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# Biological and Chemical Control Options for Geomyces Destructans and Characterization of Physiological Responses to Control Efforts

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BIOLOGICAL AND CHEMICAL CONTROL OPTIONS FOR *GEOMYCES DESTRUCTANS* AND  
CHARACTERIZATION OF PHYSIOLOGICAL RESPONSES TO CONTROL EFFORTS

by

CHRISTOPHER T. CORNELISON

Under the Direction of Sidney A. Crow Jr.

ABSTRACT

The recently identified causative agent of White-Nose Syndrome (WNS), *Geomyces destructans*, has been responsible for the mortality of an estimated 5.7 million North American bats since its emergence in 2006. A primary focus of the National Response Plan, established by US Fish and Wildlife in 2011, was the identification of biological and chemical control options. In an effort to identify potential biological and chemical control options for WNS, six previously described bacterially produced volatile organic compounds (VOCs) and multiply induced *Rhodococcus rhodochrous* DAP96253 were screened for anti-*Geomyces destructans* activity. *Geomyces destructans* conidia and mycelial plugs were exposed to the VOCs and induced *Rhodococcus* in a closed air space at 15°C and 4°C and evaluated for inhibition of conidia germination and mycelial extension. Additionally, *in situ* application methods for induced *Rhodococcus*, such as fixed cell catalyst and fermentation cell paste in non-growth conditions, were screened with positive results. *Rhodococcus* was assayed for *ex vivo* activity via exposure to bat tissue ex-

plants inoculated with *G. destructans* conidia. All VOCs inhibited radial growth of mycelial plugs and growth from conidia at both temperatures, with the greatest effect at low temperature (4°C). Induced *Rhodococcus* completely inhibited growth from conidia at 15°C and had a strong fungistatic effect at 4°C. Induced *Rhodococcus* inhibited *Geomyces destructans* growth from conidia when cultured in a shared air space with bat tissue explants inoculated with *Geomyces destructans* conidia. During the evaluation diffusible brown pigment was observed in *G. destructans* cultures exposed to induced *Rhodococcus* or select VOCs. The pigment was induced by light and oxidative challenge and hypothesized to be melanin. Traditional microbiological methods, as well as copper sulfide-silver staining and ultraviolet-visible spectroscopy, were utilized to confirm this hypothesis. This was a noteworthy result as melanin is a known virulence factor in other pathogenic fungi and may play a significant role in WNS. The identification of bacterially produced VOCs and inducible biological agents with anti-*Geomyces destructans* activity expands the pool of potential biological and chemical control options for WNS and provides wildlife management personnel with tools to combat this devastating disease.

INDEX WORDS: White-Nose Syndrome, Conidia, Mycelia, *Geomyces*, VOC, Fungistasis, Melanin, Biocontrol

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## **DEDICATION**

I would like to dedicate this dissertation to my grandmother, Donella Lowery, Ph.D. She taught me an appreciation for the natural world that is still my fundamental motivation for seeking a career in the natural sciences. Thank you for taking your grandson to national parks and museums when most children were going to movie theaters and water parks.

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First, I would like to express my sincere gratitude to Dr. Crow for allowing a young masters student to join his lab, fail, and try again. Without your mentorship I would not be the person I am today. Your willingness to allow me to learn for myself while still supporting my scientific and professional development has given me all the tools for success and the confidence to pursue my career aspirations. I would also like to acknowledge my family for their support throughout this entire experience. You have always done everything you can for me and I am very grateful for it. I would also like to thank my lovely partner, Ashley. Her support throughout this process has been amazing. I truly would not have been able to accomplish any of my goals without you. I would also like to sincerely thank my colleagues in the Pierce and Crow labs including Sarah Boyd, Kyle Gabriel, Courtney Barlament, Shashak Dakur, Ben Poodiak, Blake Cherney, Sup Du, Cui Wang, Yao Yao Liu, Katie Swensen, Ian Sarad, and everyone else who has participated in this project. This journey would not have been the same without you. I would also like to thank all the GSU and external faculty who have been so generous with their time and advice including Dr. Ahearn, Dr. Pierce, Dr. Elder, Dr. Gilbert, Dr. Chin, Dr. Simmons, Dr. Keel and Dr. Maxwell. I would also like to thank Dennis Krusak of the US Forest Service for believing in this project and working to levy support when we needed it most. I would also like to thank Katie Gillies and the rest of the team at Bat Conservation International for all the support you have given to this project. You were willing to support my research when others had already passed, I will always be grateful for that opportunity.



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**LIST OF ABBREVIATIONS**

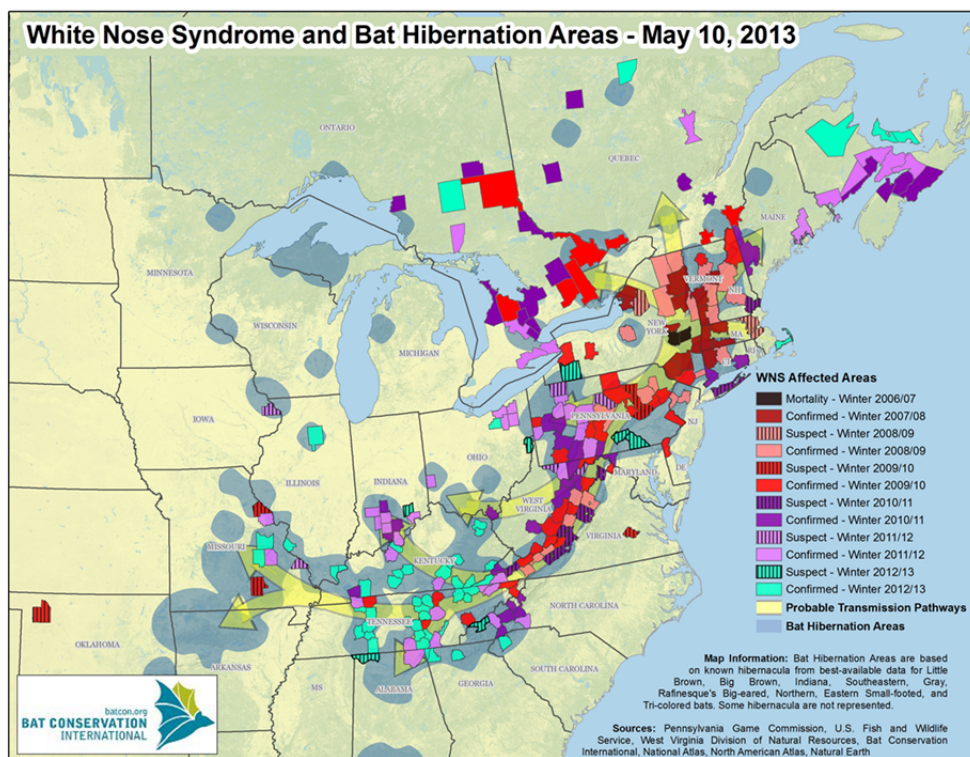
<b>ARS</b>	Agricultural Research Service (USDA)
<b>BCI</b>	Bat Conservation International
<b>DI</b>	De-ionized (water)
<b>DPI</b>	Days post inoculation
<b>GC</b>	Gas chromatography
<b>GSU</b>	Georgia State University
<b>L-DOPA</b>	L-3,4-dihydroxyphenylalanine
<b>M</b>	Molar
<b>mL</b>	Milliliter
<b>mM</b>	Millimolar
<b>mm</b>	Millimeter
<b>MS</b>	Mass spectrophotometry
<b>μM</b>	Micromolar
<b>μL</b>	Microliter
<b>NIST</b>	National Institute of Science and Technology
<b>nm</b>	Nanometer
<b>QGA-MS</b>	Quantitative Gas Analyzer Mass Spectrophotometer
<b>SDA</b>	Sabouraud Dextrose Agar
<b>SDB</b>	Sabouraud Dextrose Broth
<b>SEM</b>	Secondary electron multiplier (MS)
<b>TD</b>	Thermal de-absorption (MS)
<b>TE</b>	Tris/EDTA (buffer)
<b>UGA</b>	University of Georgia
<b>USDA</b>	United States Department of Agriculture
<b>USFS</b>	United States Forest Service

<b>USFWS</b>	United States Fish and Wildlife Service
<b>UV-VIS</b>	Ultraviolet-Visible (spectrophotometry)
<b>VOC</b>	Volatile organic compound
<b>WNS</b>	White-Nose Syndrome
<b>YEMEA</b>	Yeast extract /Malt extract agar

## 1 INTRODUCTION

### 1.1 History of White-Nose Syndrome and potential impacts

White-nose syndrome (WNS) was first identified near Albany, New York, in 2006 [21]. Since its discovery WNS has been responsible for severe declines in bat populations in the Eastern United States and Canada, with estimates of more than 5 million deaths from the disease since 2006 (Fig. 1.1) [6, 19]. Although the exact ecological and economic impact of this disease has yet to be determined, many researchers agree that continued declines in insectivorous bat populations will have a significant impact on forest management, agriculture, and insect borne disease [6, 9].



**Figure 1.1** The spread of WNS in North America since emergence in 2006. Map courtesy of Bat Conservation International.



The biodiversity<sup>2</sup> of bats has noteworthy inherent value as they are the most diverse order (Chiroptera) of mammals, representing 20% of known mammalian species [6, 7]. This high diversity may provide significant evolutionary insight and represents the most significant speciation observed in mammals. Despite the tremendous value associated with the biodiversity of bats, many scientists and legislators seek an economic value to associate with WNS in order to gain support for continued efforts to combat this rapidly spreading wildlife epidemic.

In 2010, the economic contributions of bats to North American agriculture were estimated at \$50 billion annually [7] and may represent a significant contribution to pest control and zoophily<sup>20</sup> of regionally significant agricultural and forest products [7, 17]. During peak summer feeding, a small brown bat (*Myotis lucifugus*) can consume its body weight equivalent (~10 g) of insects in a single night [6, 7]. This level of insectivorous activity may serve as a critical ecosystem service in regions with significant agricultural industries such as the Midwestern United States. Accordingly, if WNS continues to spread as predicted and mortality rates in WNS positive hibernacula<sup>10</sup> are not reduced, significant economic impacts may be seen on North American agricultural efforts [17].

In addition to pest control, many species of bats serve as pollinators for nocturnally blooming plants and trees that may have limited natural pollinators [17, 36]. Although scientific studies on the impact of chiropteraphily<sup>3</sup> in temperate North America are lacking, findings from studies in tropical regions indicate that bat-mediated nocturnal pollination serves a critical role in these ecosystems, particularly for fruit bearing vegetation [36]. Continued declines in bat populations could result in major impacts to forest services, as the combined impact of increased pest insects and reduced pollination success may result in synergistic effects compounding the broader ecological impact of WNS in North America.

Although current hypotheses support the idea that *G. destructans* is endemic to Northern Europe and was inadvertently transported to North America by recreational cavers in 2005, European bat populations do not suffer the extreme mortality rates associated with WNS in North American bats. While recent

studies are attempting to understand the difference in susceptibility of North American and European bats, many questions remain regarding this discrepancy. Due to the dormant immune function associated with torpor<sup>18</sup>, immunological adaptations are inadequate at explaining this difference in mortality. A hypothesis is emerging from the scientific community that suggests this discrepancy may be due to social behaviors in these two populations as distinct differences have been observed in their hibernation-associated behaviors [12, 40].

The colonial hibernation patterns of many North American bat species increase susceptibility to WNS as densely clustered colonies accentuate disease transmission, particularly for fungal diseases that depend on airborne conidia<sup>4</sup> for propagation. Current reports from field surveys indicate that impacted North American hibernacula are beginning to show behavioral adaptations to WNS by forming smaller, less condensed colonies [12, 40]. These behaviors have been observed in European bats that show significantly reduced susceptibility to WNS [12, 40].

The reproductive nature of most Chiroptera increases population susceptibility as females birth two or fewer pups in a year and without appropriate post-arousal fat reserves may be unable to reproduce in a given year. This ensures that population losses to WNS will take considerable time to recover and underscores the importance of protecting and maintaining currently unaffected bat hibernacula. The rapid spread of WNS and the high mortality rates associated with the disease [19], necessitate the rapid development of disease management tools. Recently the fungus *Geomyces destructans* has been identified as the causative agent of WNS [26].

*Geomyces destructans* is a psychrophilic Ascomycete with optimal growth at 7-15°C and an upper thermal tolerance of 20°C [21]. Other members of the Geomycota are common in soils in temperate regions around the planet and represent several plant pathogens but no vertebrate pathogens other than *G. destructans*. *G. destructans* has significant low temperature keratolytic activity which may play an important role in disease manifestation and progression. *G. destructans*' psychrophilic nature makes it ideal-

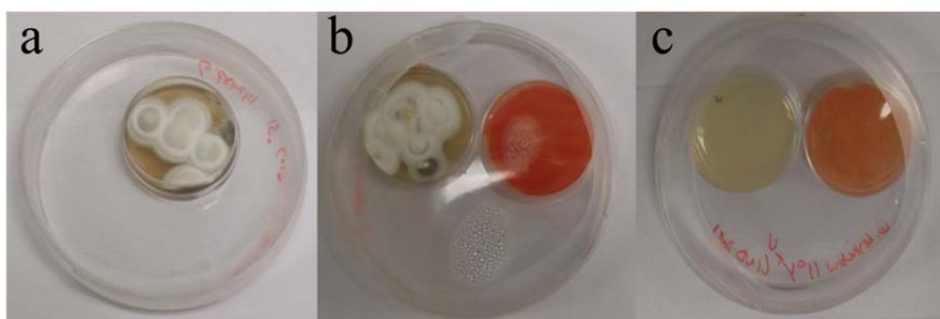
ly suited for colonization of bats in torpor, which have greatly depressed body temperatures (2-10°C), and a greatly diminished immune response [6, 21]. The clinical manifestation of *G. destructans* is characterized by fuzzy white growth on the muzzle, ears, tail, and wings of hibernating bats and results in severe physical damage to bat wing tissues [12]. The destruction of wing tissues is hypothesized to inhibit the host's ability to regulate body temperature and water retention and results in death due to starvation, dehydration, and exposure. Due to the recent identification of *G. destructans*, many ecological and physiological traits and their influence on virulence are yet to be elucidated.

The rapid spread and high mortality rates associated with WNS make the development of *in situ* treatment options for *G. destructans* a significant objective for wildlife management agencies. Accordingly, the development of biologically derived treatment options is preferred over chemical or physical treatments, since classic examples of chemical treatments in karst<sup>12</sup> environments are now a cautionary tale [2]. To this end, the US Department of the Interior and the US Fish and Wildlife Service (USFWS) released “A National Plan for Assisting States, Federal Agencies, and Tribes in Managing White-Nose Syndrome in Bats” (Appendix B) in May 2011. In this plan, significant focus was placed on the identification and development of biological and chemical control options for WNS. Accordingly, goals 3, 4, and 5 in element D as well as goal 4 in element E were directly addressed in this work (Appendix B).

## **1.2 Investigation of *Rhodococcus rhodochrous* DAP 96253 as a biological control agent for WNS**

*Rhodococcus rhodochrous* is a soil associated Actinomycete with tremendous metabolic and physiological diversity [32]. *R. rhodochrous* has been used extensively in bioremediation of nitrile containing compounds [27] and has demonstrated delayed fruit ripening activity when cultured with climacteric<sup>6</sup> fruits and vegetables [32]. Additional analysis of *R. rhodochrous* has demonstrated that it is able to inhibit the growth of select fungi associated with fresh fruits and vegetables [Pierce personal communication]. Several enzymes have been shown to increase in activity and prevalence in cells induced to delay

fruit ripening and may play a role in the observed antifungal activity [32]. Initial investigation of the potential antagonism of *G. destructans* by *Rhodococcus* indicated that, when induced under the protocol outlined in US patents 7,531,343, and 7,531,344 [30, 31], *Rhodococcus rhodochrous* DAP 96253 completely inhibited *G. destructans* growth from conidia when cultured with a shared air-space at 15°C (Fig. 1.2). Accordingly, the principal objective of this project is the evaluation of *Rhodococcus rhodochrous* DAP 96253 induced with urea for potential *in situ* application as a biological control agent for *G.destructans*.



**Figure 1.2** Shared air-space inhibition of *Geomyces destructans* conidia by *Rhodococcus rhodochrous* DAP 96253. *G. destructans* control (a), Uninduced *Rhodococcus* (b, - urea), and induced *Rhodococcus* (c, + urea at 7.5g L<sup>-1</sup>) and at 15°C.

Due to the nature of the inhibition demonstrated in the shared air-space co-culture experiments, and previous documentation of volatile based fungal inhibition by rhodococci [16, 22], the observed inhibition was hypothesized to be mediated by one or more bacterially produced volatile organic compounds<sup>19</sup> (VOCs) and/or the degradation of a fungal VOC signal. To confirm this hypothesis the identification of volatiles by mass spectral (MS) analysis of pure and co-cultures as specific volatile signatures potentially responsible for the observed inhibition of *G. destructans* conidia germination<sup>8</sup> was attempted with little success. The volatile agents elaborated are hypothesized to be similar to those detected from

other fungi, such as fatty acid esters, alcohols, and hydrocarbons, all of which are readily detected by GS/MS analysis [3, 4, 11, 14-16, 20, 23, 28, 35, 38]. The lack of separation associated with gas chromatography produces highly complex and difficult to deconvolute spectra. Subsequent analyses with software and libraries designed for GC/MS profiles are also of little use when applying strictly MS derived data. The availability of deconvolution software for exclusively MS derived data is currently limited. Future attempts to identify the VOCs involved in the observed microbial antagonism<sup>15</sup> of induced *Rhodococcus* should rely on established techniques and instrumentation in order to avoid the challenges associated with pioneering a method for reliable chemical identification in highly complex biological systems. The identification of the VOCs responsible for the observed antagonism must be elaborated in order to fully appreciate the potential impacts of applying this biological control agent in the field.

In addition to the strong evidence established via *in vitro* analysis of the observed antagonism the evaluation of the efficacy of induced *Rhodococcus* was pursued in order to establish *in vivo* efficacy at preventing fungal invasion of bat tissue. This goal was accomplished using a recently developed co-culture technique, a bat-skin explant assay, developed by Dr. Kevin Keel at the University of Georgia's (UGA) Southeastern Cooperative Wildlife Disease Study (SCWDS, Keel personal communication). The evaluation of the *in vivo* efficacy of induced *Rhodococcus* in preventing or reducing the infective potential of *G. destructans* conidia demonstrated induced *Rhodococcus* could completely inhibit the growth of *G. destructans* on living bat tissue. This is the first example of *ex vivo* efficacy for any biological control agent of WNS and represents a major milestone in this effort.

In order to optimize biocontrol efficacy and reduce potential cross contamination of karst environments various whole and fixed cell applications were investigated. The evaluation of various application methods of *Rhodococcus* induced for potential *in situ* application including whole cell application, non-growth fermentation cell paste, and fixed cell catalyst [30-32] were conducted. Non-living applications showed little inhibitory activity in all trials and were determined not to be the ideal delivery method for this biological control agent. However, non-growth fermentation cell paste demonstrated persistent

inhibitory activity and represents the most promising application method evaluated. The associated cell paste activity is a significant development as it represents multiple hallmarks of ideal biocontrol agents. The efficacy of fermentation cell paste was also evaluated using the bat tissue explant assay with tremendous success. The availability of a biological control agent that can be applied in the absence of growth media supplementation is a significant step towards *in situ* application. This contact-independent activity reduces the risk of cross contamination of *Rhodococcus* applications with native karst microflora, as well as reduces the cost associated with, and the potential for, long-term augmentation of karst ecosystems from *in situ* application

The evaluation of *Rhodococcus rhodochrous* DAP 96253 has demonstrated the tremendous potential of this organism for application as a biological control agent of *G. destructans*. This is the first and only demonstration of contact-independent antagonism of *G. destructans* and represents the most significant step towards the development of treatment tools since the emergence of WNS in 2006.

### **1.3 Evaluation of Bacterially derived Volatile Organic Compounds for management of WNS**

In addition to the evaluation of induced *Rhodococcus* as a biocontrol<sup>1</sup> agent of *G. destructans*, previously described bacterially produced antifungal volatiles [11, 16] were assayed for their *in vitro* potential to inhibit the growth and proliferation of *G. destructans*. The volatiles include benzothiazole; nonanal; decanal; 2-ethyl-1-hexanol; N,N-dimethyloctylamine; and benzaldehyde. Previous investigations of fungistatic soils were able to identify bacteria that produced anti-fungal VOCs which were later identified via SPME/GC/MS of cultures and soils. The VOCs were produced by *Pseudomonads* and *Bacillus* spp. and demonstrated broad spectrum antifungal activity [11, 16]. Volatile-based fungistasis<sup>7</sup> in soils has been observed in terrestrial environments around the globe. Due to the biological and chemical complexity of these environments the ultimate source of the active VOCs is often unknown but typically attributed to bacteria. The geology and ecology of soil make the presence of inhibitory volatiles of particular interest,

as low levels of VOCs are able to inhibit fungal growth in a dense, compartmentalized, and diverse ecosystem. Using this ecosystem as an ideal example of naturally occurring biological control of fungal proliferation I began to investigate biologically derived VOCs with known anti-fungal activity.

The influence of the VOCs on the germination and mycelial extension of *G. destructans* was evaluated using microscopic imaging techniques. In an effort to optimize the efficacy of the VOCs for potential field applications formulations of VOCs were evaluated for potential synergistic effects. Combinations of two VOCs at a total relative concentration of 4 $\mu$ M revealed several potentially synergistic blends. Accordingly, these synergistic blends were used to establish formulations of three VOCs ultimately yielding highly effective formulations with greatly increased anti-*Geomyces* activity at 4 $\mu$ M relative concentrations. The identification of biologically produced inhibitory volatiles expands the pool of potential biocontrol agents of *G. destructans* and the development of chemical formulations with significant anti-*Geomyces* activity at low concentrations provides promising chemical control options for *in situ* management of WNS.

#### **1.4 Physiological variants of *Geomyces destructans* in response to control agent exposure**

During the evaluation of biological and chemical control options for *G. destructans* the production of diffusible brown pigment was detected in several exposed cultures. To further understand this physiological variation and its potential impact on control efforts, the pigment was investigated to determine its physiological role. Initial evaluation indicated the pigment may be melanin. Due to the role of melanin as a virulence factor in other pathogenic fungi such as *Cryptococcus neoformans* and *Wangiella dermatitidis* [8, 42] significant effort was made to determine if the observed pigmentation was melanin. The production of melanin has not been previously described in *Geomyces spp.* and the production of melanin in *Geomyces destructans* may be significant in the emergence of this previously unrecognized

vertebrate pathogen. Traditional microbiological and more recently developed analytical methods of detection were used to confirm this hypothesis.

### **1.5 Forecasting the ecological impact of biocontrol agents for WNS**

A primary concern of wildlife management agencies, as outlined in the National Response Plan, was the preservation of natural cave ecosystems in the application of control agents. Karst environments are considered “biological islands” and may harbor endemic species of plants, animals, and microbes. The resilience of these unique ecosystems to control efforts is unknown but unfavorable outcomes from chemical treatment of karst environments validate these concerns [2]. In order to forecast the potential ecological effects of control agent application six common cave associated fungi were used as a model of cave associated fungal resilience. The organisms used were; *Rhizopus microsporus* var. *rhizopodiformis*, *Rhizopus oryzae*, *Absidia corymbifera*, *Eupenicillium lassenii*, *Mycelia sterilia*, and *Phycomyces blakesleanus*.



## 2 MATERIALS AND METHODS

### 2.1 Culture acquisition and maintenance

All *G. destructans* isolates used in the proposed project were provided by Dr. Kevin Keel through his WNS diagnostic work at UGA's SCWDS. Initial investigation has shown very low genetic and physiological variability amongst *G. destructans* isolates [43]. Accordingly, all assays were conducted with a small sample set of isolates ( $n \leq 3$ ). *G. destructans* cultures were maintained on Sabouraud Dextrose Agar (SDA, Difco) or in Sabouraud Dextrose Broth (SDB, Difco) at 4-15°C. *G. destructans* conidia are harvested from fungal lawns on SDA plates by adding 10 mL of conidia harvesting solution (CHS; 0.05% Tween 80, 0.9% NaCl) to the surface of the plate and gently scrapping with a sterile loop to dislodge conidia. The resulting solution is filtered through glass wool and centrifuged at 5000 rpm for 10 minutes. The resulting supernatant is removed and the spore pellet washed with 5 mL phosphate buffered saline (PBS, pH= 7), re-suspended, and filtered through glass wool. Conidia are stored in PBS at -20°C. Conidia are stored no longer than six weeks prior to use. *Rhodococcus rhodochrous* DAP 96253 cells are maintained as glycerol stock aliquots from 10 L fermentations carried out at GSU. Renewed glycerol stocks were used at the onset of each assay. The induction process was performed using the addition of urea or urea and cobalt as described in US patents 7,531,343, and 7,531,344 [30, 31].

### 2.2 Evaluation of previously described bacterially produced volatile organic compounds and Induced *Rhodococcus* for anti-*G. destructans* activity

Bacterially produced volatiles, previously described by Fernando *et al.* and Chuankun *et al.* [11, 16] were screened for anti-*G. destructans* activity via volatile exposure to conidia and mycelial plugs. The volatiles included cyclohexanol; decanal; 2-ethyl-1-hexanol; nonanal; benzothiazole; dimethyltrisulfide;

trimethylamine; benzaldehyde; and N,N-dimethyloctylamine. Anti-*G. destructans* activity was scored on a plus/minus scale for conidia inoculated plates, and radial growth from mycelial plugs will be used to determine percent inhibition as compared to unexposed controls. All experiments were conducted at 15°C or 4°C.

### ***2.2.1 Co-culture assays with Rhodococcus and VOC exposure assays***

A single-compartment Petri plate (150mm x 15mm) was used for a contained air-space to assess *G. destructans* growth characteristics in the presence of induced *Rhodococcus* or bacterially produced VOCs. A 10 µl of *G. destructans* conidia ( $10^6$  conidia/mL) in a phosphate buffer solution were spread onto SDA in Petri plates (35mm x 10mm). Multiply induced cells of *Rhodococcus* are inoculated onto Petri plates (35mm x 10mm) containing YEMEA + urea, or just YEMEA, and cultured in the contained air-space for up to 30 days. For the VOC exposure assay 30µl, 3.0 µl, and 0.3 µl of each volatile organic compound were pipetted onto a sterile filter paper disk (12.7mm) on a watch glass (75mm). These volumes represent a relative concentrations range from 0.2 mM to 1.5 µM depending on the compound and volume (Table 2.1). Each volatile, at each volume, was sealed with parafilm inside a 150mm Petri plate with a *G. destructans* -inoculated SDA. All assays were done in triplicate. Subsequent evaluations were carried out at 4 µM relative concentrations for all VOCs.

**Table 2.1** Relative concentrations of VOCs used in shared air-space experiments. Concentrations represent complete volatilization of VOC in the contained headspace of a 150mm x 15mm petri plate.

VOC	Volume ( $\mu\text{L}$ )	Relative Concentration( $\mu\text{M}$ )
Benzothiazole	0.3	2.75
	3	27.52
	30	275.18
Nonanal	0.3	1.74
	3	17.44
	30	174.42
Decanal	0.3	1.59
	3	15.94
	30	159.41
2-ethyl-1-hexanol	0.3	1.91
	3	19.12
	30	191.20
N,N-dimethyloctylamine	0.3	1.46
	3	14.59
	30	145.90
benzaldehyde	0.3	2.94
	3	29.40
	30	294.00

### ***2.2.2 Induced *Rhodococcus* and VOC germule suppression assay***

Thin layers (~ 750  $\mu\text{L}$ ) of 10% SDA were applied to standard microscope slides and 100  $\mu\text{L}$  of *G. destructans* conidia ( $10^{-6}$  mL) were spread across the agar surface. Volumes producing 4  $\mu\text{M}$  relative concentrations of each VOC were placed on an absorbent disk on a watch glass and then placed with an inoculated slide into a Petri plate (90 mm x 15 mm), sealed with parafilm, and incubated at 4 °C. For *Rhodococcus* assays small petri plates (35mm x 10mm) inoculated with *Rhodococcus* were used in place of the VOC. Controls were set up with no VOC or *Rhodococcus* exposure. All trials were conducted in duplicate. At 4 and 7 days post-inoculation, conidia were observed in a light microscope at 200X magnification for the presence of germule<sup>9</sup> formation. Germules were defined as single mycelial extensions emanating from conidia with a length equal to or greater than the intact conidia. Slides were retained and examined daily for up to 21 days after germule formation was first observed on control slides. Recovery of conidia was determined by removing the control agent (*Rhodococcus* or VOCs) after 24 hours, 72 hours, and 7 days. Slides were observed for 21 days after removal of control agent to assess recovery.

### ***2.2.3 VOC formulation assay for anti-*G. destructans* activity***

VOC formulations utilizing mixtures of two pure VOCs were created with all fifteen possible combinations of the six VOCs by applying volumes corresponding to 2  $\mu\text{M}$  relative concentrations of each VOC to an absorbent disk and arranging combinations of two disks of different VOCs on a single watch glass. Volumes corresponding to 4  $\mu\text{M}$  relative concentrations of each pure VOC were used as synergism controls. *G. destructans* mycelial plugs cut from the leading edge of actively growing colonies were inserted into fresh SDA plates (35mm x 10mm) and sealed with parafilm in large Petri plates (150 mm x 15 mm) with each formulation or pure VOC. Plates were then incubated at 15 °C for 21 days. Each test was conducted in triplicate. Area measurements were conducted with the use of digital photography and computer analysis, every two days post inoculation.

#### ***2.2.4 Preparation and evaluation of fixed cell catalyst and Fermentation cell paste in non-growth conditions***

Immobilization of whole bacteria was carried out based on the methods of DeFilippi [13] and Lopez-Gallego *et al.* [25] by Pierce lab personnel. Refinement of immobilized cells to produce active catalyst was carried out according to the methods of Pierce *et al.* [30-32] by Pierce lab personnel. Evaluation of anti-*G. destructans* activity of fixed-cell catalyst and fermentation cell paste was determined in co-culture assays with *G. destructans* conidia and mycelial plugs with various amounts of control agents (< 1g) as described previously. Efficacy was determined by observation of germination and mycelial extension as compared to unexposed controls for growth from conidia, and as percent reduction in radial growth of mycelial plugs.

#### ***2.2.5 Ex vivo anti-infectivity assay***

*In vivo* efficacy of induced *Rhodococcus* was evaluated using an *ex vivo* model of WNS developed by Dr. Kevin Keel. Living samples of bat skin, maintained in tissue culture media, were inoculated with *G. destructans* conidia. Infected explants were incubated in a shared air-space with induced *Rhodococcus*. Uninoculated control explants were incubated alone, or with uninduced *Rhodococcus*. Initial experiments were conducted at 7°C. Anti-infective efficacy was determined by visual and microscopic evaluation of bat wing membrane tissue cultures exposed to induced *Rhodococcus* as compared to unexposed, and uninduced controls.

### **2.3 Resilience of cave-associated fungi to WNS control agents**

In order to forecast the potential ecological impacts of control agent application six common cave associated fungi were selected as a model of cave associated fungal resilience. The organisms used were; *Rhizopus microsporus* var. *rhizopodiformis*, *Rhizopus oryzae*, *Absidia corymbifera*, *Eupenicillium lasse-*

*nii*, *Mycelia sterilia* (*Aspergillus flavus*), and *Phycomyces blakesleannus*. All cultures were obtained from the US Department of Agriculture's Agricultural Research Service (ARS) culture collection. Mycelial plugs and conidia were exposed to induced *Rhodococcus* in a shared air-space and monitored for radial growth inhibition and inhibition of conidia germination. Recovery of conidia was evaluated by removing conidia inoculated slides at given time points and comparing germination and mycelial extension to unexposed controls.

## **2.4 Melanin production in *Geomyces destructans***

### **2.4.1 L-DOPA melanin induction**

SDA supplemented with 0.2g/L L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma) were inoculated with 100  $\mu$ L of *G. destructans* conidia suspension ( $\sim 10^6$  conidia/mL) and incubated at 15°C for 14 days. After 14 days significant melanin production was observed and cultures were processed for additional analyses.

### **2.4.2 Copper sulfide-silver staining of control agent induced pigmented cultures**

Melanin production in *Geomyces destructans* was determined using a modified version of the method of Butler *et al* [9]. *G. destructans* conidia ( $\sim 10^4$  conidia/mL) were inoculated onto SDA and allowed to grow until mycelial were visible ( $\sim 5$  DPI) and then exposed to benzothiazole (27.5  $\mu$ M) or induced *Rhodococcus* in a closed air-space at 15°C for greater than 21 days. Exposed cultures with observable diffused pigment or increased reverse side mycelial pigmentation as well as unexposed controls were further analyzed. For initial evaluation light microscopy was used. Small slices of agar were excised and stained in 1.5 mL volumes of all solutions unless otherwise indicated. In between staining steps the agar slices were washed with de-ionized (DI) water. Agar slices were incubated overnight in 10mM copper sulfate in DI. Agar slices were then rinsed twice with DI water and pretreated in a 1.0% sodium sulfide

solution in DI water at 25 °C for 60 minutes in the absence of light. Agar slices were then washed with DI water, dried, and developed in a 5 mL solution of 22mg silver lactate and 170 mg hydroquinone in a citrate buffer (1.0 M, pH 3.7) solution for 60 minutes at 25°C in the absence of light. Staining controls lacked either the sulfide or silver treatment, and melanin induction controls were not exposed to benzothiazole or induced *Rhodococcus*. Images were captured at 200X magnification.

#### ***2.4.3 Spectral analysis of control agent induced pigmentation***

Control agent exposed (benzothiazole or induced *Rhodococcus*) cultures producing visible pigmentation were cut into sections and submerged in 10 mL of Tris-EDTA (TE) buffer for 24 hours. Brown pigment was observed to diffuse into the buffer from the agar. Negative control extracts were prepared by submerging non-control agent exposed hyaline<sup>11</sup> culture slices in TE buffer for 24 hours. All extracts were filtered twice with 0.45 µm syringe filters (Pall). Positive controls were generated by suspending synthetic melanin<sup>17</sup> (Sigma-Aldrich) in TE buffer at concentrations ranging from 1mg/mL to 1µg/mL as well as extracting diffusible melanin from L-DOPA supplemented cultures as described above. The resulting extracts were used for Ultra Violet Visible (UV VIS) spectrophotometric analysis (Nanodrop, Thermo Scientific).

#### **2.5 Mass Spectral analysis of antagonistic Volatiles**

A single SDA plate (35mm x 10mm) inoculated with *G. destructans* conidia (~10<sup>4</sup> conidia/mL) was placed into a modified 250mL carrier flasks with two plates (35mm x 10mm) of induced *Rhodococcus* and incubated at 15°C for 14 days. Control flasks contained either a single SDA plate inoculated with *G. destructans* conidia or two plates of induced *Rhodococcus*. All trials were conducted in triplicate. After 14 days mass spectral analysis was conducted using a Quantitative Gas Analyzer Quadrupole Mass Spectrophotometer (QGA-MS, Hiden Analytical) as specified by the manufacturer. The QGA-MS probe was inserted into a hole bored into a single cap of the carrier flask and sealed with parafilm. Analyses were

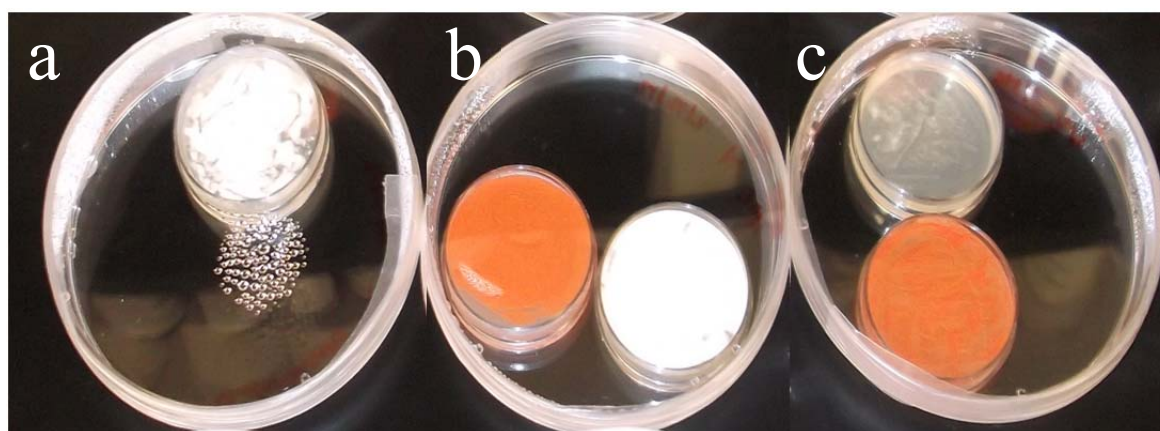
conducted under a variety of settings and multiple detectors and with varying scan lengths as well as with and without background subtraction of atmospheric gas. Mass spectra generated were exported to the National Institute of Standards and Technology/Environmental Protection Agency/National Institute of Health Mass Spectral Database (NIST 11) for evaluation. Spectra were compared to the ionization patterns contained in the NIST library and Match Factor and Probabilities of potential matches were generated and their quality was determined based on the guidelines for interpretation of results provided in NIST 11 user guide. Match factors less than 600 and probabilities less than 20% were considered unresolved.

### 3 RESULTS

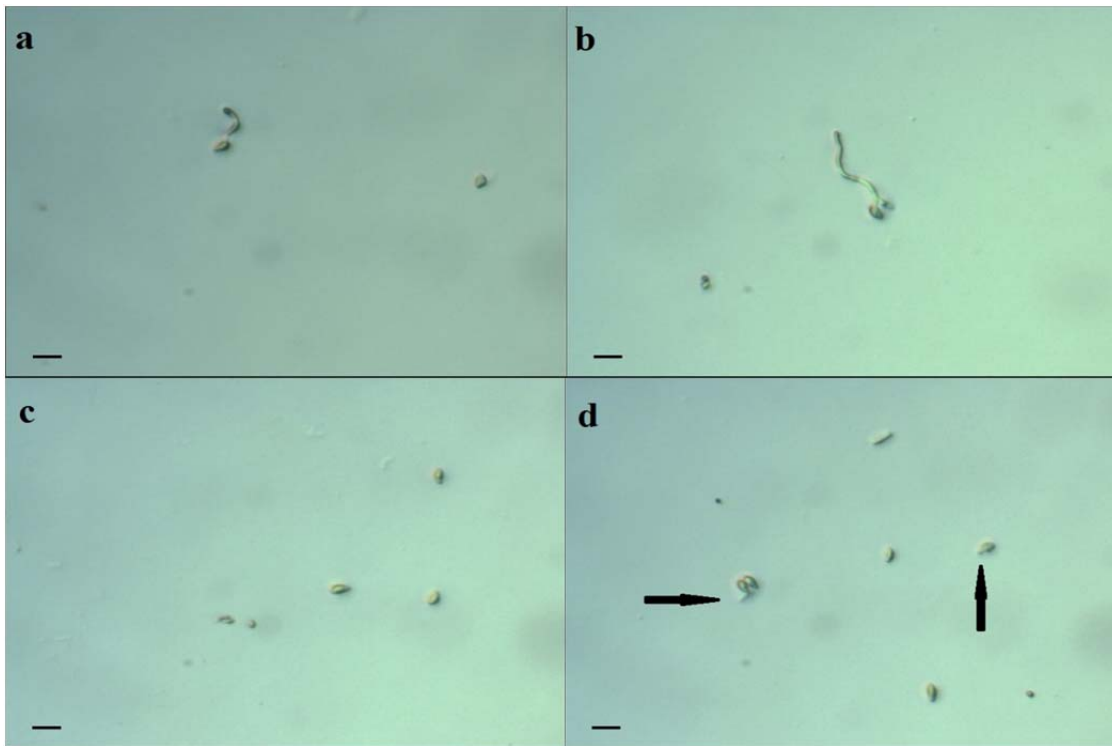
#### 3.1 Anti-*G. destructans* activity of induced *Rhodococcus*

Initial experiments with urea induced *Rhodococcus* demonstrated complete inhibition of growth from conidia of *G. destructans* when cultured with a shared air space at 15°C (Fig. 1.2). Uninduced *Rhodococcus* showed no signs of inhibition compared to unexposed controls. Subsequent testing at 7°C and 4°C demonstrated fungistatic activity of induced *Rhodococcus* and resulted in slower germination and reduced total mycelial growth as compared to uninduced and unexposed controls (Fig 3.1). Microscope slide agar overlays inoculated with *G. destructans* spores failed to form germules when exposed to induced *Rhodococcus* (Fig. 3.2). Microscopic evaluation of *G. destructans* cultures from conidia exposed to induced *Rhodococcus* at 4°C revealed an abnormal mycelial phenotype. Further analyses revealed that abnormally curly mycelia were consistently formed in *G. destructans* colonies from conidia exposed to induced *Rhodococcus* at 4°C (Fig. 3.3). Additionally, tape mounts of *G. destructans* cultures grown for conidia exposed to induced *Rhodococcus* at 7°C and 4°C indicated less conidiation<sup>5</sup> than uninduced and unexposed controls.

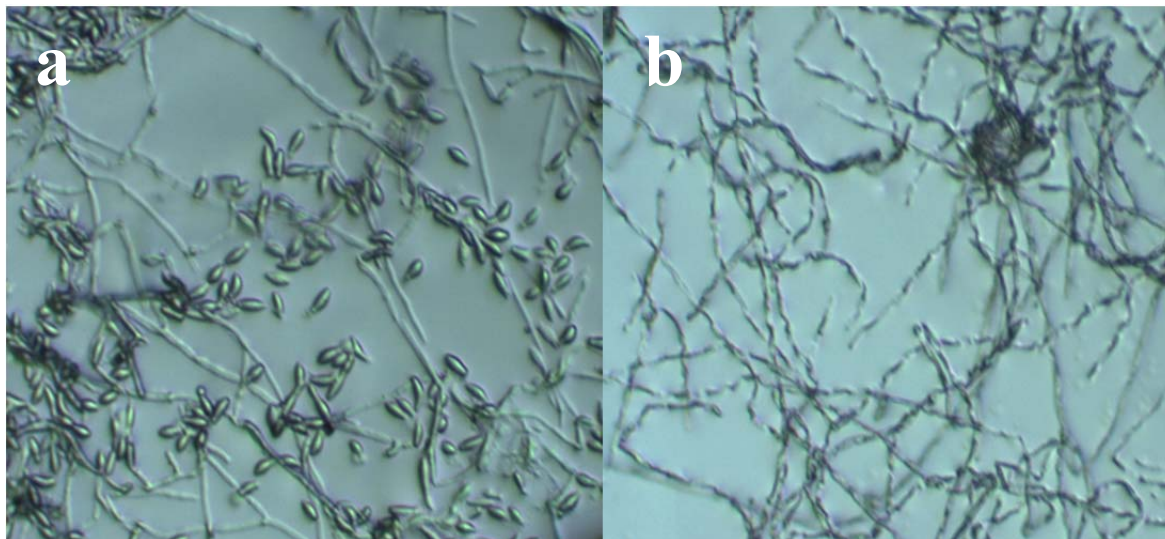




**Figure 3.1** Shared air-space co-culture of *Geomyces destructans* spores ( $10^4 \text{ mL}^{-1}$ ) with *Rhodococcus rhodochrous* DAP 96253, uninduced (b, - urea) induced (C, + urea at  $7.5 \text{ g L}^{-1}$ ) and a *G. destructans* control (a) at  $4^\circ\text{C}$ . Results representative of exposure at  $7^\circ\text{C}$ .



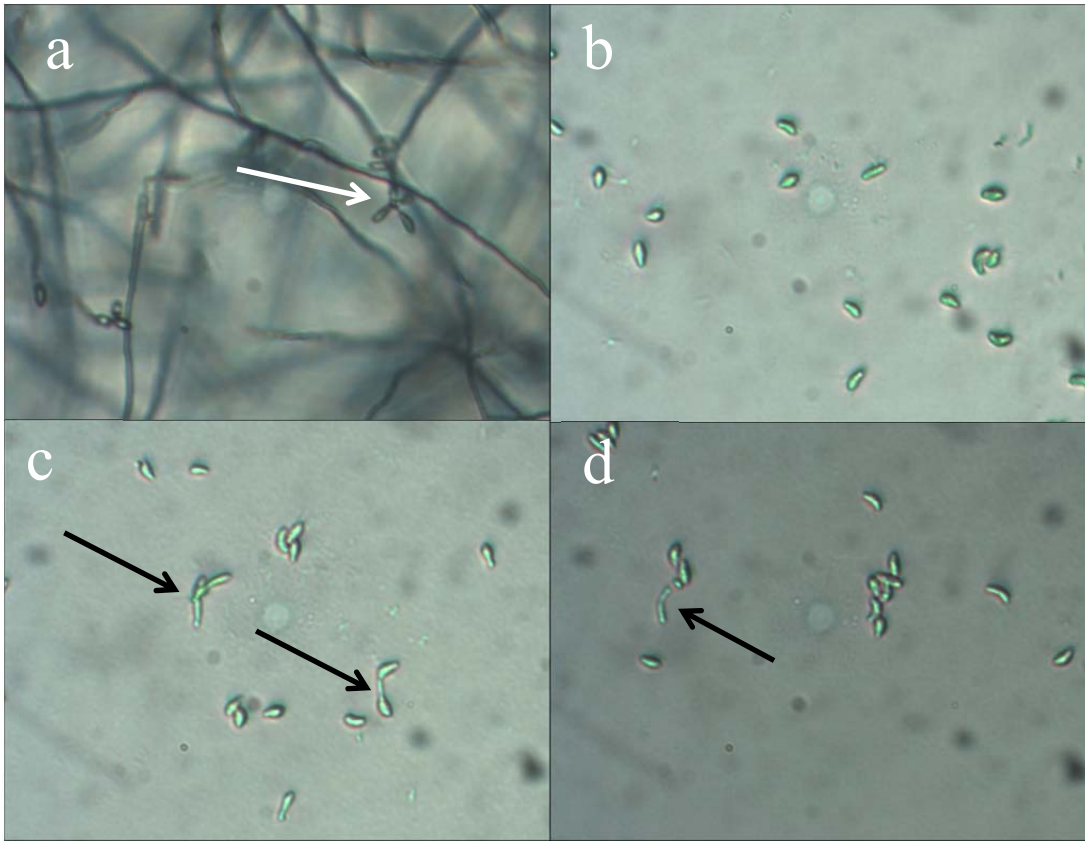
**Figure 3.2** Inhibition of *Geomyces destructans* germule formation by shared air-space co-culture with induced *Rhodococcus rhodochrous* DAP96253 at 15°C. Typical germule formation 5 days post-inoculation in unexposed controls (a and b). Exposed spores 10 days post inoculation (c) and exposed spores showing early signs of germination (arrows) but no germule formation (d). All images captured at 200X magnification. Scale bar is 10μm.



**Figure 3.3** Exposure to benzothiazole produced abnormal mycelial formation in *G. destructans*. Typical mycelial formation and conidiation of *G. destructans* exposed to benzothiazole in the presence of activated carbon (**a**). Abnormally curly mycelia and reduced conidiation of *G. destructans* exposed to benzothiazole (**b**). All images captured at 200x magnification.

### ***3.1.1 Induced Rhodococcus permanently and irreversibly inhibits conidia germination***

Slide agar overlays inoculated with *G. destructans* conidia and exposed to induced *Rhodococcus* failed to produce germules 21 days after removal of *Rhodococcus* (Fig. 3.4). Conidia exposed to induced *Rhodococcus* for 24 hours revealed no signs of germule formation, whereas conidia exposed for 4 and 7 days exhibited early signs of germination but no obvious germules (Fig. 3.4). Conidia exposed to previously described VOCs failed to prevent conidia germination, but delayed germination by up to 5 days (Data not shown).



**Figure 3.4** *G. destructans* conidia are unable to recover after 24 hour exposure to induced *Rhodococcus*. *G. destructans* control slide (a) produced significant mycelia growth and conidiation (white arrow) after 5 days. *G. destructans* conidia exposed to induced *Rhodococcus* for 24 hours (b), 72 hours (c) and 7 days (d) failed to form germules 21 days after removal of induced *Rhodococcus*. Halted germination was observed in 72 hour and 7 day exposures (black arrows). All images captured at 200X magnification.

### 3.1.2 *Ex vivo anti-infectivity assay*

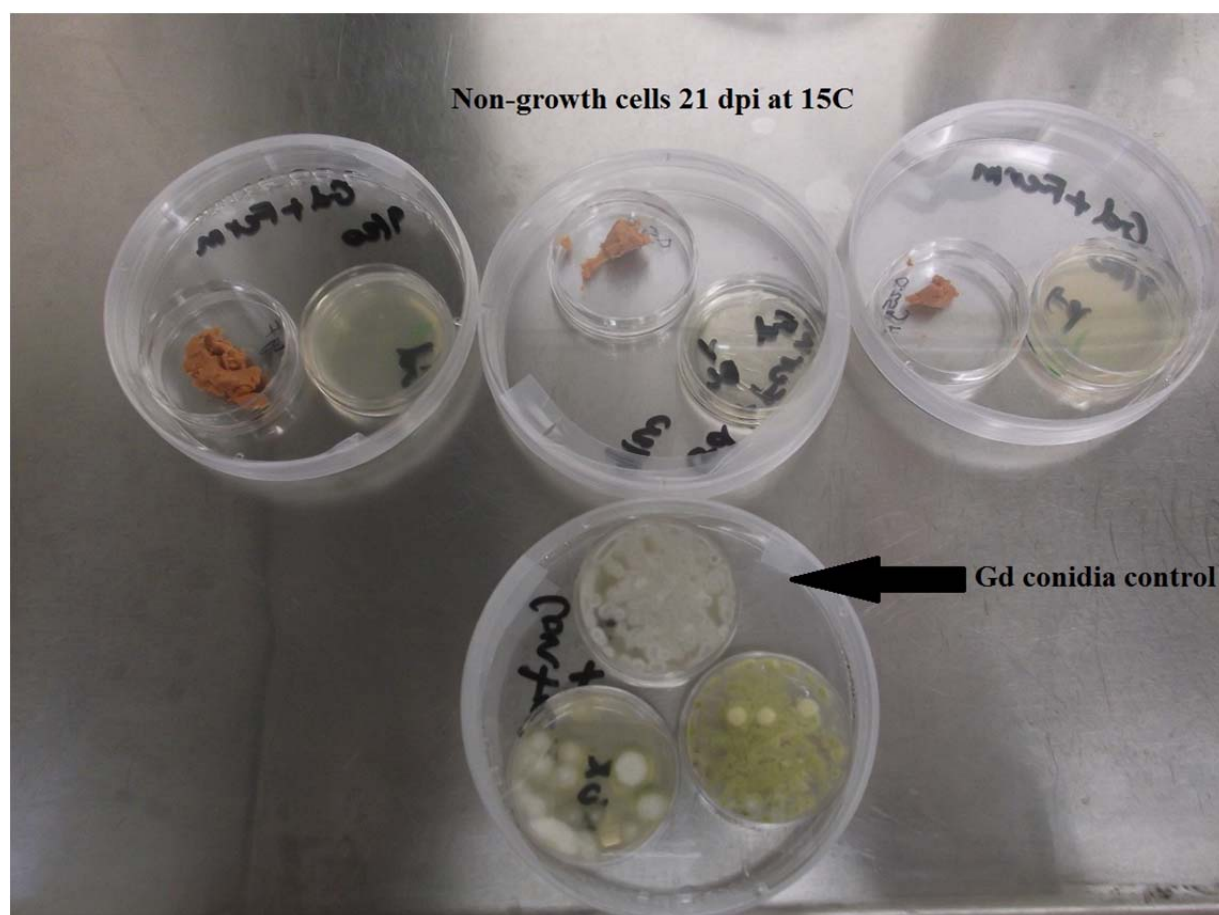
Induced *Rhodococcus rhodochrous* DAP96253 completely inhibited the colonization of bat wing explants by *G. destructans* conidia in all replicates (n=9) when incubated in a shared air space for 21 days at 7°C (Fig. 3.6). Explants exposed to uninduced *Rhodococcus* and unexposed explants were fully colonized at 14 days post inoculation.



**Figure 3.5** Bat wing tissue explants in a shared air-space with induced *Rhodococcus rhodochrous* DAP96253 14 days post-inoculation with *Geomyces destructans* conidia.

### 3.1.3 Evaluation of fixed cell catalyst and fermentation cell paste

Fixed cell catalyst [30-32] failed to inhibit or slow growth from conidia of *G. destructans* when grown in a shared air-space. Fermentation cell paste in quantities as low as 0.25g completely inhibited growth from spores of *G. destructans* for greater than 80 days (Fig. 3.7).



**Figure 3.6** Fermentation cell paste of induced *Rhodococcus rhodochrous* DAP96253 in a shared air-space with *Geomyces destructans* conidia inoculated plates. Image taken 21 days post inoculation.

### 3.2 Anti-*G. destructans* activity of previously described bacterially produced volatiles

Initial investigation demonstrated inhibitory activity for most VOCs at relative concentrations less than 4 $\mu$ M. Decanal; 2-ethyl-1-hexanol; nonanal; benzothiazole; dimethyltrisulfide; benzaldehyde; and N,N-dimethoctylamine all demonstrated anti-*G. destructans* activity when 30 $\mu$ L of the respective compound were placed adjacent to SDA plates inoculated with *G. destructans* conidia in a closed system at 15°C (Table 3.1). Control plates containing 1g activated carbon showed no inhibition for decanal; 2-ethyl-1-hexanol; and benzaldehyde; while the remaining compounds inhibitory activity persisted in the presence of activated carbon (Table 3.1). Subsequent assays with 3 $\mu$ L of each compound demonstrated similar results with only N,N-dimethoctylamine unable to completely inhibit *G. destructans* growth from conidia at 7 days (Table 3.1). The addition of activated carbon abolished all inhibitory activity of the assayed compounds at 3  $\mu$ L (Table 3.1). At 11 days of exposure to 3  $\mu$ L of each respective compound only 2-ethyl-1-hexanol, decanal, and nonanal demonstrated inhibitory activity, with all activated carbon controls abolishing the inhibitory activity (Table 3.1). Additionally, *G. destructans* cultures from conidia exposed to 3  $\mu$ L benzothiazole without activated carbon revealed unique colony morphology characterized by increased pigmentation of the underside of the culture and diffused into the growth media as compared to unexposed cultures and cultures exposed to benzothiazole in the presence of activated carbon (Fig. 3.8).

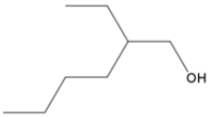
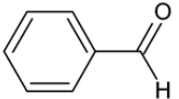
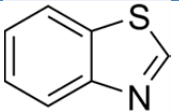
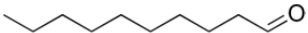
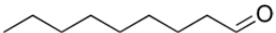
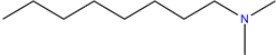
Assays using mycelial plugs cut from the leading edge of actively growing *G. destructans* colonies on SDA were exposed to the previously described bacterially produced volatiles at 30  $\mu$ L, 3  $\mu$ L, and 0.3  $\mu$ L of each respective compound and incubated in a contained air-space at 15°C. At 30  $\mu$ L all compounds completely inhibited the growth of *G. destructans* mycelia for up to 9 days (Fig. 3.9a). At 14 days of exposure only *G. destructans* plugs exposed to decanal showed any radial growth, with 83% reduction in growth as compared to unexposed controls (Fig. 3.9a). At 3  $\mu$ L of each respective compound decanal and N,N-dimethoctylamine yielded only minor reductions in radial growth, whereas the remaining compounds completely inhibited radial mycelial growth of *G. destructans* for up to 14 days (Fig.

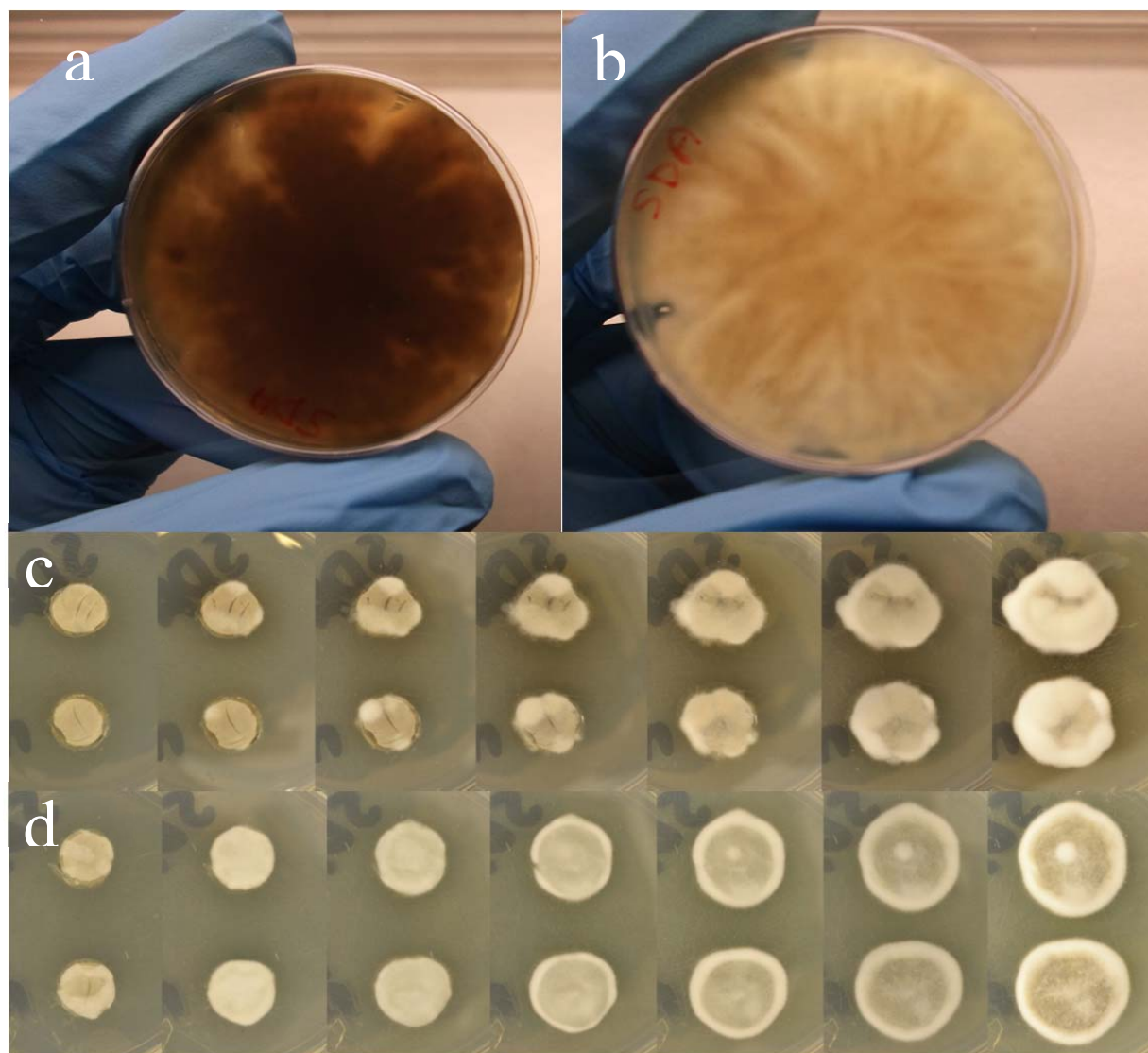
3.9b). At 0.3  $\mu\text{L}$  of each compound only benzothiazole demonstrated significant inhibitory activity with a 60% reduction in radial growth at 14 days of exposure (Fig. 3.9c). Interestingly, at 0.3  $\mu\text{L}$  N,N-dimethoctylamine induced growth as compared to unexposed controls (Fig. 3.9c). This result may be due to hormesis [37].

In order to forecast the *in situ* efficacy of the VOCs additional *in vitro* evaluation was conducted at 4°C to more accurately represent the environmental conditions of North American hibernacula. Exposure to 30  $\mu\text{L}$  or 3  $\mu\text{L}$  of each respective VOC completely inhibited radial growth of *G. destructans* for greater than 21 days (data not shown). Exposure to 0.3  $\mu\text{L}$  of each respective VOC inhibited radial growth for all VOCs except benzaldehyde (Fig. 3.9d). The greatest degree of inhibition was observed with decanal which demonstrated a greater than 99% reduction in growth area at 35 days post inoculation (Fig. 3.9d). Based on these initial results VOC exposure was standardized to 4 $\mu\text{M}$  relative concentrations for subsequent evaluations.

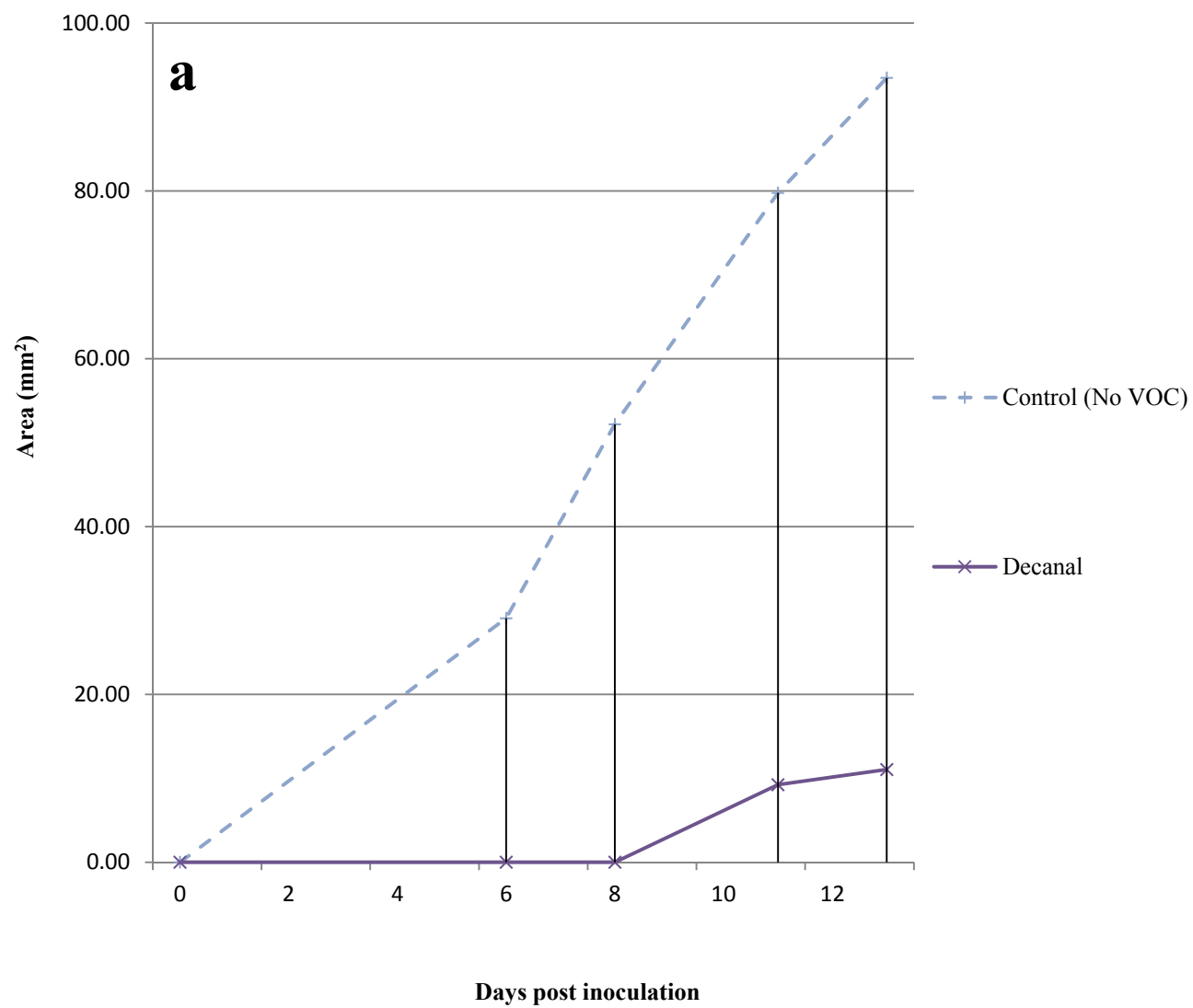


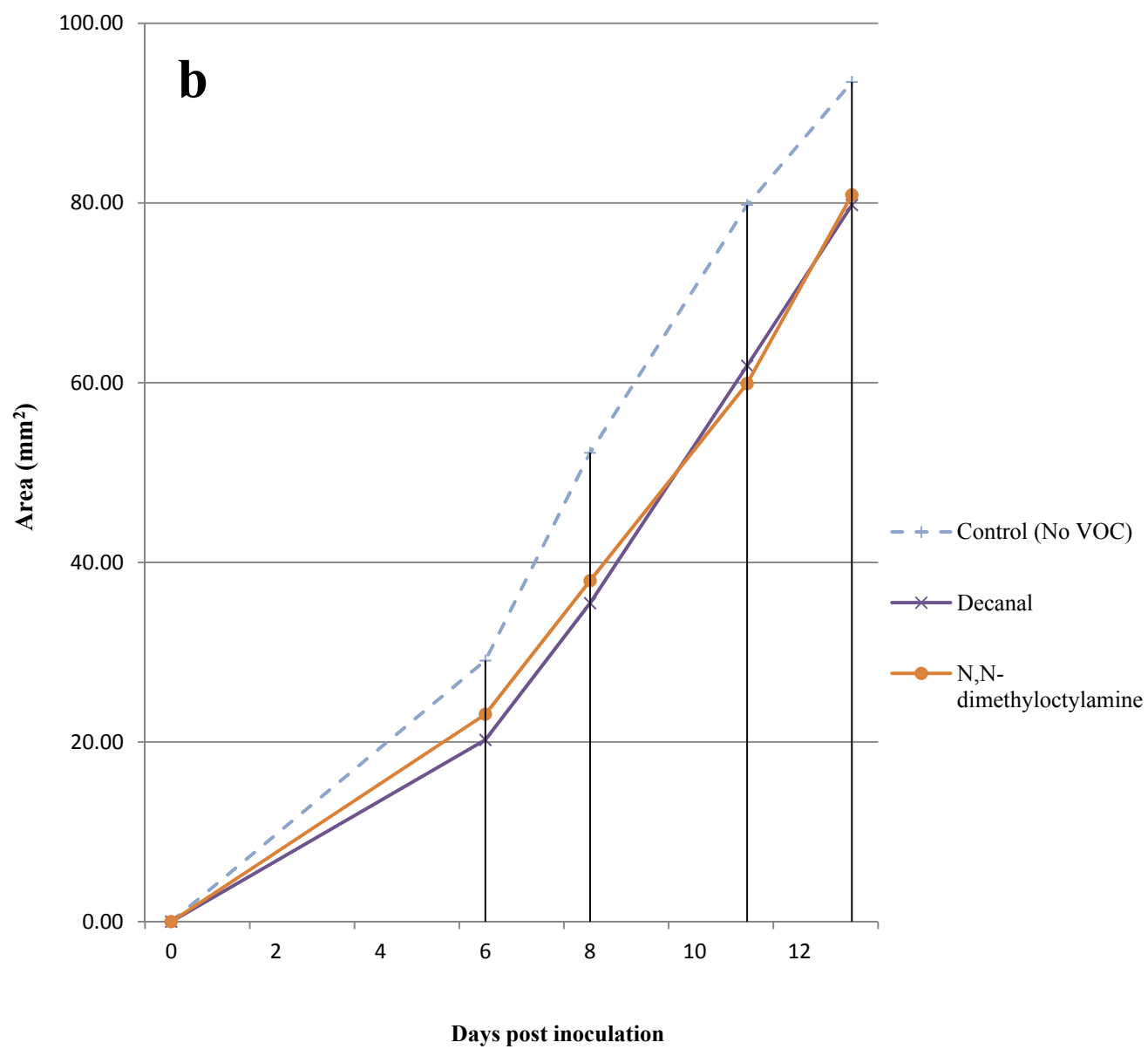
**Table 3.1** Evaluation of anti-*G. destructans* activity of bacterially produced antifungal VOCs with *G. destructans* conidia. + indicates growth from spores. – indicates no visible growth. \*\* Incubated with activated carbon, <sup>d</sup>=7 day exposure, <sup>dd</sup>=10 day exposure

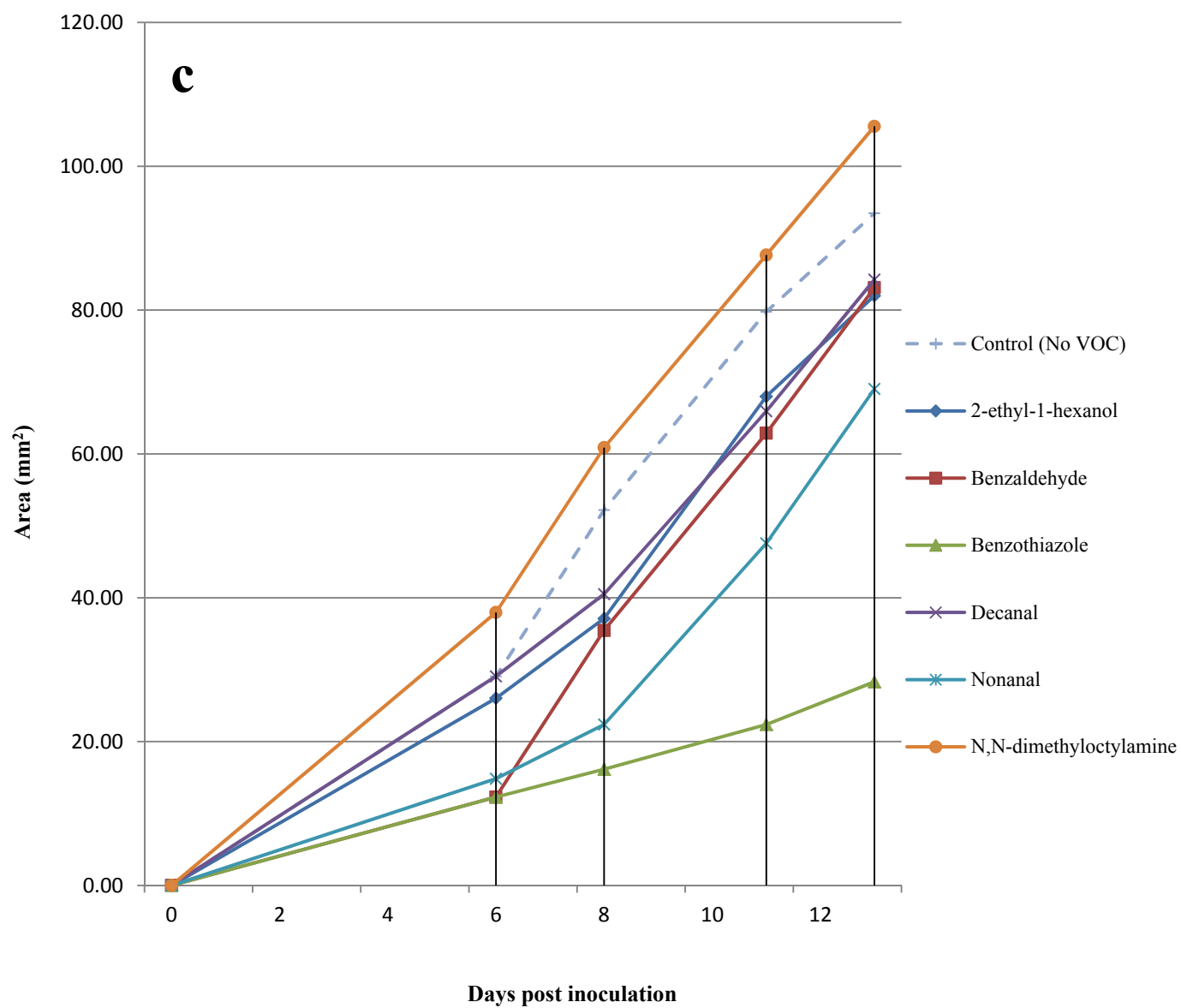
VOC	Chemical Structure	30 $\mu$ l	30 $\mu$ l **	3 $\mu$ l <sup>d</sup>	3 $\mu$ l <sup>d**</sup>	3 $\mu$ l <sup>dd</sup>	3 $\mu$ l <sup>dd**</sup>
2-ethyl-1-hexanol		-	+	-	+	-	+
Benzaldehyde		-	+	-	+	+	+
Benzothiazole		-	-	-	+	+	+
Decanal		-	+	-	+	-	+
Nonanal		-	-	-	+	-	+
N,N-dimethyloctylamine		-	-	+	+	+	+
Control		+	+	+	+	+	+

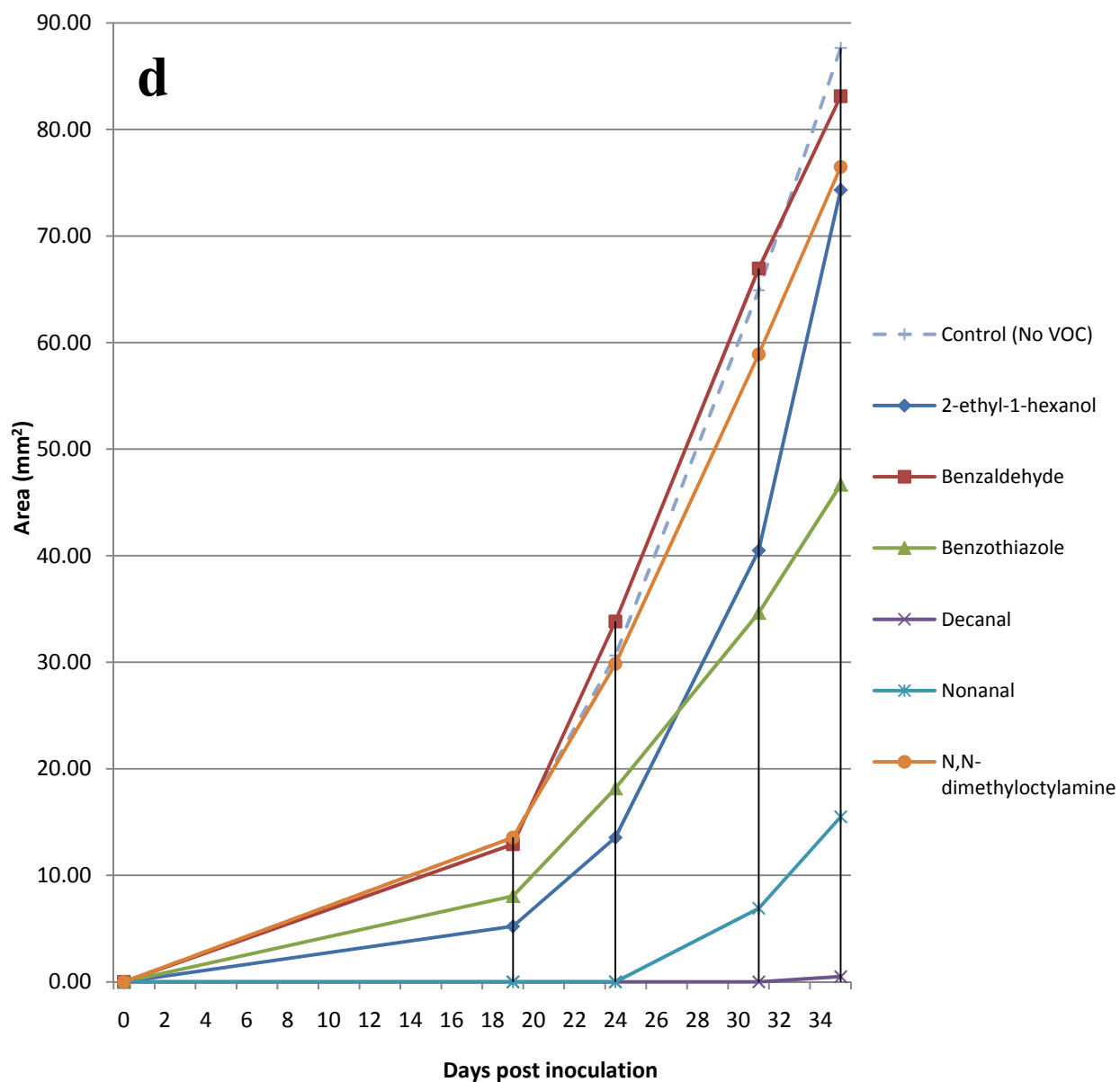


**Figure 3.7** Benzothiazole induces pigment production in *G. destructans*. Changes in reverse side pigmentation of *G. destructans* cultures from spores exposed to 0.3  $\mu\text{L}$  benzothiazole without (a) and with (b) activated carbon. Time lapse photography of benzothiazole induced *G. destructans* pigment production on SDA. 2 to 14 days (L to R) growth in the shared air-space of 4  $\mu\text{M}$  benzothiazole (c). 2 to 14 days (L to R) growth of control (d).







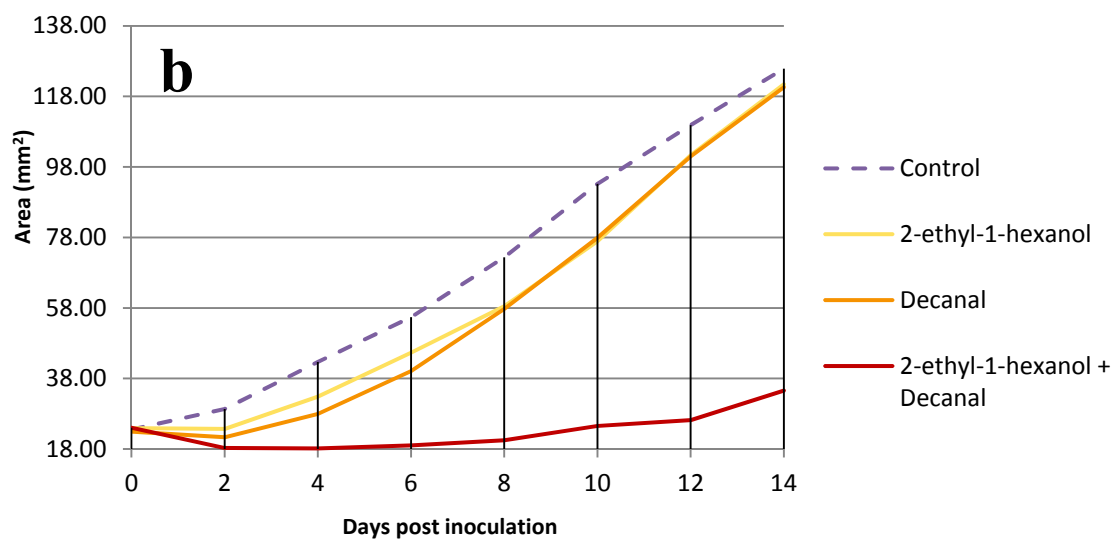
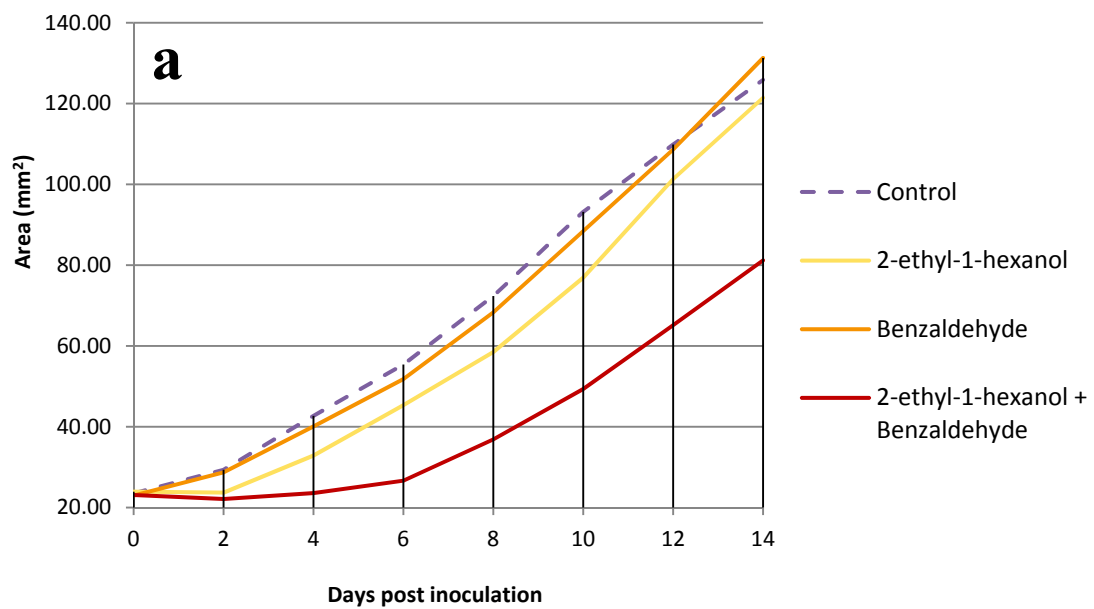


**Figure 3.8** Growth areas of *G. destructans* mycelial plugs exposed to bacterially produced VOCs at 15°C at 30  $\mu\text{L}$  (a), 3  $\mu\text{L}$  (b), and 0.3  $\mu\text{L}$  (c), respectively. Growth area of mycelial plugs exposed at 4°C to 0.3  $\mu\text{L}$  (d) of bacterially produced VOCs. VOCs not shown completely inhibited radial growth for the duration of the experiment.

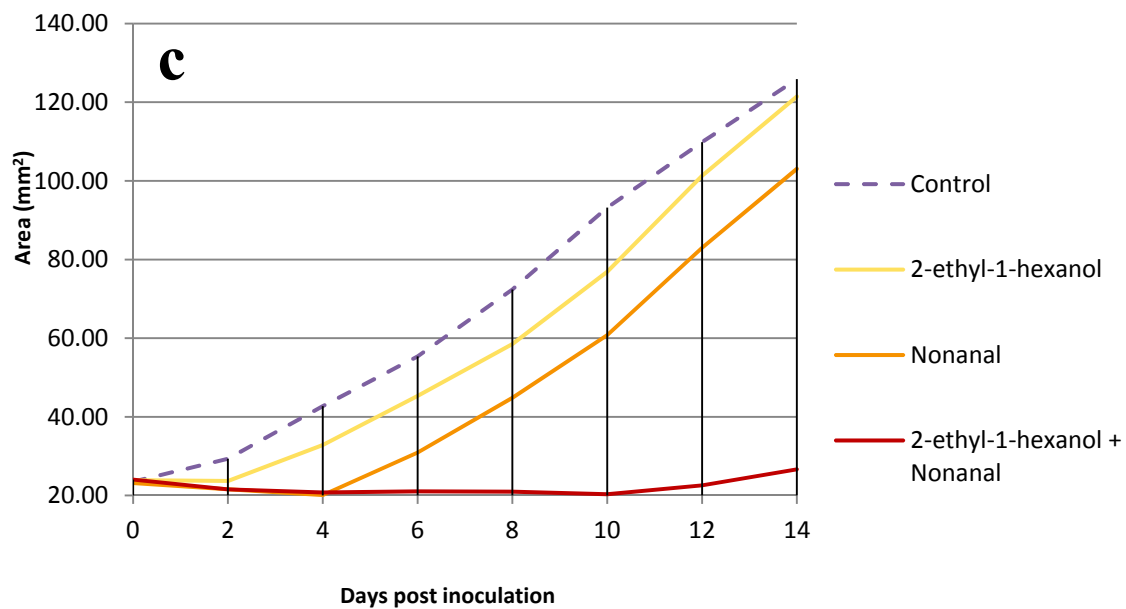
### 3.2.1 VOC formulation assay for anti-*G. destructans* activity

In addition to evaluating individual VOCs, formulations were investigated for potential synergistic effects. Three VOC formulations comprised of two VOCs were observed to synergistically inhibit the growth of *G. destructans* mycelial plugs; more than the combined inhibition of each of the pure VOCs alone. Those include 2-ethyl-1-hexanol & benzaldehyde; 2-ethyl-1-hexanol & nonanal; 2-ethyl-1-hexanol & decanal; and 2-ethyl-1-hexanol & N,N-dimethoctylamine (Figures 3.10a, 3.10b, and 3.10c respectively). The greatest inhibition by the formulation occurred with 2-ethyl-1-hexanol & nonanal, which demonstrated greater than 95% reduction in growth as compared to unexposed controls 14 days post inoculation (Fig. 3.10c).

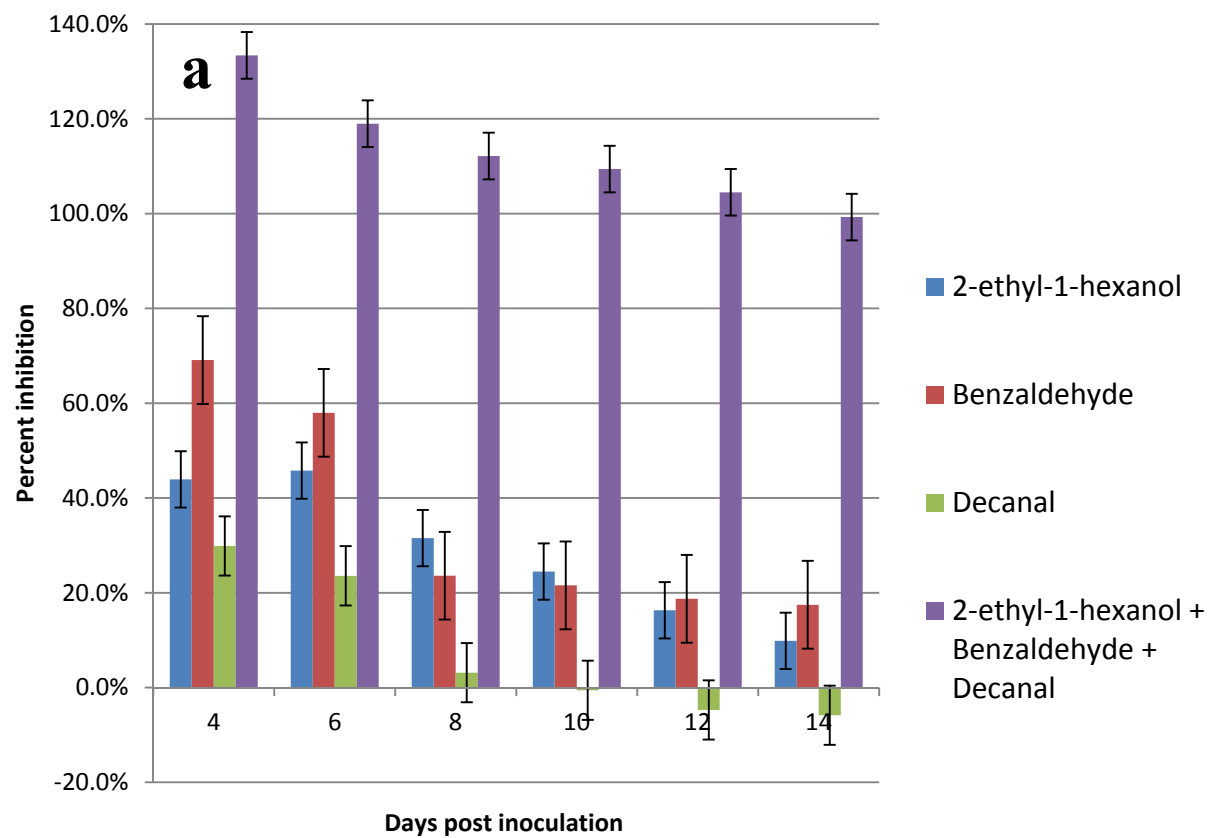
Two VOC formulations comprised of three VOCs with a total relative concentration of 4  $\mu\text{M}$  were observed to synergistically inhibit the growth of *G. destructans* mycelial plugs; more than the combined inhibition of each of the pure VOCs alone at a relative concentration of 4  $\mu\text{M}$ . Those include 2-ethyl-1-hexanol; benzaldehyde; and decanal; and 2-ethyl-1-hexanol; nonanal; and decanal with the prior completely inhibiting radial growth at 14 days post inoculation (Fig. 3.11).

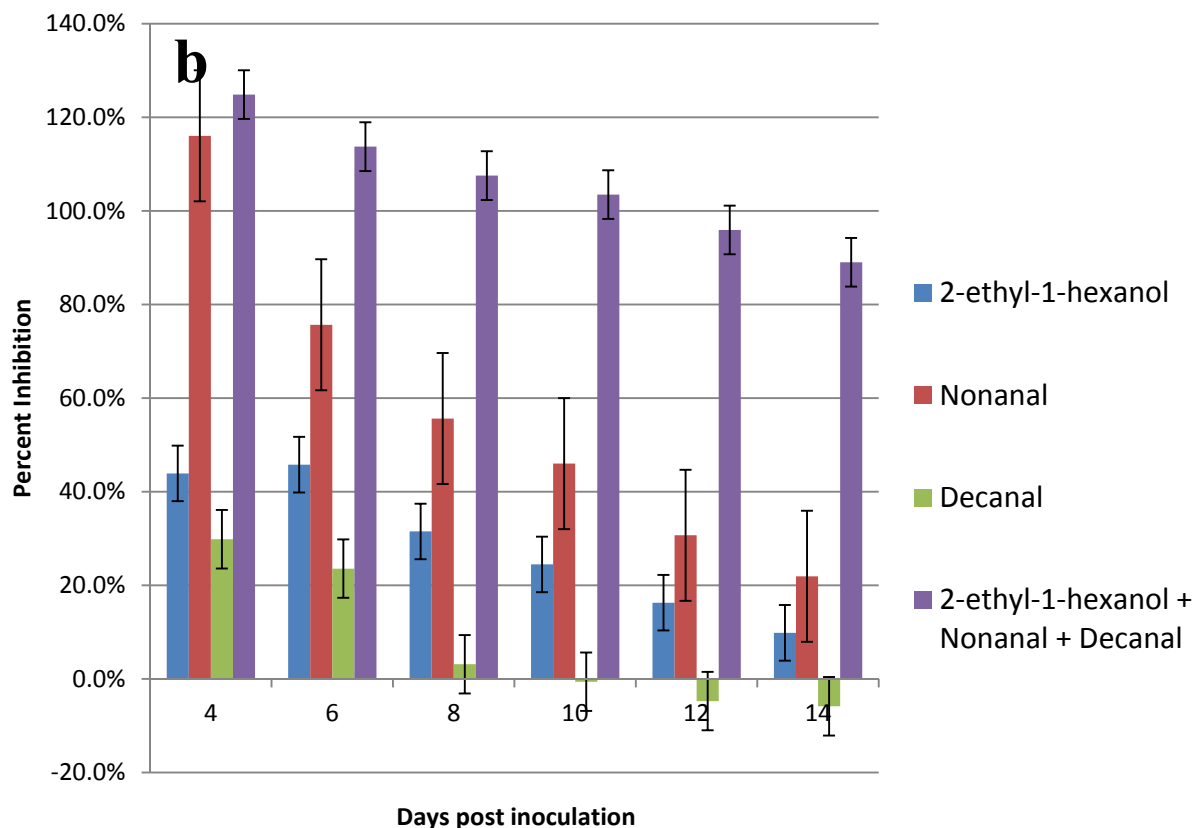






**Figure 3.9** Growth areas of *G. destructans* mycelial plugs exposed to each individual VOC as well as formulations at 15°C. Measurements taken every two days for 14 days. (a) 2-ethyl-1-hexanol & benzaldehyde, (b) 2-ethyl-1-hexanol and decanal, and (c) 2-ethyl-1-hexanol and nonanal.





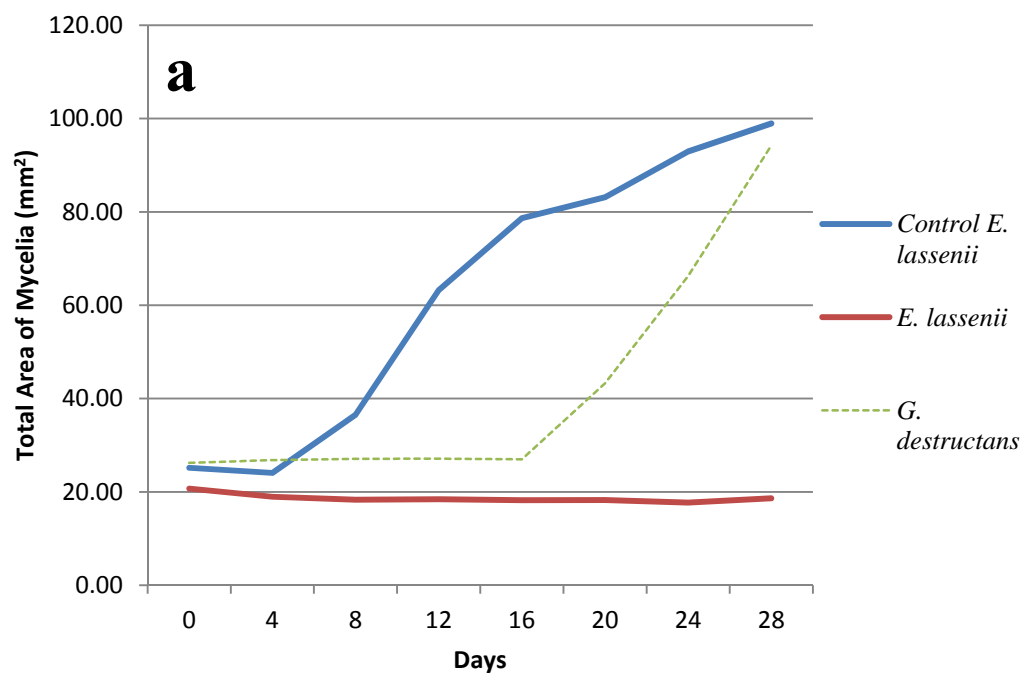
**Figure 3.10** Percent inhibition of radial growth of *G. destructans* exposed to pure VOCs and VOC formulations. Percent inhibition was determined by comparing the growth area of *G. destructans* mycelial plugs exposed to VOC formulations and the pure VOCs comprising those formulations to unexposed control plugs at 15°C. Each pure VOC was exposed at a relative concentration of 4  $\mu\text{M}$  and each formulation was comprised of each VOC at a relative concentration of 1.3  $\mu\text{M}$ . Formulations of 2-ethyl-1-hexanol, benzaldehyde, and decanal (a), as well as 2-ethyl-1-hexanol, nonanal, and decanal (b) were found to have synergistic effects, yielding greater inhibition combined than the sum of the inhibitions observed with each individual VOC.

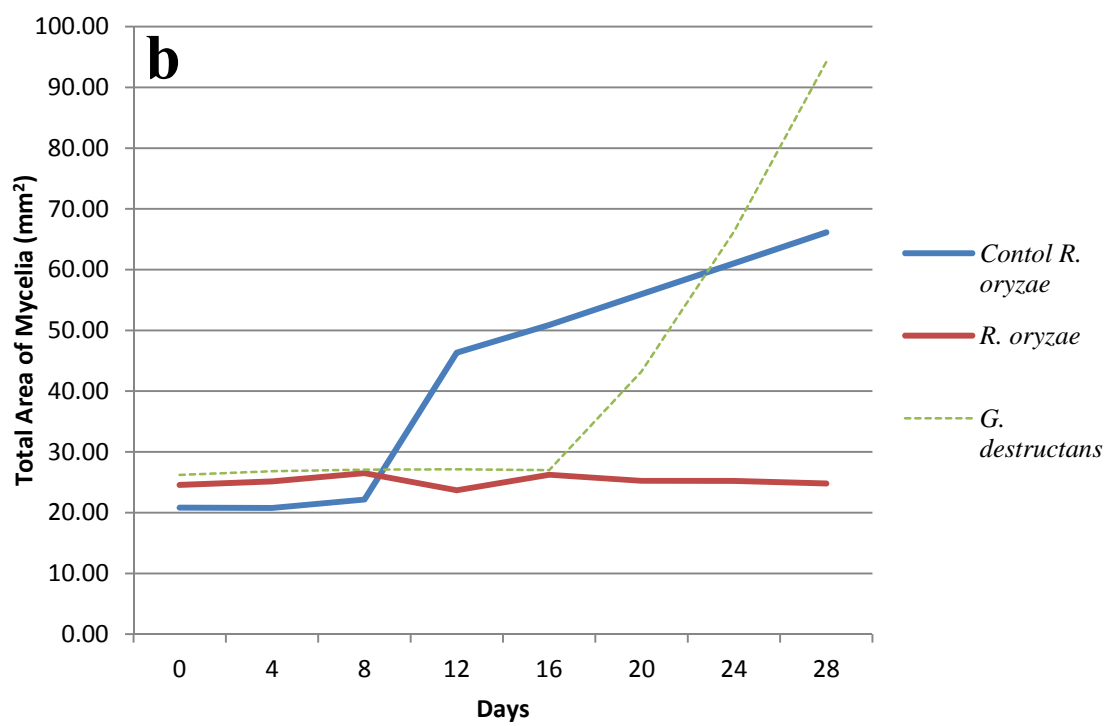
### 3.3 Mass Spectral analysis of antagonistic Volatiles

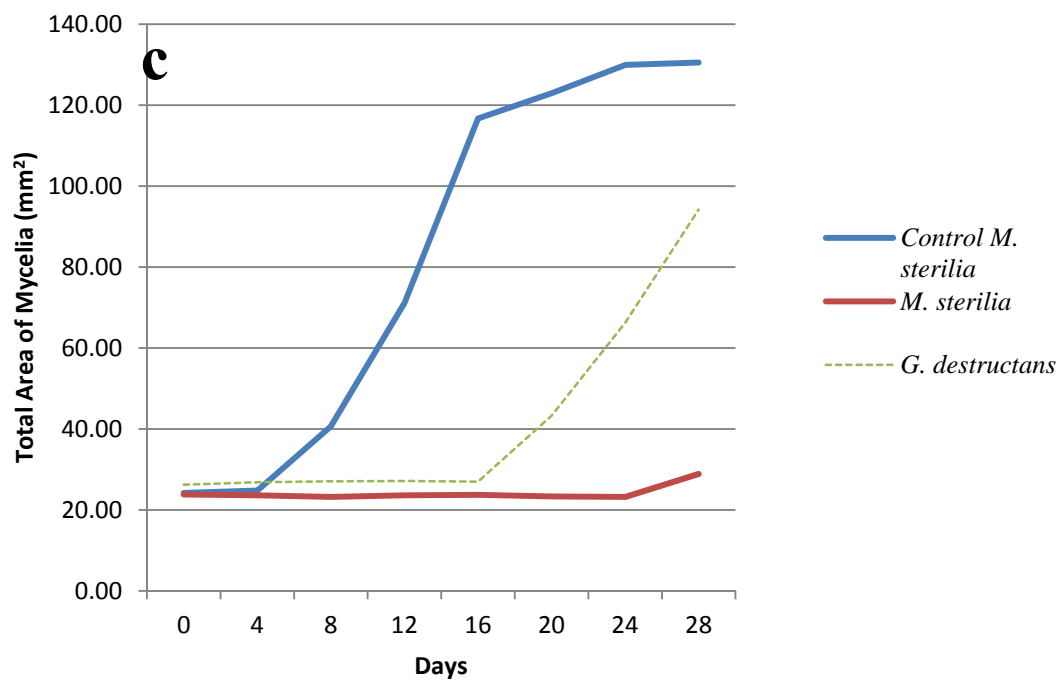
Representative pure and co-cultures of *Geomyces destructans* and *Rhodococcus rhodochrous* DAP96253 failed to produce consistent, reproducible, and interpretable spectra when analyzed using exclusively mass spectrometry. Samples were analyzed with both SEM and faraday detectors and under a wide range of settings recommended by the manufacturer in an attempt to resolve a trend in the VOC composition of the respective microbial headspaces. In all attempts identification of chemical species using the NIST library failed to generate hits above the required identification threshold.

### 3.4 Fungal resilience to control agent exposure

Induced *Rhodococcus rhodochrous* DAP 96253 significantly inhibited radial growth of *Eupenicillium lassenii*, *Rhizopus oryzae*, and *Mycelia sterilia* (*Aspergillus flavus*) at 15°C (Fig. 3.12). Subsequent evaluation of the potential for recovery after 24 hour exposure to induced *Rhodococcus* indicated that *Rhizopus oryzae*, *Rhizopus microspora*, *Mycelia sterilia* (*Aspergillus flavus*) and *Absidia corymbifera* mycelial convalesce to vegetative growth after removal of induced *Rhodococcus* while *Eupenicillium lassenii* did not (Table 3.2).







**Figure 3.11** Growth areas of select fungal plugs exposed to induced *R. rhodochrous* DAP 96253. (a) *Eu-penicillium lassenii*, (b) *Rhizopus oryzae*, and (c) *Mycelial sterilia* (*Aspergillus flavus*) failed to grow in a shared air-space with induced *Rhodococcus*. All trials conducted at 15°C.

**Table 3.2** Recovery of mycelial plugs after 24 hour exposure to induced *Rhodococcus rhodochrous* DAP96253. + indicates growth, - indicates permanent inhibition. Cultures were evaluated for 14 days after removal of induced *Rhodococcus*. All trials conducted at 25°C.\*Slowed growth after removal of control agent

	Control 1	Control 2	Experimental 1	Experimental 2
<i>R. microspora</i>	+	+	+	+
<i>R. oryzae</i>	+	+	+	+
<i>A. corymbifera</i>	+	+	+	+
<i>E. lassenii</i>	+	+	-	-
<i>M. sterilia</i>	+	+	+	+

### 3.5 Melanin production in *G. destructans*

*G. destructans* cultures incubated in the absence of light have revealed distinct colony morphology as compared to cultures grown under ambient white light. Light grown colonies at 15°C on SDA produced a brown diffusible pigment and demonstrated a greater degree of reverse side mycelial pigmentation when compared to cultures grown in the absence of ambient white light (Fig 3.13).

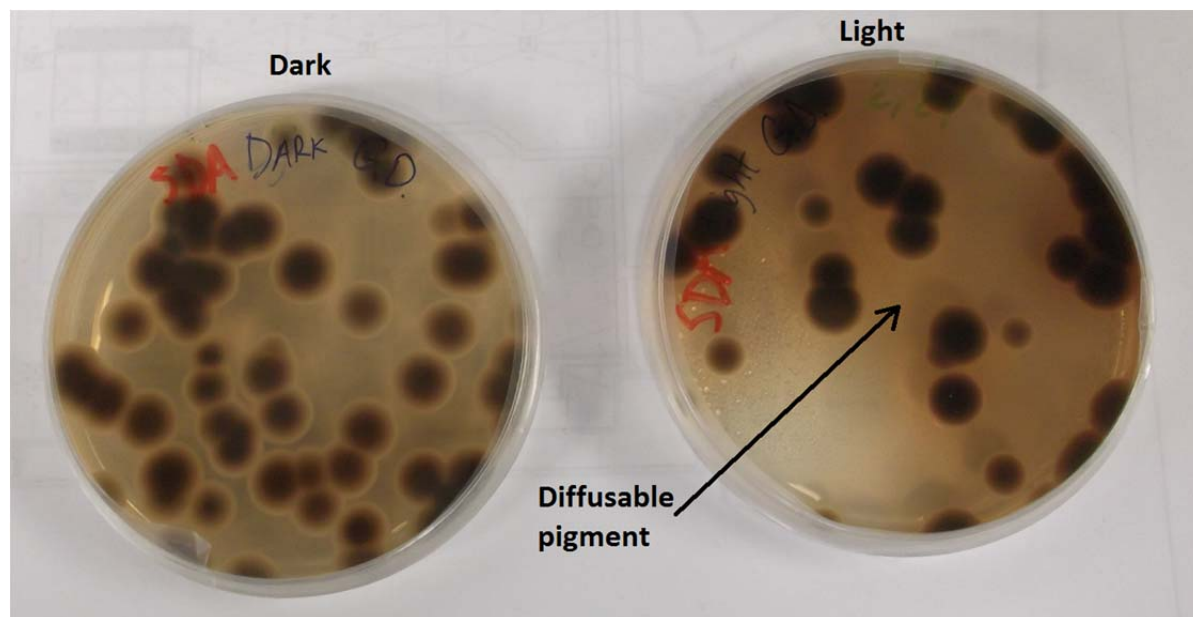
Subsequent evaluation of cultures from mycelial plugs exposed to induced *Rhodococcus* or benzothiazole revealed increased reverse side pigmentation as well as significant diffusible pigment production (Fig. 3.8, Fig. 3.14). In order to determine whether the observed pigment was melanin a copper sulfide-silver staining method [9] was employed. Samples from visibly pigmented exposed cultures and unexposed control cultures produced significantly different stains localizations when evaluated microscopically (Fig. 3.15) Exposed samples had increased staining of conidia and significant pigment associated



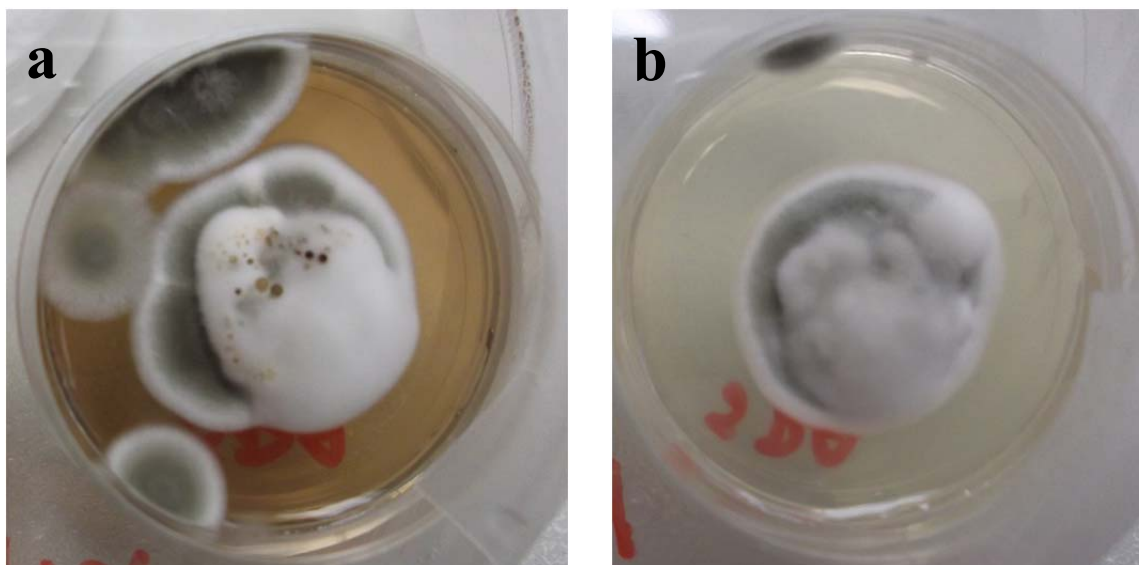
with agar as compared to hyaline control samples (Fig. 3.15) No pigment was observed to be localized to the mycelia as all samples produced hyaline mycelia regardless of preparation.

UV Vis spectroscopy of filtered diffusible pigment extracts showed strong absorption in the 200-250 nm range corresponding with synthetic melanin standards and previous studies for fungal melanin (Fig. 3.16a) [24, 42]. The log absorbance plotted for the visible spectrum (400-600nm) of control agent exposed culture extracts and synthetic melanin standards produced a negative slope characteristic of melanin while hyaline culture extracts produced a positive slope in the same region (Fig. 3.16b). The melanin standard corresponding to 0.1 mg/mL produced and highly similar absorption maxima to the  $10^{-2}$  diluted control agent exposed culture extract indicating an estimated melanin concentration of 1  $\mu\text{g/mL}$  in the original fungal extract (Fig. 3.16c).

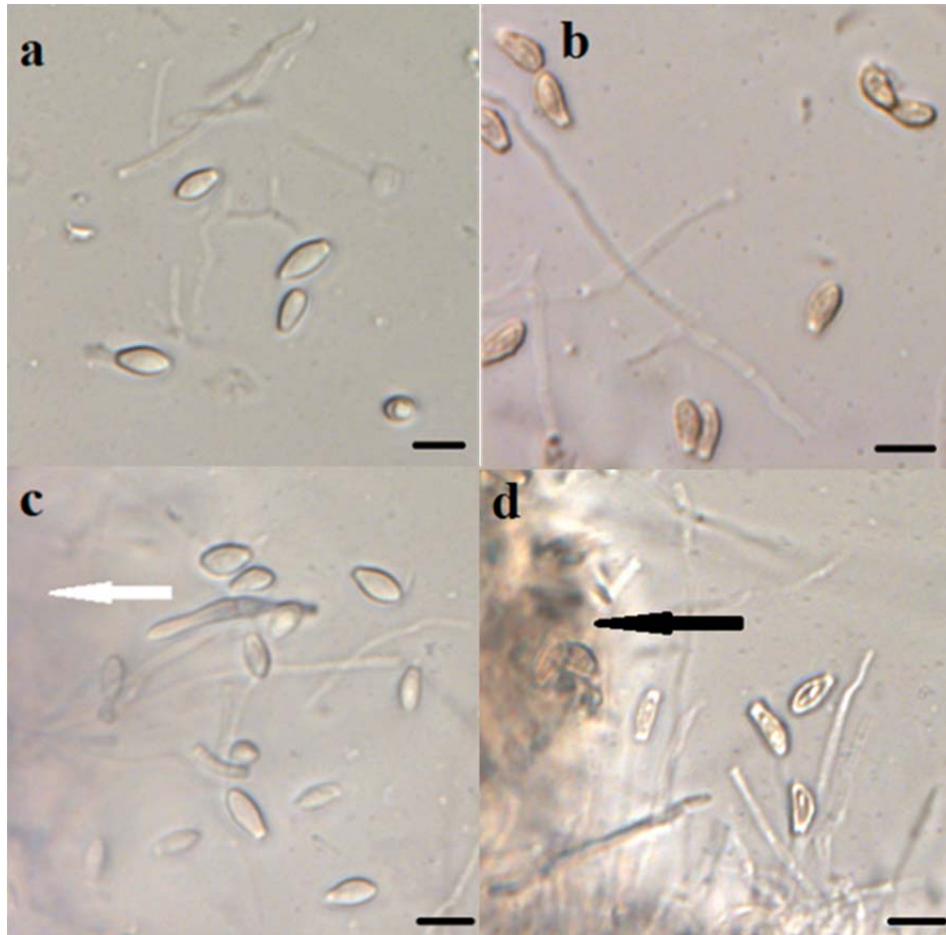
Growth on L-DOPA supplemented media produced significant melanization of *G. destructans* (Fig. 3.17). The induced melanization was observed to be secreted into the media as well as localized to the mycelia and conidia (Fig. 3.17).



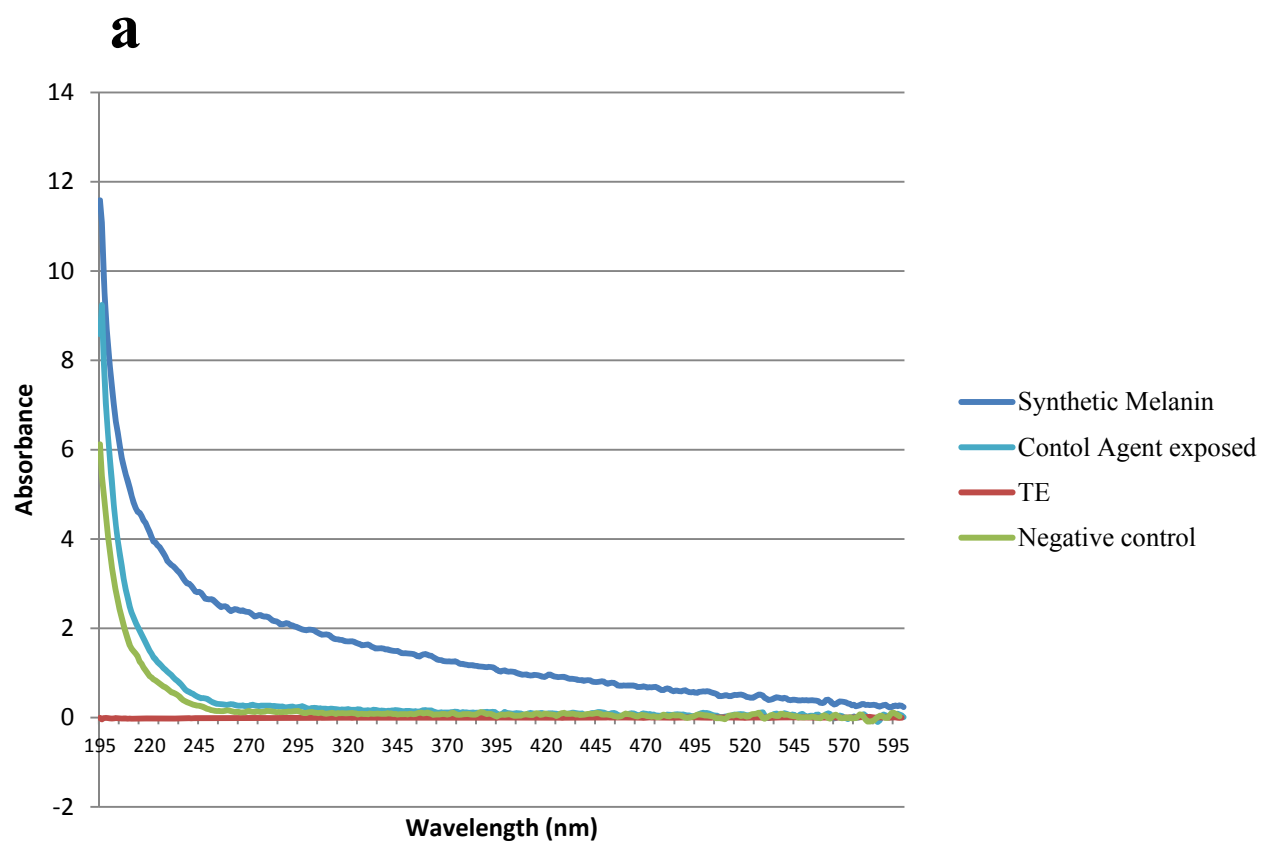
**Figure 3.12** Production of a brown diffusible pigment in *G. destructans* cultures grown from spores in ambient white light. Cultures grown in the absence of light lack the diffusible pigment.

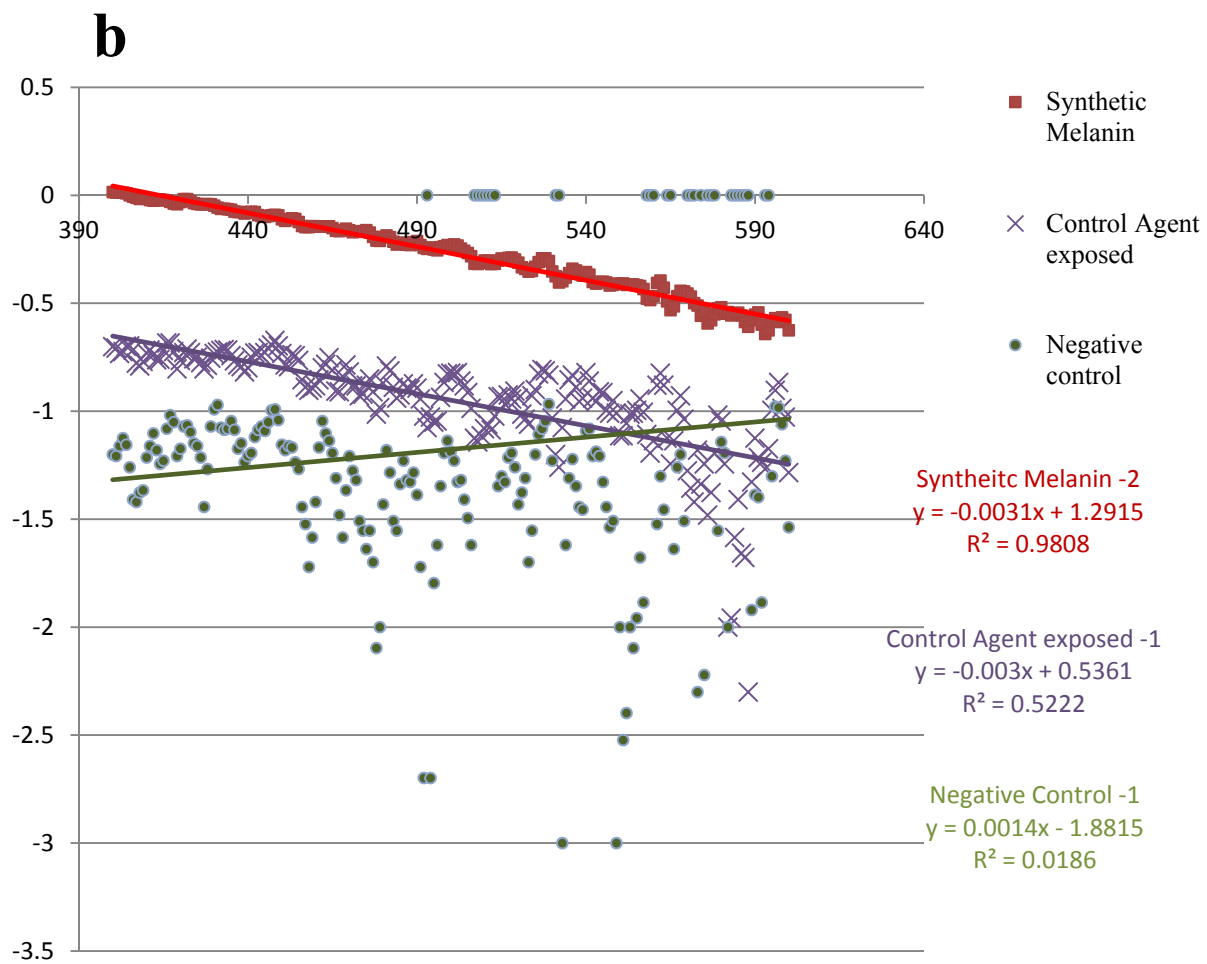


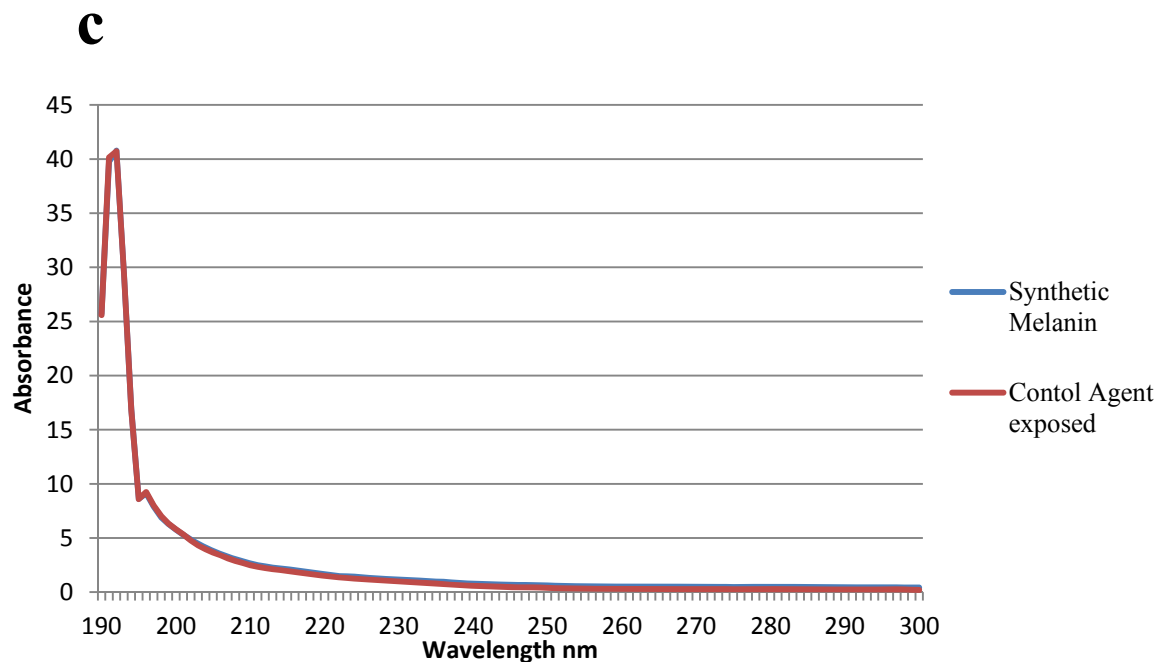
**Figure 3.13** Exposure to induced *Rhodococcus* induces diffusible pigment production in *G. destructans* (a). Unexposed cultures lack diffusible pigment (b).



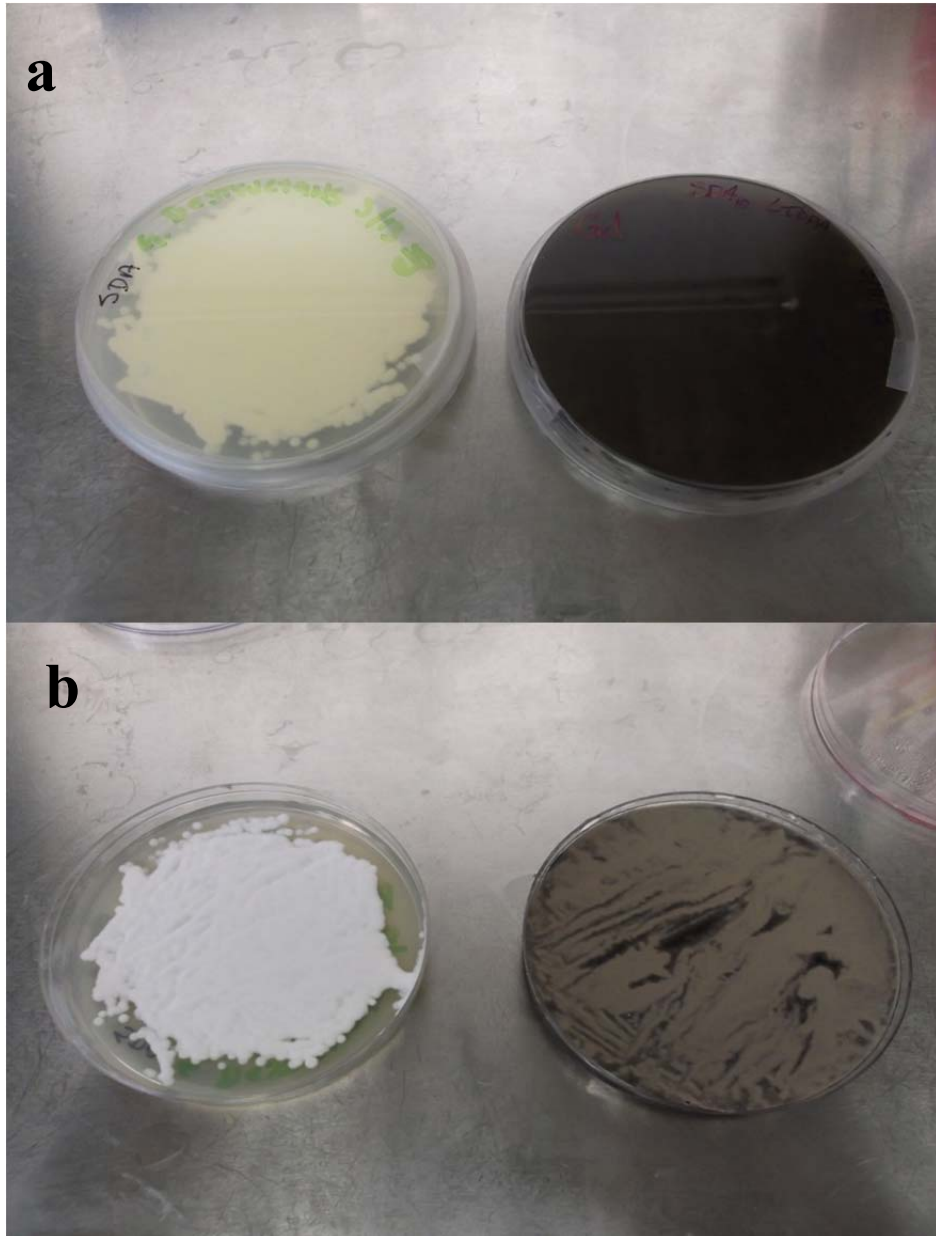
**Figure 3.14** Copper sulfide-silver staining of hyaline and pigmented culture of *G. destructans*. Unexposed cultures produced hyaline conidia (a) and no diffusible or localized pigment (c, white arrow). Control-agent exposed cultures produced pigmented conidia (b) and diffusible and localized pigment (d, black arrow). All Images taken at 200X magnification. Images corrected for white balance. Scale bar is 5 $\mu$ m.







**Figure 3.15** UV-Vis spectra of pigmented fungal extracts and synthetic melanin standards. UV-Vis spectra (a, 195–600 nm) of fungal extracts from control agent exposed and unexposed hyaline cultures of *Geomyces destructans* as well as synthetic melanin standards. Slope of linear regression (b) of log absorbance versus wave length in the visible spectrum (400–600 nm). (c) A synthetic melanin standard of 0.1mg/mL produced identical absorption maxima to the  $10^{-2}$  diluted control agent exposed extract indicating a melanin concentration of 1mg/mL for the original extract.



**Figure 3.16** SDA supplemented with L-DOPA (0.2g/L) induces significant melanin production in *Geomyces destructans*. Increased melanin is excreted into the media as seen on the reverse side of the plate (a, image right) as well as localized in the mycelia and conidia (b, image right).

## 4 DISCUSSION

Since its initial discovery in 2006 *G. destructans* has spread to twenty-four states and four Canadian provinces and is implicated in the mortality of 5.7 million bats [19]. Cave closures and culling of infected individuals appears to have little to no impact on the spread and mortality associated with this devastating disease. Classic disease management practices applied in agriculture such as vaccination and broad spectrum dissemination of antibiotics are not realistic options for management of disease in wild, highly disseminated, and migratory animal populations. Accordingly, the development of novel treatment options are needed to avert the spread of this disease, and reduce the mortality associated with currently infected hibernacula. To this end the development of biologically based control tools is the preferred option for application in karst environments.

Since the publication of the National Response Plan (Appendix B) several groups have initiated investigations to identify potential biological control agent for *Geomyces destructans* [1, 10, 18]. Several of the investigations have relied on traditional sources of biocontrol agents or probiotics such as bacilli and lactobacilli or competitive exclusion fungi such as a-toxigenic *Aspergillus* spp. and *Trichoderma* sp. as well as attempts to isolate bat-skin-associated microbes with anti-*G. destructans* activity [1, 10, 18]. While these approaches have proven successful in agricultural and human health applications [29, 33, 41] their application in the attempted remediation of WNS in bats has not been demonstrated. The requirement for contact with *G. destructans* and the bat hosts is a major hurdle for any agents reliant on competitive exclusion or non-volatile antimicrobial compound production. These potential control agents may prove to have limited efficacy against *G. destructans* *in situ* and potentially be harmful to the bat hosts. In contrast, my evaluation of induced *Rhodococcus rhodochrous* DAP 96253 for application as a biological control agent of *G. destructans* aligns ideally with the needs of wildlife management agencies tasked with combatting WNS and is the first documented contact-independent microbial antagonism of *Geomyces destructans*.



The evolutionary lineage of *Rhodococcus* lends itself to VOC based fungistasis due to its terrestrial ancestry. The global prevalence of fungistatic soils is a measure of the natural antagonisms that exists in the complex environments. Due to the ubiquity of *Rhodococcus* in soils it can be expected that *Rhodococcus* contribute to VOC based fungistasis observed in these environments. Leveraging these natural antagonisms for control efforts has many benefits particularly in the case of WNS. The complexity of soil ecology selects for antagonisms that are effective in diverse, compartmentalized environments where soluble diffusion maybe limited. Accordingly the production of antagonistic VOCs provide a viable means for soil dwelling bacteria to compete with soil dwelling fungi for resources. The ability of *Rhodococcus* to detect and interfere with volatile signals has also been demonstrated in its delayed fruit ripening activity [32] and can be anticipated to mediate the observed anti-*Geomyces destructans* activity.

While the efficacy of urea induced *Rhodococcus* under growth conditions is promising for *in situ* management of WNS, the need for growth media supplementation poses problems for field application. The long term *in vitro* efficacy of non-growth condition cell paste at 4°C allows for increased confidence in forecasting the efficacy of this biocontrol agent in managing WNS in the field. The lack of growth media reduces the costs associated with application as well as reduces the likelihood of cross contamination of control agent media with native cave flora. In addition, the contact-independent basis of the non-growth antagonism will allow for *in situ* application methods that will reduce the potential for ecological impacts associated with introducing exogenous organisms to karst environments. The ecological impacts of any potential control agent are of significant concern for wildlife management agencies, and the evaluation of potential ecological impacts must be assessed in order to circumvent ecological disaster associated with augmenting cave microflora<sup>14</sup> (i.e. Lascaux cave) [2].

The evaluation of *Rhodococcus* using *ex vivo* bat tissue explants as an indicator of anti-infective activity was paramount to establishing *Rhodococcus* as a viable biocontrol agent of *G. destructans*. This was the first demonstration of inhibition of fungal colonization of bat tissue by a biological control agent and further more is the only demonstration of biologically mediated contact-independent antagonism of

*G. destructans*. This *ex vivo* efficacy justifies further *in vivo* studies with live bats and should be pursued vigorously by federal agencies tasked with the management of this wildlife epidemic.

The ability of dormant conidia to remain viable in host-free environments increases long term impacts of fungal pathogens and renders infected environments inhospitable to re-colonization. The impact of WNS in locations such as New York has been tremendous rendering entire geographical areas vacant of insectivorous bats. In order to restore ecological balance to these devastated communities inactivation of viable *G. destructans* conidia must occur. The permanent and irreversible inhibition of conidia germination is a promising result and indicates that treatment of previously decimated hibernacula to inactivate resident conidia prior to re-colonization attempts may be feasible by applying induced *Rhodococcus* in these environments.

Attempts to determine the microbial VOCs involved in the observed antagonism using mass spectral analysis failed to produce consistent and reproducible results. Consultation with the manufacturer confirmed my concerns that in the absence of separation afforded by gas chromatography/ mass spectrometry (GC/MS) the identification of unknown VOCs in a complex headspace would not be obtainable. The small molecular mass and high diversity of microbially produced VOCs expounds this issue, and ionization of these chemical species creates significant molecular overlap and exceeds the capacity of deconvolution software [23, 35]. Future attempts to resolve the VOCs involved in this antagonism should rely on well-established techniques such as thermal desorption/gas chromatography/mass spectrometry (TD/GC/MS) that have proven successful in this endeavor and generate data compatible with the more refined deconvolution software [23, 35].

In addition to *Rhodococcus*, soil based fungistasis was investigated on a broader scale to determine the potential of this environment to harbor additional biocontrol agents as well as to identify potential chemical control agents. The co-evolution of soil microbiota has produced antagonisms ideally suited for the complex ecology of soil. Harnessing these natural antagonisms can be a powerful tool in combat-

ing WNS as many of the traits of these antagonisms yoke favorably with the ecology of hibernacula and the terrestrial heritage of the *Geomyces* warrants their susceptibility. Accordingly, the evaluation of previously described bacterially produced antifungal volatiles was investigated with great success. The long term efficacy of low relative concentration VOCs unveils the potential of these compounds for *in situ* application in the treatment of WNS. Additionally, the development of synergistic blends serves to bolster the appeal of soil based fungistasis as a source of potential control agents as VOC blends are likely responsible for the observed fungistatic activity of repressive soils [11, 20, 38]. While several pure VOCs and blends produced significant growth inhibition, compounds and/or concentrations unable to significantly inhibit growth caused noteworthy stress to *G. destructans* as determined by the abnormal phenotypes observed under these conditions. The evaluation of bacterially derived VOCs has expanded the pool of potential biological control agents as well produced several VOC formulations with excellent anti-*Geomyces* activity at low relative concentrations. The availability of volatile formulations for control of *G. destructans* growth could prove to be a powerful tool for wildlife management agencies.

My evaluation of the resilience of resident fungi in response to induced *Rhodococcus* indicates that impacts on the resident flora other than *Geomyces* spp. may be significant. Mycelial plugs exposed to induced *Rhodococcus* for 28 days at 15 °C and then removed from the presence of induced *Rhodococcus* failed to return to typical growth patterns although growth was restored when *Rhodococcus* was removed after 24 hours. Initial analysis of conidia recovery will need to take place before impacts on the native mycoflora<sup>16</sup> can be accurately forecast. The potential concern of this broad spectrum anti-fungal activity should not be overstated as limited growth of fungi in North American caves during winter is expected. In this case the application of induced *Rhodococcus* during the winter hibernation season may inhibit the growth of fungi including *G. destructans* but the long term (seasonal) impact has yet to be determined. The evaluation of resident mycoflora conidia susceptibility and recovery will be significant to understanding the potential long term ecological impacts of applying *Rhodococcus* to these environments. Permanent and irreversible inhibition of resident mycoflora conidia would be of significant concern but may be man-

ageable due to the ubiquitous distribution of resident mycoflora in the karst environment and the colonial hibernation patterns of several susceptible North American bat species.

The identification of diffusible pigment, further characterized as melanin, is significant for future investigation into the virulence of *Geomyces destructans* as well as for determining the efficacy of potential control agents. Melanin is a known virulence factor in pathogenic fungi such as *Cryptococcus neoformans* and *Wangiella dermatitidis* which increases hyphal invasiveness and reduces the host's ability to clear sub-cutaneous infection [8, 24]. In addition, melanin reduces environmental oxidative damage to fungal cellular components and may influence the efficacy of control efforts and antifungal treatments [8, 24, 39]. The absorbance maxima between 190 and 250 produced via UV VIS spectral analysis, as well as the negative slope of the log absorbance in the visible spectrum compare favorably to previous studies of fungal melanin [8, 24] as well as synthetic melanin standards. The results all indicate that the pigment is melanin, however the application of more refined analytical techniques will allow for the complete resolution of the chemical structure of the observed pigment. Nevertheless, the heavily pigmented phenotype produced by cultivation of *G. destructans* on SDA supplemented with L-DOPA demonstrated the melanogenic<sup>13</sup> potential of this vertebrate pathogen. The resulting L-DOPA induced pigmentation shows similar characteristics to the pigment produced in response to control agent exposure but withstanding more refined chemical characterization a definitive connection cannot be resolved.

The increased pigmentation of conidia from cultures exposed to control agents may be significant for recurrent applications in heavily colonized hibernacula. The increased pigmentation associated with these conidia may serve to reduce the impact of the control agents and select for resistant propagules in these environments. Further analysis of the viability and susceptibility of pigmented conidia will need to be conducted to address these concerns.

I anticipate that this project will revolutionize the way WNS is managed in the field. The data I have presented in this dissertation has direct implication on disease management, and to the authors un-

derstanding, it is the first documented case of contact-independent biological antagonism of *G. destructans*. Until recently, the prognosis for susceptible North American bat populations was bleak at best. The development biological and chemical treatment options provide wildlife management agencies with the first potential tools for control of *G. destructans* transmission and infectivity.

This project involved collaboration with Georgia State University faculty, other University System of Georgia institutions, University of California- Davis faculty, the U.S. Forest Service, and the non-profit organization Bat Conservation International. Cumulatively this project represents a significant interdisciplinary collaborative effort in response to a national crisis and has produced the first tangible biological tool for management of WNS in bats. A summary of the investigation of the anti-*G. destructans* activity of induced *Rhodococcus* was published in the Summer 2013 edition of BATS magazine under the title “The Enemy of My Enemy is My Friend: A new hope in the battle against WNS” (Appendix C). Based on the results presented in this dissertation the USFS has initiated a series of field trials with *Rhodococcus* that are scheduled to begin in Fall 2013.

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## APPENDICES

### Appendix A

#### Glossary of terms

1. **Biocontrol** – The application of biological entities to reduce or eliminate unwanted organism including exotic species, pests and pathogens.
2. **Biodiversity – 1)** The number and variety of organisms found within a specified geographic region. **2)** The variability among living organisms on the earth, including the variability within and between species and within and between ecosystems.
3. **Chiropteraphily** – A designation within pollination syndrome used to describe obligate bat-mediated pollination.
4. **Conidia** – Asexual, haploid, non-motile spores produced externally by fungi. In Ascomycetes conidia are produced by specialized structures termed conidiophores and typically dispersed in air currents. Also termed spores.
5. **Conidiation** - The biological process in which filamentous fungi reproduce asexually from conidia. Also termed sporulation.
6. **Climacteric (Botany)** - A stage of fruit ripening associated with ethylene production and cellular respiration rise. Climacteric is the final physiological process that marks the end of fruit maturation and the beginning of fruit senescence.
7. **Fungistasis** – Non-lethal inhibition of fungal growth and germination.
8. **Germination** – In filamentous fungi, germination is the process by which hyphae emerge from conidia.
9. **Germule** – The initial mycelial extension emanating from conidia that is longer than the length of the conidia from which it emerged.
10. **Hibernacula** – A zoological term to describe a shelter or abode used by animals for over wintering. Typically associated with torpor.
11. **Hyaline** – Denotes a material with a glass-like appearance, free of pigmentation.
12. **Karst** – A landscape formed from the dissolution of soluble rocks characterized by sinkholes, caves, and underground drainage systems.
13. **Melanogenic** – Capable of producing melanin or melanin containing compounds.
14. **Microflora** – A group or consortia of microorganisms present in a specific, localized location or environment.
15. **Microbial antagonism** – A type of symbioses of microorganisms which describe one microorganism's ability to kill, injure, or inhibit the growth of another microorganism.
16. **Mycoflora** - The fungi characteristic of a region or specific environment or ecosystem.
17. **Synthetic Melanin** – Laboratory synthesized melanin involving an air oxidation of tyrosine or L-DOPA in the presence of tyrosinase enzymes (extracted from fungi). The pigment obtained from the enzymatic oxidation readily precipitates.
18. **Torpor** – A state of decreased physiological activity in an animal, usually by a reduced body temperature and rate of metabolism. Torpor is used to enable animals to survive periods of reduced food availability.

19. **Volatile organic compound (VOC)** – Organic chemicals that have a high vapor pressure at ordinary, room-temperature conditions. Their high vapor pressure results from a low boiling point, which causes large numbers of molecules to evaporate or sublime from the liquid or solid form of the compound and enter the surrounding air.
20. **Zoophily** – A form of pollination whereby pollen is transferred by vertebrates, particularly by hummingbirds and other birds, and bats.

## Appendix B

Excerpts from the WNS National Response Plan. Courtesy of the WNS National Plan Writing Team and the Steering Committee to Develop the WNS National Plan.

6

### Elements of the National Plan

The steering committee for the WNS national plan identified seven elements to be addressed by the plan; each will be administered by a working group responsible for the coordination of activities within that element. Working groups will be populated based on individual expertise and not on agency or organizational representation. Therefore, working groups will be open to qualified individuals, regardless of affiliation or nationality. Each working group will designate one leader who will oversee and coordinate the activities within that group. All working group leaders will also serve on an oversight team to coordinate activities and ensure communication between the working groups. This role is of particular importance given the complementary objectives of several groups and the need for collaboration to avoid duplicative efforts between elements.

The seven elements of the national plan are:

- A. Communications
- B. Data and Technical Information Management
- C. Diagnostics
- D. Disease Management
- E. Epidemiological and Ecological Research
- F. Disease Surveillance
- G. Conservation and Recovery

**A. Communications and Outreach Working Group:** The purpose of this group is to develop and implement an effective plan for communicating information about



*Biologists meet outside Greeley Mine, Vermont*

Martin Moriarty/USFWS



Marcin Morawski/USFWS

*Biologist entering Greeley Mine, Vermont*

WNS to partners involved in the WNS investigation, to affected landowners and stakeholders, and to the public. The goals and actions outlined in this document provide the basis for a detailed national communications implementation plan.

**B. Data and Technical Information Management Working Group:** The purpose of this group is to provide a mechanism for making WNS information accessible in a timely fashion to all State, Tribal, and Federal agencies and others involved with the investigation and management of WNS. The primary goal of this group is to disseminate information about WNS by providing access to common scientific and technical information in a partner-based data system; working with States to create data standards that will allow inter-operability with existing WNS data sets; providing researchers and wildlife managers with near real-time access to WNS data and other critical information; and, ultimately, integrating WNS data from State and Federal agencies and others into a more structured national database.

**C. Diagnostics Working Group:** The purpose of this group is to establish standards on how laboratories are to conduct and interpret WNS testing so that results are accurate and comparable between laboratories; identify current laboratory capacity for processing WNS samples and project the capacity needed to support effective WNS management programs; provide timely reporting of diagnostic results to resource agencies responsible for management decisions; and support WNS research.

**D. Disease Management Working Group:** The purpose of this group is to identify a range of alternatives and best practices to prevent the introduction of WNS into new areas, prevent or slow the spread of WNS to WNS-free sites within infected areas, and attain sufficient control of the disease in affected areas so that genetic and regional diversity and the potential for recovery to pre-WNS abundance is maintained; secure the future of bats while avoiding unacceptable risks to other cave-obligate biota and natural systems; and collaborate with public health officials to establish whether a human health threat is associated with WNS and determine a course of action if the WNS agent poses such a threat.

**E. Epidemiological and Ecological Research Working Group:** The purpose of this group is to identify critical ongoing research needs relating to the origin, transmission, pathogenesis, and impact of WNS on bats and the environment. Research to inform management actions will be a priority; therefore, approaches that recognize the synergy between research and management will be emphasized to maximize the potential to achieve optimal results.

**F. Disease Surveillance Working Group:** The purpose of this group is to develop standards for WNS surveillance in affected and non-affected areas, and describe best practices and techniques for surveillance strategies. The goal of this group is, therefore, to provide a framework for consistent, coordinated WNS surveillance, focusing on early detection of the expansion of WNS or newly established epicenters and providing data on the progression of WNS within an affected hibernating colony.

**G. Conservation and Recovery Working Group:** The purpose of this group is to develop standards for determining if and when to monitor populations of bat species that are affected by WNS or *G. destructans*; establish criteria for prioritizing conservation and management activities; and describe best practices and techniques for the recovery of bat populations of greatest conservation concern. Additionally, this group will provide guidance on collection of baseline data for areas still unaffected by WNS, such as conducting a statewide accounting of caves and mines.

- suitable sample submissions for diagnostic evaluation.
- (2) Provide case definitions for suspected and confirmed cases of WNS, and classification criteria of contaminated hibernacula.

**Goal 4: Assist with timely reporting of WNS testing results to inform the appropriate resource management agencies for release to the broader WNS community.**

**Action:** Work with the Data and Technical Information Management Group to develop a secure, centralized database for tracking sample results and disease progression.

**Goal 5: Support WNS research such as epidemiology, treatment/management options, improved diagnostic assay development, etc.**

**Actions:**

- (1) Critically review current knowledge of WNS diagnosis to identify knowledge gaps and research needs.
- (2) Prioritize diagnostic research needs to fill identified knowledge gaps and determine funding requirements.
- (3) Help coordinate laboratory assistance with federally and state-funded WNS research projects requiring sample testing, and ensure that sufficient funding is allocated to support participating laboratories beyond their primary diagnostic priorities.

*Biologist studying bat with white-nose syndrome*



Marvin Moriarty/USFWS

**D. Disease Management:**

**D.1. Overview**

Disease management is composed of three complementary goals: to identify and implement science-based management actions to slow the expansion of WNS in order to delay, for as long as possible, the impacts of the disease reaching unaffected regions of the continent; to develop and employ interventional strategies to the disease that will ensure the perpetuation of susceptible bat species, and that will provide the best opportunities for their recovery to pre-WNS numbers in affected regions; and to ensure that implemented actions will not be detrimental to bat populations or have unacceptable effects on the ecosystems in which they are found. This work is in its infancy and most of the questions critical to its success have not yet been resolved. There are, as yet, no proven applications that address any of the challenges presented below, and it is unclear whether the objectives detailed below are obtainable. This group will assist State, Federal, and Tribal agencies in determining the goals of management actions taken and the most feasible management tools that can be applied.

Monitoring the effectiveness of management actions will be critical to achieving the goals outlined below. Successful coordination and monitoring of all management actions will maximize our potential to learn from them and allow managers to employ adaptive management principles to refine research and management priorities. It will be important to develop and/or maintain the necessary capacity within State agencies to support the implementation of these disease management objectives.

**D.2. Goals and Action Items**

**Goal 1: Critically review current knowledge of WNS disease management to identify knowledge gaps and research needs.**

**Actions:**

- (1) Solicit expert review of previous and current research projects and identify knowledge gaps.
- (2) Identify priority research questions and capacity not currently being addressed in the investigation of WNS, including human dimensions.
- (3) Identify high-priority laboratory and field activities needed to support research priorities.

**Goal 2: Reduce the risk of WNS transmission by humans.**

**Actions:** (1) Identify the mechanisms for WNS transmission by humans to



Marvin Moriarty/USFWS

Biologist photographing bat with white-nose syndrome

- environment to bats.
- (2) Provide guidance on regulation or restriction of human actions that are likely to pose a risk for spreading WNS.
    - (a) Develop standards for restricting use of potentially contaminated gear (both caving and bat research) at unaffected sites or regions.
    - (b) Manage cave access to minimize transmission risk.
    - (c) Work with cave owners to implement operating guidelines for commercial caves.
    - (d) Modify mist netting and harp trapping protocol/ techniques.
    - (e) Investigate the potential risks of commercial trafficking of bat guano to the spread of WNS.
  - (3) Develop, implement, and where possible, enforce decontamination/disinfection protocols to guard against human-assisted transmission of WNS to new sites or animals.

### Goal 3: Reduce inter-/intra-specific transmission and disease spread.

- Actions:
- (1) Investigate bat-to-bat transmission of WNS.
    - (a) Identify prevalence/distribution of infected animals within hibernacula/clusters.
    - (b) Develop techniques for identifying infected animals (photo/thermography).
    - (c) Determine effectiveness of in situ management actions (e.g., removal of infected and adjacent individuals, temporary barriers to infected substrates, etc.).
    - (d) Investigate the potential for tree bats to serve as carriers of *G. destructans*.

### Goal 4: Reduce environmental transmission to and from bats.

- Actions:
- (1) Investigate WNS transmission from environment-to-bat.
  - (2) Develop environmental decontamination techniques.

### Goal 5: Eliminate *G. destructans* from infected individuals.

- Actions:
- (1) Investigate means of *G. destructans* control that are effective and safe for the bats.
    - (a) Identify chemical control treatments for *G. destructans*.
    - (b) Identify biological control treatments for *G. destructans*.
    - (c) Identify effective environmental manipulations to reduce or eliminate *G. destructans* from affected bats or sites.
    - (d) Identify effective bat exclusion/inclusion of infected sites/uninfected sites.
  - (2) Reduce disturbance-related mortality associated with disease management activities.

### Goal 6: Identify and limit adverse ecological impacts of management actions, including decontamination techniques, to acceptable limits.

- Actions:
- (1) When appropriate, research the need for, conduct, and/or support human dimensions inquiries to define acceptable limits for ecological impacts.
  - (2) Monitor management action outcomes and use adaptive management iterations to improve results, in light of potential ecosystem impacts.

## E. Epidemiological and Ecological Research

### E.1. Overview

Although State, Federal, academic, and non-government organization researchers have worked collaboratively to increase understanding of WNS since its discovery, there are significant knowledge gaps regarding the fundamental dynamics and ecology of this disease. These gaps impede the development of plans to control and mitigate the disease, because effective management requires an understanding of the interactions among the disease, its host(s), and the environment. This

section identifies priority research areas in which progress must be made in order to better understand and respond to the threat of WNS. Key to managing this disease will be the guiding principle that research must primarily address management needs, and that basic research results should be applied to adaptive management decisions.

Research is still needed on relevant aspects of bat ecology and behavior, diagnostic methods, etiology, pathology, epidemiology of the disease, presence and persistence of the causative agent in the environment, risks posed to other species and environments, genetics of cave fungi, host immune response, limits of pathogen survival, mode of mortality, bat population structure, and differential susceptibility. This research will be conducted through partnerships among academic entities, non-government organizations, and State and Federal agencies. New information may shift priorities and reveal new areas of investigation. Therefore, an effective process for coordinating research is also required.

## **E.2. Goals and Action Items**

### **Goal 1: Critically review current knowledge of epidemiology and ecology of WNS to identify knowledge gaps and research needs.**

- Actions:**
- (1) Solicit expert review of previous and current research projects and identify knowledge gaps.
  - (2) Identify priority research questions and capacity not currently being addressed in the investigation of WNS.
  - (3) Identify high-priority laboratory and field activities needed to support research priorities.

### **Goal 2: Establish disease etiology.**

- Actions:**
- (1) Investigate the role of *G. destructans* as the likely primary causal agent of WNS, and increase our understanding of other potential contributing factors.
  - (2) Investigate the origins and evolution of *G. destructans*.
  - (3) Continue to consider evidence for other potential synergistic, predisposing, and/or causative agents for the suite of WNS signs observed in bats.

### **Goal 3: Enhance understanding of WNS pathogenesis.**

- Actions:**
- (1) Investigate the life cycle of *G. destructans*, including optimum environmental growth/viability conditions.
  - (2) Identify the mechanisms of transmission and infection of *G. destructans*.
  - (3) Investigate species differences in pathogenesis and susceptibility.
  - (4) Investigate whether other animal taxa are associated with WNS epidemiology.

### **Goal 4: Understand interactions of pathogen, host ecology, and environment.**

- Actions:**
- (1) Obtain basic epidemiological information (e.g., distribution, prevalence, incidence, case-fatality rates).
  - (2) Investigate critical control points in WNS dynamics.
  - (3) Collect baseline information on species presence, population sizes, and hibernacula in unaffected areas.
  - (4) Collect information on other biota at affected and unaffected hibernacula.
  - (5) Continue long-term monitoring efforts in affected areas to identify changes over time in disease infection, mortality, and population demography.
  - (6) Design and implement studies to identify and parameterize variables for disease models of transmission routes and rates, as well as species-specific infection, mortality, and carrier rates, and the impact(s) of bat density and species composition.
  - (7) Identify and employ appropriate disease models to evaluate and predict the spread and impact of WNS.



## Appendix C

BATS magazine article, “THE ENEMY OF MY ENEMY IS MY FRIEND: A new hope in the battle against WNS?” Courtesy of Bats Conservation International and BATS Magazine.



by Chris Cornelison

Graduate student Chris Cornelison, a microbiologist at Georgia State University, works in the lab during his search for biological agents that might combat White-nose Syndrome.

**F**or the past six years, the “silver bullet” sought by scientists battling White-nose Syndrome has been an ecologically acceptable tool for destroying or disabling the *Geomyces destructans* fungus, which causes this scourge that is killing millions of bats. But the search has been frustrating. While some chemical fungicides will kill the fungus, their use would likely devastate complex cave ecosystems and could contaminate water supplies.

Several teams, including ours, are exploring another, potentially more benign, option: biological agents. Now initial results from our research at Georgia State University suggest we have found a very promising candidate: a natural bacterium that in the lab is able to inhibit the fungus without actually touching the bats or the cave. More research is required to confirm this approach, but the evidence suggests we may be able to save bats and spare the caves.

My microbiology colleagues and I decided to tackle this problem after the national WNS Response Plan, published in May 2011, gave significant attention to developing biological and chemical control options. We soon noticed some intriguing activities exhibited by the bacterium *Rhodococcus rhodochrous*

strain DAP 96253.

Our initial investigation found that the bacterium, when cultivated under very specific conditions (U.S.P. 7,943,549), could inhibit the growth of two *Geomyces* species that are closely related to *G. destructans*. So we obtained samples of the *G. destructans* fungus from Kevin Keel (then at the University of Georgia and now at the University of California, Davis) to explore our bacterium’s anti-*Geomyces* abilities.

The initial test results were astonishing.

The cold-loving fungus attacks by sending out branching structures called hyphae that invade the bats’ tissue, especially the wings. *G. destructans* grows best at about 41 to 50 degrees Fahrenheit (5° to 10° Celsius) and essentially stops growing, becoming dormant, at 68° F (20° C). We demonstrated that *R. rhodochrous* bacteria completely blocked germination of *G. destructans* spores at 59° F (15° C) and strongly inhibited growth and reproduction at 39° F (5° C).

This was the first demonstration of biological antagonism to *G. destructans*, and we were eager to explore this potentially revolutionary tool for combating WNS. I nearly gave up, however, when I was unable to secure funding for the project, part

of my work toward a Ph.D. Luckily, I applied for and received a grant from Bat Conservation International's WNS Program.

So I initiated an experiment with Kevin Keel to determine whether *Rhodococcus* can prevent the WNS fungus from colonizing bat tissue. Rather than sacrificing a number of bats by exposing living animals to the fungus and bacterium, we used a groundbreaking technique developed by Keel to maintain disembodied bat-wing tissue culture in the laboratory.

We demonstrated the ability of *Rhodococcus* to prevent the colonization of bat-wing tissue by *G. destructans* spores for more than 40 days. The *Rhodococcus* was placed in close proximity to, but was not touching, the tissue and the fungus.

Our initial inquiry into the mechanism of the antagonism began with a simple question: does the contact-independent antagonism affect spore germination, mycelial elongation or both?

Next, a few simple experiments and some basic microscopy indicated that *Rhodococcus* completely and permanently inhibits spore germination (i.e., prevents it from sprouting) and significantly slows the growth of the tentacle-like hyphae. Since previous studies have shown the spores to be the primary infectious agent of *G. destructans*, these results suggest that *Rhodococcus* can prevent the initial colonization of healthy bats, and also slow progression of the disease in already-infected bats – and increase their chance of survival.

Using protocols developed by previous Georgia State University researchers, we grew *Rhodococcus* bacteria in 30-liter (about 8-gallon) fermentation vessels under conditions that activated its anti-*Geomyces* activity. Bacteria in the resulting "cell paste" no longer grow, but they nonetheless prevented germination of *G. destructans* spores for more than 80 days (at the time this article was written).

This was a vital step toward practical application, since it shows that we may have a control agent that can be mass produced, applied without any form of growth medium and provide long-term inhibition of *G. destructans* spores at 4° C. And it works without being in direct contact with the bats, the cave environment or the fungus.

The simplicity and efficacy of this microbial antagonism are not really surprising. The co-evolution of soil-associated fungi, such as *Geomyces*, and bacteria, such as *Rhodococcus*, lends itself to these natural antagonisms: these organisms have been waging war in a complex environment for billions of years. Humans are unlikely to devise a more effective weapon than what the natural competitors of *Geomyces* have evolved over eons of open hostilities.

Other biocontrol approaches would require microbes to be applied directly to the bats or the cave walls or soil. Our research indicates that the fermented *Rhodococcus* paste requires no direct

## Protecting Endangered Species

by Katie Gillies

Katie Gillies, who leads BCI's Imperiled Species Program, examines a cypress tree cavity to determine whether bats are using it as a roost.



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This year marks the 40<sup>th</sup> anniversary of the U.S. Endangered Species Act of 1973, a momentous milestone for wildlife conservation. When we think of endangered species, most of us picture such animals as gray wolves, grizzly bears or California condors – the movie stars of the environmental movement. But as Coordinator of BCI's Imperiled Species Program, my focus is on our threatened and endangered bats, which rarely reach

the spotlight. This year, however, bats are moving toward center stage.

Four bat species and three subspecies are currently listed as threatened or endangered under the ESA. The gray myotis (*Myotis grisescens*) has been listed as endangered since 1976. BCI played a pivotal role in the listing process. Our efforts to identify and protect significant cave roosts were critical in what was,

*Continued on page 4*



COURTESY OF KEVIN KEEL

This experiment demonstrates the potential of the *R. rhodochrous* bacterium to inhibit the growth of spores of the *Geomyces destructans* fungus that causes WNS. The first dish (A) shows spore growth after 21 days without the bacteria. Spores grew in Dish B with bacteria that were not activated ("induced") through a fermentation process. But in Dish C, induced bacteria completely inhibited spore growth after 21 days.



The author inspects a plate of *Rhodococcus rhodochrous* bacteria in a biological safety cabinet.

contact with the bats or the cave. It could, for example, be introduced on plastic sheets placed near hibernating bats. When bats are no longer at risk (typically in spring and summer), the bacteria could simply be removed.

Despite the exciting possibilities demonstrated by our research results, several important questions must still be answered before we can put this potential tool to work. We must, of course, assess any impacts to the bats and to cave ecosystems and organisms, then conduct small field trials before wide-scale application is feasible or ecologically responsible.

We are currently working on a simple model of the mycoflora of North American caves and are investigating their resilience to our biological control agent. We are also collaborating with federal wildlife biologists and toxicologists to determine any potential impacts to bats before testing our control agent in the field.

Early results are promising and provide optimism that the *Rhodococcus* control agent will give wildlife-management agencies a potent new tool to prevent the spread of WNS and begin the re-colonization of hibernacula that have been devastated by this disease.

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