Identification and characterization of control candidates for *Pseudogymnoascus destructans*, the fungus that causes white-nose syndrome in bats

by

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Abstract

Pseudogymnoascus destructans, the fungus that causes white-nose syndrome in hibernating bats, has caused unprecedented declines of bat populations in eastern North America and continues to threaten new populations as it spreads across the continent. In addition to growing on hibernating bats, *P. destructans* can grow as a saprotroph in the bat hibernaculum environment, which has likely contributed to the rapid spread and severity of the white-nose syndrome epidemic. In this thesis, we focus on the identification and characterization of candidate control agents that could target *P. destructans* in the hibernaculum environment. We first identify microbes that are strongly inhibitory to *P. destructans* in bioassays and show that some of these microbes act through the production of antifungal volatiles. We then use a yeast model to conduct preliminary analyses of the antifungal mode of action of the microbially-produced volatile compounds 2-methyl-1-butanol, 1-pentanol, propionic acid, and nonanal. We also simulate the hibernaculum environment with soil microcosms to assess the inhibitory activity of volatile compounds and microbial antagonists under hibernaculum-like conditions. Finally, we test the metabolites and volatile compounds that were inhibitory to *P. destructans* against a library of agriculturally-significant plant pathogens. Future research should further address the antifungal mechanisms of action of these compounds, their ability to inhibit *P. destructans* under hibernaculum conditions, and their employment as antifungals in general.

General introduction

The primary focus of this research is to identify microbes and natural products that can inhibit *Pseudogymnoascus destructans*, the fungus that causes white-nose syndrome in bats. White-nose syndrome has caused unprecedented declines of bat populations in eastern North America and continues to threaten new populations as it rapidly spreads across the continent. *P. destructans* can grow in the environment in bat hibernacula and although further research is needed, an increasing body of evidence suggests that this environmental growth has contributed to the rapid spread and severity of the white-nose syndrome epidemic.

The ability of *P. destructans* to act as a saprotroph enables it to grow and persist in the environment (Lorch *et al*., 2013a; Lindner *et al*., 2011; Lorch *et al*., 2013b). Upon introduction to a hibernaculum, *P. destructans* rapidly and broadly establishes itself, exponentially increasing its abundance with time (Frick *et al.*, 2017). The ability of *P. destructans* to rapidly colonize hibernacula has not only created pathogenic reservoirs in these sites, but has also likely accelerated the expansion of *P. destructans* across the continent, as infected bats entering sites can shed conidia that persist and grow in the cave environment (Hoyt *et al.*, 2015a; Frick *et al*, 2017). Although there have been no empirical studies of environment-to-bat transmission, several lines of evidence suggest that the environmental reservoir of *P. destructans* may contribute to infection of bats by the fungus. The fungal species found on bats reflect the assemblages in the environment (Vanderwolf *et al*., 2015) and airborne *P. destructans* spores can be detectable in bat hibernation sites (Kokurewicz *et al*., 2016), suggesting that conidia from environmental *P. destructans* can reach bats. Additionally, bats generally rid themselves of *P. destructans* in summer, and infection only becomes apparent once they begin hibernating in contaminated sites (Meteyer *et al*., 2011; Langwig *et al*., 2015; Frick *et al*., 2017). As bat

populations have declined and population density has decreased, infection rates have remained high in early winter, suggesting that infection may occur from sources in the environment (Frick *et al*., 2017). Thus, the environmental growth of *P. destructans* may enable a cycle of infection and spread where infected bats contaminate the environment, and the contaminated environment infects bats. This is further intensified because the environmental reservoir of *P. destructans* has not decreased with declining bat populations (Frick *et al*., 2017) and may persist in the environment for over a century in the absence of bats (Reynolds *et al*., 2015). This long-term persistence increases the likelihood of extinction of susceptible bat species by preventing a density-dependent decrease in the transmission of disease and preventing recolonization of sites from which bats have been extirpated (Frick *et al*., 2017; Hoyt *et al*., 2015a). When taken together, this evidence suggests that the environmental growth of *P. destructans* may enable the rapid spread of white-nose syndrome across the continent, may contribute to the high rates of bat infection with *P. destructans*, and may enable the long-term persistence of *P. destructans* in hibernacula.

Because of the apparent importance of the environmental growth of *P. destructans* in the white-nose syndrome epidemic, we began our research by trying to identify biocontrol candidates that could be considered for the control of environmental *P. destructans* in bat hibernacula (Chapter 1). We isolated microbes from diverse environmental sources and used bioassays to screen these microbes for activity against *P. destructans.* This work was published in PLOS ONE (Micalizzi *et al*., 2017). Through this study, we identified many excellent antagonistic microbes that could be considered further as biocontrol candidates; however, we also recognized that some of the observed antagonistic effects likely resulted from the production of antifungal compounds by the antagonists. Using a natural product-based control strategy to

target *P. destructans* could have some unique advantages over a biocontrol strategy, so we screened some of our top antagonists for the production of antifungal compounds and identified several antagonistic microbes that act through the production of inhibitory volatile compounds.

Volatile compounds are particularly promising for the chemical control of *P. destructans* in complex environments like bat hibernacula, so we examined the volatiles produced by selected antagonistic microbes in more detail. We identified the volatile compounds with gas chromatography mass spectrometry and then used a yeast model to conduct preliminary assessments on the antifungal mode of action of these compounds (Chapter 2).

Having identified microbial and volatile control candidates that effectively control *P. destructans* in bioassays, we also wanted to assess the efficacy of these control strategies in a simulated hibernaculum environment to assess their potential field applications (Chapter 3). We used hibernaculum soil to create hibernaculum-like microcosms and monitored the abundance of *P. destructans* over time in the presence of different control mechanisms. Surprisingly, we did not observe the decreases in abundance of *P. destructans* in these soil microcosm systems that we expected based on our earlier agar-based assays. We conducted follow-up assays to address the reason(s) for this discrepancy.

Finally, because we had identified antifungal volatile compounds, as well as microbial producers of secreted antifungal compounds, we decided to expand the scope of our project and determine if any of these compounds could inhibit agriculturally significant plant pathogens (Chapter 4). Information provided in this chapter indicate that some of the microbes that we identified produce compounds that may be useful in future work on the management of fungal pathogens in general, in addition to informing future strategies to combat the high rates of bat mortality due to white-nose syndrome.

Chapter 1: Identification of microbial antagonists of *Pseudogymnoascus destructans*

This chapter has been published (Micalizzi *et al*., 2017) and is presented here with minor modifications. I was the primary researcher on this study and designed and carried out the experiments, analysed the data, wrote the first draft of the manuscript, and acted as the corresponding author. T.J. Avis, G.P. White, and M.L. Smith provided some of the antagonist microbes tested and J.N. Mack and G.P. White helped morphologically identify fungi. T.J. Avis helped design experiments and T.J. Avis and G.P. White helped edit the manuscript. M.L. Smith supervised all aspects of the project and served as the primary editor on the paper.

Introduction

Pseudogymnoascus destructans (Blehert & Gargas) Minnis & D.L. Lindner is the fungus that causes a deadly disease in hibernating bats known as white-nose syndrome (WNS) (Warnecke *et al*., 2012). *P. destructans* is believed to have been introduced to North America from Europe and was first discovered in New York in 2006 (Zukal *et al.*, 2016; U.S. Fish and Wildlife Service, 2016). Over the past decade, *P. destructans* has caused extensive local extinctions and ten-fold reductions in affected North American bat populations (Frick *et al*., 2015). Further, *P. destructans* has rapidly spread to 33 U.S. states and 5 Canadian provinces (U.S. Fish and Wildlife Service, 2018) and is predicted to continue spreading (O'Regan *et al*., 2015), potentially threatening over half of all North American bat species (U.S. Fish and Wildlife Service, 2016). Such widespread loss of bats as prominent insectivores will undoubtedly have costly ecological, agricultural, and economic consequences (Boyles *et al*., 2011).

P. destructans causes WNS by colonizing the skin of hibernating bats, creating lesions and increasing the frequency with which bats emerge from torpor. This is often lethal as the

increased energy demands of disrupted torpor can result in dehydration and emaciation before water or food is available (Verant *et al*., 2014). Further mortality may be caused by immune reconstitution inflammatory syndrome, where bats regain immune function after a period of hibernation-induced immunosuppression and have severe, lethal immune responses to *P. destructans* infection (Meteyer *et al*., 2012). After the winter, surviving bats can rid themselves of *P. destructans* (Langwig *et al*., 2015; Meteyer *et al*., 2011) and quickly heal their skin lesions (Fuller *et al*., 2011). However, because *P. destructans* persists in hibernacula by growing saprotrophically when bats are absent (Lorch *et al*., 2013b; Reynolds and Barton, 2014), it is possible that healthy bats could be infected when entering contaminated hibernacula (Langwig *et al*., 2015; Reynolds *et al*., 2015). While the role of the saprotrophic growth of *P. destructans* in the white-nose syndrome epidemic remains uncertain, model predictions have suggested that under some circumstances, reducing the growth of *P. destructans* in hibernacula may mitigate or prevent WNS-associated colony collapse (Reynolds *et al*., 2015; Meyer *et al*., 2016). Taken together, this suggests that targeting the growth of *P. destructans* in hibernacula may be an important part of managing the WNS epidemic.

In this study, we isolated a diversity of microbes from Ontario and Quebec, Canada, and screened for ones that inhibit *P. destructans* to identify potential biocontrol candidates and microbially-derived natural products that reduce the growth of *P. destructans* in a low temperature (hibernaculum-like) environment.

Materials and methods

Pseudogymnoascus strains

P. destructans strains US-15, SH-864, and SH-991 were obtained from Agriculture and Agrifood Culture Collection, Ottawa, ON, Canada. Unless specified otherwise, all assays used *P.*

destructans strain US-15. *P. roseus* S8A2CN and *P. pannorum* S8A5ACS1 were isolated from soil samples in Gatineau, Québec. All cultures were grown and all assays were performed at 13 ± 1 °C, within the optimal temperature range for *P. destructans* (Verant *et al*., 2012). *Pseudogymnoascus* cultures were stored in Potato Dextrose Broth (PDB) amended with 15% sterile glycerol at -80 °C.

Isolation of antagonists

Antagonists used in this study were from various sources and locations as listed in Appendix 1. Axenic antagonist cultures were grown in 5 ml BD Difco Potato Dextrose Agar (PDA) slants and stored in PDB amended with 15% sterile glycerol at -80 °C. Soil samples were collected from cold soils (0 - 10 °C) in April 2015 from Ottawa, Gatineau, and Toronto areas and frozen at -20 °C until use. Approximately 1.5 g of soil was mixed with 10 ml of sterile tap water and 10- to 1000-fold dilutions were plated onto various media including plates containing 0.2% chitin and 0.1× Vogel's salts (Vogel, 1956) to isolate chitinolytic fungi (Hsu and Lockwood, 1975; Murphy and Bleakley, 2012), LB Miller agar with 150 mg L^{-1} cycloheximide to isolate bacteria and select against fungi (Farh *et al*., 2015), PDA with 70 mg L^{-1} Rose Bengal to isolate slow-growing fungi (Bragulat *et al*., 1991), PDA with 100 mg L^{-1} ampicillin, 50 mg L^{-1} chloroamphenicol, and 75 mg L^{-1} streptomycin sulphate to isolate fungi without bacteria, and PDA alone to culture microbes non-specifically. Antagonists were also isolated from decomposing wood, birch bark, and hay. Additional antagonists were obtained from foam taken from the surface of the Rideau River (Ottawa, ON) and by leaving Petri plates open to the air. All antagonists isolations in this study were performed at 13 ± 1 °C. Further antagonists were isolated from compost and compost tea (Koné *et al*., 2010; Dionne *et al*., 2012) and from environmental samples isolated with DG-18 (Dichloran-Glycerol Agar) in the Ottawa region.

Several strains of *Bacillus* and *Trichoderma* were from the culture collection of M.L. Smith (Providenti *et al*., 2009; Johnson *et al*., 2010).

Bioassay for filamentous fungal and actinobacterial antagonists

Aliquots of *P. destructans* cultures, macerated with a Waring blender and stored at -80 °C in PDB with 15% glycerol, were thawed at room temperature and diluted in PDB to 5000 CFU/ml in a sterile Eppendorf multichannel pipette reagent trough. A 50 × 75-mm flame-sterilized glass microscope slide was dipped edgewise into the trough and used to stamp a narrow line of *P. destructans* inoculum onto the surface of PDA in a Petri plate. Perpendicular lines were stamped on PDA at an appropriate distance from the centre in a 55-mm (for assaying slow growing antagonists) or 90-mm (for fast growing antagonists) Petri plate so that a given antagonist, inoculated in the centre of the plate, would contact *P. destructans* at about day seven. Control plates were created by stamping *P. destructans* inoculum without an antagonist.

Bioassay for bacterial and yeast antagonists

PDA in 55-mm diameter Petri plates was pre-inoculated with *P. destructans* using a glass slide as described above to create two parallel lines of inoculum. Three days after *P. destructans* was inoculated, 7-µl aliquots of a log phase antagonist culture in LB (bacteria) or PDB (yeast) were streaked in duplicate through both lines of *P. destructans*. LB broth and PDB were streaked through *P. destructans* on replicate plates as controls.

Assessing inhibition

Assay plates from antagonist screenings were photographed 14 days after *P. destructans* inoculation. Inhibition was quantified using image analysis to calculate the area of *P. destructans*. Images were scaled based on the size of the Petri plates and antagonist diameter was

measured using the measure feature in ImageJ (Schneider *et al*., 2012). To aid in distinguishing *P. destructans* from background features, images were cropped to exclude as much background as possible and only one line of *P. destructans* was considered. Ilastik version 1.2.0 (Sommer *et al*., 2011) was used to distinguish *P. destructans* colonies from antagonist colonies and background. Simple segmentations for each image were exported to ImageJ where the greyscale image was thresholded with a value of 2 for each parameter. *P. destructans* colony area was calculated using the analyze particles feature of ImageJ with no parameters specified for size or circularity. Thresholded images were manually checked to ensure *P. destructans* was fully and exclusively detected and the reported area for *P. destructans* was the area of one streak multiplied by 2 or 3, for plates stamped in duplicate or triplicate, respectively. Inhibition scores for each antagonist were reported as percent inhibition of *P. destructans*, relative to a no-antagonist control. Percent inhibition was calculated as $\left(1 - \frac{Area_{treatment}}{Area_{control}}\right) \times 100$, where $Area_{treatment}$ refers to the area of *P. destructans* in the presence of the antagonist and $Area_{control}$ refers to the area of *P. destructans* in the no-antagonist control. The percent inhibition for each antagonist, along with the respective day 14 area of *P. destructans* and antagonist diameter are provided in Appendix 1. The inhibition scores of *P. destructans* by an antagonist were ranked as 0 = negligible or no (less than 50%) inhibition of *P. destructans*; 1 = considerable (between 50% and 85%) inhibition of *P. destructans*; or 2 = complete or nearly complete (greater than 85%) inhibition of *P. destructans*. Additionally, if applicable, antagonistic ranks were qualified with: A = *P. destructans* inhibited the antagonist; B = the antagonist grew over *P. destructans* such that *P. destructans* colonies were no longer visible; and/or C = *P. destructans* colonies were present, but considerably smaller than colonies on the control plates. A representative of each of these classifications and a control plate is shown in Figure 1.1.

Identification

Isolates that inhibited *P. destructans* were identified, including all isolates that caused greater than 85% inhibition, by sequences of *ITS* rDNA (fungi), *16S* (bacteria) rDNA, and *beta-tubulin* (*Penicillium* sp.) DNA. Morphological identification was used to augment sequence-based identifications of filamentous fungi.

DNA extraction

DNA was extracted using a modified form of the protocol outlined by Lõoke and colleagues (2011). Antagonists were grown at room temperature in a 1.5-ml epitube containing 1 ml sterile PDB until the culture was visible throughout the tube. The cells were pelleted by centrifugation and resuspended in 100-150 µl of 200 mM lithium acetate with 1% SDS. Approximately 15 mg of 0.5 mm (for filamentous fungi or yeast) or 0.1 mm (for bacteria) glass beads were added to each epitube before the tubes were placed into a Fisher Scientific Isotemp waterbath at 70 °C for 10 minutes and subsequently cooled on ice. The epitubes were then shaken in a Retsch MM301 mixer mill at 20 Hz twice for 2 minutes each, separated by a 2-minute pause, and then 300 µl of ice-cold 95% ethanol was added before each tube was vortexed and left for 10 minutes. The epitubes were then centrifuged at 15,000 rpm for 3 minutes and the pellet was rinsed with 70% ethanol and dried in a Savant Speed Vac Concentrator before resuspending in 100 µl of distilled water. The epitubes were centrifuged at 15,000 rpm for approximately 15 seconds and 20 µl of the supernatant was removed and stored at -20 °C until use in PCR amplifications.

PCR and DNA sequencing

The *ITS* region of filamentous fungi and yeast was amplified using ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') or ITS9mun (5'-TGTACACACCGCCCGTCG-3') forward primers and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') reverse primer (Egger, 1995; Toju *et al*., 2012; White *et al*., 1990). The *16S* region of bacterial samples was amplified using Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') forward and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') reverse primer (Klindworth *et al*., 2013; Herlemann *et al*., 2011). The *beta-tubulin* gene of *Penicillium* antagonists was amplified using Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') forward primer and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') reverse primer (Glass and Donaldson, 1995). Standard PCR reactions contained approximately 2 µl each of 10 µM forward and reverse primers, 5 µl of 10× Taq buffer (BioShop, Burlington, ON), 2 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs (New England BioLabs, Whitby, ON), 1.25 units of Taq (New England BioLabs), approximately 100 ng of template DNA, and sterile Milli-Q water to 50 µl. For filamentous fungal and yeast *ITS*, the PCR schedule was 5 minutes at 94 °C, then 35 cycles each with 30 seconds at 94 °C, 56 °C, and 72 °C, then 7 minutes at 72 °C. For bacterial *16S*, the schedule was 10 minutes at 94 °C, then 35 cycles each with 60 seconds at 94 °C, 57 °C, and 72 °C, then 10 minutes at 72 °C. The *beta-tubulin* PCR schedule was 3 minutes at 95 °C, then 35 cycles each with 30 seconds at 95 °C, 60 °C, and 72 °C, then 7 minutes at 72 °C. PCR products were purified using a Geneaid PCR DNA fragments extraction kit and sent to Génome Québec (Montréal, QC) for Sanger sequencing (Applied Biosystems - 3730xl DNA Analyzer). Forward and reverse sequences were aligned using ExPASy ClustalW and the NCBI nucleotide BLAST database was

used to identify the microbes. DNA sequences were submitted to GenBank and accession numbers are given in Appendix 1.

Assessing volatile production and activity

Shared airspace experiments

A shared-airspace experiment was performed to assess if any of the antagonists that completely or uniformly inhibited *P. destructans* in bioassays acted through volatiles. A strip approximately 1 cm wide was cut out of the centre of a 90-mm PDA plate to create two separated pieces of agar. A thawed stock of *P. destructans* was diluted in PDB and approximately 1.3×10^4 CFUs were spread onto agar on one side of the plate. A small amount of antagonist (a small loopful for bacteria/yeast or a needleful for filamentous fungi) was suspended in 250 µl of liquid medium (PDB for filamentous fungi and yeast, LB for bacteria) and 200 µl of this suspension was spread onto the agar surface opposite of *P. destructans*. The plates were sealed with Parafilm and incubated at 13 ± 1 °C. The growth of *P. destructans* was monitored and compared to a control without an antagonist from 6 to 14 days after inoculation. The day 14 area of *P. destructans* and respective inhibition scores for each antagonist were calculated using ilastik (Sommer *et al*., 2011) and ImageJ (Schneider *et al*., 2012), as above. Based on inhibition scores, antagonist inhibition of *P. destructans* was scored as negligible, considerable, or complete/nearly complete, as described above.

Volatile identification and characterization

Headspace gas chromatography mass spectrometry (GC-MS) was used to analyse volatiles produced by the four microbes that had contact-independent inhibition of *P. destructans* after 14 days. *Pantoea ananatis* RFA4P2, *Oidiodendron* sp. PCA20P, *Pantoea* sp. OA1I3M, *Cystofilobasidium capitatum* RW3I1a, and a no-antagonist (blank) control were inoculated on 3-

ml PDA slants inside headspace jars and grown for 5 days at 13 ± 1 °C. The headspace jars were covered with a double layer of sterile foil for the incubation period and sealed approximately 10 minutes before performing GC-MS. The GC-MS was done with an Agilent Technologies 7697A headspace sampler coupled to an Agilent Technologies 7820A gas chromatography system and an Agilent Technologies 5977E mass spectrometer detector. The vials were sampled at 33.9 °C, the loop temperature was 45 °C and the transfer line was 80 °C. Samples were injected for gas chromatography in splitless mode. A 30 m × 250 µm × 0.5 µm DB-WAXetr column was used with helium carrier gas at a constant flow rate of 1 ml/min. The oven temperature was held at 50 °C for 2 min and then increased at a rate of 10 °C/min to 235 °C, where it was held for 5.5 minutes. Mass-spectrometry was performed with electron ionization, and identification of volatile compounds was performed by comparison to version 5 of the National Institute of Standards and Technology (NIST) spectra database.

Compounds identified by GC-MS were tested for inhibitory activity against *P. destructans* in bioassays. A lawn of approximately 2.6×10^4 CFUs of *P. destructans* was inoculated onto the surface of 15 ml of PDA in a 90-mm diameter Petri dish. Petri dishes were inverted and a 2.5-cm diameter sterile Whatman 3 paper disc was placed on the lid of each dish. The filter paper was saturated with 10 or 100 µl of 2-methyl-1-propanol (J.T. Baker Chemical Co., Phillipsburg NJ), 2-methyl-1-butanol (Sigma-Aldrich, Oakville ON), propanoic acid, or 1-pentanol (BDH Chemicals, Toronto ON). The 10 µl aliquots were diluted to 100 µl in sterile distilled water, except for 1-pentanol, which was diluted in 95% ethanol. Separate assays were done using 100 µl/disc of water or ethanol as carrier controls. Plates were sealed with Parafilm and incubated inverted at 13 ± 1 °C for 14 days and growth of *P. destructans* was assessed. To test whether the inhibition of *P. destructans* was fungistatic or fungicidal, the paper discs were

removed on day 14 and the lid was dried with a sterile Kimwipe. Plates were sealed with Parafilm and incubated at 13 ± 1 °C for an additional 14 days, after which growth of *P. destructans* was assessed.

Additional dilution series of each volatile compound were performed as above and the minimum inhibitory concentration, where no growth of *P. destructans* was visible after 14 days, was determined to be 50 µl for 1-pentanol, 10 µl for propionic acid, 75 µl for isobutyl alcohol, and 17.5 µl for 2-methyl-1-butanol. To determine the minimum exposure time required for an antifungal effect, a lawn of *P. destructans* was plated as described above and exposed to twice the MIC values of each compound for 30 minutes, 1 hour, or 2 hours and 45 minutes. Plates were sealed with Parafilm for the volatile incubation, after which the plate lid was replaced and plates were left unsealed for 14 days at 13 ± 1 °C. Percent inhibition of *P. destructans* was calculated using Image J and Ilastik, as described above.

Assessing activity of spent antagonist media

We screened antagonists for production of antimicrobial compounds that inhibit *P. destructans*. We used spent culture media from a subset of antagonists of *P. destructans* US-15 and screened these against *P. destructans* strains US-15, SH-864, and SH-991, *P. roseus* S8A2CN, *P. pannorum* S8A5ACS1, and *Saccharomyces cerevisiae* strain S288C. Thirty-five antagonists were each grown stationary in 250 ml flasks with 50 ml of PDB (fungi) or LB (bacteria) for 4 weeks at 13 ± 1 °C. After this time, the culture medium was harvested and passed through a 0.2-µm syringe filter and then 10× concentrated following lyophilisation. To assess inhibition of *Pseudogymnoascus* spp. and yeast, 50 µl of cell-free filtrate was 1:1 serially diluted in 50 µl of PDB (for *Pseudogymnoascus* spp.) or YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} D-glucose; for yeast) in a 96-well microtiter plate before the addition of 150 µl of PDB

with approximately 100 CFUs *Pseudogymnoascus* sp. or YPD with approximately 150 yeast cells. Ten-times concentrated medium (PDB or YPD, as appropriate) was used as a carrier control for antagonist filtrates. Inhibition was assessed visually after 14 days of growth at 13 ± 1 °C (*Pseudogymnoascus* sp.) or 3 days of growth at 30 °C (*S. cerevisiae*) and was defined as the lowest concentration of spent medium at which no growth was visible.

Tests for specificity of inhibitory interactions

To test the specificity of the inhibition of *P. destructans*, we repeated bioassays using 36 of our top antagonists against two additional strains of *P. destructans* (SH-864 and SH-991) and two close relatives of *P. destructans* (*P. roseus* S8A2CN and *P. pannorum* S8A5ACS1). The bioassays were conducted as described above for *P. destructans* US-15; however, the concentration of *Pseudogymnoascus* spp. inoculum stamped onto the plates was adjusted for each strain so that after 14 days a continuous line of fungal mycelium was visible for each strain. The concentrations were approximately 11,300 CFU/ml *P. destructans* SH-864, 10,000 CFU/ml *P. destructans* SH-991, 11,100 CFU/ml *P. roseus* S8A2CN, and 8,700 CFU/ml *P. pannorum* S8A5ACS1. Inhibition scores were calculated and ranked as described for *P. destructans* bioassays.

Results

To screen for potential biocontrol candidates, we first obtained microbial isolates from local environmental samples and from culture collections. We co-inoculated one isolate per plate with *P. destructans* and classified each isolate based on an ability to inhibit *P. destructans* growth 14 days after inoculation (described in Methods).

Inhibition scores against *P. destructans* by each bacterial, filamentous fungal, and yeast isolate were calculated based on *P. destructans* colony area in the presence of isolates (see Methods) and classified as negligible (less than 50% inhibition of *P. destructans*), considerable (50% to 85% inhibition), or complete/nearly complete (greater than 85% inhibition), as summarized in Table 1.1 and represented in Figure 1.1. Nearly 50% of the 301 isolates examined were antagonistic to *P. destructans*, and over 15% completely or nearly completely inhibited growth of *P. destructans* (Table 1.1, Figure 1.2). Most of the isolates that inhibited *P. destructans* did so by creating a zone of inhibition surrounding themselves where *P. destructans* did not grow. However, other modes of inhibition were also evident. For example, some fast-growing filamentous fungi grew over *P. destructans* colonies and induced a highly vacuolized appearance to *P. destructans* hyphae that was suggestive of programmed cell death (Figure 1.3; Biella *et al*., 2002), while the presence of some yeast isolates resulted in *P. destructans* colonies that remained uniformly small over the entire plate. For a tested subset of these inhibitory yeasts, this was a fungistatic effect since the inhibited *P. destructans* colonies resumed normal growth when transferred to a plate without the yeast. Only a few of the environmental isolates were inhibited by *P. destructans* (Table 1.1).

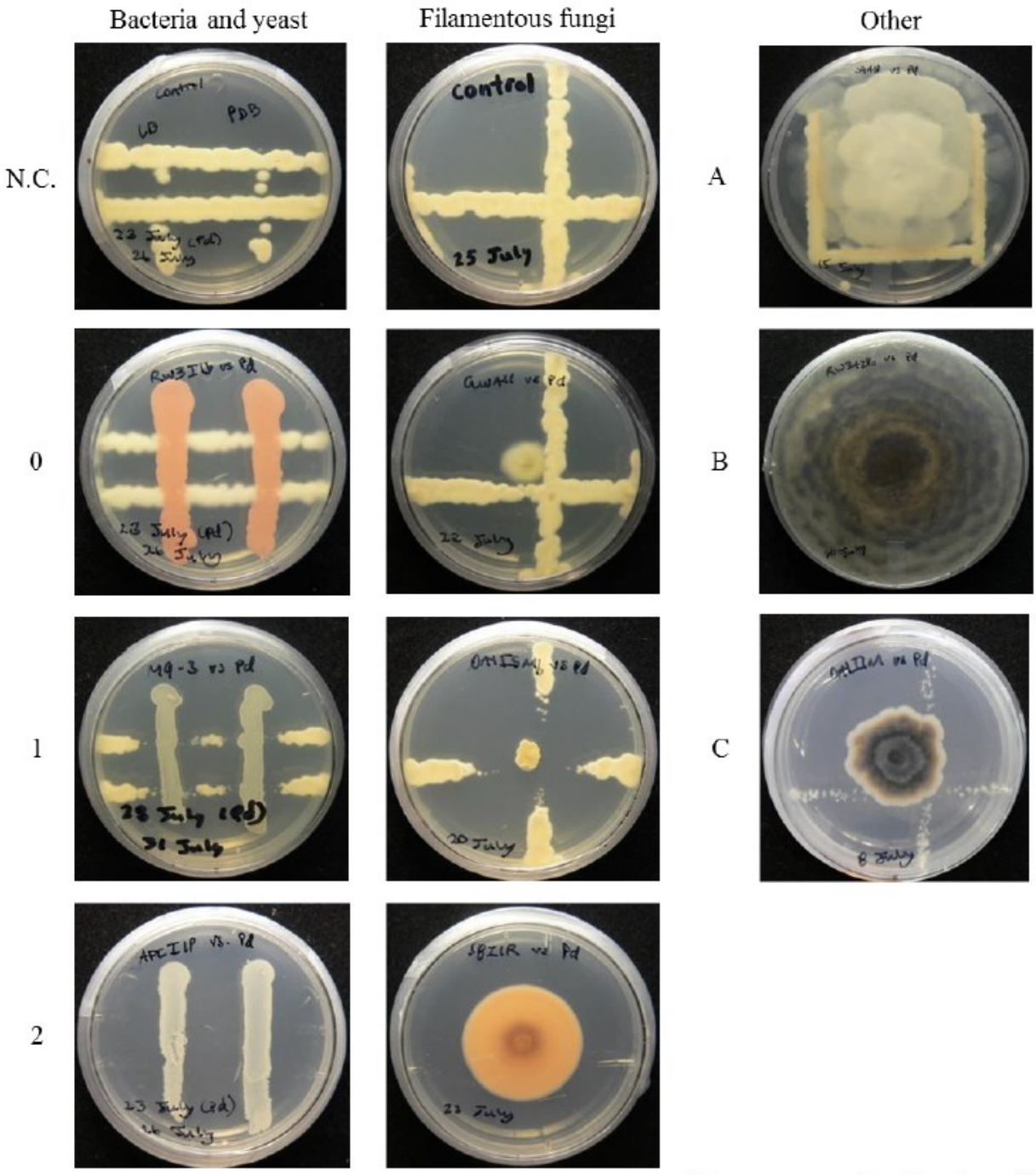


Figure 1.1. Representatives of each category of inhibition. *P. destructans* was inoculated on all plates as in the negative control (N.C.), and all photographs were taken 14 days after *P. destructans* inoculation. Classifications are (left and centre panels): (0) negligible (< 50%) inhibition, (1) considerable (50% to 85%) inhibition, (2) nearly complete/complete (> 85%) inhibition. Additionally, if applicable (right panel): (A) growth of the isolate is limited by *P. destructans*, (B) the isolate grew over *P. destructans* such that affected *P. destructans* colonies were no longer visible, (C) *P. destructans* colonies were present, but uniformly smaller than in the control plate.

Table 1.1. Isolate inhibition of *P. destructans*. Summary table showing the number of bacterial, filamentous fungal, and yeast isolates for each classification of inhibition 14 days after *P. destructans* inoculation.

	Bacteria	Filamentous fungi	Yeast	Total
Total screened	130	158	13	301
Negligible (< 50%) inhibition	75	75	6	156
Considerable (50% to 85%) inhibition	27	60	5	92
Complete/nearly complete (> 85%) inhibition	28	23	2	53
P. destructans inhibited antagonist	0	11	0	11
Antagonist grew over *P. destructans*	0	31	0	31
Reduced *P. destructans* colony size	8	25	5	38

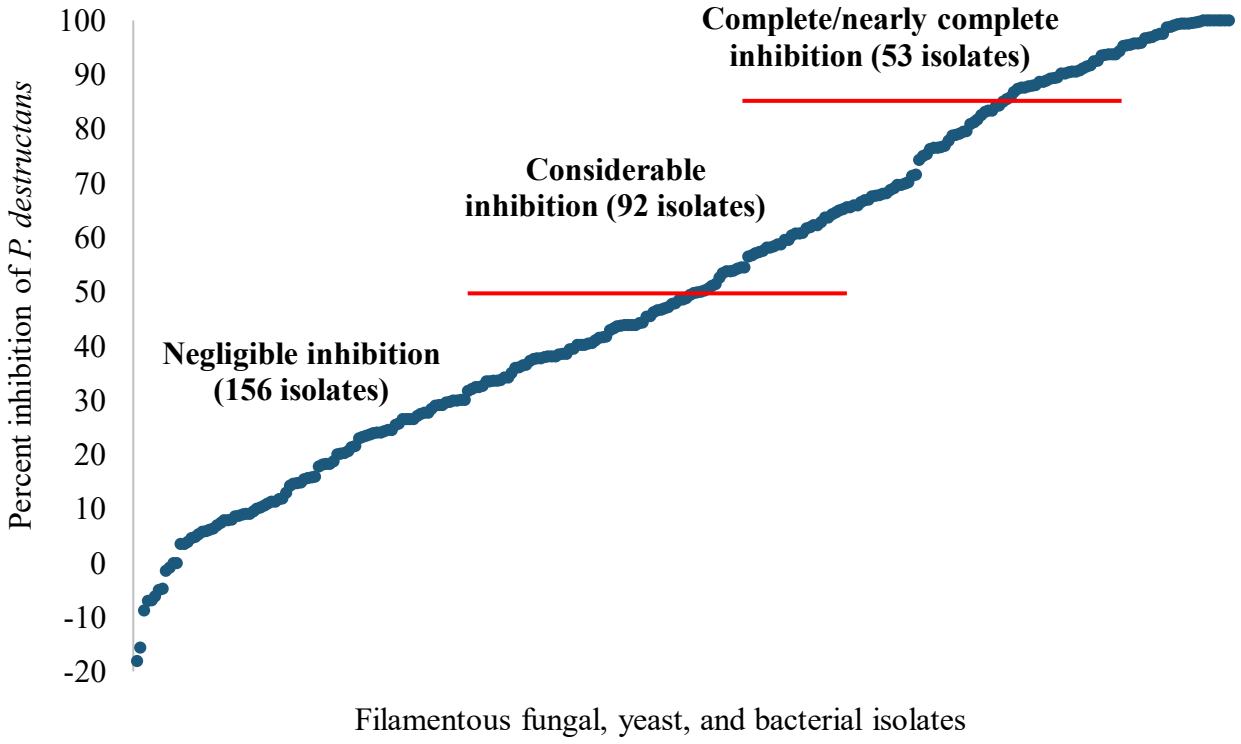


Figure 1.2. The distribution of inhibition of *P. destructans* by tested microbes. Each blue dot represents a different microbe and the red lines indicate the cut-off values for each class of inhibition.

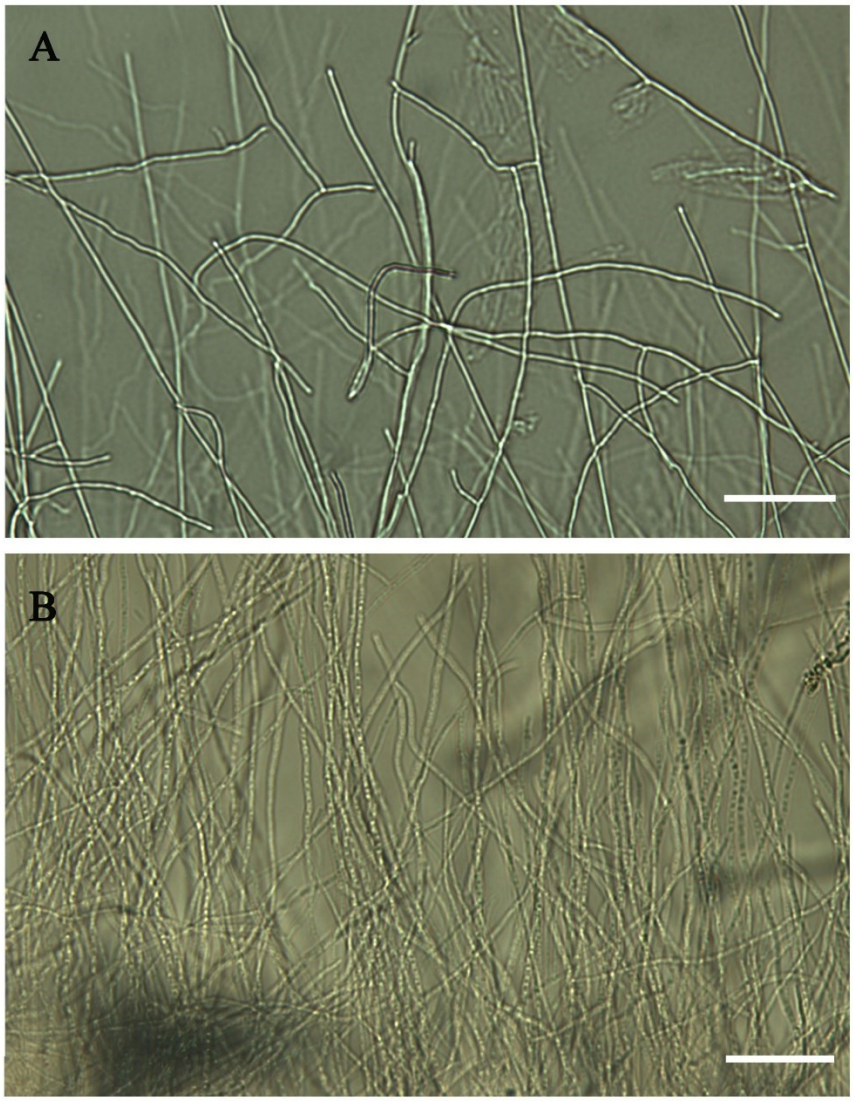


Figure 1.3. Representative image showing *P. destructans* vacuolization near antagonist hyphae. *P. destructans* was pre-inoculated for one week on a PDA-coated microscope slide before the slide was also inoculated with *Penicillium crustosum* BWA2P. After 4 days of antagonist growth, *P. destructans* hyphae had a healthy appearance on the colony side away from the antagonist (A), but had a vacuolized appearance suggestive of programmed cell death near antagonist hyphae (B). Scale bar represents 100 µm.

Isolates that inhibited the growth of *P. destructans* are henceforth referred to as 'antagonists'. The most inhibitory antagonists were taxonomically identified, where possible, to genus or species (see Methods, Appendix 1). The 28 most effective bacterial antagonists were from the genera *Bacillus* (17 strains), *Pantoea* (3 strains), *Streptomyces* (3 strains), *Pseudomonas*

(2 strains), and 1 strain each from *Rahnella*, *Arthrobacter*, and *Sphingobium*. The 23 most inhibitory filamentous fungi were primarily from the genera *Penicillium* (11 strains) and *Trichoderma* (7 strains), and the genera *Oidiodendron, Boeremia*, *Botrytis*, and *Phoma* each had 1 representative. One strongly inhibitory antagonistic filamentous fungus (isolate RW3A2Pa) could not be identified. Yeast belonging to *Cystofilobasidium* (2 strains) were also among the most inhibitory antagonists. All antagonists of *P. destructans* were preserved as frozen glycerol stocks at -80 °C.

We examined if antagonists that completely or uniformly inhibited *P. destructans* acted through volatile compounds by inoculating *P. destructans* separately from antagonists in a shared airspace (see Methods). Based on these assays, 7 of 28 antagonists tested produced volatiles that effectively reduced the growth of *P. destructans* for 6 to 10 days after inoculation. Volatiles from 2 of these antagonists (*Oidiodendron* sp. PCA20P and *Pantoea* sp. OA1I3M) caused considerable (50-85%) inhibition and 2 (*Pantoea ananatis* RFA4P2 and *Cystofilobasidium capitatum* RW3I1a) caused complete or nearly complete (greater than 85%) inhibition of *P. destructans* at 14 days after inoculation (Table 1.2). This suggests that at low temperatures, these antagonists constitutively produce volatile compounds that inhibit *P. destructans*. A representative of each class of inhibition by volatiles is shown in Figure 1.4.

Table 1.2. Screen for inhibition of *Pseudogymnoascus destructans* by volatiles produced by selected antagonists. *P. destructans* and an antagonist were inoculated on separate pieces of agar within a single plate and incubated at 13 ± 1 °C. Inhibition was assessed at 6, 10 and 14 days after inoculation and percent inhibition was calculated on day 14 as $(1 - Area_{treatment}/Area_{control}) \times 100$, based on the area of *P. destructans* in the presence of the antagonist (treatment) compared to in the no-antagonist control. The average *P. destructans* area in no-antagonist control was 2043.85 mm^2. Antagonists were ranked based on their ability to inhibit *P. destructans*: 0 = negligible (< 50%) inhibition, 1 = considerable (50% to 85%) inhibition, or 2 = complete or nearly complete (> 85%) inhibition. Asterisks (*) indicate cases where more pronounced inhibition of *P. destructans* was evident at day 6 and 10 but decreased by day 14.

Strain	*P. destructans* Area (mm^2)	Day 14 Percent Inhibition	Rank
RFA4P2	0.00	100.0	2
RW3I1a	145.96	92.9	2
PCA20P	672.15	67.1	1
OA1I3M	781.32	61.8	1*
CIB T128	1222.48	40.2	0
DUST G	1369.08	33.0	0
RW4A2P	1391.97	31.9	0
APCI1P	1394.12	31.8	0
S9A1Cs1	1398.90	31.6	0*
OA1I1M	1583.04	22.5	0
RW1I1Pa	1616.44	20.9	0
55407	1625.42	20.5	0
S4A3P	1626.44	20.4	0*
S9A1R	1699.65	16.8	0
S8A4Cs	1743.78	14.7	0*
PCI2P	1749.82	14.4	0
S8A1Cs1b	1803.02	11.8	0
S5A2LC	1818.50	11.0	0
S6A3ACS	1834.22	10.3	0
APCI2P	1922.01	6.0	0
S8I1R	1946.96	4.7	0
RW1A1P1	1949.46	4.6	0
95405	1951.98	4.5	0
BWA2P	2040.81	0.1	0
GWA3a	2047.64	-0.2	0
RW6A1P	2083.45	-1.9	0
RW1A1P2	2187.00	-7.0	0
F9-6	2209.09	-8.1	0

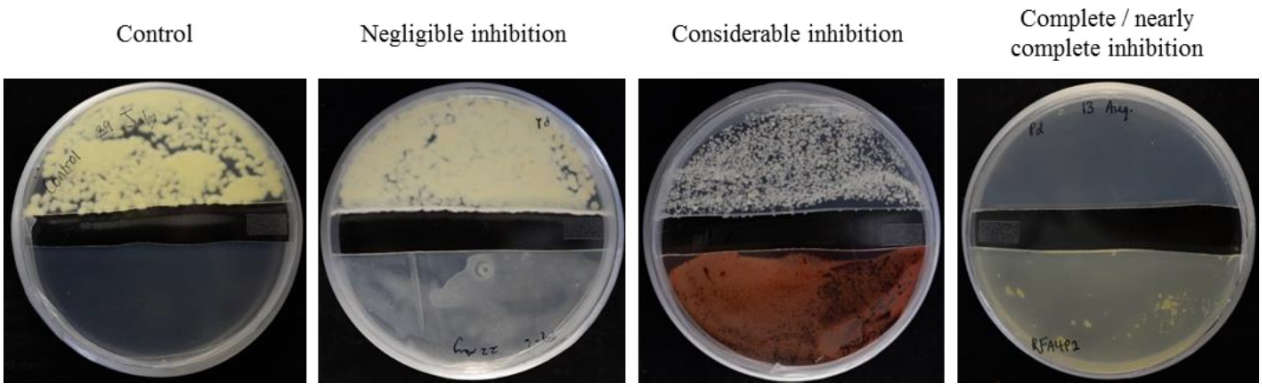


Figure 1.4. Evidence of inhibition of *P. destructans* by volatiles produced by antagonists. *P. destructans* and an antagonist were inoculated on separate pieces of agar within a single Petri plate. Photographs show *P. destructans* inoculated on the top and an antagonist on the bottom of the plate except for control plate (left), which contains *P. destructans* with no antagonist. Volatile production by antagonists was evident as considerable or complete growth inhibition of *P. destructans*. All photographs were taken 14 days after inoculation.

We conducted gas chromatography-mass spectrometry (GC-MS) to identify volatile compounds produced by the antagonists that inhibited *P. destructans* 14 days after inoculation. Volatile inhibitory compounds were identified in the headspace jars for three of the four antagonists that were analysed with GC-MS. We detected 2-methyl-1-propanol for *C. capitatum* RW3I1a and *Pantoea* sp. OA1I3M. We also detected 2-methyl-1-butanol for *C. capitatum* RW3I1a. Propanoic acid and 1-pentanol were detected for *P. ananatis* RFA4P2. Other compounds appeared to be present in samples but could not be conclusively identified. *Oidiodendron* sp. PCA20P did not produce any detectable volatiles, suggesting that unidentified factors are required to induce volatile production in this antagonist. The most probable identification for each compound, along with the corresponding match scores and probability scores are included in Appendix 2.

Compounds tentatively identified through GC-MS were screened in bioassays to determine their ability to inhibit *P. destructans*. All four compounds tested (2-methyl-1-propanol, 2-methyl-1-butanol, propanoic acid, and 1-pentanol) completely inhibited *P. destructans* growth for the 14-day experimental period when 100 µl of compound was added. Only propanoic acid was completely inhibitory when 10 µl of compound was applied. These compounds appear to be fungicidal since completely inhibited *P. destructans* did not resume growth after the compounds were removed. The ethanol control (carrier solvent for 10 µl 1-pentanol assay) also mildly affected *P. destructans* growth such that after 14 days, the average colony diameter was approximately 30% smaller than in the water control.

We also examined the effect of exposure time on the inhibition of *P. destructans* by volatile compounds. *P. destructans* was exposed to each volatile compound for 30, 60, or 165 minutes and then inhibition was assessed after 14 days of growth. 2-methyl-1-propanol strongly inhibited *P. destructans* with only 60 minutes of exposure and all compounds except 2-methyl-1-butanol caused complete or nearly complete inhibition of *P. destructans* with less than three hours of exposure (Table 1.3).

Table 1.3. The effect of exposure time on the inhibition of *P. destructans* by four volatile compounds. *P. destructans* was inoculated onto agar plates and exposed separately to twice the minimum inhibitory concentration of each volatile compound for 30, 60, or 165 minutes. The volatiles were removed and the plate was incubated at 13 ± 1 °C for 14 days. The percent inhibition of *P. destructans* in each condition is shown below and was calculated as $(1 - \text{Area}_{\text{treatment}}/\text{Area}_{\text{control}}) \times 100$, based on the day 14 area of *P. destructans* in the presence of the volatiles (treatment) compared to in a no-volatile control.

Time (min)	2-methyl-1-butanol	2-methyl-1-propanol	1-pentanol	Propionic acid
30	32.5%	31.0%	-14.3%	-7.9%
60	37.2%	85.2%	17.6%	7.8%
165	47.5%	100%	99.9%	100%

We next conducted bioassays to examine the inhibitory specificity of 36 of the most effective antagonists. For these, we repeated the bioassays that we performed with our original strain of *P. destructans* against two additional strains of *P. destructans* and representatives of the closely related species, *Pseudogymnoascus pannorum* and *Pseudogymnoascus roseus*. Of the 36 antagonists tested in bioassays, 35 inhibited both additional strains of *P. destructans*, with 29 causing complete or nearly complete inhibition in both strains. Only 16 antagonists inhibited growth of *P. roseus* and/or *P. pannorum*, with 3 of these causing complete or nearly complete inhibition in one or both strains (Appendix 3).

We also qualitatively assessed whether our best antagonists could produce antimicrobial products that inhibit *P. destructans*. We grew 35 of our top antagonists in axenic liquid cultures and screened the spent media for activity against the above mentioned five *Pseudogymnoascus* strains and, in addition, *Saccharomyces cerevisiae.* Many of our antagonists produced inhibitory compounds and, similar to bioassays, *P. destructans* strains were considerably more sensitive than other species tested (Appendix 4). Spent media from 18 of 35 antagonists tested inhibited at least one of the three *P. destructans* strains, and nine of these did not inhibit other *Pseudogymnoascus* species or brewer's yeast.

Inhibition scores for the 27 antagonists that were tested in both bioassays and spent media screens were not always congruent and some antagonists appear to have context-dependent induction and/or effects of inhibitory compounds. For example, there were 13 antagonists that inhibited all three strains of *P. destructans* in bioassays, while the respective spent culture medium caused no inhibition of *P. destructans*. Conversely, there were seven antagonists that caused negligible inhibition of *P. roseus* and/or *P. pannorum* in bioassays, while their spent culture medium was inhibitory to *P. roseus* and/or *P. pannorum*. Nevertheless, it is notable that

the culture medium from several antagonists, including *Phoma* sp. OA1I1M, *Sphingobium* sp. S8A4Cs, *Trichoderma harzianum* RW1A2P, and *Paecilomyces inflatus* PCA5P, caused very strong inhibition in all three strains of *P. destructans* while causing low inhibition of *P. roseus*, *P. pannorum*, and yeast. The inhibitory compounds produced constitutively by these antagonists could be considered further as candidate natural products to inhibit growth of *P. destructans* in bat hibernation sites.

Discussion

The white-nose syndrome that is caused by *P. destructans* has decimated eastern North American bat populations and is spreading across the continent (Warnecke *et* al., 2012; U.S. Fish and Wildlife Service, 2016; O'Regan *et al*., 2015). Saprotrophic growth of *P. destructans* in cool hibernacula may contribute to perpetuating the WNS epidemic (Reynolds *et al*., 2015) and thus may facilitate the infection or reinfection of healthy bats. In an effort to find biocontrol agents that will reduce the abundance of *P. destructans* in bat hibernacula, we isolated microbes from diverse environmental samples and tested them for inhibitory activity against *P. destructans*. We identified 145 microbes that inhibited the growth of *P. destructans* to some extent, and 53 of these completely or nearly completely inhibited *P. destructans*.

In the past decade since *P. destructans* was discovered, only a few microbes capable of inhibiting *P. destructans* have been identified: *Rhodococcus rhodochrous* (Cornelison *et al*., 2014b), *Trichoderma polysporum*, *Trichoderma harzianum* (Zhang *et al*., 2015), and *Pseudomonas* spp. (Hoyt *et al*., 2015b). These microbes are being studied for their biocontrol potential, and our study adds nearly 150 candidate biocontrol agents to this list, Additionally, we expand the list of natural products (Zhang *et al*., 2015; Cornelison *et al*., 2014a; Raudabaugh and Miller, 2015; Boire *et al*., 2016; Chaturvedi *et al*., 2011b) that could be of potential use in

controlling *P. destructans* in bat caves. We also report on 4 volatile organic compounds that appear to have a rapid fungicidal effect on *P. destructans*. All 4 of these compounds have previously been reported to be produced by fungi (e.g. Jurjevic *et al*., 2008; Sánchez-Ortiz *et al*., 2016; Stinson *et al*., 2003b) and to have at least some degree of inhibitory activity against fungi (e.g. Cruz *et al*., 2012; Yun and Lee, 2016; Zunino *et al*., 2015; Braun *et al*., 2012). Although differences in methodologies preclude making quantitative comparisons between the antagonists identified in this study and the antagonists of *P. destructans* that have been previously identified, the most notable finding from our study is the relatively high frequency of microbes that we identified that cause complete or nearly complete inhibition of *P. destructans*. While few of the previously identified antagonists have completely inhibited growth of *P. destructans*, we identified five bacterial and four filamentous fungal isolates that did so in bioassays or volatile tests. Additionally, while a bacterium has been reported that inhibits *P. destructans* through volatiles (Cornelison *et al*., 2014b), we novelly identify microbes that produce inhibitory volatiles without induction.

The microbes that we identified have several promising features as biocontrol candidates in eastern North America. First, most of our top antagonists are microbes that already occur in Ontario and Quebec that present a lower risk than introducing foreign, potentially invasive species. Second, many of our top antagonists displayed antifungal activity at about 13 °C – a temperature that represents the average temperature of North American bat hibernacula. Third, several of our top antagonists constitutively secrete compounds that inhibit *P. destructans* at concentrations that are non-inhibitory to close relatives and to *S. cerevisiae*, suggesting the possibility that these antifungal agents may have some degree of specificity towards *P. destructans*. In addition, in both bioassays and liquid media screens, *P. destructans* was more

sensitive to inhibition than close relatives, again suggesting the possibility that there may be a reduced risk of non-target effects from our antagonists. Fourth, we identified microbes that inhibit the growth of *P. destructans* by seemingly different modes. For example, *Trichoderma* and fast-growing filamentous fungal antagonists typically grew over and appeared to induce programmed cell death of *P. destructans* (Biella *et al*., 2002), indicating cell proximity/contact as a main mode of action, whereas most of our top antagonists secreted water-soluble inhibitors, indicating antibiosis as a main mode of action. Several antagonists also produced volatiles at hibernaculum-like temperatures that inhibit *P. destructans*. A diversity of modes of action provides the possibility of creating a stable biocontrol strategy that targets *P. destructans* through multiple mechanisms. Finally, our high success rate of isolating native biocontrol candidates suggests that as *P. destructans* continues to spread across the continent, additional local biocontrol candidates can be identified that may reduce the growth and persistence of *P. destructans* in hibernacula.

Another interesting aspect of our findings is that several antagonists that we identified are not known to produce antifungal compounds. To our knowledge, antifungals have not been characterized from species of *Boeremia*, *Phialosimplex, Ramularia*, or *Sphingobium*, all of which secreted inhibitors of *P. destructans*. Similarly, only preliminary characterizations of antifungals are reported for species of *Oidiodendron* (Hosoe *et al*., 1999) and *Cystofilobasidium* (Lutz *et al*., 2011). This suggests the possibility that some of our top antagonists may produce novel antifungals, which could have applications both within and beyond controlling *P. destructans*.

Although we identified many potential biocontrol agents of *P. destructans*, an important limitation of this study is that it only addressed inhibition of *P. destructans* under controlled

laboratory conditions. Future challenges to developing a biocontrol of white nose syndrome are to find antagonistic organisms that selectively inhibit growth of *P. destructans* in natural hibernacula. Considering the high proportion of microbes that inhibited *P. destructans* in our tests, it is surprising that a biocontrol of *P. destructans* has not arisen naturally. It is possible that the effects of natural antagonists are limited by an insufficient abundance and nutritional augmentation of hibernaculum sediment may be necessary to support greater antagonist growth. To explore this further, we will examine *P. destructans*-antagonist interactions in hibernaculum-like soil microcosms.

Conclusions

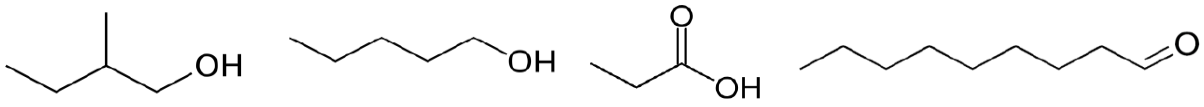
We identified over 100 microbes that inhibit the growth of *P. destructans* in a low-temperature laboratory setting. These antagonistic microbes inhibit *P. destructans* with secreted compounds, by contact inhibition, or through volatiles. Future research is needed to validate potential biocontrol strategies under hibernaculum conditions. Our results suggest that local microbes can be a source of candidate biocontrol agents to reduce the abundance of the causal agent of white-nose syndrome in bat hibernation sites and remediate bat colony collapse.

Chapter 2: Examination of antagonist metabolites

Pseudogymnoascus destructans is the fungus that causes a deadly disease in hibernating bats known as white-nose syndrome. In addition to colonizing the skin of hibernating bats, *P. destructans* can grow as a saprotroph in bat hibernacula (Lorch *et al*., 2013a; Lindner *et al*., 2011; Lorch *et al*., 2013b). A control agent that targets environmental *P. destructans* could contribute to managing the white-nose syndrome epidemic by potentially reducing the rate of bat infection and mortality, as well as the extent of *P. destructans* spread and persistence (Hoyt *et al.*, 2015a; Frick *et al*, 2017; Reynolds *et al*., 2015).

Out of many possible control strategies, the use of microbial volatile compounds is especially promising for the chemical control of *P. destructans* in bat hibernacula, due to their ability to diffuse through spaces, their biodegradability, and their low environmental persistence (Wang *et al*., 2013). Additionally, volatiles can be effective fumigants in diverse and textured environments (e.g. Strobel *et al*., 2001; Mercier and Jiménez, 2007), and have been shown to be effective for treating agricultural soils (e.g. Stinson *et al*., 2003a). We and others have previously identified several volatile compounds that inhibit *P. destructans* in bioassays (Cornelison *et al*., 2014a; Padhi *et al*., 2016; Micalizzi *et al.*, 2017) and that may be promising for the environmental control of *P. destructans*. To reduce off-target effects, these volatiles could be used as fumigants in bat hibernacula during summer months when bats are absent; however, an understanding of their antifungal mechanism of action is one of several knowledge gaps precluding field trials at this time. The focus of this chapter is to conduct preliminary characterizations of the antifungal mechanism of action of four volatile compounds that inhibit

P. destructans: 2-methyl-1-butanol, 1-pentanol, propionic acid, and nonanal. The structures of these compounds are shown in Figure 2.1.



2-methyl-1-butanol 1-pentanol Propionic acid Nonanal

Figure 2.1. Structures of the four volatile compounds examined in this chapter.

We initially detected 2-methyl-1-butanol as a volatile compound produced by the yeast *Cystofilobasidium capitatum* (Micalizzi *et al*, 2017). 2-methyl-1-butanol, also known as active amyl alcohol, is a fusel alcohol produced at low levels during fermentation and widely present in many fermented foods and beverages (Kim *et al*., 2017, Hazelwood *et al*., 2008). In *S. cerevisiae*, 2-methyl-1-butanol is produced through native amino acid synthesis pathways (Cann and Liao, 2010) or through isoleucine catabolism via the Ehrlich pathway (Hazelwood *et al*., 2008). As a by-product of amino acid catabolism, 2-methyl-1-butanol may function as a quorum-sensing compound and can induce filamentation in yeast (Hazelwood *et al*., 2008; Dickinson, 2008). There are several reports of 2-methyl-1-butanol having antifungal activity (e.g. Braun *et al*., 2012; Rezende *et al*., 2015; Fialho *et al*., 2011); however, only preliminary work has been conducted on its antifungal mode of action, which suggested that 2-methyl-1-butanol may increase membrane permeability and cause oxidative stress (Rezende *et al*., 2015).

1-pentanol, also called *n*-amyl-alcohol, is a straight-chain alcohol that we detected in volatiles from *Pantoea ananatis*. Like 2-methyl-1-butanol, 1-pentanol is considered a fusel alcohol and is found in fermented beverages (Kim *et al*., 2017); however, the natural biosynthesis of 1-pentanol is not well-understood (Cann *et al*., 2010). There are only a few reports on the antifungal activity of 1-pentanol (e.g. Liouane *et al*., 2009; Zunino *et al*., 2015),

and while high-throughput screens have been conducted to elucidate its antifungal mechanism of action, the disruption of pathways identified in these screens has not been validated with secondary assays. Nevertheless, these screens suggest that genes related to vacuolar function, cell cycle and DNA processing, gene expression, and metabolism could be required for tolerance (Fujita *et al*., 2004; Fujita *et al*., 2006). As a medium-length straight-chain alcohol, 1-pentanol could also have antifungal effects by increasing membrane fluidity and permeability (Weber and de Bont, 1996).

In addition to 1-pentanol, we detected propionic acid in the volatiles of *Pantoea ananatis*. Propionic acid is widely used fatty acid that is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (2017). Industrially, hundreds of thousands of tonnes of propionic acid are produced annually, primarily for use as a preservative in foods and animal feeds, but also as a flavouring agent and in the synthesis of perfumes, herbicides, and pharmaceuticals (Bhatia and Yang, 2017; Ahmadi *et al*., 2016). Under natural circumstances, propionic acid is produced as an end-product of fermentation in *Propionibacterium* and other bacterial genera (Bhatia and Yang, 2017; Goswani and Srivastava, 2001; Ahmadi *et al*., 2016; Liu *et al*., 2012). Although the antifungal activity of propionic acid is well-known, several different modes of action have been proposed. Many have assumed the antimicrobial activity of propionic acid occurs through its effects as a weak fatty acid, increasing membrane permeability and acidifying the cytoplasm (Ahmadi *et al*., 2016; Ferreira *et al*., 1997; Davidson *et al*., 2002). However, recent work in *Saccharomyces cerevisiae* did not find an increase in membrane permeability with propionic acid treatment and found that intracellular pH alone cannot explain inhibition by propionic acid (Ullah *et al*., 2012). Genome-wide screens have suggested that both the regulation of intracellular pH through vacuolar acidification and the degradation of proteins through the

multivesicular body pathway are important for propionic acid resistance (Mira *et al*., 2009). However, others have asserted that propionic acid acts through the generation of reactive oxygen species and mitochondrial-mediated apoptosis (Yun and Lee, 2016), while further research has shown that propionic acid inhibits glucose metabolism through indirect inhibition of the pyruvate dehydrogenase complex (Brock and Buckel, 2004). It is possible that propionic acid has multiple modes of action, or has different modes of action at different concentrations.

Cornelison *et al*. (2014a) reported on production of volatile nonanal from *Rhodococcus rhodochrous*. While we did not detect this volatile in any of our screens, we included it in further assays because it was reported to inhibit *P. destructans*. Nonanal is an aldehyde produced by plants and bacteria. While little is known about its biosynthesis, it is likely produced through the oxidation of C_{18} unsaturated fatty acids (oleic acid; Schulz and Dickschat, 2007; Dudareva *et al*., 2013). Although there are additional reports of nonanal having antifungal activity (e.g. Fernando *et al*., 2005; Kobaisy *et al*., 2001; Rajer *et al*., 2017), its mechanism of action has not been well-studied. To the best of our knowledge, there is only one report on its antifungal mode, which showed that nonanal decreased membrane stability and increased membrane permeability in *Penicillium cyclopium* (Zhang *et al*., 2017).

To contribute to developing candidate natural products that could be used to target the environmental growth of *P. destructans* in hibernacula, we conducted preliminary examinations of the antifungal mechanisms of action of these four antifungal volatiles, using brewer's yeast (*Saccharomyces cerevisiae*) as a model.

Materials and methods

Yeast deletion arrays

To detect genes involved in the volatiles' modes of action, a yeast deletion array was used. The array contained approximately 4300 haploid gene deletion strains derived from *S. cerevisiae* BY4741 (*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*), with each strain having a different non-essential gene deleted (Tong *et al*., 2001), and was pinned onto array plates containing YPD agar (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} D-glucose, and 20 g L^{-1} agar) supplemented with 200 µg ml^{-1} G418 sulphate. The lids of the array plates were lined with Whatman chromatography paper and the paper was wetted with 1 ml of water mixed with volatile compounds at the following concentrations: 100 µl of propionic acid, 150 µl of 2-methyl-1-butanol, 150 µl of 1-pentanol, or 2 µl of nonanal. To ensure that the compounds were added uniformly to the chromatography paper, 0.1% Tween 80 was used in the epitubes to emulsify the water mixtures containing 2-methyl-1-butanol, 1-pentanol, and nonanal. The plates were sealed with Parafilm and incubated inverted at 30 °C for 2 days. Plates were imaged and candidate sensitive mutants were identified using SGAtools (Wagih et al., 2013; http://sgatools.ccbr.utoronto.ca/). To confirm mutant sensitivity, mutants identified with SGAtools were grown to stationary phase in 2 ml of YPD on a shaking incubator at 30 °C for two days and diluted 0 to 10^{-4} × in YPD before spotting 15 µl aliquots onto G418 sulphate - supplemented YPD agar. Volatile compounds were applied as above and sensitivity was assessed based on a comparison to growth in a no-volatile control. Mutants that showed less than a 10^{2} × reduction in colony number were classified as insensitive, while those that showed a 10^{2} to 10^{3} × reduction were classified as sensitive and mutants that showed a 10^{4} × or greater reduction in

colony number were classified as highly sensitive (see Figure 2.2). All mutants that were confirmed as sensitive to each volatile compound were subsequently screened for sensitivity to all four volatile compounds.

Candidate genetic pathways affected by compounds were inferred based on information from gene ontology searches using Funspec (Robinson *et al*., 2002; http://funspec.med.utoronto.ca/) and DAVID Functional Annotation Tool (Huang *et al*., 2009a; Huang *et al*., 2009b; https://david.ncifcrf.gov/summary.jsp). All ontology terms that were associated with more than one gene and enriched with $p \leq 0.10$ were reported.

Follow-up to deletion arrays

To test if the pathways inferred from ontology searches were involved in compound mode of action, secondary assays were performed. Due to high levels of within-group variability when yeast cells were exposed to volatile compounds, assays were performed by exposing yeast to the liquid phase of the volatile compounds. To determine a sub-inhibitory concentration to use for each compound, yeast cells were grown overnight in YPD and their concentration was adjusted to an OD_{600} of 0.75 to 0.80. Cells were placed into 1.5 ml epitubes in 200 µl aliquots with 0.5% Tween 80 (to emulsify the compounds) before each compound was added. A dilution series of each compound was used to vary exposure concentrations. The epitubes were incubated at 30 °C for 3 hours before diluting and plating onto PDA. After two days incubation at 30 °C, colonies were counted and the concentration where cell survival was approximately 80% of the no-compound control was used for all subsequent assays.

To expose *S. cerevisiae* to the volatile compounds in secondary assays, the following protocol was used except where noted. Strain S288C was grown overnight in YPD and then

adjusted to an OD_{600} of between 0.75 and 0.80 before 200 µl aliquots of cell suspension were placed into epitubes. Sub-inhibitory concentrations of each compound (1.5 µl 1-pentanol, 1.5 µl 2-methyl-1-butanol, 0.95 µl propionic acid, 0.1 µl nonanal) were added to the cell suspensions and emulsified with 0.5% Tween 80 before incubating at 30 °C for three hours.

Oxidative stress assay

To assess if the volatile compounds caused an increase in oxidative stress, cells were stained with 2′,7′-dichlorofluorescein diacetate (DCFDA). Cells were incubated with compounds and with 10 µg ml^{-1} DCFDA dissolved in DMSO (Madeo *et al.*, 1999). As a positive control, cells were incubated with 5 mM hydrogen peroxide. To ensure that levels of oxidative stress were considered only in the live cell population, propidium iodide was added to a final concentration of 4 µg ml^{-1} and only propidium iodide negative cells were examined for DCFDA fluorescence. Cells were analysed using a BD Accuri C6 flow cytometer, with DCFDA fluorescence analysed on the FL1 channel (533/30 nm) and propidium iodide fluorescence analysed on the FL2 channel (585/40 nm). Three independent experiments were performed, each with 3 replicates and 10,000 cells counted per replicate. For statistical analysis, the average values of the replicates for each experiment were reported.

Endocytosis assay

To assess the effect of each compound on endocytosis, a lucifer yellow assay was used (Dulic *et al.*, 1991; Wiederkehr *et al.*, 2001; Motizuki *et al.*, 2008). Cells were grown overnight in YPD and adjusted to an OD of between 0.75 and 0.80 before 100 µl of the cell suspension was mixed with 100 µl of incubation buffer (12.5 mM sodium phosphate, 2.5 mM sodium fluoride) in a 1.5 ml epitube and a sub-inhibitory concentration of each compound was added separately as

described above. As a positive control, 2.5 mM sodium azide was added to the incubation buffer. Epitubes were incubated at 30 °C for 30 minutes, after which lucifer yellow was added to the cell/buffer mixture to a final concentration of 4 mg ml^{-1}. Cells were incubated at 30 °C for an additional 3 hours before washing three times and then resuspending in incubation buffer. Fluorescence was measured using the FL1 channel of a BD Accuri C6 flow cytometer. Three independent experiments were performed, each with 3 replicates and a minimum of 10,000 cells counted per replicate. For statistical analysis, the average value across the replicates for each experiment was reported. To photograph lucifer yellow uptake, an aliquot of the no-compound control and the propionic acid-treated cells were photographed under a Zeiss Axioplan 2 imaging microscope with an AxioCam HRm camera.

Cell cycle progression assay

To assess the effect of each compound on cell cycle progression, propidium iodide DNA staining was used (Wu *et al*., 2011). Yeast were exposed to compounds for 3 hours and then pelleted by centrifugation and fixed by resuspending in 500 µl of 70% ethanol. Cells were then incubated at room temperature for 2.5 hours and resuspended in 500 µl PBS for 5 to 10 minutes before pelleting and resuspending in 100 µl PBS with 1 mg/ml RNase A. Cells were incubated overnight at 37 °C and then pelleted and resuspended in 100 µl PBS with 50 µg/ml propidium iodide before incubating in the dark at 37 °C for 1 hour. Propidium iodide staining was analysed using the FL2 channel of a BD Accuri C6 flow cytometer and the percentage of cells in each phase of the cell cycle was calculated using ModFit LT. Three independent experiments were conducted, each with three replicates and 10,000 cells counted per replicate.

Permeability assay

To assess if the compounds increased membrane permeability in yeast, we analysed the proportion of cells that excluded propidium iodide as a marker of an intact membrane (Davey and Hexley, 2010). Cells were exposed to compounds before 50 µg/ml propidium iodide was added. Cells were analysed on a BD Accuri C6 flow cytometer and the percentage of cells excluding propidium iodide was calculated based on an FL2 channel threshold of 1500. This was previously determined to be a conservative minimum fluorescence value for a dead (propidium iodide-positive) cell population. The experiment was performed independently three times, each with three replicates per condition and 10,000 cells analysed per replicate.

To determine if transient membrane permeability occurred, cells were incubated with compounds, using 20% ethanol as a positive control. Cells were examined after 0, 30, 60, and 150 minutes of compound exposure by adding 50 µg/ml propidium iodide and incubating for an additional 5 minutes before using flow cytometry to determine the percentage of the population that was propidium-iodide positive, as above.

Cellular respiration assay

To assess the effect of each compound on cellular respiration, the reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was monitored (Stowe *et al.*, 1995; Sánchez and Königsberg, 2006). Yeast cells were grown overnight in YPD and then washed twice with approximately 30 ml of sterile water before starving for between 8 and 14 hours in approximately 30 ml of sterile water. Cells were then resuspended to log phase density (OD_{600} between 0.75 and 0.8) in YPD and 150 µl of cell suspension was placed into a microtiter plate with 15 µl 2.89 mM MTT, 10 µl 0.19 mM phenazine methosulfate, and 25 µl 10% Triton

X-100, before adding compounds as explained above. The microtiter plate was sealed with parafilm and placed into a BioTek Instruments Cytation 5 microtiter plate reader set to 30 °C with continuous shaking. A_{570} and A_{660} were measured every 5 minutes for 9 hours. Wells containing control conditions were placed between each compound to ensure that the potential volatilization of the compounds within the plate did not affect the results. Additionally, no-cell controls were included with each compound. Each experiment was performed independently three times, with 5 replicates per experiment. MTT reduction for yeast exposed to each compound was calculated at each time point as $\frac{A_{570}\ exp. - A_{570}\ cont.}{A_{660}\ exp.}$ where A_{570} exp. and A_{660} exp. are the absorbances of the cell suspension, and A_{570} cont. is the absorbance of the cell-free suspension.

Gene expression assay

Three strains of yeast cells stably expressing GFP (*PIN4-GFP*, *PSK2-GFP*, and *TPK2-GFP*) were grown separately overnight in YPD and adjusted to log phase before 600 µl of each were placed separate into 1.5 ml epitubes with 0.5% Tween 80. Sub-inhibitory concentrations of compounds were added to the epitubes (4.5 µl 2-methyl-1-butanol, 4.5 µl 1-pentanol, 0.3 µl nonanal, and 2.85 µl propionic acid) and 3 µl of 35 µg ml^{-1} cycloheximide in ethanol was added as a positive control. Cells were incubated in epitubes for 30 minutes before transferring to separate wells of an 8-well plate. GFP was photobleached for 20 minutes by gently agitating the plate without the lid approximately 2 cm from a 13-watt, 1050 lumen daylight-spectrum LED spotlight. Because some of the cell/compound mixture evaporated during this time, 100 µl of autoclaved distilled water was added back to each well and the cells were transferred back to epitubes with additional volatile compound (1.5 µl 2-methyl-1-butanol, 1.5 µl 1-pentanol, 0.1 µl

nonanal, and 0.95 µl propionic acid). Cells were incubated in the dark for an additional 2 hours before adding 50 µg ml^{-1} of the vital stain propidium iodide. Cells were incubated in the dark for 5 minutes and then analysed on a BD Accuri C6 flow cytometer. The fluorescence of GFP was analysed on the FL1 channel and the FL2 channel was used to exclude cells staining with propidium iodide from the analysis.

Statistical analysis

For each independent experiment, replicates were averaged and the mean values were used for statistical analysis. Statistical analyses were conducted in R. For all assays, a one-way ANOVA was conducted to test for an effect of compound treatment on phenotype. Pairwise comparisons between the control and each condition were conducted using a Tukey's HSD post-hoc test and Levene's test was used to confirm homogeneity of within-group variance. For cell cycle analysis, a separate one-way ANOVA was conducted for each phase of the cell cycle (G_1, S, and G_2) and for the MTT assay, a one-way ANOVA was conducted on the MTT reduction score at 9 hours.

Results

Mutant sensitivity to volatile compounds

We previously identified four volatile compounds that were inhibitory to *P. destructans*: 1-pentanol, propionic acid, 2-methyl-1-butanol, and isobutyl alcohol (Micalizzi *et al.*, 2017). Due to the high concentration of isobutyl alcohol required to inhibit yeast, we instead examined the mode of action of nonanal, which also inhibits *P. destructans* (Cornelison *et al*., 2014a). To examine the antifungal mode of action of these volatiles, we exposed a yeast single-gene deletion array to sub-inhibitory concentrations of each compound. We generated a set of sensitive

mutants for each compound and then compiled the sets to screen all sensitive mutants against all compounds. The sensitivity of each mutant to each compound was classified based on drop-out assays, as shown in Figure 2.2, and the sensitivity classification of each mutant to each compound is shown in Figure 2.3.

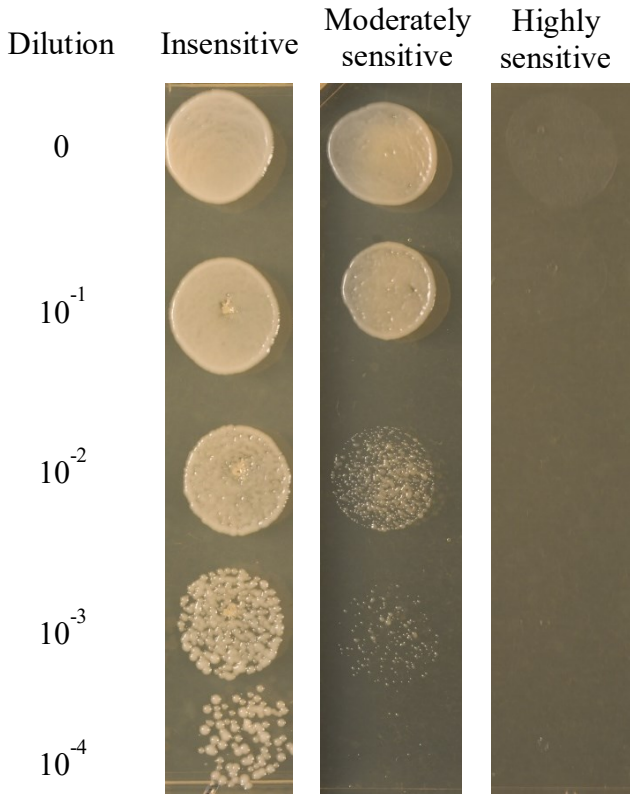


Figure 2.2. Images showing the classifications of sensitivity in yeast mutants exposed to volatile compounds. Yeast were grown to stationary phase and then serially diluted between 0 and 10^4-fold before exposure to the volatile compounds. Mutants that were sensitive between 10^{-2} and 10^{-3} × stationary phase cell density were classified as moderately sensitive and mutants sensitive at $\geq 10^{-1}$ × stationary phase cell density were classified as highly sensitive.


Gene deleted
2-methyl-1-butanol
1-pentanol
Nonanal
Propionic acid
ADH5
ADI1
ARO10
ARO9
ATG16
BAP2
BCH2
BIO3
BNA7
CHS5
COQ6
CPR5
CRP1
CSM1
CTF3
CYT1
DOT1
ECM1
EDC3
ELF1
ERC1
ERG2
ERG24
ERG3
ERG6
FSF1
GCR2
GET2
GRR1
GSF2
HIS6
HTZ1
INO4
ITR1
MCH2
MDM10
MMR1
MNN10
MRX9
MTC2
NCS6
NUP133
PAT1
PDC1
PDC5

Figure 2.3. Sensitivity of single gene mutants of *S. cerevisiae* to four volatile compounds. Mutant strains were grown to stationary phase over two days and serially diluted in YPD before plating and exposing to sub-inhibitory concentrations of volatiles. White shading indicates insensitive mutants (not sensitive or sensitive at $< 10^{-3}$ × stationary phase cell density), light grey shading indicates moderately sensitive mutants (sensitive between 10^{-1} and 10^{-3} × stationary phase cell density), and dark grey shading indicates highly sensitive mutants (sensitive at $\geq 10^{-1}$ × stationary phase cell density).

Generally, there was a high degree of overlap between yeast mutant sensitivities to each compound. Of the 82 mutants tested, 42 were at least moderately sensitive to three or more of the compounds. The groups of mutants that were sensitive to 2-methyl-1-butanol and 1-pentanol were very similar. Every mutant that was sensitive to 1-pentanol was also sensitive to 2-methyl-1-butanol; however, 12 mutants were sensitive to 2-methyl-1-butanol but not 1-pentanol. When comparing the highly sensitive mutants for both compounds, 31 out of 44 were highly sensitive to both compounds, with 10 highly sensitive only to 2-methyl-1-butanol and three highly sensitive only to 1-pentanol.

Nearly all the mutants that were sensitive to nonanal were also sensitive to the fusel alcohols 1-pentanol and 2-methyl-1-butanol; however, the fusel alcohols appeared to have additional mechanisms of action not shared with nonanal. Thirty three of 41 mutants highly sensitive to 2-methyl-1-butanol and 25 of 34 mutants highly sensitive to 1-pentanol were not highly sensitive to nonanal. Additionally, out of the 14 mutants highly sensitive to propionic acid, only four were sensitive to nonanal, while five were not highly sensitive to any other compounds.

To understand the pathways that these compounds act in, we conducted gene ontology searches of the deleted genes from highly sensitive mutants using DAVID functional analysis and Funspec. The enriched pathways for each compound are shown in Figure 2.4.

	2-methyl-1-butanol	1-pentanol	Nonanal	Propionic acid
Biological Processes				
Aromatic amino acid family biosynthetic process				
Biosynthetic process				
Carbohydrate metabolic process				
Cellular amino acid biosynthetic process				
Chromatin remodeling				
Ergosterol biosynthetic process				
Golgi to plasma membrane transport				
Lipid biosynthetic process				
Lipid metabolic process				
Metabolic process				
Mitochondrial inheritance				
Regulation of transcription (DNA-templated)				
Steroid biosynthetic process				
Steroid metabolic process				
Sterol biosynthetic process				
Transport				
Tryptophan biosynthesis				
Tryptophan metabolic process				
Vesicle fusion				
Molecular Function				
Anthranilate synthase activity				
Catalytic activity				
Lyase activity				
RNA polymerase II core promoter proximal region sequence-specific DNA binding				
SNAP receptor activity				

	2-methyl-1-butanol	1-pentanol	Nonanal	Propionic acid
Cellular Compartment				
Anthranilate synthase complex				
Cytosol				
Endoplasmic reticulum				
Exomer complex				
Integral component of membrane				
Membrane				
Mitochondrial outer membrane				
Nuclear chromatin				
KEGG Pathways				
Biosynthesis of amino acids				
Biosynthesis of antibiotics				
Biosynthesis of secondary metabolites				
Metabolic pathways				
Phenylalanine, tyrosine, and tryptophan biosynthesis				
Steroid biosynthesis				

Figure 2.4. Gene ontology terms and pathways affected by exposure to volatile compounds. Boxes show terms that were significantly ($p < 0.05$, dark blue) and moderately significantly ($0.10 \geq p \geq 0.05$, light blue) enriched for highly sensitive mutants in DAVID functional analysis or Funspec gene ontology searches. For each compound, all enriched terms are shown that were associated with more than one gene and had a p-value ≤ 0.10. White cells indicate that the pathway was not enriched for the specific compound.

Tryptophan and aromatic amino acid synthesis and metabolism was significantly enriched for all compounds. Similarly, anthranilate synthase, which catalyses an early step in tryptophan biosynthesis, was enriched for all compounds except nonanal. Steroid, sterol, and lipid biosynthesis was also significantly enriched for all compounds except for nonanal.

The most overlap in function was between 2-methyl-1-butanol and 1-pentanol, which were both enriched in terms that may be associated with vesicular trafficking (Golgi to plasma membrane transport biological process, exosome complex and membrane cellular compartments, and SNAP receptor activity molecular function [involved in vesicle fusion]). The mitochondrial outer membrane cellular compartment was also significantly enriched for 2-methyl-1-butanol and 1-pentanol. The functions of the two compounds did not completely overlap; however, and 2-methyl-1-butanol was uniquely enriched in terms that could relate to transcriptional activation (nuclear chromatin, chromatin remodeling, and RNA polymerase II core promoter proximal region sequence-specific DNA binding), while 1-pentanol was uniquely enriched in terms related to transport (transport, vesicle fusion, and integral component of plasma membrane cell compartment).

The processes enriched for propionic acid-sensitive mutants overlapped heavily with those of 1-pentanol and 2-methyl-1-butanol. The only enriched terms not shared with both 2-methyl-1-butanol and amyl-alcohol were lipid metabolic process, which was unique, and endoplasmic reticulum cellular compartment, which was also enriched for 2-methyl-1-butanol but not 1-pentanol. Nonanal had the fewest terms enriched and was uniquely enriched in the cytosol cellular compartment, catalytic activity molecular function, and carbohydrate metabolic activity biological process.

Follow-up to gene ontology searches

Because of the enrichment of the ontology term “mitochondrial outer membrane” in cells treated with 2-methyl-1-butanol and 1-pentanol, as well as literature suggesting that 2-methyl-1-butanol and propionic acid can cause oxidative stress (Rezende *et al*., 2015; Yun and Lee, 2016),

we assessed if any of the compounds caused an increase in oxidative stress using 2',7'-dichlorofluorescein diacetate (DCFDA). DCFDA is a non-fluorescent and cell permeable dye that is oxidized to the fluorescent product 2'7'-dichlorofluorescein (DCF) in the presence of reactive oxygen species and generally retained in the cell. Fluorescence in the positive control (hydrogen peroxide) was over three-fold higher than any of the four volatile compounds tested and significantly different from the negative control (mean difference (MD) = 3.59, $p = 0.032$). In contrast, the fluorescence of cells treated with 2-methyl-1-butanol, 1-pentanol, propionic acid, or nonanal were not significantly different from the negative control (all p-values > 0.99; Figure 2.5).

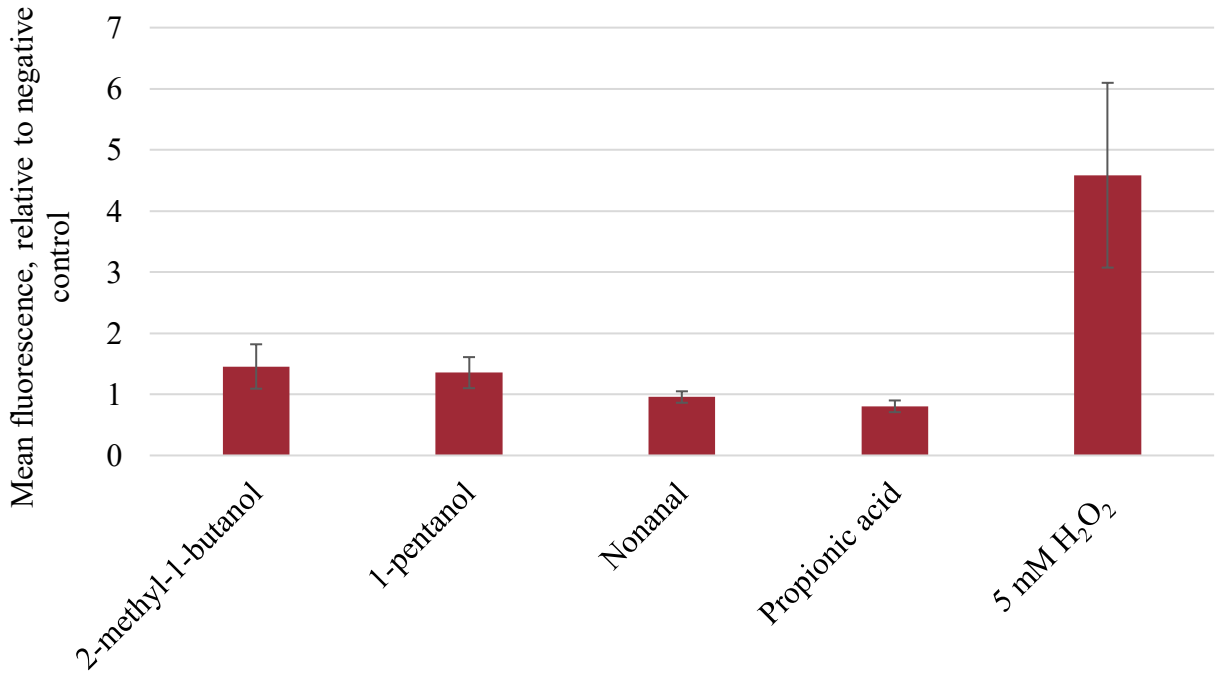


Figure 2.5. Levels of oxidative stress in yeast exposed to four volatile compounds. Graph shows mean fluorescence of live (i.e. not stained by propidium iodide) yeast cells in the presence of 2'7'-dichlorofluorescein diacetate after treatment with sub-inhibitory concentrations of 2-methyl-1-butanol, 1-pentanol, nonanal, or propionic acid. Fluorescence is expressed relative to a no-treatment control and 5 mM hydrogen peroxide was used as a positive control. Numbers are averages of three independent experiments, each with three replicates per experiment and 10,000 cells counted per replicate. Error bars represent standard error (n = 3).

Because of the enrichment of gene ontology terms associated with vesicle trafficking and the plasma membrane, we assessed if any of the compounds affected endocytosis by monitoring the uptake of lucifer yellow, a hydrophilic fluorescent dye that enters cells through endocytosis. A one-way ANOVA revealed a significant effect of compound on lucifer yellow internalization ($F(5,12) = 85.03$, $p < 0.001$). A post-hoc Tukey's HSD test showed that fluorescence in cells treated with propionic acid was significantly different than in the control (MD = 4.54; $p < 0.001$), but there were no significant differences between the control and any other condition (all p-values > 0.8; Figure 2.6, Figure 2.7).

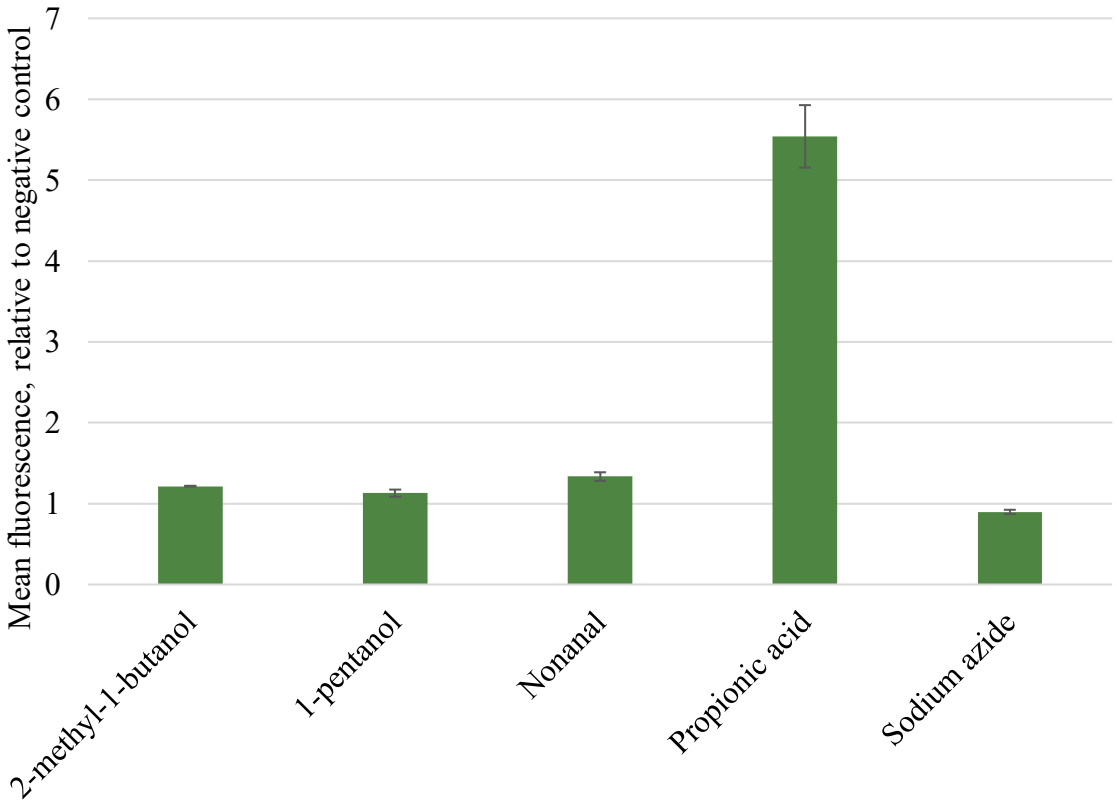


Figure 2.6. Lucifer yellow uptake in yeast cells exposed to compounds. Log-phase yeast cells were incubated for 30 minutes with a sub-inhibitory concentration of 2-methyl-1-butanol, 1-pentanol, nonanal, or propionic acid before lucifer yellow was added and cells were incubated an additional 3 hours. Sodium azide interferes with endocytosis and was used as a positive control. Lucifer yellow uptake was quantified by flow cytometry. Numbers are averages of three independent experiments, each with three replicates per experiment and 10,000 cells analysed per replicate. Error bars show standard error (n = 3).

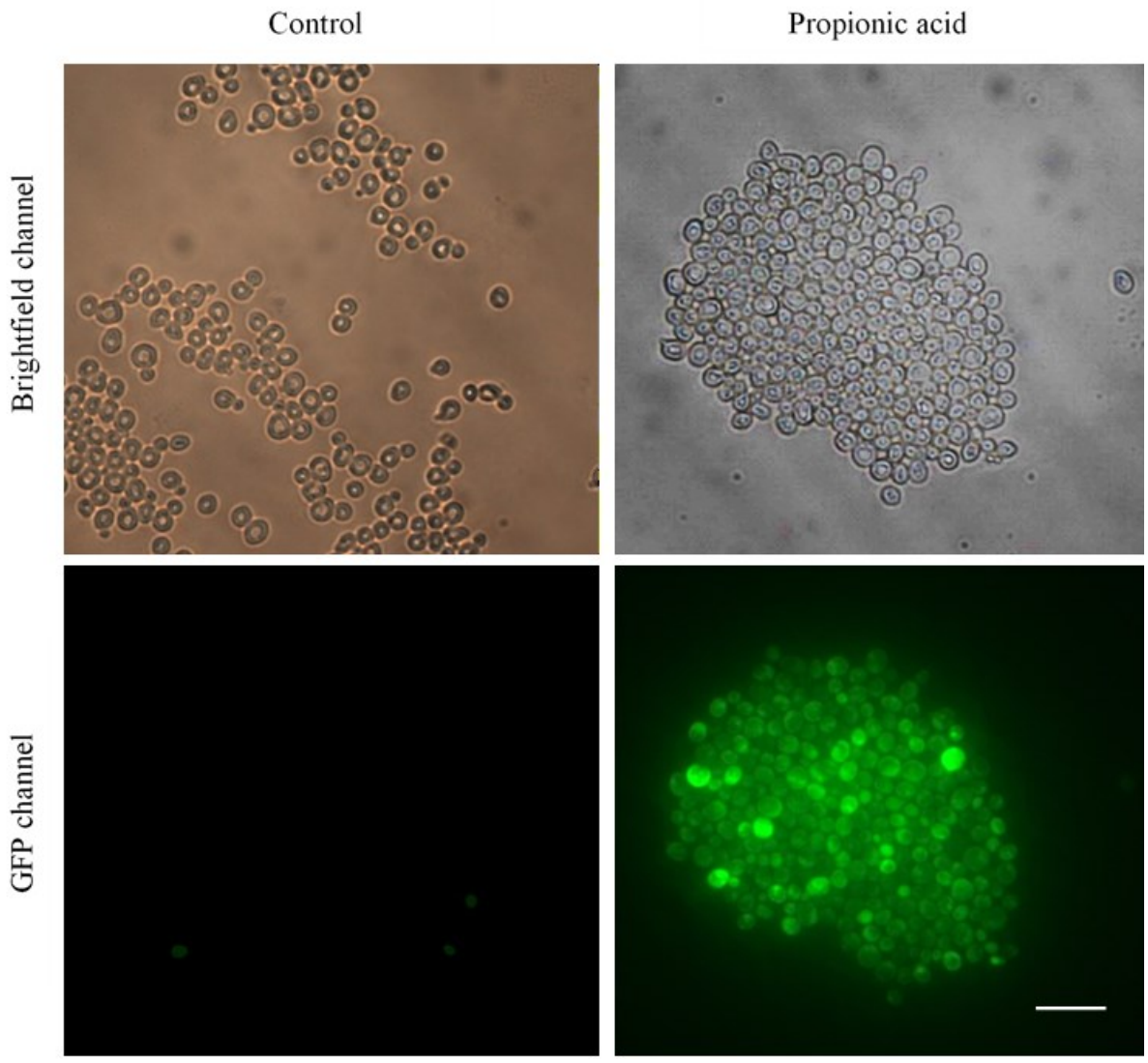


Figure 2.7. Micrographs showing lucifer yellow uptake in propionic acid-treated cells. Images show brightfield (top) and GFP (bottom) channels. Images contrast the uptake of lucifer yellow in clusters of yeast cells in a no-treatment (negative) control (left) compared to treatment with a sub-inhibitory concentration of propionic acid (right). White scale bar represents 25 µm.

Due to the enrichment of gene ontology terms related to chromatin and a high frequency of terms related to meiosis, mitosis, and cell cycle, we assessed if any of the compounds affect cell cycle progression by staining and measuring DNA content with propidium iodide and then calculating the percentage of cells in G_1, S, and G_2 phases. As shown in Figure 2.8, there was a significant effect of compound treatment on the distribution of G_1 ($F(4,10) = 21.25$; $p < 0.001$)

and G_2 ($F(4,10) = 13.83$; $p < 0.001$), but not S ($F(4,10) = 0.97$; $p = 0.47$) phase populations. A post hoc Tukey's HSD test revealed that, compared to the control, the percentage of cells in G_1 was significantly higher in yeast treated with 2-methyl-1-butanol (MD = 23.56; $p = 0.001$), 1-pentanol (MD = 26.64, $p < 0.001$), and propionic acid (MD = 25.98, $p < 0.001$). The percentage of cells in G_2 was significantly lower in yeast treated with 2-methyl-1-butanol (MD = -19.78; $p = 0.004$), 1-pentanol (MD = -22.21, $p = 0.002$), and propionic acid (MD = -25.98, $p = 0.008$). There were no significant differences in the percentage of cells in S-phase ($p \geq 0.50$ for all pairwise comparisons).

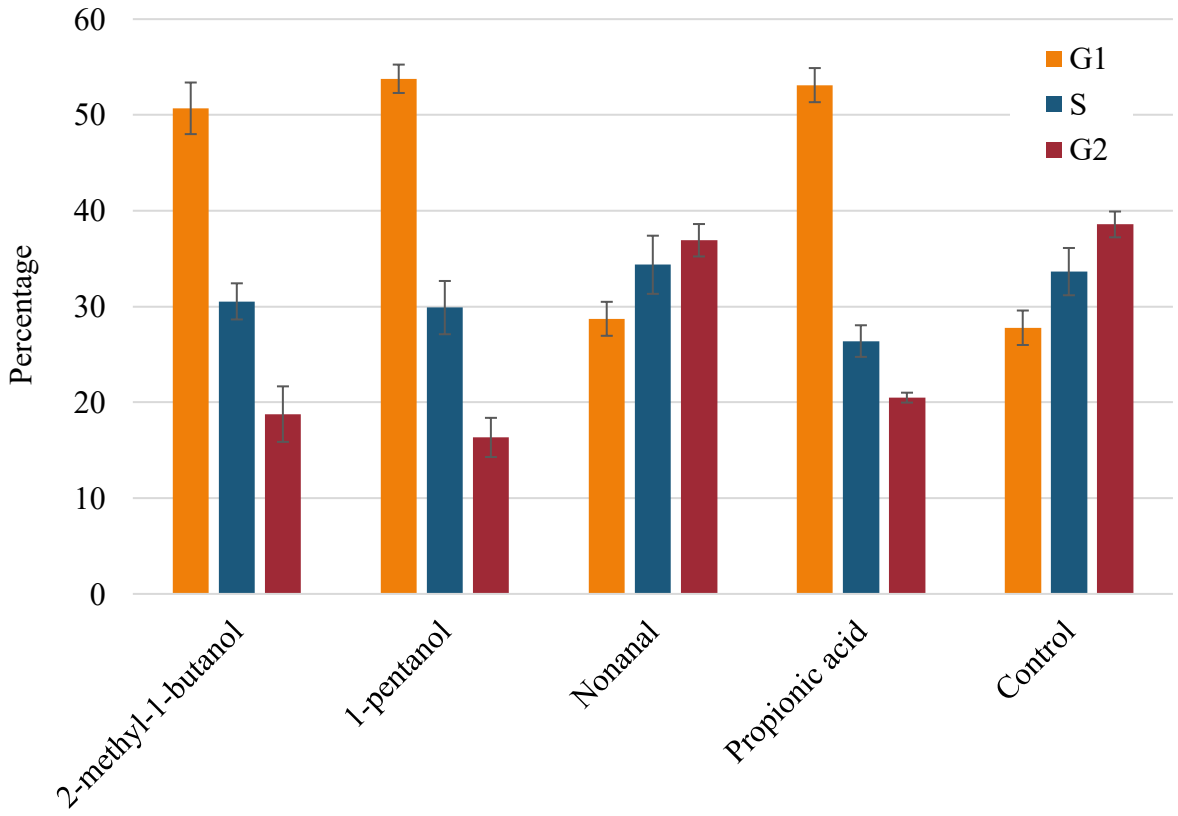


Figure 2.8. Cell cycle distributions in yeast treated with four volatile compounds. Cells were exposed to sub-inhibitory concentrations of compounds for 3 hours and then fixed before treating with RNase and staining with propidium iodide to assess DNA content. Populations were analysed using flow cytometry and the percentage of cells in each cell cycle phase was calculated in ModFit. The bar graph shows the average percentage of cells in each phase from three independent experiments, each with three replicates and 10,000 cells analysed per replicate. Error bars show standard error.

Because of the enrichment in gene ontology terms associated with membranes (membrane cellular compartment and ergosterol, lipid, steroid, and sterol biosynthesis/metabolism) and the previous indications from the literature that all four tested compounds permeabilize cell membranes, we assessed if treatment with any of the compounds increased membrane permeability by using the exclusion of propidium iodide as a marker for intact membranes. After 3 hours of treatment with volatile compounds, there was no significant effect of compound on propidium iodide fluorescence ($F(4,10) = 0.227$, $p = 0.917$) and no significant pairwise effect of any compound treatment on fluorescence ($p > 0.9$ for all comparisons; Figure 2.9). The average intracellular propidium iodide fluorescence was within 3% of the negative control uptake for all compounds tested and the greatest deviation from the control in any replicate was approximately 5%. To determine if yeast cells adapt and reverse membrane permeability over the 3-hour incubation period (Davey and Hexley, 2011), permeability was assessed at different time points and 20% ethanol was used as a positive control (Figure 2.10). Permeability was relatively consistent throughout the incubation time and all conditions caused similar levels of permeability except for the 20% ethanol positive control, which caused permeability in approximately 10% more cells than the other conditions. Taken together, this suggests that at the concentrations used, these compounds cause negligible or very transient changes in *S. cerevisiae* membrane permeability.

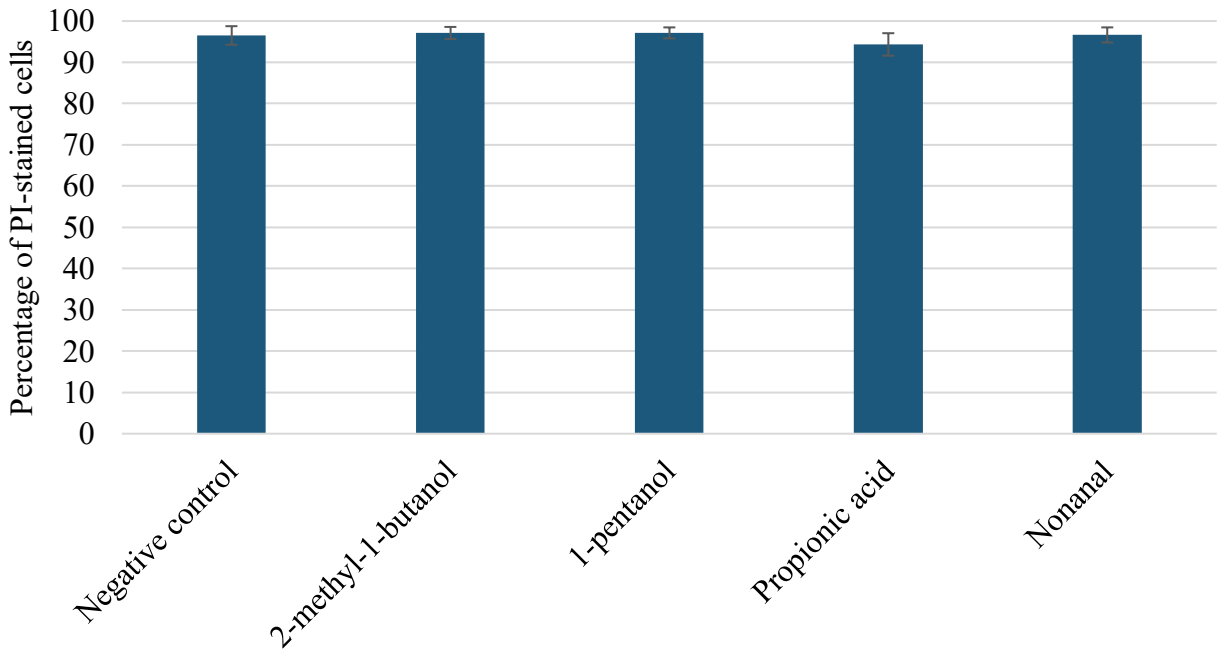


Figure 2.9. Test for yeast membrane permeabilization by compounds. Permeability was assessed by intracellular propidium iodide fluorescence. Yeast cells were exposed to a sub-inhibitory dose of each compound and incubated for 3 hours before the addition of propidium iodide. Flow cytometry was used to determine the percentage of the population that was propidium-iodide positive. Error bars show standard error from three replicates.

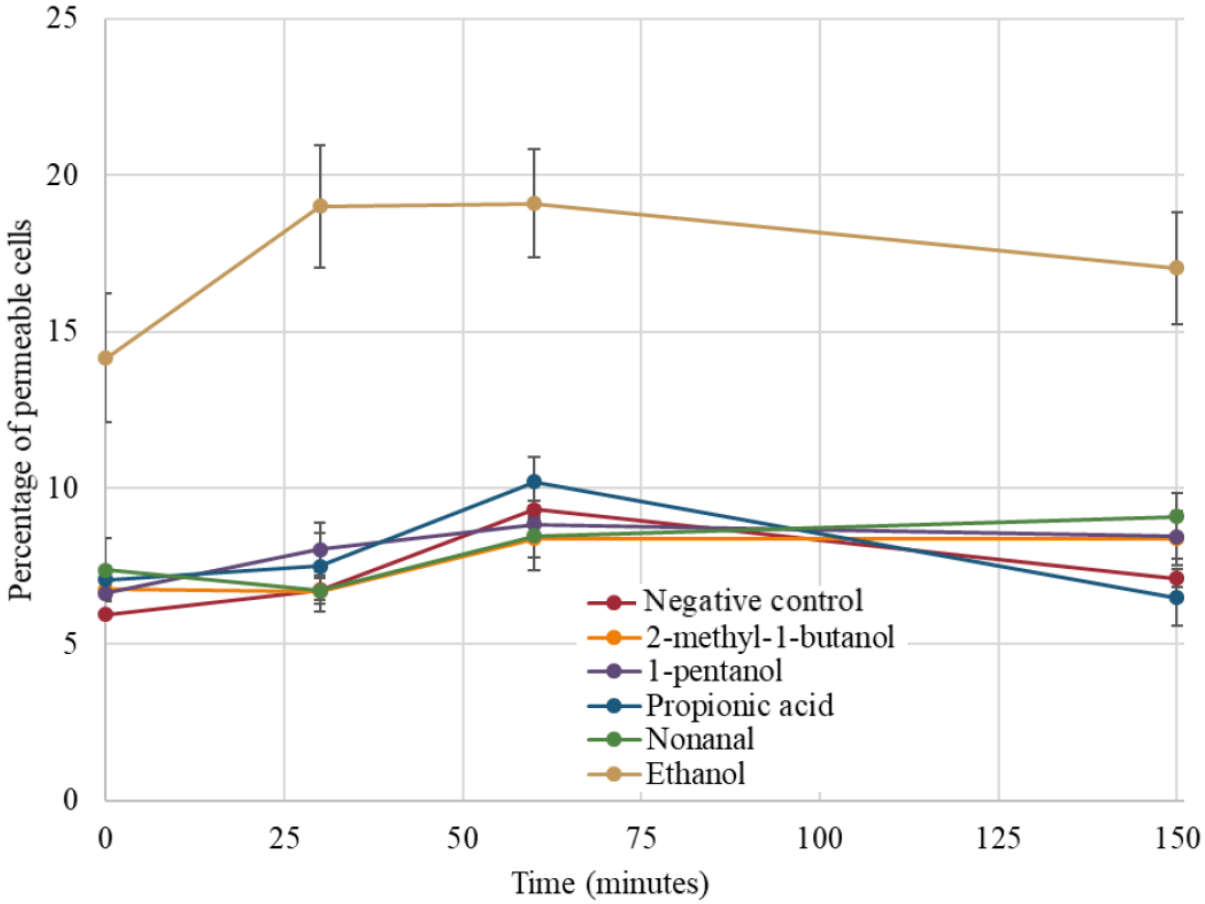


Figure 2.10. Test for yeast membrane permeabilization by compounds over time. Permeability was assessed by intracellular propidium iodide fluorescence. Yeast cells were exposed to a sub-inhibitory dose of each compound and incubated from 0 to 150 minutes before the addition of propidium iodide. After addition of propidium iodide, cells were incubated for an additional 5 minutes before using flow cytometry to determine the percentage of the population that was propidium-iodide positive. Ethanol (20%) was used as a positive control, and a no-treatment negative control was included. Error bars show standard error from three replicates.

Because of the enrichment of gene ontology terms related to metabolism and metabolic pathways, we assessed if any compounds affected cellular respiration by measuring the reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Stowe *et al.*, 1995; Sánchez and Königsberg, 2006). MTT is reduced to a purple formazan salt by NADH-dependent reactions (Riss *et al.*, 2016). A one-way ANOVA revealed that there was a significant effect of compound treatment on the reduction of MTT at 9 hours ($F(4,10) = 4.451$, $p = 0.025$). A post-

hoc Tukey HSD test showed that compared to the control, the endpoint reduction of MTT was significantly lower in cells treated with propionic acid (MD = -0.95; $p = 0.03$) but there were no significant differences in cells treated with 2-methyl-1-butanol (MD = -0.32, $p = 0.73$), 1-pentanol (MD = -0.62, $p = 0.20$), or nonanal (MD = -0.10, $p = 0.99$).

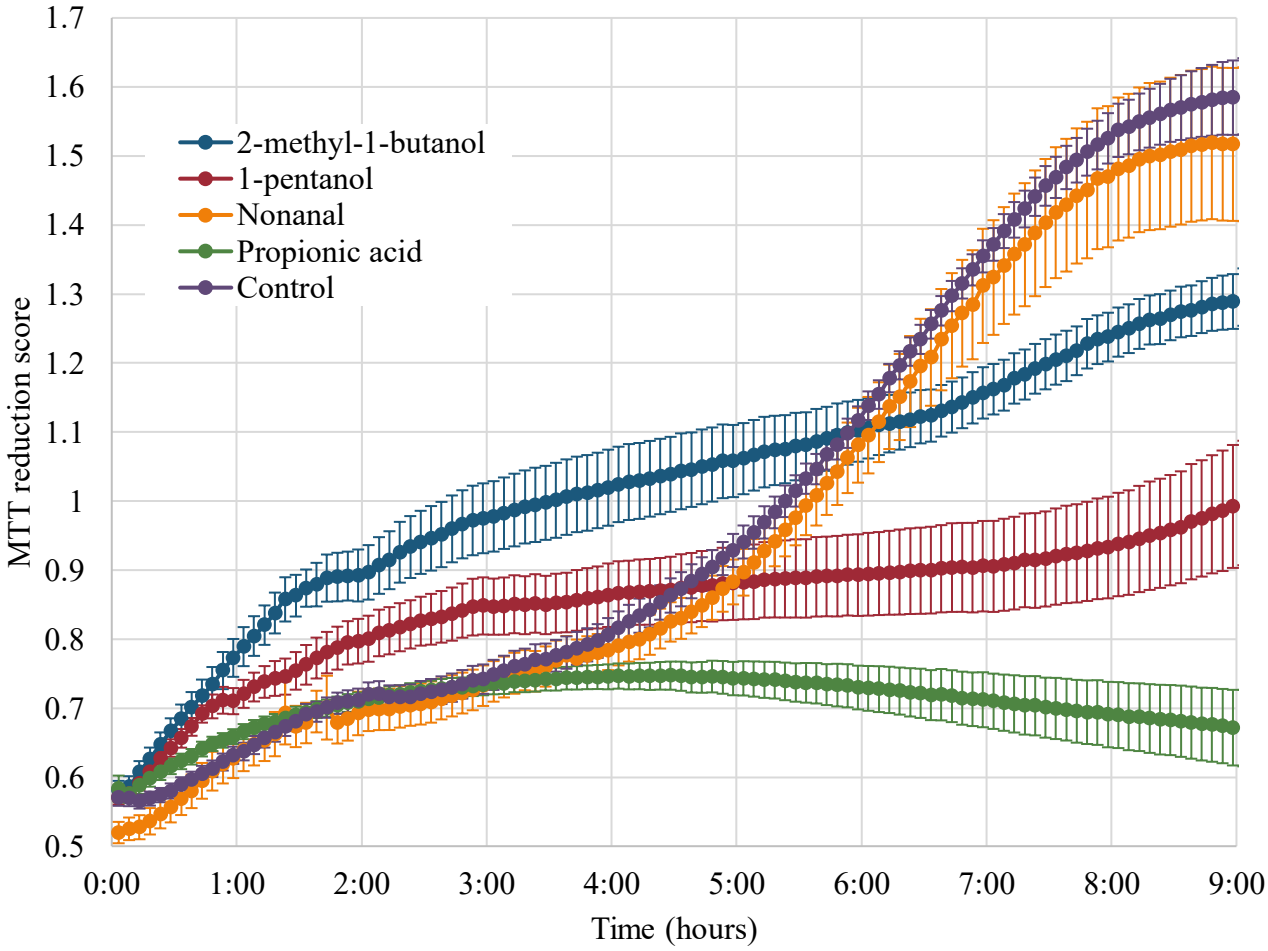


Figure 2.11. Effect of compounds on yeast reduction of MTT. Yeast were exposed to sub-inhibitory concentrations of nonanal, 1-pentanol, 2-methyl-1-butanol, and propionic acid, and the reduction of MTT was monitored spectrophotometrically for 9 hours. An MTT reduction score for yeast in the presence of each compound was calculated as $\frac{A_{570}\ exp. - A_{570}\ cont.}{A_{660}\ exp.}$ where 'A_{570} exp.' and 'A_{660} exp.' are the absorbances of the cell suspension, and 'A_{570} cont.' is the absorbance of a cell-free blank. Numbers are average scores from 3 independent experiments, each with 5 replicates. Error bars show standard error (n = 3).

Interestingly, the reduction of MTT by yeast exposed to 2-methyl-1-butanol, and to a lesser extent 1-pentanol, initially occurred at a greater rate than in the control, before decreasing to less than the control after approximately 2 hours (Figure 2.11). When strictly looking at the growth (A_{660}) of cells exposed to each compound, growth was increasingly delayed in 2-methyl-1-butanol, 1-pentanol, and propionic acid, compared to the control (data not shown), which correlates with the MTT reduction score for each compound. Additionally, the reduction of MTT in the no-cell control with propionic acid was greatly reduced compared to all other no-cell conditions (data not shown), suggesting that propionic acid interferes with the non-enzymatic background reduction of MTT.

Because of enriched gene ontology terms associated with transcriptional regulation, RNA polymerase II, and chromatin, we assessed if exposure to the compounds affects yeast gene expression. We photobleached three yeast cell lines stably expressing GFP and measured the recovery of fluorescence in the presence of the compounds. As shown in Figure 2.12, the expression of GFP varied between the three cells; however compared to a no-treatment control, GFP fluorescence was consistently lower in cells treated with propionic acid, suggesting that propionic acid may reduce global gene expression. Interestingly, propionic acid caused the most dramatic decrease in expression of *PIN4*, which promotes G_2 to M cell cycle progression and is negatively regulated during DNA damage checkpoints (Pike *et al*., 2004).

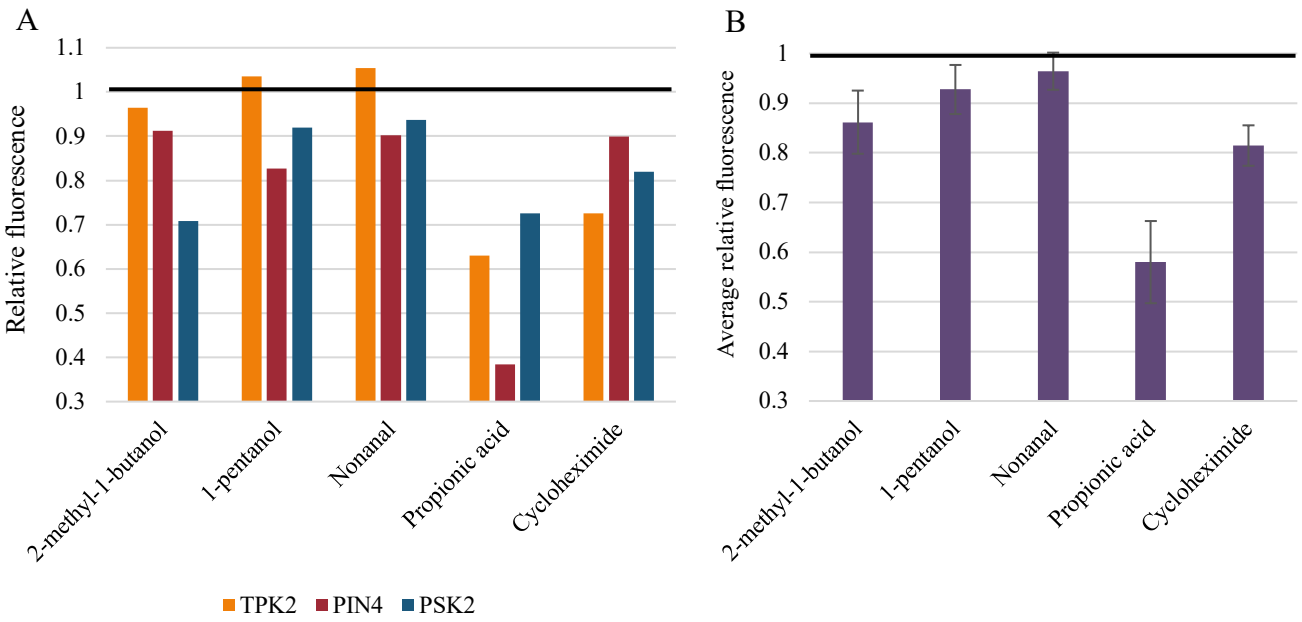


Figure 2.12. Effects of compounds on expression of GFP-tagged proteins. Three cell lines of yeast that express GFP as gene fusion constructs (*TPK2-GFP*, *PIN4-GFP*, and *PSK2-GFP*) were each separately exposed to sub-inhibitory concentrations of each volatile compound and then photobleached. Fluorescence recovery after 2 hours was measured using a flow cytometer and is expressed relative to the fluorescence in a no-treatment control (solid black line). As a positive control, 35 µg ml^{-1} cycloheximide was used. For each condition, Figure A shows the fluorescence values for each of the cells lines individually (n=1 biological replicate with 3 experimental replicates). Figure B shows the average fluorescence values across the three cell lines, with error bars showing standard error.

Discussion

In this chapter we used a yeast model to conduct a preliminary assessment of the antifungal mechanism of action of four compounds that inhibit the bat pathogen *Pseudogymnoascus destructans*. A yeast single-gene deletion array was separately exposed to sub-inhibitory concentrations of each volatile compound and sensitive mutants were identified. Gene ontology searches were performed to identify candidate pathways and the involvement of these pathways in compound response was validated with secondary assays. Based on results of

the deletion arrays, we examined the effect of the compounds on oxidative stress, endocytosis, cell cycle progression, membrane permeability, cellular respiration, and gene expression.

The sensitive mutants, enriched gene ontology pathways, and phenotypes resulting from exposure to 2-methyl-1-butanol and 1-pentanol were nearly identical and sometimes overlapped with those resulting from propionic acid exposure, while nonanal appeared to have a unique profile, suggesting a distinct mechanism of action. The structure and biosynthetic origins of these compounds reflects this pattern in activity. 2-methyl-1-butanol and 1-pentanol are both relatively short-chain fusel alcohols, whereas propionic acid is a relatively short-chain carboxylic acid. In contrast, nonanal is a long straight-chain aldehyde. In yeast, 1-pentanol and 2-methyl-1-butanol are derived from amino acid catabolism (Hazelwood *et al*., 2008; Dickinson, 2008), while propionyl-CoA, an intermediate in propionic acid metabolism, is also produced in isoleucine catabolism (Pronk *et al*., 1994). Additionally, some bacteria produce propionic acid from the catabolism of amino acids (Ganesan *et al*., 2004a; Elsden and Hilton, 1978; Ganesan *et al*., 2004b). In contrast, nonanal is formed from lipid peroxidation, which can occur during oxidative stress (O'Brien *et al*., 2005; Tagnon and Simeon, 2017). Amino acids are generally catabolized during nitrogen starvation (Ashe *et al*., 2001) and as end-products or intermediates in amino acid catabolism, 1-pentanol, 2-methyl-1-butanol, and propionic acid could act in part by signalling nitrogen starvation to the cell. While signalling roles for propionic acid are not established, 2-methyl-1-butanol and 1-pentanol have signalling roles in inducing yeast filamentous growth, which is typically a response to nitrogen starvation (Hazelwood *et al*., 2008; Dickinson, 2008).

We did not observe any treatment-mediated changes in the fluorescence of cells treated with 2′,7′-dichlorofluorescein diacetate, which is converted to a fluorescent product during oxidative stress. Previous research has shown conflicting results on whether propionic acid

causes oxidative stress (Semchyshyn *et al*., 2011; Piper, 1999; Yun and Lee, 2016); however, our findings suggest that under the conditions we explored, propionic acid does not induce oxidative stress in yeast. Similarly, nonanal is formed from lipid peroxidation during oxidative stress (O'Brien *et al*., 2005; Tagnon and Simeon, 2017), and our findings are consistent with nonanal functioning in pathways downstream of (and not causing) oxidative stress. However, our findings conflict with previous work with 2-methyl-1-butanol that reported a low-concentration of 2-methyl-1-butanol induced oxidative stress, as measured by lipid peroxidation, in *Colletotrichum* spp. (filamentous fungi; Rezende *et al*., 2015). It is possible that the delivery, dose, taxonomy, and/or method account for the differences in our observations.

We observed a striking increase in the uptake of lucifer yellow, a marker of fluid-phase endocytosis, in yeast cells treated with propionic acid, but not with the other three volatile compounds investigated. Yeast use endocytosis to uptake nutrients from their surroundings and to recycle or degrade membrane proteins (Feyder *et al*., 2015; Goode *et al*., 2015). Although the mechanisms governing the increases in endocytosis were not determined, weak acids such as propionic acid have been shown to damage membranes and disrupt the function of membrane proteins (Mira *et al*., 2010), activating quality control mechanisms that cause their endocytosis and degradation in the multivesicular body pathway (Babst, 2014; Li *et al*., 1999). Damaged membrane proteins must be removed to prevent the accumulation of damage to the cell (Li *et al*., 1999) and genes involved in protein catabolism through the multivesicular body pathway contribute to propionic acid resistance (Mira *et al*., 2009). This suggests that endocytosis of damaged surface proteins may be part of an adaptive response to propionic acid. In addition to ubiquitination and endocytosis of membrane proteins (Mira *et al*., 2010), a region of damaged membrane itself may be endocytosed. In animal cells, endocytosis increases dramatically after

cells are exposed to permeabilizing agents as the cells removal of regions of damaged membrane (Andrews *et al*., 2014). Although this is not well-studied in yeast, it is possible that membrane lesions are removed in a similar manner.

We observed perturbations in the cell cycle of yeast exposed to sub-inhibitory concentrations of propionic acid, 1-pentanol, and 2-methyl-1-butanol, suggestive of a G_1 or S-phase arrest. Cell cycle perturbations have been observed after exposure to the fusel alcohols isoamyl alcohol (Martinez-Anaya *et al*., 2003) and butanol (La Valle and Wittenberg, 2001); however, these activities have resulted in delayed progression from G_2 to M during fusel alcohol-induced filamentous growth. We did not observe a G2 arrest; instead, both the fusel alcohols and propionic acid caused an increase in the G_1 and decrease in the G_2 populations. It is well-established that cells can be arrested in G_1- or S-phase due to small cell size, DNA damage, or DNA replication stress (Bertoli *et al*., 2013; Gerald *et al*., 2002; Barnum and O'Connell, 2014); however, G_1 arrests have also been observed due to membrane permeabilization (Kono *et al*., 2016) and amino acid starvation (Unger and Hartwell, 1976; Saldanha *et al*., 2004). In animal cells, isoleucine and leucine deprivation prevent entry into S-phase (Everhart, 1972) and an amino acid availability checkpoint has been proposed in late G_1 (Saqcena *et al*., 2013). It is possible that in yeast, 2-methyl-1-butanol, 1-pentanol, and propionic acid could trigger a metabolic cell cycle arrest by signalling nitrogen or amino acid starvation.

Contrary to our expectations, we did not observe increases in membrane permeability (quantified by propidium iodide entry into live cells) after treatment with any of the compounds. There are previous reports that straight-chain alcohols (Weber and de Bont, 1996), weak organic acids (Ullah *et al*., 2012), and nonanal (Zhang *et al*., 2017) all increase membrane permeability, and there is considerable overlap between the downstream effects of membrane permeabilization

and the effects that we observed. Cells can repair their membranes from permeabilizers within minutes (Idone *et al*., 2008) or even seconds (McNeil *et al*., 2000), and the efficacy of this process is proportional to the concentration of the permeabilizer (Idone *et al*., 2008). It is possible that the concentrations of compounds that we used were below a threshold required for permeabilization, or that at these concentrations, the time required for propidium iodide to enter a permeabilized cell is greater than the time required for the cell to repair permeabilized membranes.

We used the reduction of MTT as a marker for metabolic activity and observed significantly less MTT reduction in yeast treated with propionic acid. Propionic acid has been shown to indirectly inhibit the pyruvate dehydrogenase complex, which links glycolysis and the citric acid cycle by converting pyruvate into acetyl-CoA (Brock and Buckel, 2004). This could cause end-product inhibition of glycolysis and prevent citric acid cycle entry, ultimately decreasing the generation of NADH necessary to reduce MTT. Endpoint MTT reduction was non-significantly lower in cells treated with the fusel alcohols 2-methyl-1-butanol and 1-pentanol; however, it is interesting to note that initially, treatment with these compounds increased the rate of MTT reduction. Fermentation is the preferred mode of metabolism in yeast; however, if fusel alcohols signal nitrogen starvation, then the large energy demands of a nitrogen starvation response (Thomsson *et al*., 2005) could cause upregulation of genes associated with energy generation and oxidative respiration (Mendes-Ferreira *et al*., 2007). This is supported by the observation that treatment with isoamyl alcohol (a different fusel alcohol) causes an increase in the activity of succinate dehydrogenase (Kern *et al*., 2004), an enzyme involved in oxidative metabolism that appears to be important for MTT reduction (Riss *et al*., 2016; Slater *et al*., 1963). An increase in oxidative metabolism could generate additional NADH and thus increase

MTT reduction. However, after 1 to 3 hours of exposure to both the fusel alcohols and to propionic acid, the rate of MTT reduction decreased, suggesting that a subsequent inhibition of oxidative metabolism may occur. Additionally, our results also show that the inhibitory effects of propionic acid on metabolism are greater than the effects of 2-methyl-1-butanol and 1-pentanol, suggesting separate modes of action.

We found that treatment with propionic acid appears to decrease gene expression, which could result from inhibition of transcription or translation. Signalling of amino acid starvation can inhibit translation by causing the phosphorylation of eIF2α (Taylor *et al*., 2010). However, it is interesting that we did not see a significant decrease in GFP expression in yeast treated with 2-methyl-1-butanol or 1-pentanol. Straight chain alcohols cause a downregulation of genes associated with translation and protein synthesis (Fujita *et al*., 2004) and fusel alcohols have been shown to block translation initiation by inhibiting eIF2B (Taylor *et al*., 2010; Smirnova *et al*., 2005). It is possible that the concentration that we used was below a threshold required for an effect on gene expression.

Based on the results that we observed in yeast treated with 1-pentanol, 2-methyl-1-butanol, and propionic acid, it would be interesting to examine if these compounds interfere with Target Of Rapamycin (TOR) signalling. TOR is a highly conserved pathway that regulates a diverse set of cellular factors to coordinate growth and development. In yeast, TOR has two paralogs which form signalling complexes: TOR complex 1 and 2 (TORC1 and TORC2; review in: Loewith and Hall, 2011). TORC1 signalling responds to nutritional and stressor cues in the environment to regulate growth, including translation, ribosome biogenesis, cell cycle progression, nutrient uptake, metabolism, autophagy, and the cell wall integrity pathway. While

less is known about TORC2, it appears to function independently of nutrient cues and regulates the actin cytoskeleton, endocytosis, and sphingolipid metabolism.

The TORC1 pathway responds to nitrogen and amino acid deficiencies, and inhibition of TOR signalling creates a phenotype similar to that of nitrogen starvation (Loewith and Hall, 2011; Loewith 2011). Under natural conditions, amino acid starvation induces inhibition of TORC1, which leads to increased protein turnover via increased endocytosis and processing through the multivesicular body pathway (Jones *et al*., 2012). In response to low nutrient availability, inhibition of TORC1 can lead to phosphorylation of eIF2-α and subsequent blocking of translation. This in turn can result in a G_1 arrest due to decreased translation of the cyclin Cln3, which is required for cells to commit to DNA synthesis (Barbet *et al*., 1996; Loewith and Hall, 2011; Barnum and O'Connell, 2014). Additionally, in human cells, an amino acid availability checkpoint mediated by mammalian TOR has been proposed in late G_1 (Saqcena *et al*., 2013). Inhibition of TORC1 signalling can also change the phosphorylation state of several key enzymes in glycolysis and nitrogen metabolism (Loewith, 2011). Further, inhibition of TORC1 signalling causes an upregulation of glycolysis and fermentation genes and a down-regulation of mitochondrial genes (Ring *et al*., 2012). Inhibition of these TORC1-associated functions is reminiscent of our observations in cells treated with the fusel alcohols and propionic acid. If 2-methyl-1-butanol, 1-pentanol, and propionic acid cause inhibition of TORC1 signalling, this could account for the phenotypes of increased endocytosis, cell cycle arrest, decreased gene expression, and perturbed metabolism that we observed with these compounds. However, the differences in activity between the compounds, such as the increase in endocytosis with propionic acid but not 1-pentanol or 2-methyl-1-butanol, remains to be explained. Nevertheless, it is striking how many different modes of activity we observed for propionic acid,

suggesting the possibility that propionic acid may target a central regulator of cellular homeostasis.

Importantly, as this was a preliminary investigation, there are likely other pathways involved in the response to these compounds that we did not investigate. For example, an ontology search with the deleted genes from sensitive mutants showed that terms related to tryptophan and other aromatic amino acid metabolism were significantly enriched for all compounds. In yeast, tryptophan uptake is often inhibited and can become a limiting factor for growth under a variety of adverse conditions (Abe and Iida, 2003), and interestingly, inhibition of TOR signalling can cause increased turnover and degradation of tryptophan permeases (Schmidt *et al*., 1998). Additionally, ontology terms related to ergosterol and sterol biosynthesis were significantly enriched in yeast treated with 2-methyl-1-butanol, 1-pentanol, and propionic acid. Ergosterol biosynthesis rigidifies the plasma membrane and can be part of the response to increased membrane fluidity due to exposure to straight-chain alcohols (Weber and de Bont, 1996). It is possible that these and other pathways are involved in responses to treatment with these compounds; however, it is worth noting that the ontology terms were not strong predictors for performance in secondary assays. For example, the ontology terms relating to regulation of RNA polymerase II, nuclear chromatin, and chromatin remodelling were enriched exclusively for 2-methyl-1-butanol, and regulation of transcription was enriched for both 2-methyl-1-butanol and 1-pentanol; however, the only effects on gene expression that we observed with our secondary assay (based on photobleaching of GFP-tagged proteins) were for cells treated with propionic acid. It is possible that this discrepancy arises from treating the deletion array screens with volatile compounds and the secondary assays with liquid compounds or from misinterpreting the pathways that ontology terms represent. In particular, we were unable to

observe any effects of nonanal in our secondary assays. Nonanal, therefore, appears to have a mechanism of action that is distinct from 2-methyl-1-butanol, 1-pentanol, or propionic acid.

There are many outstanding questions on the mechanisms of action of the tested volatile compounds and several limitations to our research that future work should address. Importantly, our assays were conducted with yeast because of the availability of a yeast deletion array; however, these compounds may not have the same mechanisms of action in *P. destructans*. There could be variability due to taxonomic differences or due to concentration-dependent mechanisms of action, since yeast is more resistant to these compounds than *P. destructans*. Future research should address if the effects of the volatile compounds that we observed in yeast also occur in *P. destructans*.

Conclusions

In summary, we used a yeast model to examine the antifungal mechanism of action of four volatile compounds that inhibit *P. destructans*: 2-methyl-1-butanol, 1-pentanol, propionic acid, and nonanal. We found that none of the compounds appear to increase oxidative stress or membrane permeability; however, propionic acid, 2-methyl-1-butanol, and 1-pentanol cause changes to the cell cycle. Additionally, propionic acid appears to cause a dramatic increase in endocytosis and disrupt metabolism and gene expression, while nonanal appears to have a mode of action independent from the other compounds. Although future research is needed to fully elucidate the antifungal mechanisms of action of these interesting volatile compounds, we provide a preliminary assessment of how they may inhibit fungi.

Chapter 3: Testing the efficacy of microbial and volatile inhibitors of *Pseudogymnoascus destructans* in hibernaculum-like soil microcosms

Since it was first detected in New York in 2006, *Pseudogymnoascus destructans*, the causal agent of white-nose syndrome in bats, has spread across the continent and killed millions of bats (Frick *et al*., 2015; U.S. Fish and Wildlife Service, 2016). The ability of *P. destructans* to grow as a saprotroph enables it to persist in the hibernaculum environment (Lorch *et al*., 2013a; Lorch *et al*., 2013b; Lindner *et al*., 2011), and this persistence likely contributes to the infection of bats, the expansion of white-nose syndrome across the continent, and the extirpation or extinction of susceptible bat species (Hoyt *et al.*, 2015a; Frick *et al*, 2017, Reynolds *et al*., 2015).

Targeting the environmental growth of *P. destructans* could aid in managing white-nose syndrome and could occur through a biocontrol strategy, where hibernacula are inoculated with antagonist microorganisms, and/or through the use of natural products, where antifungal compounds are applied to the hibernaculum environment. We have previously identified several microbial antagonists and microbially-derived volatile compounds that show excellent inhibition of *P. destructans* in bioassays and could be considered as candidate biocontrols and natural products to control *P. destructans* in hibernacula. However, the survival and activity of a microbial biocontrol agent in the environment, as well as the nature of its activity, can be highly variable and depends on complex interactions between biotic and abiotic factors. This may include interactions with the existing microbial community, the density at which the biocontrol agent is inoculated in the environment, substrate features such as texture, moisture, pH, and nutrient sources, as well as environmental features such as oxygen availability and temperature (Alabouvette and Steinberg, 1995; Hoitink and Boehm, 1999; Raaijmakers *et al*., 2002).

Additionally, the susceptibility of fungi to antifungals can be affected by environmental factors (e.g. Buchta and Otcenásek, 1996), and the ability of volatile inhibitory compounds to move through hibernaculum sediment and interact with *P. destructans* is unknown. Thus, to understand the effectiveness of our previously identified microbial and volatile control candidates, it is important to examine their ability to reduce the *P. destructans* reservoir in a hibernaculum-like context.

Microcosm studies have been used to simulate the environment and study a wide variety of microbial interactions in soil (e.g. Providenti *et al*., 2004; Trevors *et al*., 1990; Grenni *et al*., 2012), including the environmental fate of *P. destructans* after exposure to biocontrol candidates (Zhang *et al*., 2015). DNA-based environmental monitoring is one way to assess the persistence of *P. destructans* in treated microcosms and has been widely used to determine the fate of microbes in the environment, particularly for microbes released from industrial fermentations (e.g. Providenti *et al*., 2004; Wendt-Potthoff *et al*., 1994), in bioterrorism simulations (Johnson *et al*., 2010), and released as biocontrol agents (e.g. Pujol *et al*., 2006; Oskiera *et al*., 2017). These methods often involve the extraction of total DNA from environment samples, followed by the PCR-based amplification of a sequence specific to the organism of interest (e.g. Hynes *et al*., 2006; Providenti *et al*., 2004; Oskiera *et al*., 2017).

The use of hibernaculum-like soil microcosms may provide a small-scale simulation of the hibernaculum environment, where *P. destructans*-contaminated hibernaculum sediment can be treated with a natural product or antagonistic control candidate and the abundance of *P. destructans* DNA can be monitored over time. However, soil microcosms cannot truly emulate the natural environment. Microcosms only simulate a defined set of conditions and do not account for the heterogeneity of biotic and abiotic factors between and within hibernacula (e.g.

Bilecki, 2003; Vanderwolf *et al*., 2012; Zhang *et al*., 2014; Reynolds *et al*., 2015). It is possible that control candidates that perform well in microcosms would fail to have activity under field conditions, and that candidates that do not have activity in microcosms would be effective under natural conditions. Nevertheless, microcosms can provide an informative assessment prior to undertaking field trials.

Our initial screens on agar medium in Petri dishes of microbes co-inoculated with *Pseudogymnoascus destructans* revealed several excellent biocontrol candidates that, in these bioassays, strongly inhibited *P. destructans* and appeared to act with some degree of specificity (Micalizzi *et al.*, 2017). We also identified four volatile compounds that inhibit *P. destructans* and conducted preliminary assessments of their mechanisms of action. To better assess the capacity of these antagonists to act as biocontrol and chemical control candidates in bat hibernacula, we tested their ability to inhibit *P. destructans* in hibernaculum-like soil microcosms.

Materials and methods

Preparation of frozen stocks

For accurate quantitation, frozen stocks were used to inoculate hibernaculum soil. All assays used *P. destructans* strain US-15. *P. destructans* and antagonists were separately grown in 15-ml falcon tubes in approximately 10 ml PDB (for *P. destructans* and fungal antagonists) or LB (for the bacterial antagonist). The liquid cultures of fungi were homogenized using a Waring blender and stored in approximately 15% glycerol at -80 °C before enumerating by plating a dilution series.

Microcosms

Microcosm soil

Soil was collected in early August 2016 from hibernacula located approximately 60 km north of Grand Rapids, Manitoba, and shipped to Ottawa, ON in double Ziploc bags. The bags were slightly opened after receiving and stored at 4 °C until use in microcosm experiments (ranging from one week to 15 months). The hibernacula from which soil was collected are not known to be contaminated by *P. destructans*.

Microcosms with microbial antagonists

Hibernaculum soil was homogenized by hand in a plastic bag before 25 ± 1 g (approximately 20 ml) of soil was added into a 125-ml flask that was then sealed with a double-layer of aluminum foil. Microcosms were prepared in duplicate with no *P. destructans* added (negative control), *P. destructans* added without an antagonist (positive control), and in experimental conditions where *P. destructans* was co-inoculated with *Pantoea ananatis* RFA4P2, *Penicillium* sp. S9A1R, or *Trichoderma harzianum* RW1A2P antagonists. Frozen stocks of *P. destructans* and antagonists were centrifuged at 5000 × g for 5 min and the supernatant was replaced with autoclaved distilled water. A total volume of 500 µl of *P. destructans* containing approximately 50 x 10^6 CFU and a total volume of 500 µl of antagonist containing approximately 25 x 10^6 CFU were inoculated, as evenly as possible, into the soil. An equivalent amount of autoclaved distilled water was added to the control flasks. Flasks were sealed with a double layer of foil and placed at 13 ± 1 °C for approximately 4 months. Sterile distilled water was added to saturate the soil in the flasks approximately every 40 days.

Microcosms with volatile compounds

To assess if volatile compounds could inhibit *P. destructans* in soil microcosms, hibernaculum soil was homogenized by hand in a plastic bag before 10 ± 0.5 g of soil was placed into a 125-ml flask and inoculated with 10×10^6 CFU *P. destructans* in 1 ml of water. A plastic disk was cut to cover the opening of the flask and a cotton applicator stick was bored into the plastic such that the cotton was suspended in the flask approximately 1 cm above the soil. The cotton was treated with 67.5 µl 1-pentanol, 67.5 µl 2-methyl-1-butanol, 55 µl propionic acid, or 0.875 µl nonanal and the top of the flask was covered with foil. No-inoculum and no-treatment controls were included and flasks were placed at 13 ± 1 °C for approximately one month.

Culture of volatile-treated microcosm soil

To assess viability of *P. destructans* after volatile treatment, 0.5 g of the hibernaculum soil that was treated with volatile compounds as above was inoculated in triplicated into 5 ml of PDB two weeks after treatment. Tubes were covered, but allowed for gas exchange, and placed at 13 ± 1 °C for approximately one month.

Detection of *P. destructans* DNA in soil microcosms

Hibernaculum soil or garden soil (from Ottawa, ON) were homogenized by hand in plastic bags before 5 g of each were added separately to 50 ml flasks. To inoculate flasks with *P. destructans* DNA, approximately 50 mg of 0.5 mm glass beads were added to an epitube with 2.5×10^6 CFU *P. destructans*. The tubes were shaken in a Retsch MM301 mixer mill at 30 Hz three times for 2 minutes, each separated by a 2-minute pause, and then 300 µl of ice-cold 95% ethanol was added before each tube was vortexed and left for 10 minutes. The epitubes were then centrifuged at 15,000 rpm for 3 minutes and the pellet was rinsed with 70% ethanol and resuspended in distilled water. For each soil type, microcosms were inoculated with 1400 µl of

distilled water containing 2.5×10^6 CFU live *P. destructans*, *P. destructans* DNA from 2.5×10^6 CFU, or an uninoculated negative control. Flasks were covered with a double layer of foil and placed at 13 ± 1 °C.

qPCR detection of *P. destructans* in microcosms

For all microcosms, total DNA extractions were performed with a Macherey-Nagel NucleoSpin Genomic DNA from Soil kit and quantified using Quant-iT PicoGreen dsDNA Assay Kit, according to the manufacturers' instructions. In the microcosm culture experiment, some replicates formed a floating mat of mycelia on top of the liquid culture, in which case a proportional amount of the mat and liquid fractions were sampled for DNA extraction.

To quantify the abundance of *P. destructans* in microcosm soil, qPCR was performed with primers targeting the Alpha-L Rhamnosidase gene of *P. destructans* (Chaturvedi *et al*., 2011a). qPCR reactions contained 50 ng of total soil DNA, 10 µl of 2 × KAPA SYBR FAST Bio-Rad iCycler Master Mix, and 0.4 µl each of 10 µM V1958 forward (5′-GGAAGCCGTGGAATGTGAA-3′) and 1959a reverse (5′-TGCGTTGATACTATTGTCTTTTCTGAA-3′) primers (Chaturvedi *et al*., 2011a), in a final reaction volume of 20 µl. The amplification was performed in a BioRad CFX Connect Real-Time qPCR machine with an initial denaturation at 95 °C for 3 minutes followed by 40 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. The threshold was arbitrarily set to 100 RFU and cycle at threshold was used as a quantitation measure.

Results

We co-inoculated hibernaculum soil with *P. destructans* and one of *Trichoderma harzianum* RW1A2P, *Pantoea ananatis* RFA4P2, or *Penicillium* sp. S9A1R antagonists. The abundance of *P. destructans* was monitored over 118 days and compared to controls. For all three antagonists tested, over time, the abundance of *P. destructans* did not differ from the abundance of *P. destructans* in the no-antagonist control (Figure 3.1). All soil samples that were inoculated with *P. destructans* contained a considerably larger amount of *P. destructans* than the uninoculated control, where *P. destructans* remained below the limit of detection for the duration dof the experiment.

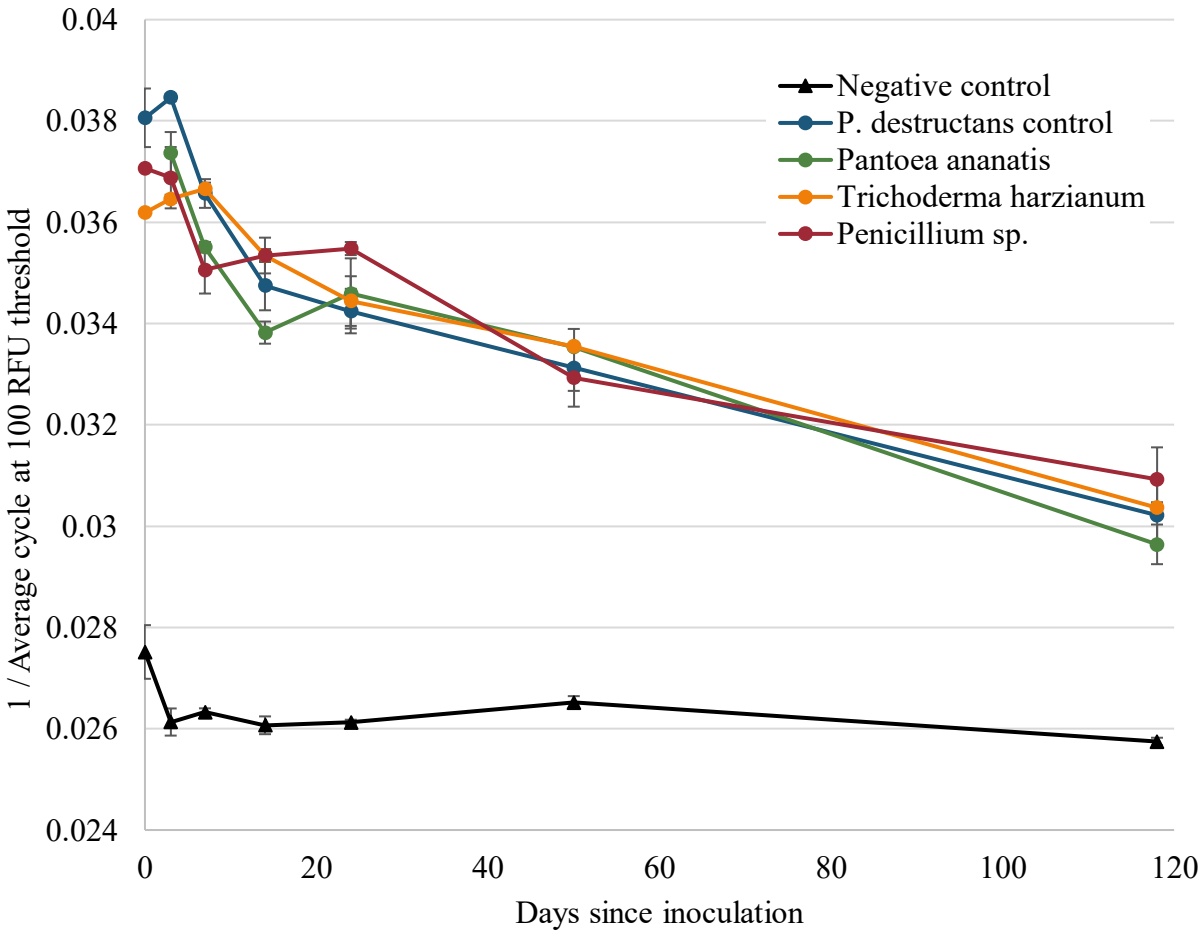


Figure 3.1. Microcosm abundance of *P. destructans* in the presence of microbial antagonists. Microcosms were uninoculated (control), inoculated with *P. destructans* and no antagonist (*P. destructans* control), or inoculated with one of three antagonists, and abundance was quantified with qPCR over a 118-day period. *P. destructans* abundance was quantified by the qPCR cycle at an arbitrary 100 RFU threshold. Conditions were performed in duplicate and error bars represent standard error. Triangle markers indicate samples that were below the limit of detection (no amplicon band visible following gel electrophoresis).

We tested four antifungal volatile compounds for the ability to inhibit *P. destructans* in hibernaculum-like soil microcosms. At the concentrations used, the volatile compounds did not reduce the abundance of *P. destructans* DNA below that of a no-volatile control, and all inoculated conditions contained a much greater abundance of *P. destructans* than the uninoculated control (Figure 3.2). Interestingly, while the abundance of *P. destructans* DNA in soil treated with nonanal was almost identical to the abundance in a no-volatile control, the abundance of *P. destructans* DNA in soil treated with 2-methyl-1-butanol, 1-pentanol, and propionic acid was greater than that in a no-volatile control.

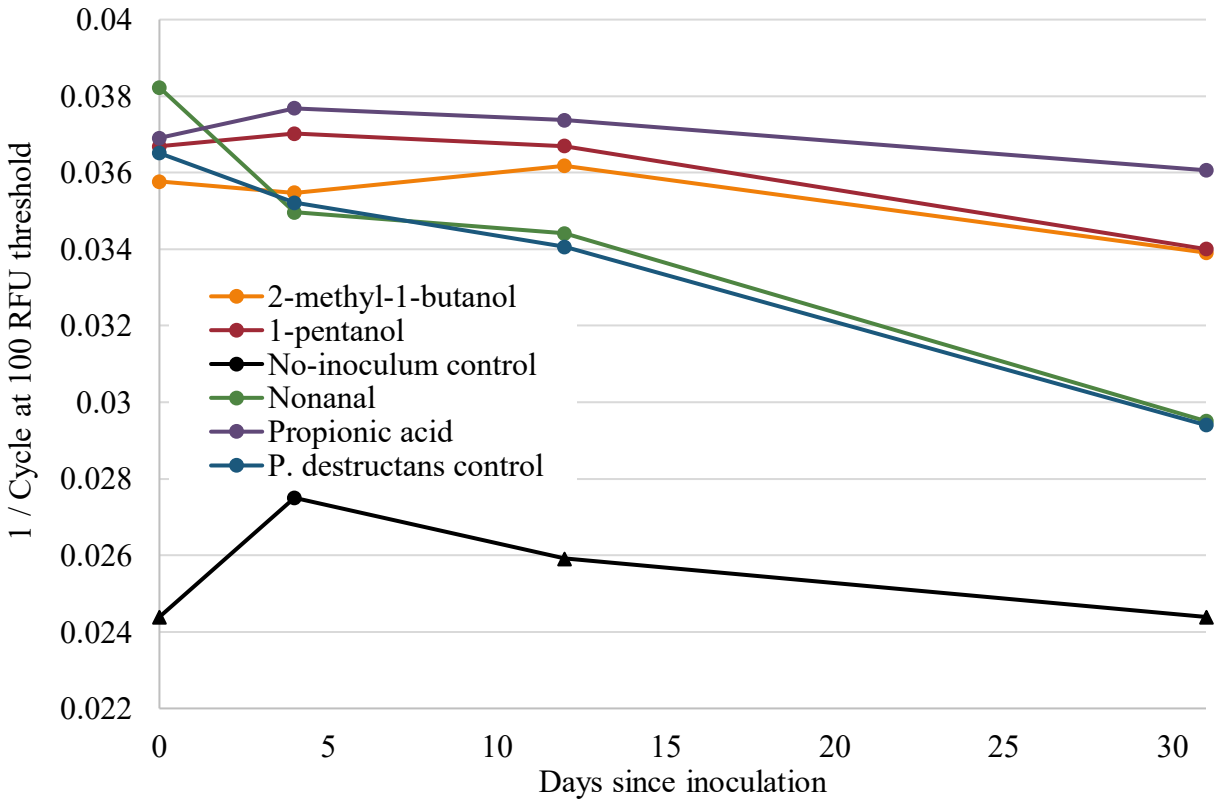


Figure 3.2. Detection of *P. destructans* in hibernaculum-like soil microcosms after fumigation with volatile compounds. *P. destructans* was inoculated into hibernaculum soil in a flask and 1-pentanol, 2-methyl-1-butanol, propionic acid, or nonanal was placed onto a cotton applicator stick suspended above the soil within the flask. Controls without *P. destructans* (no inoculum control) and with *P. destructans* but without volatiles (*P. destructans* control) were included. *P. destructans* abundance was quantified by the qPCR cycle at an arbitrary 100 RFU threshold. Triangle markers indicate that no amplicon band was visible after gel electrophoresis.

To assess if the persistence of *P. destructans* DNA in the volatile-treated hibernaculum soil was due to the persistence of live *P. destructans*, we inoculated soil from each of the volatile-treated microcosms and their respective controls into potato dextrose broth (PDB) and measured the relative abundance of *P. destructans* over time in the broth (Figure 3.3). The *P. destructans* control showed a slight decrease in the relative abundance of *P. destructans* over time, while the no-inoculum control remained relatively consistent. The relative abundance of *P. destructans* in cultured soil that was treated with 2-methyl-1-butanol and 1-pentanol rapidly increased in the first week after culturing and then gradually decreased over the rest of the experimental period. In contrast, the relative abundance of *P. destructans* in cultured soil that was treated with nonanal gradually decreased over the first two weeks and then increased again. The relative abundance of *P. destructans* in soil treated with propionic acid decreased at an approximately linear rate over time, and that rate appeared to be greater than in the no-volatile control. The changes in relative *P. destructans* abundance could be attributed to growth of *P. destructans* or microbial degradation of *P. destructans* DNA. Additionally, because the quantification of *P. destructans* in this system was expressed relative to the total abundance of all organisms, a change in the background microbial abundance could alter the relative abundance of *P. destructans*.

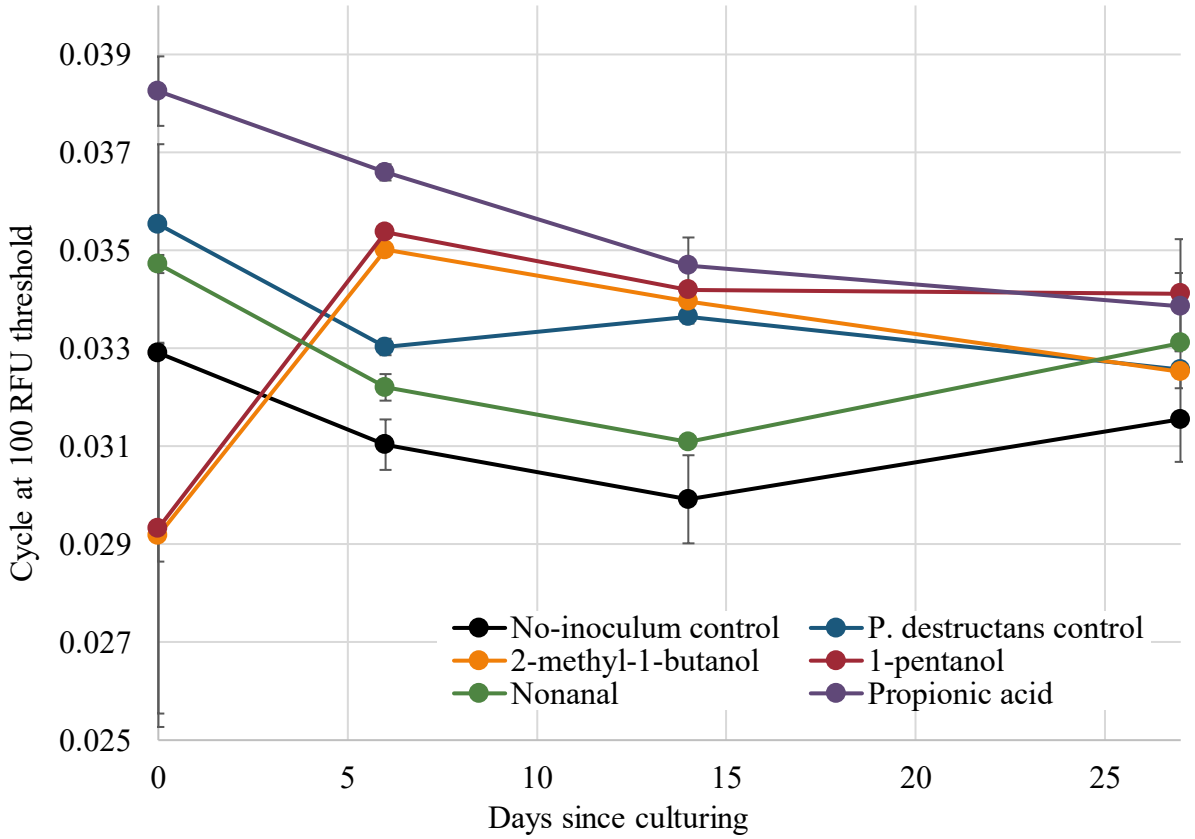


Figure 3.3. Detection of *P. destructans* in microcosm soils cultured in potato dextrose broth over time. Soils that were treated with volatile compounds and then left for 2 weeks were subsequently inoculated into potato dextrose broth and incubated at 13 ± 1 °C for approximately 1 month. Values reported are the averages of 3 replicates and error bars show standard error.

To assess whether our quantification of *P. destructans* could include DNA from dead *P. destructans*, we inoculated hibernaculum soil with live *P. destructans* and with extracted *P. destructans* DNA and monitored the abundance of *P. destructans* DNA over time (Figure 3.4a). To assess how the substrate affected the trends that we observed, we repeated the experiment in garden soil (Figure 3.4b).

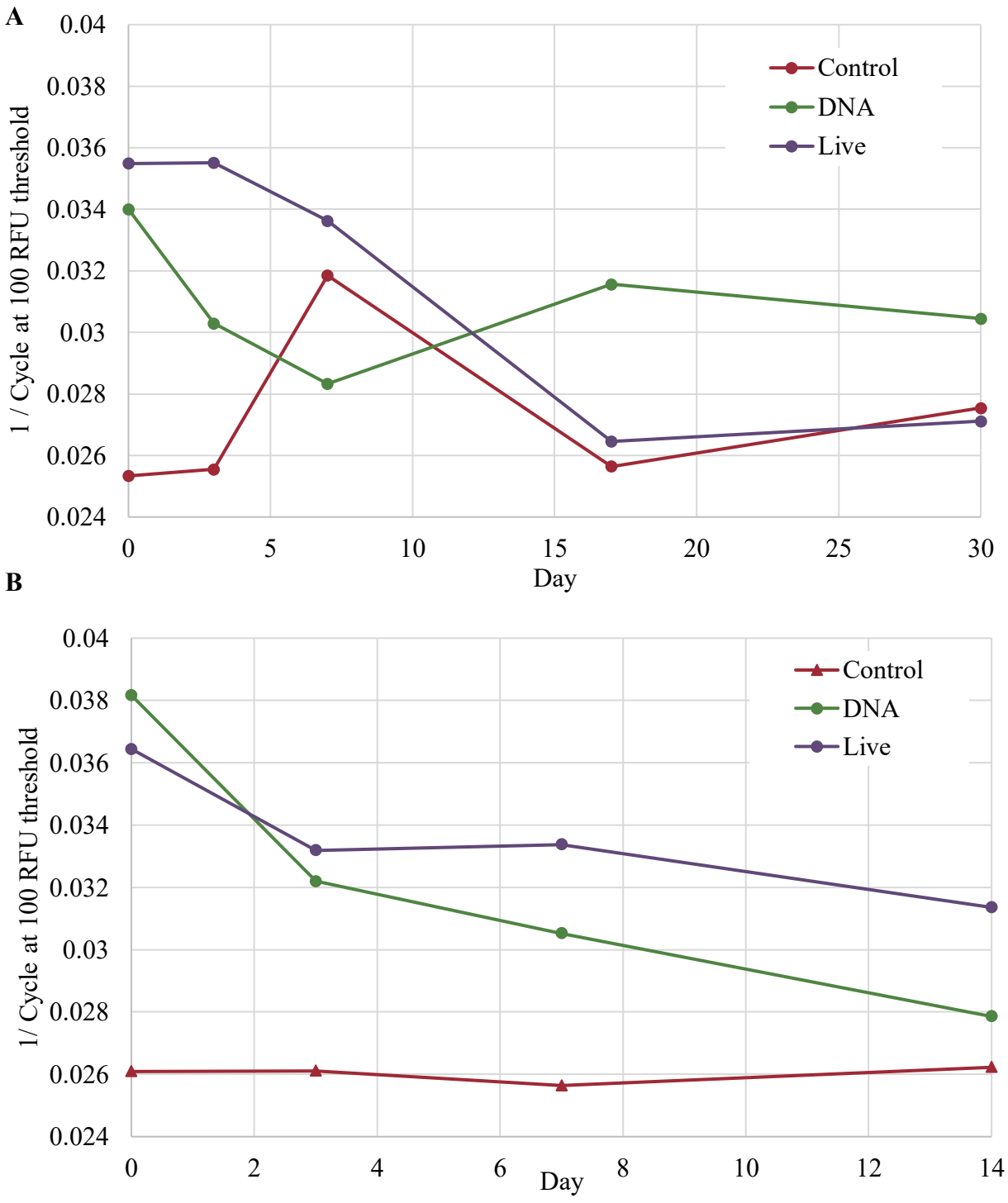


Figure 3.4. Detection of *P. destructans* in hibernaculum soil (A) and garden soil (B) microcosms. Microcosms were uninoculated (control), inoculated with *P. destructans* DNA, or inoculated with live *P. destructans*. The abundance of *P. destructans* DNA was quantified by the qPCR cycle at an arbitrary 100 RFU threshold. Triangle markers indicate that no amplicon band was visible after gel electrophoresis.

In garden soil, *P. destructans* remained below the limit of detection in an uninoculated control and extracted *P. destructans* DNA that was added directly to uninoculated soil gradually decreased over time. The abundance of live *P. destructans* also slowly decreased over time. In hibernaculum soil, the abundance of extracted *P. destructans* DNA, as well as the abundance of *P. destructans* in the no-inoculum control fluctuated but remained at levels near the initial abundance at the end of the experiment, while the abundance of live *P. destructans* decreased to near the limit of detection.

Discussion

We have previously identified microbial antagonists and volatile treatments that inhibit the growth of *P. destructans* in bioassays. As a follow-up to those tests, we used hibernaculum-like soil microcosms to assess if these agents cause inhibition of *P. destructans* in conditions more closely resembling a hibernaculum. Overall, we did not observe any notable decreases in the abundance of *P. destructans* when this bat pathogen was co-inoculated with any one of three microbial antagonists, *Trichoderma harzianum* RW1A2P, *Pantoea ananatis* RFA4P2, or *Penicillium* sp. S9A1R. These antagonists all caused greater than 90% inhibition of *P. destructans* in bioassays on agar plates (Chapter 1). Similarly, compared to a control, we did not observe any decreases in *P. destructans* abundance in soil microcosms treated with any one of the four volatile compounds previously shown to inhibit *P. destructans* in bioassays (Chapter 1; Cornelison *et al.*, 2014a).

Several explanations may account for the relatively low efficacy of our treatments in soil microcosms. It is possible that insufficient amounts of biocontrol agents or compounds were used to inhibit *P. destructans* in the soil microcosms. Despite the environmental sources of these

antagonists (soil, rotten wood, and river foam), nutritional supplementation may be required to support the level of environmental growth required for a biocontrol to affect *P. destructans* abundance (George White, personal communication), and we did not assess antagonist survival in our experiments. For the volatile compounds, we used at least three times the minimum inhibitory concentration (per unit of airspace), required to inhibit *P. destructans* on agar plates; however, *P. destructans* was inoculated at a high density and microcosm flasks were loosely sealed with foil, allowing the diffusion of compounds out of the flask. Further, we do not know the extent to which the volatile compounds permeate through the soil; however, some reduction of *P. destructans* near the soil surface would be expected regardless of soil permeability, due to the fungicidal nature of 2-methyl-1-butanol, 1-pentanol, and propionic acid (Chapter 1).

In the volatile-treated microcosms, propionic acid was used at the highest concentration (relative to the MIC) and in previous bioassays, appeared to be the least volatile of the four compounds. When microcosms were treated with propionic acid, *P. destructans* persisted in microcosms more than in any other condition. However, when the treated hibernaculum soil was inoculated into PDB and monitored over time, the relative abundance of *P. destructans* decreased more in propionic acid-treated soil than in any other treatment. This could suggest that propionic acid reduces the viability, and thus abundance, of *P. destructans* in the microcosms, and that our qPCR assay did not effectively quantify the abundance of viable *P. destructans*.

To assess these discrepancies, we monitored microcosms following the addition of extracted *P. destructans* DNA and found that in hibernaculum soil, this DNA only slightly decreased over 30 days. When we repeated the experiment using garden soil, we observed a greater, more rapid decrease in detectable *P. destructans* DNA, suggesting that properties of the hibernaculum soil may contribute to the observed persistence of DNA. Both the mineral

composition and the microbial activity of soil can affect the degradation of DNA in soil. While DNA in soil is primarily degraded by prokaryotic DNases, degradation can be greatly delayed if DNA binds to soil particles (Blum *et al*., 1997; Pietramellara *et al*., 2009; Levy-Booth *et al*., 2007). The type of soil affects the capacity and kinetics of DNA binding, and clay content is a primary factor that enables soil to bind DNA (Blum *et al*., 1997; Levy-Booth *et al*., 2007). Although no chemical analysis of the hibernaculum soil was performed, its clay-like texture could partly explain the persistence of DNA compared to the garden soil that we tested. Likewise, the hibernaculum soil used had low organic material evident in comparison to the garden soil, which would presumably harbour fewer microbes that could degrade dead *P. destructans*. Reduced growth of the soil microbial community, either due to a low capacity to support growth or treatment-mediated microbial death, could increase the persistence of *P. destructans* DNA by resulting in decreased production of microbial DNases and decreased microbial degradation of the *P. destructans* cell wall.

It is interesting that Zhang *et al*. (2015) were able to use qPCR-based monitoring in soil microcosms to show that strains of *Trichoderma* can inhibit *Pseudogymnoascus destructans* in a simulated hibernaculum environment. One possible explanation for the discrepancy between our findings could be that Zhang *et al*. inoculated soil with a conidial suspension of *P. destructans*, while we used macerated colonies. Our macerated fragments of *P. destructans* were very dense and ranged from approximately 0.001 to 0.2 mm^2, while single *P. destructans* conidia from our strains were approximately 2×10^{-5} mm^2. Compared to conidia, the large, highly dense colony forming units that we used would likely be more resistant to compound treatment and microbial antagonism, as well as to degradation of cells and DNA. Additionally, Zhang *et al*. described a granular consistency to the hibernaculum soil that they used, contrasting with the clay-like

consistency of our hibernaculum soil. Thus, it is possible that there were differences in the capacity of the soils to bind DNA and protect it from degradation.

It is worth noting that several qPCR-based detection methods of *P. destructans* have been proposed to monitor the environmental abundance of *P. destructans* without controls to determine if they are detecting dead *P. destructans* (e.g. Shuey *et al*., 2014; Verant *et al*., 2016). Our results suggest that under certain conditions, these methods may overestimate the true abundance of viable *P. destructans*.

Conclusions

Taken together, our results suggest that the qPCR-based detection of *P. destructans* was not a reliable way to assess the survival of *P. destructans* after treatment with different antagonists and volatile compounds. Conditions other than viability, including the composition of soil, the survival and abundance of the microbial community, inoculum density, and rate of degradation of cells and/or DNA may obscure estimates of the abundance of *P. destructans*. While conditions may exist where these techniques can be used successfully, we suggest that environmental monitoring of *P. destructans* should include controls to assess if detection of dead *P. destructans* is occurring.

Chapter 4: Antifungal applications to plant pathogens

It is estimated that to feed the predicted global population, food production will have to increase by over 50% by 2050 (Royal Society, 2009). Globally, approximately 8 to 15% of staple crops (wheat, rice, corn, and potatoes) (Oerke, 2006) and a further 25 to 50% of harvested fruits and vegetables are lost to plant and postharvest pathogens (Sharma *et al*., 2009; Tripathi and Dubey, 2004). Controlling the growth of agricultural pathogens and food spoilage microbes could contribute to addressing the food requirements of a growing human population.

The most common treatments to control plant pathogens are synthetic contact fungicides, which are increasingly used in Canada due to reduced crop rotation, increased pathogen introduction, increased yield targets, and climate change-induced changes in pathogen distribution (Gossen *et al*., 2014). However, broad-scale fungicide use is increasingly regulated due to risks of off-target toxicity, carcinogenicity, and persistence (Tripathi and Dubey, 2004; Gossen *et al*., 2014). Natural products from microbes have been considered as a possible alternative to the use of synthetic fungicides to target plant pathogens. These compounds tend to be biodegradable in the environment, which reduces the risk of environmental toxicity and non-target effects, and may have novel mechanisms of action, which can help ameliorate the growing problem of multi-class fungicide resistance (Kim and Hwang, 2007; Deising *et al*., 2008).

We have previously identified antifungal natural products that inhibit *Pseudogymnoascus destructans* and yeast (*Saccharomyces cerevisiae*). Because of the importance of controlling the growth and spread of plant pathogens, we screened microbial volatiles and broths containing secreted microbial metabolites against a collection of agriculturally significant plant pathogens.

Materials and methods

Plant pathogen strains

The following strains of plant pathogens were used in volatile and liquid culture assays: *Botrytis cinerea* 189076, 231370, and RW1A6P, *Fusarium oxysporum* DAOM 215464, *Alternaria alternata* 196949, *Alternaria solani* DAOM 229596 and 229590, *Elsinoë brasiliensis* 395825, *Sclerotinia sclerotiorum* 241671, *Fusarium avenaceum* RW1A5P, *Verticillium dahliae* 176 (Laboratoire de diagnostic en phytoprotection; MAPAQ, Québec, Canada), *Cryphonectria parasitica* EP155, P88-8, and P74-3, *Pseudomonas syringae* P4326 and Pst DC3000. Morphological examination was used to confirm the identity of the fungal pathogens.

Preparation of plant pathogen stocks

Fungal plant pathogens were grown separately on potato dextrose agar plates and then removed from the plate and macerated in PDB using a Waring commercial blender. Bacterial plant pathogens were grown in LB broth. Stocks were amended with approximately 15% glycerol and frozen in 1 ml aliquots at -80 °C. Thawed stocks were enumerated by plating a dilution series onto potato dextrose agar.

Test of antagonist broths for activity against plant pathogens

Antagonistic strains previously shown to have antifungal activity in bioassays against *P. destructans* were grown stationary at 13 ± 1 °C for 4 weeks in PDB (for fungi and yeast) or LB (for bacteria). The media that the strains grew in was then passed through a Fisher P8 filter paper, 10× concentrated by lyophilisation, and separated from cells through a 0.22 µm syringe filter. The resulting cell-free broths were stored in 100 µl aliquots at -20 °C until use in screens

for antifungal activity against the collection of plant pathogens. All screens were conducted in microtiter plates by 1:1 serially diluting 50 µl of 10× broth in 50 µl of PDB (for fungi) or LB (for bacteria) and then adding 150 µl of PDB or LB containing approximately 100 CFU of plant pathogen. As a negative control, 10× PDB (for broths from fungal antagonists) or 10× LB (for broths from bacterial antagonists) was used.

Test of antagonist broth activity over time

Thirteen of the broths most inhibitory to plant pathogens in initial trials were tested against three of the most sensitive plant pathogens after broths were stored at -20 °C for 221 or 292 days. Frozen aliquots of broth were thawed and screened as above, against *S. sclerotiorum* 241671, *Elsinoë brasiliensis* 395825, and *Cryphonectria parasitica* P74-3 and the lowest concentration required for complete inhibition of the plant pathogens was compared to that on day 1 (freshly prepared broths).

Test of volatile activity against plant pathogens

Plant pathogen stocks were diluted in potato dextrose broth (PDB) and a 5 µl drop containing approximately 50 CFU was aliquoted into the centre of a BD Difco Potato Dextrose Agar (PDA) plate. Plates were left at room temperature and growth was monitored daily until mycelial growth was visible. The plate was then inverted and a 2-cm disk of Whatman filter paper was placed into the lid and 50 µl of 2-methyl-1-butanol, 50 µl of 1-pentanol, 25 µl of propionic acid, or 1 µl of nonanal was added to the filter paper. Plates were then sealed with parafilm and left for 4 hours at room temperature. After the incubation, the plate lids were replaced to remove the source of volatile, and the plates were left unsealed at room temperature.

Growth was monitored daily for one week after exposure and compared to a no-exposure control.

Results

We grew antagonists of *P. destructans* stationary in liquid media for one month and screened the concentrated cell-free broth against a collection of 16 plant pathogens spanning 10 species. All plant pathogens were sensitive to at least five of the 33 broths that we tested. Generally, *Fusarium* spp. and *Alternaria alternata* were the least sensitive, while strains of *Cryphonectria parasitica* and *Pseudomonas syringae* were the most sensitive. Different strains of the same pathogen were usually similar in their sensitivity, with the exception of *Alternaria solani*, where strain 229590 was resistant to most broths and strain 229596 was among the most sensitive strains that we tested. The three broths with the greatest activity were from *Lecanicillium* sp. S8I1CN, an unidentified fungus RW3A2Pa, and *Trichoderma atroviride* RW4A2P, which were strongly inhibitory to most pathogen strains. A few strains were mildly sensitive to the concentrated LB broth control. The inhibition of each pathogen by each antagonist broth, relative to the original broth concentration, is shown in Appendix 5.

We tested a subset of the most inhibitory broths for stability over time. Thirteen broths were stored at -20 °C for over 220 days after which their activity against three of the most sensitive plant pathogens was compared to their original activity. Mild variation (activity at 0.5 to 2 × original broth concentration was expected due to minor differences in pipetting and pathogen density, and generally, the activities of the broths did not change with storage. However, the stored broth from *Penicillium* sp. S8I2ACS had considerably lower activity against *Elsinoë brasiliensis* than the fresh broth, while after storage, the broth from *Bacillus* sp. 14807

and *Pseudomonas* sp. APCI2P had considerably higher activity against *Elsinoë brasiliensis* and *Cryphonectria parasitica*, respectively. The relative activity over time of each broth against each plant pathogen is shown in Table 4.1.

Table 4.1. Antifungal activity of broths over time. Thirteen microbial broths were screened for inhibition of *Sclerotinia sclerotiorum* 241671, *Elsinoë brasiliensi*s 395825, and *Cryphonectria parasitica* P74-3. Stocks were prepared freshly and then prepared again after over 220 days of storage at -20 °C, Numbers reflect $\frac{C_{fresh\ broth}}{C_{stored\ broth}}$, where *C* is the concentration required for complete inhibition (i.e. < 1 reflects a loss of activity with storage). Numbers with an asterisk indicate that there was no complete inhibition at the highest concentration with the freshly prepared broth, and for calculation purposes, $C_{original}$ was considered twice the highest concentration used.

Broth antagonist strain	*S. sclerotiorum*	*E. brasiliensis*	*C. parasitica*
Pseudomonas sp. APCI2P	2	2	4
RFA6P	2	2	0.5
Lecanicillium sp. S8I1CN	2	1	1
Trichoderma atroviride RW4A2P	1	2	1
Bacillus sp. APCI1P	1	2	1
Bacillus sp. 14807	1	16	1
Bacillus sp. B-14324	1	1	0.5
Pantoea ananatis RFA4P2	1	1	1
Bacillus sp. 55407	0.5	1	1
Trichoderma sp. S3A2ACS	4*	2	2
Penicillium yarmokense S8A1ACS	1	2	1
Penicillium sp. S8I2ACS	2*	0.125	1

We assessed the sensitivity of 12 strains of plant pathogens to a short treatment with volatile compounds that were previously demonstrated to be inhibitory to *P. destructans* and *S. cerevisiae*. Actively growing pathogens were exposed to volatile compounds for 4 hours, after

which their growth was monitored for one week. The growth of each pathogen before and after exposure to each volatile compound is shown in Figure 4.1. Treatment with nonanal was the least inhibitory to pathogen growth, ranging from having no effect on *B. cinerea* RW1A6P growth to delaying *V. dahliae* 176 growth for approximately 4 days. Treatment with 1-pentanol and 2-methyl-1-butanol typically caused similar pathogen growth delays, arresting growth for an average of 3.1 and 2.3 days, respectively. This effect was fungistatic and when growth resumed, the rate was comparable to that before treatment. Treatment with propionic acid appeared to have a fungicidal effect on the pathogens and growth typically failed to resume after treatment. In Figure 4.1, propionic acid does not appear to be fungicidal against *A. solani* 229596 and *B. cinerea* RW1A6P; however, in both cases the filter paper to which propionic acid was applied was not centered in the petri dish. In previous assays, propionic acid appeared to have a low volatility, and in these cases, pathogen growth only resumed on the colony edge furthest from the propionic acid source.

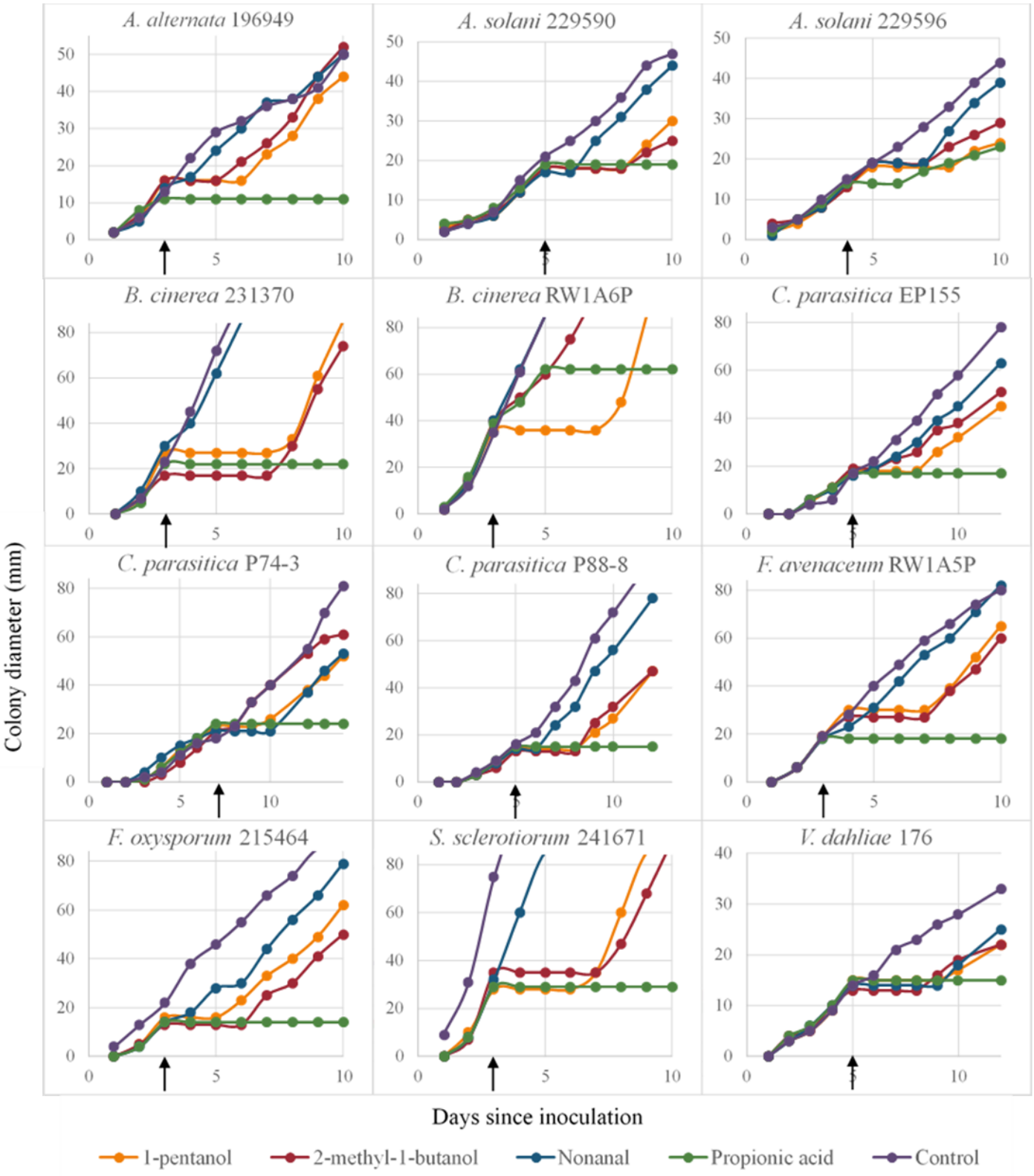


Figure 4.1. Growth of 12 strains of plant pathogens exposed to four volatile compounds. Plant pathogens were incubated for 4 hours with 50 µl 1-pentanol, 50 µl 2-methyl-1-butanol, 1 µl nonanal, 25 µl propionic acid, or a no-volatile control. Black arrows indicate the time of volatile exposure. Approximately 50 CFU were inoculated onto PDA plates and exponentially growing colonies were treated with volatiles for 4 hours, after which growth was monitored for 1 week.

Discussion

In this work, we identified fungi that constitutively secrete compounds with broad activity against a collection of plant pathogens. The most inhibitory extracts were derived from the spent broth of *Trichoderma atroviride*, *Lecanicillium* sp., and an unidentified fungus. The antifungal activity of members of the *Trichoderma* genus is well established. *Trichoderma* spp. are known to produce cell wall-degrading enzymes (reviewed in Gruber and Seidl-Seiboth, 2012) and many antibiotic secondary metabolites (reviewed in Zeilinger *et al*., 2016). Similarly, members of the *Lecanicillium* genus have been shown to produce lytic and cell wall-degrading enzymes (Nguyen *et al*., 2015; Bidochka *et al*., 1999; Rocha-Pino *et al*., 2011). Based on ITS sequencing, the unidentified antagonist most likely belongs to the genus *Phacidium*; however, it was sterile in culture and this identification could not be morphologically confirmed. To the best of our knowledge, there are no reports on antifungal activity by extracts of the *Phacidium* genus, suggesting the possibility of novel antifungal compounds. While it is perhaps not surprising that these fungi were able to produce inhibitory compounds, it is interesting that under the incubation conditions, they were constitutively secreted without induction. Additionally, their apparent stability over time when stored at -20 °C makes them attractive candidates for further investigation into their potential industrial use.

In addition to screening crude liquid broths, we showed that under laboratory conditions, short treatments with volatile compounds can have fungistatic or fungicidal activity against a collection of plant pathogens. Volatile natural products are promising candidates for controlling plant pathogens due to their potential to be used as fumigants. These volatile compounds could provide alternatives to traditionally used fumigants such as methyl bromide and metam sodium, which are highly toxic to vertebrates (Yang *et al*., 1995; Pruett *et al*., 2001). 1-pentanol and 2-

methyl-1-butanol showed promising fungistatic inhibition of plant growth and it is possible that this inhibition could be fungicidal at higher concentrations. Propionic acid was particularly effective and had an at least partially fungicidal effect on all tested pathogens. Propionic acid is generally regarded as safe by the U.S. Food and Drug Administration (2017), and while it has been researched and used widely as a food preservative, there has been less research into its potential agricultural use. Our results suggest that volatile natural products could be used to control the growth of plant pathogens in agricultural systems; however, further research is needed to examine the efficacy and safety of these strategies *in vivo* and under field conditions.

Conclusions

We conducted preliminary screens of antifungal microbial natural products to determine if any compounds were able to inhibit the growth of a collection of plant pathogens. We identified 33 broths that inhibit a subset of the plant pathogen collection., and these broths were from antagonists that constitutively secrete compounds under the incubation conditions. Most of these compounds appear to be stable for a prolonged period at -20 °C. We also demonstrate that the plant pathogen collection is sensitive to treatment with three volatile compounds, of which propionic acid was the most promising because it had a fungicidal effect and it is generally regarded as safe. While future research is needed to identify and characterizes these compounds, our work suggests that they could potentially have applications in controlling agriculturally significant plant pathogens.

General conclusion

In this set of experiments, we primarily focused on the identification and characterization of candidate control agents for the environmental growth of *P. destructans*, the fungus that causes white-nose syndrome in hibernating bats. We first identified a collection of microbes that were strongly inhibitory to *P. destructans* in bioassays. Further characterization of these antagonistic microbes revealed that they act through contact inhibition, or through secretion of liquid or volatile antifungal compounds (Chapter 1). We examined the microbially-produced volatile compounds 2-methyl-1-butanol, 1-pentanol, propionic acid, and nonanal in further detail and conducted preliminary analyses of the antifungal mode of action of these compounds using a yeast model (Chapter 2). 2-methyl-1-butanol, 1-pentanol, and propionic acid caused an accumulation of cells in the G_1 phase of the cell cycle, while propionic acid further caused an increase in endocytosis and a perturbation of cellular respiration and gene expression. We then simulated the hibernaculum environment with soil microcosms to assess the inhibitory activity of the volatile compounds and a subset of our top antagonists under hibernaculum-like conditions (Chapter 3). Surprisingly, in this system, we did not detect inhibition of *P. destructans* commensurate with our agar-based assays and propose that this may be due to features of the environment and/or treatment that enable the persistence of DNA from dead *P. destructans*. We suggest that currently employed qPCR-based assays for the detection and quantitation of environmental *P. destructans* should include negative controls to ensure that they are not detecting DNA from dead *P. destructans*. Finally, we tested the metabolites and volatile compounds that we found to be inhibitory to *P. destructans* against a library of agriculturally significant plant pathogens (Chapter 4). There was a high degree of cross-over of strains that had antifungal activity against *P. destructans* and also against the plant pathogen collection. The

inhibitory compounds appear to be stable over time under -20 °C storage conditions. Future research should further address the antifungal mechanisms of action of these compounds, their ability to inhibit *P. destructans* under hibernaculum conditions, and their employment as antifungals in general.

Appendix 1. Identification of microbial antagonists of *Pseudogymnoascus destructans*. Isolates are sorted by percent inhibition, which reflects the degree of inhibition of *P. destructans* by an antagonist. Isolates were ranked as follows: 0 = negligible (< 50%) inhibition, 1 = considerable (50% to 85%) inhibition, 2 = complete or nearly complete (> 85%) inhibition. Additionally, if applicable, ranks were qualified with: A = growth of the antagonist is limited by *P. destructans*, B = the antagonist grew over *P. destructans* such that affected *P. destructans* colonies were no longer visible, C = *P. destructans* colonies were present, but uniformly smaller than in the control plate. *P. destructans* area and isolate diameter refer to sizes 14 days after *P. destructans* was inoculated. Genbank accession numbers refer to sequences of 16S rDNA (bacteria), ITS rDNA (fungi), and beta-tubulin DNA (*Penicillium* sp.).

Isolate	Type	Source[1]	Source Location	Rank	Percent Inhibition[4]	*P. destructans* Area (mm^2)	Isolate Diameter[5] (mm)	Identification	Genbank Accession Number		
									16s	ITS	β-tubulin
S7A3CVb2	Bacteria	Soil site 7	Gatineau, QC	0	3.4	499.38	11.01				
DUST E	Bacteria	M.L. Smith	Ottawa, ON	0	3.4	499.34	7.78	*Bacillus thuringiensis*			
S4A1P	Bacteria	Soil site 4	Gatineau, QC	0	5.8	487.42	10.95				
DUST H	Bacteria	M.L. Smith	Ottawa, ON	0	5.8	487.21	6.40	*Bacillus pumilus*			
M9-1B	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	6.1	485.69	6.81	*Bacillus megaterium*	KT382244		
B-14471	Bacteria	M.L. Smith	Ottawa, ON	0	6.3	484.52	5.21	*Bacillus subtilis*			
M9-18	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	7.0	481.16	9.97	*Brevibacillus borstelensis*	KT382254		
S2A2LC	Bacteria	Soil site 2	King City, ON	0	7.3	479.26	13.23				
DUST I	Bacteria	M.L. Smith	Ottawa, ON	0	7.8	476.62	4.91	*Bacillus pumilus*	KY305270		
S9A4Pb	Bacteria	Soil site 9	Gatineau, QC	0	7.9	476.28	12.15				
DUST F	Bacteria	M.L. Smith	Ottawa, ON	0	7.9	476.09	7.95	*Bacillus megatarium*			
S9I1P	Bacteria	Soil site 9	Gatineau, QC	0	8.6	472.48	9.89				
DUST C	Bacteria	M.L. Smith	Ottawa, ON	0	8.9	471.09	4.35	*Bacillus sphaericus*			
IC23	Bacteria	Sheep manure compost tea (T.J. Avis)	Quebec, QC	0	9.0	470.60	8.46	*Bacillus subtilis*	KJ689307		
DUST B	Bacteria	M.L. Smith	Ottawa, ON	0	9.6	467.76	8.01	*Bacillus megatarium*			
S8I3P	Bacteria	Soil site 8	Gatineau, QC	0	10.5	462.72	9.26				
14308	Bacteria	M.L. Smith	Ottawa, ON	0	10.9	460.95	10.09	*Bacillus* sp.			

S3A2LCb	Bacteria	Soil site 3	King City, ON	0	11.2	459.04	10.08				
S1A1ACS	Bacteria	Soil site 1	King City, ON	0	11.8	456.39	11.26				
GWA16	Bacteria	G.P. White	Ottawa, ON	0	13.0	450.15	10.11				
CU12	Bacteria	Soil (T.J. Avis)	Ottawa, ON	0	14.6	441.76	7.50	*Bacillus subtilis*	JX489167		
S9A2P2	Bacteria	Soil site 9	Gatineau, QC	0	14.7	441.19	8.85				
DUST J	Bacteria	M.L. Smith	Ottawa, ON	0	14.9	440.33	8.09	*Bacillus megatarium*	KY305271		
S3A2P	Bacteria	Soil site 3	King City, ON	0	15.4	437.59	15.08				
S4A3LC	Bacteria	Soil site 4	Ottawa, ON	0	15.8	435.48	9.16				
S4I1P	Bacteria	Soil site 4	Ottawa, ON	0	15.8	435.29	15.30				
PCI5P	Bacteria	Plate contaminant	Ottawa, ON	0	17.8	407.47	6.25	*Streptomyces* sp.	KY305242		
842	Bacteria	M.L. Smith	Ottawa, ON	0	18.1	423.39	5.18	*Bacillus polymyxa*			
S8A1P	Bacteria	Soil site 8	Gatineau, QC	0	18.3	422.60	11.86				
S3A1P	Bacteria	Soil site 3	King City, ON	0	20.0	413.83	11.33				
S7A7Pb	Bacteria	Soil site 7	Gatineau, QC	0	20.1	413.25	9.96				
F9-9	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	20.2	412.69	6.71	*Pseudomonas koreensis*	KT382238		
S7A8P	Bacteria	Soil site 7	Gatineau, QC	0	20.6	410.79	8.72				
S3A2LC	Bacteria	Soil site 3	King City, ON	0	21.6	405.66	12.48				
M9-17	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	23.0	398.46	10.48	*Arthrobacter psychrophenolicus*	KT382253		
DUST D	Bacteria	M.L. Smith	Ottawa, ON	0	23.9	393.65	10.48	*Bacillus* sp.	KY305269		
S7A3P	Bacteria	Soil site 7	Gatineau, QC	0	24.2	392.00	7.99				
M9-19	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	24.5	390.62	8.47	*Rummeliibacillus pycnus*	KT382255		
F9-2	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	25.4	385.68	9.97	*Bacillus subtilis*	KT382234		
B9-8	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	0	25.7	384.52	5.45	*Bacillus subtilis*	KT382230		
14581	Bacteria	M.L. Smith	Ottawa, ON	0	26.4	380.45	12.75	*Bacillus megaterium*			
B9-9B	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	26.5	380.37	8.93	*Bacillus megaterium*	KT382232		
F9-7	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	27.5	375.07	7.18	*Pseudomonas arsenicoxydans*	KT382236		
M9-7	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	29.1	366.67	4.86	*Bacillus subtilis*	KT382248		

12713	Bacteria	M.L. Smith	Ottawa, ON	0	29.7	363.61	8.92	*Bacillus licheniformis*			
M9-8	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	29.9	362.41	12.66	*Arthrobacter humicola*	KT382249		
S7A2CV	Bacteria	Soil site 7	Gatineau, QC	0	32.4	349.82	10.47	*Bacillus* sp.	KY305272		
RW1I2P	Bacteria	Rotten wood	Ottawa, ON	0	32.4	349.40	10.59				
S7A1P	Bacteria	Soil site 7	Gatineau, QC	0	33.5	343.69	6.39				
S3A4LC	Bacteria	Soil site 3	King City, ON	0	33.6	343.41	12.37				
B-4378	Bacteria	M.L. Smith	Ottawa, ON	0	35.0	336.34	7.15	*Bacillus* sp.	KY305268		
F9-13	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	36.0	330.99	10.55	*Pseudomonas brenneri*	KT382242		
S8A5ACS2	Bacteria	Soil site 8	Gatineau, QC	0	37.2	324.93	7.40				
8010	Bacteria	M.L. Smith	Ottawa, ON	0	37.6	322.62	8.05	*Arthrobacter globiformis*			
PCA7P	Bacteria	Plate contaminant	Ottawa, ON	0	38.0	307.10	8.23	*Streptomyces* sp.	KY305241		
S4I1LC	Bacteria	Soil site 4	Ottawa, ON	0	38.4	318.34	11.62				
S7A2P	Bacteria	Soil site 7	Gatineau, QC	0	39.5	312.89	13.42				
M9-16	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	40.4	308.35	6.76	*Pseudomonas gessardii*	KT382252		
B9-4	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	0	41.0	304.95	8.60	*Paenibacillus favisporus*	KT382227		
B9-1	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	0	41.5	302.61	8.38	*Bacillus subtilis*	KT382225		
F9-10	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	41.6	302.15	7.91	*Pseudomonas brenneri*	KT382239		
S7A6P	Bacteria	Soil site 7	Gatineau, QC	0	43.8	290.72	10.36				
M9-20	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	43.8	290.71	9.39	*Bacillus badius*	KT382256		
S3A1LC	Bacteria	Soil site 3	King City, ON	0	44.3	288.30	13.85				
4329	Bacteria	M.L. Smith	Ottawa, ON	0	45.5	282.09	12.24				
S8A4P	Bacteria	Soil site 8	Gatineau, QC	0	46.1	278.57	8.80				
B-354	Bacteria	M.L. Smith	Ottawa, ON	0	46.7	275.64	13.03	*Bacillus subtilis*			
RFA1P1	Bacteria	Foam from Rideau River	Ottawa, ON	0	46.9	274.50	12.61				
S7A3CV1a	Bacteria	Soil site 7	Gatineau, QC	0	47.1	273.36	10.65				
S3A4LCb	Bacteria	Soil site 3	King City, ON	0	47.8	269.94	8.70				
GHA5	Bacteria	Hay (G.P. White)	Ottawa, ON	0	48.6	265.99	10.94				

S3A2LCAP	Bacteria	Soil site 3	King City, ON	0	48.8	264.98	7.67				
B9-3	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	0	49.3	261.96	11.70	*Pseudomonas gessardii*	KT382226		
F9-8	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	0	49.7	259.99	7.29	*Bacillus subtilis*	KT382237		
M9-1A	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	0	49.9	258.97	9.01	*Arthrobacter humicola*	KT382243		
13563-0	Bacteria	M.L. Smith	Ottawa, ON	1	50.5	256.03	7.81	*Bacillus amyloliquefaciens*			
B-23049	Bacteria	M.L. Smith	Ottawa, ON	1	51.4	251.25	9.58	*Bacillus subtilis*			
PCI4P	Bacteria	Plate contaminant	Ottawa, ON	1	53.4	230.88	7.09	*Streptomyces* sp.	KY305263		
S1A2LC	Bacteria	Soil site 1	King City, ON	1	56.7	223.92	9.48				
S8A1Cs1	Bacteria	Soil site 8	Gatineau, QC	1	57.3	220.71	11.08				
IB2	Bacteria	Sheep manure compost tea (T.J. Avis)	Quebec, QC	1	58.3	215.58	13.93	*Aminobacter aminovorans*	KJ689311		
S8A6P	Bacteria	Soil site 8	Gatineau, QC	1	58.7	213.68	16.31				
S8I5P	Bacteria	Soil site 8	Gatineau, QC	1	59.5	209.25	10.18				
F9-11	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	1	60.4	204.81	7.24	*Pseudomonas moraviensis*	KT382240		
9500	Bacteria	M.L. Smith	Ottawa, ON	1	60.7	203.24	10.29	*Bacillus circularns*			
S1A1LC	Bacteria	Soil site 1	King City, ON	1	60.7	202.99	9.93				
F9-12	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	1	60.9	202.45	9.68	*Bacillus subtilis*	KT382241		
DUST A	Bacteria	M.L. Smith	Ottawa, ON	1	61.6	198.49	11.55	*Bacillus thuringiensis*			
B9-14	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	1	63.7	187.75	8.36	*Bacillus subtilis*	KT382233		
6051A	Bacteria	M.L. Smith	Ottawa, ON	1	64.2	184.98	7.72	*Bacillus subtilis*	KY305264		
S8A3Pb	Bacteria	Soil site 8	Gatineau, QC	1	64.6	183.26	12.56				
941	Bacteria	M.L. Smith	Ottawa, ON	1	65.2	179.88	7.21	*Bacillus subtilis*			
4698	Bacteria	M.L. Smith	Ottawa, ON	1	65.9	176.47	9.57	*Micrococcus luteus*			
B-363	Bacteria	M.L. Smith	Ottawa, ON	1	66.5	173.32	6.03	*Bacillus subtilis*	KY305267		
M9-3	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	1	69.6	157.09	9.83	*Bacillus subtilis*	KT382246		
55407	Bacteria	M.L. Smith	Ottawa, ON	1,C	71.5	147.33	11.68	*Bacillus* sp.	KY305244		
PCI6P	Bacteria	Plate contaminant	Ottawa, ON	1	76.3	117.56	8.34	*Streptomyces* sp.	KY305273		
RW1A1P2	Bacteria	Rotten wood	Ottawa, ON	1,C	76.6	121.00	8.63	*Rahnella aquatilis*	KY305246		

S5A1P	Bacteria	Soil site 5	Ottawa, ON	1	79.0	108.40	11.82				
NRS 213	Bacteria	M.L. Smith	Ottawa, ON	1	79.6	105.45	15.98	*Bacillus subtilis*			
S4I1ACS	Bacteria	Soil site 4	Ottawa, ON	1	82.5	86.47	8.28	*Streptomyces* sp.	KY305251		
S4A3P	Bacteria	Soil site 4	Ottawa, ON	1,C	83.4	85.99	13.96	*Arthrobacter* sp.	KY305247		
S4A2LCb	Bacteria	Soil site 4	Ottawa, ON	2,C	85.5	75.16	14.56	*Bacillus* sp.	KY305249		
DUST G	Bacteria	M.L. Smith	Ottawa, ON	2,C	85.9	73.07	11.18	*Bacillus* sp.	KY305245		
M9-4	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	2	86.8	68.43	7.47	*Bacillus subtilis*	KT382247		
PCI1P	Bacteria	Plate contaminant	Ottawa, ON	2	88.6	56.49	18.74	*Streptomyces* sp.	KY305262		
S9A1Cs1	Bacteria	Soil site 9	Gatineau, QC	2,C	89.2	55.70	14.04	*Bacillus subtilis*	KY305248		
B9-7	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	2	89.4	55.05	9.57	*Bacillus subtilis*	KT382229		
S6A3ACS	Bacteria	Soil site 6	Ottawa, ON	2	90.2	48.60	4.49	*Streptomyces laeteviolaceus*	KY305253		
13540-4	Bacteria	M.L. Smith	Ottawa, ON	2	90.5	49.24	9.74	*Bacillus subtilis*	KY305265		
14807	Bacteria	M.L. Smith	Ottawa, ON	2	90.6	48.77	9.40	*Bacillus* sp.	KY305255		
55405	Bacteria	M.L. Smith	Ottawa, ON	2,C	91.5	43.81	8.39	*Bacillus subtilis*	KY305250		
IC10	Bacteria	Sheep manure compost tea (T.J. Avis)	Quebec, QC	2	93.4	33.89	9.54	*Bacillus subtilis*	KJ689307		
B9-9A	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	2	93.7	32.48	8.84	*Bacillus subtilis*	KT382231		
M9-14	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	2	94.3	29.60	9.84	*Bacillus subtilis*	KT382251		
M9-9	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	2	95.4	23.85	7.98	*Bacillus subtilis*	KT382250		
OA1I3M	Bacteria	Outdoor air	Ottawa, ON	2,C	96.7	17.20	7.32	*Pantoea* sp.	KY305243		
B9-5	Bacteria	Bovine manure compost (T.J. Avis)	Quebec, QC	2	96.8	16.47	7.09	*Bacillus subtilis*	KT382228		
B-14324	Bacteria	M.L. Smith	Ottawa, ON	2	97.3	13.93	9.00	*Bacillus* sp.	KY305258		
GWA3c	Bacteria	G.P. White contaminant	Ottawa, ON	2	97.5	12.83	12.91	*Bacillus subtilis*	KY305259		
S5A2LC	Bacteria	Soil site 5	Ottawa, ON	2	98.7	6.57	18.43	*Pantoea* sp.	KY305261		
APCI1P	Bacteria	Assay plate contaminant	Ottawa, ON	2	99.2	3.94	10.24	*Bacillus* sp.	KY305256		
RFA4P2	Bacteria	Foam from Rideau River	Ottawa, ON	2	99.3	3.47	18.61	*Pantoea ananatis*	KY305260		
55406	Bacteria	M.L. Smith	Ottawa, ON	2	99.4	2.98	9.35	*Bacillus* sp.	KY305266		
M9-2	Bacteria	Marine compost (T.J. Avis)	Quebec, QC	2	99.4	2.98	7.18	*Arthrobacter humicola*	KT382245		

GWA3a	Bacteria	G.P. White contaminant	Ottawa, ON	2	99.6	2.24	8.25	*Rahnella aquatilis*	KY305240		
APCI2P	Bacteria	Assay plate contaminant	Ottawa, ON	2	100.0	0.00	8.52	*Pseudomonas* sp.	KY305257		
F9-6	Bacteria	Forestry compost (T.J. Avis)	Quebec, QC	2	100.0	0.00	9.57	*Pseudomonas moraviensis*	KT382235		
S8A1Cs1b	Bacteria	Soil site 8	Gatineau, QC	2	100.0	0.00	7.91	*Streptomyces* sp.	KY305254		
S8A4Cs	Bacteria	Soil site 8	Gatineau, QC	2	100.0	0.00	7.83	*Sphingobium* sp.	KY305252		
PCA6P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	-18.1	585.19	6.20				
CPV	Filamentous Fungus	Cassava (Georgette Briggs)		0	-15.7	573.00	3.48				
PCA19P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	-8.7	538.78	6.62				
S6A2ACSb	Filamentous Fungus	Soil site 6	Ottawa, ON	0	-7.0	530.22	9.01				
GHA4	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	-6.9	529.83	6.39				
GWA11	Filamentous Fungus	G.P. White	Ottawa, ON	0	-6.2	526.26	9.45	*Aspergillus* sp.[2]			
PCA1P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	-5.0	520.05	8.31				
S7A1R	Filamentous Fungus	Soil site 7	Gatineau, QC	0	-4.8	519.10	8.50				
LAA3P	Filamentous Fungus	Lab air	Ottawa, ON	0	-1.5	502.80	7.11				
CPVI	Filamentous Fungus	Cassava (Georgette Briggs)		0	-0.8	499.50	4.87				
GHA2	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	-0.1	495.72	9.98				
GHA2	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0,A	-0.1	495.72	9.98	*Aspergillus* sp.[2]			
S9A4P	Filamentous Fungus	Soil site 9	Gatineau, QC	0	3.8	476.60	6.94				
PCA15P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	4.5	473.06	5.49				
RW3I2Pb	Filamentous Fungus	Rotten wood	Ottawa, ON	0	4.8	471.75	12.53				
S3A1ACS	Filamentous Fungus	Soil site 3	King City, ON	0	5.2	469.61	13.98				
PCA16P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	8.5	453.29	5.46				
OA3I2P	Filamentous Fungus	Outdoor air	Ottawa, ON	0	9.1	450.51	16.55				
PCA18P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	10.0	445.92	6.74				
PCA2P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	10.2	444.66	8.48				

PCA3P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	11.3	439.52	7.36				
PCI7P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	11.8	436.83	6.05				
S9A5R	Filamentous Fungus	Soil site 9	Gatineau, QC	0	14.2	789.83	74.60				
PCI3P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	15.6	418.14	10.46				
RW5A3P	Filamentous Fungus	Rotten wood	Ottawa, ON	0	18.2	752.71	62.06				
S4A2LC	Filamentous Fungus	Soil site 4	Ottawa, ON	0	18.7	402.60	6.76				
PCA11P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	21.3	390.07	10.19	*Paecilomyces inflatus*[2]		KY305057	
PCI8M	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	23.2	380.54	7.25				
GHA3	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	23.5	379.19	10.90				
PCA17P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	23.7	378.19	10.52				
RW3A2Pb	Filamentous Fungus	Rotten wood	Ottawa, ON	0	24.0 [6]						
S8A3Pb	Filamentous Fungus	Soil site 8	Gatineau, QC	0	24.0 [6]						
S9A3P	Filamentous Fungus	Soil site 9	Gatineau, QC	0	26.5	364.33	20.04				
S8A6ACS	Filamentous Fungus	Soil site 8	Gatineau, QC	0	26.5	364.10	21.04				
RW1I1P	Filamentous Fungus	Rotten wood	Ottawa, ON	0	27.1	671.06	44.88				
S8A1R	Filamentous Fungus	Soil site 8	Gatineau, QC	0	27.6	358.56	17.11				
GHA7	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	27.7	358.25	11.74	*Scopulariopsis brumptii*		KY305028	
S3A1R	Filamentous Fungus	Soil site 3	King City, ON	0	28.4	354.93	10.21				
S9A5P	Filamentous Fungus	Soil site 9	Gatineau, QC	0	29.0	351.88	18.34				
OA3I1P	Filamentous Fungus	Outdoor air	Ottawa, ON	0	29.1	652.70	32.74				
GWA17	Filamentous Fungus	G.P. White	Ottawa, ON	0	29.6	348.83	9.91	*Graphium*sp.[2]			
GHA6	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	29.9	347.30	17.80				
PCA8P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	30.1	346.52	19.53				
RW1A3P	Filamentous Fungus	Rotten wood	Ottawa, ON	0	31.7	529.95	86.00				

PCA14P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	32.1	336.61	11.96	*Paecilomyces inflatus*[2]		KY305080	
S8A3R	Filamentous Fungus	Soil site 8	Gatineau, QC	0	32.6	334.10	21.27				
S9A1P	Filamentous Fungus	Soil site 9	Gatineau, QC	0,A	33.4	612.93	66.60				
GWI3	Filamentous Fungus	G.P. White	Ottawa, ON	0	33.5	329.59	14.21	*Stachybotrys* sp.[2]			
PCI9P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	33.7	328.55	7.71				
S7A3CVb	Filamentous Fungus	Soil site 7	Gatineau, QC	0	35.9	317.50	18.58				
OA2I3M	Filamentous Fungus	Outdoor air	Ottawa, ON	0	36.4	585.55	33.49	*Cladosporium* sp.		KY305029	
GWA14	Filamentous Fungus	G.P. White	Ottawa, ON	0	36.6	314.27	19.25	*Paecilomyces variotii*[2]			
S7A7P	Filamentous Fungus	Soil site 7	Gatineau, QC	0	37.7	308.75	9.64				
PCA9P	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	37.8	308.40	17.03				
GHA1	Filamentous Fungus	Hay (G.P. White)	Ottawa, ON	0	38.0	307.24	11.86				
S8A5ACS1	Filamentous Fungus	Soil site 8	Gatineau, QC	0	38.0	307.21	19.47	*Pseudogymnoascus pannorum*[2]		KY305034	
S6A1R	Filamentous Fungus	Soil site 6	Ottawa, ON	0	38.1	570.07	69.50				
S8A5P	Filamentous Fungus	Soil site 8	Gatineau, QC	0	38.5	304.52	22.98				
S8A4R	Filamentous Fungus	Soil site 8	Gatineau, QC	0	38.6	304.41	18.42				
S7A5P	Filamentous Fungus	Soil site 7	Gatineau, QC	0	39.4	300.39	28.10	*Paecilomyces lilacinus*[2]			
S8I3CN	Filamentous Fungus	Soil site 8	Gatineau, QC	0	40.2	296.46	17.95				
S7A4P	Filamentous Fungus	Soil site 7	Gatineau, QC	0	40.2	296.20	6.35				
OA3I1Pb	Filamentous Fungus	Outdoor air	Ottawa, ON	0	40.6	546.90	25.83				
PCA21M	Filamentous Fungus	Plate contaminant	Ottawa, ON	0	42.9	282.81	20.54				
S4A1CV	Filamentous Fungus	Soil site 4	Ottawa, ON	0,A	43.3	522.19	39.69	*Mortierella alpina*[2]		KY305049	
S8I1ACS	Filamentous Fungus	Soil site 8	Gatineau, QC	0,A	43.6	518.60	44.00				
S8A1Cs	Filamentous Fungus	Soil site 8	Gatineau, QC	0	43.7	278.86	6.70				
LAA2P	Filamentous Fungus	Lab air	Ottawa, ON	0	43.8	517.29	38.46	*Cladosporium* sp.[2]		KY305056	

S3I1ACS	Filamentous Fungus	Soil site 3	King City, ON	0	43.9	278.03	14.30	*Paecilomyces carneus*[2]		KY305032	
OA1I5Mb	Filamentous Fungus	Outdoor air	Ottawa, ON	0	44.2	276.42	5.58	*Ramularia vizellae*[2]		KY305079	
GWA1	Filamentous Fungus	G.P. White	Ottawa, ON	0	45.3	270.88	14.72				
S6A3R	Filamentous Fungus	Soil site 6	Ottawa, ON	0	46.5	492.20	86.00				
BWA2P	Filamentous Fungus	Birch bark	Ottawa, ON	0	47.7	481.02	41.05	*Penicillium crustosum*[2]		KY305052	KY305094
S8A1CN	Filamentous Fungus	Soil site 8	Gatineau, QC	0	48.4	255.62	21.83	*Penicillium sp.*[2]			
GWA3b	Filamentous Fungus	G.P. White	Ottawa, ON	0	49.9	248.41	27.12				
QM	Filamentous Fungus	Soil	Ottawa, ON	1,A,B	50.3	246.47	36.55	*Cladosporium* sp.[2]		KY305065	
GWA8	Filamentous Fungus	G.P. White	Ottawa, ON	1	51.0	450.54	76.15				
S8A4ACS	Filamentous Fungus	Soil site 8	Gatineau, QC	1,A	52.6	436.15	72.23	*Mortierella* sp.[2]			
PCA22M	Filamentous Fungus	Plate contaminant	Ottawa, ON	1	53.7	229.34	13.66	*Phialosimplex caninus*[2]		KY305081	
GWA15	Filamentous Fungus	G.P. White	Ottawa, ON	1,B	53.9	228.37	12.75				
LAA1P	Filamentous Fungus	Lab air	Ottawa, ON	1	54.2	226.67	35.34				
GWA9	Filamentous Fungus	G.P. White	Ottawa, ON	1	54.4	419.53	25.60	*Aspergillus niger*[2]			
S6A2CV	Filamentous Fungus	Soil site 6	Ottawa, ON	1	54.5	419.05	86.00				
JB PERI-2	Filamentous Fungus	M.L. Smith	Peru	1,B	56.4	401.16	86.00	*Trichoderma harzianum 2*			
RW5A1P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,A,B	57.1	212.63	36.05				
RW1A4P	Filamentous Fungus	Rotten wood	Ottawa, ON	1	57.6	390.62	74.50				
S4A1ACS	Filamentous Fungus	Soil site 4	Ottawa, ON	1,B	58.1	385.82	45.35	*Microsphaeropsis* sp. [2]		KY305064	
RFA7P	Filamentous Fungus	Foam from Rideau River	Ottawa, ON	1,B	58.1	207.46	54.05	*Microdochium bolleyi*[2]		KY305060	
S3A2CV	Filamentous Fungus	Soil site 3	King City, ON	1	58.7	204.58	22.39				
PCA4P	Filamentous Fungus	Plate contaminant	Ottawa, ON	1	59.5	200.83	25.30	*Paecilomyces inflatus*[2]		KY305082	
GWI5	Filamentous Fungus	G.P. White	Ottawa, ON	1,B	62.2	187.26	27.19	*Penicillium chrysogenum*[2]		KY305055	KY305087
S6A1RQM	Filamentous Fungus	Soil site 6	Ottawa, ON	1	62.3	347.36	70.74				

S7A1CVb	Filamentous Fungus	Soil site 7	Gatineau, QC	1	62.8	342.13	86.00				
LAI1P	Filamentous Fungus	Lab air	Ottawa, ON	1,A,B	63.6	334.99	35.87	*Cladosporium* sp.[2]		KY305066	
CPK 882	Filamentous Fungus	M.L. Smith	Iran	1,B,C	65.0	322.26	86.00	*Trichoderma pleuroticola 2*			
S3A1CV	Filamentous Fungus	Soil site 3	King City, ON	1	65.5	317.45	75.12				
S8A2CN	Filamentous Fungus	Soil site 8	Gatineau, QC	1	65.6	170.59	18.68	*Pseudogymnoascus roseus*[2]		KY305084	
PCA5P	Filamentous Fungus	Plate contaminant	Ottawa, ON	1	65.8	169.28	7.06	*Paecilomyces inflatus*[2]		KY305083	
S4A2P	Filamentous Fungus	Soil site 4	Ottawa, ON	1	66.8	164.50	7.06				
GWA12	Filamentous Fungus	G.P. White	Ottawa, ON	1	67.0	163.65	16.06	*Simplicillium* sp. [2]		KY305078	
GWI2	Filamentous Fungus	G.P. White	Ottawa, ON	1	67.6	160.60	35.10	*Penicillium* sp.[2]		KY305033	
RFA2P	Filamentous Fungus	Foam from Rideau River	Ottawa, ON	1,A,B	67.6	160.34	37.74	*Cladosporium* sp.[2]		KY305058	
RFA1P	Filamentous Fungus	Foam from Rideau River	Ottawa, ON	1,B	67.8	296.26	70.15				
RW2A2P	Filamentous Fungus	Rotten wood	Ottawa, ON	1	68.0	294.25	86.00				
GWI6	Filamentous Fungus	G.P. White	Ottawa, ON	1	68.1	158.16	37.57				
WSF 2925	Filamentous Fungus	M.L. Smith	United States	1	68.7	287.77	86.00	*Trichoderma harzianum*			
S6A2ACSa	Filamentous Fungus	Soil site 6	Ottawa, ON	1	69.1	152.95	10.71				
RW5A2P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,B	69.7	150.13	38.88	*Cladosporium* sp.[2]		KY305063	
RFA3P	Filamentous Fungus	Foam from Rideau River	Ottawa, ON	1,B	69.9	148.94	38.35	*Cladosporium* sp.[2]		KY305059	
GWA13	Filamentous Fungus	G.P. White	Ottawa, ON	1	70.1 [6]			*Aspergillus ochraceus*[2]		KY305086	
GWA7	Filamentous Fungus	G.P. White	Ottawa, ON	1,B	71.3	264.42	56.71	*Alternaria* sp.[2]		KY305053	
OA2I6M	Filamentous Fungus	Outdoor air	Ottawa, ON	1	74.3	236.60	86.00				
S7A1ACS	Filamentous Fungus	Soil site 7	Gatineau, QC	1	74.9	230.82	86.00				
TUB F-1005	Filamentous Fungus	M.L. Smith	Brazil	1	75.3	226.97	86.00	*Trichoderma harzianum*			
BWA4P	Filamentous Fungus	Birch bark	Ottawa, ON	1,C	76.5	216.59	86.00	*Trichoderma koningiopsis*[2]		KY305042	
RW1A5P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,A,B	76.5	215.93	62.97	*Fusarium avenaceum*[2]		KY305067	

PCA13P	Filamentous Fungus	Plate contaminant	Ottawa, ON	1	76.8	114.71	8.54	*Oidiodendron* sp.[2,3]		KY305068	
GWA2	Filamentous Fungus	G.P. White	Ottawa, ON	1	77.8	203.96	86.00	*Trichoderma* sp.[2]			
RW4A3P	Filamentous Fungus	Rotten wood	Ottawa, ON	1	78.7	196.06	86.00				
JB NZ2-4	Filamentous Fungus	M.L. Smith	New Zealand	1,B,C	78.9	194.24	86.00	*Trichoderma harzianum 2*			
S8I1CN	Filamentous Fungus	Soil site 8	Gatineau, QC	1	79.4	102.00	17.86	*Lecanicillium* sp.[2,3]		KY305085	
GWA18	Filamentous Fungus	G.P. White	Ottawa, ON	1	80.9	94.82	34.76				
RFA6P	Filamentous Fungus	Foam from Rideau River	Ottawa, ON	1,B,C	81.2	92.95	26.13				
RW2A1P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,B,C	81.7	168.31	86.00	*Trichoderma* sp.[2]		KY305045	
OA2A1M	Filamentous Fungus	Outdoor air	Ottawa, ON	1,C	83.1	83.58	86.00	*Trichoderma atroviride*[2]		KY305043	
S9A3R	Filamentous Fungus	Soil site 9	Gatineau, QC	1,B,C	83.4	82.42	27.50	*Penicillium hoeksii*[2]		KY305048	
RW1I1Pa	Filamentous Fungus	Rotten wood	Ottawa, ON	1,C	84.2	145.67	49.01	*Penicillium yezoense*[2]		KY305038	KY305091
S9A1CV	Filamentous Fungus	Soil site 9	Gatineau, QC	1,C	84.3	144.92	86.00	*Mortierella hyalina*[2]		KY305040	
OA1I4M	Filamentous Fungus	Outdoor air	Ottawa, ON	1,B,C	87.4	116.39	57.15	*Alternaria alternata*[2]		KY305051	
RW4A1P	Filamentous Fungus	Rotten wood	Ottawa, ON	1	87.6	114.35	86.00				
S6A1CV	Filamentous Fungus	Soil site 6	Ottawa, ON	1,C	87.8	112.37	74.14	*Mortierella gamsii*		KY305027	
RW3A1P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,C	87.9	111.01	86.00	*Trichoderma atroviride*[2]		KY305039	
RW4A2P	Filamentous Fungus	Rotten wood	Ottawa, ON	1,B	88.1	109.51	86.00	*Trichoderma atroviride*[2]		KY305062	
PCI2P	Filamentous Fungus	Plate contaminant	Ottawa, ON	1,C	88.7	56.14	15.08	*Oidiodendron* sp.[2,3]		KY305036	
TUB F 945	Filamentous Fungus	M.L. Smith	Thailand	1,A,B,C	89.5	96.35	86.00	*Trichoderma harzianum* [2]			
PCA20P	Filamentous Fungus	Plate contaminant	Ottawa, ON	2,C	90.2	48.60	13.30	*Oidiodendron* sp.[2,3]		KY305035	
CIB T128	Filamentous Fungus	M.L. Smith	Colombia	2,C	90.5	87.36	86.00	*Trichoderma harzianum*[2]			
RMF-7876	Filamentous Fungus	M.L. Smith	Ottawa, ON	2,B,C	90.8	84.61	86.00	*Trichoderma harzianum* [2]			
S5A1CV	Filamentous Fungus	Soil site 5	Ottawa, ON	2,B,C	91.1	82.03	86.00	*Trichoderma harzianum*[2]		KY305046	
GWI1	Filamentous Fungus	G.P. White	Ottawa, ON	2,B,C	91.7	41.11	41.75	*Penicillium chrysogenum*[2]		KY305050	KY305088

S8I2ACS	Filamentous Fungus	Soil site 8	Gatineau, QC	2	92.5	37.32	19.10	*Penicillium* sp.[2,3]		KY305073	
RW1A2P	Filamentous Fungus	Rotten wood	Ottawa, ON	2,B	92.5	69.04	86.00	*Trichoderma harzianum*[2]		KY305061	
OA1I1M	Filamentous Fungus	Outdoor air	Ottawa, ON	2,C	93.6	31.95	20.98	*Phoma* sp.[2]		KY305031	
S8A1ACSa	Filamentous Fungus	Soil site 8	Gatineau, QC	2	93.7	31.00	14.31	*Penicillium janczewskii*[2]		KY305069	
S8A3Cs	Filamentous Fungus	Soil site 8	Gatineau, QC	2	95.3	23.53	20.23	*Penicillium canescens* [2]		KY305071	KY305092
GWI4	Filamentous Fungus	G.P. White	Ottawa, ON	2,B	95.5	41.36	45.14	*Penicillium chrysogenum*[2]		KY305054	KY305093
S8A1ACSb	Filamentous Fungus	Soil site 8	Gatineau, QC	2	95.7	21.34	18.22	*Penicillium janczewskii*[2]		KY305070	
S8A1ACS	Filamentous Fungus	Soil site 8	Gatineau, QC	2	95.8	21.04	26.92	*Penicillium yarmokense*[2]		KY305077	KY305090
RW6A1P	Filamentous Fungus	Rotten wood	Ottawa, ON	2,C	95.8	38.65	28.28	*Boeremia exigua*[2]		KY305037	
S7I1ACS	Filamentous Fungus	Soil site 7	Gatineau, QC	2,B,C	97.5	22.69	80.17	*Trichoderma koningii*[2]		KY305047	
S9A2R	Filamentous Fungus	Soil site 9	Gatineau, QC	2	98.8	5.72	40.90	*Penicillium hoeksii*[2]		KY305076	
S8I2R	Filamentous Fungus	Soil site 8	Gatineau, QC	2	99.0	5.03	20.79	*Penicillium janczewskii*[2]		KY305074	
TUB F 703	Filamentous Fungus	M.L. Smith	India	2,B,C	99.5	4.65	86.00	*Trichoderma* sp.[2]			
S8I1R	Filamentous Fungus	Soil site 8	Gatineau, QC	2	99.7	1.46	25.99	*Penicillium janczewskii*[2]		KY305072	
RW1A6P	Filamentous Fungus	Rotten wood	Ottawa, ON	2,B,C	100.0	0.00	86.00	*Botrytis cinerea*[2]		KY305044	
RW3A2Pa	Filamentous Fungus	Rotten wood	Ottawa, ON	2,B	100.0	0.00	86.00				
S3A2ACS	Filamentous Fungus	Soil site 3	King City, ON	2,C	100.0	0.00	86.00	*Trichoderma* sp.[2,3]		KY305041	
S9A1R	Filamentous Fungus	Soil site 9	Gatineau, QC	2	100.0	0.00	33.10	*Penicillium* sp.[2,3]		KY305075	KY305089
S2A1LC	Yeast	Soil site 2	Ottawa, ON	0	24.5	390.26	6.44				
RW3I1b	Yeast	Rotten wood	Ottawa, ON	0	30.0	361.99	8.97				
BY	Yeast	Dishwasher (G.P. White)	Ottawa, ON	0	34.1 [6]			*Exophiala dermatitidis*[2]		KY305030	
RFA4P1	Yeast	Foam from Rideau River	Ottawa, ON	0	34.1 [6]						
RFA8P	Yeast	Foam from Rideau River	Ottawa, ON	0	40.2	550.27	30.53				
S8I4P	Yeast	Soil site 8	Gatineau, QC	0	41.7	301.29	7.32				
OA1I5M	Yeast	Outdoor air	Ottawa, ON	1	53.7	239.35	9.87				

BWA3P	Yeast	Birch bark	Ottawa, ON	1,C	61.9	196.93	10.03	*Cystofilobasidium capitatum*		KY305024	
9950	Yeast	M.L. Smith	Ottawa, ON	1	85.0	77.62	10.76	*Candida utilis*			
OA2I4M	Yeast	Outdoor air	Ottawa, ON	1,C	87.6	63.94	24.68	*Aureobasidium pullulans*		KY305026	
OA1I2M	Yeast	Outdoor air	Ottawa, ON	1,C	88.8	57.74	25.77	*Aureobasidium* sp.		KY305025	
RW3I1a	Yeast	Rotten wood	Ottawa, ON	2,C	93.7	32.55	15.91	*Cystofilobasidium capitatum*		KY305023	
RW1A1P1	Yeast	Rotten wood	Ottawa, ON	2,C	96.9	16.00	13.04	*Cystofilobasidium* sp.		KY305022	

[1]Site1: cattails and soil from a frozen cattail marsh, Site 2: sediment from a thawing pond, Site 3: frozen soil from a mixed forest, Site 4: frozen soil from a Eastern White Cedar forest, Site 5: thawed soil from an ephemeral pond, Site 6: thawed soil from a pine forest, Site 7: thawed and frozen soil from a grass field, Site 8: soil from rock outcropping, Site 9: thawed soil from a maple forest.

[2]Genetic identification was supplemented with morphological examination.

[3]Morphology and/or sequencing data suggests that this is an undescribed species.

[4]Percent inhibition was calculated as $(1 - \text{Area}_{\text{treatment}}/\text{Area}_{\text{control}}) \times 100$. The average *P. destructans* area in no-antagonist controls was 307 mm^2 for filamentous fungi and actinobacteria on small plates with 2 stamps of *P. destructans*, 920 mm^2 for filamentous fungi on large plates with 3 stamps, and 517 mm^2 for bacteria and yeast on small plates with 2 stamps.

[5]Diameter for bacterial and yeast isolates refers to the sum of the width of both streaks through *P. destructans* inoculum.

[6]Day 14 photographs were not available to quantify. Rank is based on a qualitative assessment at the time of bioassay and percent inhibition corresponds to the average percent inhibition for the given rank.

Appendix 2. Most probable identifications for each volatile compound detected through gas chromatography-mass spectrometry analysis of fungal and bacterial antagonists. Antagonists were inoculated on 3-ml PDA slants inside of headspace jars and volatiles were identified. Also listed is the corresponding retention time, molecular formula, match factor (MF), reverse match factor (RMF), probability of match (Prob), and in library (InLib) score for each proposed identification. Italicized entries were tested against *P. destructans* in further assays.

Antagonist	Retention time (min)	Most probable identification	Molecular Formula	MF, RMF	Prob	InLib Score
Cystofilobasidium capitatum RW3I1a	5.76	Ethanol, 2-(1-methylethoxy)-	$C_5H_{12}O_2$	808, 808	63.5%	-240
	6.23	*1-Propanol, 2-methyl-*	*$C_4H_{10}O$*	*890, 940*	*86.5%*	*191*
	7.93	*1-Butanol, 2-methyl-*	*$C_5H_{12}O$*	*933, 933*	*69.2%*	*113*
	20.83	12-Crown-4	$C_8H_{16}O_4$	588, 620	25.2%	-1424
	22.35	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	$C_{19}H_{25}NO_5$	771, 884	21.1%	-539
Pantoea sp. OA1I3M	5.76	Hexane, 3-methoxy	$C_7H_{16}O$	656, 782	32.1%	-1103
	6.22	*1-Propanol, 2-methyl-*	*$C_4H_{10}O$*	*832, 878*	*73.5%*	*-161*
	7.93	1-Pentanol	$C_5H_{12}O$	891, 893	29.1%	-138
	9.36	Urea, ethyl-	$C_3H_8N_2O$	772, 841	61.9%	-324
Pantoea ananatis RFA4P2	5.76	2-Propanone, 1-(1-methylethoxy)-	$C_6H_{12}O_2$	685, 873	32.5%	-941
	6.23	*Propanoic acid*	*$C_3H_6O_2$*	*891, 891*	*70.5%*	*146*
	7.94	*1-Pentanol*	*$C_5H_{12}O$*	*939, 943*	*52.5%*	*184*
	9.36	Morpholine, 4-[[(dimethylamino)thioxomethyl]thio]-	$C_7H_{14}N_2OS_2$	811, 811	29.1%	-371
Oidiodendron sp. PCA20P	n/a	n/a	n/a	n/a	n/a	n/a
PDA-only control	5.76	Acetone, ethyl methyl acetal	$C_6H_{14}O_2$	704, 766	76.6%	-190

Appendix 3. Inhibition scores for 36 selected antagonists against different *Pseudogymnoascus* species. Two strains of *P. destructans* and one strain each of *P. roseus* and *P. pannorum* were used. Percent inhibition was calculated as (1 - $Area_{control}$ / $Area_{experimental}$) × 100. *Pseudogymnoascus* spp. areas refer to the total area of *Pseudogymnoascus* spp. 14 days after inoculation. Antagonists were ranked as: 0 = negligible (< 50%) inhibition, 1 = considerable (50% - 85%) inhibition, 2 = nearly complete/complete (> 85%) inhibition, relative to a no-antagonist control. Additionally, if applicable, ranks were qualified with: A = growth of the antagonist is limited by *Pseudogymnoascus* spp., B = the antagonist grew over colonies such that affected *Pseudogymnoascus* spp. colonies were no longer visible, C = *Pseudogymnoascus* spp. colonies were present, but uniformly smaller than in the control plate.

Antagonist	*P. destructans*						*P. roseus*			*P. pannorum*		
	SH864			SH991			S8A2CN			S8A5ACS1		
	***P. destructans* Area (mm^2)**	**Percent Inhibition**	**Rank**	***P. destructans* Area (mm^2)**	**Percent Inhibition**	**Rank**	***P. roseus* Area (mm^2)**	**Percent Inhibition**	**Rank**	***P. pannorum* Area (mm^2)**	**Percent Inhibition**	**Rank**
14807	5.62	98.9	2C	72.03	87.8	2C	805.96	22.2	0A	865.09	19.7	0A
APCI2P	0.00	100.0	2	0.00	100.0	2	241.99	76.6	1	487.66	54.7	1
B-14324	0.60	99.9	2	4.74	99.2	2	729.72	29.5	0A	538.86	50.0	0A
B9-5	35.94	93.3	2	68.42	88.4	2	806.20	22.1	0	657.35	39.0	0A
GWA3a	2.23	99.6	2	5.00	99.2	2	601.37	41.9	0	468.99	56.5	1
GWA3c	0.00	100.0	2	5.99	99.0	2	468.12	54.8	1	536.53	50.2	1
IC10	58.16	89.1	2	92.21	84.4	1	710.87	31.3	0	527.38	51.0	1A
M9-14	42.72	92.0	2	122.17	79.3	1	814.32	21.4	0	571.57	46.9	0
M9-2	48.10	91.0	2	140.63	76.2	1	831.85	19.7	0	687.24	36.2	0
M9-4	42.48	92.1	2	48.48	91.8	2	669.81	35.3	0A	739.85	31.3	0A
M9-9	78.31	85.4	2	77.83	86.8	2	402.49	61.1	1A	698.67	35.1	0
OA1I3M	7.08	98.7	2	22.25	96.2	2C	496.67	52.0	1	459.50	57.3	1
PCA13P	187.80	64.9	1	329.35	44.3	0	893.31	13.7	0	967.29	10.2	0
PCA20P	24.16	95.5	2	29.80	95.0	2	892.63	13.8	0	945.29	12.2	0
PCI2P	53.23	90.0	2	72.94	87.7	2	1018.52	1.6	0	922.27	14.4	0
RFA4P2	20.25	96.2	2C	6.72	98.9	2C	617.23	40.4	0	704.14	34.6	0
RW1A1P1	54.60	89.8	1C	78.04	86.8	2C	698.48	32.5	0	791.66	26.5	0
RW1A2P	36.73	95.4	2C	139.59	84.3	1C	1193.26	23.2	0A	1553.52	3.8	0A
RW3A2Pa	0.00	100.0	2	0.00	100.0	2	0.00	100.0	2	728.03	54.9	1A
RW3I1a	19.31	96.4	2C	39.80	93.3	2C	595.26	42.5	0	595.90	44.7	0

Antagonist	*P. destructans*						*P. roseus*			*P. pannorum*		
	SH864			SH991			S8A2CN			S8A5ACS1		
	P. destructans Area (mm^2)	Percent Inhibition	Rank	*P. destructans* Area (mm^2)	Percent Inhibition	Rank	*P. roseus* Area (mm^2)	Percent Inhibition	Rank	*P. pannorum* Area (mm^2)	Percent Inhibition	Rank
RW4A2P	0.00	100.0	2B	0.00	100.0	2B	506.51	67.4	1	490.43	69.6	1
RW6A1P	3.70	99.5	2	10.60	98.8	2C	1258.45	19.0	0	1194.88	26.0	0
S4I1ACS	8.91	98.3	2	11.18	98.1	2	487.40	52.9	1	531.98	50.6	1
S5A2LC	4.17	99.2	2	19.98	96.6	2	782.98	24.4	0	581.96	46.0	0
S6A3ACS	0.00	100.0	2	0.00	100.0	2	493.51	52.3	1	647.11	39.9	0
S8A1ACS	4.24	99.2	2	28.10	95.2	2	805.08	22.2	0	863.74	19.8	0
S8A1ACSa	1.75	99.7	2	11.67	98.0	2	887.94	14.2	0	774.39	28.1	0
S8A1ACSb	1.81	99.7	2	15.34	97.4	2	797.24	23.0	0	719.64	33.2	0
S8A1Cs1b	0.00	100.0	2	0.00	100.0	2	154.77	85.1	2	158.72	85.3	2
S8A3Cs	10.64	98.0	2	45.19	92.4	2	365.62	64.7	1	861.00	20.1	0
S8A4Cs	0.00	100.0	2	0.00	100.0	2	481.33	53.5	1	585.57	45.6	0
S8I1R	2.78	99.5	2	7.82	98.7	2	738.41	28.7	0	149.85	86.1	2
S8I2ACS	31.82	94.1	2	21.12	96.4	2	510.14	50.7	1	549.07	49.0	0
S8I2R	2.31	99.6	2	16.82	97.2	2	942.53	9.0	0	769.21	28.6	0
S9A1R	0.00	100.0	2	5.78	99.0	2	752.16	27.4	0	862.07	20.0	0
S9A2R	41.77	92.2	1	149.04	74.8	1	1112.22	-7.4	0	448.39	58.4	1

Appendix 4. Inhibitory concentrations for the filtered, spent media from each of 35 antagonist cultures. Antagonists were grown at 13 ± 1 °C for 28 days and screened against three strains of *Pseudogymnoascus destructans* and one strain each of *P. roseus*, *P. pannorum*, and *Saccharomyces cerevisiae*. Inhibitory concentrations were recorded after 14 days of *Pseudogymnoascus* growth or 3 days of *S. cerevisiae* growth and are expressed relative to the original concentration of the day 28 antagonist medium. NI indicates that complete inhibition was not evident even at highest concentration of 2.5× antagonist medium and ND indicates that inhibitory concentration was not assessed.

	P. destructans			*P. roseus*	*P. pannorum*	*S. cerevisiae*
Antagonist	**US15**	**SH864**	**SH991**	**S8A2CN**	**S8A5ACS1**	**S288C**
14807	NI	2.5	NI	2.5	NI	NI
APCI1P	2.5	NI	NI	NI	NI	NI
APCI2P	0.08	0.08	0.08	0.16	0.16	ND
BWA3P	NI	NI	NI	NI	NI	ND
GWA3a	NI	NI	NI	NI	NI	NI
GWA3c	NI	NI	NI	NI	NI	ND
OA1I1M	0.08	0.08	0.08	NI	NI	NI
OA1I3M	NI	2.5	NI	2.5	NI	NI
OA1I5Mb	NI	NI	NI	NI	NI	NI
PCA13P	NI	NI	NI	NI	NI	ND
PCA20P	NI	NI	NI	NI	NI	ND
PCA22M	NI	NI	NI	NI	NI	ND
PCA5P	0.31	0.08	0.16	2.5	NI	NI
PCI2P	NI	NI	NI	NI	NI	ND
RFA4P2	NI	1.25	2.5	1.25	NI	ND
RFA6P	0.63	0.31	0.31	NI	NI	ND
RW1A1P1	NI	NI	NI	NI	NI	ND
RW1A2P	0.63	0.16	0.16	2.5	NI	NI
RW3A2Pa	0.01	<0.005	0.01	0.63	1.25	ND
RW3I1a	NI	NI	NI	NI	NI	NI
RW4A2P	0.63	0.16	0.31	1.25	1.25	2.5
S4I1ACS	NI	NI	NI	NI	NI	ND
S5A2LC	NI	NI	NI	NI	NI	NI
S6A3ACS	NI	NI	NI	NI	NI	NI
S8A1ACS	NI	NI	NI	NI	NI	ND
S8A1ACSa	0.63	0.63	0.63	NI	NI	ND
S8A1ACSb	NI	NI	NI	NI	NI	NI
S8A1Cs1b	NI	NI	NI	NI	NI	NI
S8A4Cs	0.31	0.16	0.16	NI	NI	NI
S8I1CN	2.5	1.25	1.25	NI	NI	NI
S8I1R	2.5	NI	NI	NI	NI	NI
S8I2ACS	1.25	0.63	0.63	2.5	NI	NI
S8I2R	NI	2.5	NI	NI	NI	NI
S9A1R	2.5	1.25	2.5	NI	NI	ND
S9A2R	NI	NI	NI	NI	NI	ND

Appendix 5. Inhibition of plant pathogens by cell-free broth from different microbial antagonistic strains. Numbers reflect the concentration, relative to the original broth, required for complete inhibition of the pathogen, and >2.5 reflects that no inhibition was seen at the highest concentration. Day indicates how many days the pathogens grew before recording the growth and ND indicates that inhibition was not assessed.

Broth antagonist strain	***V. dahliae* 176**	***B. cinerea* 231370**	***B. cinerea* 189076**	***A. solani* 229590**	***C. parasitica* EP155**
Day	***3***	***3***	***5***	***3***	***5***
Alternaria alternata OA1I4M	0.625	>2.5	>2.5	>2.5	>2.5
Aureobasidium pullulans OA2I4M	>2.5	>2.5	>2.5	>2.5	>2.5
Bacillus sp. 14807	>2.5	0.078	>2.5	0.313	0.010
Bacillus sp. 55407	>2.5	0.625	>2.5	0.625	0.156
Bacillus sp. APCI1P	>2.5	0.625	>2.5	0.625	0.313
Bacillus sp. B-14324	>2.5	0.313	>2.5	0.625	0.156
Bacillus subtilis S9A1Cs1	>2.5	>2.5	*0.156*	>2.5	*0.156*
Boeremia exigua RW6A1P	>2.5	>2.5	>2.5	>2.5	>2.5
Elsinoë brasiliensis CABI 395825	>2.5	>2.5	>2.5	>2.5	>2.5
Lecanicillium sp. S8I1CN	<0.002	<0.002	<0.002	>2.5	<0.002
Paecilomyces inflatus PCA5P	>2.5	>2.5	>2.5	>2.5	>2.5
Pantoea ananatis RFA4P2	>2.5	0.313	>2.5	0.625	0.313
Pantoea sp. OA1I3M	>2.5	0.625	>2.5	0.625	0.313
Penicillium canescens S8A3CS	0.156	>2.5	0.156	1.250	1.250
Penicillium janczewskii S8A1ACSa	>2.5	>2.5	>2.5	>2.5	>2.5
Penicillium janczewskii S8I2R	>2.5	>2.5	>2.5	>2.5	1.250
Penicillium sp. S8I2ACS	0.156	>2.5	>2.5	1.250	0.078
Penicillium sp. S9A1R	>2.5	>2.5	>2.5	>2.5	>2.5
Penicillium yarmokense S8A1ACS	0.313	1.250	1.250	>2.5	1.250
Phoma sp. OA1I1M	>2.5	>2.5	>2.5	>2.5	>2.5
Pseudogymnoascus roseus S8A2CN	>2.5	>2.5	>2.5	>2.5	>2.5
Pseudomonas sp. APCI2P	>2.5	0.625	>2.5	0.625	0.156
Ramularia vizellae OA1I5Mb	>2.5	>2.5	>2.5	>2.5	>2.5
RFA6P	0.625	0.313	0.313	>2.5	0.625
RW3A2Pa	0.078	0.625	0.156	0.156	0.625
S4A2P	>2.5	>2.5	>2.5	>2.5	>2.5
S8I1ACS	0.156	>2.5	>2.5	>2.5	>2.5
Simplicillium sp. GWA12	>2.5	>2.5	0.156	>2.5	1.250
Sphingobium sp. S8A4Cs	>2.5	>2.5	>2.5	>2.5	1.250
Streptomyces laeteviolaceus S6A3ACS	>2.5	>2.5	>2.5	>2.5	>2.5
Trichoderma atroviride RW4A2P	0.156	0.156	0.078	>2.5	0.078
Trichoderma harzianum RW1A2P	0.625	>2.5	0.625	>2.5	1.250
Trichoderma sp. S3A2ACS	0.625	0.625	0.313	>2.5	0.625
10 × LB	>2.5	>2.5	>2.5	>2.5	1.250
10 × PDB	>2.5	>2.5	>2.5	>2.5	>2.5
2 mg ml^{-1} Hygromycin B	<0.002	<0.002	0.156	<0.002	<0.002

Broth antagonist strain	***F. avenaceum* RW1A5P**	***B. cinerea* RW1A6P**	***S. sclerotiorum* 241671**	***A. alternata* 196949**	***P. syringae* 4326**
Day	***3***	***3***	***3***	***3***	***3***
Alternaria alternata OA1I4M	>2.5	>2.5	>2.5	>2.5	>2.5
Aureobasidium pullulans OA2I4M	>2.5	>2.5	>2.5	>2.5	>2.5
Bacillus sp. 14807	>2.5	0.039	0.313	>2.5	0.625
Bacillus sp. 55407	>2.5	>2.5	0.313	>2.5	0.625
Bacillus sp. APCI1P	>2.5	>2.5	0.625	>2.5	0.625
Bacillus sp. B-14324	>2.5	0.625	0.313	>2.5	0.625
Bacillus subtilis S9A1Cs1	>2.5	>2.5	>2.5	>2.5	>2.5
Boeremia exigua RW6A1P	>2.5	>2.5	>2.5	>2.5	0.625
Elsinoë brasiliensis CABI 395825	>2.5	>2.5	>2.5	>2.5	>2.5
Lecanicillium sp. S8I1CN	0.156	<0.002	0.005	0.010	0.156
Paecilomyces inflatus PCA5P	>2.5	>2.5	>2.5	>2.5	>2.5
Pantoea ananatis RFA4P2	>2.5	0.625	0.313	>2.5	0.625
Pantoea sp. OA1I3M	>2.5	>2.5	0.625	>2.5	0.625
Penicillium canescens S8A3CS	0.625	1.250	1.250	1.250	0.156
Penicillium janczewskii S8A1ACSa	>2.5	>2.5	>2.5	>2.5	>2.5
Penicillium janczewskii S8I2R	>2.5	>2.5	>2.5	>2.5	>2.5
Penicillium sp. S8I2ACS	>2.5	>2.5	>2.5	1.250	1.250
Penicillium sp. S9A1R	>2.5	>2.5	>2.5	>2.5	1.250
Penicillium yarmokense S8A1ACS	0.625	>2.5	1.250	1.250	0.313
Phoma sp. OA1I1M	>2.5	>2.5	>2.5	>2.5	0.625
Pseudogymnoascus roseus S8A2CN	>2.5	>2.5	>2.5	>2.5	>2.5
Pseudomonas sp. APCI2P	>2.5	0.625	0.625	>2.5	0.625
Ramularia vizellae OA1I5Mb	>2.5	>2.5	>2.5	>2.5	>2.5
RFA6P	>2.5	0.313	0.625	>2.5	0.625
RW3A2Pa	0.313	0.156	0.156	0.625	0.078
S4A2P	>2.5	>2.5	>2.5	>2.5	0.625
S8I1ACS	>2.5	>2.5	>2.5	>2.5	0.625
Simplicillium sp. GWA12	>2.5	>2.5	>2.5	>2.5	>2.5
Sphingobium sp. S8A4Cs	>2.5	>2.5	>2.5	>2.5	0.625
Streptomyces laeteviolaceus S6A3ACS	>2.5	>2.5	>2.5	>2.5	0.625
Trichoderma atroviride RW4A2P	0.625	0.156	0.313	>2.5	0.078
Trichoderma harzianum RW1A2P	>2.5	>2.5	0.625	>2.5	1.250
Trichoderma sp. S3A2ACS	>2.5	0.625	>2.5	>2.5	0.625
10 × LB	>2.5	>2.5	>2.5	>2.5	0.625
10 × PDB	>2.5	>2.5	>2.5	>2.5	>2.5
2 mg ml^{-1} Hygromycin B	<0.002	<0.002	<0.002	<0.002	0.020

Broth antagonist strain	*C. parasitica* P88-8	*E. brasiliensis* 395825	*C. parasitica* P74-3	*F. oxysporum* 215464	*P. syringae* Pst 3000	*A. solani* 229596
Day	***5***	***5***	***6***	***3***	***3***	***5***
Alternaria alternata OA1I4M	>2.5	>2.5	>2.5	>2.5	ND	ND
Aureobasidium pullulans OA2I4M	>2.5	>2.5	>2.5	>2.5	0.313	1.250
Bacillus sp. 14807	0.039	0.313	0.010	>2.5	>2.5	0.313
Bacillus sp. 55407	0.156	0.313	0.156	>2.5	>2.5	0.156
Bacillus sp. APCI1P	0.156	0.313	0.156	>2.5	>2.5	>2.5
Bacillus sp. B-14324	0.156	0.313	0.020	>2.5	0.625	0.313
Bacillus subtilis S9A1Cs1	0.078	0.313	0.078	>2.5	>2.5	0.313
Boeremia exigua RW6A1P	>2.5	>2.5	>2.5	>2.5	1.250	>2.5
Elsinoë brasiliensis CABI 395825	>2.5	>2.5	1.250	>2.5	>2.5	>2.5
Lecanicillium sp. S8I1CN	<0.002	<0.002	<0.002	>2.5	0.313	<0.002
Paecilomyces inflatus PCA5P	>2.5	>2.5	>2.5	>2.5	0.625	>2.5
Pantoea ananatis RFA4P2	0.313	0.313	0.156	>2.5	>2.5	0.313
Pantoea sp. OA1I3M	0.313	0.313	0.156	>2.5	>2.5	0.625
Penicillium canescens S8A3CS	0.625	1.250	0.625	1.250	>2.5	1.250
Penicillium janczewskii S8A1ACSa	>2.5	>2.5	>2.5	>2.5	1.250	>2.5
Penicillium janczewskii S8I2R	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
Penicillium sp. S8I2ACS	0.078	0.156	0.078	>2.5	0.625	0.625
Penicillium sp. S9A1R	>2.5	>2.5	>2.5	>2.5	0.625	>2.5
Penicillium yarmokense S8A1ACS	1.250	1.250	1.250	1.250	0.625	1.250
Phoma sp. OA1I1M	>2.5	>2.5	>2.5	1.250	1.250	>2.5
Pseudogymnoascus roseus S8A2CN	>2.5	>2.5	1.250	>2.5	>2.5	>2.5
Pseudomonas sp. APCI2P	0.156	0.313	0.078	>2.5	>2.5	1.250
Ramularia vizellae OA1I5Mb	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
RFA6P	0.039	0.625	0.020	1.250	0.625	0.625
RW3A2Pa	0.625	0.156	0.625	0.625	0.078	>2.5
S4A2P	>2.5	>2.5	>2.5	>2.5	1.250	>2.5
S8I1ACS	>2.5	>2.5	0.625	>2.5	0.625	>2.5
Simplicillium sp. GWA12	>2.5	1.250	>2.5	>2.5	0.020	0.625
Sphingobium sp. S8A4Cs	1.250	1.250	0.625	>2.5	>2.5	1.250
Streptomyces laeteviolaceus S6A3ACS	>2.5	>2.5	>2.5	>2.5	1.250	>2.5
Trichoderma atroviride RW4A2P	0.078	0.156	0.078	>2.5	0.156	0.625
Trichoderma harzianum RW1A2P	1.250	>2.5	1.250	>2.5	0.625	0.625
Trichoderma sp. S3A2ACS	0.625	0.625	0.625	>2.5	0.039	0.625
10 × LB	1.250	>2.5	>2.5	>2.5	>2.5	0.625
10 × PDB	>2.5	>2.5	>2.5	>2.5	1.250	>2.5
2 mg ml^{-1} Hygromycin B	<0.002	<0.002	<0.002	0.005	0.156	<0.002

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