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Biological control of boxwood blight by *Pseudomonas protegens* recovered from recycling irrigation systems

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ABSTRACT

Boxwood blight caused by *Calonectria pseudonaviculata* (*Cps*) is an emerging disease in the United States. It has caused significant loss to the ornamental nursery and landscape industries. In this study, 1547 bacterial strains recovered from recycling irrigation systems were evaluated *in vitro* and *in planta* for their biological control efficacy against *Cps*. Three most potent stains 13A3, 14D5, and 34B6 identified all belonged to *Pseudomonas protegens*. Strains 13A3 and 14D5 reduced *Cps* mycelial growth by 95 to 100% in two sets of *in vitro* experiments including a 48-well plate assay and a dual culture assay. Strain 34B6 also reduced *Cps* growth by 93% in the 48-well plate assay and 66 to 72% in dual culture plates. *In planta*, strain 14D5 reduced *Cps* infection by more than 50%, while strains 13D3 and 34B6 by 40 to 46%. *Pseudomonas protegens* strains produced antifungal secondary metabolites that reduced *Cps* growth and infection. Other possible modes of action against boxwood blight are discussed.

1. Introduction

Boxwood (*Buxus* spp.) is an important landscape plant and a major nursery crop in the United States. The national boxwood industry has a wholesale value of $126 million in 2014 (USDA, 2015). Numerous cultivars of boxwood varying in their color, size, shape, and preference for shade are popularly used as hedges, topiaries, and globe forms in gardens, homes, and historic landmarks. Boxwood was considered a generally low maintenance plant that was susceptible to only a few diseases and pests such as boxwood decline, root rot, Volutella blight, and boxwood leafminer (Hansen, 2009) until boxwood blight began to devastate the boxwood nursery and landscape industries (Palmer and Shishkoff, 2014).

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. Its major diagnostic symptoms include dark brown leaf spots, black streaks on stem, and rapid defoliation. Since 2011, boxwood blight has spread to more than 24 states in the U.S. (Gauthier et al., 2016; Iriarte et al., 2016; Ivors et al., 2012; Malapi-Wight et al., 2014).

Current management measures of boxwood blight have included rapid detection followed by eradication of *Cps* (Dart et al., 2014; Gehesquière et al., 2013; Malapi-Wight et al., 2016), fungicide application (Baudoin et al., 2015; Henricot et al., 2008; Henricot and Wedgwood, 2013; LaMondia, 2014; LaMondia, 2015), host resistance (Ganci et al., 2013; Henricot et al., 2008; LaMondia, 2015; Shishkoff et al., 2015; Thammina et al., 2017), and other cultural practices such as use of sanitizers (Dart et al., 2015b; Shishkoff, 2016). One underdeveloped component within the current disease management framework is biological control (biocontrol).

A few studies have focused on the biocontrol of boxwood blight. First, Hébert et al. found that *Trichoderma* species identified from the rhizosphere of boxwood plants reduced *Cps* growth by up to 99.4% (Hebert et al., 2014). Second, a *Trichoderma koningiopsis* isolate Mb2 recovered from a collapsing wild mushroom significantly suppressed the mycelial growth of *Cps* (Kong and Hong, 2017). It also reduced the *Cps* infection by 85% on cuttings of *B. sempervirens* 'Suffruticosa' and 54 to 63% on containerized *B. sempervirens* 'Justin Brouwers' plants (Kong and Hong, 2017). Third, several commercial biofungicide products were evaluated for their potential as alternatives for boxwood blight mitigation (Yang and Hong, 2017). The product RootShield PLUS WP reduced disease severity for up to 44.4% (Yang and Hong, 2017).

Bacteria are often used as biocontrol agents (BCAs) of fungal plant pathogens. Of the most widely used are *Agrobacterium*, *Bacillus*, *Pseudomonas*, and *Streptomyces* species (McSpadden Gardener and Fravel, 2002). Mechanisms by which these bacteria act against plant pathogens include antibiosis, competition, parasitism, and induction of host resistance (McSpadden Gardener and Fravel, 2002). Whether any bacteria are capable of controlling boxwood blight is yet to be

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

Isolate	Alternative code	Host	Tissue	Location	Year	GenBank Accession
11A01	VA11-232	*Buxus sempervirens*	Leaf petiole	Virginia, USA	2011	KX601058
11A03	BB137	*Buxus* sp.	Leaf petiole	Virginia, USA	2013	KX601060
11A04	BB188	*B. sempervirens*	Stem	Virginia, USA	2013	KX601059

determined.

Objectives of this study were to screen diverse bacterial strains recovered from recycling irrigation systems against *Cps* in dual culture, to identify promising strains to species level by DNA sequencing, and to further evaluate them using *in planta* assays for their potential as BCAs of boxwood blight.

2. Materials and methods

2.1. Isolation of bacterial strains and selection of Cps isolates

A total of 1547 bacterial cultures were evaluated in this study. They were originally isolated from recycling irrigation reservoirs at three ornamental plant production nurseries in the eastern Virginia from April 2012 to April 2013 (Table S1). Subcultures were recovered from long-term storage at −80 °C where original cultures grown in nutrient broth (NB) (Difco; Becton, Dickinson & Co., Sparks, MD, USA) were mixed with a quarter volume of 80% glycerol in 2-mL microtubes. Bacterial cultures were maintained on nutrient agar (NA) (Difco; Becton, Dickinson & Co., Sparks, MD, USA) and in NB during this study.

Three *Cps* isolates used in this study (Table 1) were maintained in potato dextrose agar (PDA). Agar blocks with actively growing cultures in PDA were also stored in distilled deionized water (diH_2O) in 2-mL microtubes for long-term storage at 15 °C.

2.2. In vitro screening in 48-well plates

All 1547 bacterial cultures were evaluated for their potential to suppress the mycelial growth of three *Cps* isolates (Table 1) in a 48-well plate assay. Each bacterial strain was cultured in 1-mL NB in a 2-mL microcentrifuge tube at 30 °C with moderate shaking (approx. 200 rpm) for 48 h. Two 6 to 8-week-old *Cps* cultures grown in PDA in 10-cm Petri dishes were blended in a Waring Laboratory Blender for 1 min at low speed (< 10,000 rpm) with 500-mL diH_2O. The slurry was poured through nested sieves of 355 and 125-μm pore size and washed three times with diH_2O. *Cps* propagules on the 125-μm sieve were re-suspended in diH_2O then diluted to make a final concentration of 1000 *Cps* propagules per mL. Then 10-μL *Cps* suspension containing approximately 10 *Cps* propagules was added into each well of pre-poured 48-well plates (0.5-mL NA per well). The agar surface was air-dried for approximately 30 min in a Class II Biological Safety Cabinet. The plates were then incubated at 20 °C in the dark. After 48 h, 10-μL bacterial culture in NB was added to each well, three replications for each bacterial culture. Ten-μL sterile NB was added into each of three control wells per plate. Fifteen bacterial strains plus one control were included in each 48-well plate. The agar surface was then air-dried. Plates were lidded and incubated at 20 °C in the dark.

The growth of *Cps* in each well was assessed 5 and 10 days after the addition of bacterial cultures using a 0–5 scale (Fig. 1). Evaluations against two other *Cps* isolates were performed and repeated once for promising bacterial strains identified in the initial screening experiment. Bacterial strains that consistently reduced the mycelial growth of all three *Cps* isolates by ≥90% in both experiments (Table S1) were selected for identification to species level by DNA sequencing and evaluation *in planta*.

2.3. DNA extraction, amplification, and sequencing

Promising bacteria cultures from the *in vitro* screening in 48-well plates were identified by sequencing the 16S rRNA gene. Each was cultured in 1.5-mL NB in a 2-mL microcentrifuge tube at 30 °C with moderate shaking for 48 h. Bacterial pellets were collected by centrifugation then re-suspended in lysis buffer included in the Maxwell® 16 FFS Nucleic Acid Extraction System (Custom) kit (Promega, Madison, WI, USA). Bacterial suspensions were then transferred to MP Biomedicals Lysing Matrix A, 2 mL tubes. Bacterial cells were lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA, USA). Bacterial genomic DNA was extracted using the Maxwell® 16 FFS Nucleic Acid Extraction System (Custom) kit in combination with a Maxwell® Rapid Sample Concentrator (Promega, Madison, WI, USA).

Amplifications were performed with forward primer 27F and reverse primer 1492R for the 16S rRNA gene (Marchesi et al., 1998). Sequencing was performed in both directions with the same primers as for PCR by the University of Kentucky Advanced Genetic Technologies Center (Lexington, Kentucky).

Sequences of both directions were viewed with Finch TV 1.4.0. and edited manually to correct obvious errors to produce a consensus sequence. These sequences were then compared with those of type strains using the EzTaxon Server (Yoon et al., 2017). In addition to species ID, all sequences were aligned using Clustal W (Larkin et al., 2007) and assigned into genotypes. All sequences produced in this study have been deposited in GenBank under accession numbers MG269573 to MG269719 (Table S1).

2.4. Cps-bacteria interactions in dual culture plates

Twenty-one bacteria strains selected by genotype were evaluated in this assay. A cultural plug (5 mm) of *Cps* isolate 11A01 was placed at the center of each plate containing 12-mL mixed NA and PDA (1:1 in volume) media. Three streaks of 48-h bacterial culture in NB were made equidistantly on the agar surface in each plate. Control plates were streaked with NB without any bacteria. Triplicate plates were used for each bacterial strain. The plates were arranged upside down and placed in an incubator set at 25 °C in the dark. The interactions between bacteria and *Cps* were characterized into three distinct types (Fig. 2) and radial growth of *Cps* were measured using a digital caliper after 14 days. This experiment was repeated once.

2.5. In planta evaluation of selected bacterial strains

Approximately 6-inch tall boxwood starter plants (*B. sempervirens* 'Justin Brouwers') grown in 3-inch pots were obtained from a commercial ornamental producer. Plants were maintained in a greenhouse (*c.* 25 °C). They were irrigated daily or as needed.

Selected bacterial strains were cultured in 7-mL NB in 15-mL centrifuge tubes at 30 °C with moderate shaking for 48 h. Then the liquid cultures were diluted by adding diH_2O to make a final concentration of 10^7–10^8 cfu/mL as determined by plating on NA. Boxwood plants were treated twice with the same bacterial cultures. Twenty mL diluted bacterial liquid culture was sprayed on the foliage of each plant 14 days and 6 h prior to *Cps* inoculation, respectively. Triplicate plants were treated with each bacterial strain. NB was sprayed onto the control plants.