Microsclerotial Enumeration, Size, and Survival of *Calonectria pseudonaviculata*

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Abstract

Boxwood blight caused by *Calonectria pseudonaviculata* (*Cps*) is an emerging disease in the United States. Understanding the biology and survival of microsclerotia, the resting propagules of *Cps*, is important to managing the disease and restoring *Cps*-infested sites. In this study, an image-analyzing software (OpenCFU) was adapted to enumerate microsclerotia produced in culture plates. Microsclerotial counts increased with time up to 42 days while their size continued to grow for

70 days. Additionally, younger microsclerotia were less sensitive to extreme temperatures of -10 and 40°C than older ones. For instance, 21-day-old microsclerotia survived at almost twice the rate of 70-day-old ones after a 24-h exposure to -10°C . Likewise, the 21-day-old microsclerotia survived at much greater rates than 70-day-old ones (50 to 70% versus $<10\%$) after a 24-h exposure to 40°C . The practical implications of these results are discussed.

Boxwood blight caused by *Calonectria pseudonaviculata* (*Cps*) (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous (anamorph: *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenew. & C.F. Hill) is an emerging disease. This disease was first observed in the United Kingdom in 1994 (Henricot 2006) and in the United States in 2011 (Ivory et al. 2012). It has spread to more than 24 states in the U.S. (Ivory et al. 2016; Ivory et al. 2012; Malapi-Wight et al. 2014; Ward Gauthier et al. 2016) and resulted in severe losses to the ornamental horticulture industry, historical gardens, and landscapes (Avenot et al. 2017; Palmer and Shishkoff 2014; Tripp 2016). Its major diagnostic symptoms include dark brown leaf spots, black streaks on stem, and rapid defoliation.

Microsclerotium as the survival structure of *Cps* is important to the disease cycle of boxwood blight. Henricot et al. (2008) reported that *Cps* could survive in the litter for at least 5 years. *Cps* microsclerotia were first captured under the microscope by Weeda and Dart (2012). In a follow-up study, Dart et al. (2015) demonstrated that after being buried in the soil for 40 weeks, *Cps* microsclerotia had no sign of decline, suggesting this pathogen has a significant soil phase. Shishkoff and Camp (2016) also reported that *Cps* microsclerotia survived for more than 30 months at 0 to 20°C , but they died off after 2 and 7 months at 30 and -10°C , respectively. Furthermore, Shishkoff (2016) tested microsclerotia of various sizes for their survival after exposure to the sanitizer ZeroTol. The medium (177 to $249\text{ }\mu\text{m}$) and small (125 to $177\text{ }\mu\text{m}$) microsclerotia had the highest and lowest survival rates, respectively, with large (250 to $353\text{ }\mu\text{m}$) ones in the middle (Shishkoff 2016). It is unclear whether and how *Cps* survival under adverse conditions may be associated with its microsclerotial size. More investigations into the biology and survival of *Cps* microsclerotia are needed in order to develop sustainable solutions to managing boxwood blight.

Production, enumeration, and standardization are among the most important factors affecting *Cps* microsclerotia research quality and productivity. A method reported by Shishkoff and Camp (2016) is widely used for producing *Cps* microsclerotia in culture plates. Currently, enumeration on *Cps* microsclerotia is done manually, which is labor intensive and time consuming. The size of *Cps* microsclerotia produced in culture plates ranged from 125 to $353\text{ }\mu\text{m}$ as reported by

Shishkoff (2016). How microsclerotial size may be related to the culture age, and consequently to *Cps* survival under extreme environmental conditions are yet to be determined. Thus, the objectives of the present study were to develop a rapid enumeration method for *Cps* microsclerotia in culture plates and determine how microsclerotial size might change with culture age, as well as how it might affect the survival of microsclerotia under extreme temperatures.

Materials and Methods

Isolate selection and production of microsclerotia. Five *Cps* isolates recovered from *Buxus* and *Sarcococca* spp. in Virginia and North Carolina, U.S.A., were selected as representative isolates in this study (Table 1). Microsclerotia were produced using a modified method with cellophane (Nakajima et al. 1988; Shishkoff and Camp 2016). An agar block (5 mm in diameter) taken from the actively growing area of a 7- to 14-day-old culture was placed at the center of an autoclaved cellophane sheet (Sigma-Aldrich Co., MO, U.S.A.) covering the surface of 15-ml freshly prepared glucose-yeast extract-tyrosine agar (GYETA) (Hunter 1992) in a 10-cm Petri dish. The culture plates were sealed with Parafilm and incubated in darkness at 20°C .

Selection of threshold value in OpenCFU. Use of OpenCFU software to count microsclerotia began with photographing the culture plates. The software then recognized and counted circular objects on the digital images while the counting accuracy was improved by a user-defined thresholding procedure (Geissmann 2013). Selection of threshold value in OpenCFU (Geissmann 2013) in this study was accomplished by counting the *Cps* microsclerotia in the same plates manually and using the software and comparing the counts. Two *Cps* isolates 11A01 and 11A08 recovered from different hosts (Table 1) and three culture ages of 21, 42, and 70 days were included in this comparison. On each day, microsclerotia in triplicate plates per isolate were

Table 1. Origin of five representative isolates of *Calonectria pseudonaviculata* used in this study

Isolate	Alternative code	Host	Tissue	Location	Year
11A01	VA11-232	<i>Buxus sempervirens</i>	Leaf petiole	Virginia, U.S.A.	2011
11A03	BB137	<i>Buxus</i> sp.	Leaf petiole	Virginia, U.S.A.	2013
11A04	BB188	<i>B. sempervirens</i>	Stem	Virginia, U.S.A.	2013
11A05	BBRC1	<i>Buxus</i> sp.	n.a. ^z	North Carolina, U.S.A.	2014
11A08	RL1	<i>Sarcococca hookeriana</i> 'Humilis'	Leaf	Virginia, U.S.A.	2015

^z Not available.

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Fig. 1. Comparison of microsclerotial counts obtained manually and using OpenCFU with various threshold settings at 0, 1, 2, and 3. Columns topped with a same letter within each isolate and individual culture ages did not differ significantly according to Waller-Duncan k-ratio *t*-test at $P = 0.05$.

Fig. 2. Colony morphology variations between *Calonectria pseudonaviculata* isolates 11A01 and 11A03 on day 42. Isolate 11A01 produced limited to no aerial mycelia, while 11A03 produced abundant aerial mycelia, which made photo-analysis by OpenCFU and manually counting impossible.

manually counted under a dissecting microscope using a click-counter. Cellophane surface of the same plates were photographed 10 times each using a Nikon D90 camera. The camera was stabilized on a stand and set uniformly (manual, ISO 400, F5.6 w/ 40s, incandescent in white balance) throughout the experiment. The culture plates were placed in a photography light tent with background light. Photos were analyzed using OpenCFU (Geissmann 2013) with the radius minimum set at “1,” and threshold values at “0,” “1,” “2,” and “3.” The resulting

counts at various threshold values in OpenCFU were then compared with manual counts. The threshold value at which microsclerotial counts using OpenCFU did not differ from the manual counts was then set in OpenCFU as the standard in the following experiments.

Enumeration of microsclerotia. Five culture plates of each isolate were photographed on days 14, 21, 28, 42, 56, and 70 using the Nikon D90 camera as described above. On each day, 10 photos were taken on the cellophane surface of each culture plate. Microsclerotia captured

Fig. 3. Microsclerotial production by four *Calonectria pseudonaviculata* isolates over a 70-day period in two experiments. Microsclerotial counts marked with a same letter within each isolate and experiment did not differ significantly according to Waller-Duncan k-ratio *t*-test at $P = 0.05$.

Fig. 4. Morphology of microsclerotia produced by isolate 11A01. A, Globose. B, Ellipsoid. C and D, Irregular-shaped. Bars = 100 μm .

in each photo were enumerated in OpenCFU (Geissmann 2013) with threshold value determined above. This experiment was conducted twice.

Size of microsclerotia. Microsclerotia were harvested from triplicate culture plates of isolates 11A01, 11A04, 11A05, and 11A08 on days 21, 28, 42, 56, and 70. Two milliliters of distilled water (diH₂O) were added to each culture plate. A sterile cotton tipped applicator was used to brush the cellophane surface. The slurry was poured into a 50-ml centrifuge tube. The above procedure was repeated until all microsclerotia on the cellophane were collected. Then, diH₂O was added to the centrifuge tube until the total volume reached 25 ml. The tube containing *Cps* cultural suspension was vortexed at the maximum speed for 1 min. The agitated slurry was run through two sieves with 355 and 125 μ m mesh size. The sieves were washed through with diH₂O several times to remove mycelia and other nontarget structures. Microsclerotia on the 125- μ m sieve were resuspended in diH₂O in a 50-ml tube. Fifty randomly selected microsclerotia harvested from each culture plate were examined using a HDMI-2 camera (Southern Microscope, Inc., NC, U.S.A.) connected to a Nikon Labophot-2 microscope and measured for their size using TCApture version 3.9.0.6001. Two measurements were taken perpendicularly for each microsclerotium with the first along its longest axis (length) and the second (width) in the middle on that axis in order to best estimate its size. This experiment was conducted twice.

Survivals of microsclerotia at various temperatures. Microsclerotial suspensions harvested on days 21, 28, 42, 56, or 70 were

pipetted onto Whatman Grade 1 filter papers. The wet filter papers were then blow-dried in a biosafety cabinet for 2 h. Each filter paper with approximately 500 microsclerotia harvested from each culture plate was cut into three equal sections, which were then incubated for 24 h at -10, 20, and 40°C, respectively. Microsclerotia were then separated from each filter paper section using sterile cotton tipped applicators and plated onto triplicate Petri dishes containing 15 ml of freshly prepared acidified peptone-dextrose agar (PEDA). The PEDA plates containing microsclerotia were placed under ambient light at room temperature (~23°C) for 10 days. The microsclerotial survival was determined by counting the percentage of germinated microsclerotia among 100 randomly selected microsclerotia on each PEDA plate. This experiment was conducted twice.

Statistical analysis. Basic calculations and descriptive statistics were performed using Microsoft Excel 2016. Analysis of variance was performed using R statistical software version 3.1.3 (R Core Team 2015) to determine the difference in results between repeat experiments, among representative isolates, replicates, and other factors. Waller-Duncan k-ratio *t*-test was performed to provide grouping using the package 'agricolae' (de Mendiburu 2015) in R. The significance levels in this study were set at 0.05 (5%).

Results

Threshold value in OpenCFU. There was no difference in microsclerotial counts using OpenCFU among the replicate culture plates

Fig. 5. Microsclerotial size (length and width) of four *Calonectria pseudonaviculata* isolates increasing with age.

($P = 0.32$) and photos ($P = 0.99$). Thus, the average microsclerotial count of 30 photos at each threshold setting in OpenCFU was compared with the manual count of microsclerotia by isolate and culture age.

Microsclerotial counts decreased as the threshold value increased from 0 to 3 in OpenCFU (Fig. 1). For both isolates, microsclerotial counts using OpenCFU at threshold 0 were significantly higher than manual counts at all culture ages (Fig. 1). There was no significant difference in the microsclerotial count between the OpenCFU at threshold 1 and manual counting (Fig. 1). For isolate 11A01, microsclerotial counts using the OpenCFU were significantly lower when threshold was set at 2 and 3 than respective manual counts at all three culture ages. For isolate 11A08, no significant difference was observed between manual counts and those using OpenCFU at threshold values 1, 2, and 3 (Fig. 1). Based on these results, threshold value was set at 1 in OpenCFU in the following enumeration.

Enumeration of microsclerotia. All isolates produced abundant microsclerotia on cellophanes. Isolates 11A01 (Fig. 2), 11A04, 11A05, and 11A08 produced limited to no aerial mycelia. However, isolate 11A03 produced abundant aerial mycelia that completely covered the cellophane surface (Fig. 2). Thus, microsclerotia produced by isolate 11A03 could not be counted either manually or using OpenCFU. This isolate was excluded from subsequent experiments.

For the remaining four isolates, there was no difference in the microsclerotial count among five replicate culture plates within isolates ($P = 0.21$) and 10 photos within plates ($P = 0.71$). Microsclerotial counts were significantly different between two repeating experiments ($P < 0.01$), and among four isolates ($P < 0.01$). Microsclerotia produced by all isolates in both experiments rapidly increased with time from day 14 to 28 (Fig. 3). The speed increase slightly declined between days 28 and 42, although a significant

Fig. 6. Length distribution of the 21- to 70-day-old microsclerotia produced by four isolates of *Calonectria pseudonaviculata*. Columns and moving average trendlines (period set at 2) were plotted in Excel.

difference in the microsclerotial count was still present between these two culture ages (Fig. 3). Thereafter, the count stabilized between days 42 and 70. The only exception was that the count of isolate 11A05 on day 56 that was significantly higher than on day 42 in the first experiment (Fig. 3).

Size of microsclerotia. Microsclerotia varied greatly in shape (Fig. 4). They could be globose (Fig. 4A), ellipsoid (Fig. 4B), and irregular (Figs. 4C and D). The average length to width ratios were approximately 1.5 on day 21, and 1.3 on day 70.

Both microsclerotial length ($P < 0.01$) and width ($P < 0.01$) were significantly different between two experiments. There was no difference in the length among three replicate culture plates ($P = 0.56$) and 50 replicate microsclerotia ($P = 0.96$). Similarly, no difference in the microsclerotial width was observed among culture plates ($P = 0.14$) and replicates ($P = 0.18$).

Microsclerotial length and width increased with time in both experiments. In experiment 1, the average length of microsclerotia

increased from $198 \pm 47 \mu\text{m}$ to $237 \pm 62 \mu\text{m}$ from day 21 to 70 with a $0.8 \mu\text{m}$ daily increase. Similarly, the average width increased from $130 \pm 38 \mu\text{m}$ to $174 \pm 49 \mu\text{m}$ during the same period with a $0.9 \mu\text{m}$ daily increase (Fig. 5). In experiment 2, the average length increased from $203 \pm 41 \mu\text{m}$ to $256 \pm 61 \mu\text{m}$ with a daily increase of $1.1 \mu\text{m}$ and the width increased from $132 \pm 37 \mu\text{m}$ to $193 \pm 49 \mu\text{m}$ with a daily increase of $1.2 \mu\text{m}$ (Fig. 5).

Distributions of microsclerotial length and width are presented in Figures 6 and 7, respectively. On day 21, the most common length ranged from 180 to 190 μm in experiment 1 and 190 to 200 μm in experiment 2 (Fig. 6) and the most common width ranged from 110 to 120 μm in experiment 1, and 100 to 110 μm in experiment 2 (Fig. 7). As expected, the occurrence of larger microsclerotia increased with time. On day 70, the most common length range was 200 to 230 μm in experiment 1 and 240 to 250 μm in experiment 2 (Fig. 6). The most common width range was 140 to 170 μm in experiment 1 and 150 to 190 μm in experiment 2 (Fig. 7).

Fig. 7. Width distribution of the 21- to 70-day-old microsclerotia produced by four isolates of *Calonectria pseudonaviculata*. Columns and moving average trendlines (period set at 2) were plotted in Excel.

Microsclerotial survival at various temperatures. Microsclerotial survival as measured by the germination rate was significantly different among culture ages ($P < 0.01$) and incubation temperatures ($P < 0.01$). There was no difference in the germination rate between two repeat experiments ($P = 0.86$), among three culture plates ($P = 0.95$) and replicate PEDA plates ($P = 0.93$).

Microsclerotial survival decreased with increasing culture age at 20°C (Fig. 8). For instance, the 21- and 28-day-old microsclerotia produced by all four isolates survived at rates of >98% (Fig. 8), while those 70-day-old ones produced by isolates 11A04, 11A05, and 11A08 survived at lower rates of 90.9 to 94.3%, except for 11A01 at the rate of 98.1% (Fig. 8).

Exposures to extreme temperatures greatly reduced the microsclerotial survival and this negative impact was more profound on older microsclerotia. After a 24-h exposure to -10°C, 69.4 to 82.0% of the 21-day-old microsclerotia survived, while only 31.5 to 52.9% of the 70-day-old ones did (Fig. 8). Likewise, after a 24-h exposure to 40°C, 52.4 to 67.3% of the 21-day-old microsclerotia survived, while only 0.9 to 9.8% of the 70-day-old ones did (Fig. 8).

Discussion

This study adapted the image-analyzing software OpenCFU (Geissmann 2013) for improving the enumeration of *Cps* microsclerotia. The count of microsclerotia produced in culture plates increased with time up to 42 days. While microsclerotia continued to grow in size for up to 70 days, their survival decreased with culture age. These results have several practical implications in future studies on *Cps* microsclerotium biology and the development of management strategies for boxwood blight.

Adapting OpenCFU greatly expedited microsclerotial enumeration and improved its accuracy. It took more than 2 h to count 2,000 to 3,000 microsclerotia manually in a single culture plate. It was almost impossible to count the microsclerotia in 20 plates manually on each designated assessment time without sacrificing the counting accuracy. With the adapted OpenCFU setting threshold at 1 in this study, only <30 min was required to count microsclerotia in a culture plate. By allowing all required microsclerotial enumeration in a timely manner, this new method certainly will improve research data quality. It also may reduce potential human error that is likely associated with manual counting. This method could be easily

adapted for enumeration of (micro)sclerotia produced by other fungi. It must be noted that selection of threshold value is crucial to the accuracy of microsclerotial enumeration when adapting this method, and therefore so is the initial manual counts upon which the threshold value is set.

Substantial variation was found in the size of *Cps* microsclerotia. The microsclerotia length ranged from approximately 100 to 450 µm (Fig. 6) and their width from approximately 50 to 350 µm (Fig. 7). This size range appeared wider than the previous report (Shishkoff 2016). Also, microsclerotial size increased with time up to 70 days in this study. It would be interesting to measure the size of microsclerotia that are older than 70 days to determine their maximum size. Additionally, the microsclerotial size distributions (Figs. 6 and 7) indicate that culture age is critical to the productivity in experiments that harvest microsclerotia of certain sizes. Furthermore, Shishkoff (2016) reported that microsclerotia produced in leaf tissues were significantly smaller than those produced on cellophane sheets in culture plates. Future studies are warranted to determine the size distribution of microsclerotia produced in plant tissues.

Survival of *Cps* microsclerotia could be reduced by a short exposure to high temperatures. For instance, the survival rates of microsclerotia were significantly reduced to <10% after a 24-h exposure to 40°C (Fig. 8). Such temperature influence on the survival of *Cps* microsclerotia was also reported by Shishkoff and Camp (2016). These results indicate that heat treatments, such as solarization and composting, are promising measures to eradicate *Cps* microsclerotia and restore infested sites. Solarization has been used to eradicate resting propagules of other *Calonectria* spp. (Polizzi et al. 2003; Vitale et al. 2013), while a composting system to eradicate *Cps* has been under evaluation (Harvey et al. 2015). Additional studies are needed to determine the specifics for developing a heat-treatment protocol, such as the most timely and economically effective combination of temperature and duration, as both a 2-month incubation at 30°C (Shishkoff and Camp 2016) and a 24-h exposure to 40°C in this study eradicated or greatly reduced the survival of *Cps* microsclerotia. It is worth mentioning that microsclerotia in plant tissues appeared to survive better than those produced in vitro (Shishkoff and Camp 2016). Because 40°C was not included as a treatment in Shishkoff and Camp (2016), nor microsclerotia produced in plant tissues in this study, future studies are warranted to provide additional

Fig. 8. Germination rates of 21- to 70-day-old microsclerotia of four *Calonectria pseudonaviculata* isolates after a 24-h exposure to 20, -10, and 40°C. Germination rates marked with a same letter within each isolate and temperature treatment did not differ significantly according to Waller-Duncan k-ratio *t*-test at $P = 0.05$.

information on the survival of microsclerotia produced in situ and their sensitivity to 40°C.

Although microsclerotial survival was impaired at –10°C, this low temperature was not as efficient as the high temperature in reducing microsclerotial survival. After a 24-h exposure to –10°C, *Cps* microsclerotia survived at the rate of 40 to 80% (Fig. 8). In another study, it took 7 months to eradicate *Cps* microsclerotia at –10°C, while only 2 months at 30°C (Shishkoff and Camp 2016). Furthermore, it is unknown whether this low temperature truly resulted in mortality or merely induced the dormancy of microsclerotia. Previous studies reported that low temperatures could induce dormancy in fungal resting propagules such as microsclerotia of *Calonectria crotalariae* (Griffin et al. 1978) and sclerotia of *Sclerotinia sclerotiorum* (Dillard et al. 1995). It remained unknown if non-germinated microsclerotia in this study were dead or became dormant after exposure to –10°C, knowing that special measures could be required to break such dormancy (Dillard et al. 1995).

The fact that younger microsclerotia survived better at extreme temperatures than older ones clearly indicates that longer exposure times in the heat treatment are required to eradicate younger microsclerotia than the older ones. Considering the older microsclerotia are larger in size, this finding may also indicate a negative correlation between microsclerotial size and their survival at extreme temperatures, although more investigations are warranted to further explore the potential correlation between the microsclerotia size and survival under other adverse environmental conditions.

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