Evaluating Boxwood Susceptibility to *Calonectria pseudonaviculata* Using Cuttings from the National Boxwood Collection

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Accepted 25 December 2014. Published 26 January 2015.

ABSTRACT

Shishkoff, N., Daughtrey, M., Aker, S., and Olsen, R. T. 2014. Evaluating boxwood susceptibility to *Calonectria pseudonaviculata* using cuttings from the National Boxwood Collection. Plant Health Progress doi:10.1094/PHP-RS-14-0033.

Accessions in the National Boxwood Collection of the U.S. National Arboretum were inoculated with *Calonectria pseudonaviculata* in order to determine susceptibility to boxwood blight as part of longer-term evaluations of whole plants. Terminal unrooted cuttings were inoculated with *C. pseudonaviculata* and symptoms rated. Cuttings showed a wide range in susceptibility. There were significant differences in percent diseased leaves and percent defoliation among the 42 species and cultivars. Cuttings of some *Buxus sempervirens* cultivars were among those with the highest percent diseased leaves, with eight cultivars showing as much disease as *B. sempervirens* 'Suffruticosa': 'Scupi', 'Pendula', 'Rotundifolia', 'Denmark', 'Handsworthiensis', 'Northland', 'Arborescens', and 'Northern New York'. All others showed significantly less disease, as measured by percent diseased leaves. A number of accessions were contrasted to the less susceptible *B. sinica* var. *insularis* 'Pincushion' and showed a similarly low level of disease: *Buxus* 'Green Ice', *B. sempervirens* 'Decussata', *B. sinica* var. *insularis* 'Wintergreen', *Buxus,* sp. (57950*H), *Buxus* 'Green Mound', *B. sinica* var. *insularis* 'Winter Beauty', and *B. microphylla* var. *japonica* 'Winter Gem'. A diverse array of germplasm is available in the genus *Buxus,* and identifying acceptable levels of disease tolerance in cultivars that represent this diversity will contribute to its continued use in ornamental landscapes.

INTRODUCTION

Boxwood blight, a disease causing leaf spots and stem lesions on *Buxus* species, is caused by *Calonectria pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous (syns. *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenew. & C.F. Hill and *C. buxicola* B. Henricot), an invasive pathogen first detected in the 1990s in the UK and New Zealand (Henricot et al. 2002; Ridley 1998) and first observed in the United States in 2011 (Douglas 2012; Ivors et al. 2012). This disease causes concern in the nursery and landscape industry because it attacks an economically and historically important ornamental plant formerly considered to have few important arthropod pests or pathogens. Boxwood blight kills young plants and weakens and disfigures older ones (Douglas 2012; Henricot and Culham 2002). It is unclear at this time whether the planting of tolerant cultivars will be a successful strategy in fighting the disease, but use of tolerant cultivars may earn a place on the menu of research-based integrated management practices, now wanted by boxwood nurseries, home gardeners, and landscape maintenance firms. For this laboratory study, we collected cuttings from 42 accessions in the National Boxwood Collection of the National Arboretum. Additional cuttings collected at the same time were rooted for later planting at sites in Connecticut, North Carolina, New Jersey, and New York, where they will be inoculated or exposed to natural infection by *C. pseudonaviculata*. Some of the unrooted cuttings were immediately tested in vitro for their susceptibility to *C. pseudonaviculata*, and these results are presented here.

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doi:10.1094/PHP-RS-14-0033

COLLECTION OF CUTTINGS

All cuttings were collected from boxwood at the U.S. National Arboretum (Washington, DC) in late July when new growth was mature. Plants had not been sheared and had received no fungicide treatments. Cuttings were shipped to propagation sites and the Fort Detrick laboratory for receipt 24–25 July 2013.

PRODUCTION OF CONIDIA

A representative isolate (cbs114417) from Great Britain was used for inoculum. This isolate did not differ in virulence or cultural characteristics from isolates collected from Connecticut, Delaware, or North Carolina (Shishkoff, *unpublished*) and, as the type for *Cylindrocladium buxicola* (a taxonomic synonym of *Calonectria pseudonaviculata*), has been widely distributed among researchers. All of these isolates fall into population type "G-1" typical of *C. pseudonaviculata*. Cellophane sheets (Biorad GelAir cellophane support, Bio-Rad Laboratories Inc., Hercules, CA) were cut into 8.2-cm diameter circles and autoclaved. Glucose-yeast extract-tyrosine (GYET; Hunter 1992) was prepared and poured to 9-cm petri plates. After the agar had solidified, the surface of the medium in each plate was topped with a piece of sterile cellophane. Following inoculation with the pathogen, plates were incubated for 1–2 months at 20°C until the surface of the cellophane was covered with microsclerotia. The cellophane was then peeled from the surface of the culture and placed on fresh GYET agar (without cellophane), causing the microsclerotia to germinate and produce copious numbers of conidia. These were collected in water (with 0.1% v/v Tween-20) and adjusted to 2000 spores/ml.

INOCULATION OF BOXWOOD

The apical portion of each unrooted cutting was dipped in a 500-ml beaker containing the spore suspension for about 5 sec and then the cut basal end was placed in a 50-ml centrifuge tube filled with water. In each of two consecutive trials, four cuttings from each cultivar were inoculated, and one was dipped in water rather than spore solution to serve as a negative control. Inoculated and control cuttings were placed in a mist tent overnight and exposed to the fog produced from a model DK625 ultrasonic fogger (Mainland Mart Corp., El Monte, CA). Cuttings were then moved to a mist tent in a greenhouse at constant 25°C and lightly misted every 10 min (Fig. 1). Ratings of symptoms were made 7 days and 11 days after inoculation. At 7 days, the number of spots per leaf was counted (Table 1). Any fallen leaves were also rated and counted. At 11 days, the number of infected leaves and fallen leaves were counted, as was the number of lesions per stem; the cumulative percentage of fallen leaves is reported in Table 1.

STATISTICS

Data for the two trials were combined for analysis. Analysis of variance was tested using General Linear Models in SAS (PROC GLM, SAS Institute Inc., Cary, NC) and means separated using Fisher's Least Significant Difference. When analyzing cultivar differences in the percentage of diseased leaves, the rating from day 11 could be normalized after an arc sine transformation and exclusion of three cultivars with very low disease ratings. The "Contrast" function was used to compare all cultivars to the positive control, *B. sempervirens* 'Suffruticosa' (English boxwood) and to a cultivar with few symptoms, *B. sinica* var. *insularis* 'Pincushion'. The number of spots per leaf and lesions per twig could not be normalized, while defoliation could be normalized and analyzed only for the 15 cultivars with the greatest amount of defoliation.

SUSCEPTIBILITY OF INOCULATED BOXWOOD CUTTINGS

Cuttings of the boxwood accessions showed a wide range in susceptibility to dip inoculation with *C. pseudonaviculata* under the optimal conditions for infection provided. There were significant differences in the percent diseased leaves ($P < 0.0001$) and percent defoliation ($P < 0.0001$) among species and cultivars. Table 1 lists the accessions tested ranked by the percentage of diseased leaves after 11 days. Cuttings of *Buxus sempervirens* L. (common or American boxwood) cultivars were among those with the highest percentage of diseased leaves, with eight cultivars showing as much disease as our positive control *B. sempervirens* 'Suffruticosa': 'Scupi', 'Pendula', 'Rotundifolia', 'Denmark', 'Handsworthiensis', 'Northland', 'Arborescens' (31793*H), and 'Northern New York'. All other cultivars of *B. sempervirens* and remaining *Buxus* species showed significantly less disease. A number of accessions were contrasted to the less

FIGURE 1

Unrooted cuttings of boxwood accessions were dip-inoculated and set upright in 50-ml centrifuge tubes filled with water. They were exposed overnight to ultrasonic fog then moved to a mist tent in a greenhouse at constant 25°C and lightly misted every 10 minutes (pictured).

susceptible *B. sinica* (Rehder & E.H.Wilson) M.Cheng. var. *insularis* 'Pincushion' and showed a similarly low level of disease: *Buxus* 'Green Ice', *B. sempervirens* 'Decussata', *B. sinica* var. *insularis* 'Wintergreen', *Buxus* sp. (57950*H), *Buxus* 'Green Mound', *B. sinica* var. *insularis* 'Winter Beauty', and *B. microphylla* Siebold & Zucc var. *japonica* 'Winter Gem'. Figures 2 and 3 show symptoms on A (adaxial) and B (abaxial) leaf surfaces for two *Buxus* species.

Leaf drop for English boxwood was 6.5% and leaf drop for common boxwood was 14.3% on inoculated cuttings. No lesions or abscised leaves were observed in the controls. Leaf drop on the cuttings which exhibited lower leaf spotting than English and common boxwoods ranged from 0–13.2%.

CONCLUSIONS

Henricot et al. (2008) tested 11 species of *Buxus* and found all of these species to be susceptible to the disease, by rating inoculated plants for the proportion of leaves with spotting. The most susceptible plants included *B. sempervirens* 'Suffruticosa', *B. sinica* var. *insularis*, and *B. harlandii*, although there was some difference in severity depending on the isolate used. Ganci et al. (2013a; 2013b) tested a number of cultivars as entire container-grown plants inoculated outdoors and found generally high susceptibility in cultivars of *B. sempervirens* (including English boxwood) and lower susceptibility in cultivars of *B. microphylla*. Ganci et al. (2014) also performed tests of intact plants under laboratory conditions and found significant correlation between susceptibility ratings of plants inoculated in the field and in the lab. Further experiments will use plants grown from cuttings taken at the same time as those in the lab study, placed at sites where they will be exposed to natural or introduced inoculum.

FIGURE 2

Disease symptoms on a cutting of *Buxus sempervirens* 'Northland'. (**A**) Adaxial leaf surfaces with discolored lesions; (**B**) abaxial leaf surfaces with lesions covered with conidiophores.

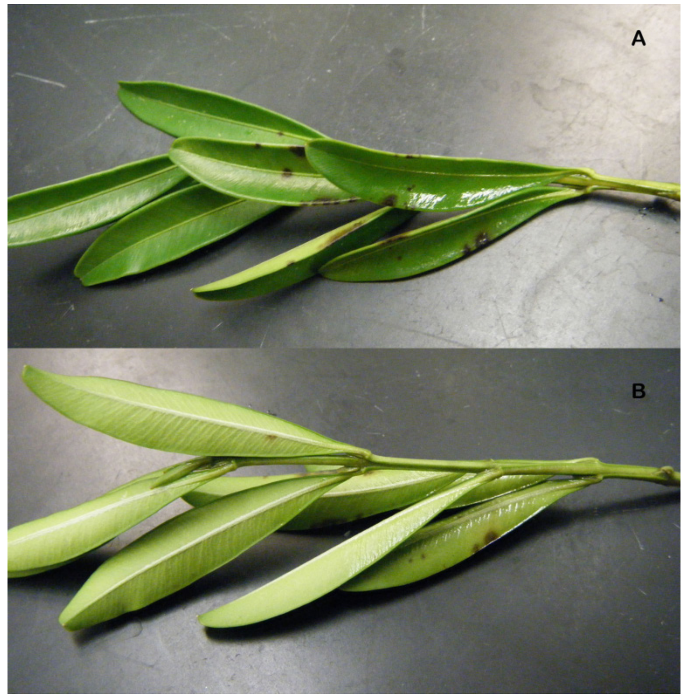

FIGURE 3

Disease symptoms on a cutting of *Buxus harlandii*. (**A**) Adaxial leaf surfaces with discolored lesions; (**B**) abaxial leaf surfaces.

Detached-leaf studies can give different results from whole-plant assays and from plants infected naturally, and care has to be taken to interpret such results. However, detached-organ assays are often chosen because they are faster, cheaper, and take up less space than whole-plant assays or field tests. Sometimes detached-leaf assays work very well because they give similar results to whole-plant assays (Anteneh et al. 2013) are more uniform and more easily reproducible than whole plant assays (Arraiano et al. 2001; Azmat et al. 2013), protect against cross-contamination when different pathogen isolates are used (Jackson et al. 2008), or can accurately predict disease on a different plant part (Pettitt et al. 2011). However, sometimes they work less well because not all resistance is expressed in detached leaves (Browne et al. 2005; Michel et al. 2010.), susceptibility is variable in differently-aged leaves (Ergon and Tronsmo 2006; Nowakowska et al. 2014; Osman-Ghani 1982), or the pathogen behaves differently on detached leaves (Townley et al. 2002). Sometimes detached leaves must be intentionally wounded to study secondary pathogens (Shi and Hsiang 2014). Akhtar et al. (2012) compared 82 tomato genotypes against *Phytophthora infestans* using detached-leaf and whole-plant assays and rated 41 susceptible and 40 highly susceptible using the detached-leaf assay, while 18 were susceptible and 63 highly susceptible using the whole-plant assay. With *Phytophthora ramorum*, a pathogen that can infect leaves, stems, and roots, no single assay (detached leaf dip, whole plant dip, stem-wound inoculation assay or log inoculation) predicted the full range of symptoms observed in the field (Hansen et al. 2005). Foolad et al. (2000) compared field, greenhouse and detached-leaflet assays to determine susceptibility of tomato genotypes to early blight, using area under the disease progress curve and percent defoliation for field experiments, percent defoliation for greenhouse tests and lesion radius and rate of lesion expansion in detached leaf tests. There was significant correlation between field and greenhouse assays, but leaf assays were inconsistent and correlated with neither. The use here of detached cuttings takes into account differently-aged leaves in a way that should allow comparison with field trial results.

In addition, laboratory assays have advantages of speed, uniformity of inoculum concentration, and consistent environmental conditions for infection and disease development. Furthermore, inoculating cuttings in controlled environments prevents introduction of harmful pathogens into pathogen-free areas. Cuttings take up much less space than whole plants and cost less to procure (an important consideration for studies on a high-cost ornamental). An additional advantage of our study was that the identities of boxwood plants are linked to a reference collection, i.e., the National Boxwood Collection at the U.S. National Arboretum. Boxwood cultivars can be misidentified in the nursery trade, and old selections such as *B. sempervirens* 'Arborescens' and *B. sempervirens* 'Suffruticosa' may represent populations of individuals with diverse genotypes.

In our laboratory tests, accessions of English and some common boxwood showed over 71% spotted leaves and defoliation rates up to 14%. English and common boxwood currently account for the majority of *Buxus* used in the nursery trade in the United States. Thus, identifying cultivars or species with lower spotting and defoliation than these would provide an option for improved boxwood performance in U.S. gardens where *C. pseudonaviculata* is present. In this trial, 29 of the accessions from the U.S. National Arboretum showed statistically fewer spotted leaves than the English or the most susceptible of the common boxwood (along with 3 others which could not be included in the analysis because of the very low number of leaves infected). These less susceptible accessions showed spotting on only 6.3–36.2% of leaves.

TABLE 1
Susceptibility of unrooted cuttings of 42 accessions of boxwood from the U.S. National Arboretum to *Calonectria pseudonaviculata*.

No.[a]	*Buxus* species & cultivar	Diseased Lvs (%)[b]	Spots/ leaf[c]	Lesions/ stem[d]	Fallen Lvs (%)[e]
9548*H	*sempervirens* ‘Scupi’	80.9 A	2.75	10.63	12.2
59820*H	*sempervirens* ‘Pendula’	76.4 AB	2.33	0.63	1.3
29703*H	*sempervirens* ‘Suffruticosa’	74.2 AB	1.99	1.50	6.5
36365*J	*sempervirens*	71.5 ABC	2.22	2.75	14.3
35494*H	*sempervirens* ‘Rotundifolia’	70.4 ABC	1.74	6.88	34.7 AB
34196*H	*sempervirens* ‘Denmark’	67.5 ABCD	2.83	3.38	15.2 CDEF
4233*H	*sempervirens* ‘Handsworthiensis’	63.0 ABCDE	1.81	2.38	18.3 CDE
51910*H	*sempervirens* ‘Northland’	62.1 ABCDE	1.47	5.38	21.5 BCD
31793*H	*sempervirens* ‘Arborescens’	59.2 BCDEF	2.48	5.00	17.2 CDEF
29701*H	*sempervirens* ‘Northern New York’	59.5 BCDEF	1.88	1.75	15.7 CDEF
18834*H	*harlandii*	52.5 CDEFG	3.93	1.88	20.8 CD
29694*H	*sempervirens* ‘Marginata’	52.5 DEFG	1.19	1.25	4.2
54327*H	*sempervirens* ‘Newport Blue’	49.2 DEFGH	1.04	2.13	10.5
57953*H	*sempervirens* ‘Arborescens'	48.4 EFGHI	2.88	12.50	40.4 A
51907*H	‘Green Velvet’	48.1 EFGHIJ	2.25	3.00	5.4
68631*H	*sempervirens* ‘Dee Runk’	46.5 EFGHIJK	2.65	3.88	22.3 BC
33789*H	*sempervirens* ‘Graham Blandy’	46.6 FGHIJK	2.93	7.25	6.6 F
35487*H	*sempervirens* ‘Edgar Anderson’	44.0 FGHIJKL	1.97	2.63	8.2 EF
29224*H	*microphylla* ‘Grace Hendrick Phillips’	42.9 FGHIJKLM	2.51	1.75	9.0
51905*H	‘Green Mountain’	41.5 GHIJKLMN	1.67	1.63	16.8 CDEF
34198*H	*sempervirens* ‘Myrtifolia’	41.5 GHIJKLMN	0.96	1.88	9.6 DEF
7025*H	*microphylla* var. *japonica* ‘National’	40.4 GHIJKLMN	2.06	3.13	26.8 ABC
33810*H	*microphylla* ‘John Baldwin’	39.8 GHIJKLMN	1.22	1.25	9.1
72213*H	*microphylla* var. *japonica* ‘Jim Stauffer’	37.4 GHIJKLMNO	1.70	0.63	7.1
52423*H	*bodinieri*	36.2 HIJKLMNOP	2.29	0.75	13.9
51904*K	‘Green Gem’	34.8 HIJKLMNOPQ	1.91	0.38	7.3
68273*H	‘Glencoe’	33.3 IJKLMNOPQ	1.81	2.88	7.6
51896*H	*wallichiana*	31.7 JKLMNOPQ	1.16	1.00	6.8
6395*H	*sempervirens* ‘Vardar Valley’	31.8 KLMNOPQR	0.98	1.88	3.0
69558*H	*sempervirens* ‘Ohio’	31.8 KLMNOPQR	1.50	3.25	0.0
78079*H	*microphylla* var. *japonica* ‘Gregem’	28.5 LMNOPQRS	1.89	2.38	0.9
71429*H	‘Green Ice’	28.6 MNOPQRS	1.06	5.00	0.0
17078*H	*sempervirens* ‘Decussata’	26.4 NOPQRS	2.56	3.63	16.2 CDEF
37772*H	*sinica* var. *insularis* ‘Wintergreen’	23.8 OPQRS	1.14	4.50	8.9
57950*H	*Buxus* sp.	21.6 PQRS	2.11	3.63	0.5
51906*H	‘Green Mound’	20.4 QRST	1.00	1.50	1.3
51900*H	*sinica* var. *insularis* ‘Winter Beauty’	17.5 RST	1.66	4.25	3.7
51898*H	*sinica* var. *insularis* ‘Pincushion’	16.6 ST	1.10	0.25	5.7
54326*H	*microphylla* var. *japonica* ‘Winter Gem’	7.3 T	0.63	1.88	4.6
4899*CH	*microphylla* ‘Compacta’	14.1	0.13	2.63	0.0
4227*R	*microphylla* var. *japonica*	19.3	0.73	2.88	9.3
60705*H	*sinica* var. *aemulans*	6.3	0.33	1.13	4.7

[a] Accession number for the U.S. National Arboretum collection.

[b] The percentage of diseased leaves 11 days after inoculation. Numbers followed by the same letter do not differ significantly by General Linear models with LSD. Data not followed by a letter had to be excluded from the dataset because of excessive zeros preventing the normalization of the dataset.

[c] Spots counted on infected leaves 7 days after inoculation.

[d] Lesions counted on each stem piece 11 days after inoculation.

[e] The percentage of leaves that had dropped off over the 11-day period after inoculation. Numbers followed by the same letter do not differ significantly by General Linear models with LSD. Data not followed by a letter had to be excluded from the dataset because of excessive zeros preventing the normalization of the dataset.

Future field planting studies with the same accessions will be helpful for assessing boxwood landscape performance when *C. pseudonaviculata* is present, and will also help us to see how closely field performance with boxwood plants parallels results in this laboratory assay on mature cuttings. It remains to be seen what measurable symptom factors are most important in field performance. To select plants with the best field tolerance to the disease, is it important to select only for reduced leaf infection, or is reduced shoot infection equally important? Will defoliation be the factor most critical to consider when selecting plants with good landscape performance? Are stem lesions a better indicator of long-term susceptibility? We anticipate that the best ornamental boxwood will very likely be those that have minimal infection of leaves, and thus will show little defoliation. A diverse array of germplasm is available in the genus *Buxus*, and includes plants with differences in leaf form and color, size and habit, and temperature tolerances. The continued use of boxwood in ornamental landscapes is dependent on identifying acceptable levels of boxwood blight tolerance in cultivars that represent this diversity. For nursery production and hedging purposes, for example, relatively fast growth and an upright, dense habit are highly desirable.

Although the planting of tolerant cultivars may seem like a good strategy for integrated management, it may at times lead to pathogen spread on boxwood showing few or no symptoms. Ganci et al. (2013b) inoculated whole plants of *B. harlandii*, a partially resistant species, and placed healthy but highly susceptible *B. sempervirens* 'Suffruticosa' plants approximately 25 cm distant from the *B. harlandii.* After six weeks, the non-inoculated *B. sempervirens* were exhibiting dramatic symptoms, while *B. harlandii* showed only minor leaf spotting. Thus, species and cultivars exhibiting minimal symptoms have been shown to transmit boxwood blight to more susceptible boxwood plants: a similar scenario could take place in nurseries or landscapes. Tolerant cultivars bought from an outside source are therefore probably better planted at a site without pre-established boxwood. Based on this study, cultivars with different levels of susceptibility to boxwood blight can be identified for use in additional tests with different isolates of the pathogen, in order to see if differences in virulence exist for *C. pseudonaviculata*. The ability to compare *Calonectria* isolates for virulence is especially important because two distinct populations have been identified in Europe (Douglas 2012), which are now thought to comprise separate species (K. Heungens, *personal communication*), only one of which is currently known in the United States. Continued use of boxwood in American landscapes will depend on the successful integration of disease management practices and breeding of novel, boxwood blight-tolerant cultivars.

ACKNOWLEDGMENTS AND DISCLAIMERS

This research was partially supported by funding from the USDA-APHIS-CPHST through the FY2012 and FY2013 Farm Bills The use of trade, firm, or corporation names on this page is for the information and convenience of the reader. Such use does not constitute an official endorsement of approval by the USDA Agricultural Research Service, NAL or BIC of any product or service to the exclusion of others that may be suitable. The U.S. Department of Agriculture (USDA) prohibits discrimination in all its programs and activities.

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