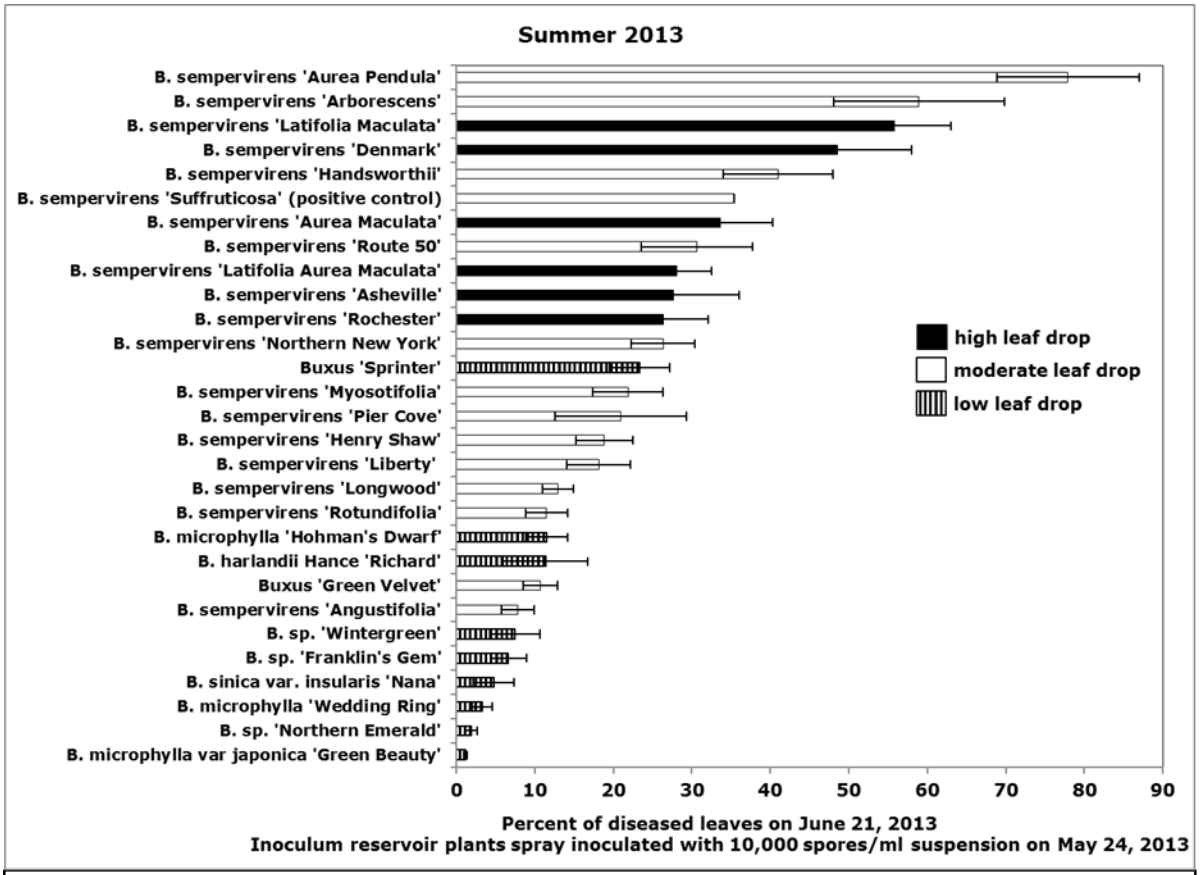

Fig. 6. Susceptibility of commercial *Buxus* cultivars to boxwood blight in 2013. Cultivars were exposed to the pathogen by sitting next to inoculum reservoir plants for 23 days. Experiment conducted on an outdoor shaded container pad with overhead irrigation at the Mountain Horticultural Crops Research and Extension Center. Error bars represent standard error of the mean. Leaf drop ratings are indicated by the color of the bar.

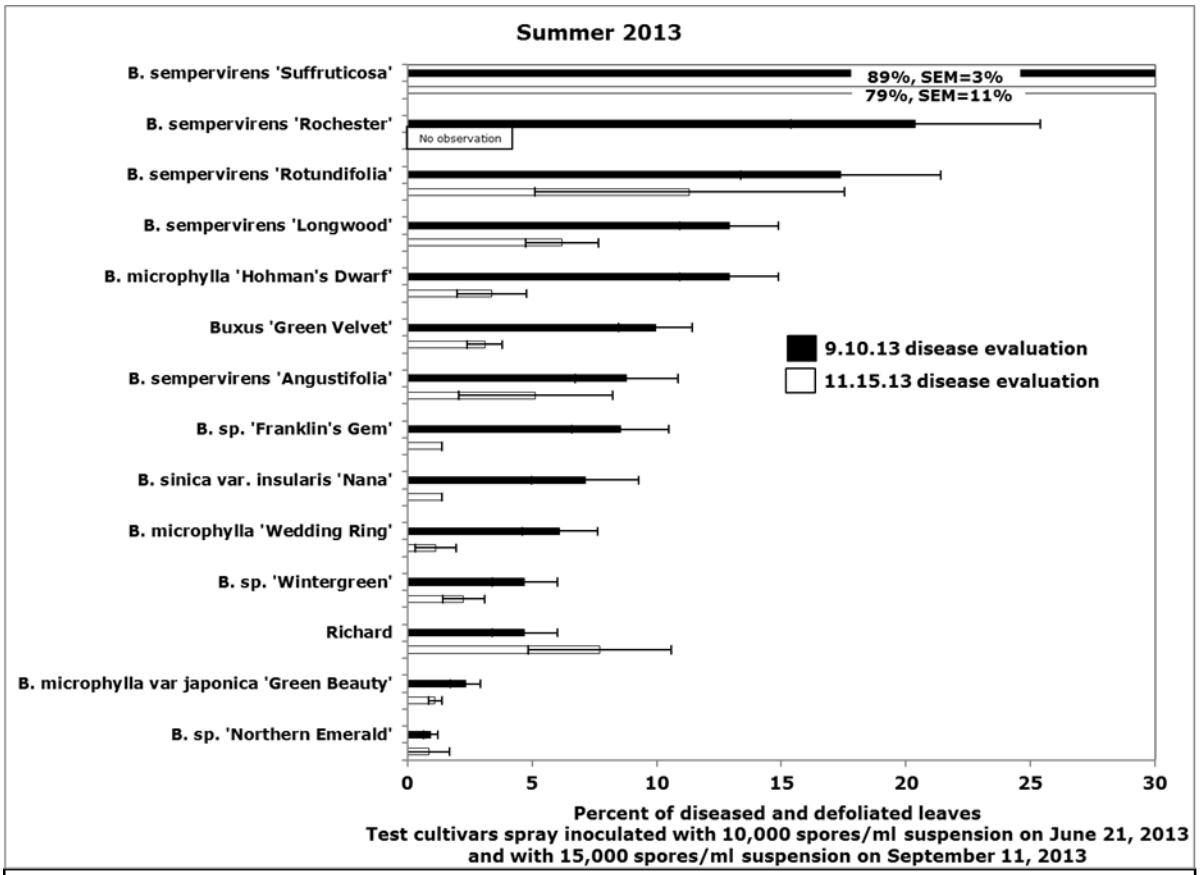

Fig. 7. Susceptibility of *Buxus* cultivars to boxwood blight in 2013 at 81 (9.10.13) and 147 (11.15.13) days post the inoculation performed on June 21, 2013. Cultivars were also direct inoculated with the pathogen on September 11, 2013. Experiment conducted on an outdoor shaded container pad with overhead irrigation at the Mountain Horticultural Crops Research and Extension Center. Error bars represent standard error of the mean (SEM).

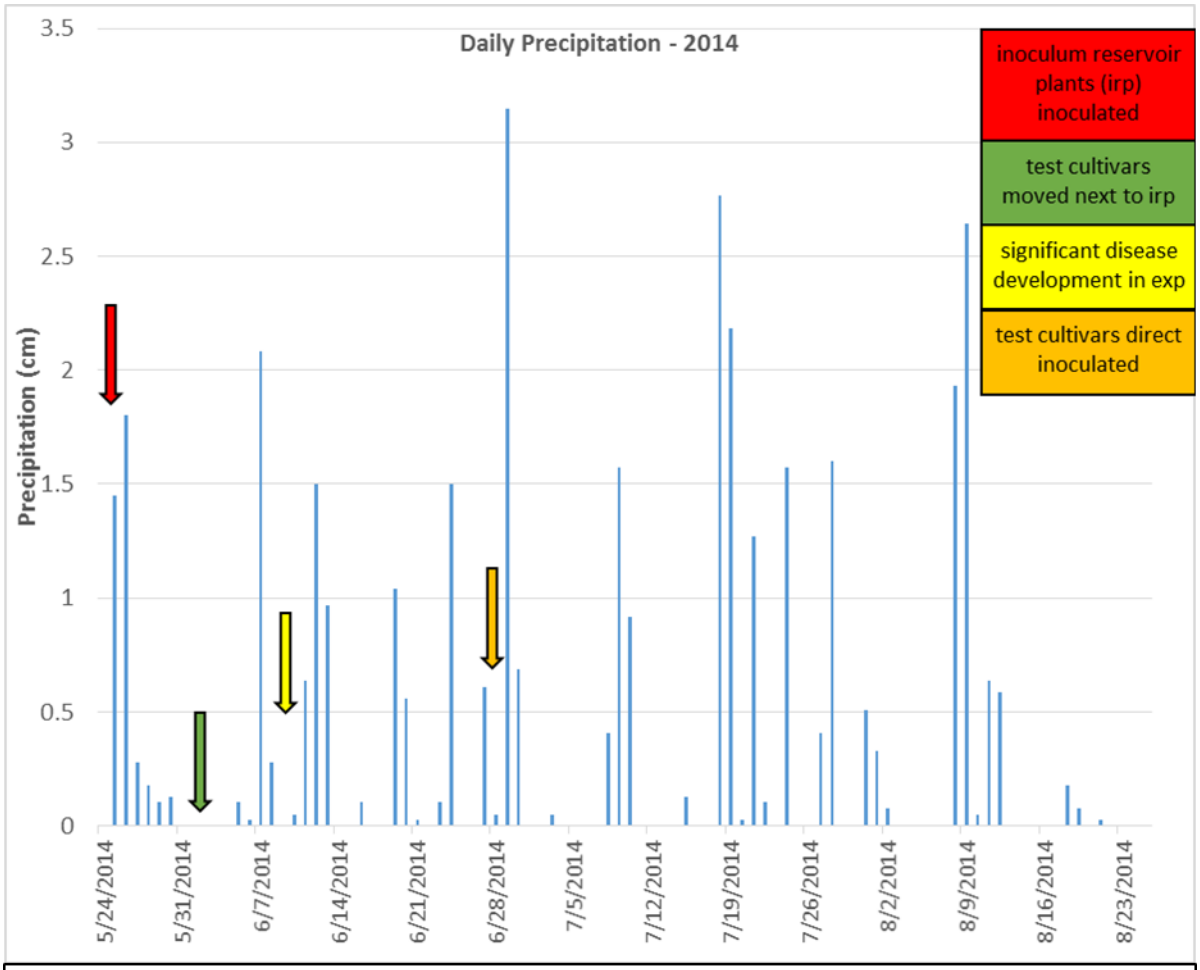

Fig. 8. Daily precipitation during the 2014 container pad susceptibility trial at the Mountain Horticultural Crops Research and Extension Center, Mills River, NC. Arrows indicate events during the experiment.

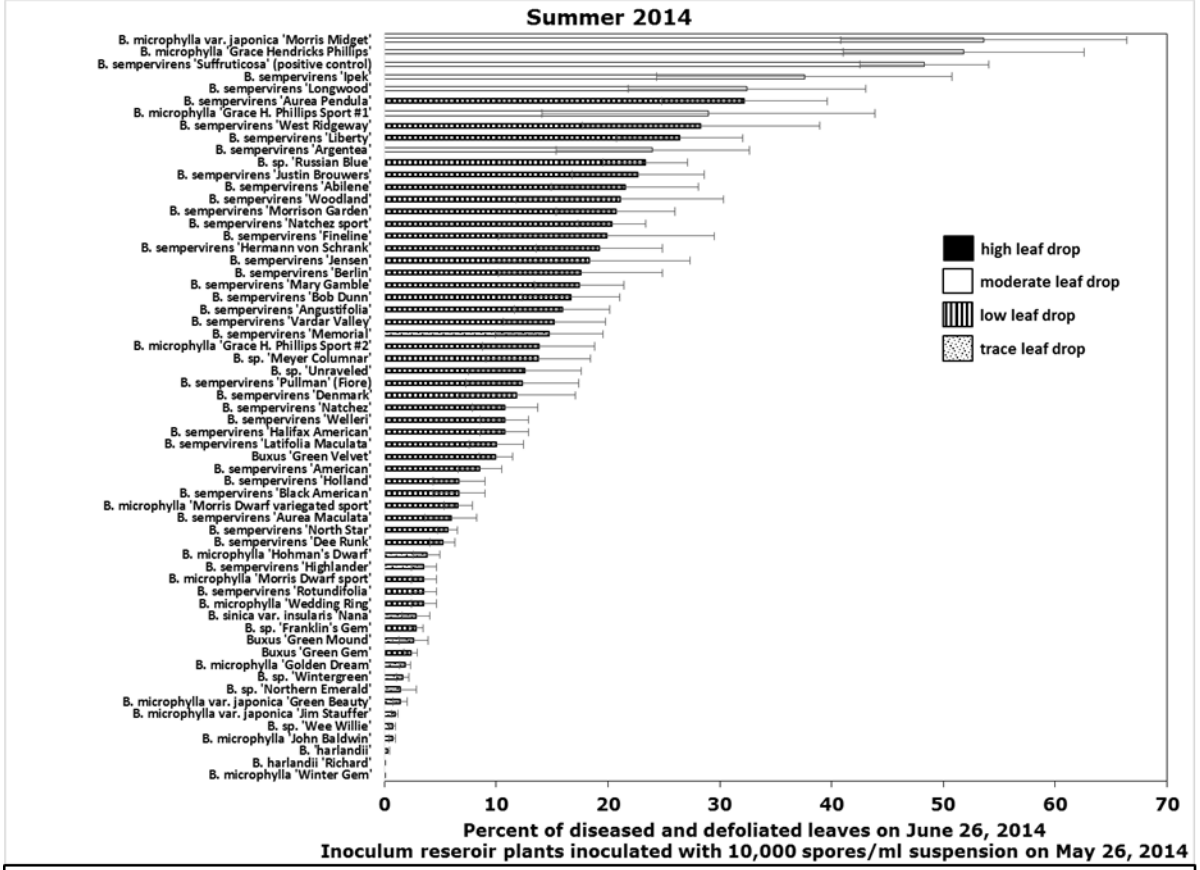

Fig. 9. Susceptibility of commercial *Buxus* cultivars to boxwood blight in 2014. Cultivars were exposed to the pathogen by sitting next to inoculum reservoir plants for 24 days. Experiment conducted on an outdoor shaded container pad with overhead irrigation at the Mountain Horticultural Crops Research and Extension Center. Error bars represent standard error of the mean. Leaf drop ratings are indicated by the color of the bar.

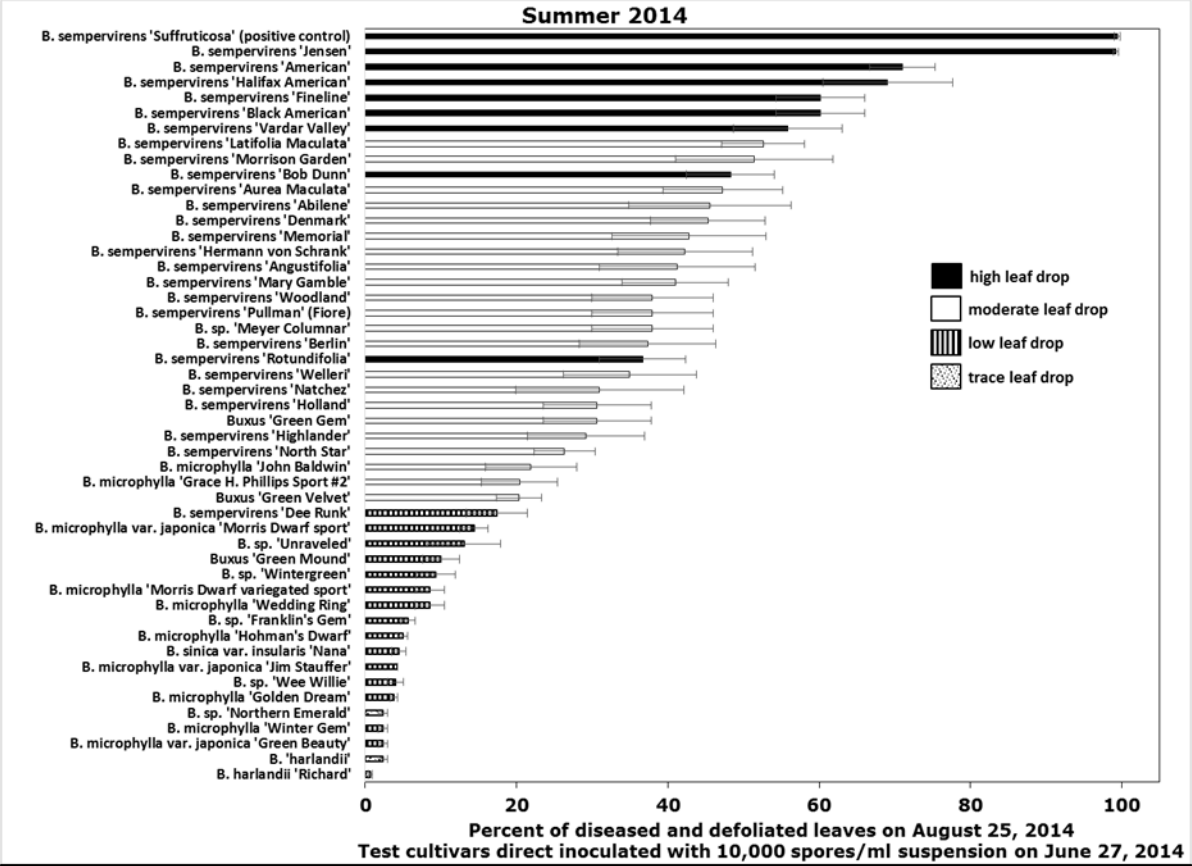

Fig. 10. Susceptibility of *Buxus* cultivars to boxwood blight in 2014 at 59 (8.25.14) days post the inoculation performed on June 27, 2014. Experiment conducted on an outdoor shaded container pad with overhead irrigation at the Mountain Horticultural Crops Research and Extension Center. Error bars represent standard error of the mean. Leaf drop ratings are indicated by the color of the bar.

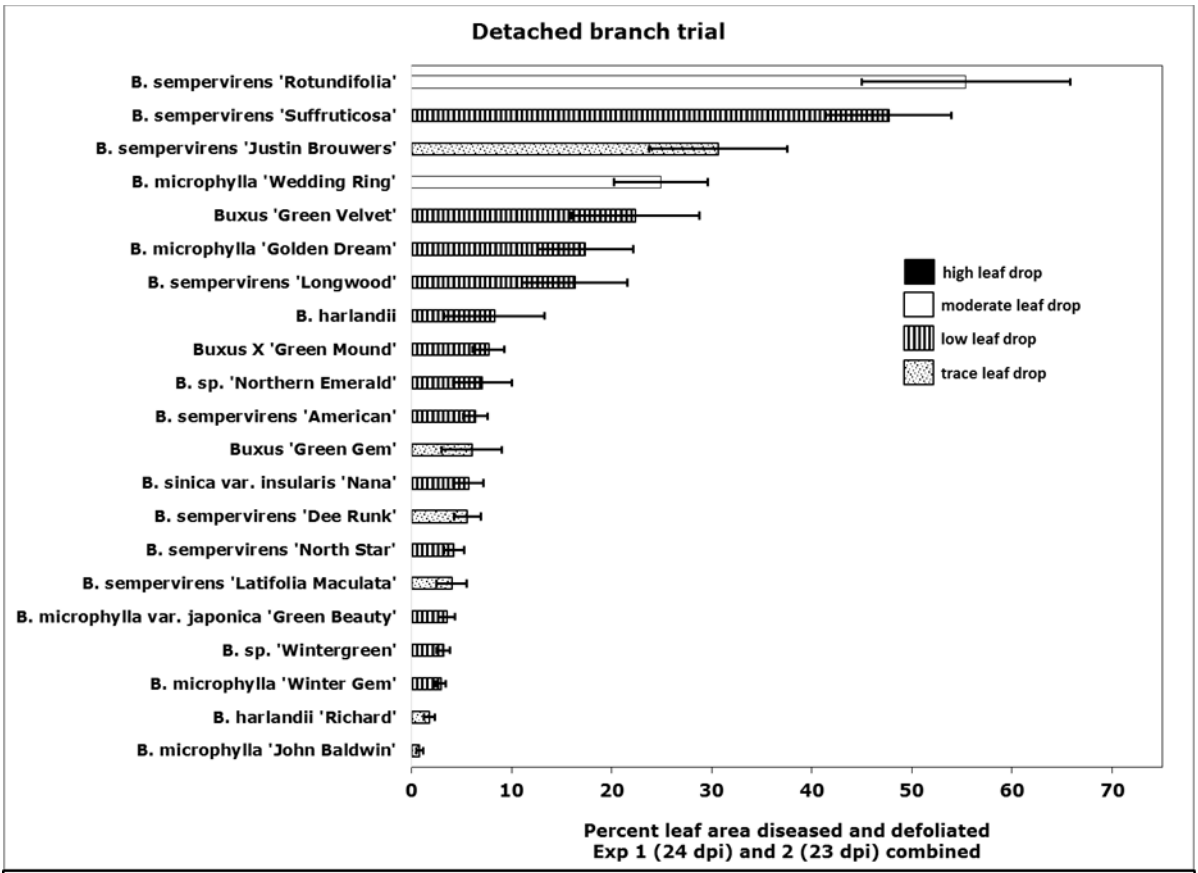

Fig. 11. Susceptibility of detached branches of *Buxus* cultivars to boxwood blight. Disease severity evaluated at 24 days post inoculation (dpi) in experiment 1 and 23 dpi in experiment 2. Disease severity data combined from both experiments. Error bars represent standard error of mean. Leaf drop ratings are indicated by the color of the bar.

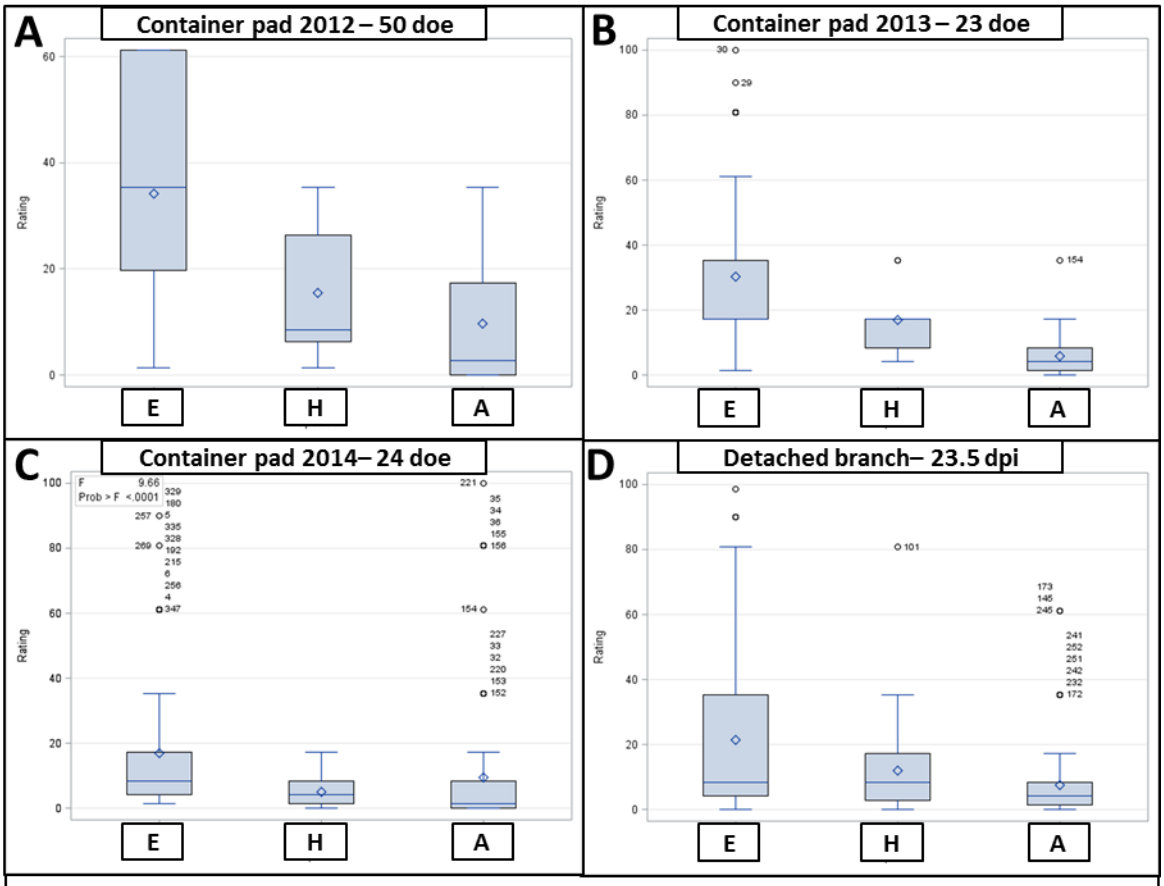

Fig. 12. Box plots show the differences in disease severity ratings between *Buxus* species differing in geographic origin. Y-axis indicates disease severity rating. X-axis indicates species; E=European, H=Hybrid, A=Asiatic. Susceptibility of commercial *Buxus* species to boxwood blight determined in a container pad susceptibility trial in 2012 (**A**), in 2013 **(B),** in 2014 (**C**), and in a detached branch trial **(D).** doe = days of exposure to the inoculum reservoir plants.

CHAPTER 3. Components of quantitative resistance to boxwood blight in *Buxus* cultivars

INTRODUCTION

Boxwood blight is a devastating foliar disease on *Buxus*, caused by the fungal pathogen *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum*, *Cylindrocladium buxicola*). It became apparent that boxwood blight was a seriously economically important disease in the late 1990s and early 2000s in the United Kingdom (5). Some of the most popular and commonly grown boxwood are *B. sempervirens* 'Suffruticosa' (also known as English boxwood) and 'American'. The latter is the common cultivar name in the United States, but in Europe it is referred to as simply *B. sempervirens*. This species is indigenous to Europe and it is thought that the first *B. sempervirens* plants were brought to the US in 1653 (1). Both 'American' and 'Suffruticosa' cultivars of *B. sempervirens* are susceptible to boxwood blight disease, and infection can result in complete defoliation.

Even though *B. sempervirens* is very popular, there are approximately 25 to 30 additional species of *Buxus* that have been well described and about 95 to 100 species in *Buxus* overall. Boxwood species are indigenous all over the world and in a variety of climates (9) (Fig. 1). Cultivars of *B. sempervirens* from Europe and the Asiatic species and cultivars *B. harlandii*, *B. microphylla*, and *B. sinica* are the most commonly grown and distributed *Buxus* spp. in the nursery trade. Given the variety of boxwood species available host resistance has been investigated as a boxwood blight management strategy.

As of 2008, only *B. sempervirens, B. microphylla, and B. sinica* were reported as hosts for *C. pseudonaviculata* in *Buxus* species. However, since then other species of *Buxus*

have been reported as hosts (2-4). In a detached leaf pathogenicity experiment performed on 11 species, *B. sempervirens* 'Suffruticosa' and *B. sinica* var. *insularis* were the most susceptible while *B. balearica* and *B. riparia* were the most resistant in terms of leaf spotting. In this study, no boxwood species were shown to have immunity to *C. pseudonaviculata*, and species expressed a range of susceptibility to the pathogen (4).

Additional cultivar susceptibility tests were conducted on whole plants in greenhouse and field environments in Europe and a wide range of susceptibility in *Buxus* spp. to the boxwood blight pathogen was observed (2, 3). In these experiments, the Asiatic types, in general, expressed more host resistance to boxwood blight in comparison to the European types.

The discovery of boxwood blight into the U.S. in 2011 (7) prompted researchers in the U.S. to begin investigating host resistance in *Buxus* spp. Experiments were conducted on a shaded outside container pad with overhead irrigation in western North Carolina and the experiment was designed so that cultivars were exposed to *C. pseudonaviculata* in a way that simulated natural pathogen spread. The goal of the experiments was to identify field resistance in *Buxus* to the boxwood blight pathogen. Cultivars that express acceptable resistance under naturally varying field environments may not perform well in an environment which is very conducive for pathogen growth and disease development. In addition, field resistance cannot be readily distinguished from avoidance or tolerance. However, if adult plants display an acceptable level of field resistance this would allow growers and boxwood enthusiasts alike to grow boxwood cultivars without the devastating losses caused by boxwood blight.

Studies conducted in the U.S. also identified that the Asiatic species tended to be more resistant than the European species (Ganci, *unpublished*, M. S. Thesis, Chapter 2). However, there were exceptions; for example, *B. sempervirens* 'Dee Runk' expressed resistance in the field. This cultivar has an upright and moderately compact plant architecture and a columnar growth pattern. Since the pathogen thrives in warm and humid environments it is thought that the dense canopy of *B. sempervirens* 'Suffruticosa' and 'American' could contribute to intense disease development on these cultivars (4). It is possible that there is less humidity (and therefore less disease development) in the canopy of 'Dee Runk' compared to the dense canopies of the susceptible cultivars, *B. sempervirens* 'American' and 'Suffruticosa'. In contrast, *B. microphylla* 'Morris Midget' was more susceptible than most of the Asiatic types. It has a very dense and compact plant architecture, which suggests that high humidity within the plant canopy contributes to disease development in this cultivar.

Host resistance is characterized as either qualitative (complete resistance) or quantitative (partial resistance). Partial disease resistance results in a reduction in the rate of disease development in the host. The following factors contribute to partial resistance to fungal pathogens; long incubation period (time from inoculation until symptom development), long latent period (time from inoculation until pathogen spore production), minimal infection frequency, minimal infectious period (length of time of spore production), and minimal spore production (number of spores produced per unit area)(8). Investigation of the components that lead to partial resistance in a particular pathosystem can help in the evaluation and selection of partially resistance cultivars and an improved understanding of disease epidemiology.

The objective of this study was to quantify boxwood blight disease development in *Buxus* cultivars in order to identify components that can be attributed to host resistance. Variables measured included disease severity, incubation period, latent period, lesion area, and conidia production.

MATERIALS AND METHODS

Components of quantitative disease resistance to boxwood blight in *Buxus* cultivars were evaluated in a controlled environment chamber at the North Carolina State University Phytotron; variables measured included disease severity, incubation period, latent period, lesion area, and conidia production. Two separate experiments were performed in the fall of 2013. Each experiment consisted of a randomized complete block design with subsampling (individual leaves served as subsamples) and each of four blocks included a single plant of each of eight cultivars. The cultivars included in the study were chosen because they expressed a range of adult plant resistance reactions to the boxwood blight pathogen, *C. pseudonaviculata* (Ganci, *unpublished,* M. S. Thesis, Chapter 2) in experiments conducted in an outdoor environment on a shaded container pad with overhead irrigation. The cultivars and species *B. harlandii, B. microphylla* 'John Baldwin', *B. microphylla* var. *japonica* 'Green Beauty', *B. sempervirens* 'Dee Runk', and *B. sinica* var. *insularis* 'Nana' expressed partial resistance in the container pad trial. While the cultivars *B. microphylla* var. *japonica* 'Morris Midget', *B. sempervirens* 'American' and 'Vardar Valley' were susceptible to the boxwood blight pathogen. Whole plants used in the study were approximately three years old. 'Dee Runk', 'John Baldwin', 'Morris Midget', 'Nana', and 'Vardar Valley' were in 3.8

liter containers. ‘American’, *B. harlandii*, and ‘Green Beauty’ were in 11.4 cm square containers.

Light and temperature could be regulated in the controlled environment chamber in the Phytotron, but humidity could not be controlled. Temperature and humidity data were obtained from a digital weather station (AcuRite). Ambient humidity levels were approximately 40 to 60% which was not high enough for pathogen conidia production. In order to increase humidity during the experiments, one plant of each cultivar was placed inside each of four clear plastic boxes (Cambro Manufacturing Company, 45.72 cm x 66.04 cm x 38.1 cm); these represented the blocks. Saturated absorbent towels (Mr. Clean, Cham-it cloth) were placed inside of each plastic box. Humidity levels ranged from approximately 75 to 99% inside the boxes. Due to the size of the Phytotron chamber, 2 plastic boxes were placed on top of the other two plastic boxes and the position of each plastic box was rotated daily.

1st Experiment

Prior to inoculation the plants were watered until the substrate in the pots was saturated. Cultivars were spray inoculated with a 10,000 conidia/ml suspension until run-off inside of plastic boxes. Approximately 275 ml were used to inoculate the 8 cultivars within each of four plastic boxes. The inoculum consisted of a combination of *C. pseudonaviculata* isolates collected from North Carolina. There was a 12 h photoperiod within the chamber. After inoculation the temperature inside of the boxes was approximately 22°C during the day and 20°C during the night. Boxwood blight lesion development began on immature leaves 3 days post inoculation (dpi). After lesion development, the temperature conditions were

changed to approximately 20°C during the day and 16°C during the night. These temperature conditions were chosen because they reflect temperatures experienced during boxwood blight disease epidemics observed in the field in North Carolina.

After inoculation, plants were spritzed with water until run-off with a hand held spray bottle one to two times daily. After pathogen conidia production was observed macroscopically on leaves, 4 days post inoculation, plants were no longer watered. Due to the high relative humidity inside of the plastic boxes plant substrate remained moist throughout the duration of the experiment.

Upon lesion development, 20 individual leaves were tagged with jewelry hang tags to allow for continual monitoring of specific leaves. Twenty symptomatic mature leaves were tagged per plant with the intention of recording and measuring incubation period for 20 subsamples per plant, latent period and conidia production for 10 subsamples per plant, and lesion area for 10 subsamples per plant. In addition, 25 to 32 leaves were collected from some cultivars in anticipation that they would defoliate before all data could be collected. Previous experiments (data not shown) revealed that leaves on the cultivars *B. harlandii*, *B. microphylla* var. *japonica* 'Green Beauty', and *B. sinica* var. *insularis* 'Nana' were prone to drop from the plant shortly after initial stages of disease development.

Leaves were observed daily after inoculation. Incubation period was recorded as the time from inoculation to lesion appearance on each of the tagged leaves. In previous experiments (data not shown) production of conidia was most readily observed by illumination with a flashlight without magnification. Conidia are produced on the abaxial surface of leaves. Latent period was recorded as the time from inoculation to sporulation on

each of the tagged leaves. If a leaf was abscised it was monitored for detection of conidia production even though the leaf would not be used to record latent period. Abscission of tagged leaves occurred on all cultivars and not only those that were mentioned earlier. Approximately 24 h after conidia production was detected on abscised leaves, leaves were collected and the adaxial surface was immediately photographed for later measurement of lesion and leaf area. Leaf and lesion area were calculated with plant disease quantification image analysis software (Assess v. 2.0, APS Press). Lesion area was only calculated on leaves which positive pathogen sporulation was detected; either after leaf collection from the experiment or after incubation in a humid chamber. In some instances, abscised leaves were lost from the identification tags, resulting in missing data.

Approximately 24 h after sporulation was detected, leaves were removed from the plant. Sporulation was verified with a stereoscope (Nikon SMZ-2T) at 10X to 40X magnification. There were cases of false positive sporulation detection. Fungal tissue was present on these leaves; some of the tissue appeared to be mycelium but often the tissue appeared to have some structure. It is hypothesized that these structures were either differentiating conidiophores or germinating microsclerotia (Fig. 2). The structures appeared to be an aggregation of cells and in some cases an elongation extended from the aggregation which appeared to be an immature conidiophore. Microsclerotia have been detected on the surface of leaves even though it is more common for the microsclerotia to form within leaves. In cases where false positive sporulation was detected the recorded latent period was removed from the data set. The incidence of false positive sporulation detection essentially

resulted in the erroneous removal of leaves from the experiment. Nevertheless, the adaxial leaf surface of these leaves was photographed for later measurement of lesion and leaf area.

In cases where positive sporulation was verified microscopically, the abaxial leaf surface was photographed and used for later measurement of leaf area and calculation of conidia produced per leaf and per mm^2 of leaf area. The adaxial surface was not photographed in order to reduce chances of disturbing and removing conidia on leaves. Furthermore, it was only necessary to measure leaf area and it was not necessary to measure the lesion area on individual leaves upon which conidia production was quantified. Conidia production is not limited to the lesion area and therefore it was not appropriate to calculate conidia production per lesion area (Fig. 3). After being photographed, individual leaves were placed inside a 1.5 ml microcentrifuge tube containing 1 ml of 1% Tween 20 (Fisher Scientific) solution and stored at -20 °C for future quantification of conidia production.

After thawing, individual leaves were removed from the microcentrifuge tube and washed with 500 µl of 1% Tween 20 solution. The wash went into the microcentrifuge tube and the leaf was discarded. Before disposal, a subsample of leaves were viewed with a stereoscope (Olympus SZX7) at 10X to 40X to confirm that only a negligible amount of conidia were disposed of with the leaf. The suspension was centrifuged at 14,000 *g* for 10 min (Accuspin 400, Fisher Scientific) and the supernatant was disposed. Before disposal, a selection of supernatant samples were viewed with a stereoscope at 10X to 40X before disposal to confirm that only a negligible amount of conidia were disposed of with the supernatant. The pellet containing conidia was re-suspended with 40 µl of distilled water; this brought the total volume up to 100 µl. Samples were stored at -20°C. After thawing the

conidia suspension, two subsamples were removed with a Pasteur pipet from each microcentrifuge tube for quantification of conidia with a hemocytometer. In some cases, the conidia count with the hemocytometer was zero. Presumably, the density of conidia in suspension was so low that some aliquots did not contain conidia. These were cases of a false negative result because the presence of conidia had been verified before the leaves were placed inside the microcentrifuge tube and processed for conidia quantification. If either of the two subsample hemocytometer counts of conidia were zero then the result was changed to one. Conidia per leaf and conidia per mm^2 of leaf area were calculated.

The experiment was designed so that incubation period would be recorded for 20 leaves, latent period and conidia production would be recorded for 10 leaves, and lesion area would be recorded for 10 leaves per cultivar per block. However, due to the frequency of leaf abscission and false positive sporulation detection the number of subsamples collected per cultivar for each of the disease response variables analyzed varied greatly.

Disease severity was evaluated as percent leaf area diseased and defoliated with a modified Horsfall-Barratt scale (6) (0 = 0, 1 = 1 lesion, 2 = 0.6 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, 8 = 75 to 87%, 9 = 87 to 94%, 10 = 94 to 97%, 11 = 97 to 100%, 12 = 100%) at 4, 7, 12, and 20 days post inoculation. At 20 days post inoculation, 50 out of 696 tagged leaves (7%) remained attached. Additionally, sporulation was detected for the first time on only three leaves between 16 and 19 days post inoculation and thus the experiment was concluded 20 days post inoculation. The tagged leaves which were removed from cultivars at the end of the experiment were placed inside of a humid chamber. Leaves were monitored for 25 days. If the pathogen produced conidia then those

leaves collected at the end of the experiment were recorded with a latent period of 20 days and zero conidia production. Also, at 20 days post inoculation, up to five leaves with conidia were collected from each plant in order to compare spore production on different cultivars at a specific time point.

2nd Experiment

It was thought that the constant relative high humidity throughout the duration of the first experiment contributed to cases of false positive sporulation detection on leaves. Due to the prevalence of cases of false positive sporulation detection in the first experiment, the environmental conditions were altered in the second experiment such that the lids were not kept on the plastic boxes for the duration of the second experiment. The cultivar *B. harlandii* was removed from one of the blocks. It became apparent that the plant had an incorrect identification tag and the plant did not have the same appearance as the *B. harlandii* plants in the other blocks; this resulted in this cultivar being included in three blocks rather than in four blocks in the second experiment. The cultivars were inoculated as described above and kept in the plastic boxes for three days. After three days lesion development had occurred and the cultivars were removed from the plastic boxes and placed onto plastic trays within the growth chamber. Individual symptomatic leaves were tagged and incubation period was recorded as described previously. Plants were spritzed with water until run-off one to two times daily. Inside of the Phytotron chamber, temperature was approximately 20°C during the day and 16°C during the night and relative humidity ranged from 40 to 60%. The cultivars were moved back into the plastic boxes 8 days post inoculation. Temperature conditions remained the same but relative humidity in the plastic boxes was increased to 75

to 99%. The cultivars were no longer watered after being moved back into the plastic boxes. Leaves were observed daily and plastic boxes and trays were rotated daily.

Upon detection of pathogen sporulation, on tagged leaves latent period was recorded as previously described. In this experiment, abscised leaves were collected immediately and the adaxial surface was photographed for later measurement of lesion and leaf area. Leaf and lesion area was calculated with plant disease quantification image analysis software. As described previously, leaves were removed from the plant approximately 24 h after pathogen sporulation was observed. Sporulation was verified with a stereoscope (Nikon SMZ-2T) at 10X to 40X. As in experiment one, there where cases of false positive sporulation detection in experiment two and the appearance of fungal tissue was similar (Fig. 2). In cases where false positive sporulation was detected the recorded latent period was removed from the data analysis. The adaxial leaf surface was photographed for later measurement of lesion and leaf area. In cases where positive sporulation was detected, the abaxial leaf surface was photographed for later measurement of leaf area. After being photographed the leaves were processed and pathogen conidia production was quantified as described previously.

Disease severity was evaluated at 4, 8, 12, 19, and 23 days post inoculation. At 23 days post inoculation the experiment was concluded and 50 out of 620 tagged leaves (8%) remained attached. The tagged leaves which were removed from plants at the end of the experiment were placed inside of a humid chamber. Leaves were monitored for 8 days. If the pathogen produced conidia then those leaves collected at the end of the experiment were recorded with a latent period of 23 days and zero conidia production. Additionally, at 15 days post inoculation and at 23 days post inoculation up to 10 leaves with conidia were collected

from each plant in order to compare spore production on different cultivars at a specific time point.

Additional variables were analyzed to further investigate the resistance response for each cultivar. These response variables included: incidence of leaf abscission, incidence of a false positive sporulation detection on leaves, and incidence of a possible hypersensitive reaction. Subsamples were considered to display a hypersensitive reaction if the lesion area was below 10% of leaf area and *C. pseudonaviculata* was not able to form conidia on leaves after incubation in a humid chamber after the conclusion of the experiments.

Statistical analysis

In the first and second experiment, the prevalence of leaf abscission, false positive sporulation detection, and the absence of pathogen sporulation on some leaf samples resulted in an unbalanced data set for the response variables incubation period, latent period, spore production per leaf, spore production per mm^2, and lesion area. A review of the structure of the data sets revealed that for each response variable, variances of the mean for each cultivar were unequal and outliers were present. For these reasons an analysis of variance was performed using the GLIMMIX procedure of SAS (version 9.4; SAS Institute) to test the effects of *Buxus* cultivars on the response variables. The GLIMMIX procedure takes into account separate residual variances for each cultivar. To improve normality in the data distribution the following equation was used to transform fungal sporulation data: transformed response = ln(response+1). Reported means for the fungal sporulation response variables, conidia per leaf and conidia per mm^2 of leaf area were derived from back transforming the means and standard deviations according to the following equation: back

transformed response = $((e^{\text{transformed mean or standard deviation}}) - 1)$. The Tukey-Kramer option in the GLIMMIX procedure was used to group effects of cultivars on the response variables. This procedure uses least squares means to separate group effects and thus is an appropriate separation procedure to use on an unbalanced data set. This approach conservatively groups effects of cultivars essentially reducing the risk of type 1 errors (falsely indicating significant differences between cultivars).

The FREQ procedure of SAS was used to compare the frequency of occurrence of incidence of leaf abscission, incidence of a false positive sporulation detection on leaves, and incidence of a hypersensitive reaction for each cultivar.

Disease severity ratings were back transformed to the midpoint of the corresponding disease severity range. The GLM procedure of SAS (version 9.4; SAS Institute) was used to perform an analysis of variance to test the effect of *Buxus* cultivars on the disease severity response variable and the Waller Duncan mean separation procedure was used.

RESULTS

1st Experiment

Differences in components of partial resistance were found across boxwood cultivars tested; an analysis of variance indicated the effect of cultivar had a significant effect on latent period ($P < 0.0001$), conidia production per leaf ($P = 0.0013$), and conidia production per mm^2 ($P = 0.0275$). However, the effect of cultivar did not have a significant effect on incubation period or lesion area (Table 1). Among all cultivars tested, incubation period ranged from 4 days for *B. sempervirens* 'American' to 4.6 days for *B. microphylla* var.

japonica ‘Green Beauty’ (Fig. 4). The Asiatic cultivars *B. sinica* var. *insularis* 'Nana' and *B. harlandii* had the longest latent period at 16.1 and 10.8 days, respectively. The European cultivars *B. sempervirens* ‘American’, ‘Dee Runk’, and ‘Vardar Valley’ had the shortest latent period at 5.1, 5.9, and 6.2 days, respectively (Fig. 5A). There was a wide range of conidia production by *C. pseudonaviculata* on the cultivars in the experiment. Conidia production was greatest on the cultivar *B. microphylla* var. *japonica* ‘Green Beauty’ (3,502 conidia per leaf and 49 conidia per mm^2 of leaf area) and the least on *B. sinica* var. *insularis* 'Nana' (17 conidia per leaf and 4 conidia per mm^2 of leaf area) (Fig. 5 C&E). Lesion area ranged from 26% of leaf area on *B. harlandii* to 49% of leaf area on *B. sinica* var. *insularis* 'Nana', but were not statistically different (Fig. 5G).

The effect of cultivar had a significant effect on disease severity as indicated by an analysis of variance ($P < 0.0001$) (Table 1). The European cultivars all had a disease severity rating of 90% or greater while the Asiatic cultivar *B. microphylla* var. *japonica* ‘Morris Midget’ had a rating of 65%. The remaining Asiatic cultivars, *B. microphylla* ‘John Baldwin’, ‘Green Beauty’, and *B. harlandii* had ratings below 40% and *B. sinica* var. *insularis* 'Nana' had the lowest rating of 6% (Fig. 6).

The frequency of occurrence of incidence of leaf abscission, incidence of false positive sporulation detection on leaves (Table 2), and incidence of a possible hypersensitive reaction was evaluated for all cultivars tested. In the first experiment tagged leaves were most commonly abscised from the Asiatic cultivars while the majority of the tagged leaves remained on the European cultivars. Leaf abscission of tagged leaves occurred at a rate of 55.1% for *B. microphylla* var. *japonica* ‘Green Beauty’, 46.36% for *B. sinica* var. *insularis*

'Nana', and 37.36% for *B. harlandii* (Table 2). In the first experiment, a hypersensitive reaction occurred on 10.91% of *B. sinica* var. *insularis* 'Nana' tagged leaves, on 2.2% of *B. harlandii* tagged leaves, and 1.25% of *B. microphylla* 'John Baldwin' tagged leaves and did not occur on any of the other cultivars evaluated (data not shown).

At the end of experiment one (20 dpi), symptomatic leaves with conidia production were collected to compare the effect of cultivar on conidia production at a specific time point. The Asiatic cultivars *B. microphylla* 'John Baldwin' and *B. microphylla* var. *japonica* 'Morris Midget' had the most pathogen conidia production per leaf and per mm^2 (Fig. 7 A&B).

2nd Experiment

As in experiment one, differences in components of partial resistance were found across boxwood cultivars tested. An analysis of variance indicated the effect of cultivar had a significant effect on all variables analyzed; incubation period ($P < 0.0001$), latent period ($P < 0.0001$), conidia production per leaf ($P = 0.0028$), conidia production per mm^2 ($P = 0.0032$), and lesion area ($P < 0.0001$) (Table 1). For all cultivars tested, the incubation period ranged from 3.1 days for *B. sempervirens* 'American' to 6.5 for *B. harlandii* (Fig. 4). The latent period was shortest for the Asiatic cultivar *B. microphylla* var. *japonica* 'Morris Midget' at 10.8 days and longest for *B. sinica* var. *insularis* 'Nana' and *B. harlandii* at 17.4 days and 17.8 days, respectively (Fig 5B). Similarly to experiment one, *B. microphylla* var. *japonica* 'Green Beauty' had the most pathogen conidia production (22,876 conidia per leaf and 239 conidia per mm^2 of leaf area) (Fig. 5 D&F). However, spore production in the second experiment was 10 fold higher than in the first. The cultivars *B. sempervirens* 'American'

and *B. microphylla* ‘John Baldwin’ had 8,941 and 5,041 conidia per leaf and 124 and 96 conidia per mm^2 of leaf area, respectively. The cultivars *B. sinica* var. *insularis* ‘Nana’ and *B. microphylla* var. *japonica* ‘Morris Midget’ had the least spore production per leaf with 127 and 393 conidia, respectively. The cultivars *B. microphylla* var. *japonica* ‘Morris Midget’ and *B. sempervirens* ‘Dee Runk’ had the least spore production per mm^2 of leaf area with 8 and 10 conidia, respectively (Fig. 5 D&F). Lesion area was greatest on *B. microphylla* var. *japonica* ‘Morris Midget’ (68%) and least on *B. microphylla* var. *japonica* ‘Green Beauty’ (26%) and *B. harlandii* (22%) (Fig. 5H).

The effect of cultivar had a significant effect on disease severity as indicated by an analysis of variance ($P = 0.0002$) (Table 1). Similar to experiment one, the European cultivars all had a disease severity rating of 90% or greater. The Asiatic cultivars *B. microphylla* var. *japonica* ‘Morris Midget’ and *B. microphylla* ‘John Baldwin’ had ratings of 53% and 40%, respectively, while *B. harlandii*, and *B. microphylla* var. *japonica* ‘Green Beauty’ both had ratings below 20% and *B. sinica* var. *insularis* ‘Nana’ had the lowest rating of 9.6% (Fig. 6).

The frequency of occurrence of incidence of leaf abscission, incidence of false positive sporulation detection on leaves (Table 2), and incidence of a possible hypersensitive reaction was evaluated for all cultivars tested. In the second experiment leaf abscission occurred at a high rate (at least 40%) for all cultivars tested (Table 2). In the second experiment a hypersensitive reaction occurred on 18% of *B. sinica* var. *insularis* ‘Nana’ tagged leaves, on 13.89% of *B. microphylla* var. *japonica* ‘Green Beauty’ tagged leaves, on 10.42% of *B. harlandii* tagged leaves, and on 1.25% of *B. microphylla* ‘John Baldwin’

tagged leaves. The tagged leaves collected at the end of experiment one were incubated in a humid chamber and monitored for conidia production for 20 days but there was only eight days of incubation for the tagged leaves collected from experiment two. At the end of experiment one, 33 of the 50 remaining leaves in the experiment had pathogen spore production while only 15 of the remaining 50 in the second experiment did. This indicates that most likely the report of hypersensitive reactions for the second experiment is an overestimate.

In experiment two, symptomatic leaves with conidia production were collected at 15 and 23 dpi to compare the effect of cultivar on conidia production at a specific time point. There was only one subsample leaf that was collected from *B. harlandii* at 15 dpi and zero collected at 23 dpi; this was because there were not any symptomatic leaves with evident sporulation to collect. Thus, *B. harlandii* was not included in the analysis. At 15 dpi, the Asiatic cultivar, *B. microphylla* var. *japonica* 'Morris Midget' had the most pathogen conidia production per leaf and per mm^2 (Fig. 7 C&D). At 23 dpi, the Asiatic cultivar, *B. microphylla* 'John Baldwin' had the most pathogen conidia production per leaf and per mm^2 (Fig. 7 E&F).

DISCUSSION

Investigation of the components that may contribute to partial resistance in *Buxus* spp. and cultivars to the boxwood blight pathogen revealed some expected and some surprising results. Disease severity rankings in the first and second experiment revealed similar levels of susceptibility and resistance for the cultivars in this study. In this study, the Asiatic cultivar *B. microphylla* var. *japonica* 'Morris Midget' was not as resistant as the other

Asiatic cultivars; this is similar to the results found in previous host resistance studies (Ganci, *unpublished,* M. S. Thesis, Chapter 2). The European cultivar *B. sempervirens* 'Dee Runk' was as susceptible as the European cultivars, *B. sempervirens* 'American' and 'Vardar Valley' in this study; this is in contrast with the results obtained from an outdoor container pad trial where *B. sempervirens* 'Dee Runk' expressed the most resistance compared to other European cultivars (Ganci, *unpublished,* M. S. Thesis, Chapter 2). The goal of the experiments was to identify if a resistant reaction in the Asiatic cultivars could be attributed to one or more components of partial resistance.

In the first experiment, the cultivars were exposed to high relative humidity (75 to 99%) for the duration of the experiment. Under these conditions, the effect of cultivar did not have a significant effect on incubation period ($P = 0.27$). The higher humidity in the first experiment may have masked the variation of incubation period for the cultivars, which would have been expected given the range of susceptibility of the cultivars. However, in the second experiment the cultivars were removed from the high humidity of the inoculation plastic box after lesion development (3 dpi) and into ambient humidity levels of 40 to 60% before being moved back into the plastic boxes (8 dpi). In this experiment, the effect of cultivar did have a significant effect ($P = <0.0001$) on incubation period. In the second experiment, the European cultivars had the shortest incubation periods: 3.1 days (*B. sempervirens* 'American'), 3.2 days (*B. sempervirens* 'Dee Runk') and 3.4 days (*B. sempervirens* 'Vardar Valley'). The cultivar *B. microphylla* var. *japonica* 'Morris Midget' had an incubation period of 3.7 days however, the incubation period for *B. microphylla* 'John Baldwin' was only 3.8 days. It would seem that short incubation period does contribute to

susceptibility in the European cultivars but the comparable incubation periods for *B. microphylla* var. *japonica* 'Morris Midget' and *B. microphylla* 'John Baldwin' does not explain the differences in susceptibility in terms of disease severity for these two cultivars.

It was expected that the European cultivars, especially *B. sempervirens* 'American', would have the shortest latent period. In the first experiment, the three European cultivars experienced the shortest latent period, however, only *B. sempervirens* 'American' had a latent period that was significantly shorter than all of the Asiatic cultivars. The Asiatic cultivars *B. sinica* var. *insularis* 'Nana', *B. harlandii,* and *B. microphylla* var. *japonica* 'Green Beauty' had the longest latent periods in experiment one and they were statistically different from the European cultivars.

Some tagged leaves (subsamples) became abscised or had false positive sporulation detection. These events most likely altered our ability to make unbiased assessments of cultivar effect on latent period. In the first experiment tagged leaves were most commonly abscised from the Asiatic cultivars. This was expected as mentioned earlier but not to the extent that was observed and recorded in the first experiment. Defoliation of infected and symptomatic leaves is most likely a defense response by the Asiatic cultivars. In the first experiment, the majority of the tagged leaves remained on the European cultivars and this contributed to their susceptibility because symptomatic leaves remained on the plants and allowed further infection on adjacent leaves. However, in the second experiment leaf abscission occurred at a high rate for all cultivars and this event most likely masked the true effect of cultivar on latent period due to lost data points. Even so, the effect of cultivar did have a significant effect ($P < 0.0001$) on latent period and the most susceptible Asiatic

cultivar *B. microphylla* var. *japonica* ‘Morris Midget’ had the shortest latent period. In the second experiment, the cultivars were exposed to a change in humidity twice, as described earlier. This may have promoted leaf drop on all cultivars. Even so, the leaf abscission experienced by the Asiatic cultivars *B. microphylla* var. *japonica* ‘Green Beauty’, *B. sinica* var. *insularis* ‘Nana’, and *B. harlandii* may still indicate a resistance response. These cultivars had the lowest disease severity ratings indicating that the resistant cultivars drop their relatively few infected and symptomatic leaves, while the European cultivars incur more infected and symptomatic leaves which eventually become necrotic and drop as disease progresses.

Unexpectedly, in the first and second experiment, the highest rates of pathogen conidia production occurred on the cultivar *B. microphylla* var. *japonica* ‘Green Beauty’. However, this was not statistically significantly different from conidia production on the majority of the other cultivars. This is not surprising given the large amount of within-group variance present and the unbalanced nature of the data set. Henricot et al. (4) also found low spore production was not consistently associated with reduced disease severity in *B. riparia*. Given the relatively high rates of false positive sporulation detected in the first and second experiment our methodology may not represent an accurate reflection of conidia production for all cultivars examined. Additionally, many of the leaves collected and observed 24 h after the initial macroscopic detection of conidia actually had fungal growth with an unknown fungal structure. Perhaps the relative humidity or time of collection after sporulation detection should be altered in future experiments to provide a more rigorous assessment and comparison of conidia production on different cultivars.

The occurrence of a hypersensitive reaction is considered an indicator of a resistance response and the frequency of a hypersensitive reaction was compared between cultivars. The fact that these leaves remained on the plants despite the frequency of leaf abscission in the experiments provides further evidence that a hypersensitive reaction occurred on some cultivars. The Asiatic cultivars *B. sinica* var. *insularis* 'Nana', *B. microphylla* var. *japonica* 'Green Beauty', *B. harlandii* and *B. microphylla* 'John Baldwin' are more likely to have a hypersensitive reaction resistance response than *B. microphylla* var. *japonica* 'Morris Midget' or the European cultivars.

At the end of experiment one (20 dpi) symptomatic leaves with conidia production were collected to compare the effect of cultivar on conidia production at a specific time point. The Asiatic cultivars *B. microphylla* 'John Baldwin' and *B. microphylla* var. *japonica* 'Morris Midget' had the greatest number of conidia per leaf but the difference was not statistically different than other cultivars. Additionally, *B. microphylla* var. *japonica* 'Morris Midget' had the greatest number of conidia per mm^2 but it was only statistically significantly greater than *B. harlandii*. In experiment two, leaves were collected at 15 and 23 dpi. At 15 dpi, even though it appeared that *B. microphylla* var. *japonica* 'Morris Midget' had the highest pathogen conidia production, based on a mean comparison, there was not a significant difference between cultivars. Given that there was great variance in the data set it is not surprising that cultivars could not be statistically significantly separated. At 23 dpi, both *B. microphylla* 'John Baldwin' and *B. microphylla* var. *japonica* 'Morris Midget' had some of the highest rates of pathogen conidia production per leaf and per mm^2. Additionally, the European cultivar *B. sempervirens* 'American' had a significantly higher rate of conidia

production than *B. sempervirens* ‘Vardar Valley’ and *B. sinica* var. *insularis* ‘Nana’ at 23 dpi. Interestingly, Gehesquière (3) also identified that *B. microphylla* var. *japonica* ‘Morris Midget’ was the only cultivar out of 32 cultivars evaluated where *C. pseudonaviculata* had a statistically higher rate of conidia production.

The relative rate of pathogen spore production on leaves was much higher when leaves were collected at 15, 20, and 23 dpi compared to 24 h after conidia were detected macroscopically. The results in this study indicate that in order to capture the true effect of cultivar on conidia production the experiment methodology must account for optimum humidity conditions, leaf collection at an appropriate time and an appropriate amount of subsamples in order to decrease variance from the mean in the response variables examined. Other researchers should be aware of these suggestions for experiments conducted in the future.

The goal of this study was to analyze the effect of cultivar on boxwood blight disease response variables. Despite the issues incurred with this study in collecting and analyzing data, it is apparent that some of the response variables analyzed were indicators of resistance. For example, resistance in the cultivars *B. sinica* var. *insularis* ‘Nana’, *B. harlandii*, and *B. microphylla* var. *japonica* ‘Green Beauty’ can be attributed to their minimal disease severity, longer incubation period, and longer latent period. The Asiatic cultivar which expressed susceptibility in the field, *B. microphylla* var. *japonica* ‘Morris Midget’ had higher disease severity, shorter incubation and latent period, and moderately higher conidia production than other cultivars in this study. The cultivar *B. sempervirens* ‘Dee Runk’ was the least susceptible European cultivar in container pad susceptibility trials (Ganci, *unpublished,* M. S.

Thesis, Chapter 2). This study revealed that in humid conditions *B. sempervirens* 'Dee Runk' had a disease severity rating as high as the other European cultivars. However, overall, this study did not reveal what accounted for the apparent partial resistance of *B. sempervirens* 'Dee Runk' observed in the container pad evaluations. Furthermore, the susceptibility of *B. sempervirens* 'American' can be attributed to shorter incubation and latent periods, high percent lesion area, and high disease severity. In this experiment the pathogen did not produce significantly more conidia on this cultivar compared to other cultivars. Other studies conducted in Europe have identified that conidia production was higher on *B. sempervirens* (similar to the plant referred to as 'American' in the U.S.) and *B. sempervirens* 'Suffruticosa' compared to other cultivars (3, 4).

The high spore production on some of the Asiatic cultivars such as 'Green Beauty' and 'John Baldwin' was unexpected. The experiment was conducted in a very high humidity environment; some leaves had mycelial growth, instead of, or in addition to spores. In general, this was more common on the *B. sempervirens* cultivars and thus the frequency of false positive sporulation detection on the *B. sempervirens* cultivars could have inhibited a true measurement of conidia production on the cultivars tested. Even if there is high spore production on some leaves of resistant cultivars there are much fewer number of diseased leaves overall. This study has provided some evidence for the components which contribute to boxwood blight resistance in *Buxus* cultivars; however, the experiment should be repeated to verify the results and to further our understanding of which components of quantitative resistance contribute to field resistance.

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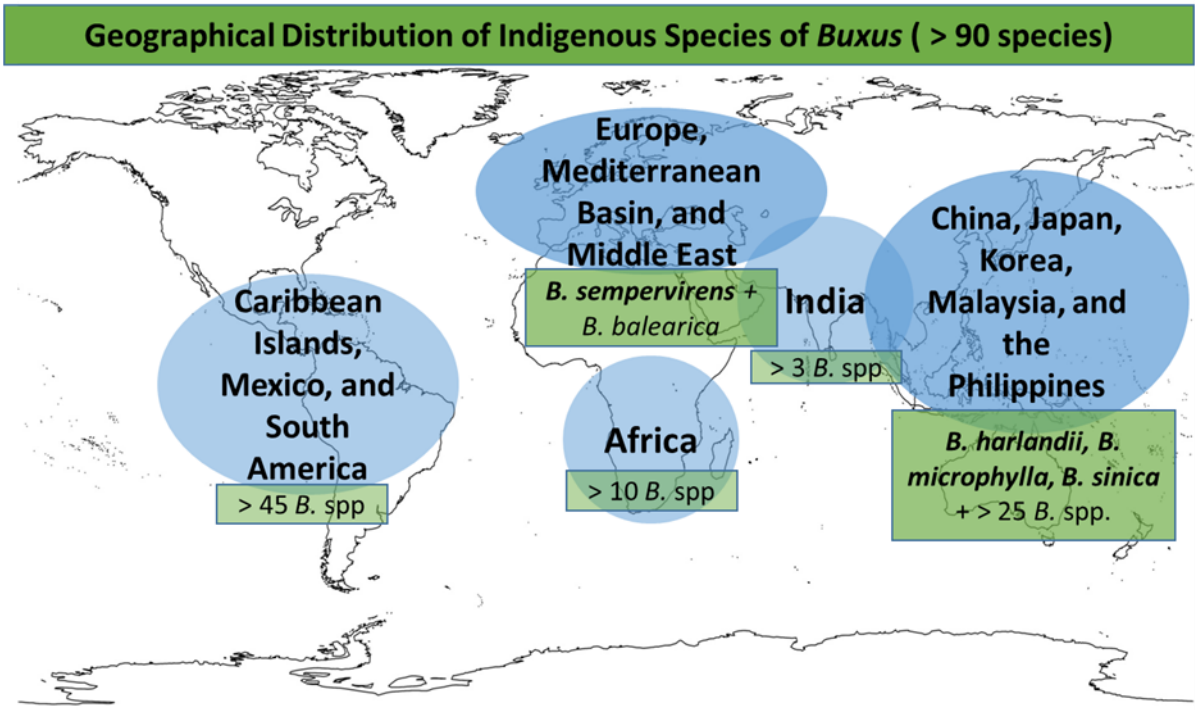

Fig. 1. Indigenous species of *Buxus* are distributed across the globe. The species most commonly grown and distributed in the nursery trade are the European and Asiatic types which are highlighted in bold.

Fungal structures formed by *Calonectria pseudonaviculata*

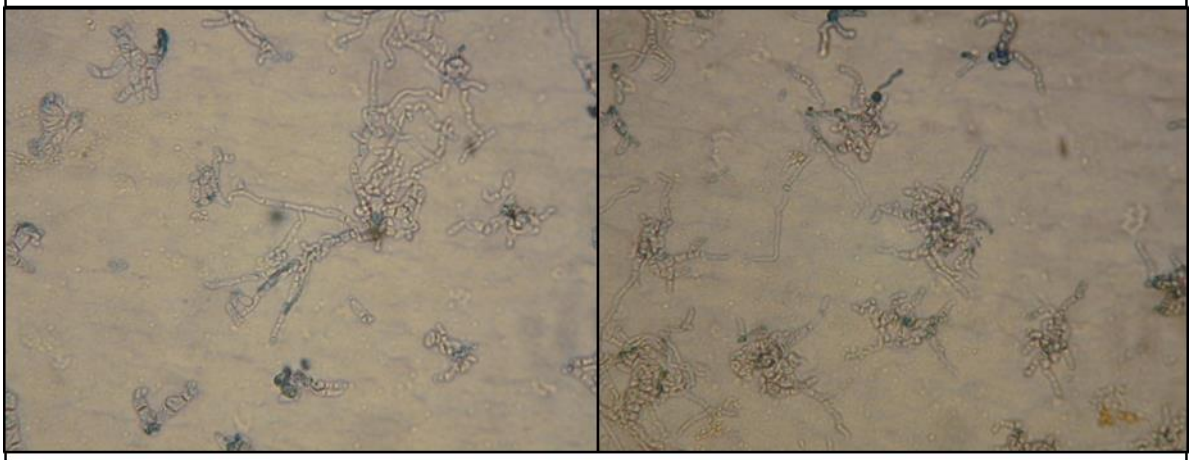

Fig. 2. Structures formed by the fungal pathogen *Calonectria pseudonaviculata* approximately 24 hours after fungal tissue was identified on the abaxial leaf surface of *Buxus* cultivars. Photographs taken at 200X magnification under a compound microscope 10 days after inoculation.

Conidia production by *Calonectria pseudonaviculata* on abaxial leaf surface

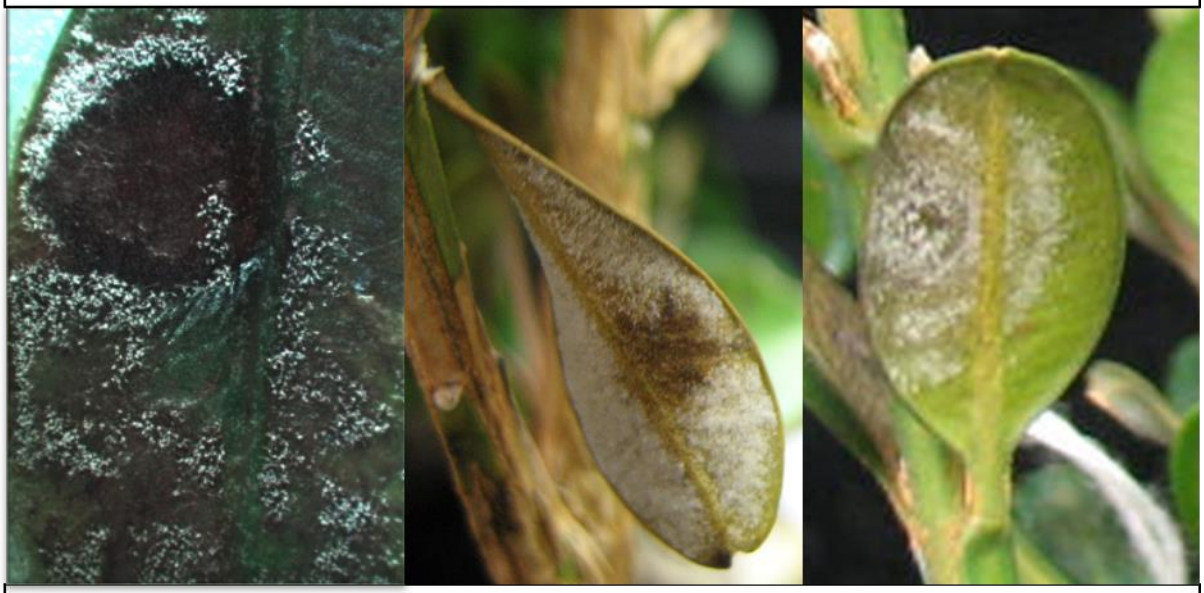

Fig. 3. Conidia production by *Calonectria pseudonaviculata* is not limited to lesion area on leaves of *Buxus* cultivars.

Table 1. ANOVA for the effect of cultivar on the variables analyzed in experiment 1 and 2.

	Experiment 1				Experiment 2			
Variable analyzed	**Num df**	**Den df**	***F***	***P > F***	**Num df**	**Den df**	***F***	***P > F***
Incubation Period (days)	7	21	1.37	0.27	7	20[d]	8.56	<0.0001*
Latent Period (days)	7	21	32.35	<0.0001*	7	20[d]	9.66	<0.0001*
Conidia production per leaf	7	21	5.29	0.0013*	7	19[e]	4.87	0.0028*
Conidia production per mm^2	7	21	2.9	0.0275*	7	19[e]	4.75	0.0032*
Lesion Area	7	21	0.72	0.6598	7	20[d]	13.43	<0.0001*
Disease Severity[abc]	.	.	21.54	<0.0001*	.	.	7.03	0.0002*

*=*P*<0.05.

[a] In experiment 1, SS was 5.15 and MS was 0.74. In experiment 2 SS was 5.38 and MS was 0.77

[b] df was 7

[c] The effect of cultivar on disease severity was tested at 20 dpi for experiment 1 and 23 dpi for experiment 2

[d] Den df was 20 because the cultivar *B. harlandii* was missing from 1 of the blocks

[e] Den df was further reduced to 19 because there was not any data collected for the response variable for the cultivar *B. sempervirens* 'Vardar Valley' from 1 of the blocks

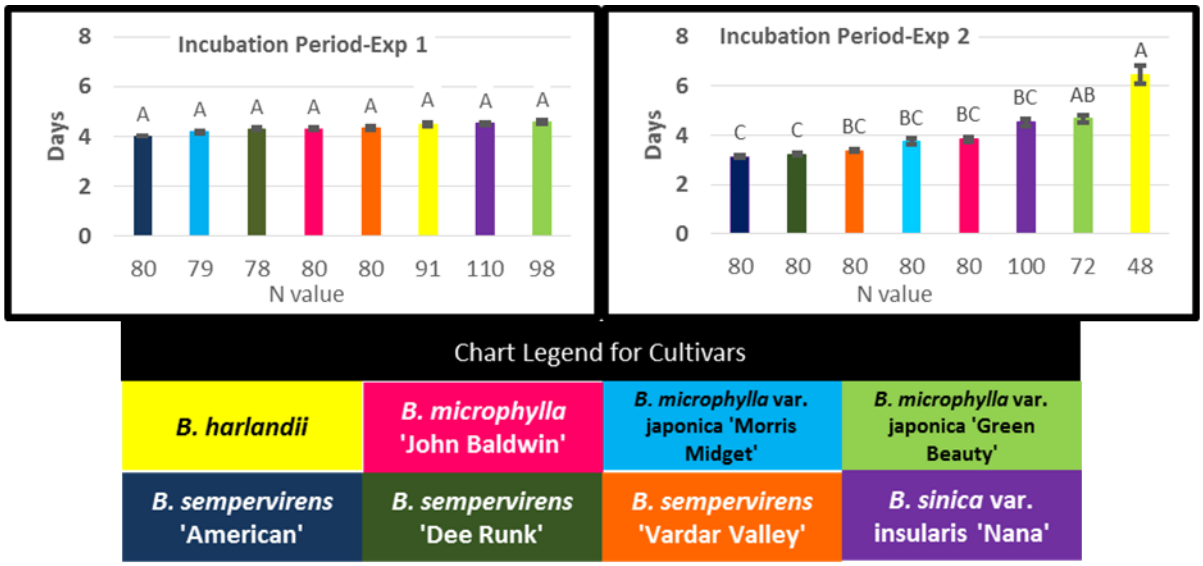

Fig. 4. Incubation period for *Buxus* cultivars in experiment 1 (left) and 2 (right). The X axis displays N value. N = number of leaf subsamples. Error bars indicate standard error of the mean. The Tukey-Kramer procedure was used to group effects of cultivars

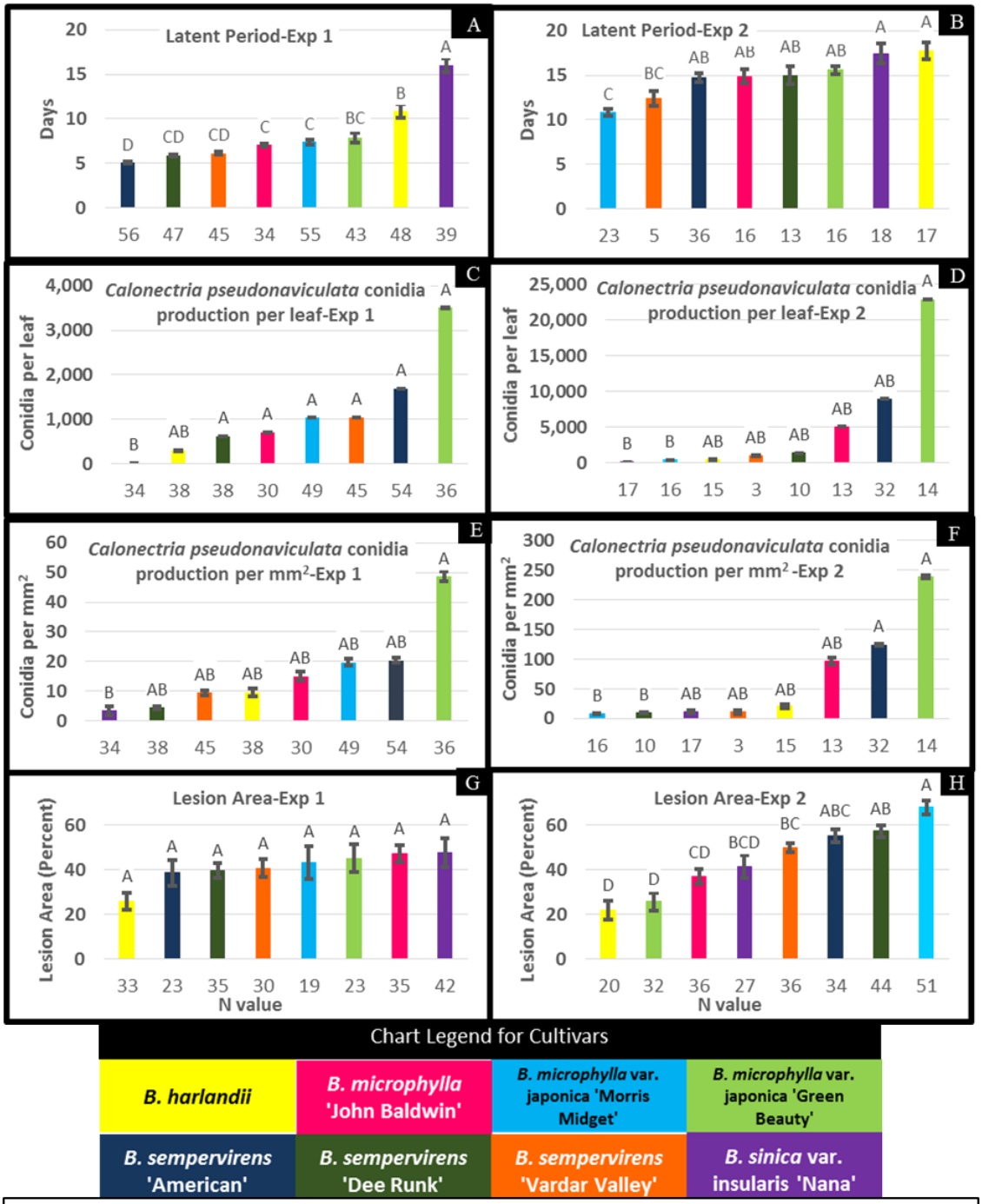

Fig. 5. Boxwood blight disease response variables for the effect of cultivar in experiments 1 and 2. The X axis displays N values. N = number of leaf subsamples. Error bars indicate standard error of the mean. The Tukey-Kramer procedure was used to group effects of cultivars. **A** and **B**, latent period; **C, D, E** and **F,** pathogen conidia production; and **G** and **H**, lesion area.

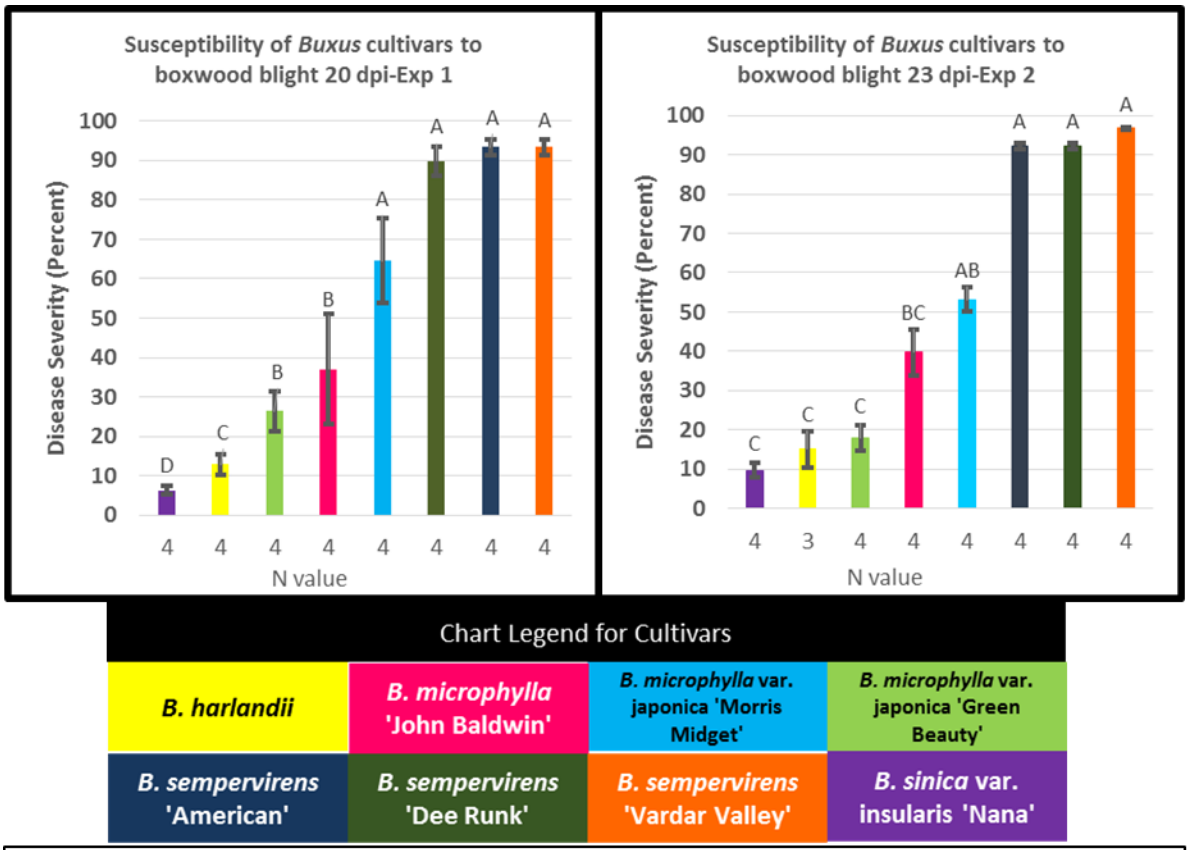

Fig. 6. Susceptibility of *Buxus* cultivars to *Calonectria pseudonaviculata* in experiments 1 (left) and 2 (right). Disease severity evaluated as percent leaf area diseased and defoliated. The X axis displays N value. N = number of observations. Error bars indicate standard error of the mean. A Waller Duncan mean separation procedure was used.

Table 2. The frequency of leaf abscission and false positive sporulation detection.

		Cultivar							
		B. sempervirens 'American'	*B. sempervirens* 'Dee Runk'	*B. sempervirens 'Vardar Valley'*	*B. harlandii*	*B. microphylla* 'John Baldwin'	*B. microphylla* var. *japonica* 'Green Beauty'	*B. microphylla* var. *japonica* 'Morris Midget'	*B. sinica* var. *insularis* 'Nana'
Initial count of subsamples per cultivar[a]	**Experiment 1**	80	80	80	92	80	98	80	111
	Experiment 2	80	80	80	51[b]	80	74	80	101
Percent of abscised subsamples	**Experiment 1**	2.5	5.13	6.25	37.36	18.75	55.1	17.72	46.36
	Experiment 2	47.5	67.5	81.25	50	63.75	63.89	41.25	62
Percent of false positive sporulation detection on subsamples	**Experiment 1**	27.5	34.62	38.75	13.19	41.25	15.31	16.46	11.82
	Experiment 2	12.5	18.75	16.25	8.33	17.5	2.78	35	5

[a] After symptom detection most commonly 20 leaves were tagged per cultivar for each of 4 blocks; these served as subsamples.

[b] In the 2nd experiment 3 blocks included *B. harlandii* instead of 4

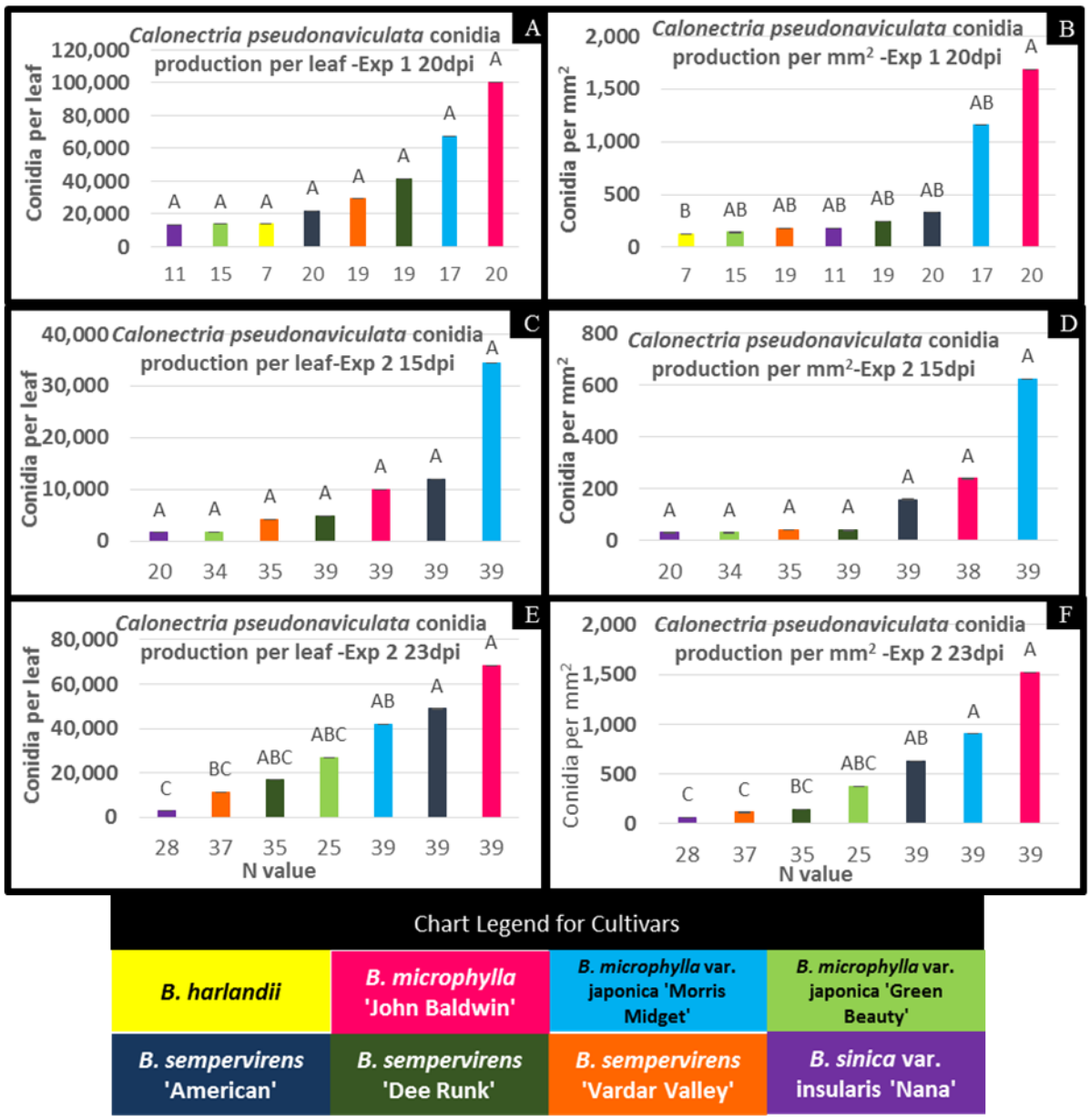

Fig. 7. *Calonectria pseudonaviculata* conidia production on cultivars. The X axis displays N values. N = number of leaf subsamples. Error bars indicate standard error of the mean. The Tukey-Kramer procedure was used to group effects of cultivars. **A** and **B**, pathogen conidia production 20 days post inoculation (dpi) in experiment 1; **C** and **D,** 15 dpi in experiment 2; and **E** and **F**, 23 dpi in experiment 2.

APPENDICES

Appendix 1. The Trojan horse experiment: understanding reservoirs of boxwood blight inoculum

Boxwood blight is a foliar disease of boxwood caused by the fungal pathogen *Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum*, *Cylindrocladium buxicola*). Prior research has shown that the popular boxwood cultivars *Buxus sempervirens* 'American' and 'Suffruticosa' (English) are highly susceptible to the pathogen. In the summer of 2012, we performed a study in order to identify boxwood cultivars with resistance to boxwood blight. Several commercial cultivars were identified as partially resistant (or tolerant) to the disease, due to minimal symptom and disease development. When exposed to *C. pseudonaviculata*, these cultivars remained healthy and expressed limited leaf lesions, stem cankers, and dieback.

The Trojan Horse study was conducted in order to determine the ability of these partially-resistant boxwood cultivars to serve as sources of inoculum for the pathogen, thus being capable of initiating infection in blocks of susceptible boxwood cultivars. We decided to call this the 'Trojan Horse Experiment' because we wanted to identify if boxwood blight could be introduced to a landscape or production area disguised like the Greeks in the infamous Trojan horse story. We hypothesized that apparently healthy, partially resistant cultivars with *C. pseudonaviculata* infection could serve as inoculum reservoirs which would contribute to infections and boxwood blight outbreaks in more susceptible cultivars.

The trial was conducted in a self-contained, shaded lathe house with overhead irrigation in Raleigh, NC during April and May 2013. The partially resistant test plants included *B. harlandii*, *B. microphylla* 'Golden Dream', *B. microphylla* 'John Baldwin', *B. microphylla* var. japonica 'Green Beauty', *B. microphylla* 'Winter Gem', *B. sinica* var

insularis ‘Nana’, and *Buxus* ‘Green Gem’. These test plants in the study were exposed to the fungus in two different ways: 1) prior exposure to C. *pseudonaviculata* by both direct spray (1,000 spores per ml) and indirect (test plants were placed next to heavily infected ‘Suffruticosa’ plants) inoculation during the summer of 2012; and 2) recent exposure to *C. buxicola* on 11 April by direct spray-inoculation of 10,000 spores per ml until run-off. The study included ‘Suffruticosa’ positive and negative control plants which were direct spray-inoculated until run-off with either 10,000 spores per ml, 1,000 spores per ml, or water. Healthy, uninfected ‘Suffruticosa’ plants were placed on both sides of each partially-resistant test cultivar to serve as disease indicator plants. Disease evaluations were performed on 2 May, 10 May, and 28 May using a modified Horsfall-Barratt rating scale. Partially-resistant test plants which were recently inoculated with the pathogen in April 2013 were generally more effective at transmitting the pathogen to healthy ‘Suffruticosa’ plants than the test plants that were exposed to the pathogen during the summer of 2012.

Between 18 May and 23 May, the Raleigh area received over two inches of rain with an average daily temperature of 72°F, therefore the conditions were extremely conducive for boxwood blight over this time period. Due to the climate, high inoculum production on infected plants in the experiment, and inoculum transmission via wind-driven rain, some of the non-inoculated ‘Suffruticosa’ negative control plants (spaced 3 ft. from infected reservoir plants) were also infected with *C. pseudonaviculata*. Inter-plot interference from the inoculated ‘Suffruticosa’ positive control plants may have contributed to some disease development on the negative controls and the other disease indicator plants in the experiment. However, our data suggests that the partially-resistant boxwood cultivars we

tested with minimal boxwood blight symptom development may be capable of transmitting the pathogen to healthy, susceptible cultivars (Fig. 1&2).

Fig 1. Experiment design. The center plant, partially resistant cultivar *Buxus harlandii*, was direct inoculated with 10,000 *Calonectria pseudonaviculata* spores per ml until runoff on 11 April. Then healthy, uninfected *B. sempervirens* 'Suffruticosa' were placed next to the test plant. This photograph was taken 28 May.

Fig. 2. Up close photograph of the partially resistant cultivar, *Buxus harlandii*, from Fig. 1. The dark circular lesions with yellow halos are boxwood blight symptoms. This photograph was taken 28 May. Although this cultivar produces minimal box blight symptoms after infection, the fungus can still sporulate on the plant.

Appendix 2. Components of partial resistance investigation in outdoor and detached branch trials.

Components of partial resistance to the boxwood blight pathogen, *C. pseudonaviculata* was investigated for *Buxus* cultivars on an outdoor container pad and on detached branches in humid chambers. Disease response variables included incubation period, latent period, conidia production (detached branch trial only), lesion area and disease severity (container pad trial only). The cultivars in the trials had expressed a range of resistance in previous cultivar susceptibility trials (Ganci, *unpublished,* M.S. Thesis, Chapter 2). Seven cultivars and species were included in both trials; *B. harlandii*, *B. microphylla* 'John Baldwin', *B. microphylla* var. *japonica* 'Green Beauty', *B. sempervirens* 'Dee Runk', and *B. sinica* var. *insularis* 'Nana' expressed field resistance in previous trials. While the cultivars *B. microphylla* var. *japonica* 'Morris Midget', *B. sempervirens* 'American' were susceptible. Both experiments consisted of a randomized complete block design with three blocks with a single plant replication in each. Plants in experiment were approximately three years old.

Container pad trial

Cultivars were direct inoculated on a shaded outdoor container pad with a 10,000 conidia/ml suspension until run off on 17 Jul 2013. Plants were covered with plastic bags overnight. After removal of plastic bags, plants were over-head irrigated every 2 h from 6:00 am to 10:00 pm for a 10 min duration. Irrigation output was approximately 0.5 cm/10 min. Lesion development began on some cultivars 20 Jul 2013 (3 days post inoculation (dpi)). Symptomatic leaves were tagged with jewelry hang tags and incubation period was recorded (Fig. 1). The leaves served as subsamples. Up to 20 leaves were tagged per plant. In some

instances there were less than 20 symptomatic leaves per plant and this resulted in lower subsample numbers for some cultivars. On 21 Jul 2013 irrigation was changed to twice daily for a 10 min duration. Leaves were observed daily and latent period was recorded when spore production was observed on tagged leaves (Fig. 1). Spore production was not observed on *B. harlandii*, *B. microphylla* 'John Baldwin', *B. microphylla* var. *japonica* 'Green Beauty', or *B. sinica* var. *insularis* 'Nana'. Disease severity was evaluated as percent leaf area diseased on 24 Jul 2013 (7 dpi) (Fig. 2). On 8 Aug 2013 (22 dpi) leaves with lesions were collected from each plant. There were only three symptomatic leaves present on the cultivar *B. harlandii*, for this reason it was excluded from the lesion area analysis. Leaves were photographed and leaf and lesion area were calculated with plant disease quantification image analysis software (Assess v. 2.0, APS Press) (Fig. 2). Disease severity ratings were back transformed to the midpoint of the corresponding disease severity range. In order to improve normality in the data sets, disease severity and lesion area response values were transformed according to the following equation; transformed response = log base 10(response + 1). The transformed response variables were used for a Waller Duncan mean separation procedure in the GLM procedure of SAS (version 9.4; SAS Institute). Graphical presentation of disease severity and lesion area represents means from the non-transformed data set.

Detached branch trial

Detached branches of cultivars were placed into individual plastic boxes and direct inoculated with a 10,000 conidia/ml suspension until run off on 17 Jul 2013. Plastic boxes contained saturated paper towels and served as humid chambers. The lids and paper towels in the humid chamber were spritzed with water daily. Lesion development began on some

cultivars 19 Jul 2013 (2 days post inoculation (dpi)). Symptomatic leaves were tagged with jewelry hang tags and incubation period was recorded (Fig. 3A). The leaves served as subsamples. Up to 10 leaves were tagged per plant. Leaves were observed daily and latent period was recorded when spore production was observed on tagged leaves (Fig. 3B). Approximately, Twenty-four h after latent period was recorded leaves were collected for spore production analysis. Leaves were photographed and leaf area was calculated with plant disease quantification image analysis software (Assess v. 2.0, APS Press). Conidia was counted for each individual leaf subsample with a hemocytometer and conidia production per leaf and per mm2 was calculated (Fig. 3 C&D). In order to improve normality in the data distribution the following equation was used to transform fungal sporulation data; transformed response = natural log(response+1). Reported means for the fungal sporulation response variables, conidia per leaf and conidia per mm^2 of leaf area were derived from back transforming the means according to the following equation; back transformed response = $((e^{\text{transformed mean}}) - 1)$.

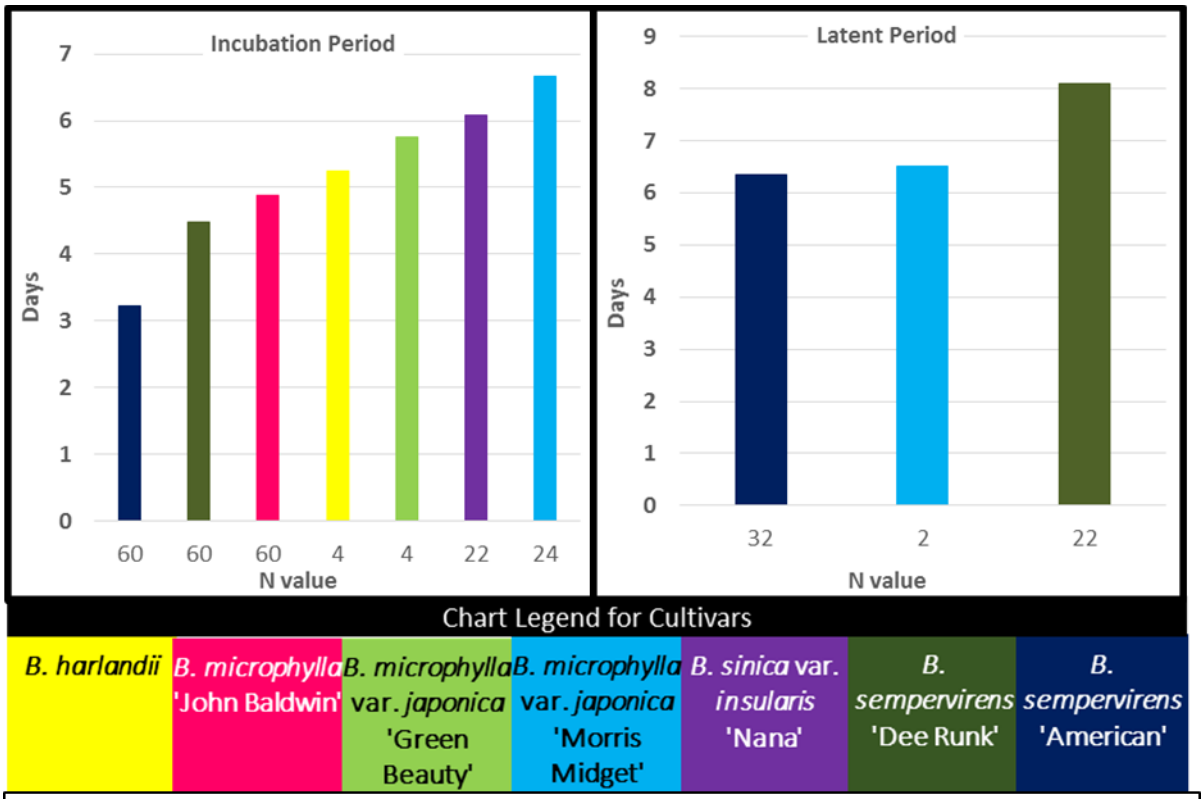

Fig. 1. Incubation period (left) and latent period (right) for *Buxus* cultivars. Experiment conducted on an outdoor shaded container pad with overhead irrigation.

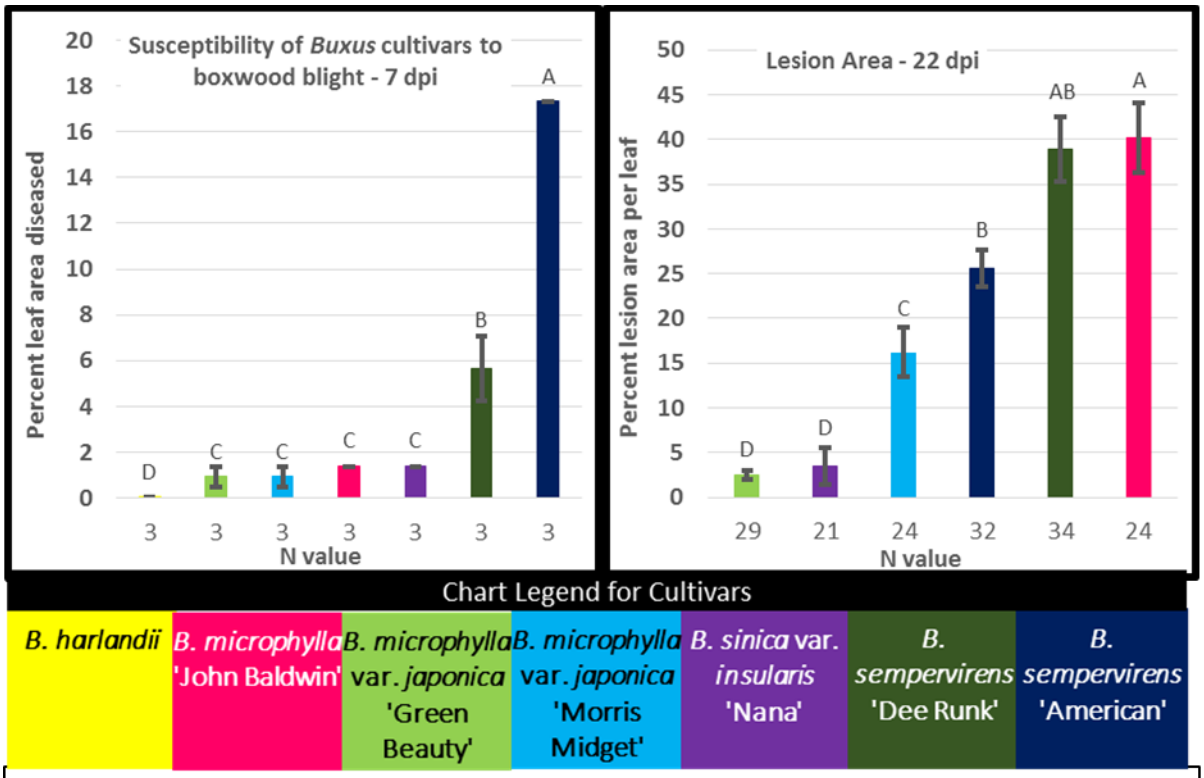

Fig. 2. Susceptibility of *Buxus* cultivars to boxwood blight (left) and lesion area (right). Error bars represent standard of the mean. A Waller Duncan mean separation procedure was used. *B. harlandii* was not included in lesion area analysis. Experiment conducted on an outdoor shaded container pad with overhead irrigation.

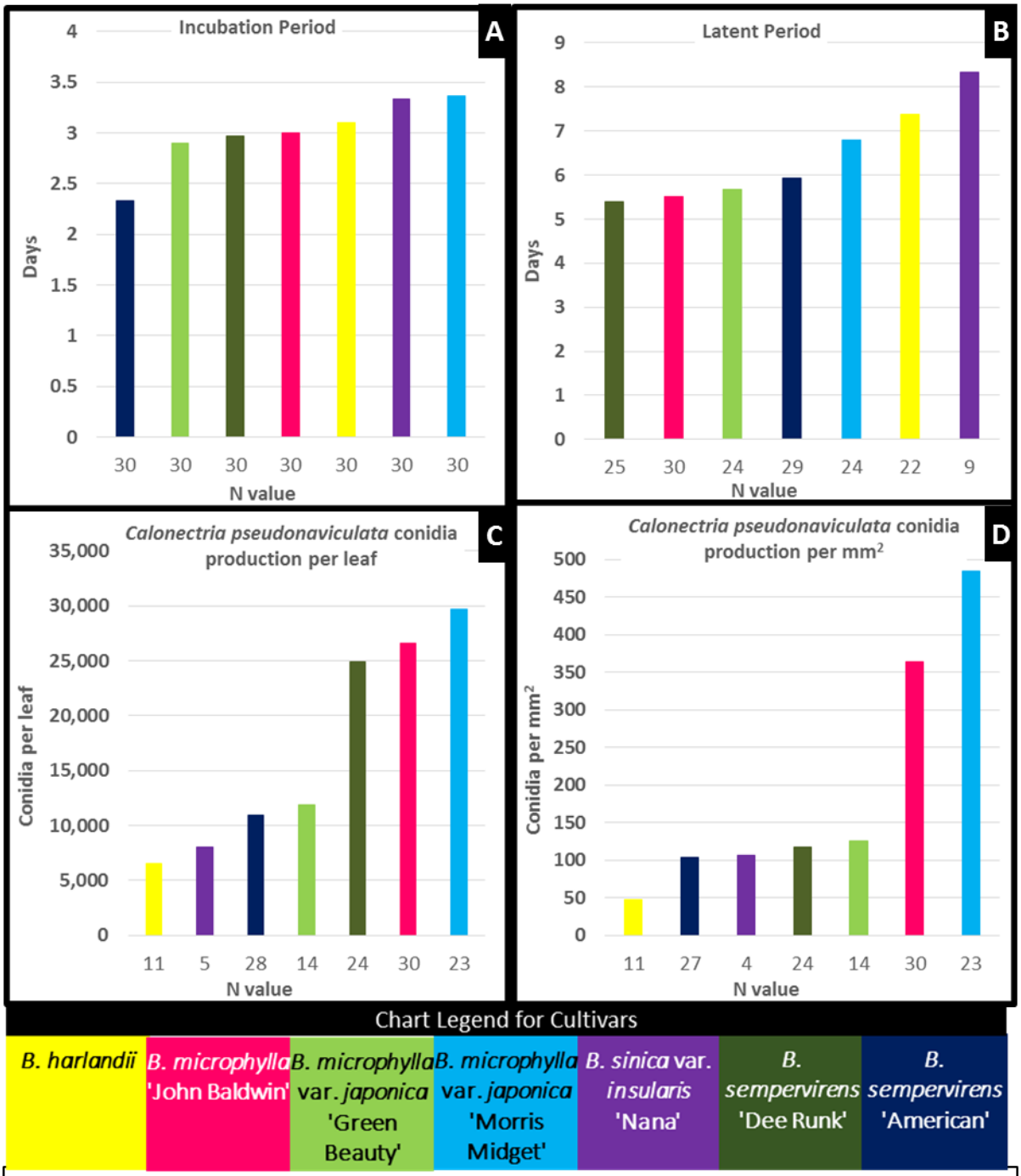

Fig. 3 Boxwood blight disease response variables for the effect of cultivar in a detached branch experiment conducted in humid chambers. **A**, incubation period; **B**, latent period; **C** and **D,** pathogen conidia production.