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Conidial production and viability of *Calonectria pseudonaviculata* on infected boxwood leaves as affected by temperature, wetness, and dryness periods

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Abstract

Calonectria pseudonaviculata causes lesions on boxwood leaves and twigs. Controlled-environment experiments were conducted to determine the effects of temperature and leaf wetness period on *C. pseudonaviculata* sporulation on diseased (cv. Suffruticosa) leaves and of dryness periods and high temperature on conidial survival. Infected leaves were incubated in moist chambers and subjected to six temperatures (9, 13, 17, 21, 25, and 29°C) and six leaf wetness periods (0, 12, 24, 40, 48, and 72 h). Spore production was influenced significantly by wetness period, temperature, and their interaction. Increasing duration of leaf wetness and increasing temperature generally increased sporulation, with no sporulation occurring at 29°C or 9 and 13°C, except at 72 h of wetness exposure, while it was optimal at 21°C. Detached leaves with profuse conidia were subjected to a range of drying (relative humidity at 65%) times (0, 2, 4, 6, and 8 h) at two temperatures of 21 and 29°C. Conidia were then harvested and plated on water agar. Germinating conidia were counted to measure the spore viability. Spore mortality increased with increasing dryness duration at both temperatures but occurred more quickly and severely at 29 than 21°C. Overall, this study extended biological knowledge of conditions required for crucial stages of the *C. pseudonaviculata* disease cycle and the obtained results will be vital for developing boxwood blight forecasting and management tools.

KEYWORDS
Cylindrocladium buxicola, spore survival, sporulation potential, weather parameters

1 | INTRODUCTION

Boxwood blight is caused most commonly by *Calonectria pseudonaviculata*, and less commonly by *C. henricotiae*, a second species that occurs in Europe but has not been seen in the United States (Gehesquière et al., 2016). Over the past 25 years, boxwood blight has been reported for many countries (Daughtrey, 2019; Henricot, 2006), initially from the UK and New Zealand, and subsequently from Belgium, Croatia, Czech Republic, Denmark, France, Georgia, Germany, Ireland, Italy, the Netherlands, and other countries (Daughtrey, 2019). The disease has also been reported for Canada and 30 US states (Daughtrey, 2019). It is a major concern in the boxwood nursery and landscape industry because the causal pathogen can be lethal and attacks an economically and historically important ornamental plant (Gehesquière et al., 2013; Palmer & Shishkoff, 2014; Shishkoff et al., 2015).

The current approach for management of this disease depends largely upon use of less susceptible cultivars and fungicide protection. Although some cultivars are more tolerant than others, no boxwood cultivar is immune to the blight disease (Ganci et al., 2013; LaMondia & Shishkoff, 2017; Shishkoff et al., 2015). Chemical protection, although effective (Baudoin et al., 2015; Henricot et al.,

2008; LaMondia, 2015), is costly due to high fungicide prices, a long season of susceptibility, short retreatment intervals needed for effective control, and the required labour.

Knowledge of pathogen basic biology is useful for developing a more rational strategy of managing this disease. There have been several in planta studies about the influences of temperature and leaf wetness on some components of *C. pseudonaviculata* disease cycle under laboratory and controlled environments (Avenot et al., 2015, 2017; Gehesquière, 2014; Gehesquière et al., 2016; Henricot, 2006). These studies revealed moderate temperatures and prolonged leaf wetness period as an important factor driving the infection and spread of boxwood blight (Avenot et al., 2015; Gehesquière, 2014; Henricot, 2006). For instance, Avenot et al. (2017) reported that disease incidence on inoculated plants increased with increasing temperature (18–25°C), with the optimal temperature for disease symptom development estimated at 23.7°C. Similarly, Gehesquière (2014) reported that disease incidence was greatest when inoculated boxwood plants were incubated at either 19.5 or 22.4°C, the maximum temperatures tested in that study. Work by Henricot (2006) also suggested that infection by the fungus is rapid in warm (18–25°C) and humid conditions. Avenot et al. (2017) further reported that dry interruptions of wet periods reduced the amount of disease development on inoculated plants compared to those plants exposed to continuous wetness. In vitro studies of the impacts of abiotic factors on *C. pseudonaviculata* development have characterized this fungus as a low-temperature pathogen because it is able to grow below 10°C. In contrast, its growth is inhibited at 30°C and it is killed at 33°C (Henricot, 2006). From a more recent temperature-dependent in vitro study, no pathogen growth was observed at temperatures of 3 and 31°C, while optimal growth was observed at 25°C for all *C. henricotiae* and *C. pseudonaviculata* isolates tested (Gehesquière, 2014; Gehesquière et al., 2016). When the effect of temperature on sporulation of isolates was investigated, the optimal temperature was only 18°C, with reduced sporulation at 21.1 and 23.2°C (Gehesquière, 2014; Gehesquière et al., 2016).

Pathogen reproduction is a key element of epidemic development, and understanding the conditions that influence sporulation is of critical importance to timing fungicide application (Anco et al., 2013). For most polycyclic diseases, sporulating lesions are the largest source of inoculum for the temporal and spatial spread of disease during the growing season, with fungal sporulation mainly influenced by the interactions of leaf wetness and temperature (Anco et al., 2013; Lalancette et al., 2003; Zadoks & Schein, 1979). However, the specific set of conditions favouring or affecting the production of secondary inoculum of *C. pseudonaviculata* on boxwood leaves is poorly understood. In several fungal species, desiccation has been found to reduce the ability of conidia to germinate (Bradley et al., 2003). How dry period may interrupt *C. pseudonaviculata* spore production and affect spore survival at different temperatures is not known.

The objectives of this study were to (a) determine the effects of temperature and leaf wetness period on *C. pseudonaviculata* sporulation; and (b) assess the influence of high temperature and dry period on spore survival by measuring conidial germination.

2 | MATERIALS AND METHODS

2.1 | Generating diseased boxwood leaves for sporulation study

Two single-spored isolates of *C. pseudonaviculata*, CP-137 and CP-160, recovered from diseased boxwood leaves from a boxwood nursery in Carroll County, VA, and a private property in Richmond, VA, were used in this study. Three 5-mm diameter potato dextrose agar (PDA) plugs from the actively growing margin of the pathogen colonies were placed onto new PDA Petri dishes (10 Petri dishes for each isolate) and allowed to grow for up to 2 weeks. To stimulate conidia formation, Petri dishes were flooded with approximately 4–5 mm of sterile deionized (DI) water for 2 h, and then mycelia were scraped with a sterile spatula (Dart et al., 2015). Loose mycelia were rinsed off the agar by spraying the Petri dishes with sterile DI water. Thereafter, the Petri dishes were incubated upside down at 22–24°C in ambient light for up to 5 days. Freshly formed spores were harvested by spraying each Petri dish with approximately 30 ml of DI water, the force of the water helping to release the conidia into a beaker. The resulting stock conidial suspension was shaken vigorously and filtered through three layers of cheesecloth. Two-year-old *Buxus sempervirens* 'Suffruticosa' plants ($n = 2$) in 1-gallon pots were inoculated with a conidial suspension of an equal mix of the above two isolates at 2×10^4 conidia/ml, using a hand sprayer (PRO16RW; Contico) until run-off. The inoculated plants and one uninoculated control (sprayed with water) were immediately moved into a growth chamber (M-12; Environmental Growth Chambers) set at 23°C and 100% relative humidity, with a photoperiod of 14 h light (55 $mmol \cdot m^{-2} \cdot s^{-1}$) and 10 h of darkness. They were incubated for 24 h to facilitate infection and then moved and held for 2 weeks in dry conditions (65%–75% RH) in the greenhouse with natural lighting and an average temperature of 25 ± 3°C to allow symptom development in the absence of sporulation.

2.2 | In-vivo sporulation on diseased leaves as impacted by temperature and wetness period

Temperatures assessed in this study were 9, 13, 17, 21, 25, and 29°C, while leaf wetness periods were 0, 12, 24, 40, 48, and 72 h. A completely randomized factorial design with temperature and wetness period as treatments was used to examine their effects on sporulation of *C. pseudonaviculata* on artificially infected boxwood leaves.

Leaves with nonsporulating boxwood blight lesions of similar sizes (approximately 3–6 mm^2) were arbitrarily selected, surface sterilized with a 0.8% solution of sodium hypochlorite for 30 s, rinsed in sterile DI water, and blotted dry. Ten infected leaves were placed in individual 9-mm Petri dishes with abaxial surface up and were subjected to

designated temperature and wetness period treatments. The open Petri dishes with leaves were placed in four clean plastic containers (crispers; 30 × 24 × 12 cm high) with DI water at the bottom to ensure high relative humidity (100% RH) was maintained. Four Petri dishes were used for each temperature and wetness treatment, with one Petri dish inside each crisper representing each of the six leaf wetness periods. The experiment used four replicates and was conducted twice with a total of 144 Petri dishes (1440 leaves) used in each trial. Humidity and leaf wetness were maintained by positioning a moistened, Grade 2 qualitative filter paper (Cytiva Whatman) at the bottom of each Petri dish. The four containers were then sealed and randomly assigned to available M12-growth chambers. Only one or two growth chambers were available at the same time during these experiments, and each was randomly assigned to and set at a constant and designated temperature of 9, 13, 17, 21, 25, or 29°C and a 14-h light (55 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/10-h dark cycle. To keep the leaf wet but with minimal water droplet runoff, both leaves and filter papers were sprayed for about 5 s with DI water as needed using a hand-held sprayer. Ten leaves from the uninoculated control plant were placed in one dish in one crisper and exposed to the above conditions. After each wetness period, all 10 leaves in each Petri dish in each crisper were collected and placed into Falcon tubes containing a 5-ml solution of sterile DI water with 0.02% Tween 20 (Croda Inc.). The tubes were shaken to dislodge spores from each set of leaves. Each resulting spore suspension was vortexed for 30 s and the number of spores per millilitre for each temperature and wetness treatment was immediately counted with the aid of a haemocytometer and compound microscope.

2.3 | In-vivo spore survival on diseased leaves as affected by dry period at two temperatures

A split-plot experimental design was used to examine the effects of post-sporulation temperature and dry period on in-vivo survival of conidia on boxwood leaves. Temperature (9, 13, 17, 21, 25, or 29°C) was used as the main-plot effect while drying time (0, 2, 4, 6, or 8 h) was used as the subplot effect. Two growth chambers were available during the experiments, and each was randomly assigned to one temperature during both trials. Conidia used in this study were all freshly produced on diseased leaves (10 per Petri dish) in moistened conditions as described above, and then incubated at 21°C with uninterrupted leaf wetness for 58 h to allow significant sporulation. Leaves bearing conidia were then transferred from the moistened environment to dry filter paper lining the bottom of new Petri dishes. The Petri dishes were placed in plastic containers as described above, which were left open and placed in growth chambers with 65% RH at either 21 or 29°C in the dark for 0, 2, 4, 6, or 8 h. Three replicate Petri dishes were used for each combination of temperature and drying time, and the experiment was carried out twice. At the end of each designated dry period, all leaves in each Petri dish were recovered and washed in 1.5 ml sterile DI water to dislodge the conidia. One hundred microlitres of each resultant conidial suspension (>20,000 spores/ml) were spread onto a Petri dish with 2% water agar. After 24 h incubation at approximately 24°C in the dark, conidia in each Petri dish were examined with the aid of a compound microscope. The number of germinated conidia out of the first 100 observed was used to measure the survival rate at each temperature and dry period treatment assessed.

2.4 | Data analysis

Differences between data from both trials in sporulation and germination experiments were tested using two-sample *t* tests. The effects of temperature and wetness period on in-vivo sporulation data were analysed using a two-way analysis of variance (ANOVA) for a two-factor completely randomized design. Multiple comparison with Fisher's LSD test at $\alpha = 0.05$ was subsequently used to test for differences among the six temperatures, six wetness durations, and the interaction of temperature × wetness duration. Prior to the analysis, the sporulation count data were transformed using a $\log(y + 1)$ transformation to improve normality and homogeneity of variance.

The effects of temperature, dry period, and the interaction of dry period × temperature on spore germination rate of *C. pseudonaviculata* on boxwood leaves were examined using ANOVA for a split-plot design, with temperature as the main-plot factor and drying time as the subplot, followed by multiple comparison with Fisher's LSD at $\alpha = 0.05$. The response variable (spore germination rate) was transformed using an arcsine $\sqrt{y}$ transformation to satisfy the normality and homogeneity requirements. All statistical analyses were performed with Statistix 10 (Analytical Software).

3 | RESULTS

3.1 | In-vivo sporulation on diseased leaves as impacted by temperature and wetness period

There were no significant differences among the means from both trials ($p = 0.7075$). The results of the ANOVA showed that there was a significant effect of temperature ($p < 0.0001$), wetness period ($p < 0.0001$), and the interaction of temperature × wetness period ($p < 0.0001$) on sporulation of *C. pseudonaviculata* on boxwood leaves. An examination of the interaction effect showed that spore production was greatest at 72 h and 21°C. Little sporulation occurred under wetness periods of 0, 12, and 24 h regardless of the temperatures tested, and minor sporulation was observed at 29°C regardless of the wetness periods assessed (Figure 1). Overall, sporulation under wetness periods of 40, 48, and 72 h was significantly greater than under wetness periods of 0, 12, and 24 h at all temperatures, except 9 and 29°C, when the amount of sporulation was similar across all leaf wetness periods. In addition, mean number of spores, at all tested temperatures except 29°C, was also significantly

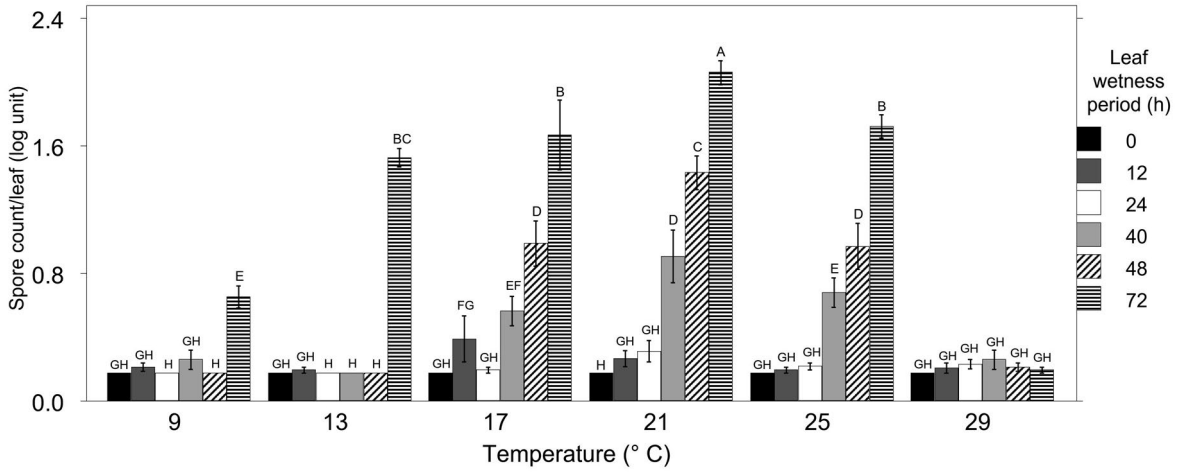

FIGURE 1 Effects of temperature and leaf wetness period on sporulation of *Calonectria pseudonaviculata* on boxwood leaves. Each bar (spore count/leaf in log unit) represents the mean of eight replicates from two experimental runs. Significant differences are indicated by different letters (α = 0.05)

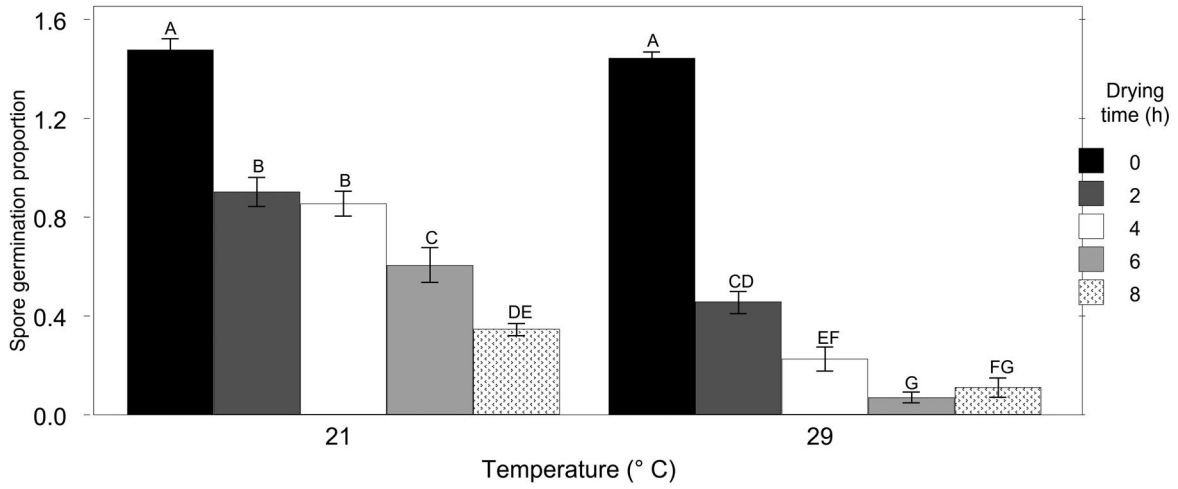

FIGURE 2 Effects of temperature and dry period on spore survival of *Calonectria pseudonaviculata* on boxwood leaves as measured by colony count. Each bar (germination proportion unit) represents the mean of six replicates from two experimental runs. Spores were produced for 58 h in wet conditions prior to drying. Significant differences are indicated by different letters (α = 0.05)

greater at 72 h wetness period compared with the other five wetness periods between 0 and 48 h (Figure 1).

3.2 | In-vivo spore survival on diseased leaves as affected by dry period at two temperatures

There were no significant differences among the means from both trials ($p = 0.8244$). The results of the ANOVA showed that there was a significant effect of temperature ($p < 0.0001$), dryness period ($p < 0.0001$), and the interaction of temperature × dryness period ($p < 0.0001$) on spore survival of *C. pseudonaviculata* on boxwood leaves. Spore survival decreased with increasing dry period and this decline was significantly sharper at 29 than 21°C (Figure 2). Spore survival was lowest when spores were exposed to a minimum of 6–8 h dry period after the initial wet period (Figure 2).

4 | DISCUSSION

This study improves our understanding of boxwood blight epidemiology by documenting the interactive effects of wetness/dryness duration and temperature exposures on the ability of *C. pseudonaviculata* to reproduce and germinate.

Our in vivo study of the effects of various temperature and leaf wetness regimes on *C. pseudonaviculata* sporulation revealed that it occurred abundantly under long periods of free moisture, and quantifies its modulation by temperature, increasing to an optimum at 21°C, and then decreasing faster at higher temperatures. An earlier

temperature sporulation study, which was conducted in vitro at three temperatures, found that sporulation of most *C. pseudonaviculata* isolates tested was highest at 18 and lower at 21.1 and 23.3°C (Gehesquière et al., 2016). In vitro studies of the factors that induce sporulation sometimes produce more variable results than sporulation patterns of the same fungus in vivo (Rodrigues et al., 2010; Rotem, 1994; Vloutoglou, 1994). Our study with infected leaves incorporates the influence of the host on pathogen reproduction concurrently with the effects of various temperatures and leaf wetness durations, and provides critical data documenting the need for long wetness periods for profuse pathogen sporulation, which can be used to elaborate epidemiological models (Anco et al., 2013; Bradley et al., 2003; De Wolf & Isard, 2007; Graça et al., 2009; Lalancette et al., 2003).

Our study also provides information about the very reduced sporulation at temperatures of 9 and 13°C for wetness periods less than 72 h and the detrimental effect of excessively high temperature on *C. pseudonaviculata* inoculum production on diseased leaves, with a temperature of 29°C strongly inhibiting sporulation, regardless of wetness duration. These findings parallel the observed effects of these variables on other biological components of the *C. pseudonaviculata* disease cycle: vegetative growth was also found to be significantly reduced at low temperature, with an optimum at 25°C, and strongly inhibited at high temperatures (Gehesquière, 2014; Henricot, 2006; LaMondia, 2014). Also, *C. pseudonaviculata* infection studies have shown that disease incidence increased with longer wetness periods (Avenot et al., 2017; Gehesquière, 2014), but infection rates decreased rapidly at temperatures higher than 25°C and are completely inhibited at 29°C, even in high-moisture conditions (Avenot et al., 2017).

Our second study, on the effect of temperature and leaf dryness duration on spore viability, revealed that *C. pseudonaviculata* spores germinated profusely following exposure to optimal sporulation temperature and moisture conditions. In contrast, exposure to high temperature and several hours of dryness kill *C. pseudonaviculata* spores and thus reduce or halt spore germination. Differences in relative humidity and solar radiation are likely to modify the rate of viability decline and should be examined in future studies. In several fungal species, desiccation and high temperatures have been found to reduce the ability of conidia to germinate (Bradley et al., 2003; Miller et al., 2018; Shishkoff et al., 2021). Miller et al. (2018), investigating the thermal sensitivity of aqueous conidial suspensions of *C. pseudonaviculata* and *C. henricotiae*, found that 90% of conidia of both boxwood blight pathogens exposed to 45°C for 35.4 min were killed. Similarly, exposures of diseased, detached boxwood leaves of cv. Justin Brouwers to hot water treatments significantly reduced conidial production and increased mortality of *C. pseudonaviculata* and *C. henricotiae* (Shishkoff et al., 2021).

Our findings demonstrate that *C. pseudonaviculata* spores neither reproduce well nor survive very long under dry conditions at temperatures ≥29°C. These growth chamber data are also in agreement with our field data, which showed that *C. pseudonaviculata* sporulation on infected tissues collected following periods of dryness throughout the growing season was either absent or limited (authors' unpublished data). Furthermore, these results are consistent with the lethal effect of high temperature and dry period on other major stages of the pathogen's disease cycle (Avenot et al., 2017; Henricot, 2006). For instance, interrupted wetness periods were shown to significantly reduce infection by *C. pseudonaviculata* (Avenot et al., 2015, 2017).

The findings of the present study add detail to our understanding of the weather conditions that allow boxwood blight to develop. The Virginia climate is characterized by variable temperatures with rainy periods alternating with or interrupted by warm and dry conditions, especially during the summer, and both the fluctuating reservoir of inoculum and conditions favouring infection will affect disease development. The information reported here will allow improvement of the boxwood blight infection risk model (Coop, 2013) into a more comprehensive forecasting system that will allow more effective interventions.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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