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Susceptibility of Boxwood Accessions from the National Boxwood Collection to Boxwood Blight and Potential for Differences between *Calonectria pseudonaviculata* and *C. henricotiae*

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Abstract. **Forty *Buxus* accessions from the U.S. National Arboretum National Boxwood Collection were evaluated as potted plants and detached leaves for susceptibility to *Calonectria pseudonaviculata* (Crous et al.) L. Lombard et al., and nine boxwood cultivars were evaluated against both species of *Calonectria* causing boxwood blight, C. *pseudonaviculata* and *C. henricotiae*. Accessions of *B. harlandii* Hance, *B. sinica* (Rehder and E.H.Wilson) M.Cheng, and *B. microphylla* Siebold and Zucc. had less disease than *B. microphylla* ×*sempervirens*, and all had fewer lesions per plant than the 20 *B. sempervirens* L. accessions evaluated. Variation within species was observed. Of the individual accessions, *B. sinica* var. *aemulans* (accession 60705*H), *B. sempervirens* (36365*J), and *B. harlandii* (18834*H) were least susceptible, with <10 lesions per plant. *B. sempervirens* 'Scupi' (9548*H), *B. microphylla* 'Compacta' (4899*CH), *B. sempervirens* 'Arborescens' (57953*H), *B. sinica* var. *insularis* 'Pincushion' (51898*H), and *B. microphylla* var. *japonica* 'Jim Stauffer' (72213*H) each had <20 lesions. These rankings differ from previous studies that used detached leaf and unrooted cutting assays. Normalizing to account for plant size effects on inoculation and disease increased variability for individual accession rankings but did not result in significant differences in the most and least susceptible accessions or species ranking. Nine boxwood cultivars evaluated against both pathogen species exhibited a range of susceptibility against four pooled isolates each of *C. pseudonaviculata* and *C. henricotiae*. Although small differences in disease severity were observed on boxwood inoculated with the two pathogens, there was no interaction of cultivar and pathogen species, suggesting that a cultivar rated resistant to one species was resistant to the other. These results may aid boxwood breeders to develop resistance to boxwood blight.**

Boxwood blight is a new disease in North America (Ivors et al., 2012; Palmer and Shishkoff, 2014). The disease, incited by the pathogen *Calonectria pseudonaviculata* (Crous et al.) L. Lombard et al., was previously reported in the United Kingdom, many countries in Europe, and New Zealand (Gehesquière et al., 2016). A second species causing boxwood blight disease, *C. henricotiae* Gehesquière, Heungens, and J.A. Crouch, was recently described from Europe (Gehesquière et al., 2016). *Calonectria pseudonaviculata* has now been reported from 22 states (AL, CA, CT, DE, FL, IL, IN, KS, KY, MA, MD, NC, NH, NJ, NY, OH, OR, PA, RI, SC, TN, and VA) and three Canadian provinces (BC, ON, and QC). Boxwood is a major ornamental in the USA with more than $118 million in wholesale nursery production value in 2014 (USDA-NASS Census of Horticultural Specialties, https://www.agcensus.usda.gov/Publications/2012/Online_Resources/Census_of_Horticulture_Specialties/hortic_1_019_020.pdf). It is a slow-growing, long-lived plant and can be extremely valuable; economically and historically important plantings exist in many locations. The boxwood blight pathogen affects all *Buxus* species including Japanese [*Pachysandra terminalis* (LaMondia et al., 2012)] and Allegheny spurge [*P. procumbens* (LaMondia and Li, 2013)], as well as *Pachysandra axillaris* (J.A. LaMondia, unpublished data) and sweet box [*Sarcococca* spp. (Henricot et al., 2008; Kong et al., 2017b; Malapi-Wight et al., 2016)]. To date, *Pachysandra* infection has only been found in landscapes with infected boxwood; no nursery infection has been detected (Kong et al., 2017a; J.A. LaMondia, unpublished data). Boxwood blight symptoms include brown to black leaf spots and stem lesions leading to defoliation and shoot death (Douglas, 2012). The disease has resulted in serious losses of more than $5.5 million in dead or destroyed boxwood plants to date in Connecticut alone, $3 million in the first year after identification of the pathogen in field-grown and container nurseries (LaMondia, 2015), and has also resulted in significant losses in landscape plantings.

Best management practices for boxwood blight have focused on exclusion and sanitation (Douglas, 2012), as well as fungicide application tactics (Henricot and Wedgwood, 2013; LaMondia, 2015; Palmer and Shishkoff, 2014). Long-term management approaches will require the identification or development of resistant or partially resistant boxwood. The *Buxus* genus is diverse, with 91 species (Batdorf, 2004) and large numbers of hybrids and cultivars. This diversity may offer a source of resistance to *C. pseudonaviculata.* Several studies using different methods and isolates of the pathogen have shown differences in susceptibility to boxwood blight, sometimes with contradictory results (Ganci et al., 2013; Gehesquière et al., 2016; Guo et al., 2015; Henricot et al., 2008; LaMondia, 2015). The objectives of the current study were to evaluate a wide range of boxwood plants from a known reference collection, the National Boxwood collection at the U.S. National Arboretum, and to compare different disease evaluation methods and fungal isolates or species. The use of resistant or tolerant cultivars may become part of integrated management practices for nurseries, home gardens, and landscapes. Breeding efforts in the United States have thus far concentrated on *C. pseudonaviculata* (Thammina et al., 2016), but because it is possible that *C. henricotiae* will be introduced into this country at some point, it is important to know if resistance to the fungal species is correlated. Cultivars showing a wide range of susceptibility to *C. pseudonaviculata* were chosen for side-by-side comparisons of symptoms caused by the two pathogens to determine whether breeding for tolerance to one might confer tolerance to the other.

Materials and Methods

Whole-plant assays. Forty *Buxus* accessions from the National Boxwood Collection of the U.S. National Arboretum were evaluated for boxwood blight susceptibility. Cuttings were taken at the U.S. National Arboretum in Washington, DC, in late July 2013 and shipped to the Cornell Long Island Horticultural Research and Extension Center in Riverhead, NY, for rooting. The numbers of available plants were limited; rooted plants in 1- or 2-L capacity containers were brought to Connecticut in Sept. 2014 and again in Apr. 2015, resulting in two experiments that were conducted with three to five replicate plants each conducted at the Connecticut Agricultural Experiment Station Valley Laboratory in Windsor, CT. The accessions evaluated are listed in Table 1. Some of these accessions T1

were previously evaluated as detached cuttings (Shishkoff et al., 2015) before the remainder of the cuttings collected at the same time were rooted for use in these experiments.

The anamorph of *C. pseudonaviculata* (syn ≡ *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenew., and C.F. Hill, = *Cylindrocladium buxicola* Henricot) was used in these experiments. Conidial suspensions of two Connecticut isolates, Cps-CT-L1 (ATCC MYA-4891) and Cps-CT-S1 (ATCC MYA-4890), were prepared from cultures grown on half-strength potato dextrose agar (Benton, Dickinson and Co., Sparks, MD) (LaMondia, 2015). The conidial suspension was adjusted to 0.5×10^5 conidia/mL, and conidia were inoculated to wet boxwood foliage using a hand-held hand pump spray bottle using a coarse setting. Four sprays per plant (0.75 mL/spray) from each of four directions were used to uniformly inoculate plants. White polyethylene sheeting was suspended over the inoculated plants for 24 h at ambient temperatures of 20–25 °C to increase humidity. The plants were not watered for 24 h. After removal of the polyethylene, the plants were grown in the greenhouse and overhead watered by hand twice per day. Plant size was rated on a scale of 1 to 5 where a rating of 1 represented the smallest plants at ≈10 cm in height with 5–6 stems, and the largest plants were rated as 5 with about five times the shoot volume of the plants rated as 1. The numbers of discrete lesions on leaves and stems were counted 3 weeks (Spring 2015) or 4 weeks (Fall 2014) after inoculation.

Detached leaf assays. Ten medium-sized fully matured and hardened leaves per cultivar (less if plants were very small or did not have mature leaves) were collected from greenhouse-grown plants in Connecticut and surface sterilized by placing the leaves one cultivar at a time between two fine mesh rings (one at the bottom to hold the leaves and one on top to keep the leaves submerged and contained), submerging the mesh rings in an aluminum pie pan full of 10% bleach solution for 30 s, then rinsing with tap water for an additional 30 s. The smallest and largest leaves and the oldest leaves from the very bottom of the plant were avoided. The leaves were air-dried and placed on labeled glass slides, five leaves with the adaxial surface and five with the abaxial surface facing upward. The slides were placed in clear humidity chambers (Rubbermaid Commercial Products, Winchester, VA) (45.7 × 30.5 × 15.2 cm; 13.2-L capacity) with wire mesh false bottoms 2.5 cm above the bin bottom. Inoculum of *C. pseudonaviculata* was prepared as before and adjusted to 300 conidia per drop (0.04 mL). One drop was placed on the surface of each leaf. Water (60 °C) was added to the bottom of the bin to 1.5 cm, and the bin was closed to create humid conditions. After 24 h, leaves were individually tipped to shake off drops and returned to humidity chambers. The chambers were held at 22–25 °C, misted with water daily, and the leaves evaluated for infection, percent leaf area affected, and sporulation 7 d after inoculation. The detached leaf experiment was performed four times. The accessions evaluated are listed in Table 3.

Leaf and stem lesion data from the two whole-plant experiments were similar with no interaction between boxwood accession and experiment, so data were combined for analysis as were normalized data that adjusted for plant size (lesions divided by plant size/volume rating). The data were nonnormal and therefore analyzed by the nonparametric Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks with mean separation by the Kruskal–Wallis multiple comparison Z-value test (Number Cruncher Statistical System 2000) (Hintze, 1998). The percentage of inoculated detached leaves with disease on the adaxial or abaxial surfaces and the percentage of the abaxial surface with visible symptom development and *C. pseudonaviculata* sporulation were also nonnormal and analyzed by the Kruskal–Wallis one-way ANOVA on ranks. Means were separated by the Kruskal–Wallis Z-value test.

Comparison of Calonectria species. Cultivars were chosen based on the results of Shishkoff et al. (2015), where cuttings of the same 42 accessions of boxwood growing at the U.S. National Arboretum were evaluated for susceptibility to one isolate of *Calonectria pseudonaviculata* (CBS114417) several days after harvesting from outdoor-grown plants. Cultivars used in the current experiment (*Buxus sempervirens* 'Dee Runk', *B. sempervirens* 'Thomas Jefferson', *B. sempervirens* 'Vardar Valley', *B. sempervirens* 'Justin Brouwers', *Buxus* × 'Green Velvet', *B. harlandii* 'Richard', *B. microphylla* 'Little Missy' and two additional experimental breeding lines SB108 and SB17) were expected to display a range of susceptibility to *C. pseudonaviculata* from high (for *B. sempervirens* cultivars) to low (for *B. microphylla*). Four isolates of *Calonectria pseudonaviculata* (CBS114417 from the United Kingdom, CpsCT1 from Connecticut, 13. DE.01b.1 from Delaware, and NCBB1 from North Carolina) and four isolates of *Calonectria henricotiae* from Europe (JKI 2106 from Germany, CB045 from Belgium, and NL009 and bg209a from the Netherlands) were used in these experiments. They were chosen to represent a diverse genotypic range for each species (Gehesquière et al., 2016). For each pathogen species, the mixture of four isolates per species was used to inoculate whole plants in 10-cm pots. To produce spores, 1–2-month-old cultures in 9-cm-diam petri dishes formed microsclerotia on 8-cm cellophane disks (Biorad GelAir cellophane support; Bio-Rad Laboratories, Inc.) covering the surface of glucose–yeast extract–tyrosine agar (GYET; Hunter, 1992). To produce conidia from the microsclerotia, the cellophane was peeled from the surface of the culture and placed on fresh GYET agar, which stimulated sporulation after 4–6 d. Resulting conidia were collected in water with 0.1% v/v Tween-20 and adjusted to 2000 spores/mL. Equal volumes of each of the four isolates per species were combined (and sampled with a hemocytometer to confirm spore concentration) to create the suspension used for inoculation of plants. Each spore suspension (2000 spores/mL) was sprayed onto a set of the nine cultivars using a DeVilbiss bottle connected to an air compressor until good coverage was achieved and the spore suspension was beginning to drip from foliage. Six plants of each cultivar were sprayed with inoculum suspensions of each of the two fungal species for a total of 108 inoculated plants, with three plants of each cultivar (27 plants total) sprayed with water alone to serve as noninoculated controls, an important treatment because the secondary pathogen *Pseudonectria buxi* is very common and superficially morphologically similar to *Calonectria*. All plants inoculated with *C. pseudonaviculata* were placed on one side of a 1.0 × 1.8-m dew chamber set at 20 °C, and those inoculated with *C. henricotiae* were placed on the other side. Dew forms on the plants spontaneously in a dew chamber. Plants were placed in the same dew chamber (with a 30-cm gap) so that the infection conditions were as similar as possible with no cross-contamination. Control plants were placed in a smaller (0.70 × 0.76 m) dew chamber set at 20 °C. The plants were exposed to dew for 48 h and then placed in a plastic mist tent with

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Table 1. Susceptibility of potted boxwood accessions from the U.S. National Arboretum to *Calonectria pseudonaviculata*.

Accession no.[z]	*Buxus* species and cultivar	Size rating[y]	Lesions per plant[x]	Normalized[w]
68273*H	*B.* 'Glencoe'	2	61.6 FGH	30.3 GHIJK
51904*K	*B.* 'Green Gem'	1.3	27.3 ABCDEFGH	25.5 CDEFGHIJK
71429*H	*B.* 'Green Ice'	4.3	83.0 H	19.6 BCDEFGHIJ
51906*H	*B.* 'Green Mound'	2	23.1 ABCDEF	12.4 ABCDE
51905*J	*B.* 'Green Mountain'	1.1	30.4 ABCDEFGH	29.8 GHIJK
51907*H	*B.* 'Green Velvet'	2.1	55.6 DEFGH	28.5 FGHIJK
18834*H	*B. harlandii*	1.4	7.4 ABC	7.0 ABC
52423*H	*B. harlandii* (=*Buxus bodinieri*)	1.4	28.1 ABCDEFGH	26.3 DEFGHIJK
4899*CH	*B. microphylla* 'Compacta'	1	14.5 ABCD	18.0 ABCDEFGHI
29224*H	*B. microphylla* 'Grace Hendrick Phillips'	1	61.0 FGH	61.0 M
33810*H	*B. microphylla* 'John Baldwin'	2.9	26.0 ABCDEFG	9.4 ABC
78079*H	*B. microphylla* var. *japonica* 'Gregem'	2	50.4 CDEFGH	24.6 CDEFGHIJK
72213*H	*B. microphylla* var. *japonica* 'Jim Stauffer'	2.5	17.4 ABCDEF	10.0 ABC
7025*H	*B. microphylla* var. *japonica* 'National'	2.9	24.1 ABCDEF	9.4 ABC
54326*H	*B. microphylla* var. *japonica* 'Winter Gem'	3.3	34.3 BCDEFGH	11.4 ABCD
36365*J	*B. sempervirens*	1.2	4.6 AB	2.6 AB
31793*H	*B. sempervirens* 'Arborescens'	4.1	159.5 H	39.6 KL
57953*H	*B. sempervirens* 'Arborescens'	1	16.3 ABCDE	16.3 ABCDEFGHI
17078*H	*B. sempervirens* 'Decussata'	2.8	34.1 BCDEFGH	14.2 ABCDEF
68631*H	*B. sempervirens* 'Dee Runk'	3.9	58.4 EFGH	15.6 ABCDEFGH
34196*H	*B. sempervirens* 'Denmark'	3.3	57.9 EFGH	18.5 BCDEFGHIJ
35487*H	*B. sempervirens* 'Edgar Anderson'	4.1	64.8 GH	15.8 ABCDEFGH
33789*J	*B. sempervirens* 'Graham Blandy'	3.6	75.5 H	20.8 CDEFGHIJ
4233*H	*B. sempervirens* 'Handsworthiensis'	3.5	41.8 CDEFGH	11.4 ABCD
29694*H	*B. sempervirens* 'Marginata'	2.4	47.6 CDEFGH	21.3 CDEFGHIJ
34198*J	*B. sempervirens* 'Myrtifolia'	3.8	85.1 H	22.2 CDEFGHIJ
54327*H	*B. sempervirens* 'Newport Blue'	2.9	62.0 GH	21.9 CDEFGHIJ
29701*H	*B. sempervirens* 'Northern New York'	3.6	89.5 H	26.0 DEFGHIJK
51910*H	*B. sempervirens* 'Northland'	4	110.6 H	27.7 FGHIJK
69558*H	*B. sempervirens* 'Ohio'	4	107.0 H	27.1 EFGHIJK
59820*H	*B. sempervirens* 'Pendula'	3.6	93.0 H	26.1 DEFGHIJK
35494*H	*B. sempervirens* 'Rotundifolia'	2.8	75.0 H	33.7 IJKL
9548*H	*B. sempervirens* 'Scupi'	1.3	14.4 ABCD	14.4 ABCDEFG
29703*H	*B. sempervirens* 'Suffruticosa'	2	91.4 H	45.5 LM
6395*H	*B. sempervirens* 'Vardar Valley'	2	38.1 CDEFGH	23.8 CDEFGHIJK
60705*H	*B. sinica* var. *aemulans*	1.4	2.4 A[v]	2.3 A
51898*H	*B. sinica* var. *insularis* 'Pincushion'	1	17.0 ABCDEF	17.0 ABCDEFGHI
51900*H	*B. sinica* var. *insularis* 'Winter Beauty'	1	22.8 ABCDEF	22.8 CDEFGHIJK
57950*H	*Buxus* sp.	3.3	21.9 ABCDEF	7.1 ABC
51896*H	*Buxus wallichiana*	4.3	160.1 H	37.3 IJKL
	P =	0.00001	0.00001	0.00001

[z]Accession number from the U.S. National Arboretum collection.
[y]Size rating based on a scale of 1 to 5: Plant size was rated on a scale of 1 to 5 where a rating of 1 represented the smallest plants at ≈10 cm in height with 5–6 stems, and the largest plants were rated as 5 with about five times the shoot volume of the plants rated as 1.
[x]Number of leaf and stem lesions counted per plant.
[w]Normalized data; lesions divided by size rating.
[v]Data were analyzed by the nonparametric Kruskal–Wallis one-way ANOVA on ranks and means were separated by the Kruskal–Wallis multiple comparison Z-value test. Means within columns followed by the same letter are not significantly different ($P = 0.05$).

a cardboard partition in a greenhouse at 22 ± 2 °C with 30 s of overhead misting every 10 min. Ratings of symptoms were made 11 d after inoculation. At that time, the numbers of infected leaves, fallen leaves, and total leaves (both on plant and fallen) were counted. The experiment was performed three times. Data for the three trials were examined separately and combined for analysis. Analysis of variance was tested using General Linear Models in SAS (PROC GLM; SAS Institute, Cary, NC) using the model: arcsin transformation of percent diseased leaves = trial isolate cultivar trial*isolate trial*cultivar cultivar*isolate. Means were separated using Fisher's least significant difference. Percentage of fallen leaves were normalized with an arcsin transformation and analyzed the same way.

Results

Whole-plant assays. We observed significant differences in the number of boxwood blight lesions per plant that developed under disease-conducive conditions for 40 *Buxus* accessions from the National Boxwood Collection of the U.S. National Arboretum (Table 1). *B. sinica* var. *aemulans* (60705*H), *B. sempervirens* (36365*J), and *B. harlandii* (18834*H) were the least susceptible accessions tested, with fewer than 10 lesions per plant. Data were normalized to account for plant size (total number of lesions per plant/plant size rating), and in most cases, plant susceptibility did not change dramatically. Numbers of leaf and stem lesions per plant were significantly correlated with numbers of lesions normalized by plant shoot volume ($r = 0.71$, $P = 0.0001$).

Disease susceptibility within *Buxus* species was compared, ranging from a single accession for *B. wallichiana* to 20 accessions for *B. sempervirens* (Table 2). Whereas only a single accession of *B. wallichiana* was available for evaluation, it was the most susceptible accession (51896*H), followed by the *B. sempervirens* accessions tested. *B. sinica* (three accessions) and *B. harlandii* (accession 18834*H and synonym *B. bodinieri* 52423*H) were the least susceptible species tested, and *B. microphylla* and *Buxus hybrids* (*microphylla koreana* × *sempervirens*) were intermediate. There was significant variation within species: whereas most *B. sempervirens* accessions were very susceptible, *B. sempervirens* accession 36365*J was among the least susceptible plants evaluated. Two accessions of *B. sempervirens* 'Arborescens' reacted very differently: accession 31793*H was among the most susceptible plants tested, and accession 57953*H was among the least susceptible plants.

Detached leaf assays. The susceptibility of the same boxwood accessions as determined by detached leaf assays was different from that of whole plants (Table 3). The number of lesions per plant for whole potted plants was poorly correlated with the percentage of

Table 2. Susceptibility of whole-plant *Buxus* species and hybrids from the U.S. National Arboretum collection to boxwood blight incited by *Calonectria pseudonaviculata*.

Buxus species	Number of accessions evaluated	Lesions per plant[z]	Normalized lesions by plant size[y]
B. harlandii (*B. bodinieri*)	2	19.5 AB[x]	18.3 AB
Buxus hybrid (*microphylla koreana* ×*sempervirens*)	6	48.6 C	24.2 BC
B. microphylla	7	31.1 BC	16.7 AB
B. sempervirens	20	69.6 D	22.9 BC
B. sinica	3	11.1 A	11.1 A
B. wallichiana (51896*H)	1	160.1 E	37.3 C
P =		0.00001	0.0001

[z]Number of leaf and stem lesions counted per plant.
[y]Normalized data; lesions divided by size rating.
[x]Data were analyzed by the nonparametric Kruskal–Wallis one-way ANOVA on ranks and means were separated by the Kruskal–Wallis multiple comparison Z-value test. Means within columns followed by the same letter are not significantly different ($P = 0.05$).

detached leaves infected ($r = 0.27$, $P = 0.09$) but significantly correlated with percentage of leaf area diseased for the same accessions, although with a low r value ($r = 0.39$, $P = 0.01$). Lesion number normalized by plant shoot size was not significantly correlated with either percentage of detached leaves infected or percentage of leaf area diseased for the same accessions ($r = –0.04$, and $r = 0.11$, respectively). Infection of detached leaves in these experiments ranged from 43.7% to 91.8%. Lesions on infected leaves expanded to cover most of the leaf surface, and sporulation under conducive conditions was significantly different between the accessions, ranging from less than 10% of leaves to 88%.

The susceptibility of detached leaves within *Buxus* species for the accessions that we evaluated (ranging from a single accession to 20 accessions) was similarly ranked to the whole-plant evaluations (Table 4). *Buxus wallichiana* was the most susceptible accession (51896*H) for percentage of inoculated detached leaves infected and for percent leaves with sporulation. *Buxus harlandii* (accession 18834*H and synonym *B. bodinieri* 52423*H) were the least susceptible species. Infection as a result of inoculation with the same number and cohort of conidia was much more successful on the abaxial than the adaxial leaf surface. On the abaxial surface, germinated conidia most often penetrated stomates, but appressoria and direct penetration were also observed. No symptoms developed on the adaxial surface of *B.* 'Green Velvet', but with the aid of a microscope, we observed sparse hyphal growth and periodic direct penetration of a single cell that resulted in the production of a conidiophore and a few conidia. No stomates were observed on the adaxial surface of 'Green Velvet'.

Comparison of Buxus cultivar susceptibility and virulence of Calonectria species. Boxwood cultivars showed a wide range in susceptibility to inoculation with *C. pseudonaviculata* or *C. henricotiae*. Table 5 lists the cultivars tested ranked by the percentage of diseased leaves after 11 d. The GLM model was significant at $P < 0.0001$. The variable "cultivar" was significant at $P < 0.0001$; cultivars with the highest and lowest amount of disease remained roughly the same from trial to trial, with *Buxus sempervirens* 'Dee Runk' and *B. sempervirens* 'Thomas Jefferson' showing the greatest proportion of diseased leaves (13.3% to 22.2%) and *Buxus* 'SB108' and *B. microphylla* 'Little Missy' showing the least (3.6% to 4.8%). The interaction "cultivar*trial" was significant at $P < 0.0001$; for cultivars in the middle rankings, there was considerable shifting from trial to trial with disease ratings varying from 5.4% to 13.8% in the moderately susceptible cultivars. The variable "trial" was significant at $P < 0.0001$; plants in trial 3 showed significantly greater disease for both isolates compared with the other two trials. The variable "isolate" was significant at $P = 0.0008$, although the difference in severity was usually not more than a few percent points. The interaction "isolate*trial" was significant at $P < 0.0001$; over all the trials, plants inoculated with *C. henricotiae* had significantly more disease, but in trial 2, plants inoculated with *C. pseudonaviculata* showed more disease. The interaction "cultivar*isolate" was not significant ($P = 0.13$), indicating that the susceptibility of cultivars to disease did not differ for the two species of *Calonectria*.

Defoliation was not high at 11 d after inoculation, but the GLM model for defoliation was significant ($P < 0.0001$), and all the variables and interactions were significant ($P \leq 0.0002$). Table 6 lists the cultivars tested and their defoliation after 11 d. Defoliation was significant and highest in *Buxus sempervirens* 'Vardar Valley' (3.1% to 4.1%), and lowest in *Buxus* 'SB108' and *B. microphylla* 'Little Missy' (1.5% to 1.7%), but the degree of defoliation by cultivars varied widely from trial to trial. Defoliation was slightly greater in plants inoculated with *C. henricotiae*. The interaction "cultivar*isolate" was significant, indicating a different pattern of defoliation when cultivars were inoculated with different pathogen species, and the most noticeable difference was a slightly greater leaf loss in *B. sempervirens* 'Vardar Valley' when inoculated with *C. pseudonaviculata.*

Discussion

It is important to determine the boxwood blight susceptibility of boxwood species, cultivars, and accessions for several reasons. Knowledge of the relative susceptibility to blight will influence which species and cultivars will be selected for landscape plantings because it will affect the intensity of management programs for control of the disease. Differences in susceptibility will be used by plant breeders to develop new cultivars tolerant or resistant to boxwood blight.

Determining the relative susceptibility of different boxwood to this disease is no small task as there are 95–100 species of *Buxus*, of which several species and hybrids of species are widely grown as ornamentals (Batdorf, 2004). The most important ornamental species, *B. sempervirens*, has more than 400 named cultivars (Neimera, 2012), and there can be significant genetic variation within cultivars as a result of selection from a variable source originating from seed, vegetative propagation from sports, misidentification, or even mislabeling (Thammina et al., 2016). In addition, some names such as 'Arborescens' or 'Prostrata' are used for multiple accessions with diverse genotypes that represent either treeform or prostrate growth habits (Thammina et al., 2016).

There have been several evaluations of boxwood susceptibility to boxwood blight published. Despite some conflicting results that may be due to cultivar variability, or differences in evaluation techniques using whole plants or detached stems or individual leaves, information is accumulating over time to allow some general assumptions regarding differences in species susceptibility as a starting point for further evaluation. In general, whether studies have used detached cuttings (Guo et al., 2015, 2016; Henricot et al., 2008; Shishkoff et al., 2015) or whole plants in the field or nursery (Ganci et al., 2013; Guo et al., 2016; LaMondia, 2015), all studies have found *B. sempervirens* cultivars to be generally very susceptible and *B. microphylla* cultivars to be less so. Henricot et al. (2008) used detached leaves to conclude that the most susceptible plants (based on percentage of leaf spotting) included *B. sempervirens* 'Suffruticosa', *B. sinica* var. *insularis* and *B. harlandii* (accession not specified), with *B. microphylla* less susceptible, although there was some difference in severity depending on the isolate used. *B. balearica* was the most resistant species evaluated, in contrast with the results from Guo et al. (2016). Ganci et al. (2013) observed a wide range of susceptibility and concluded that *B. sempervirens* cultivars were generally most susceptible and that *B. microphylla* var. *japonica* 'Green Beauty', *B. sinica* var. *insularis* 'Nana', *B. harlandii*, and *B. microphylla* 'Golden Dream' were least susceptible.

Guo et al. (2016) recently evaluated screening techniques and the susceptibility of eight boxwood cultivars to boxwood blight and concluded that either mycelium or spores could be used and that detached leaf or whole-plant assays gave comparable results. They also found that *B. microphylla* 'John Baldwin' and *B.* '*Suffruticosa*' clustered in the most susceptible group whereas 'Jim Stauffer' and *B. harlandii* clustered in the

Table 3. Susceptibility of boxwood accessions from the U.S. National Arboretum to *Calonectria pseudonaviculata* as determined by a detached leaf assay.

Accession no.[z]	*Buxus* species and cultivar	Disease on adaxial leaf surface[y]	Disease on abaxial leaf surface	Percent abaxial leaf symptomatic	Percent abaxial leaves with sporulation
68273*H	*B.* 'Glencoe'	7 AB	56 AB	44.0 ABC	20.0 ABCD
51904*K	*B.* 'Green Gem'	33 D	80 CDEF	73.8 CDEF	48.0 DEFGH
71429*H	*B.* 'Green Ice'	7 AB	92 EF	92.6 FG	56.0 FGH
51906*H	*B.* 'Green Mound'	0 A	72 BCDE	74.5 CDEF	48.0 DEFGH
51905*J	*B.* 'Green Mountain'	33 D	72 BCDE	63.7 CDE	40.0 CDEFG
51907*H	*B.* 'Green Velvet'	0 A	72 BCDE	62.8 CDE	32.0 BCDEF
18834*H	*B. harlandii*	33 D	72 BCDE	26.3 A	20.0 ABCD
52423*H	*B. harlandii* (=*Buxus bodinieri*)	0 A	90 DEF	68.6 CDE	25.0 ABCDE
4899*CH	*B. microphylla* 'Compacta'	13 ABC	60 ABC	56.8 ABCDE	28.0 ABCDE
29224*H	*B. microphylla* 'Grace Hendrick Phillips'	0 A	46 A	44.3 ABC	34.6 BCDEF
33810*H	*B. microphylla* 'John Baldwin'	13 ABC	96 F	88.5 FG	68.0 GHI
78079*H	*B. microphylla* var. *japonica* 'Gregem'	50 E	92 EF	80.0 EFG	61.5 FGHI
72213*H	*B. microphylla* var. *japonica* 'Jim Stauffer'	0 A	76 BCDEF	78.0 DEFG	8.0 A
7025*H	*B. microphylla* var. *japonica* 'National'	0 A	77 BCDEF	80.5 EFG	42.3 CDEFG
54326*H	*B. microphylla* var. *japonica* 'Winter Gem'	0 A	68 ABCD	50.0 ABCD	16.0 ABC
36365*J	*B. sempervirens*	0 A	75 BCDEF	58.8 ABCDE	18.8 ABCD
57953*H	*B. sempervirens* 'Arborescens'	33 D	84 DEF	87.8 EFG	64.0 GHI
31793*H	*B. sempervirens* 'Arborescens'	33 D	84 DEF	87.8 EFG	64.0 GHI
17078*H	*B. sempervirens* 'Decussata'	7 AB	92 EF	77.0 DEFG	32.0 BCDEF
68631*H	*B. sempervirens* 'Dee Runk'	20 BCD	80 CDEF	41.5 AB	52.0 EFGH
34196*H	*B. sempervirens* 'Denmark'	0 A	96 F	82.3 EFG	36.0 BCDEF
35487*H	*B. sempervirens* 'Edgar Anderson'	0 A	92 EF	84.5 EFG	68.0 GHI
33789*J	*B. sempervirens* 'Graham Blandy'	7 AB	84 DEF	71.3 CDEF	48.0 CDEFGH
4233*H	*B. sempervirens* 'Handsworthiensis'	13 ABC	80 CDEF	70.5 CDEF	52.0 EFGH
29694*H	*B. sempervirens* 'Marginata'	20 BCD	96 F	73.0 CDEF	32.0 BCDEF
34198*J	*B. sempervirens* 'Myrtifolia'	13 ABC	96 F	87.8 EFG	88.0 I
54327*H	*B. sempervirens* 'Newport Blue'	27 CD	80 CDEF	78.8 DEFG	32.0 BCDEF
29701*H	*B. sempervirens* 'Northern New York'	21 BCD	88 DEF	79.8 EFG	52.0 EFGH
51910*H	*B. sempervirens* 'Northland'	13 ABC	80 CDEF	87.0 EFG	64.0 GHI
69558*H	*B. sempervirens* 'Ohio'	13 ABC	72 BCDE	85.0 EFG	52.0 EFGH
59820*H	*B. sempervirens* 'Pendula'	20 BCD	92 EF	74.5 CDEF	40.0 CDEFG
35494*H	*B. sempervirens* 'Rotundifolia'	13 ABC	88 DEF	73.5 CDEF	36.0 BCDEF
9548*H	*B. sempervirens* 'Scupi'	0 A	84 DEF	74.3 CDEF	20.0 ABCD
29703*H	*B. sempervirens* 'Suffruticosa'	13 ABC	72 BCDE	55.3 ABCD	44.0 DEFG
6395*H	*B. sempervirens* 'Vardar Valley'	27 CD	68 ABCD	55.3 ABCD	12.0 AB
60705*H	*B. sinica* var. *aemulans*	82 F[x]	83 CDEF	44.6 ABC	38.9 BCDEFG
51898*H	*B. sinica* var. *insularis* 'Pincushion'	13 ABC	76 BCDEF	70.5 CDEF	56.0 FGH
51900*H	*B. sinica* var. *insularis* 'Winter Beauty'	29 D	77 BCDEF	70.5 CDEF	46.2 DEFGH
57950*H	*Buxus* sp.	0 A	72 BCDE	54.3 ABCD	44.0 DEFG
51896*H	*Buxus wallichiana*	7 AB	76 BCDEF	91.8 FG	76.0 HI
	P =	0.0001	0.0002	0.0001	0.0001

[z]Accession number from the U.S. National Arboretum collection.
[y]Percent leaves with lesions resulting from inoculation with ≈300 conidia in a single drop on the adaxial or abaxial surface.
[x]Data were analyzed by the nonparametric Kruskal–Wallis one-way ANOVA on ranks and means were separated by the Kruskal–Wallis multiple comparison Z-value test. Means within columns followed by the same letter are not significantly different ($P = 0.05$).

most resistant group. *B. sempervirens* 'Vardar Valley' and *B. balearica* were intermediate. Guo et al. (2016) also concluded that the time of year of pathogen inoculation (spring, summer, or winter) did not affect the relative symptom expression observed for the most resistant or susceptible boxwood, and our current studies concur.

The different screening techniques each have advantages and disadvantages concerning ease of screening, amount of plant material required, laboratory or greenhouse space requirements, and time. Whole-plant screening is more expensive, slower, and uses more space. If all plants are not of the same size, questions exist about how best to inoculate. In the current experiments, all plants regardless of size were inoculated with same number of conidia, which was not an overwhelming number, such that we could expect a similar number of lesions per plant if disease susceptibility and conidial deposition were similar. However, as we questioned whether larger plants might allow for more effective conidium deposition on leaves when using a directed spray inoculation technique, we also normalized for plant size. This had some effect on individual accessions but did not change the conclusions about best or worst performing accessions or differences observed between *Buxus* species. Our results suggest that simple numbers of leaf and stem lesions per plant were a better measure of susceptibility than attempts to normalize by plant size or volume as normalizing did not result in significant differences in species ranking, and nonnormalized data were better correlated with detached leaf disease incidence and severity. Defoliation was not found to be a useful measure of disease in this trial with whole plants or in a study using detached cuttings (Shishkoff et al., 2015), although in both cases, defoliation was measured only 11 d after inoculation, and these data might have been more informative if collected on a later date. LaMondia (2015) evaluated defoliation in field and greenhouse trials after 42 d and concluded that Korean and 'Winter Gem' (*Buxus sinica* var. *insularis*) were least susceptible, common boxwood (*B. sempervirens*) and True Dwarf (*B. sempervirens* 'Suffruticosa') were most susceptible, and the hybrids 'Green Mountain' (*B. sinica* var. *insularis* × *B. sempervirens* 'Suffruticosa') and 'Green Velvet' (*B. sinica* var. *insularis* × *B. sempervirens* 'Suffruticosa') were intermediate. Leaves with lesions defoliate and timing of data collection can be important as leaves with multiple lesions tend to drop earlier.

We also observed a very large difference in disease resulting from inoculation of top or bottom leaf surfaces, as did Guo et al. (2016) and Shishkoff et al. (2015). Our microscopic observation of infection showed that *C. pseudonaviculata* most often infected through stomates but could produce appressoria and directly infect the leaf. The boxwood leaves that we examined had stomates at the bottom of the leaf, likely explaining the increased efficiency of infection on that surface. Other factors related to the extensiveness of infection may be involved as well, as we also observed that conidia on the

Table 4. Susceptibility of detached leaves of *Buxus* species and hybrids from the U.S. National Arboretum collection to boxwood blight incited by *Calonectria pseudonaviculata*.

Buxus species	Number of accessions evaluated	Percent leaves with lesions[z]	Percent leaf symptomatic[y]	Percent leaves with sporulation[x]
B. harlandii (*B. bodinieri*)	2	43.7 A[w]	80.0 AB	22.2 A
Buxus hybrid (*microphylla koreana* ×*sempervirens*)	6	68.4 B	74.0 A	40.7 B
B. microphylla	7	67.5 B	71.6 A	35.8 AB
B. sempervirens	20	73.9 B	84.4 B	44.6 B
B. sinica	3	62.4 B	78.6 AB	48.2 B
B. wallichiana (51896*H)	1	91.8 C	76.0 AB	76.0 C
P =		0.00002	0.006	0.0002

[z]Percentage of leaves with lesions resulting from inoculation with ≈300 conidia in a single drop on the abaxial surface.
[y]Percentage of abaxial leaf surface symptomatic.
[x]Percentage of leaves with conidia of *C. pseudonaviculata* present.
[w]Data were analyzed by the nonparametric Kruskal–Wallis one-way ANOVA on ranks and means were separated by the Kruskal–Wallis multiple comparison Z-value test. Means within columns followed by the same letter are not significantly different ($P = 0.05$).

Table 5. Boxwood cultivars tested ranked by the percentage of diseased leaves 11 d after inoculation with either *Calonectria pseudonaviculata* or *C. henricotiae*.

	Percent diseased leaves per plant[z]		
Boxwood cultivars	*C. pseudonaviculata*[a]	*C. henricotiae*	Overall susceptibility[y]
Buxus microphylla 'Little Missy'	3.6	4.3	A
SB17	4.8	4.6	A
SB108	6.3	8.5	B
Buxus harlandii 'Richard'	5.4	9.3	B
Buxus × 'Green Velvet'	6.3	13.5	C
Buxus sempervirens 'Justin Brouwers'	10.5	11.5	CD
Buxus sempervirens 'Vardar Valley'	12.9	13.8	D
Buxus sempervirens 'Thomas Jefferson'	13.3	13.3	D
Buxus sempervirens 'Dee Runk'	18.1	22.2	E

[z]The proportion of diseased leaves 11 d after inoculation. Inoculation was done with spore suspensions made from four isolates of each species.
[y]Statistics were done on data from three trials using General Linear Model analysis. Numbers followed by the same letter do not differ significantly by least significant difference ($P = 0.05$).

Table 6. Boxwood cultivars tested ranked by the percentage of fallen leaves 11 d after inoculation with either *Calonectria pseudonaviculata* or *C. henricotiae*.

	Percent defoliation per plant[z]		
Boxwood cultivars	*C. pseudonaviculata*[a]	*C. henricotiae*	Overall susceptibility[y]
Buxus microphylla 'Little Missy'	1.5	1.5	A
SB17	1.6	1.7	AB
SB108	2.5	3.5	CD
Buxus harlandii 'Richard'	0.8	3.0	ABC
Buxus × 'Green Velvet'	1.1	4.1	BCD
Buxus sempervirens 'Justin Brouwers'	1.5	1.8	AB
Buxus sempervirens 'Vardar Valley'	4.1	3.1	D
Buxus sempervirens 'Thomas Jefferson'	1.7	3.1	BC
Buxus sempervirens 'Dee Runk'	2.3	3.1	BCD

[z]The proportion of fallen leaves 11 d after inoculation. Inoculation was done with spore suspensions made from four isolates of each species.
[y]Statistics were done on data from three trials using General Linear Model analysis. Numbers followed by the same letter do not differ significantly by least significant difference ($P = 0.05$).

adaxial surface could directly infect a single cell and produce a few strands of hyphae growing across the outside of the leaf with an occasional conidiophore with conidia without producing macroscopic symptoms.

In general, in our current experiments, we found that similar results were observed for *Buxus* species and hybrids using either detached leaves or whole plants; however, like Guo et al. (2016), we also observed instances where there were differences between whole-plant response to inoculation vs. detached leaves using clonal cuttings of the same plants and the same pathogen isolates. Our results were similar to Guo et al. (2016) in that 'John Baldwin' was more resistant when tested as a whole plant than as detached leaves. These discrepancies also point out the need to use accession numbers or have specific cultivars of known provenance to compare methods as variation in plants has previously been discussed. Thammina et al. (2016) evaluated genetic relationships and noted discrepancies in boxwood identities for several accessions. Shishkoff et al. (2015) used detached leaves and stems to evaluate the same boxwood accession clones used in the current experiments and concluded that *B. sinica* and *B. microphylla* were least symptomatic with *B. sinica* var. '*aemulans*' most resistant, similar to our results, but they concluded that *B. sempervirens* 'Scupi' was the most susceptible accession tested and that *B. harlandii* grouped with most of the susceptible *sempervirens* accessions, quite different results from those reported here.

These results question the utility of only using detached leaf assays for screening for disease reaction and suggest that some components of resistance may be systemic (Browne et al., 2005) and involve more than cuticle thickness as suggested by Henricot et al. (2008). Detached leaves may not fully express resistance present in whole plants because of induced systemic resistance or other mechanisms. Plant architecture is not considered and resistance ranking might be different after several disease cycles rather than a single infection period as we also found differences in lesion size and pathogen sporulation. More studies regarding the mechanism(s) involved in disease response between closely related boxwoods are required. Detached leaf or stem assays are faster, less expensive, and can be useful as initial screens and for looking at the expression of components of resistance, but they should likely be paired or followed up with whole-plant evaluation of susceptibility.

It is also imperative to determine whether boxwoods screened against *C. pseudonaviculata* would also respond in a similar manner to *C. henricotiae*. We found *B. sempervirens* 'Dee Runk' to be the most susceptible cultivar tested in our current trial with both *Calonectria* species. Ganci et al. (2013) ranked it as "moderately tolerant" based on the diseased leaf area. Shishkoff et al. (2015) ranked it as "moderately susceptible" based on the proportion of diseased leaves. The disease rank in this study of cultivars or species exposed to both pathogens was roughly the same as the rank of those plants in Shishkoff et al. (2015) for their susceptibility to *C. pseudonaviculata* alone. There, *B. sempervirens* 'Dee Runk' and *Buxus* 'Green Velvet' were more susceptible than *B. sempervirens* 'Vardar Valley', which was more susceptible than various cultivars of *B. sinica* and *B. microphylla*. Although cultivar susceptibility differed in this whole-plant laboratory study compared with whole-plant field studies (Ganci et al., 2013), some of the variability might have been due to differences in environment or plant architecture. *Buxus sempervirens* 'Thomas Jefferson' was considered by its breeders to be tolerant to boxwood blight because of its open structure and upright habit (Patrick, 2013), but it's doubtful that either of these features would have reduced infections in our tests using dew chambers and mist tents.

In Europe, the two pathogen species can be found together in Belgium, Germany, the Netherlands, Slovenia, and the United Kingdom (Gehesquière et al., 2016), but some evaluations of cultivar susceptibility were

performed before it was known that two species of *Calonectria* were present. Henricot et al. (2008) tested 11 species or cultivars of *Buxus* with four isolates of *Calonectria* [subsequently identified as *C. pseudonaviculata* in Gehesquière et al. (2016)]. Gehesquière et al. (2016) tested 37 *Buxus* cultivars as 2-yr cuttings in greenhouse trials against five isolates of the two *Calonectria* species, four of *C. pseudonaviculata*, and one of *C. henricotiae*; the experiment was run at 16.2 ± 3.5 °C evaluating percent infected leaves and found no difference in virulence and no interaction of isolate*cultivar.

We found small but significant differences in disease severity between the two species under the conditions tested (inoculation at 20 °C and incubation at 22 ± 2 °C), but it is not clear whether this result would be seen at other temperatures. The introduction of the two pathogens to Europe is so recent that the geographical range of the two pathogens is not fully understood; therefore, it is not yet clear if differences in temperature tolerance between the two species will be reflected in the future in differences in geographic range. It is also unclear whether the similar reaction of cultivars to the two species would also be observed at colder or warmer temperatures. However, based on results so far, a cultivar rated resistant to one species was resistant to the other.

Our current results demonstrated significant differences in susceptibility between boxwood species, cultivars within species, and even between different accessions of the same species (*B. sempervirens*) or designation (*B. sempervirens* 'Arborescens'). This suggests that there is the potential for breeders to select for reduced susceptibility and develop cultivars with improved resistance to boxwood blight.

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