Survival of *Microsclerotia* of *Calonectria pseudonaviculata* and *C. henricotiae* Exposed to Sanitizers

Nina Shishkoff, Foreign Disease-Weed Science Research Unit, ARS/USDA, Frederick, MD 21702

Accepted for publication 8 January 2016. Published 14 January 2016.

ABSTRACT

Shishkoff, N. 2016. Survival of microsclerotia of *Calonectria pseudonaviculata* and *C. henricotiae* exposed to sanitizers. Plant Health Prog. 17:13-17.

Discrete microsclerotia of *C. pseudonaviculata* from culture were treated with eight commercially available sanitizers (ZeroTol 2.0, Oxidate, Sanidate, X³, Greenshield, and Lysol Concentrate at maximum label rates, plus 70% Ethanol and 0.6% sodium hypochlorite) to determine effective methods of disinfestation of nursery beds and benches. Ethanol proved most effective, killing microsclerotia in less than 5 min. ZeroTol, 10% bleach, and 70% ethanol were used in comparison

tests of microsclerotia of *C. pseudonaviculata* and *C. henricotiae*. Microsclerotia of *C. henricotiae* were found to be more sensitive regardless of size. This finding, along with other research that has found differences in fungicide sensitivity and thermotolerance between the two species, suggests that differences between the species should be elucidated.

INTRODUCTION

Boxwood blight is caused by two species of *Calonectria*: *C. pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous; and *C. henricotiae* Gehesquière, Heungens and J.A. Crouch. The symptoms, leaf and stem lesions on boxwood, were first noticed in the 1990s in Great Britain (Henricot and Cullum 2002) and New Zealand (Ridley 1998), then eventually throughout Europe thereafter: in Belgium (Crepel et al. 2003); Ireland (Henricot 2006); Germany (Brand 2005); The Netherlands (Henricot 2006); France (Saurat et al. 2012); Spain (Pintos Varela et al. 2009); Italy (Sarachi et al. 2008); Croatia (Cech et al. 2010); the Czech Republic (Safrankova et al. 2012); Georgia (Gorgiladze et al. 2011); Abkhazia (Gasich et al. 2013); and Turkey (Akilli et al. 2012). In North America, the disease was first observed in 2011 in the U.S. states of Connecticut, North Carolina, and Oregon (Douglas 2012; Ivors et al. 2012), and in the province of British Columbia, Canada (Elmhirst et al. 2013); it is currently reported in more than 14 U.S. states (Malapi-Wight et al. 2014). The fungal pathogen was named *Calonectria pseudonaviculata* (Lombard et al. 2010). The original isolates examined (by amplified fragment length polymorphism analysis) were genetically similar, suggesting an introduced organism of limited genetic diversity (Henricot and Culham 2002). Later, some European populations were determined to differ significantly. They were phylogenetically distinct, could not interbreed with the other population despite being of the opposite mating type, and had different sensitivities to temperature and fungicides (Gehesquière et al. 2016). Gehesquière et al. recognized the second clade as a separate species, *C. henricotiae*, and considered it another introduced organism. So far, only *C. pseudonaviculata* is known to be present in the United States (Gehesquière et al. 2015). Boxwood blight has caused concern in

the nursery industry not only because it can kill young boxwood plants and weaken and disfigure older ones, but because infected boxwood leaves and stem tissue contain microsclerotia (Weeda and Dart 2012) that remain viable in soil and debris for long periods of time (Dart et al. 2013; Dart et al. 2015; Ganci 2014), making replanting of boxwood in affected sites problematic. In a previous study (Douglas 2013), a number of commercially available sanitizing agents were tested for their ability to kill mycelium and spores of the pathogen. In this study, discrete microsclerotia of *C. pseudonaviculata* were treated to determine effective methods of disinfestation of nursery beds and benches. The most effective were used to compare efficacy against microsclerotia of both species.

PRODUCTION OF MICROSCLEROTIA

Two isolates of *C. pseudonaviculata* were used in experiments (CBS114417 from Great Britain and CpsCT1 from Connecticut) and three of *C. henricotiae* (Bp55, CB045, and Bp78). Experimentation with all isolates was restricted to a Biosafety Level 3 greenhouse at Fort Detrick, MD. Microsclerotia were produced by placing culture plugs of the pathogen onto the surface of autoclaved cellophane sheets (Biorad GelAir cellophane support, Bio-Rad Laboratories, Inc., Hercules, CA), which covered the surface of glucose yeast-extract tyrosine (GYET) agar (Hunter 1992) in 9-cm Petri dishes. After 2 months of incubation at 20°C, the surface of the cellophane was covered with microsclerotia that were easily detached by adding 2 ml of water and gently rubbing the cellophane with a gloved finger. Pooled microsclerotial suspensions were run through a series of nested sieves to capture microsclerotia in the 125 to 350 µm range. These microsclerotia were suspended in sterile water and added to a Buchner funnel under vacuum fitted with an 11-cm No. 5 Whatman filter paper. After removal from the funnel, the filter paper was cut into small squares (ca. 1 cm²) that contained approximately 50 microsclerotia. Two squares were placed in 90- × 20-mm mesh bags (25-µm mesh size, 100% polyamide sefar nitex bags, Sefar Inc., Buffalo, NY) for use in sanitizer assessments.

Corresponding author: N. Shishkoff. Email: nina.shishkoff@ars.usda.gov.

doi:10.1094/PHP-RS-15-0038
© 2016 The American Phytopathological Society

Microsclerotia of *C. pseudonaviculata* (isolate CBS114417) exposed to sanitizing agents for 0, 15, 30, and 60 min. ZeroTol 2.0 (hydrogen dioxide, 27.1%) was used at the maximum labeled rate for uncleaned surfaces and work areas (1:50 dilution), while Oxidate and Sanidate (hydrogen peroxide, 0.108%) and X³ (benzalkonium chloride, 0.13%) were used at their ready-to-use full strength rate. Ethanol was used at a 70% rate and bleach (sodium hypochlorite, 6.0%) at a 10% rate. Greenshield (quaternary ammonium compounds, 20%) was used at the product label rate for pots, flats, and benches (1 tbsp/gal). All dilutions were made using water with Tween 20 as a wetting agent (1 drop/liter) and this solution was used as the control disinfectant solution. Mesh bags containing microsclerotia were placed in 50-ml centrifuge tubes containing 40 ml of the test solutions. Two bags from each test solution were removed at 0, 15, 30, and 60 min. Upon removal from the test solution, each pair of mesh bags was placed in a 50-ml centrifuge tube containing sterile water and agitated for several minutes before being placed in a second tube of water for 20 to 30 min. The mesh bags were then split at the seam and inverted onto a plate of GYET agar so that the microsclerotia were spread over the surface of the agar. After 3 to 4 days at 20°C, plates were examined for germinating microsclerotia that produced mycelium with a characteristic chestnut-brown pigment in agar and conidiophores of *Calonectria*. Percent germination was recorded for each plate. This experiment was repeated 3 times. Percent germination of microsclerotia was arcsine-square root-transformed to normalize data and then analyzed using the General Linear Model procedure in SAS (SAS Institute Inc., Cary, NC). Treatments were compared using Fisher's Least Significant Difference test at each sampling interval. Analysis showed model variables "sanitizer," "time," and "sanitizer × time" to be significant at $P < 0.0001$ and that some treatments differed significantly at each time interval by LSD analysis. In these experiments, both 70% ethanol and ZeroTol at the 1:50 rate significantly reduced germination of microsclerotia to 0 after 15 min. No germination was observed at time 0 for ZeroTol treatments, but due to the difficulty of quickly removing so many samples, time zero was realistically more like 30 to 60 sec. Sanidate and 10% bleach reduced microsclerotial germination to ca. 20% after 1 h, and the other treatments were less effective (Fig. 1). Microsclerotia treated with bleach eventually whitened, but we found that if any brown tissue remained in the microsclerotium, it was able to germinate (Fig. 2).

Microsclerotia of *C. pseudonaviculata* (isolate CBS114417) exposed to sanitizing agents at 0, 5, 10, and 15 min. Bleach, ethanol, and Sanidate were used at the same rates; ZeroTol was used at the rate for cleaning pots, trays, flats, for drenching soil, or for sanitizing clean surfaces and cutting tools (1:100 v/v); and Lysol Concentrate (o-benzyl-p-chlorophenol, 5.5%) was used at the rate for disinfecting surfaces (2.5 oz/quart). The experiment was repeated 3 times. Percent germination of microsclerotia was arcsine-square-root transformed to normalize data and then analyzed using the General Linear Model procedure. Treatments were compared using Fisher's Least Significant Difference (LSD) test at each sampling interval. Analysis showed variables "sanitizer," "time," and "sanitizer × time" to be significant at $P < 0.0001$ and some treatments differed significantly at each time interval by LSD analysis. As in the first experiment, the most effective sanitizer was 70% ethanol, reducing germination to zero after 5 min. The lower rate of ZeroTol was still fairly effective after 15 min, but the Lysol treatment did not differ from the control (Fig. 3). Bleach significantly reduced germination

compared to the control, but was not as effective as ethanol or ZeroTol.

Exposing microsclerotia of two isolates of *C. pseudonaviculata* and two of *C. henricotiae* to sanitizing agents at 0, 5, 10, and 15 min. Microsclerotia (*C. pseudonaviculata* isolates CBS114417 and CpsCT1, and *C. henricotiae* isolates CB045 and bp78) were exposed to ZeroTol (1:100 v/v), 10% bleach, and 70% ethanol for up to 15 min. The experiment was repeated two times. Percent germination of microsclerotia was arcsine-square-root transformed to normalize data and then analyzed using the General Linear Model procedure. Treatments were compared using Fisher's Least Significant Difference test at each sampling interval. All treated microsclerotia showed significantly decreased germination compared to the control, but the *C. henricotiae* isolates were significantly more affected by ZeroTol and bleach than isolates of *C. pseudonaviculata* (Fig. 4b and c). All microsclerotia were killed by exposure to ethanol for 5 min (Fig. 4a).

Exposing different size classes of microsclerotia of *C. pseudonaviculata* and *C. henricotiae* to ZeroTol for 0, 5, 10, or 15 min. Microsclerotia of *C. pseudonaviculata* isolate CBS114417 and *C. henricotiae* isolate bp55 were captured in nested sieves in three size categories—large (250 to 353 µm), medium (177 to 249 µm) and small (125 to 177) — and each size class was tested for sensitivity to ZeroTol (1:100 v/v). The experiment was run 3 times, but one trial lacked small microsclerotia for *C. pseudonaviculata*. Percent germination of microsclerotia was arcsine-square-root transformed to normalize data and then analyzed using the General Linear Model procedure. Treatments were compared using Fisher's Least Significant Difference test at each sampling interval. Analysis showed the model to be significant at $P < 0.0001$, with isolate, sanitizer, and time significant at $P < 0.0001$ and size ("large," "medium," or "small") of microsclerotia significant at $P < 0.02$. Microsclerotia of *C. henricotiae* were more sensitive to ZeroTol than those of *C. pseudonaviculata* regardless of size category (Fig. 5).

FIGURE 1

Microsclerotia of *C. pseudonaviculata* (isolate CBS114417) exposed to sanitizing agents for 0, 15, 30, and 60 minutes. Microsclerotia from culture were exposed to ZeroTol 2.0 (1:50 v/v), Oxidate (full strength), Sanidate (full strength) X³ (full strength), ethanol (70%), sodium hypochlorite (0.6%), or Greenshield (1 tbsp/gal). Water with Tween 20 as a wetting agent (1 drop/1 liter) served as the control. Treatments were compared using T tests (Least Squares Difference) at each sampling interval; symbols followed by different letters differed significantly ($P < 0.05$).

FIGURE 2

Microscerotia treated with sodium hypochlorite (0.6%) for (A) 0 min (B) 15 min (C) and (D) 60 minute. The microscerotium in (C) still has a brown core and has germinated; the microscerotium in (D) is completely bleached.

FIGURE 3

Microscerotia of *C. pseudonaviculata* (isolate CBS114417) exposed to sanitizing agents at 0, 5, 10, and 15 min. Microscerotia were exposed to Sanidate (full strength), ethanol (70%), sodium hypochlorite (0.6%), ZeroTol(1:100 v/v), and Lysol Concentrate (2.5 oz/quart). Treatments were compared using T tests (Least Squares Difference) at each sampling interval; symbols followed by different letters differed significantly ($P < 0.05$).

Determining the size of microscerotia in leaf tissue.

Boxwood plants (*B. sempervirens* ‘Suffruticosa’) were inoculated with spore suspensions (1×10^4 spores/ml) of the 5 isolates used in this experiment, placed in a dew chamber set at 20°C for 4 days until symptoms began to be visible, and incubated in the greenhouse until large lesions were present on leaves. Symptomatic leaves were allowed to rest on a bed of moist sand for a month, then 10 leaves were collected, cleared in 10% KOH then stained in Trypan blue. Microscerotium diameter for each isolate was measured at three or more locations in each leaf using measuring software (ACT-1 software, Nikon, Inc.), on a Nikon DS-L1 digital camera. Diameters of microscerotia were measured and analyzed without transformation using variables “isolate” and “leaf” (nested within “isolate”) in General Linear Models with differences among isolates compared using Fisher’s Least Squares Difference. Analysis showed variables “isolate” and “leaf” to be significant at $P < 0.0001$. *C. pseudonaviculata* isolate CBS114417 had microscerotia significantly larger than those of all other isolates (with an average diameter of $62.13 \pm 13.0 \mu\text{m}$); microscerotia of *C. pseudonaviculata* isolate CpsCt1 did not differ significantly in size from *C. henricotiae* isolate Bp55 ($52.32 \pm 12.0 \mu\text{m}$ and $51.60 \pm 10.4 \mu\text{m}$, respectively) but were larger than those of *C. henricotiae* isolates CB045 and

Bp78, with CB045 significantly smaller than any other isolate (average $43.5 \pm 8.8 \mu\text{m}$).

CONCLUSIONS AND RECOMMENDATIONS

Microsclerotia used in experiments were from culture under relatively clean, soil-free conditions. Microsclerotia from culture, while easy to use in quantitative experiments, cannot replicate natural conditions. They were larger in general than those formed in leaf tissue, which might make them harder to kill using sanitizers; on the other hand, microsclerotia embedded in leaf tissue might have added protection from sanitizers because of the leaf tissue. These experiments should be considered a starting point to evaluate candidate sanitizers that are commercially available. Once promising candidates have been found, the next logical step is to try them out in naturally infested sites. Peroxides will react with any organic material, so under natural conditions one might see less efficacy for ZeroTol, Sanidate, and Oxidate. ZeroTol was most effective at the highest rate (1:50), at which it is a powerful oxidant. Ethanol (70%) was extremely effective for microsclerotia, but might be less effective on spores (Douglas

2013). While bleach was shown in these trials to be a reasonable sanitizer, few growers will want to dip tools in a powerful oxidant or expose workers to fumes for extended periods of time. Some products may be too expensive for large-scale use. All of this suggests that remediation of microsclerotia-infested soil will be a challenge in fighting this disease.

There appeared to be a significant difference in the sensitivity of microsclerotia of the two species to some sterilants. In culture, the microsclerotia of *C. henricotiae* are somewhat smaller than those of *C. pseudonaviculata*, but in leaf tissue the difference is not as extreme and microsclerotial size also varied from leaf to leaf. During a concurrent survival study of *C. pseudonaviculata* in litter sampled over time, numerous infected leaves were examined, and microsclerotial size varied considerably; when they developed in substomatal cavities in the mesophyll, they were fairly uniform in size unless they coalesced into larger masses or erupted through the epidermis to form larger masses (Shishkoff, *unpublished data*). However, in the current tests the difference in effect of sterilants was mainly correlated to species, not size. Gehesquière et al. (2015) determined a number of differences between *C. pseudonaviculata* and *C. henricotiae*, including lower sensitivity to fungicides in *C. henricotiae* sufficient “to cause *in vivo* insensitivity to standard doses of ... two commercial products containing active ingredients registered for use against box blight in several countries, illustrating the potential threat of *C. henricotiae* to the international boxwood industry.” *C. henricotiae* was also found to have a higher thermotolerance. These factors suggest that differences between the species should be elucidated.

ACKNOWLEDGMENTS

Thanks to Kristine Johnson and Dr. Farivar Eskandari for technical assistance. Funding support was provided from the 2013 Farm Bill project through APHIS. 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

FIGURE 4

Exposing microsclerotia of 2 isolates of *C. pseudonaviculata* (on chart abbreviated as Cps) and two of *C. henricotiae* (Che) to sanitizing agents at 0, 5, 10, and 15 minutes. For clarity, results for the different sanitizers were presented in separate graphs of percent germination over time, with the controls for the entire experiment (one control for each isolate) shown in A. (A) Microsclerotia of four isolates exposed to 70% ethanol compared to microsclerotia of the four isolates exposed to a water control; (B) Microsclerotia exposed to ZeroTol (1:100 v/v); (C) microsclerotia exposed to 10% bleach. Treatments were compared using T tests (Least Squares Difference) at each sampling interval; symbols followed by different letters differed significantly ($P < 0.05$). Each isolates exposed to each sanitizer differed significantly in percent germination from its control after time zero (letters not shown).

FIGURE 5

Exposing different size classes of microsclerotia of *C. pseudonaviculata* (Cps) and *C. henricotiae* (Che) to ZeroTol for 0, 5, 10, or 15 minutes. Microsclerotia of *C. pseudonaviculata* and *C. henricotiae* were captured in nested sieves in 3 size categories: large (250 to 353 μm), medium (177 to 249 μm), and small (125 to 177 μm) and each size class was tested for sensitivity to ZeroTol (1:100 v/v). Treatments were compared using T tests (Least Squares Differences) at each sampling interval; symbols followed by different letters differed significantly ($P < 0.05$).

LITERATURE CITED

- Akilli, S., Katircioğlu, Y. Z., Zor, K., and Maden, S. 2012. First report of box blight caused by *Cylindrocladium pseudonaviculatum* in the Eastern Black Sea region of Turkey. New Dis. Rep. 25:23. <http://dx.doi.org/10.5197/j.2044-0588.2012.025.023>
- Brand, T. 2005. Auftreten von *Cylindrocladium buxicola* B. Henricot an Buchsbaum. Nordwest-Deutschland. Nachrichtenbl. Dtsch. Pflanzenschutzdienstes (Braunschweig, Ger.) 57:237-240.
- Cech, T., Diminic, D., and Heungens, K. 2010. *Cylindrocladium buxicola* causes common box blight in Croatia. Plant Path. 59:1169.
- Crepel, C., and Inghelbrecht, S. 2003. First report of blight on *Buxus* spp. caused by *Cylindrocladium buxicola* in Belgium. Plant Dis. 87:1539.
- Dart, N., and Hong, C. 2014. An improved leaf disc bioassay for detecting *Calonectria pseudonaviculata* in soil and potting media. Plant Dis. 98:1626-1631.
- Dart, N., Hong, C., Craig, C. A., Fry, J. T., and Hu, X. 2015. Soil inoculum production, survival, and infectivity of the boxwood blight pathogen, *Calonectria pseudonaviculata*. Plant Dis. 99:1689-1694. <http://dx.doi.org/10.1094/PDIS-12-14-1245-RE>
- Douglas, S. M. 2012. Boxwood blight - A new disease for Connecticut and the U.S. Boxwood Blight Information Page, Connecticut Agriculture Experiment Station, New Haven, CT. Retrieved from http://www.ct.gov/caes/lib/caes/documents/publications/fact_sheets/plant_pathology_and_ecology/boxwood_blight_a_new_disease_for_connecticut_and_the_u.s._07-20-12_r.pdf
- Douglas, S. M. 2013. Efficacy of sanitizing agents to refine best management practices for the boxwood blight pathogen *Calonectria pseudonaviculata*. Phytopathology 103:S2.36.
- Elmhirst, J. F., and Auxier, B. E. 2013. First report of box blight caused by *Cylindrocladium pseudonaviculatum* (*C. buxicola*) in British Columbia, Canada. Plant Dis. 97:559.
- Ganci, M., Benson, D. M., LaMondia, J. A., and Ivors, K. 2014. The show must go on: Boxwood and beyond. Phytopathology 104:S3:147.
- Gasich, E. L., Kazartsev, I. A., Gannibal, P. B., Koval, A. G., Shipilova, N. P., Khlopunova, L. B., and Ovsyannikova, E. I. 2013. *Calonectria pseudonaviculata* - a new for Abkhazia species, the causal agent of boxwood blight. Mikol. Fitopatol. 47:129-131.
- Ghesquière, B., Crouch, J. A., Marra, R. E., Van Poucke, K., Rys, F., Maes, M., Gobin, B., Höfte, M., and Heungens, K. 2016. Characterization and taxonomic reassessment of the box blight pathogen *Calonectria pseudonaviculata*, introducing *Calonectria henricotiae* sp. nov. Plant Pathol. 65:37-52. <http://dx.doi.org/10.1111/ppa.12401>
- Gorgiladze, L., Meparishvili, G., Sikharulidze, Z., Natsarishvili, K., and Davitadze, R. 2011. First report of box blight caused by *Cylindrocladium buxicola* in Georgia. New Dis. Rep. 23:24.
- Henricot, B., and Culham, A. 2002. *Cylindrocladium buxicola*, a new species affecting *Buxus* spp., and its phylogenetic status. Mycologia 94:980-997.
- Hunter, B. B. 1992. *Cylindrocladium*. Pages 107-110, 248 in: Methods for Research on Soilborne Phytopathogenic Fungi. L. L. Singleton, J. D. Mihail, and C. M. Rush, eds. APS Press, St. Paul, MN.
- Ivors, K. L., Lacey, L. W., Milks, D. C., Douglas, S. M., Inman, M. K., Marra, R. E., and LaMondia, J. A. 2012. First report of boxwood blight caused by *Cylindrocladium pseudonaviculatum* in the United States. Plant Dis. 96:1070.
- Lombard, L., Crous, P. W., Wingfield, B. D., and Wingfield, M. J. 2010. Phylogeny and systematics of the genus *Calonectria*. Stud. Mycol. 66:31-69.
- Malapi-Wight, M., Hébert, J. B., Buckley, R., Daughtrey, M. L., Gregory, N. F., Rane, K., Tirpak, S., and Crouch, J. A. 2014. First report of boxwood blight caused by *Calonectria pseudonaviculata* in Delaware, Maryland, New Jersey, and New York. Plant Dis. 98:698.
- Pintos Varela, C., González Penalta, B., Mansilla Vázquez, J. P., and Aguín Casal, O. 2009. First Report of *Cylindrocladium buxicola* on *Buxus sempervirens* in Spain. Plant Dis. 93:670.
- Ridley, G. S. 1998. New plant fungus found in Auckland box hedges (*Buxus*). Forest Health News 77:1.
- Safrankova, I., Kmoch, M., and Holkova, L. 2012. First report of *Cylindrocladium buxicola* on box in the Czech Republic. New Dis. Rep. 25:5.
- Sarachi, M., Rocchi, F., Pizzatti, C., and Cortesi, P. 2008. Box blight, a new disease of *Buxus* in Italy cause by *Cylindrocladium buxicola*. J. Plant Pathol. 90:581-584.
- Saurat, C., Fourrier, C., and Ioos, R. 2012. First report of blight disease on *Buxus* caused by *Cylindrocladium buxicola* in France. Plant Dis. 96:1069.
- Weeda, S. M., and Dart, N. L. 2012. Histological evidence that microsclerotia play a significant role in disease cycle of the boxwood blight pathogen in southeastern United States and implications for disease mitigation. Plant Health Prog. <http://dx.doi.org/10.1094/PHP-2012-0403-01-BR>.