GENETIC DIVERSITY AND BOXWOOD BLIGHT SUSCEPTIBILITY OF *SARCOCOCCA*

LINDL.

by

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(Under the Direction of Donglin Zhang)

ABSTRACT

Sarcococca Lindl. is a genus of woody shrubs that have valuable landscape characteristics including fragrant winter flowers, evergreen foliage, few pests and diseases, and shade adaptability. Nonetheless, they are underutilized as landscape plants. Additionally, a relatively new disease called boxwood blight, caused by *Calonectria pseudonaviculata* (Crous & al.) L. Lombard & al., is causing severe damage to members of the Buxaceae, including *Sarcococca*. Genetic diversity of *Sarcococca* accessions available in the United States was analyzed using flow cytometry and eight Inter-Simple Sequence Repeat molecular markers. Data from both techniques show that *Sarcococca* taxa are commonly misidentified. The first studies assessing *Sarcococca* susceptibility to boxwood blight using detached leaf and detached stem assays showed that *Sarcococca* are less susceptible to the disease than known susceptible boxwood controls. These studies provide basic genetic and disease resistance information for plant breeders, horticulturists, and plant pathologists interested in *Sarcococca* or boxwood blight management.

INDEX WORDS: *Sarcococca*, sweet box, *Calonectria pseudonaviculata*, boxwood blight, genome size, flow cytometry, inter simple sequence repeat, disease resistance

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CHAPTER 1

LITERATURE REVIEW

The Genus *Sarcococca*

Sarcococca Lindl. is a genus of approximately 20 evergreen woody species in the Buxaceae native primarily to East Asia, though one species, *S. conzattii* (Standl.) I. M. Johnst., has been reported (and disputed) in Central America (eFloras, 2008; Sealy, 1986). They are underutilized as landscape shrubs, and few species are well-represented in American horticulture. *Sarcococca* are noted for their evergreen foliage, shade preference, drought adaptability, and fragrant winter flowers and come in a wide range of sizes, from spreading groundcover no taller than 30 cm to shrubs 1-2.5 m tall (Dirr, 2009). Bloom time is typically in early to late winter, though *S. wallichii* Stapf and *S. saligna* (D. Don) Müller Argoviensis can bloom from approximately October to January (Wynn-Jones, 2005). Winter hardiness appears to be best for *S. confusa* Sealy, *S. hookeriana* Baill, and *S. orientalis* C.Y. Wu (Dirr, 2009). *S. hookeriana/humilis* has six cultivars: 'Ghorepani, 'PMOORE03' Winter Gem™, 'Purple Stem', 'Sarsid1' Fragrant Valley™, 'Sarsid2' Fragrant Mountain™, and 'Western Hills' (Botanic Gardens Conservation International, n.d.; Moore, 2016). *S. ruscifolia* var. *chinensis* has one cultivar called 'Dragon Gate' (Botanic Gardens Conservation International, n.d.).

Taxonomic Relationships

The most comprehensive taxonomic treatments of *Sarcococca* are by Sealy (1986) and eFloras (2008), which described 11 species (plus 9 subspecific taxa) and 9 species (plus 2

subspecific taxa), respectively. There is no current monograph of the genus, though there are an estimated 20 species (eFloras, 2008). The taxonomy of the genus is still confused. Distinguishing species by morphological features is difficult and carpel number is often useful for making a distinction (Sealy, 1986).

The taxonomic treatments of *S. hookeriana* and *S. ruscifolia* are worth noting. Sealy (1986) recognized two varieties of *S. hookeriana*: var. *digyna*, and var. *humilis*. eFloras (2008) only recognized var. *digyna*, absorbing *S. hookeriana* var. *humilis* into var. *digyna*. eFloras (2008) also did not recognize *S. ruscifolia* var. *chinensis*, while Sealy (1986) did. The lumping of all *S. ruscifolia* into a single species with no lower taxa aligns with Dirr (2009), who was unable to distinguish between var. *ruscifolia* and var. *chinensis*.

The molecular taxonomic relationships of the Buxaceae were investigated by von Balthazar et al. (2000) using nuclear internal transcribed spacers (ITS) and plastid *ndhF* gene sequences. In their study, they found two clades within *Sarcococca* using ITS spacers, one containing *S. hookeriana* var. *humilis*, *S. ruscifolia*, and *S. confertiflora* Sealy, and one containing *S. saligna* and *S. wallichii*. Using *ndhF* sequences, in the tree they present (of 150 equally most-parsimonious trees), all species except *S. wallichii* fall under the same clade. In the most parsimonious tree using combined *ndhF* and ITS sequences, *S. hookeriana* and *S. ruscifolia* grouped together and were sister to *S. confertiflora*. *Sarcococca saligna* and *S. wallichii* are sister to each other on a separate part of the *Sarcococca* clade. These discrepancies left room to further explore species relatedness and to verify the identities of cultivated species and cultivars. Several taxa not included in their study are in cultivation in the United States, including *S. confusa*, *S. hookeriana* var. *digyna*, *S. orientalis*, *S. ruscifolia* var. *chinensis*, and *S. vagans*.

Denaeghel et al. (2017) used amplified fragment length polymorphisms (AFLP), flow cytometry, and basic cytology to investigate genetic relationships among cultivated *Sarcococca* genotypes acquired from breeders and gardens in Belgium, the Netherlands, or Scotland. Caution must be used when interpreting the results presented here because cultivated specimens could be misidentified, but the authors tried to control for this by using many references to verify the identity of the plants used in the study. Using AFLPs from four fluorescent EcoRI and MseI primer combinations with six selective bases, they found similar results to von Balthazar et al. (2000), with *S. ruscifolia* and *S. hookeriana* clustering together and one accession of *S. saligna* clustering with an *S. wallichii* accession. Additionally, they found *S. orientalis* to cluster most closely with *S. ruscifolia* and *S. confusa*. They were also able to separate accessions of two varieties of *S. hookeriana*, though they still clustered together. *S. saligna* clustered in two different places on the dendrogram, with some accessions being more closely related to *S. wallichii* and some being more closely related to *S. hookeriana*.

Denaeghel et al. (2017) published the first major work regarding genome size and chromosome numbers in *Sarcococca*. Basic chromosome number has been previously reported in *S. humilis*, *S. saligna*, *S. pruniformis*, *S. ruscifolia*. All have a base chromosome number of 14 and all are diploid except *S. ruscifolia* and *S. confusa*, which are tetraploid (Darlington and Wylie, 1955; Saggoo, 2011). Denaeghel et al. (2017) supported their conclusion, finding diploid and tetraploid groups of *Sarcococca*. *Sarcococca hookeriana* var. *digyna*, *S. hookeriana* var. *humilis*, *S. saligna*, *S. orientalis*, *S. coriacea*, *S. vagans*, and *S. wallichii* were found also to be diploid (2n = 2x = 28). *S. hookeriana* accessions and two *S. saligna* accessions had 2C nuclear DNA content values from 4.11 to 4.20 pg. All other diploid accessions ranged from 7.25 to 9.63 pg/2C. This included one *S. saligna* accession, which had a 2C nuclear DNA content value of

9.63 ± pg. Denaeghel et al. (2017) noted morphological differences among their *S. saligna* accessions. The *S. saligna* with the larger 2C genome size resembled *S. wallichii,* while the *S. saligna* with the smaller genome size resembled *S. hookeriana.* These differences may indicate a misidentified or new species and warrant further investigation.

Knowing genome size and chromosome number is useful for predicting crossability of related taxa, but little genetic and cytogenetic information is known regarding *Sarcococca* in the United States. The only known interspecific hybrid *Sarcococca* on the market is a cross between *Sarcococca hookeriana* var. *digyna* and *Sarcococca humilis*, two closely related species (*S.* 'PMOORE03') (Moore, 2016). Because *Sarcococca humilis* is often considered a variety of S. *hookeriana* (*S. hookeriana* var. *humilis*), intervarietal hybrid may be the more appropriate term. Seedlings were obtained from crosses between European accessions of *S. hookeriana* var. *digyna* and *S. hookeriana* var. *humilis*, *S. hookeriana* var. *digyna* and *S. ruscifolia* var. *chinensis*, *S. hookeriana* var. *digyna* and *S. saligna*, *S. hookeriana* var. *digyna* and *S. confusa*, *S. ruscifolia* var. *chinensis* and *S. wallichii*, and *S. ruscifolia* var. *chinensis* and *S. confusa*. The parents of progeny from most crosses were verified using AFLPs, and 82% of screened progeny were determined to be true hybrids (containing > 25% of unique male parental markers), while the remainder were hypothesized to contain substitutions or additions of chromosomes from one parent. The only progeny not verified with AFLPs were those between *S. hookeriana* var. *digyna* (2x, 2C = 3.74 pg) and *S. ruscifolia* var. *chinensis* (4x, 2C = 6.81 pg), but these progenies were presumed interspecific hybrids because they were putative triploids (2C = 5.33-5.84 pg) based on flow cytometry (Denaeghel et al., 2017). Seeds were not obtained following many other crosses attempted by Denaeghel et al. (2017). This may indicate that embryo rescue is necessary to obtain interspecific hybrid progeny from some crosses. Because interspecific and intraspecific

crossing is possible, but most hybrids are not represented on the American market, understanding genetic relatedness of American *Sarcococca* accessions is worthwhile.

One taxon, *Sarcococca confusa*, has a fittingly confusing history, as it is only known from cultivation, possibly through an E. H. Wilson collection from China for Arnold Arboretum or Veitch Nurseries (Sealy, 1986). Sealy (1986) postulated that it could be a hybrid between *S. hookeriana* var. *digyna* and *S. ruscifolia* var. *chinensis*, two species Wilson collected in or near Wa-shan in China on similar dates in 1908. Crossability within the genus *Sarcococca* is not well understood, though a reported cross between *S. hookeriana* var. *digyna* and *S. ruscifolia* var. *chinensis* yielded putative triploids (Denaeghel et al., 2017). *Sarcococca confusa* accessions are tetraploids, not triploids which would be expected from a 2x × 4x interploid cross. *S. confusa* may still be an interspecific hybrid, but it came true from seed (Sealy, 1986). This deserves further exploration, as members of *Sarcococca* are reported apomicts through adventitious polyembryony, meaning seedlings may be clones of the female parent (Naumova, 1992). Polyembryony was observed in *Sarcococca confusa* plants in our collection at UGA Horticulture Farm (unpublished).

Genetic Techniques for Under-studied Crops

For crops that do not have the benefit of available genomic information, research progress can still be made using molecular markers and other genetic techniques. Many marker systems, including AFLPs, RAPDs (Random Amplification of Polymorphic DNA), RFLPs (Restriction Fragment Length Polymorphisms), and ISSRs (Inter-Simple Sequence Repeats), do not require genomic information but can be useful for genotyping, DNA fingerprinting,

parentage determination, and genetic diversity assessment (Bello-Bello et al., 2014; Denaeghel et al., 2017; Eldredge et al., 1992; Kafkas & Perl-Treves, 2002; Zhang et al., 2000).

ISSR (Inter-simple sequence repeat) markers are our lab's marker of choice due to their ease of use, relatively low cost, and high reproducibility. They are commonly used for genomic fingerprinting, genetic diversity determination, and phylogenetic analysis (Ferreira et al., 2015; Galván et al., 2003; Shen et al., 2006). ISSR primers flank microsatellites, and these regions can be amplified via polymerase chain reaction (PCR) (Ng & Tan, 2015). These amplified regions can then be run on an agarose gel and scored for genetic analysis (Ng & Tan, 2015).

Under-studied crops typically lack genomic information, but they also often lack basic cytological information including chromosome number and ploidy. Basic cytology and flow cytometry are frequently used to understand chromosomal genetic relationships in ornamental crops (Lattier & Contreras, 2017; Parris et al., 2010; Ranney et al., 2018). Flow cytometry is a technique in which genome size can be estimated using a flow cytometer and standards of known genome size. Following nuclei extraction and staining with a DNA fluorochrome, a sample of interest can be run through a flow cytometer at the same time as a known standard, and the ratio of fluorescent peaks can be used to determine a relative genome size in picograms.

Boxwood Blight Caused by *Calonectria pseudonaviculata*

Sarcococca is reputed as a genus of plants in the Buxaceae resistant to insects and disease. However, boxwood blight is a new, fast-spreading fungal disease in the United States causing rapid defoliation and potentially death of members of the Buxaceae. It was first reported in the United States in 2011 after being known in the United Kingdom since the 1990s (Crous, Groenwald, & Hill, 2002; Henricot & Culham, 2002). In the United States, the pathogen

(*Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculatum = C. buxicola*)) was

first discovered in North Carolina and soon after found in Connecticut, both times on *Buxus*

sempervirens (Ivors et al., 2012). In 2013 boxwood blight was found in Georgia, and as of

December 2015, was known to exist in at least 25 states (LaMondia & Shishkoff, 2017;

Williams-Woodward, 2015). The disease has also been reported in much of Europe, the Republic

of Georgia, Iran, and Turkey (Leblanc et al., 2018). A reported second species causing similar

symptoms, *Calonectria henricotiae*, has only been found in Europe (Gehesquière et al., 2016).

Research assessing the host range of the *C. pseudonaviculata* has focused on the genus *Buxus*,

starting with Henricot et al. (2008), who found nine *Buxus* species and an unidentified

Sarcococca capable of serving as hosts following stem dipping inoculations. They also found a

wide range of susceptibility, with *Buxus balearica* and *Sarcococca* sp. exhibiting the lowest

sporulation following inoculation. They speculated this low sporulation was the reason boxwood

blight had not been found on either species in the field. However, *Sarcococca hookeriana* was

reported to have been infected by *C. pseudonaviculata* in a home landscape in Maryland in 2014

and was again observed infected by the blight in a home landscape in Virginia in 2015,

indicating that field infection is possible (Kong et al., 2017; Malapi-Wight et al., 2016). To the

best of my knowledge, no other *Sarcococca* species have been reported to be infected with

boxwood blight in the field. Beyond the single species tested by Henricot et al. (2008), no

susceptibility testing has been done for *Sarcococca*, in vitro or ex vitro. The lack of infection in

other species is probably a result of less frequent landscape use. In addition to *Buxus* and

Sarcococca, *C. pseudovaniculata* has been reported to cause stem blight and leaf spot in other

members of the Buxaceae, including three *Pachysandra* species (LaMondia & Li, 2013;

LaMondia, 2017; LaMondia et al., 2012). Susceptibility screening of *Pachysandra axillaris* (one

cultivar), *P. procumbens* (one commercial selection), and *P. terminalis* (five cultivars) using whole plants and detached leaves had shown that *Pachysandra* selections could harbor the disease to varying degrees, though *P. procumbens*, a U.S. native species, appeared to be the most susceptible (LaMondia, 2017).

Initial symptoms of infection by the boxwood blight pathogen on *Buxus* (*Calonectria pseudonaviculata* (syn. *Cylindrocladium pseudonaviculata = C. buxicola*)) include light to dark brown spots, often with dark borders. These spots can enlarge and coalesce, turning the whole leaf a tan color before dropping and leaving bare stems (IVORS et al., 2012; WILLIAMS-WOODWARD, 2015). This defoliation is rapid and a useful identification characteristic for boxwood blight. Other diseases do not cause defoliation or act as quickly as boxwood blight (Williams-Woodward, 2015). Black streaks and cankers can also form on stems.

Researchers at North Carolina State University, the United States National Arboretum, and elsewhere have done extensive screening of *Buxus* taxa for susceptibility to the boxwood blight pathogen. Using leaf drop and plant dieback following spray or direct inoculation of the blight pathogen to quantify susceptibility, Ganci et al. (2013) found that susceptibility to the blight varies among 32 cultivated *Buxus* taxa. They sprayed *Buxus sempervirens* 'Suffruticosa' plants with a *Calonectria pseudonaviculata* conidia solution until runoff and then placed them near cultivars of *Buxus* in a nursery environment. They then allowed infection to occur via splash dispersal from overhead irrigation. In one experiment, they found susceptibility in *Buxus sempervirens* cultivars alone varies from high to low (~80% to ~5% diseased leaves) (Ganci et al., 2013).

At the United States National Arboretum, researchers have developed protocols for testing blight susceptibility in a laboratory environment using unrooted cuttings of *Buxus* taxa

(Guo et al., 2015; Shishkoff et al., 2015). In these studies, unrooted cuttings or individual leaves were sprayed with or dipped in a conidia solution. Then various parameters (infected leaf area, percent leaf drop, spots per leaf, lesions per stem) were used to quantify infection rates. In preliminary testing, Guo et al. (2015) found that abaxial leaf surface inoculation provided higher rates of infection than adaxial surface inoculation, possibly due to greater stomatal density on the abaxial surface. They noted that leaf surface inoculations required less inoculum and leaf surface while allowing better control over number of conidia, while stem spray inoculations were better for screening many genotypes and for using a smaller sample size. Presumably, these techniques could be applied to *Sarcococca* taxa.

No other diseases have been reported to significantly affect *Sarcococca*. However, because *Sarcococca* is underutilized in American landscapes, it might be worth investigating susceptibility to other fungal diseases that are known to infect *Buxus*, such as Macrophoma leaf spot caused by *Macrophoma candollei*, Phytophthora root rot caused by *Phytophthora parasitica* and *P. cinnamomi*, boxwood decline caused by *Paecilomyces buxi*, and Volutella blight caused by *Pseudonectria buxi* (Moorman, 2017).

Conclusions and Goals

Sarcococca is a genus of plants with many traits that are valuable to the nursery and landscape industry including attractive foliage, fragrant flowers, landscape adaptability, ease of propagation, and few problems with pests and diseases. However, a lack of genetic and disease susceptibility information regarding the genus limits the progress that can be made through breeding. Through this thesis, I hope to clarify the identities and genetic relationships among American *Sarcococca* accessions to guide use of these plants by breeders, landscape

professionals, and gardeners. By understanding *Sarcococca* susceptibility to boxwood blight

caused by *Calonectria pseudonaviculata*, we can provide better management advice for the

disease as it relates to all members of the Buxaceae, and we can hopefully identify accessions

that show promise for creating *Sarcococca* resistant to this devastating disease.

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CHAPTER 2

GENETIC DIVERSITY OF AMERICAN *SARCOCOCCA* ACCESSIONS ASSESSED USING

INTER-SIMPLE SEQUENCE REPEAT MARKERS AND FLOW CYTOMETRY[1]

[1]Ryan, C.F. and D. Zhang. To be submitted to *HortScience.*

Abstract

Inter-simple sequence repeat (ISSR) markers were used to assess the species and genetic relationships among 38 accessions of *Sarcococca* found in the United States. A total of 97 loci were generated from 8 primers with an average of 12 bands per primer. These primers produced a range of 8 to 20 loci, and 95.8% of them were polymorphic. An unweighted pair group method with arithmetic mean (UPGMA) tree was assembled using Jaccard's similarity coefficient, and five clades were recognized. *Sarcococca vagans* from Far Reaches Farm and *S. hookeriana* var. *digyna* 'Purple Stem' had the lowest Jaccard similarity coefficient (0.34), while several *S. orientalis* accessions were very similar (Jaccard similarity coefficient > 0.9). Overall, relationships in the presented dendrogram were similar to published trees, suggesting ISSRs can be used for determining genetic relationships among *Sarcococca* taxa. Flow cytometry with the fluorochrome DAPI (4′,6-diamidino-2-phenylindole) was used to determine relative genome size of select accessions from clusters on the dendrogram. Relative holoploid (2C) genome size ranged from 3.59 pg to 15.51 pg. Some relative genome sizes differed from published values, likely due to DNA staining with DAPI instead of propidium iodide and different base pair compositions between internal standards. The combination of ISSR data and genome sizes allowed for the identification of species, and new names for mislabeled accessions are suggested. Taxa with the name *Sarcococca hookeriana* were found in three clusters. However, differences in morphology, ISSR profiles, and relative 2C genome size suggest some of these plants are mislabeled.

Introduction

     Sarcococca is a genus of evergreen shrubs in the Buxaceae native almost exclusively to Asia, though one species, *S. conzattii* (Standl.) I. M. Johnst., has been reported in Mexico and Guatemala (eFloras, 2008; Sealy, 1986). Members of the genus have many redeeming ornamental qualities including evergreen foliage, fragrant winter flowers, pest and disease resistance, attractive fruits, and adaptability to dry shade. There are roughly 20 species in the genus, of which about 7 species are grown in the United States (Dirr, 2009; eFloras, 2008; personal observation).

     Sarcococca has seen little work regarding breeding and selection, and consequently only about six named selections are found at nurseries and gardens in the United States: *S. hookeriana* var. *digyna* 'Purple Stem', *S. hookeriana* var. *digyna* 'Western Hills', *S. hookeriana* var. *humilis* 'Sarsid1' (Fragrant Valley™), *S. hookeriana* var. *humilis* 'Sarsid2' (Fragrant Mountain™), *S. hookeriana* var. *humilis* 'PMOORE03' (Winter Gem™), and *S. ruscifolia* var. *chinensis* 'Dragon Gate'(Botanic Gardens Conservation International, n.d.; Moore, 2016; personal observation). As a result of the lack of named selections, the majority of *Sarcococca* in cultivation are distributed by species names alone, making the identification of unique genotypes difficult. Additionally, the identity of certain clones is questioned.

     Sealy (1986) described *Sarcococca* as a "natural" genus, meaning all species have uniform floral characteristics and all are evergreen shrubs. He also noted that identification using flower features is difficult due to small flower size and that vegetative features can be useful but are variable. Thus, molecular taxonomic work would help clarify the diversity and species boundaries of the genus. The molecular taxonomic relationships of the Buxaceae were investigated by von Balthazar et al. (2000) using nuclear internal transcribed spacers (ITS) and

plastid *ndhF* sequences. This study included 5 species of *Sarcococca*. Using cultivated *Sarcococca* germplasm and amplified fragment length polymorphisms (AFLP), Denaeghel et al. (2017) found similar relationships among *Sarcococca* taxa to those of von Balthazar et al. (2000). They also included four more species. To date, however, only European *Sarcococca* accessions have been surveyed. Nearly all species and named accessions studied by Denaeghel et al. (2017) and von Balthazar et al. (2000) are in cultivation in the United States.

Molecular taxonomic relationships for crops with little published genomic information are often analyzed using molecular marker systems that do not require prior genomic information. Common polymerase chain reaction (PCR) based markers include random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR) and inter-Simple Sequence Repeats (Reddy et al., 2002; Zhang et al., 2000; Zhao et al., 2007). Inter-simple sequence repeat markers have benefits over other PCR-based marker systems due to their relatively low cost, greater reproducibility, and the lack of a need for knowledge of flanking sequences (Reddy et al., 2002).

Determining genome size and chromosome number in plants is important for understanding species relationships and crossability. Chromosome number and genome size have been reported for several *Sarcococca* species, though only for Asian and European accessions. Base chromosome number is x = 14; most species are reported as diploids, though *S. confusa* and *S. ruscifolia* are only known as tetraploids (Darlington & Wylie, 1955; Denaeghel et al., 2017; Saggoo et al., 2011). For diploids, holoploid genome size exists in smaller (4.11-4.20 pg/2C) and larger (7.25-9.63 pg/2C) groupings, while tetraploids are all relatively similar in genome size (7.91-8.18 pg/2C).

In addition to supporting breeding activities, knowledge of genetic relationships among *Sarcococca* clones may prove valuable for the management and control of an emerging disease called boxwood blight caused by *Calonectria pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous. This disease is known to infect ornamental members of the Buxaceae, causing leaf spots, stem lesions, and rapid defoliation. The disease was first reported in the United States in 2011 and has since spread to at least 25 states (Ivors et al., 2012; LaMondia & Shishkoff, 2017; Williams-Woodward, 2015). The disease has been found to infect *Sarcococca* in the field, but only one *Sarcococca* species has been screened for susceptibility (Henricot et al., 2008; Kong et al., 2017; Malapi-Wight et al., 2016). A better understanding of species relationships may enable researchers to more effectively assess susceptibility of *Sarcococca* to boxwood blight.

The purpose of this study was to survey genetic diversity and genome size in United States accessions of *Sarcococca* taxa to better understand the relatedness and identities of taxa and their potential applications to breeding and boxwood blight management. Using these results, we make recommendations regarding the classification of certain taxa and provide baseline genetic information for *Sarcococca* cultivated in the United States.

Materials and Methods

Plant Materials

A total of 40 *Sarcococca* accessions (Table 2.1) were collected for this study. These plants span seven species, three botanical varieties, and five cultivars, and to the best of our knowledge represent all species and cultivars in cultivation in the United States, except *S. hookeriana* 'PMOORE03' (Winter Gem™). These plants were acquired from nurseries and

botanical gardens across the United States and cultivated at the University of Georgia Durham Horticulture Farm, Watkinsville, GA.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

Newly developed leaves for each accession were collected and ground with liquid nitrogen using a mortar and pestle. If not immediately being used for DNA extraction, ground leaf tissue was stored at -20°C. Genomic DNA was extracted from ground tissue using the DNEasy Plant Mini Kit (Qiagen, Hilden, Germany). Extracted DNA concentration (ng/μl) and purity (A260/A280) was determined using a spectrophotometer (NanoDrop Lite; Thermo Scientific, Waltham, MA).

Extracted DNA fragments were then PCR-amplified using a thermocycler (Mastercycler Pro; Eppendorf, Hamburg, Germany) and ISSR primers (UBC Primer Set #9, University of British Columbia). A 20 µl reaction volume was used consisting of 10 µl AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA), 20 ng of template DNA suspension, 2 µl primer, and the remaining volume sterile distilled H_2O. The PCR program consisted of initial denaturation at 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, primer annealing at a temperature dependent on the primer (Table 2.2) for 45 seconds, and extension at 72°C for 2 minutes; and a final extension at 72°C for 7 minutes. PCR products were then held at 4°C until removal from the thermocycler, after which they were run through gel electrophoresis or stored at -20°C. PCR products were electrophoresed in an agarose gel (1.2% agarose with 0.5x Tris-Borate-EDTA buffer) with 1-2 drops of ethidium bromide (10 mg/ml). Gels were run at 80-90V for 2-3 hours. A 100 base pair DNA ladder (TrackIt; Invitrogen, Carlsbad, CA) was included in

electrophoresis runs to have a molecular weight standard to compare bands from different gels. Gels were photographed under UV light using a digital camera.

DNA Marker Data Analysis

Bands that were defined and repeatable were scored as dominant markers, with a 1 indicating presence of a band and a 0 indicating absence. A UPGMA (unweighted pair group method with arithmetic mean) dendrogram (Figure 2.1) based on Jaccard's similarity coefficient was constructed in R using the hclust function (The Project for Statistical Computing, 2018). A heat map using Jaccard similarity coefficient was constructed using the ggplot2 package in R.

Flow Cytometry

Newly emerged leaf tissue for each accession (Table 2.1) was collected and stored in a resealable bag at 4°C until use. Approximately 2 cm^2 leaf area for each sample and a standard of known genome size were co-chopped using a razor blade in a Petri dish containing 500 μL of nuclei extraction buffer (Cystain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex, Görlitz, Germany). The standards with known genome size were either *Pisum sativum* 'Ctirad' (2C = 9.09 pg) or *Zea mays* 'CE-777' (2C = 5.43 pg) (Doležel et al., 1998; Lysak & Doležel, 1998) and were chosen based on sample expected genome size and previous results using the same standards (Denaeghel et al., 2017). The extracted nuclei and chopped leaves in buffer were filtered through a 50 μm nylon mesh filter (CellTrics; Partec, Görlitz, Germany) into a 3.5 mL plastic tube. The filtered buffer-nuclei suspension was then stained with 1500 μL of 4′,6-diamidino-2-phenylindole (DAPI, CyStain UV Precise P Staining Buffer; Partec, Münster, Germany). Stained nuclei were processed using a flow cytometer (Beckman Coulter CyAn;

Beckman Coulter, Brea, CA), with approximately 10,000 counts per subsample, at least two subsamples per accession, and a CV of approximately 10% or less.

Relative 2C genome size was calculated as follows:

$$2C \; Genome \; Size$$

$$= 2C \; Genome \; Size \; of \; Standard \; (pg) \times \frac{Mean \; Fluorescence \; of \; Sample}{Mean \; Fluorescence \; of \; Standard}$$

Results and Discussion

Flow Cytometry

Relative 2C genome size for the *Sarcococca* used in this study ranged from 3.58 to 15.50 pg (Table 2.3). The results for those accessions with relatively low genome size (~4 pg) are similar to those of Denaeghel et al. (2017), who found *S. hookeriana* accession genome sizes ranged from 4.11 to 4.20 pg/2C using propidium iodide (PI). Caution should be used when comparing genome sizes determined using DAPI (AT preferential) versus those determined using propidium iodide (DNA intercalating) because genome size estimations using a base preferential fluorochrome can over- or underestimate 2C genome size (Doležel et al., 1998 & 1992; Parris et al., 2010). Nonetheless, determination of genome size using different fluorochromes can produce results that are not significantly different from each other or underestimate genome size (Doležel et al., 1992; Parris et al., 2010).

DAPI is frequently used as a fluorochrome primarily because using it is faster, costs less, and produces less variable fluorescence peaks (Contreras and Ruter, 2011; Ranney et al., 2018). Accessions with a genome size of ~12+ pg showed peaks in the same region as *Pisum sativum* 'Ctirad', so *Zea mays* 'CE-777' was used instead to calculate their relative genome size. *Sarcococca ruscifolia* and *S. confusa* accessions had a relative genome size of approximately 12

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pg/2C, which is much larger than the expected ~8 pg/2C determined by Denaeghel et al. (2017). This may be due to a significant difference in % AT base pair composition between *Z. mays* 'CE-777' and *Sarcococca*. A %AT in Z. mays 'CE-777' smaller than the %AT in *Sarcococca* accessions would overestimate 2C genome size in the *Sarcococca* accessions when using DAPI (AT preferential) as a DNA stain.

AT frequency in our *Sarcococca* could be calculated by repeating flow cytometry analysis of these accessions using propidium iodide and using previously published equations (Barow & Meister, 2002). When *Sarcococca* accessions with DAPI 2C = ~12 pg are co-chopped with accessions with DAPI 2C = ~ 4 pg and run through the flow cytometer, the ratio of mean fluorescent values (~12 pg accession/ ~4 pg accession) is approximately 2:1 (data not shown), suggesting that these larger genome accessions have a genome size approximately two times that of the smaller genome accessions. This fits with reported genome sizes of ~8 pg and ~4 pg in most *Sarcococca* taxa screened using propidium iodide, respectively. (Denaeghel et al., 2017).

Inter-Simple Sequence Repeat Markers

A total of 8 primers (Table 2.2) were used to generate 97 total bands, with an average of 12 bands per primer. The range of loci was from 8 to 20 and 95.8% of them were polymorphic. We were able to successfully develop a dendrogram using the UPGMA method and eight ISSR markers (Table 2.2, Figure 2.2). The 8 primers were used to generate 97 total scorable loci, ranging from 8 to 20 loci per marker for an average of 12 and 95.8% polymorphism. Because ISSR markers are dominant and thus represent a molecular phenotype and not genotype, there is not a recommended similarity index for comparing taxa. Jaccard's

similarity index is a common measure of similarity used in ISSR studies, and it calculates

similarity based on the presence of shared bands (Kosman & Leonard, 2005). It is impossible to

determine the true similarity of individuals because ISSRs cannot detect if an allele comes from

homozygous or heterozygous loci and thus similarity estimates should be regarded as rough

estimates (Kosman & Leonard, 2005).

A heat map representing taxon similarity based on Jaccard's similarity coefficient was

developed (Figure 2.2). This is a graphical alternative to sharing long and convoluted similarity

tables. Excluding the *Buxus sempervirens* 'Suffruticosa' outgroup, similarity values ranged from

1.0 for *S. orientalis* (HF 160001 and HF 160002) to 0.34 (*S. hookeriana* var. *digyna* 'Purple

Stem' JCN and *S. vagans* FRF) (Figure 2.2).

The dendrogram produced using ISSR data from 8 ISSR markers (Figure 2.1) agrees with

trees produced in other studies involving *Sarcococca* and the Buxaceae (Denaeghel et al., 2017;

von Balthazar et al., 2000). Five notable clusters were produced (Figure 2.1): *S. wallichii*, *S.

saligna*, and *S. vagans* (A); small genome (~4 pg/2C) accessions of *S. hookeriana* (B); *S.

orientalis* (C); large genome *S. confusa* and *S. ruscifolia* (D); and small genome *S. ruscifolia* var.

chinensis (E). Almost all *Sarcococca hookeriana* clustered together, with all small genome *S.

hookeriana* var. *humilis* and *S. hookeriana* var. *digyna* clustering in separate but sister groups.

Sarcococca hookeriana accessions that clustered along with *S. orientalis* have a larger 2C

genome size and larger, thicker leaves. For the cluster containing *S. ruscifolia and S. confusa*,

genome size is approximately 12 pg/2C, while for the cluster containing *S. ruscifolia* 'Dragon

Gate' and others *S. ruscifolia* var. *chinensis*, genome size is approximately 4 pg/ 2C for

accessions tested. *Sarcococca ruscifolia* var. *chinensis* 'Dragon Gate' was reported to be a

tetraploid (2n = 4x = 56) with a relative 2C genome size of 7.91 determined using PI (Denaeghel

et al., 2017). Counting chromosomes and flow cytometry using PI should be completed to verify the identity of our *S. ruscifolia* var. *chinensis* 'Dragon Gate' accessions. There are two clones being distributed under the name 'Dragon Gate'. Our accession from Far Reaches Farm is a clone of the original *S. ruscifolia* var. *chinensis* from Roy Lancaster obtained via Cistus Nursery. The low genome size of this accession and other plants in cluster E suggest a new cytotype for *S. ruscifolia*.

Sarcococca *wallichii*, *S. saligna*, and *S. vagans* all clustered together, which is plausible based on prior study, genome size, and morphology (Denaeghel et al., 2017; Sealy, 1986). Because our *Sarcococca saligna* accessions have a large relative genome size and grouped with *S. wallichii* and *S. vagans*, it is probable that these accessions are more similar to the large genome *S. saligna* accessions of Denaeghel et al. (2017) and not their small genome *S. saligna* accession.

Prior to this study, we hypothesized that some accessions were mislabeled. The *S. hookeriana* accessions that clustered within the *S. orientalis* group have a larger genome (Table 2.3) and different morphological features (personal observation) than other *S. hookeriana* accessions, supporting the hypothesis that these accessions are mislabeled *S. orientalis*. Other mislabeled *S. hookeriana* accessions clustered among some *S. ruscifolia* var. *chinensis* genotypes and sister to *S. ruscifolia* and *S. confusa*. This suggests that these accessions may represent *S. ruscifolia* instead of *S. hookeriana*. Crosses were made using *S. hookeriana* (HF) as a female parent. Fruits produced from this cross were red, which is a feature of *S. ruscifolia* and not *S. hookeriana*, further suggesting this plant is mislabeled (eFloras, 2008).

Sarcococca wallichii (WN) grouped most closely with *S. vagans*, suggesting this plant may also be mislabeled. This *S. wallichii* accession has larger leaves and a wider habit much more in line with *S. vagans* (FRF) than *S. wallichii* (FRF).

One accession, *S.* sp. (ABG) had a relative 2C genome size of 8.72 and was sister to *S. orientalis* accessions. The difference in genome size and location on the dendrogram suggest that this plant, which was wild-collected in China, may represent a new species to American horticulture. This plant has yet to flower, making identification following published dichotomous keys difficult (eFloras, 2008; Sealy, 1986).

Lastly, both *Sarcococca hookeriana* 'Purple Stem' accessions clustered together, but had a Jaccard similarity value of 0.83, suggesting that this cultivar comprises more than one genotype (Figure 2.2).

The identity of *Sarcococca* confusa is questionable because *Sarcococca confusa* is not recognized in the Flora of China and is only known in cultivation. It may have been collected in China by Ernest H. Wilson in 1908 along with *S. hookeriana* var. *digyna* and *S. ruscifolia* var. *chinensis* (eFloras, 2008; Sealy, 1986). Sealy (1986) suggested it might be an interspecific hybrid between *S. hookeriana* var. *digyna* and *S. ruscifolia* var. *chinensis* in part because *Sarcococca hookeriana* var. *digyna* is strictly bicarpellary and *S. ruscifolia* var. *chinensis* is strictly tricarpellary (Sealy, 1986). Denaeghel et al. (2017) found *S. confusa* to be sister to *S. ruscifolia* var. *chinensis*, but they did not include many of the *S. ruscifolia* accessions used in our study.

Many *S. ruscifolia* accessions screened in this study were genetically similar to *S. confusa* accessions (e.g. Jaccard similarity of 0.93 between *S. ruscifolia* (JCN) and *S. confusa* (GPN)). It may be that many of our *S. ruscifolia* accessions are misidentified *S. confusa* or that *S. confusa* is actually *S. ruscifolia* that produces bi- and tri-carpellary female flowers.

Sarcococca ruscifolia, *S. wallichii*, and *S. vagans* can also produce bi- and tri-carpellary flowers, which suggests that this feature may not be as reliable as previously reported (Denaeghel et al., 2017; eFloras, 2008; Sealy, 1986).

Genome size is approximately the same among many of our *S. ruscifolia* and *S. confusa* at about 12 pg/2C (Table 2.3). Vegetative morphology among these accessions is also similar. An investigation of floral morphology of *S. confusa* and *S. ruscifolia* accessions is warranted to better understand the identities of these plants.

The results of this study provide new information regarding identity and relatedness of cultivated *Sarcococca* accessions in the United States. Our study indicates that several plants are misidentified, so caution should be used regarding given names for *Sarcococca*. Coupled with other studies, this research provides a baseline for *Sarcococca* breeding and further research. By understanding relatedness of taxa and genome size, breeders can make better informed decisions regarding *Sarcococca* crosses.

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Table 2.1. *Sarcococca* accessions used for the ISSR study and recommended reclassification based on ISSR and flow cytometry results and morphological observations.

Source[X]	Accession Number	Taxon	Suggested name (if warranted)
ABG	160006	*Sarcococca hookeriana* var. *digyna* Variegated	*S. orientalis* [Δ]
ABG	160007	*S.* aff. *ruscifolia* var. *chinensis*	*S. ruscifolia* var. *chinensis* [Δ]
ABG	160008	*S.* sp.	*
DK	160025	*S. hookeriana* var. *hookeriana*	*S. orientalis* [Δ]
FRF	160009	*S. wallichii*	*
FRF	160010	*S. ruscifolia* var. *chinensis* 'Dragon Gate'	*
FRF	160010	*S. vagans*	*
FRF	160010	*S. hookeriana*	*
GPN	160011	*S. confusa*	*
HF	160001	*S. orientalis*	*
HF	160002	*S. orientalis*	*
HF	160003	*S. confusa*	*
HF	160004	*S. hookeriana*	*S. ruscifolia* var. *chinensis* [Δ]
JCN	160011	*S. saligna*	*
JCN	160012	*S. ruscifolia*	*
JCN	160013	*S. hookeriana* var. *digyna* 'Purple Stem'	*

Table continued from previous page.

Source[X]	Accession Number	Taxon	Suggested name (if warranted)
JCRA	41763	*S. hookeriana* var. *digyna* 'Western Hills'	*S. ruscifolia* var. *chinensis* 'Western Hills' [Δ]
JCRA	980363	*S. saligna*	*
JCRA	xx0884	*S. hookeriana*	*
JCRA	41718	*S. ruscifolia* var. *chinensis* 'Dragon Gate'	*
JCRA	980362	*S. ruscifolia*	*
JCRA	980361	*S. confusa*	*
NC	160014	*S. hookeriana* var. *humilis* 'Sarsid 1' Fragrant Valley™	*
NC	160015	*S. hookeriana* var. *humilis*	*
PDN	160016	*S. hookeriana* var. *humilis* 'Purple Stem'	*
PDN	160017	*S. ruscifolia* var. *chinensis*	*
RFN	160018	*S. hookeriana* var. *humilis* 'Sarsid 2' Fragrant Mountain™	*
SBG	160026	*S. ruscifolia*	*
SBG	19940807	*S. ruscifolia*	*
SBG	19940806	*S. orientalis*	*
SBG	20010702	*S. confusa*	*

Source[X]	Accession Number	Taxon	Suggested name (if warranted)
		Table continued from previous page.	
SBG	20010688	*S. hookeriana* var. *humilis*	*
SFN	150000	*Buxus sempervirens* 'Suffruticosa'	*
WH	160019	*S. saligna*	*
WH	160020	*S. ruscifolia*	*
WH	160021	*S. hookeriana* var. *humilis*	*
WN	160022	*S. confusa*	*
WN	160023	*S. wallichii*	*S. vagans* [Δ]
WN	160024	*S. orientalis*	*

[X]**Source Abbreviations: HF**= University of Georgia Durham Horticulture Farm, Watkinsville, GA; **ABG**=Atlanta Botanical Garden, Gainesville, GA; **DK**= David Klemm, Fairfax, VA; **FRF**=Far Reaches Farm, Port Townsend, WA; **GPN**=Griffith Propagation Nursery, Watkinsville, GA; **JCRA**=J.C. Raulston Arboretum, Raleigh, NC; **NC**= Nurseries Caroliniana, North Augusta, SC; **JCN**=Joy Creek Nursery, Scappoose, OR; **PDN**=Plant Delights Nursery, Raleigh, NC; **RFN**=Rarefind Nursery, Jackson, NJ; **SBG**=State Botanical Garden of Georgia, Athens, GA; **SFN**= Silver Falls Nursery, Salem, OR; **WH**=Willis Harden, Commerce, GA; **WN**=Woodlanders Nursery, Aiken, SC.

*Our results suggest that these accessions do not warrant reclassification.

[Δ]Our results suggest that these accessions warrant reclassification. (These accessions are marked with the same symbol in Figure 2.1.)

Table 2.2 Inter-Simple Sequence Repeat primers used for this study.

Name	Sequence (5' to 3')	Annealing Temperature	Number of Scored Loci	% Polymorphic
UBC808	AGA GAG AGA GAG AGA GC	53°C	8	87.5
UBC810	GAG AGA GAG AGA GAG AT	53°C	8	87.5
UBC815	CTC TCT CTC TCT CTC TG	53°C	20	100
UBC823	TCT CTC TCT CTC TCT CC	53°C	13	100
UBC835	AGA GAG AGA GAG AGA GYC	56°C	11	90.9
UBC836	AGA GAG AGA GAG AGA GYA	54°C	10	100
UBC840	GAG AGA GAG AGA GAG AYT	53°C	14	100
. /UBC842	GAG AGA GAG AGA GAG AYG	52°C	13	92.3
Mean			12 (97)	95.8%

Table 2.3 Relative genome size and standard error of the mean for select *Sarcococca* accessions determined using DAPI.

Source[X]	Accession Number	Taxon	Relative 2C Genome Size ± SEM	Internal Standard [Y]
HF	160003	*Sarcococca confusa*	12.23 ± 0.14	Corn
JCR	980361	*S. confusa*	12.06 ± 0.14	Corn
SBG	20010702	*S. confusa*	11.90 ± 0.09	Corn
WN	160022	*S. confusa*	12.28 ± 0.06	Corn
NC	160015	*S. hookeriana* var. *humilis*	4.04 ± 0.09	Pea
WH	160021	*S. hookeriana* var. *humilis*	3.83 ± 0.08	Pea
JCRA	xx0884	*S. hookeriana*	3.59 ± 0.43	Pea
HF	160004	*S. hookeriana*	3.76 ± 0.18	Pea
RFN	160018	*S. hookeriana* var. *humilis* 'Sarsid 2' Fragrant Mountain™	3.96 ± 0.13	Pea
NC	160014	*S. hookeriana* var. *humilis* 'Sarsid 1' Fragrant Valley™	4.09 ± 0.11	Pea
PDN	160016	*S. hookeriana* var. *humilis* 'Purple Stem'	4.12 ± 0.05	Pea

Source[X]	Accession Number	Taxon	Relative 2C Genome Size ± SEM	Internal Standard [Y]
SBG	20010688	*S. hookeriana* var. *humilis*	4.09 ± 0.19	Pea
WN	160024	*S. orientalis*	11.93 ± 0.04	Corn
ABG	160008	*S.* sp.	8.72 ± 0.24	Corn
PDN	160017	*S. ruscifolia* var. *chinensis*	11.67 ± 0.03	Corn
SBG	19940807	*S. ruscifolia*	11.49 ± 0.04	Corn
FRF	160010	*S. ruscifolia* var. *chinensis* 'Dragon Gate'	4.11 ± 0.05	Pea
JCN	160012	*S. ruscifolia*	11.85 ± 0.16	Corn
SBG	160026	*S. ruscifolia*	11.79 ± 0.00	Corn
ABG	160007	*S.* aff. *ruscifolia* var. *chinensis*	3.95 ± 0.05	Pea
WH	160020	*S. ruscifolia*	12.10 ± 0.03	Corn
JCRA	980363	*S. saligna*	15.40 ± 0.23	Corn
JCN	160011	*S. saligna*	15.33 ± 0.19	Corn
WH	160019	*S. saligna*	15.51 ± 0.01	Corn
FRF	160010	*S. vagans*	13.89 ± 0.05	Corn
WN	160023	*S. wallichii*	13.92 ± 0.20	Corn

Table continued from previous page.

[X]See footnote for Table 2.1. (Source abbreviations are the same for Table 2.1 and Table 2.3)

[Y]**Corn**=Zea may 'CE-777', 2C = 5.43 pg; **Pea**= *Pisum sativum,* 2C = 9.09 pg

Figure 2.1. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Jaccard's similarity coefficient developed using binary data from 8 ISSR markers. These markers produced a total of 97 scorable loci, of which 95.8% were polymorphic. Letters indicate five clusters of *Sarcococca* accessions that differ based on marker profiles, morphology, and 2C genome size.

Δ Our results suggest that these accessions warrant reclassification. (See Table 2.1.)

Figure 2.2. A heatmap based on Jaccard similarity coefficients for 38 *Sarcococca* accessions. The darker the individual rectangle, the more closely related the taxa that share the rectangle are.

CHAPTER 3

SUSCEPTIBILITY OF *SARCOCOCCA* TAXA TO BOXWOOD BLIGHT EVALUATED

USING DETACHED STEMS[1]

[1]Ryan, C.F., Williams-Woodward, J., and Zhang, D. To be submitted to *Plant Health Progress*.

Abstract

Boxwood blight caused by *Calonectria pseudonaviculata* is relatively new disease to the United States and causes devastating damage to members of the boxwood family (Buxaceae). Many boxwoods (*Buxus*) and some *Pachysandra* have been screened for susceptibility to the disease, but *Sarcococca* has been neglected. Twenty-two *Sarcococca* representing at least seven species and two *Buxus* accessions were evaluated using detached stem inoculations for susceptibility to boxwood blight caused by *Calonectria pseudonaviculata*. Results of two rounds of stem inoculations showed significantly different amount of disease expression between both rounds, probably due to different locations used for disease incubation and differences in duration of disease development. For example, *Sarcococca hookeriana* var. *humilis* (WH) had a diseased area of 13% after the first round but 70% after the second round. A range of susceptibility was found, with boxwoods tending to exhibit high susceptibility relative to the *Sarcococca* across both trials at 15.8 to 22.3 and 77.8 to 95.4 percent diseased area for trials one and two, respectively. *Sarcococca* accessions were much more variable in susceptibility, with nearly 1.5% to 100% symptomatic leaf area, depending on accession and trial. Some accessions of *Sarcococca ruscifolia* exhibited very little symptom development (as low as 1.5% diseased leaf area) and may represent the taxon of *Sarcococca* least susceptible to the disease. *Sarcococca vagans* showed high susceptibility 3-5 days after inoculation, developing 14 water-soaked spots per cm^2, 7 more spots per cm^2 than the next highest *Sarcococca* accession. But only 31% of its total leaf area developed into symptomatic brown spots. This may represent a separate mechanism of resistance. This is the first study of *Sarcococca* susceptibility to boxwood blight using detached stems and is part of a larger body of work trying to understand this new disease.

Introduction

Boxwoods (*Buxus*) are among the most valuable and commonly grown ornamental shrubs in the United States, worth an estimated $126 million in 2014 (United States Department of Agriculture, 2015). Currently an emerging disease called boxwood blight is causing severe defoliation, dieback, and death in infected boxwoods and other members of the boxwood family (Buxaceae). The disease was first discovered in 1994 in the United Kingdom but has since been found in many countries around the world (Henricot and Culham, 2002). It is caused by at least two related pathogens, *Calonectria pseudonaviculata* (Crous et al.) L. Lombard et al. and *C. henricotae* Gehesquière, Heungens, and J.A (Gehesquière et al., 2016). *Calonectria pseudonaviculata* is known in North America, Europe, and New Zealand, while *C. henricotae* is only known in Europe (Gehesquière et al., 2016). In the United States boxwood blight was first discovered in North Carolina and Connecticut in 2011 and is now found in at least 25 states (Ivors et al., 2012; LaMondia and Shishkoff, 2017; Williams-Woodward, 2015).

Research assessing the host range of the *C. pseudonaviculata* has focused on the genus *Buxus*, starting with Henricot et al. (2008), who found nine *Buxus* species capable of serving as hosts following stem dipping inoculations. The disease has also been found to infect three species of *Pachysandra* (Kong et al., 2017a; LaMondia, 2017; LaMondia et al., 2012). Many boxwood taxa and seven *Pachysandra* taxa have since been evaluated for susceptibility to boxwood blight, with varying results but no immunity found (Ganci, 2013; Guo et al., 2015; LaMondia, 2017; LaMondia and Shishkoff, 2017; Shishkoff et al., 2015). Methods of evaluation have included whole plants, detached stems, and detached leaf inoculations using conidial sprays, stem dips, single droplets, mycelium, or actively growing fungal plates to inoculate the plants (Ganci et al., 2013; Guo et al., 2015; Guo et al., 2016; Shishkoff et al., 2015).

Despite intensive screening of boxwood taxa and some screening of *Pachysandra* taxa for susceptibility to boxwood blight, the other ornamental genus in the Buxaceae *Sarcococca* has been neglected. The only *Sarcococca* known to be susceptible to boxwood blight was an unidentified species, which showed low sporulation following laboratory inoculations, causing the researchers to speculate that low sporulation was the reason boxwood blight had not been found on *Sarcococca* in the field (Henricot et al., 2008). However, *Sarcococca hookeriana* was reported to have been infected by *C. pseudonaviculata* in a home landscape in Maryland in 2014 and was again observed infected by the blight in a home landscape in Virginia in 2015 (Kong et al., 2017b; Malapi-Wight et al., 2016). Because *Sarcococca* is now known to be a host to the boxwood blight pathogen and because *Sarcococca* is often cultivated among or near other members of the Buxaceae, it is important to understand how different *Sarcococca* taxa available in the horticulture industry respond to the disease. In this study, we used detached stems to assess susceptibility of *Sarcococca* accessions to boxwood blight caused by *Calonectria pseudonaviculata*.

Materials and Methods

Plant Materials

Cultivated *Sarcococca* and *Buxus* taxa were acquired from several nurseries and gardens (Table 3.1) and grown at University of Georgia Durham Horticulture Farm, Watkinsville, GA. Stems with leaves that were sufficiently hardened off were collected from these plants and nearby gardens in June 2017 and March 2018 and stored at 4°C until use. Collected stems were trimmed so that all *Sarcococca* had approximately 10 leaves per stem and all *Buxus* had approximately 15 leaves per stem.

Inoculum

Single-conidium isolates of *Calonectria pseudonaviculata* from naturally-infected boxwoods from 5 locations were incubated on potato dextrose agar (PDA) (BD Difco, Sparks, MD) amended with ampicillin trihydrate (250 mg/L) (Sigma Aldrich; St. Louis, MO) for 2 weeks at room temperature in ambient light. Seventeen total isolates were used (Cp1 (A-D), Cp2 (A-D), Cp3 (A-D), Cp4 (A-B), Cp5 (A-C)) but Cp4 isolates were not included in the second trial. Culture plates were flooded with sterilized deionized water (SDW) for 2 hours, decanted, scraped with a sterile metal spatula to remove aerial hyphae, and rinsed with SDW by swirling and decanting. Plates were then incubated upside-down for 5 days at 23°C under a 12 hr light/dark cycle. Conidia were harvested by washing plates with a stream of SDW while the plate was held over a beaker. The resulting conidial suspension was filtered through three layers of sterile cheesecloth to remove clumps of hyphae. Conidia were counted with a hemocytometer and adjusted to a final concentration of 20,000 conidia/ml.

Stem Inoculations

Four stems for each accession were dipped in a 1000 ml beaker containing the 20,000 conidia/ml solution and occasionally swirled for 3-4 minutes. An additional two stems were dipped in sterile distilled water to serve as negative controls. Following dipping in conidial suspension, the stems were inserted into 50 ml plastic centrifuge tubes filled with 1.2% bacto agar (first trial) or 1.0% bacto agar (second trial). The bacto agar served to provide moisture to the stems while firmly holding them in place. The stems were then placed in cardboard holding trays, inserted into large plastic bags whose interiors were spritzed with water, and allowed to

incubate under ambient laboratory conditions. Number of spots on each leaf was recorded three (first trial) to five (second trial) days after inoculation. After spots were counted, stems were placed in a growth chamber with a 12 hr light/dark cycle (first trial) or placed back into moist plastic bags (second trial), and disease was allowed to develop further. Three days after counting spots in the second trial, the bags were opened. Nine (first trial) or fourteen (second trial) days after inoculation leaves from the stems were collected and the abaxial leaf surface of each was photographed. Leaves were collected for each stem where possible, but excessive leaf drop and human error led to most leaves being collected and then randomly placed in four groups of 10 (*Sarcococca*) or 15 (*Buxus*) leaves per accession.

Infected leaf area and total leaf area were calculated using Assess 2.0, the Image Analysis Software for Plant Disease Quantification (APS Press, St. Paul, MN) and used to determine the percentage of infected leaf area. Percentage of leaves showing symptoms per stem was also calculated by dividing the number of leaves with water-soaked spots by the number of leaves (10 or 15 for *Sarcococca* or *Buxus*, respectively) and multiplying by 100. For the second trial, number of water-soaked spots per average leaf size was calculated by dividing the average number of spots per leaf of each stem replicate by the average area (cm^2) of each set of 10-15 leaves.

Statistical Analysis

Data were analyzed following a completely randomized experimental design. In the first trial, 29 total taxa were screened, while in the second trial, only 24 taxa were screened. This difference was mainly due to a lack of availability of stems from certain accessions for the second trial. To effectively compare the two trials, the extra taxa included in the first trial were

removed from analysis. The effect of measurement date was determined using analysis of variance (ANOVA) and was determined to be significant ($p < 0.00622$). Thus, data for both dates were not combined. Mean percent of infected leaves three or five days after inoculation and percentage of symptomatic area 9 or 14 days after inoculation for trials one and two, respectively, were calculated, Differences in means were assessed using an ANOVA. Means were separated using Tukey's Honest Significant Difference test. All data analysis was performed using R (R Core Team, 2017) with the built-in function aov and HSD.test from the agricolae package (Mendiburu, 2017).

Results

All screened accessions exhibited boxwood blight symptoms following inoculation with the disease, indicating that no immunity is present in the accessions tested. The effect of date between trials was significant ($p < 0.00622$) for all comparable variables. This was not anticipated but suggests that the duration between inoculation and counting spots (3 vs. 5 days), duration between inoculation and photographing leaves (9 vs. 14 days), and the treatment of stems following counting spots (growth chamber vs. ambient laboratory conditions) were different enough to significantly change results. Mean percent diseased area for all taxa was 8.9 in the first trial and 59.9 in the second trial. Mean percent of leaves with spots was 50.8 in the first trial and 66.0 in the second trial. Boxwood blight caused by *Calonectria pseudonaviculata* performs differently depending on environmental conditions. Increased periods of leaf wetness up to 20 hours and temperatures of about 25°C are highly conducive to disease development in boxwood (Avenot et al., 2017). Different times of data collection and environmental conditions

of the two trials likely explain much of why percent infection and percentage of symptomatic leaves differed so much between the two trial dates.

Stem Dip Inoculation: June 2017

Three days after inoculation in June 2017, the number of symptomatic leaves was determined. This ranged from 2.5% to 87.5% (Figure 3.2), though most *Sarcococca* and *Buxus* accessions were not significantly different from each other. Twenty-three of twenty-nine total accessions were not significantly different from each other. These 23 accessions included the boxwood positive controls. Most notably, two *S. ruscifolia* var. *chinensis* accessions (ABG, FRF) showed almost no symptoms three days after infection. Twelve days after infection when the leaves were photographed, percent diseased area was calculated. Percent diseased area was relatively low for most accessions (Figure 3.3). *Sarcococca humilis* Fragrant Valley™ had the largest percentage of symptomatic area at 58.9%. Outside the boxwood accessions which were expected to be highly symptomatic, the most symptomatic accessions were of *S. hookeriana*, ranging from 12.4 to 58.9% diseased area. Many accessions had very little symptomatic area, including the *S. ruscifolia* var. *chinensi*s accessions that had almost no symptomatic leaves three days after inoculation. The low amount of symptom development in most accessions relative to trial two is likely a consequence of different disease incubation environments following water-soaked spot counting.

Stem Dip Inoculation: March 2018

Symptoms were allowed to develop for two extra days after inoculation in March 2018 compared to March 2017. This appeared to lead to a greater amount of symptom development in

nearly every accession (Figures 3.1, 3.3). The greatest percentage of symptomatic leaves was *Sarcococca vagans* with 97.5% of its leaves developing water-soaked spots, though this may be somewhat misleading because *S. vagans* (FRF) and also *S. wallichii* (FRF, 82.5%) have the largest leaves of all accessions. Thus, there was more surface area to be infected. Most significantly, three *S. ruscifolia* var. *chinensis* accessions (HFa, PDN, FRF) had the lowest amount of infected leaves, ranging from 0% (FRF) to 30% (HFa). *Sarcococca ruscifolia* var. *chinensis* 'Dragon Gate' (FRF) is the only accession that had almost no symptom expression over both dates. Variability among accessions in response to this disease is something we have experienced consistently through our susceptibility experiments.

Percentage of symptomatic leaf area (Figure 3.5) also increased from June 2017, likely due to the different environments and time periods of disease incubation 3 to 5 days after inoculation. *Sarcococca orientalis* (HFa, ABG, and SBG), *S. ruscifolia* (WH), and *S. wallichii* FRF were the only accessions to have greater % diseased area than at least one boxwood accession. Leaves each of those accession had a % diseased area greater than 77%. *Sarcococca vagans*, of which 97.5% of its leaves had water-soaked spots 5 days after inoculation, was only 31.1% diseased. This corresponds with the results of a detached leaf assay (Chapter 4) in which *S. vagans* and a few other species appeared more capable of developing discrete spots that spread far less than other accessions. However, the *S. saligna* and *S. wallichii* accessions that developed discrete spots in that study did not develop as discrete spots in this study. The *S. ruscifolia* accessions that showed low amounts of water-soaked spots 5 days after inoculation also show a relatively small amount of disease spread, with 'Dragon Gate' being almost asymptomatic at 2% diseased leaf area.

Leaf area in cm^2 was measured for leaves in the March 2018 experiment, and average number of water-soaked spots per average leaf area was calculated (Figure 3.6). The boxwood species, which only developed a combined average of 7.7 spots per leaf are among the accessions that developed the most spots when scaled by leaf area. The boxwoods were positive controls in this experiment and produced statistically more spots per square centimeter than 14-20 *Sarcococca* accessions. *Sarcococca vagans* developed the most water-soaked spots at 14.0 spots/cm^2. This was statistically different from all *Sarcococca* except *S. saligna* (JCN). In general, the number of water-soaked spots produced per cm^2 was comparable to the percent infected area 14 days after inoculation, excluding *S. vagans* (FRF), which was among the lowest in percent infected area despite producing so many spots. Several accessions that produced few spots per square centimeter (e.g. the bottom three *S. ruscifolia* var. *chinensis*) also developed very little symptomatic area.

Comparisons Among Trial Dates

Four accessions responded similarly for percent diseased area between both trials: *Sarcococca hookeriana* var. *humilis* (Fragrant Valley and Fragrant Mountain) and *S. ruscifolia* var. *chinensis* (PDN and 'Fragrant Valley') (Figure 3.1). Fragrant Valley sweet box was the only accession to have a greater percent diseased area in the first trial than the second trial (58.9% vs. 52.4%). Fragrant Valley sweet box and Fragrant Mountain sweet box were the only *Sarcococca* accessions to develop significantly more % diseased area in the first trial than both boxwood accessions, but in the second trial both accessions' % diseased areas were significantly lower than *Buxus sempervirens* 'Suffruticosa'. Only *S. hookeriana* var. *humilis* Fragrant Valley was statistically similar to *Buxus* 'Green Gem'. *Sarcococca orientalis* (ABG, HFa, SBG), *S.*

wallichii (FRF), and *S. ruscifolia* (WH) changed drastically between dates. On the first date, these accessions were among the lowest in terms of % diseased area (0.83%-2.1%), while on the second date, these accessions were among the highest (92.3%-98.9%).

Percent symptomatic leaves was more similar between dates than percent diseased area, possibly due to similar treatment of plants after inoculation, though the extra two days between dates for counting water-soaked spots likely explains the increase in total percent of symptomatic leaves for the second trial. *Sarcococca ruscifolia* var. *chinensis* 'Dragon Gate' was the only accession to have very low amounts of spotting for both dates. Only one detached stem of eight total across both dates developed spots for this accession. The amount of spotting in the boxwoods was much higher relative to other accessions in the first trial compared to the second trial, though in both trials they were not statistically different from most accessions. In general, numerical trends changed among accessions and dates for percent symptomatic leaves, but statistically differences were hard to detect.

Discussion

Detached stem assays have been used frequently and successfully to assess susceptibility of boxwoods and *Pachysandra* to boxwood blight caused by *Calonectria pseudonaviculata* (Guo et al., 2015; Henricot et al., 2008; LaMondia and Shishkoff, 2017; Shishkoff et al., 2015). Other assays, such as detached leaf assays have also been effective at assessing disease (Guo et al., 2015; Guo et al., 2016). Detached plant organs are easy to obtain and screen, making them common choices for plant disease studies, including in non-boxwood blight studies (Miller-Butler et al., 2018; Mondal et al., 2004). Still, detached stems should only be used as part of a series of different susceptibility assays evaluating different types of inoculum (e.g. hyphae,

microsclerotia, different isolates, *C. henricotiae*), detached plant organs, and whole plants. Whole plant assays are valuable because factors like plant architecture and systemic resistance may be missed when only using detached stems and leaves (Conrath, 2006; Robert et al., 2018).

The only published studies regarding susceptibility of *Sarcococca* to boxwood blight have used single species, including one that was unidentified, to compare disease development and susceptibility (Henricot et al., 2008; Kong and Hong, 2018). In both studies, the authors found *Sarcococca* were less susceptible to boxwood blight than the boxwoods to which they compared. However, a declaration that boxwoods in general are more susceptible to boxwood blight may be premature, considering only one known *Sarcococca* species has been compared to other members of the Buxaceae, and in that study it was compared to the highly susceptible *Buxus sempervirens* 'Suffruticosa' (Kong and Hong, 2018). In general *Buxus* accessions were routinely among the most symptomatic plants in our study. However, this was not always the case, and in some cases highly symptomatic boxwoods could not be statistically separated from *Sarcococca*. Additionally, one of the boxwoods we used as a positive control was the highly susceptible *Buxus sempervirens* 'Suffruticosa'. Thus, it is not surprising that it among the most susceptible plants in our study. To fully understand how members of the Buxaceae compare regarding boxwood blight susceptibility, studies should be completed in which a wide range of taxa across all three susceptible ornamental genera are assessed for susceptibility to the disease.

Some plants in our study did appear to exhibit lower susceptibility to boxwood blight than other *Sarcococca*. One hypothesis regarding the reduced disease symptoms in several of the less symptomatic *Sarcococca ruscifolia* var. *chinensis* accessions is that the leaf surfaces deterred conidia from entering the leaves. The abaxial and adaxial leaf surfaces on these plants are remarkably smooth and may have caused water droplets containing conidia to easily slide off

the leaf surface before any conidia could germinate and enter the leaves through the cuticle or stomata. Many of these *S. ruscifolia* var. *chinensis* accessions showed similar susceptibility to other *Sarcococca* species during a detached leaf assay (Chapter 4). However, in a detached leaf assay, the leaves are placed horizontally, making it impossible for conidial solution to slide off. This suggests that it could be a leaf physical feature that contributes to resistance and not an existing physiological or inducible resistance mechanism. *Sarcococca vagans* (FRF) appeared to be less susceptible than other *Sarcococca* by being able to compartmentalize disease. It has very large leaves relative to most *Sarcococca* species, yet even after developing many water-soaked spots the disease did not expand like it did on other *Sarcococca*. These results are similar to those of a detached leaf assay in which *S. vagans* (FRF) tended to produce much more discrete spots than other *Sarcococca* (Chapter 4).

This study contributes to the growing body of work regarding susceptibility of members of the Buxaceae to boxwood blight. We have demonstrated that at least 7 *Sarcococca* species are susceptible to boxwood blight, and they can display symptoms as severe as boxwoods. An understanding of boxwood blight susceptibility in the Buxaceae is vital to disease management because members of this family are frequently cultivated together. In fact, the first two *Sarcococca* found to be infected with boxwood blight in the field were both found growing with *Buxus sempervirens* (Kong et al., 2017b; Malapi-Wight et al., 2016). An understanding of *Sarcococca* susceptibility to boxwood blight should be incorporated into any future disease management plants, and breeders should try to find and incorporate resistance to this disease in any future releases.

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Table 3.1. *Sarcococca* and *Buxus* accessions used to assess susceptibility to boxwood blight.

Plants were collected from gardens and nurseries in the United States and grown at the

University of Georgia Horticulture Farm, Watkinsville, GA.

Taxon	Source [X]
Buxus 'Green Gem'	HF
Buxus sempervirens 'Suffruticosa'	SFN
S. confusa	HF
S. confusa	SBG
S. hookeriana var. *digyna* 'Purple Stem'	PDN/JCN
S. hookeriana var. *humilis*	SBG
S. hookeriana var. *humilis*	WH
S. hookeriana var. *humilis* 'Sarsid 1' Fragrant Valley™	NC
S. hookeriana var. *humilis* 'Sarsid 2' Fragrant Mountain™	RFN
S. orientalis	ABG
S. orientalis	HFa
S. orientalis	SBG

Table continued from previous page.	
Taxon	**Source** [X]
S. ruscifolia	JCN
S. ruscifolia	SBGa
S. ruscifolia	WH
S. ruscifolia var. *chinensis*	ABG
S. ruscifolia var. *chinensis*	HFa
S. ruscifolia var. *chinensis*	PDN
S. ruscifolia var. *chinensis* 'Dragon Gate'	FRF
S. saligna	JCN
S. saligna	WH
S. sp.	ABG
S. vagans	FRF
S. wallichii	FRF

[X]**Source Abbreviations: HF**= University of Georgia Durham Horticulture Farm, Watkinsville,

GA; **ABG**=Atlanta Botanical Garden, Gainesville, GA; **FRF**=Far Reaches Farm, Port

Townsend, WA; **JCN**=Joy Creek Nursery, Scappoose, OR; **PDN**=Plant Delights Nursery,

Raleigh, NC; **RFN**=Rarefind Nursery, Jackson, NJ; **SBG**=State Botanical Garden of Georgia,

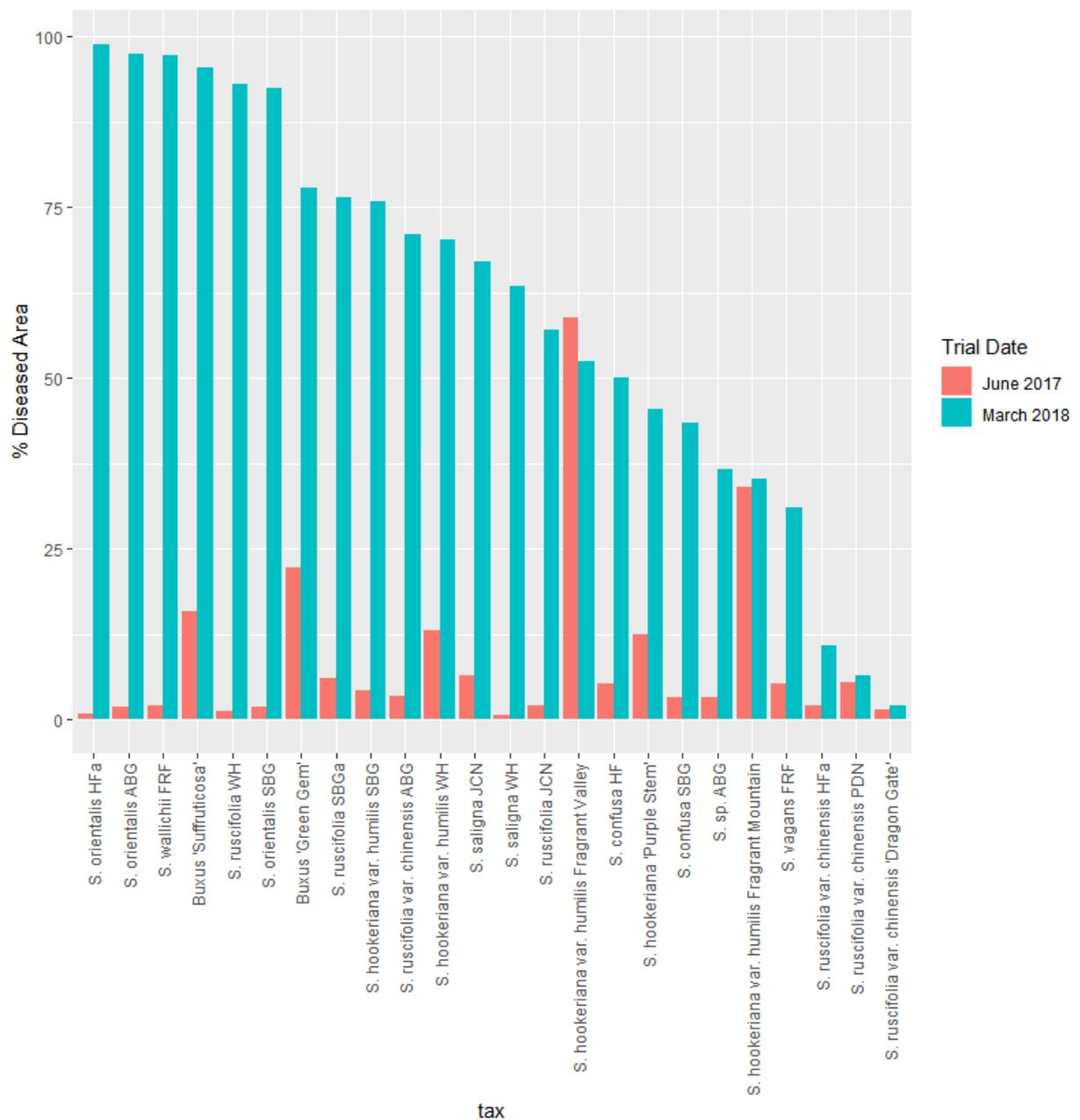Athens, GA; **SFN**= Silver Falls Nursery, Salem, OR; **WH**=Willis Harden, Commerce, GA

Figure 3.1 Percent diseased area for trial one (June 2017) and trial two (March 2018) nine to fourteen days after inoculation with *Calonectria pseudonaviculata* in a 20,000 conidia/ml solution. Trials were significantly different from each other (p <2 x 10^{-16}). Accesssions only present in the first trial are not presented in this figure.
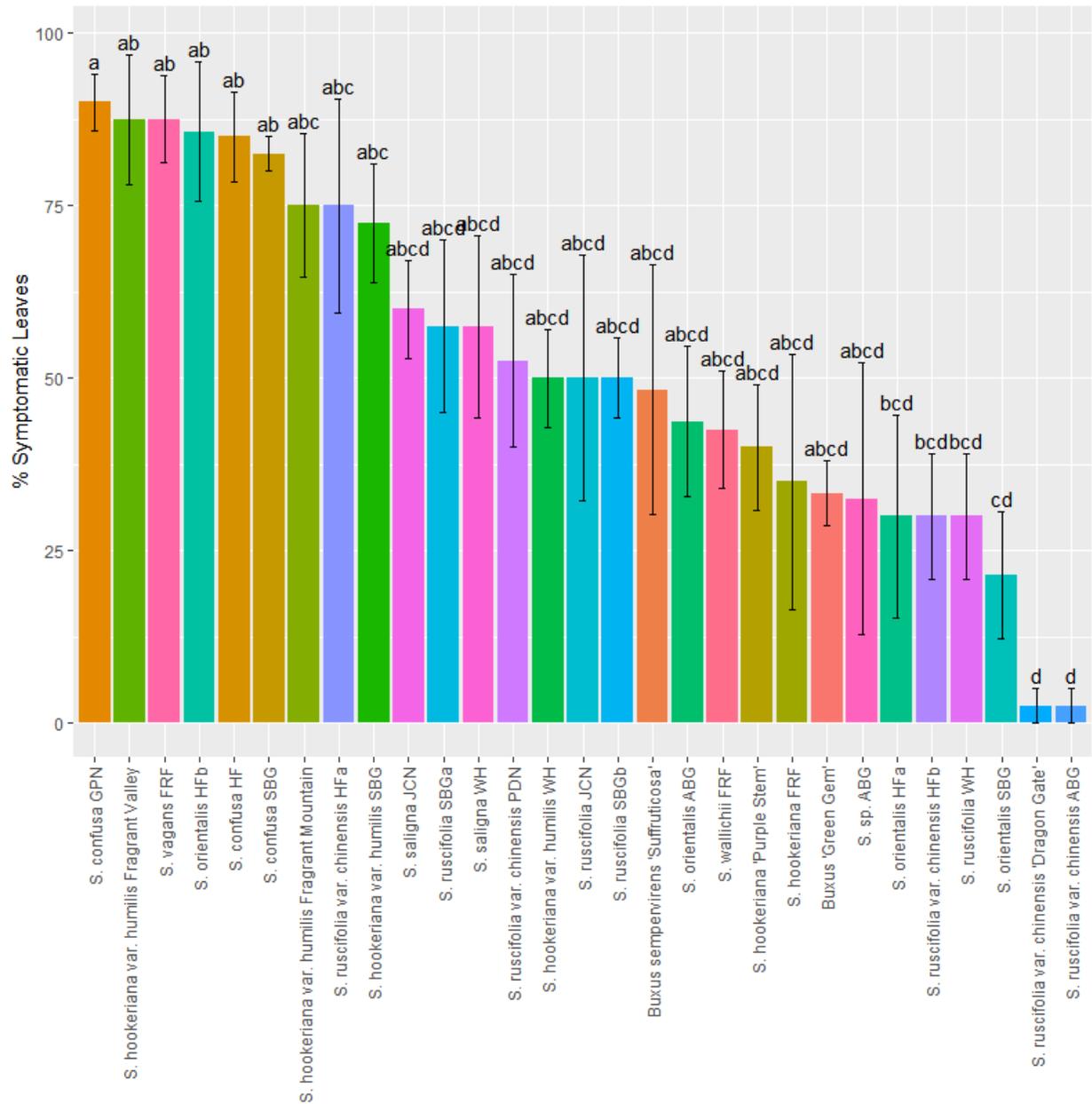
Figure 3.2. Percent of total number of leaves that developed water-soaked spots with a 95% confidence interval 3 days after stem dip inoculation with *Calonectria pseudonaviculata* conidia (20,000 conidia/ml) in June 2017. Shared letters above bars indicate no significant differences (p > 0.05). Trial date 1 includes five more accessions than trial date 2.
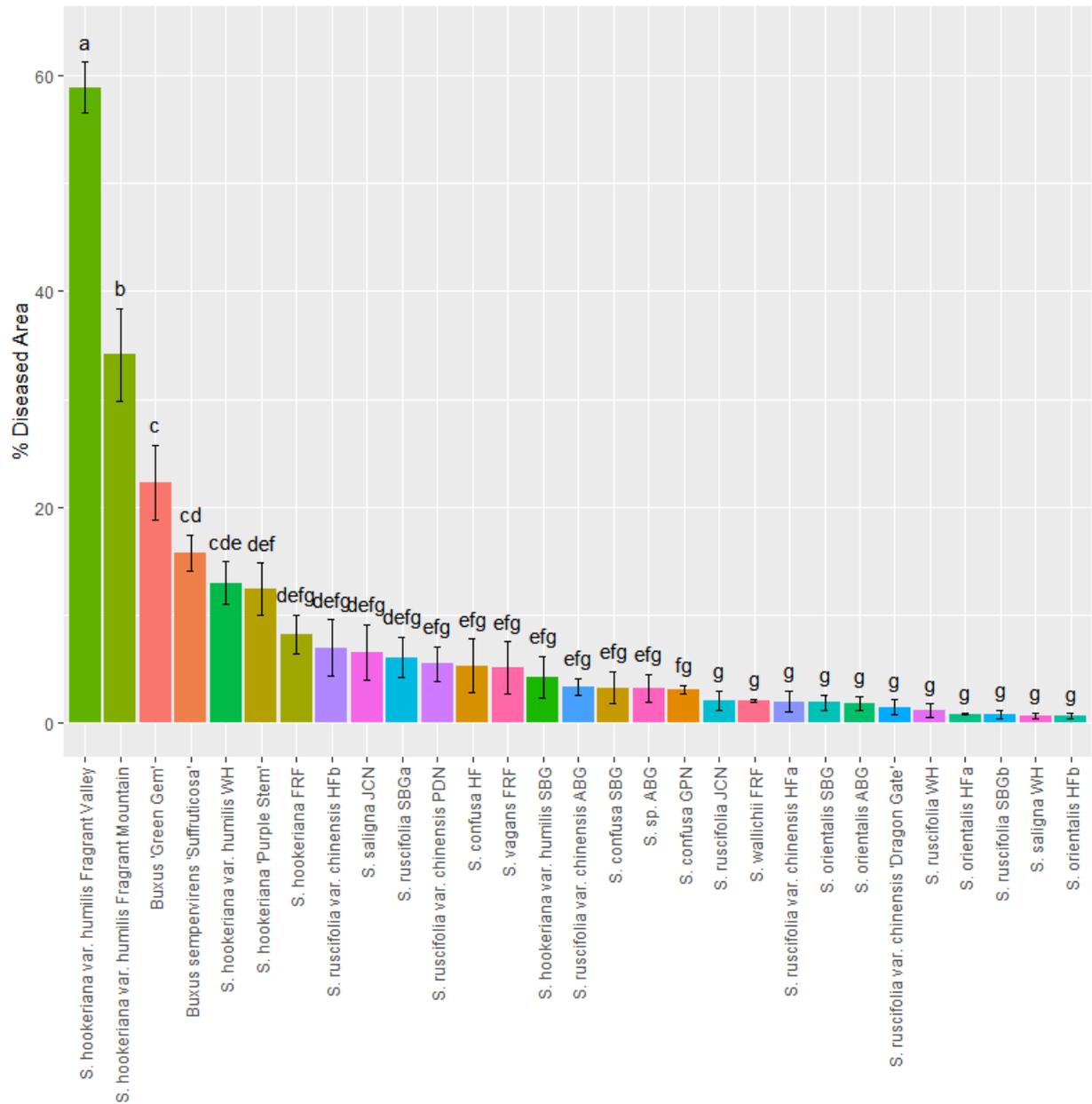
Figure 3.3. Percent of total leaf area showing boxwood blight symptoms with a 95% confidence interval 9 days after inoculation with *Calonectria pseudonaviculata* in a 20,000 conidia/ml solution in June 2017. Area was measured using Assess 2.0 and shared letters above bars indicate no significant differences (p > 0.05). Trial date 1 includes five more accessions than trial date 2.
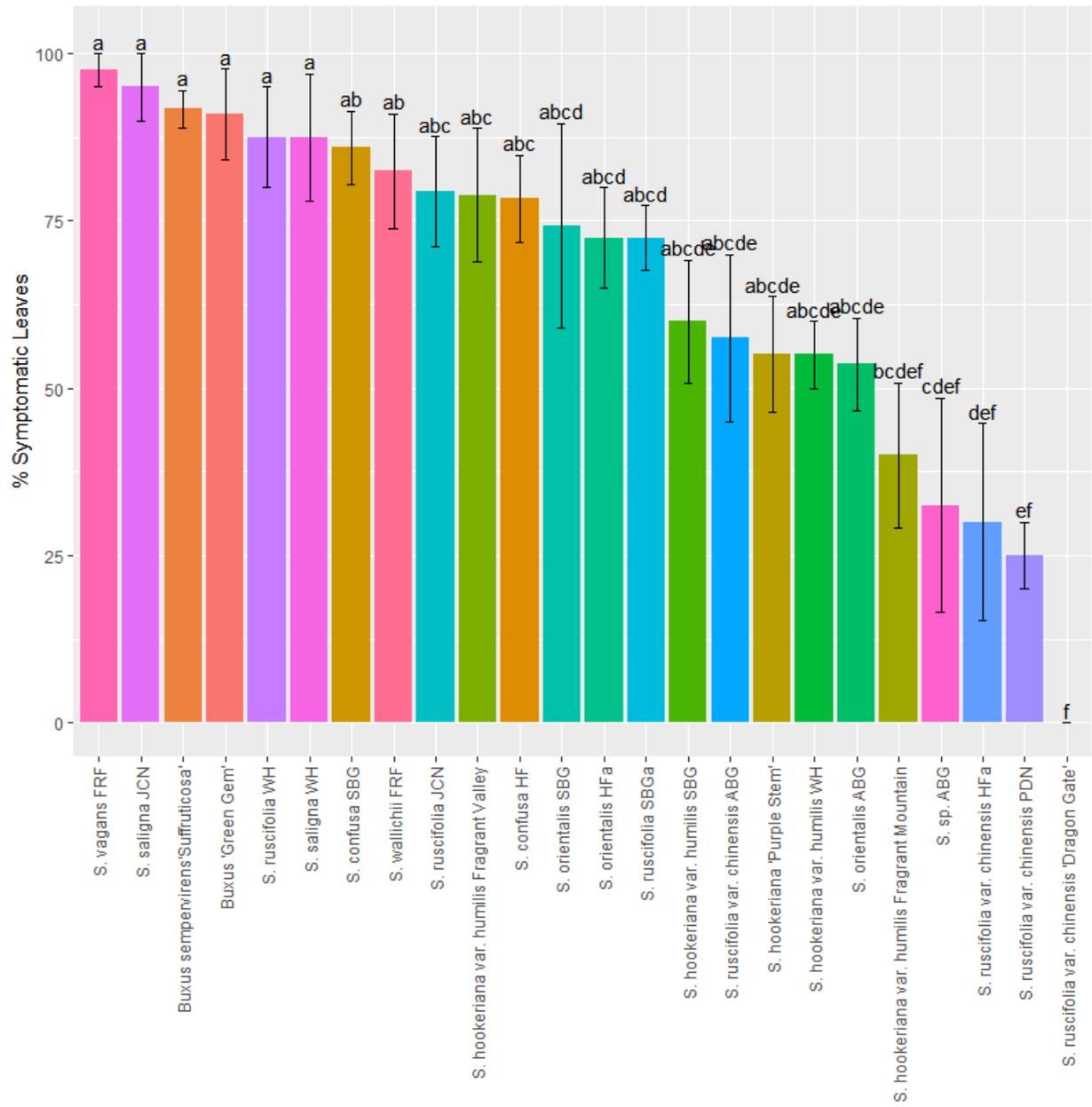
Figure 3.4. Percent of total number of leaves developing water-soaked spots with a 95% confidence interval 5 days after stem dip inoculation with *Calonectria pseudonaviculata* conidia (20,000 conidia/ml) in March 2018. Shared letters above bars indicate no significant differences (p > 0.05).
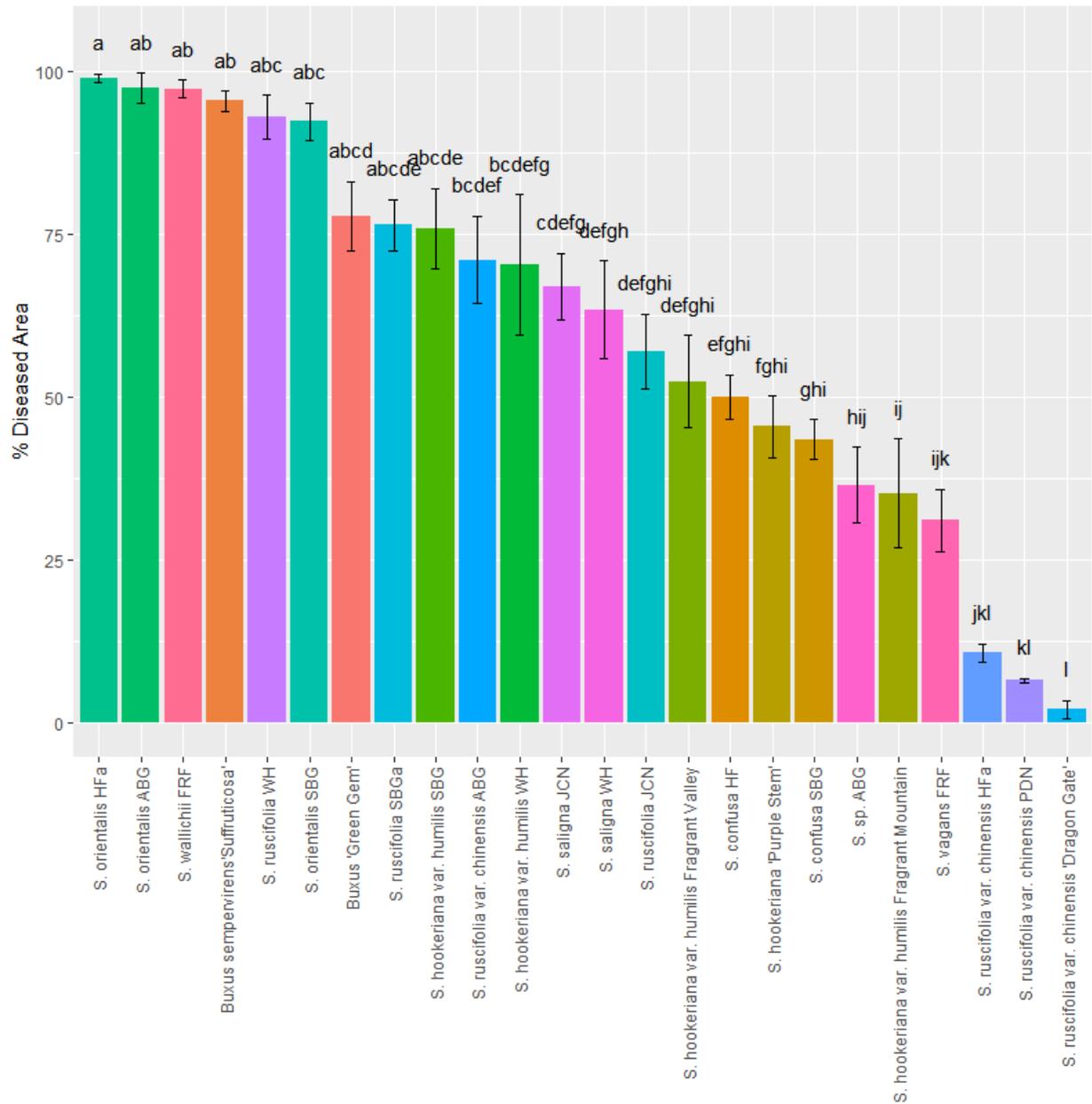
Figure 3.5. Percent of total leaf area showing boxwood blight symptoms with a 95% confidence interval 14 days after inoculation in a 20,000 conidia/ml solution in March 2018. Area was measured using Assess 2.0 and shared letters above bars indicate no significant differences (p > 0.05).
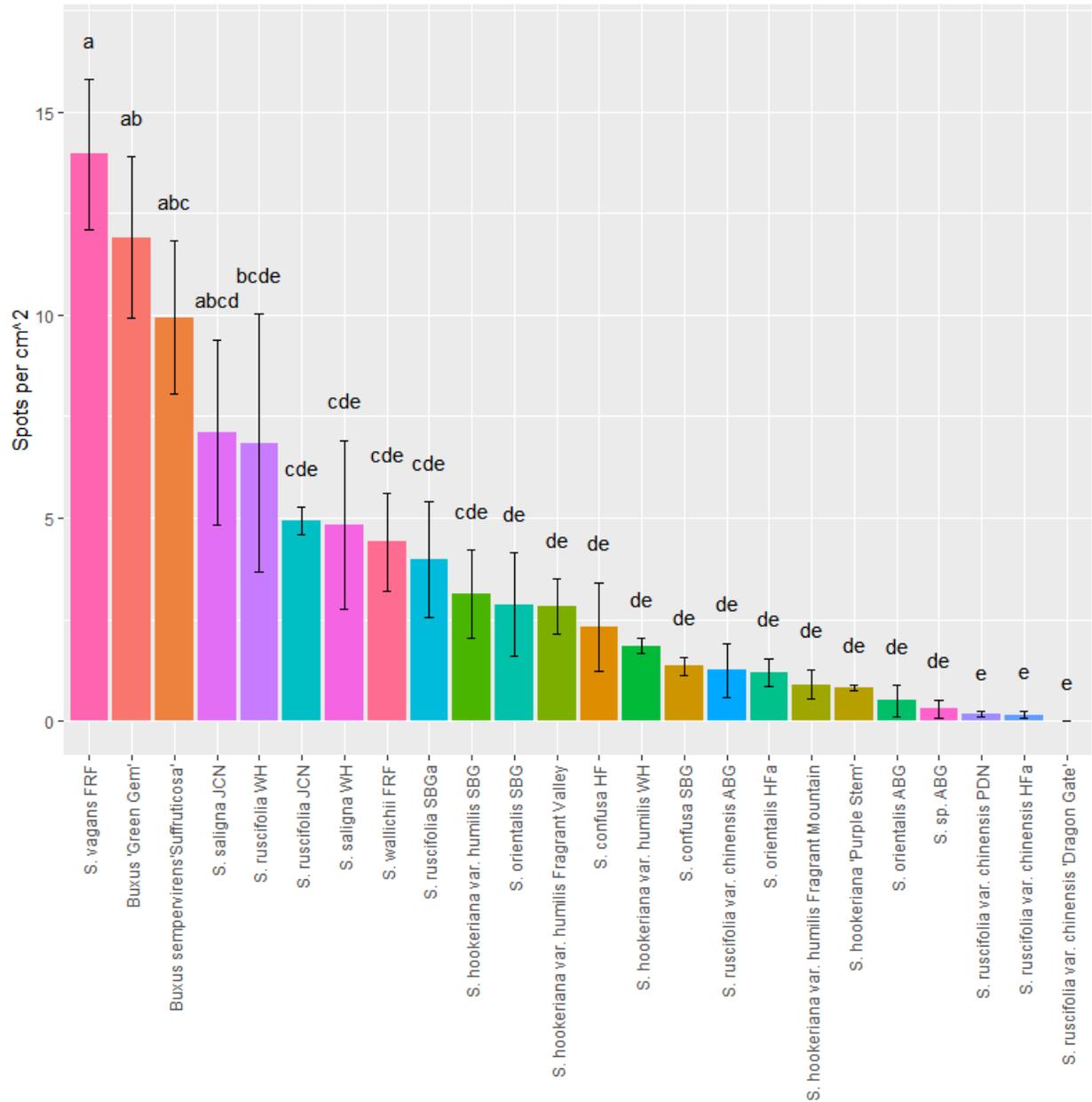
Figure 3.6. Number of water-soaked spots per leaf per average leaf area (cm^2). Total leaf area was measured 14 days after inoculation using Assess 2.0 in March 2018. Number of spots per leaf area was calculated by dividing the mean number of spots counted 5 days after inoculation by the average leaf size. Shared letters above bars indicate no significant differences (p > 0.05)., and error bars are 95% confidence intervals.

CHAPTER 4

SPORULATION OF *CALONECTRIA PSEUDONAVICULATA* FOLLOWING INOCULATION

OF *SARCOCOCCA* AND *BUXUS* TAXA[1]

Abstract

Boxwood blight caused by *Calonectria pseudonaviculata* was introduced to the United States in 2011. It is known to affect members of the Buxaceae, including *Buxus*, *Pachysandra*, and *Sarcococca*. A detached leaf assay was used to assess the boxwood blight susceptibility and conidia production on 26 *Sarcococca* accessions and 2 *Buxus* accessions. Sixteen to seventeen days following inoculation with drops of conidial solution containing approximately 200 conidia each, total disease area and conidia production were measured. Boxwoods showed the highest amounts of conidia production (>64,000 conidia per cm^2) and diseased area (>90% total area), while *Sarcococca* accessions produced an estimated 2,926 to 52,623 conidia and had diseased area ranging from 5 to 74% of total area. Few statistical differences were detected for spore production and disease susceptibility for the *Sarcococca* accessions except that they appeared to sporulate less than the boxwood controls. Some individual accessions produced a significant amount of conidia overall, but these conidia only came from a few plates. Leaf spots on three closely related species (*S. saligna, S. wallichii,* and *S. vagans* ranged from 10-18% of total diseased area and may represent some disease resistance. Other species, especially several *S. ruscifolia* var. *chinensis* accessions, produced conidia and developed diseased area in this assay, contrary to results in the detached stem assay in Chapter 3. This is the first detached leaf screening of multiple *Sarcococca* accessions for conidia production and boxwood blight susceptibility. Understanding the susceptibility of non-boxwoods to boxwood blight is an important aspect of disease management and should be considered moving forward.

Introduction

Boxwoods (*Buxus* spp.) are among the most common landscape plants in the United States and commercially were valued at an estimated $126 million in 2014 (United States Department of Agriculture, 2015). Currently a new disease that defoliates and kills boxwood and other members of the Buxaceae is spreading worldwide, with known reports in Europe, North America, the Middle East, and New Zealand (Crous et al., 2002; Henricot and Culham, 2002; Ivors et al., 2012; Lehtijärvi et al., 2014).The disease is now known in at least 25 states (LaMondia and Shishkoff, 2017; Williams-Woodward, 2015).

The causal pathogen of boxwood blight is in the genus *Calonectria*. There are two known species, both of which cause similar symptoms, though only *C. pseudonaviculata* has been found in the United States (Gehesquière et al., 2016). The disease is known to infect members of the Buxaceae, including *Buxus*, *Pachysandra*, and *Sarcococca* (Ivors et al., 2012; Kong et al., 2017; LaMondia et al., 2012). Disease susceptibility screening for the disease has been performed on many boxwood taxa and some *Pachysandra*, but *Sarcococca* has been neglected (Ganci, 2013; Guo et al., 2015; LaMondia, 2017; LaMondia and Shishkoff, 2017; Shishkoff et al., 2015). Only one unidentified *Sarcococca* species and *S. hookeriana* var. *humilis* have been screened for susceptibility to the disease (Henricot et al., 2008; Kong and Hong, 2018), despite at least 7 species in cultivation in the United States and more grown worldwide (Dirr, 2009; personal observation). Understanding how *Sarcococca* taxa respond to the disease is important for understanding this disease and how to manage it.

Disease assays have varied with different combinations of host plant material and inoculum used. Detached leaves and stems are space- and cost- effective means of evaluating susceptibility to boxwood blight and have been used regularly for susceptibility evaluations.

(Guo et al., 2015; Guo et al., 2016; LaMondia and Shishkoff, 2017; Shishkoff et al., 2015). Inoculation methods have included pipetting a conidial solution onto detached leaves and spraying whole plants with a mycelium or conidial solution (Guo et al., 2016; LaMondia and Shishkoff, 2017). Similar results have been found between whole plant and detached leaf inoculations, though detached leaves tended to have higher amounts of disease. Consequently, we used detached leaves to assay boxwood blight susceptibility in *Sarcococca* and estimate the ability of cultivated accessions to produce conidia following infection.

Materials and Methods

Plant Materials

Cultivated *Sarcococca* and *Buxus* taxa were acquired from several nurseries and gardens (Table 4.1) and were grown at University of Georgia Durham Horticulture Farm, Watkinsville, GA. Stems with leaves that were sufficiently hardened off were collected from plants in our collection and nearby gardens in October 2018 and stored at 4°C until use. *Sarcococca* accessions are the focus of these experiments, but two boxwood taxa, *Buxus sempervirens* 'Suffruticosa' and *B.* 'Green Gem', were selected to serve as positive controls. Leaves were detached from stems, rinsed free of debris using double distilled water, blotted dry using paper towels, and then trimmed using a sterile scalpel to lay flat in 15mm x 90 mm plastic plates containing 1% water agar (10 g bactoagar/ per liter of deionized water).

Inoculum

Single-conidial isolates of *Calonectria pseudonaviculata* collected from four locations (Cp1-C, Cp2-C, Cp2-5, Cp2-6, Cp4-B, Cp5-A) were incubated on potato dextrose agar (PDA)

(BD Difco, Sparks, MD) amended with ampicillin trihydrate (250 mg/L) (Sigma Aldrich; St.

Louis, MO) for 2 weeks at room temperature in ambient light. Culture plates were flooded with

sterilized deionized water (SDW) for 2 hours, decanted, scraped with a sterile metal spatula to

remove aerial hyphae, and rinsed with SDW by swirling and decanting. Plates were then

incubated upside-down for 5 days at 23°C under a 12 hr light/dark cycle. Conidia were harvested

by spritzing plates with SDW using a handheld spray bottle while the plate was held over a

beaker. The resulting conidial suspension was filtered through three layers of sterile cheesecloth

to remove clumps of hyphae. Conidia were counted using a hemocytometer and adjusted to a

final concentration of 2×10^4 conidia/ml.

Detached Leaf Inoculations

Detached leaves were placed with their abaxial surface facing upward in three plastic

plates total, two for boxwood blight inoculations and one for a sterile distilled water negative

control. For large leaves, two leaves were placed on each plate, and for small or narrow leaves,

four leaves or leaf segments were placed on each plate. The 20,000 conidia/ml solution was

pipetted onto the abaxial surface of detached leaves in single 10 µl drops (approximately 200

conidia per drop). Large leaves/leaf sections received two drops diagonally opposite each other

on either side of the leaf midvein and small leaves/leaf sections received a single drop each,

meaning the leaves on each plate received a total of four drops of conidial solution.

The plates were placed on metal trays and then covered in plastic wrap to maintain high

moisture levels. Disease was allowed to develop under ambient laboratory conditions. Twelve

days after inoculation, leaves were photographed, and infected leaf area and total leaf area were

determined using Assess 2.0, the Image Analysis Software for Plant Disease Quantification (APS

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Press, St. Paul, MN). Sixteen to seventeen days after inoculation, leaves on each plate were forcefully sprayed with a spray bottle containing distilled water to dislodge conidia. Number of conidia per the collected volume of sprayed water per plate was estimated using a hemocytometer. Number of conidia per infected leaf area was then calculated (Table 4.1).

Statistical Analysis

Leaves were randomly chosen to be inoculated with the boxwood blight pathogen or SDW. The data were analyzed based on a completely randomized experimental design with a plate representing an experimental unit. The effect of date on conidia per infected area and percentage of total infected area was determined not to be significant (p>0.05) following two-sample t-tests, so data for both dates were combined. We assessed the differences in mean number of conidia per infected square centimeter and percentage of total infected area using a simple one-way analysis of variance (ANOVA) in R (R Core Team, 2017). Means were separated using Tukey's Honest Significant Difference and were grouped using the HSD.test function from the R package agricolae. For both conidia/cm^2 and percentage of total area infected, boxwoods had the highest values, though not always with statistical significance (p≤0.05). To see if there were any trends solely among *Sarcococca*, the boxwood accessions were removed from the analysis and an ANOVA was run again for each dependent variable. To assess the susceptibility and conidia production of *Sarcococca* and *Buxus* by species, accessions were grouped by taxon. Species were grouped primarily by species name, though there is evidence that some of our *S. ruscifolia* accessions (SBGa, WH, JCN) are genetically more similar to *S. confusa* than to *S. ruscifolia* var. *chinensis* (Chapter 2). Thus, some *S. ruscifolia* accessions were sorted into the *S. confusa* group and the rest were grouped with plants labeled *S.*

ruscifolia var. *chinensis*. Additionally, *Sarcococca hookeriana* var. *humilis* is occasionally

elevated to species level (*S. humilis*) but was grouped with all *S. hookeriana* for this analysis.

Due to uneven sample sizes, means were separated using the nonparametric Kruskal-Wallis test

in the agricolae package in R. This analysis uses Fischer's least significant difference criterion

with the function kruskal. Data were considered statistically significant at $p \leq 0.05$.

Results

Response of Accessions to Boxwood Blight Following Detached Leaf Inoculations

All plants screened in this study showed boxwood blight symptoms, and *Calonectria*

pseudonaviculata conidia were produced from each accession, indicating that immunity to the

disease is not present in any taxa used in this study (Table 4.1). Water-soaked spots appeared on

inoculated leaves as soon as two days after inoculation, and total infected area gradually spread

over the course of the experiments (data not shown).

Boxwood accessions produced significantly more conidia per cm^2 (>64,000 conidia/cm^2)

than all *Sarcococca* accessions. The boxwoods also had significantly greater infected area

overall (>90% of total area) than 14 different *Sarcococca* accessions (Table 4.1). However,

Buxus 'Green Gem' was the only boxwood that showed significant difference ($p \leq 0.05$) from all

or most *Sarcococca* taxa for both conidia/cm^2 and percent symptomatic area, despite *Buxus*

sempervirens 'Suffruticosa' being repeatedly found to be highly susceptible to boxwood blight

(Ganci et al., 2013; Guo et al., 2016; Shishkoff et al., 2015). *Sarcococca confusa* (HF) produced

52,623 conidia/cm^2 of diseased area, which was the most of all *Sarcococca*, yet this was not

significantly different from any *Sarcococca* accessions when boxwoods were included in the

analysis. *Sarcococca confusa* (HF) was also among the *Sarcococca* with the greatest percentage

of symptomatic leaf area at 64%, behind *S. confusa* (GPN, 67%), *S. hookeriana* var. *humilis* (WH, 66%), and *S. hookeriana* 'Purple Stem' (74%). These and 6 other *Sarcococca* accessions did not differ significantly from the boxwoods for percentage of symptomatic leaf area. No obvious trend could be discerned regarding which species showed the greatest infection percentage. Most *Sarcococca* accessions produced similar conidia/cm^2 infected tissue as *Buxus sempervirens* 'Suffruticosa', indicating equal susceptibility.

With the boxwoods removed, *Sarcococca* accessions produced similar conidia/cm^2 (Table 4.1). Additionally, *S. confusa* (HF) was the only accession found to be statistically significantly different from other *Sarcococca* accessions for conidia/cm^2. It was significantly different from 5 accessions (Table 4.1): three *S. ruscifolia* (JCN, SBGb, PDN), *S. orientalis* (HFb), and *S. vagans* (FRF). Conidia/cm^2 for these six accessions ranged from 2969 to 7922. All other accessions were determined not to be statistically different from each other. For percent diseased area, the 18 accessions with greatest percent symptomatic were not significantly different from each other, though percentage ranged from 26 to 74%.

Species Relationships for Disease Susceptibility

Trends have been reported for susceptibility of different *Buxus* species groups to boxwood blight, so accessions were grouped by species to assess species relationships (Henricot et al., 2008; LaMondia and Shishkoff, 2017). Grouping accessions by taxonomic classification did reveal some trends regarding species relationships. Boxwoods were statistically different from all *Sarcococca* for conidia/cm^2 (Figure 4.1). *Sarcococca* species groupings ranged from 7345 to 22825 conidia/cm^2, and *S. hookeriana*, *S. ruscifolia/confusa*, and *S. ruscifolia* var.

chinensis were significantly different from *S.* sp. and *S. vagans*. For percent symptomatic area, no *Sarcococca* species were significantly different from each other (Figure 4.2).

The boxwood leaves were almost completely diseased, with percent symptomatic area ranging from 92 to 98%. The boxwoods were statistically different from all *Sarcococca* except *S. sp.*, which had a percent diseased area of 60.5%. *Sarcococca* sp. was the had the 7th most diseased area of all taxa screened, while producing the 6th least conidia/cm^2. Percent diseased area in *S. wallichii* (18%), *S. saligna* (17%), and *S. vagans* (10%) was relatively low and significantly different from all other groups for *S. saligna* and *S. vagans*. These species produced very discrete spots relative to other accessions following inoculation (Figure 4.3). Most accessions became increasingly brown and covered in hyphae, while the diseased areas on *S. wallichii*, *S. saligna*, and *S. vagans* remained very intact and defined. These three species appear to be closely related (Denaeghel et al., 2017; von Balthazaar et al., 2002; Chapter 2) and may represent a group of taxa more capable of slowing the spread of infection than others.

Discussion

This is the first study comparing susceptibilities of multiple *Sarcococca* taxa to boxwood blight caused by *Calonectria pseudonaviculata*. While it was difficult to discern any trends regarding certain accessions or taxonomic groups, this study demonstrates that a wide range of *Sarcococca* are susceptible to boxwood blight, though seemingly less so than some boxwoods. Prior to this study, the susceptibility of one unidentified *Sarcococca* species and eleven boxwood accessions was assessed using detached leaves from whole stems that had been dipped in 10^6 conidia/ml suspension (Henricot et al., 2008). This study included *Buxus sempervirens* 'Suffruticosa' and *Buxus* 'Green Gem', both of which were also included in our study. The

unidentified *Sarcococca* accession produced statistically fewer conidia per leaf area across two (of three) different isolates than nine of ten boxwood species screened. The only boxwood species with comparable conidia production for those two isolates was *B. balearica*. Similarly, Kong and Hong (2018) compared the susceptibility of *Buxus sempervirens* 'Suffruticosa', *Pachysandra terminalis*, and *Sarcococca hookeriana* var. *humilis* to boxwood blight following inoculation with *Cps* isolates collected from all three taxa. Significantly smaller lesions, fewer conidia, and fewer microsclerotia were produced on non-boxwood hosts than on *Buxus sempervirens* 'Suffruticosa'. These two studies, coupled with our results, suggest that conidia production and disease symptoms are greater on boxwoods than on *Sarcococca*. Still, a study containing Buxaceae taxa of many susceptibilities should be performed before any blanket statements regarding comparisons of susceptibility of the different genera are made.

Detached leaf assays for boxwood blight susceptibility are a space and cost-saving method for disease evaluation and in general produce similar results to whole plant and detached stem assays, though disease incidence is often greater for detached leaves (Guo et al., 2015; Guo et al., 2016). Results of boxwood blight disease assays are not always uniform, however, and assay method and measured variables can influence susceptibility ratings. Using a detached leaf assay similar to ours, *Buxus* 'Green Gem' was found to not be significantly different from *Buxus sempervirens* 'Suffruticosa' for percent of leaves showing lesions on abaxial leaf surface, percent abaxial leaf symptomatic, and percent abaxial leaves with sporulation (LaMondia and Shishkoff, 2017). It was, however, found to be significantly different for percent of leaves showing lesions on the adaxial leaf surface and was numerically more susceptible for all variables measured in the detached leaf assay. In that same study, *Buxus* 'Green Gem' and *Buxus sempervirens* 'Suffruticosa' were determined to not significantly differ in number of lesions per plant

following whole plant inoculations. They did significantly differ once number of lesions per plant was normalized for plant size. In another study, *Buxus suffruticosa* 'Suffruticosa' and *Buxus* 'Green Gem' did not differ significantly regarding sporulation on leaves following stem inoculations with three boxwood blight isolates, though they did significantly differ in the percentage of leaves showing greater than 50% spotting (Henricot et al., 2008). In a 2012 study, *Buxus* 'Green Gem' was rated as moderately tolerant versus *Buxus sempervirens* 'Suffruticosa', which was rated as highly susceptible following whole plant inoculations (Ganci et al., 2012). Susceptibility rating clearly depends on the method of inoculation and the variables measured. This may explain why *Buxus* 'Green Gem' produced more conidia and had a greater diseased area than the reported highly susceptible *Buxus sempervirens* 'Suffruticosa' in our study. To fully represent disease susceptibility, different susceptibility assays should be viewed in conjunction with each other. Using leaves alone can exclude important aspects of plant disease resistance including plant form and systemic resistance (LaMondia and Shishkoff, 2017; Orlowska et al., 2013). Conversely, using a susceptibility scale based solely on plant appearance leaves out important disease factors such as amount and rate of conidia production.

Because *Sarcococca* is an ornamental plant, tolerance of the disease is an important factor when assessing how a plant responds to the disease. All taxa in this study developed significant spots and leaf damage following inoculation, though some were able to isolate the damage better than others. The ability of a plant to compartmentalize disease may be a key factor in identifying selections tolerant to boxwood blight. *Sarcococca saligna*, *S. wallichii*, and *S. vagans* accessions (Figure 4.3) showed discrete spots relative to other accessions and may indicate a higher level of resistance to boxwood blight. On the other hand, only *S. vagans* produced a number of conidia/cm^2 statistically different from some other species groups. This

type of relationship between conidia production and size of symptomatic area may mislead those trying to manage this disease and may lead to greater infection in plantings of more than one plant. This type of relationship, in which the intensity of symptoms does not represent the amount of sporulation has been found in a different devastating disease, *Phytophthora ramorum* Werres, De Cock & Man in 't Veld (Jinek et al., 2011). In a detached stem assay for disease resistance (Chapter 3), the *S. saligna* and *S. wallichii* accessions developed a high degree of diseased leaf area, contrary to results presented here, suggesting other factors such as environment, inoculated plant organ, plant architecture, or inoculation method may play a role in how disease develops.

Several plants from the detached stem assay (Chapter 3) that showed low amounts of water-soaked spot production or low amounts of diseased area had high amounts of conidia production and diseased leaf area in this study. *Sarcococca ruscifolia* var. *chinensis* 'Dragon Gate' had less than 2% diseased area between both studies and was the only accession to show very low susceptibility for both dates, yet it produced 17,605 conidia/cm^2 and had 31% diseased area by the end of this study. Conidia production and infected area for this plant is not significantly different from most *Sarcococca* accessions tested, suggesting the low disease symptoms in the stem inoculations may be a result of plant physical features and not existing physiological or inducible resistance mechanisms. *Sarcococca ruscifolia* var. *chinensis* (ABG and PDN) also exhibited low symptom development in at least one of the detached stem trials. *Sarcococca ruscifolia* var. *chinensis* (ABG) produced 19,328 conidia across 59% diseased area, while *S. ruscifolia* var. *chinensis* (PDN) produced the second least amount of conidia/cm^2 (6314) and was among the lowest accessions to diseased area.

Some taxa did not develop significant amounts of diseased area. *Sarcococca ruscifolia* (SBGb) had a mean diseased area of 0.94 cm^2, in part because some leaves developed almost no diseased area at all or the diseased area did not expand beyond a few water-soaked spots. This accession did produce a total of 91,664 conidia, though they all came from a single plate which had a total diseased area of 3.07 cm^2. This trend was also observed on *S. ruscifolia* var. *chinensis* (PDN), which produced 113,333 of a total 133,333 conidia on leaves from a single plate that also made up 66% of total diseased area. This variability in infectability of leaves may indicate some resistance to boxwood blight. It also suggests that if the infected leaves do sporulate, they can produce a large amount of conidia.

Boxwood blight susceptibility of boxwood relatives like *Sarcococca* underscores the importance of understanding the host range of this disease for effective disease management. *Sarcococca* and *Pachysandra* are commonly planted among and near boxwoods. If infected, these taxa can provide multiple sources of inoculum to manage and be aware of in addition to boxwoods. Even though sporulation and percent symptomatic area for *Sarcococca* was less than in boxwoods in our study, it is important to be aware that these plants still support the boxwood blight pathogen. Species that are less symptomatic but still support the disease can serve as a "Trojan Horse" in which they still produce inoculum that can be spread to nearby plants even though they may not appear to be highly infected (Ganci, 2014). Growers and gardeners need to consider and be aware of all possible hosts and the symptomology if they wish to effectively manage this disease.

This study provides evidence that at least seven *Sarcococca* species are susceptible to boxwood blight. We did not detect many trends among species and accessions but did find the *Sarcococca* to produce less conidia/cm^2 than the boxwoods. In preparing for this experiment, we

found that spraying inoculated leaves with a spray bottle and then allowing disease to develop for several more weeks increased conidia production (data not shown). It may be that *Sarcococca* accessions need more of an environmental trigger to increase disease activity, and this may explain the lack of significance we found for disease parameters measured. Future experiments such as whole plant and detached stem inoculations, as well as different types of inoculum, would help better our understanding of how *Sarcococca* respond to this emerging pathogen.

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Table 4.1 Conidia production and infected leaf area of *Buxus* and *Sarcococca* accessions following detached inoculation with boxwood blight caused by *Calonectria pseudonaviculata*. Leaves were inoculated with drops containing approximately 200 conidia, and disease was allowed to develop for 16 to 17 days.

Taxon	Source[X]	Conidia/ diseased cm^2 with *Buxus* spp.	Conidia/ diseased cm^2 without *Buxus* spp.	Percent Infected Area with *Buxus* spp.	Percent Infected Area without *Buxus* spp.
Buxus 'Green Gem'	HF	165077 a	165077 NA	98 a	98 NA
Buxus sempervirens 'Suffruticosa'	SFN	64562 b	64562 NA	92 ab	92 NA
S. confusa	HF	52623 bc	52623 a	64 abcde	64 abc
S. ruscifolia var. *chinensis*	HFb	26527 bc	26527 ab	55 abcdefg	55 abcde
S. hookeriana var. *humilis* 'Sarsid 1' Fragrant Valley™	NC	26365 bc	26365 ab	47 bcdefgh	47 abcdef
S. confusa	GPN	26297 bc	26297 ab	67 abcd	67 ab
S. confusa	SBG	20725 bc	20725 ab	14 fgh	14 def
S. hookeriana var. *humilis* 'Sarsid 2' Fragrant Mountain™	RFN	20060 bc	20060 ab	54 abcdefg	54 abcdef
S. hookeriana 'Purple Stem'	PDN/JCN	19503 bc	19503 ab	74 abc	74 a
S. ruscifolia var. *chinensis*	ABG	19328 bc	19328 ab	59 abcdef	59 abcde
S. hookeriana var. *humilis*	SBG	17877 bc	17877 ab	26 cdefgh	26 abcdef

Table continued from previous page.					
Taxon	**Source**[X]	**Conidia/ diseased cm^2** [Y]	**Conidia/ diseased cm^2 without _Buxus_ spp.**	**Percent Infected Area**[Y]	**Percent Infected Area without _Buxus_ spp.**
S. ruscifolia var. _chinensis_ 'Dragon Gate'	FRF	17605 bc	17605 ab	31 cdefgh	31 abcdef
S. ruscifolia	SBGa	17015 bc	17015 ab	46 bcdefgh	46 abcdef
S. hookeriana var. _humilis_	WH	15662 bc	15662 ab	66 abcd	66 ab
S. saligna	JCN	15653 bc	15653 ab	21 defgh	21 bcdef
S. wallichii	FRF	14686 bc	14686 ab	18 defgh	18 bcdef
S. orientalis	SBG	12942 bc	12942 ab	16 efgh	16 cdef
S. ruscifolia	WH	12370 bc	12370 ab	34 cdefgh	34 abcdef
S. orientalis	HFa	10929 bc	10929 ab	31 cdefgh	31 abcdef
S. saligna	WH	9986 bc	9986 ab	13 fgh	13 def
S. ruscifolia var. _chinensis_	HFa	9950 bc	9950 ab	51 abcdefgh	51 abcdef
S. orientalis	ABG	9525 bc	9525 ab	54 abcdefg	54 abcdef
_S._ sp.	ABG	8918 bc	8918 ab	61 abcdef	61 abcd
S. ruscifolia	JCN	7922 bc	7922 b	41 cdefgh	41 abcdef
S. ruscifolia	SBGb	7465 c	7465 b	5 h	5 f
S. vagans	FRF	7345 c	7345 b	10 gh	10 ef
S. ruscifolia var. _chinensis_	PDN	6314 c	6314 b	15 fgh	15 cdef
S. orientalis	HFb	2969 c	2969 b	34 cdefgh	34 abcdef

[X]**Source Abbreviations: HF**= University of Georgia Durham Horticulture Farm, Watkinsville,

GA; **ABG**=Atlanta Botanical Garden, Gainesville, GA; **FRF**=Far Reaches Farm, Port

Townsend, WA; **GPN**=Griffith Propagation Nursery, Watkinsville, GA; **NC**= Nurseries

Caroliniana, North Augusta, SC; **JCN**=Joy Creek Nursery, Scappoose, OR; **PDN**=Plant Delights

Nursery, Raleigh, NC; **RFN**=Rarefind Nursery, Jackson, NJ; **SBG**=State Botanical Garden of

Georgia, Athens, GA; **SFN**= Silver Falls Nursery, Salem, OR; **WH**=Willis Harden, Commerce,
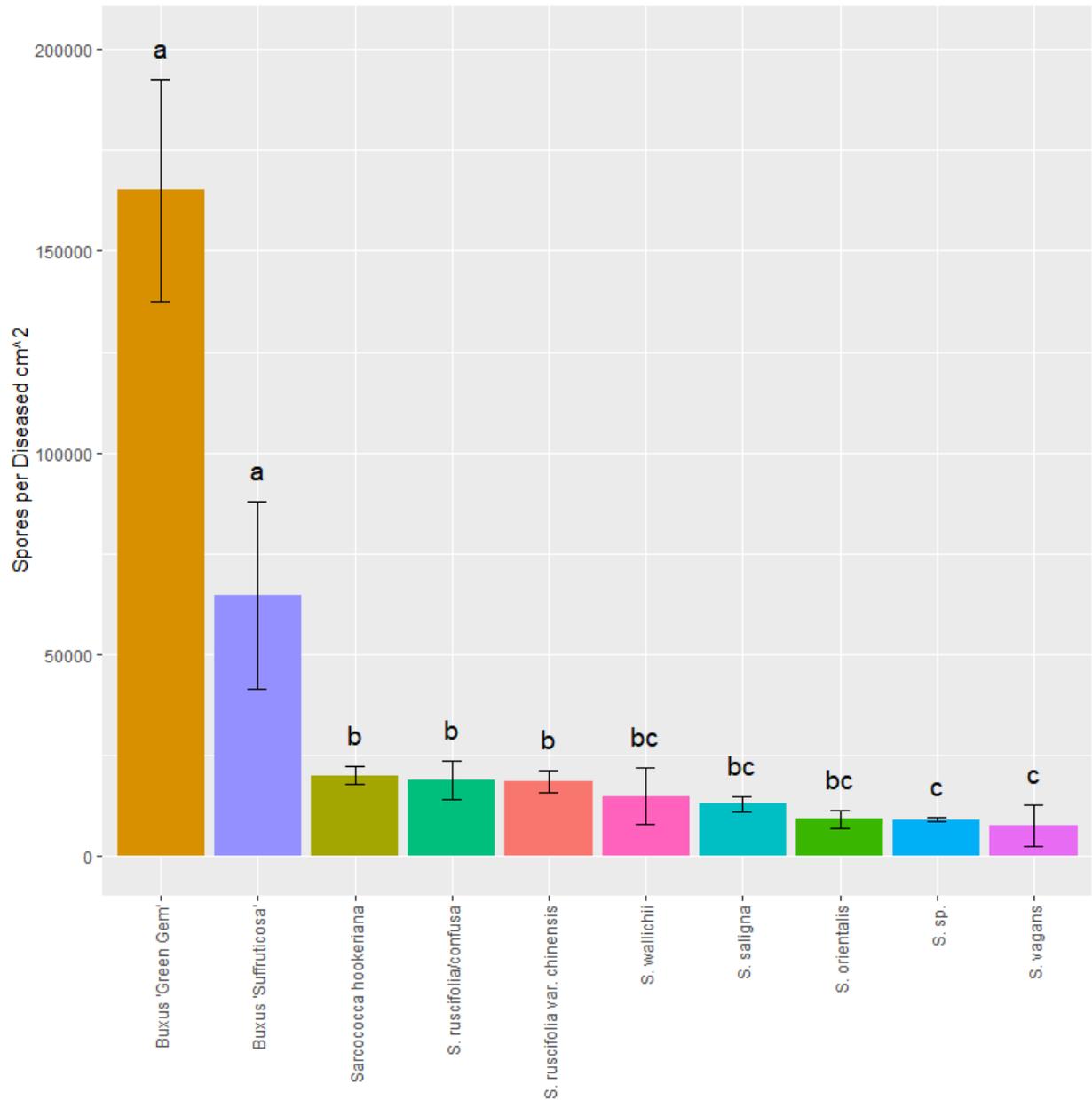
GA

Figure 4.1. Conidia production per diseased leaf area (cm^2) 16-17 days after inoculation with a 20,000 conidia/ml solution. Letters indicate Fischer's least significant difference groupings following mean separation using the Kruskal-Wallis test. Bars containing the same letters are not considered statistically different ($p > 0.05$). A 95% confidence interval is included on each bar. Accessions were grouped by species. *Sarcococca ruscifolia* (SBGa, WH, JCN) were grouped with *S. confusa* based on genetic data from Chapter 2.

Figure 4.2 Percent diseased leaf area 16-17 days after inoculation following inoculation with a 20,000 conidia/ml solution. Letters indicate Fischer's least significant difference groupings following mean separations using the Kruskal-Wallis test. Bars containing the same letters are not considered statistically different ($p > 0.05$). A 95% confidence interval is included on each

bar. Accessions were grouped by species. *Sarcococca ruscifolia* (SBGa, WH, JCN) were

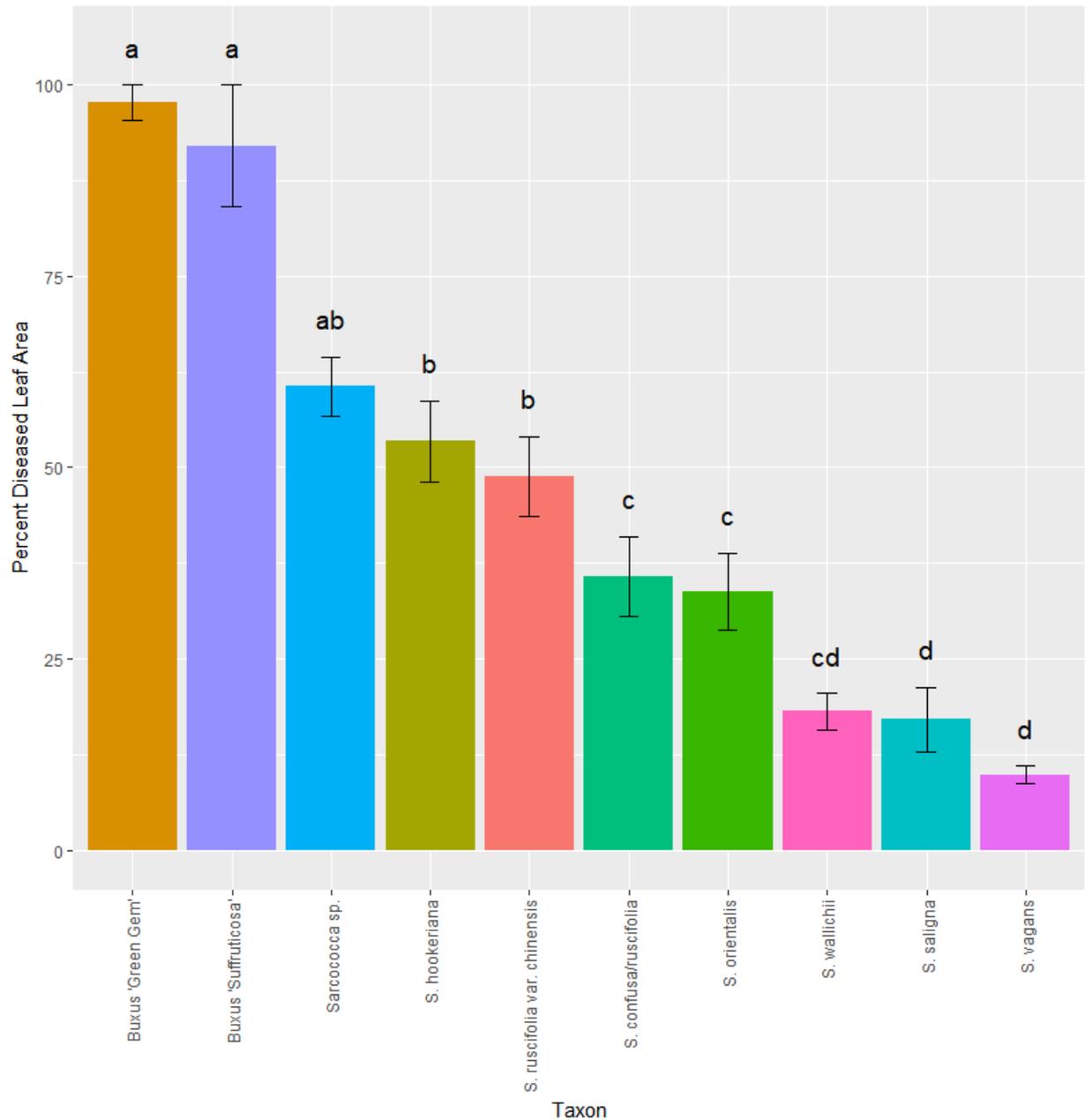grouped with *S. confusa* based on genetic data from Chapter 2.

Figure 4.3. *Sarcococca vagans* (a), *S. saligna* (b), and *S. wallichii* (c) 12 days after inoculation.

Accessions of these species tended to have smaller, more discrete spots compared to other

accessions.

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