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Can fungal epiphytes reduce disease symptoms caused by *Phytophthora ramorum*?

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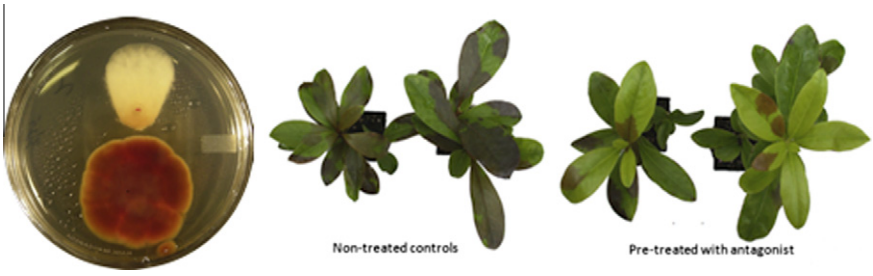
HIGHLIGHTS

- Selected fungi demonstrate antagonism towards *Phytophthora ramorum*.
- Fungal epiphytes reduce necrosis on rhododendrons caused by *P. ramorum*.
- A method was developed to screen fungi in planta for biological control of *P. ramorum*.

GRAPHICAL ABSTRACT



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ABSTRACT

Leaf infection of ornamental species by *Phytophthora ramorum* has a significant impact on the spread of this disease. Fungicides have had limited success at controlling this disease. With increasing concerns that repeated fungicide applications will exacerbate the potential for fungicide resistance and mask symptoms, alternative control measures are desired. The potential of biological control has not been thoroughly examined. Fungi, isolated from soil, were screened in dual culture with *P. ramorum* for antagonistic activity. Three isolates, identified as *Penicillium daleae*, *Metarhizium anisopliae*, and *Penicillium herquei*, were selected for further testing on the aerial plant parts of rhododendrons. Different factors, including culture age, application timing, dose response, and additives in the formulation were studied to determine their effects on the antagonists to reduce leaf necrosis. Although responses were variable for the different antagonists, this study showed that fungi applied to the leaf surface could reduce necrosis caused by *P. ramorum*. The method developed can be used for screening potential antagonists *in planta*.
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1. Introduction

Ramorum blight, caused by *Phytophthora ramorum* Werres, de Cock, and Man in't Veld, is considered to have only a moderate impact on ornamental plants since it usually is not lethal to these hosts (Kliejunas, 2011). Symptoms on rhododendrons, camellias, viburnums, and other hosts are usually non-distinctive leaf necrosis and blights that are not easily distinguishable from other diseases. However, ramorum blight does have a serious impact on the nursery industry. Because long distance dispersal of *P. ramorum* is facilitated by shipments of infected nursery plants across the country (Parke and Lucas, 2008) and concerns exist that the pathogen could spread from nurseries into natural habitats (Davidson et al., 2005), strict guidelines have been imposed on nurseries in areas known to have *P. ramorum*. These restrictions have had substantial economic consequences on the nursery industry (Kliejunas, 2011).

In order to remove restrictions on individual nurseries, these establishments must prove that they no longer have *P. ramorum* by testing the soil and plant material (USDA/APHIS, 2010). If *P. ramorum* infected plant material is found, the required protocol dictates that all host and associated host plants within a defined destruction block be destroyed (USDA/APHIS, 2010). In addition, a 10-meter radius around the destruction block is designated as a quarantine block, where plants cannot be moved in or out, and must be maintained in that status for a minimum period of 90 days to determine if *P. ramorum* has spread beyond the border of the destruction block. Ornamental plants often are cultivated under high density conditions that could easily facilitate

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plant-to-plant spread of *P. ramorum* (Englander and Tooley, 2003) outside the initial destruction block. *P. ramorum* has been demonstrated to spread plant-to-plant in aerial infections by physical contact and splash from rain and overhead irrigation (Heungens et al., 2010; Tjosvold et al., 2010) with symptoms often taking some time to be noticed.

There have been numerous studies examining the use of fungicides to control *P. ramorum* on ornamentals (Heungens et al., 2006; Linderman and Davis, 2008; Tjosvold et al., 2008; Pérez-Sierra et al., 2011). Although fungicides are effective to some degree when applied as a preventative (Heungens et al., 2006; Tjosvold et al., 2008), they require repeated applications. Linderman and Davis (2008) found that all chemicals tested in their study were fungistatic and not fungicidal. Besides environmental concerns, repeated applications potentially could lead to fungicide resistance by the pathogen as has been observed by other *Phytophthora* spp. (Grünwald et al., 2006). In addition, U.S. and European Union regulations prohibit the application of fungicides in the quarantine zones (USDA/APHIS, 2010; Pérez-Sierra et al., 2011) due to potential masking of symptoms.

A few studies have investigated the use of biological control to manage leaf diseases caused by *Phytophthora* spp. (Li et al., 1997; Daayf et al., 2003; Tondje et al., 2007). In addition, several studies have included basic experiments to assess the potential of biological organisms to inhibit *P. ramorum* infection (Linderman and Davis, 2006; Widmer 2008). However, no studies have examined the potential of biological control to specifically reduce damage to ornamentals caused by *P. ramorum*. The main objective of this study was to determine whether fungi applied to plant material as epiphytes can reduce disease symptoms caused by *P. ramorum*. Methodology developed will enable the screening of more candidate antagonists *in planta*.

2. Materials and methods

2.1. Culture isolation, maintenance, and identification

Soil was collected from three locations within Frederick County, MD. Microorganisms were isolated from soil from each location. A soil dilution was prepared by adding 0.33 g of soil to 1 L sterile water stirring for at least 10 min. Ten 1-ml samples from each suspension were dispensed into a sterile, 90-mm-diameter Petri plates. To isolate general fungi, molten potato dextrose agar (PDA) containing 100 mg per L streptomycin, 50 mg per L chloramphenicol, and 1 ml per L Tergitol NP-14 was poured into each plate and mixed by swirling the plate. The plates were allowed to solidify at room temperature, wrapped with Parafilm, and maintained in the dark at 24 °C. To another set of 10 plates containing 1 ml of the soil dilution aliquot, molten chitin-based agar (4 g bleached chitin, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4$ 5 H_2O, 0.01 g $FeSO_4$ 7 H_2O, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, 20 g agar, 1 L H_2O, pH 8.0) was poured into the plates and swirled to mix well to facilitate the isolation of actinomycetes (El-Nakeeb and Lechvalier, 1963). After 4 d, cultures growing on the agar medium were individually transferred from hyphal tips to fresh half-strength PDA (1/2PDA). The cultures were stored on 1/2PDA at 24 °C in the dark until ready to use further.

Cultures that showed in vitro inhibitory activity and were selected for in vivo screening, were identified through a molecular technique described by Berner et al. (2005). Genomic DNA was extracted from 7-day-old mycelial cultures with the DNEasy Plant Mini kit (QIAGEN, Valencia, California). Approximately 100 mg of mycelium was ground in liquid nitrogen with a mortar and pestle for the extraction. The genomic DNA was quantified on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). DNA, at a concentration of 10 ng per 100 uL, was used to amplify the internal transcribed spacer (ITS) 1, the 5.8 ribosomal RNA gene and the ITS spacer 2. Polymerase chain reaction was carried out on the GeneAmp 9700 (Applied Biosystems, Foster City, California) at these parameters: denaturing at 94 °C for 2 min; followed by 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min for a total of 30 cycles; then a final extension of 72 °C for 10 min. The primers used in the amplification were ITS5 and ITS4 (White et al., 1990) at a final concentration of 1 μM. PCR products were sequenced directly with Big Dye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI 3130 Genetic Analyzer (Applied Biosystems) according to manufacturers' guidelines in 20 μL reactions containing 100 ng of PCR template. The ITS5 and ITS4 primers were used in the sequencing reaction at a concentration of 3.2 pM. The sequences generated from reactions with the ITS5/ITS4 primer set were aligned with the BLAST algorithm of the National Center for Biotechnology Information.

P. ramorum isolate WSDA-1772 (NA1 lineage, originally isolated from *Viburnum plicaturm* "Mariessii" (Veitch) Rehder in Oregon) was maintained on 20% clarified V8 agar and used for all plant inoculation studies. To maintain pathogenicity, the isolate was inoculated on and recovered from rhododendron leaves approximately every 6 months on a *Phytophthora*-selective medium (Ferguson and Jeffers, 1999). Other *P. ramorum* isolates used in initial screening tests were 0–16 (NA1 lineage, originally isolated from Coast Live Oak, Marin Co., CA), PRG-6 (EU1 lineage, originally isolated from *Viburnum* x *bodnantense* Aberc. ex. Gard., Germany), PRG-1 (EU1 lineage, originally isolated from *Rhododendron* sp. L. "Schneewolke", Germany), 0–217 (NA1 lineage, originally isolated from *Rhododendron* sp. "Gomer Waterer", Felton, CA), PRG-2 (EU1 lineage, originally isolated from *Rhododendron catawbiense* Michx., Germany), BBA 15/01–18 (EU1 lineage, originally isolated from *V.* x *bodnantense* "Dawn", Germany), P195–46 (NA1 lineage, originally isolated from Coast Live Oak, Santa Rosa, CA), OR-03-74-2 (NA1 lineage, originally isolated from *V.* x *bodnantense*, Clackamas, Co., OR), and 05–166 (NA2 lineage, originally isolated from *Rhododendron* sp., WA). The isolates also were maintained in liquid nitrogen as part of the international collection of plant pathogens at the National Cancer Institute's Central Repository in Frederick, MD.

Zoospores were prepared by modification of the method of Mitchell and Kannwischer-Mitchell (1992). Five 10-mm plugs of each isolate were added to separate 90-mm plates containing sterile 20% V8 broth. The cultures were allowed to grow for 3 days at 20 °C in the dark. The mycelium was rinsed three times in sterile 0.1 mM 2-[N-morpholino]ethanesulfonic acid buffer, pH 6.2 (herein referred to as MES buffer) and then placed back in the 20 °C incubator in the dark overnight. Zoospores were induced to release from the formed sporangia by placing the cultures at 4 °C for 30 min and then incubating at room temperature. After 30–45 min, zoospores were released and the concentration was determined by diluting the suspension in MES buffer, vortexing to induce encystment, and then counting with a hemacytometer. The zoospores were diluted to the final concentrations by slowly pipetting a specific amount in MES buffer. Motility of the zoospores was verified after dilution through a dissecting microscope.

2.2. Plant material

Potted *Rhododendron* "Cunningham's White" cuttings were originally received from Brigg's Nursery (Elma, Washington), from which additional plants were propagates by rooted cuttings under a mist tent. All plants were maintained in the greenhouse in 5-cm pots. Plants for each experiment were selected for uniform size and age. If necessary, the plants were trimmed back so that each plant had 10–20 total leaves.