Research

Calonectria pseudonaviculata Conidia Dispersal and Implications for Boxwood Blight Management

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Abstract

We investigated *Calonectria pseudonaviculata* conidial dispersal from sporulating lesions on boxwood leaves and sporulating cultures on half-strength PDA (1/2 PDA). *Botrytis cinerea*-infected blossoms were used as a control. Dispersal of *C. pseudonaviculata* or *Botrytis* conidia was confirmed by capture using an Allergenco air sampler at 15 liters/min and by microscopic observation of conidia and *C. pseudonaviculata* growth on 15-cm-diameter 1/2 PDA Petri dishes. *C. pseudonaviculata* conidia were not dispersed by either dry or moist air currents directed at conidia and conidiophores from 2 mm away at air speeds of 19.8 m/s for 10 min or by a fine mist with water droplets (mean diameter 20 μm) with air speeds of 1.7 m/s. *C. pseudonaviculata* spores were dispersed by splash of water droplets at air speeds of 9.0 to 19.8 m/s. *C. pseudonaviculata* conidia released from phialides by water could not be wind dispersed after the water had evaporated. Secondary water dispersal was reduced because conidia strongly adhered to a surface after drying. Boxwood leaves dropped from heights of 15, 33, or 66 cm landed with more than 60% of leaves facing abaxial surface up. The cupped shape of most boxwood leaves may result in the abaxial surface with sporulation facing up. That orientation may also aid in retention of water films to wet and release conidia for splash dispersal. This is consistent with observations of increased disease severity in lower boxwood canopies and reinforces suggestions for best management practices including mulching and pruning lower branches to reduce the incidence and severity of disease.

Keywords: *Buxus*, boxwood blight, *Calonectria pseudonaviculata*, spread

Introduction

Boxwoods (*Buxus* spp.) are the most economically important evergreen shrubs in the United States. Boxwoods represent more than $126 million in annual wholesale value (USDA-NASS 2014) and are extremely valuable components of many landscape plantings, including many historically important gardens (Batdorf 2005). Boxwood blight disease is caused by the fungal pathogen *Calonectria pseudonaviculata* syn. ≡ *Cylindrocladium pseudonaviculatum* Crous, J.Z. Groenew. & C.F. Hill, = *Cylindrocladium buxicola* (Henricot) (Crous & al.) L. Lombard & al. in the United States. The pathogen produces cylindrical two-celled conidia ranging from 48 to 62 × 4 to 6 μm in size. The conidia are held side by side above the leaf surface on phialides in sticky clusters that can be approximately 50 × 50 μm. Henricot (2006) postulated that wind dispersal of the sticky conidia was not likely. Gehesquière et al. (2013) developed real-time polymerase chain reaction assays to detect *C. pseudonaviculata* and quantified conidia in air and water samples. They concluded that *C. pseudonaviculata* was consistently detected in high concentrations in water samples but only sporadically and in low concentrations in air samples. Dispersal was also investigated under simulated nursery conditions (Gehesquière 2014); windborne dispersal was unlikely, and both direct and indirect water splash dispersal was demonstrated. Windborne movement of *C. pseudonaviculata* conidia may be physically limited by the large size of conidia and the sticky nature of conidial aggregations (LeBlanc et al. 2018). However, size may limit distance of movement more than mechanism of dispersal, because large spores of *Alternaria brasiliensis* (>150 × 28 μm) (Simmons 2007) and large pollen grains such as corn (*Zea mays*) with diameter ranging from 80 to 125 μm are readily wind dispersed (Hofmann et al. 2014). In addition, sticky clumps of pollen such as *Ambrosia* can also be distributed by wind (Martin et al. 2009). No direct investigations of windborne dispersal of *C. pseudonaviculata* conidia have been reported.

The *C. pseudonaviculata* pathogen causes leaf and stem lesions and significant defoliation of diseased leaves. Sporulation of the fungus occurs primarily on the abaxial surface of the leaves. We noted that many dropped diseased leaves surrounding infected plants had abaxial surfaces facing up with visible sporulation, so we conducted experiments to examine whether boxwood leaves dropped from different heights tended to land in a biased orientation. The goal of this research was to test dispersal of *C. pseudonaviculata* conidia or clumps of conidia via windborne versus splash mechanisms, and to measure the bias of leaf drop orientation, because this would affect splash dispersal.

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Pathogen

Anamorphic isolates of *C. pseudonaviculata* from Connecticut were used in these experiments. Inocula consisted of conidia sporulating on infected leaves of *Buxus microphylla* var. *koreana* × *B. sempervirens*

‘Green Mountain’ or on agar blocks excised from sporulating cultures grown on half-strength potato dextrose agar (1/2 PDA) (LaMondia 2015).

Experimental Methods

We conducted replicated experiments to investigate *C. pseudonaviculata* conidial dispersal from both sporulating lesions on infected Green Mountain boxwood leaves and sporulating cultures on 1.0- or 0.2-cm^2 blocks of 1/2 PDA. Air streams were directed from a two-channel Air Delivery System (Analytical Research Systems, Gainesville, FL) at 138 kPa with a 1.3 liters/min flow rate through a 1-mm inner diameter Pasteur pipette to create a jet of air at 71.3 kph (19.8 m/s) (Aylor 1975) (Fig. 1). Dry or saturated air jets were achieved by use of a desiccant (Drierite, calcium sulfate anhydrous, W. A. Hammond Drierite, Xenia, OH) or by bubbling air through water to achieve saturation (part of the Air Delivery System). The first five iterations of the experiment were conducted with dry air, and 12 additional experiments were conducted with saturated air. Air was directed to the side of conidiophores protruding from leaves or plugs from distances of 2 mm while the pipette orifice and spores were observed through a stereo microscope (50 to 60× magnification) over a 10-min period. Airborne particles were collected over a 10-min period using an Allergenco MK-3 air sampler (Environmental Monitoring Systems, Charleston, SC) at a 15 liters/min air flow rate placed 5 to 20 cm downstream from the source of conidia (Fig. 2). Samples collected on slides (25 × 75 mm) with transparent double-coated tape (Scotch 9425, 3M, St. Paul, MN) were examined using a compound microscope at 400× magnification. Individual petals from *Botrytis cinerea*-infected geranium blossoms were used as a positive control at distances of 3 cm from the pipette orifice with the air sampler placed 20 cm downstream. Dispersal of airborne conidia was also attempted by directing air over sporulating leaf lesions or agar plugs held at the edge of and 1.5 cm above 1/2 PDA plates with air directed toward the agar surface. Additionally, the effects of water and water movement/splash were examined as a drop of sterile distilled water was either splashed past a sporulating leaf lesion or placed on the surface of a sporulating leaf lesion or on an agar plug and blown by air at 9 or 19.8 m/s or tipped to transfer the drop to 1/2 PDA plates (Figs. 3 and 4). Agar plates were observed using a stereo microscope for presence of conidia and for *C. pseudonaviculata* colony growth over time. Data were collected as positive or negative conidial transfer (binomial data) and analyzed by Fisher’s exact test.

A fine mist was generated by an ultrasonic humidifier (Vicks model V4600, Kaz USA, Marlborough, MA) with an air flow of 1.7 m/s. Mist droplet diameter was measured by placing water-sensitive paper (TeeJet, Wheaton, IL) 5 cm from the orifice, and droplet sizes were measured using an ocular micrometer. Sporulating boxwood leaves were held in the mist at 5 cm from the orifice, and airborne particles were collected over a 10-min period using an Allergenco

FIGURE 1

Attempted dispersal of *Calonectria pseudonaviculata* conidia with air currents from a two-channel Air Delivery System at 138 kPa with a 1.3 liters/min flow rate through a 1-mm inner diameter Pasteur pipette to create a jet of air at 71.3 kph (19.8 m/s).

MK-3 air sampler placed 30 cm downstream as described above (Fig. 5). The experiment was conducted eight times.

Sporulating boxwood leaves were touched to drops of water on glass microscope slides to transfer conidia to the water drop. The water drop was allowed to air dry, at which time saturated air currents (air speed of 19.8 m/s) were used to try to dislodge conidia from the slide surface at distances of 1 to 2 mm as described above. Conidia were observed microscopically (40× magnification), and 100 conidia per drop were scored as being either displaced or adhering to the slide. After 30 min, a water drop was placed on top of conidia adhering to the slide, and the air jet was used to blow away the drop. Conidia were observed microscopically (40× magnification) during this process, and 100 conidia per drop were scored as being displaced or adhering to the slide. The experiment was conducted 10 times, and data were combined.

To confirm casual observations that most of the leaves in pots were facing abaxial surface up, we counted 1,000 dropped leaves on the surface of pots of 'Dee Runk', 'Green Velvet', 'Richard', and 'Vardar Valley' boxwood plants and scored them as facing abaxial or adaxial surface facing up. In addition, mature Green Mountain boxwood leaves of various sizes were held horizontally adaxial side up at 66, 33, or 15 cm and dropped onto a cotton pad. One hundred leaves were dropped one at a time and recorded as landing with either the adaxial or abaxial side facing up. The experiment was conducted five times at each height. Data were analyzed by the binomial Clopper–Pearson exact test (Clopper and Pearson 1934).

Dispersal of *C. pseudonaviculata*

C. pseudonaviculata conidia and conidiophores subjected to either dry or saturated air streams at 19.8 m/s held as close as 2 mm from the pipette orifice were observed using a microscope. No detachment of conidia or conidial aggregates was observed. Rather, they bent over and remained in place on conidiophores. *C. pseudonaviculata* conidia were not collected from air sampler traces in any of the 17 attempts to disperse and collect spores, regardless of whether air was dry or saturated (Table 1). In contrast, large numbers of *B. cinerea* conidia were observed in traces when air was directed at *B. cinerea*-infected blossoms as a positive control. Similar results were obtained using 1/2 PDA Petri dishes to collect spores. Water drops added to sporulating leaves or agar plugs released conidia from the conidiophores, and movement of the water by air streams readily dispersed conidia. Fine mists generated by an ultrasonic humidifier in air currents of 1.7 m/s averaged 20 μm in diameter (maximum = 50 μm, minimum = 5 μm) and did not result in release of conidia and subsequent collection by the air sampler. Data were analyzed by Fisher's exact test contingency table differences between *C. pseudonaviculata* dispersal in air or water ($P < 0.00001$).

Conidia from sporulating boxwood leaves were transferred to a water drop on glass microscope slides and allowed to air dry. Conidia were not displaced by saturated air currents at 19.8 m/s that were used to try to dislodge them at distances of 1 to 2 mm (Table 2). Most conidia observed microscopically were single with only a few remaining in contact with each other. When a new water drop was placed on the dried conidia after 30 min, air currents at 19.8 m/s dislodged the drop but did not dislodge 90% (90.4; SE = 5.7) of the conidia adhering to the slide.

An average of 60.2% of 1,000 dropped leaves on the surface of pots of Dee Runk, Green Velvet, Richard, and Vardar Valley boxwood plants were facing abaxial surface up. Boxwood leaves dropped from heights of 15, 33, or 66 cm landed with more than

FIGURE 2

Airborne particles were collected over a 10-min period using an Allergenco MK-3 air sampler at an air flow rate of 15 liters/min placed 5 to 20 cm downstream from the source of conidia.

60% of leaves facing abaxial surface up (Table 3). Data were analyzed by the binomial Clopper–Pearson exact test and were significantly different from a theoretical value of 0.5 ($P < 0.0001$).

Conclusions

This is the first direct demonstration that *C. pseudonaviculata* conidia were not detached from conidiophores and dispersed by air currents. Henricot (2006) speculated that sticky spores were not wind dispersed but did not report investigations or data. The sporadic recovery of *C. pseudonaviculata* at low concentrations in air samples by qPCR assays (Gehesquière et al. 2013) represented six conidial equivalents per 12-h air sampling by Burkard spore traps and may have been due to rain events and water splash or arthropod activity rather than wind dispersal of dry conidia. Additional epidemiological results suggested that *C. pseudonaviculata* is likely a water- and not a wind-dispersed pathogen, because dispersal was correlated with rain events in simulated nursery experiments (Gehesquière 2014). The data reported herein confirm this conclusion.

Our results suggest that *C. pseudonaviculata* conidia did not become airborne, because they did not detach from conidiophores when dry or saturated air currents at speeds of up to 19.8 m/s were directed at them. Nor were they detected to be dispersed to air by air samplers or by observation or growth on 1/2 PDA Petri dishes. As previously reported (Gehesquière et al. 2013; Henricot 2006), water placed on leaves detached the conidia from the conidiophores, and conidia were readily moved from sporulating leaves with movement of that water by air or by other means. However, a fine mist moving across leaves was not sufficient to release conidia to the air.

We also investigated the possibility of secondary wind dispersal of dry conidia after they were detached from phialides by water, after the water had evaporated. This secondary form of wind dispersal was shown not to be likely, because conidia released in water subsequently remained firmly attached to a glass surface once the water had evaporated, and they did not become airborne when later impacted by fast-moving air currents; when a second drop of water was added 30 min later and blown off by an air stream at 19.8 m/s, more than 90% of the conidia still remained attached to the surface.

Our results indicate that conidia will not move from a sporulating plant by air currents unless they are detached and moved by rain, rain splash, or irrigation water. If drops with conidia dry, the sticky conidia will likely remain attached to a surface and not be wind distributed. These conidia will also not readily spread by additional water drops after drying. In the absence of other means of spread

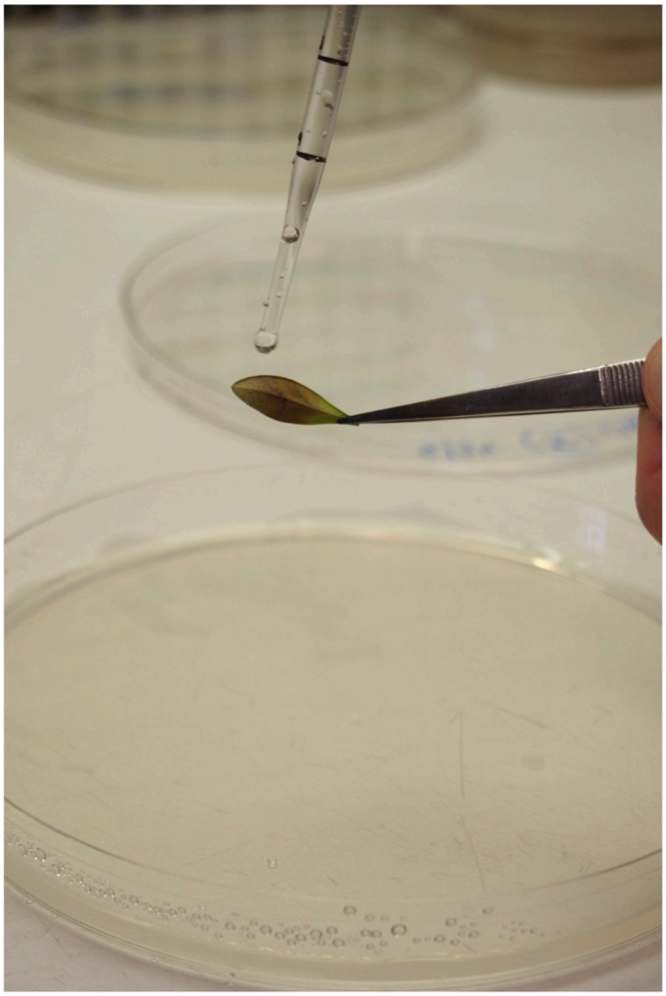

FIGURE 3

Leaf splash *Calonectria pseudonaviculata* conidial dispersal from sporulating leaves onto half-strength PDA.

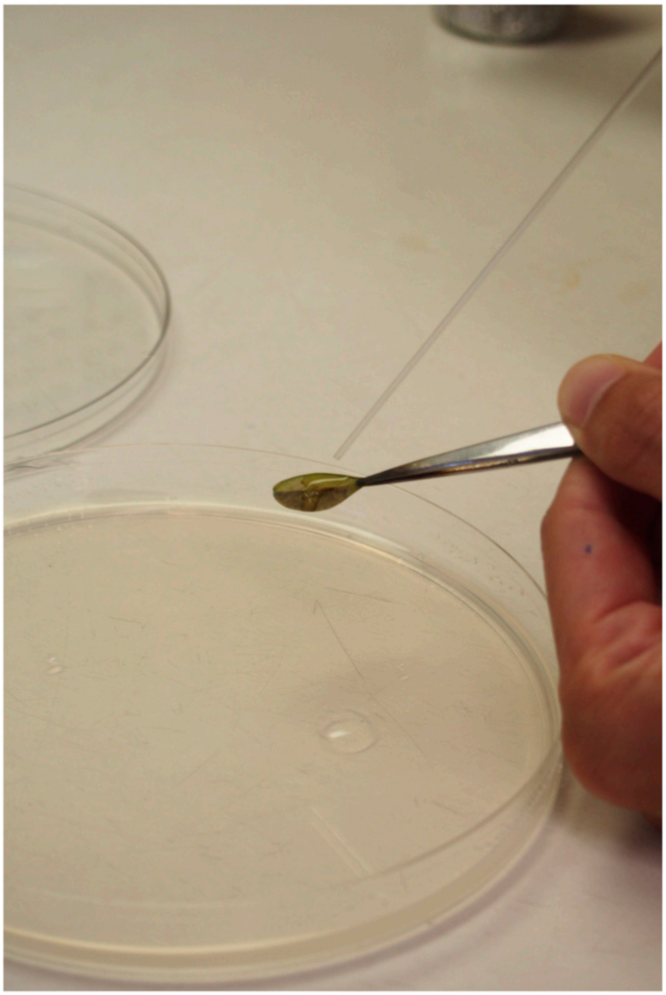

FIGURE 4

Air-dispersed *Calonectria pseudonaviculata* conidia in water drops on sporulating leaves onto half-strength PDA.

(such as moving infected boxwood, movement of infected plant parts such as detached diseased leaves, contact distribution while shearing/pruning, and physical movement by contact with animals or other means), models of rain intensity on splash dispersal of fungal conidia can be developed and used to estimate dispersal distances and aid with management decisions (Madden et al. 1996; Ntahimpera et al. 1997).

It was observed during our evaluation of other boxwood blight experiments that dropped diseased leaves seemed to have a higher percentage of leaves with sporulating abaxial surfaces facing up than down. This observation that over 60% of Dee Runk, Green Velvet, Richard, and Vardar Valley boxwood leaves faced abaxial surface up was consistent with our results herein that over 60% of leaves dropped from 15 to 66 cm landed with the abaxial surface facing up. Although different boxwood cultivars with different leaf shapes and sizes remain to be studied, this may suggest important implications for dispersal of the pathogen, because boxwood blight infection and sporulation occur primarily on abaxial leaf surfaces. Because conidia are not wind dispersed but instead detached and moved by water and water splash, production of conidia on the abaxial surface of leaves remaining on plants seems an inefficient manner of spread. However, diseased plants are often heavily defoliated. Fallen leaves with sporulating surfaces facing soil or the potting mix would also seem to reduce splash dispersal. The cupped shape of most boxwood leaves may be responsible for the orientation of leaves after dropping, with the abaxial surface and sporulation facing up. The curved surface of leaves in that

TABLE 1
Dispersal of *Calonectria pseudonaviculata* conidia by air streams (19.8 m/s, 2 mm distant) or fine mist (1.7 m/s) directed at conidia and conidiophores or water drops on sporulating leaves and agar

	Number of positive detects/number of attempts	
Source of conidia	Air sampler	Half-strength PDA Petri plate
Infected boxwood leaf[a]	0/17[b]	0/15
Agar plug culture[a]	0/20	0/20
Infected leaf – fine mist[c]	0/8	…
Water drop on leaf	…[d]	40/40
Water drop on agar plug	…	39/40
Botrytis on blossom[e]	5/5	…

[a] The first five attempts were with dry air and the remainder with saturated air streams.

[b] Data were collected as positive or negative conidial transfer (binomial data) to slide traces or as conidial transfer and growth. Data were analyzed by Fisher's exact test contingency table differences between *C. pseudonaviculata* dispersal in air or water ($P < 0.00001$).

[c] Fine mist (water droplets 5 to 50 μm, mean 20 μm) generated by an ultrasonic humidifier and air speed of 1.7 m/s were directed at sporulating leaves from 5 cm.

[d] Not done.

[e] *Botrytis cinerea* conidia on geranium blossoms were used as a positive control.

FIGURE 5
Attempted dispersal of *Calonectria pseudonaviculata* conidia with air currents and fine mists (5 to 50 μm diameter).

TABLE 2
Secondary dispersal of *Calonectria pseudonaviculata* conidia by air streams

Treatment[a]	Number (of 1,000) displaced by air
Conidia after water evaporation	0/1,000
Conidia displaced with a second drop	96/1,000

[a] *C. pseudonaviculata* conidia in suspension were allowed to air dry on a glass slide, at which time saturated air currents (19.8 m/s) were used to try to dislodge them from the slide surface at distances of 1 to 2 mm. Conidia were observed microscopically (40× magnification), and 100 conidia per drop were scored as being displaced or adhering to the slide. After 30 min, a water drop was placed on top of conidia adhering to the slide, and the air jet was used to blow away the drop. One hundred conidia per drop were scored as being displaced or not during this process. The experiment was conducted 10 times, and data were combined.

TABLE 3
Orientation of *Buxus* × 'Green Mountain' leaves dropped from different heights

	Leaf surface facing up[a]	
Height of leaf release (cm)	Abaxial	Adaxial
15	58.8[b]	41.2
33	68.2	31.8
66	59.5	40.5

[a] One hundred leaves were dropped one at a time and recorded as landing with either the adaxial or abaxial side facing up. The experiment was conducted five times at each height.

[b] Data were analyzed by the binomial Clopper–Pearson exact test and were significantly different from a theoretical value of 0.5 ($P < 0.0001$).

orientation may aid in retention of water films to wet and release conidia and, further, contribute to splash dispersal biomechanics similar to bird's nest fungi (Hassett et al. 2013). This is consistent with observations of increased disease severity in lower canopies, and suggestions for best management practices include mulching to reduce infection (Likins et al. 2019) and pruning lower branches of plants to reduce the most easily infected areas and increase air flow and drying (LaMondia et al. 2019).

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