THE EFFECT OF VOLATILE ORGANIC COMPOUNDS ON *PSEUDOGYMNOASCUS DESTRUCTANS* THE CAUSATIVE AGENT OF WHITE NOSE SYNDROME IN BATS

By

VICTORIA LOIS KORN

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Joan W. Bennett

And approved by

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ABSTRACT OF THE THESIS

The effect of Volatile Organic Compounds on *Pseudogymnoascus destructans* the causative agent of White-Nose Syndrome in bats

By VICTORIA LOIS KORN

Thesis Director:

Joan W. Bennett

The causative agent of White-Nose Syndrome (WNS) in bats is the psychrophilic fungus, *Pseudogymnoascus destructans*. Since its discovery in the winter of 2006-2007 there has been a flurry of research to better understand this pathogenic fungus as well as find a treatment to save the bats. There is promise in finding a potential treatment through the use of safe Volatile Organic Compounds (VOC). There was potential for the use of 1-octen-3-ol, mushroom alcohol as a treatment for WNS. The first chapter shows the effectiveness of the R and S enantiomers and the racemic form of 1-octen-3-ol (mushroom alcohol) as well as *trans*-2-hexenal (leaf aldehyde), on mycelial plugs and conidiospores at temperatures of 5, 10 and 15˚C. 1-octen-3-ol was able to inhibit mycelial and conidiospore growth of *P. destructans* at 0.4 and 0.8 μmol/mL and the R enantiomer of this compound was more effective than the S enantiomer, supporting the finding that biological systems can be sensitive to stereochemistry. *trans*-2-hexenal was more effective than 1-octen-3-ol and showed fungicidal activity at 0.05 μmol/mL to both conidiospores and mycelia of *P. destructans*. The second chapter is a transcriptomics

study to understand a potential pathway *trans*-2-hexenal takes in inhibiting *P. destructans* growth. *P. destructans* was treated with *trans*-2-hexenal at sublethal concentrations, and RNA was extracted for Illumina sequencing. It was found that *trans*-2-hexenal was able to inhibit growth, possibly through downregulating the production of essential genes, like Superoxide Dismutase. Also discovered was the downregulation of known endopeptidase enzyme, Destrucin-2, indicating *trans*-2-hexenal may also able to reduce virulence while inhibiting growth.

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1. Introduction

1.1 White-Nose Syndrome

The causative agent of white-nose syndrome in bats is the psychrophilic fungus *Pseudogymnoascus destructans* (Lorch 2011, Zukal et al. 2014). Since the discovery of the fungus in the Northeastern region of North America during the winter of 2006 to 2007 there has been a flurry of research to better understand this fungus and why it has such a dramatic effect on our native bat species, leading to an overwhelming decline in bat populations (Frick et al. 2010, Blehert 2009). *P. destructans* has existed for many years in Eurasia, but was only identified after its discovery in North America as an invasive species (Leopardi et al. 2015, Blehert et al. 2009).

P. destructans was originally of the *Geomyces* genus, and renamed after careful genetic evaluation comparing soil species (Minnis and Linder 2013). The genome of *P. destructans* has been sequenced (Palmer et al. 2018, Drees et al. 2016). *P. destructans* is cold loving, growing optimally from 12.5-15.8˚C, the typical temperature range of bat hibernacula in winter (Verant et al. 2012). It is spread from bat to bat contact as well as from the environment forming a white fuzzy growth on the wings and noses of bats while in hibernation, giving rise to the White-Nose Syndrome name (Blehert et al. 2009, Lorch et al. 2011). The fungus grows saprophytically as well as parasitically on a bat host. It has the ability to utilize enzymes to survive in cave soil or for survival on a host bat (Reynolds and Barton 2014, O'Donoghue et al. 2015). *P. destructans* is heterothallic but only one of the mating types has been found in North America (Palmer et al. 2014).

Bats infected with WNS are typically seen with the namesake fungal growth on the nose and wings, eventually leading to necrosis of wing tissue and hyphae replacing

hair follicles and in sebaceous glands (Chaturvedi et al. 2010). There are behavioral symptoms fsuch as bats waking more frequently from torpor, which is linked to mortality. Spelunkers also have observed bats crawling on the ground and diving through piles of snow in what is believed to be an attempt to find nutrients (Reeder et al. 2012). Unfortunately, there are no nutrients such as insects available for these bats, since hibernation occurs during the winter season (Reynolds and Barton 2014). There is also study into the production of enzymes from *P. destructans.* Some endopeptidases discovered are named Destructin-1, Destructin-2, and Destructin-3, and are collagen degrading (O'Donoghue et al. 2015). These endopeptidases are likely causing the necrosis of wing tissue in infected bats, which has been linked to be the leading cause death by hypertonic dehydration (Warnecke et al. 2013).

The quest for a prevention or cure for WNS is fueled by the importance of bats in many ecosystems. There are many different species of bats native to different regions of the world; some species are pollinators of certain plants (Kunz et al. 2011), a phenomenon known as chiropterophily (Tschapka and Dressler 2002). The majority of bats are insectivorous, and fly at night, utilizing their famous echolocation technique to eat insects, including large numbers of mosquitoes. It has been found that the Little Brown Bat (*Myotis lucifigus*) consumes 61 different species of insect and 5 species of arachnids (Kunz et al. 2011).

In North America, WNS has been found in eleven bat species: Big brown bat (*Eptesicus fuscus*), Cave bat (*Myotis velifer*), Eastern small-footed bat (*Myotis leibii*), Gray bat (*Myotis grisescens*), Indiana bat (*Myotis sodalis*), Little brown bat (*Myotis lucifugus*), Long-legged bat (*Myotis volans*), Northern long-eared bat (*Myotis*

septentrionalis), Southeastern bat (*Myotis austroriparius*), Tricolored bat (*Perimyotis subflavus*), and the Yuma bat (*Myotis yumanensis*). The fungus has also been found without symptomatic infection on six bat species: Eastern red bat (*Lasiurus borealis*), Silver-haired bat (*Lasionycteris noctivagans*), Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), Virginia Big-Eared Bat (*Corynorhinus townsendii virginianus*), Cave bat (*Myotis velifer*), and the Townsend's big-eared bat (*Corynorhinus townsendii*). An additional six species in North American have non-symptomatic infections. Although, WNS affects mostly North American bats, fourteen bat species with symptomatic infections and seven with atopic infections have been found in Europe and Asia (WhiteNoseSyndrome.org).

Currently, WNS in North America is distributed mostly in the northeastern region. However, WNS has been confirmed in 33 states (Alabama, Arkansas, Connecticut, Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Maine, Maryland, Massachusetts, Michigan, Minnesota, Missouri, New Hampshire, Nebraska, New Jersey, New York, North Carolina, Ohio, Oklahoma, Rhode Island, Pennsylvania , South Carolina, South Dakota, Tennessee, Vermont, Virginia, Washington, West Virginia, and Wisconsin), 7 Canadian provinces (Manitoba, New Brunswick, Newfoundland and Labrador, Nova Scotia, Ontario, Prince Edward Island, and Quebec), and the fungus has been discovered in 3 additional states (Mississippi, Texas, and Wyoming) (WhiteNoseSyndrome.org). Due to a long incubation period of ~120 days, the fungus requires a long winter to infect and kills bats (Lorch et al. 2011); therefore, WNS is less likely to occur in the warmer, southern parts of North America. In Eurasia, where it is believed that *P. destructans* originated, the infection is not deadly. It is thought that

bats have coevolved with the fungus over the many years it has existed there (Warnecke et al. 2012, Zukal et al. 2016, and Puechmaille et al. 2011). Therefore, since the fungus is invasive in North America bats native to this area do not have the same coevolved traits or immunity to protect themselves from this fungal disease.

1.2 Proposed Controls

Attempts to prevent or mitigate WNS have taken a wide variety of approaches, most of them focused on finding a way to inhibit the growth of *P. destructans*. They include UV treatments, exposure to a cocktail of volatile organic compounds (VOCs) and chitosan. Low dose exposure to a few seconds of UV-C light from a hand-held UV-C light source resulted in 85% mortality of *P. destructans* while a moderate dose exposure killed more than 99% of the fungus (Palmer et al. 2018). It is hypothesized that the fungus evolved in the absence of the sun and genetic evaluation has found that the fungus is unable to repair itself from UV damage. Thus, research scientists have come up with a possible plan to treat caves with small tanning beds to administer UV in small flashes (Palmer et al. 2018). In another approach, several volatile organic compounds produced by bacteria inhibit the growth of *P. destructans* (Cornelison et al. 2014a, Cornelison et al. 2014b). The volatile sesquiterpene trans, trans-farnesol emitted by the yeast *Candida* has shown promise (Raudabaugh and Miller 2015). In a biocontrol strategy, *Pseudomonas* spp. isolated from the skin of bats were found effective in inhibiting the growth of *P. destructans* in vitro (Hoyt et al. 2015).

According to a number of announcements in print, on-line media, and an unpublished Master's thesis (Kulhanek, 2016) chitosan treatments show promise as a

treatment in experimental trials with *Myotis* sp. against white nose syndrome. Chitosan treatments require direct contact with bats. A group in Michigan is working on a spraying method and believe the chitosan will work not only to prevent WNS infection, but also as a treatment to heal wounds caused by *P. destructans* (BatCon 2017).

1.3 Public awareness and education about WNS

It is believed that WNS was introduced to North American by human activity, perhaps something as simple as unclean hiking and/or spelunking equipment brought into a New York cave (Blehert et al. 2009, Zukal et al. 2016, Leopardi et al. 2015). In recent years, there has been increasing mention of WNS on social media platforms to warn those who enjoy hiking or spelunking to clean their equipment in case they came into contact with *P. destructans*. The WNS Decontamination Team, part of the Disease Management Working Group, produced a national decontamination protocol in 2012 and an updated protocol in 2016 (WNS Decontamination Team). There is also an excellent website dedicated to keeping up-to-date information on all things pertaining to WNS, WhiteNoseSyndrome.org. This site is sponsored by the U. S. Fish and Wildlife Service with support from 100 US state and Canadian provincial agencies and institutions, including universities, tribes and non-governmental agencies. The site has sections that follow the distribution of WNS on multiple maps over time and is constantly updated in order to help the community stay united while trying to understand and combat WNS. For example, many informational pieces are posted such as the WNS Fact Sheet from the US Fish and Wildlife Service, and a "White Nose Syndrome Response Logo" in order to symbolize a united front against the ongoing epidemic. There is also a White-Nose

Syndrome Workshop that allows scientists to share their work and see what others may be doing. The dates and locations of professional meetings are posted on their website so scientists so can stay up-to-date on the latest research about WNS, including potential treatments.

Articles and news stories on White-Nose Syndrome can be seen on many platforms, such as National Geographic which featured a story on the work of Cornelison et al. 2014a, Cornelison et al. 2014b, as well as The New York Times, The Nature Conservancy, the U.S. National Park Service and the Student Conservation Association, which is a branch of the U.S. National Park Service (USNPS, SCA, TNC, Robbins 2019, Lee 2015).

1.4 Volatile Organic Compounds (VOC)

Volatile organic compounds, also known as VOC or aroma compounds have been used to combat *P. destructans* (Cornelison et al. 2014a, Cornelison et al. 2014b, Padhi et al. 2016, Padhi et al. 2018). VOCs are low molecular weight and low vapor pressure carbon compounds, and are chemically classified as alcohols, aldehydes, hydrocarbons, aromatics, nitrogen-containing compounds, sulfur- containing compounds, terpenoids and various derivatives (Korpi et al. 2009, Herrmann 2011, Morath et al. 2012, Bennett et al. 2013). Chemical standards of VOCs can be purchased in liquid form from several chemical supply houses. In nature many bacteria, plants, and fungi produce VOCs as biosynthetic products or breakdown products of metabolism (Korpi et al. 2009, Hermann 2011).

We come across VOCs in everyday life, from the unpleasant odors emitted by the bacteria and fungi found in damp homes to the fumes that come from paint and vehicles on the street. Yet, some researchers feel the human ability to smell is underutilized (Shepherd 2004, McGann 2017). Some of the best-known volatile liquids are industrial and laboratory solvents such as benzene, ethanol, chloroform and methylene chloride, all of which are liquid compounds that easily enter the gas form at normal room temperature and pressure. Numerous synthetic and non-synthetic chemicals used in cleaning products, cosmetics, disinfectants, paints, varnishes, as well as fuels, also are volatiles. There is literature on these products, which sometimes emphasize adverse health effects (McFee and Zavon 1988, Samet and Spengler 1991, Meyer 2014, Weschler and Nazaroff 2013). The Environmental Protection Agency has regulations for VOC emissions which vary by state. The Environmental Protection Agency (EPA) describes regulations in New England and discusses their Reasonably Available Control Technology (RACT) which considers the lowest level of emissions based on the economic and technological capabilities of the state (EPA). This helps to limit the emission of harmful VOCs that can lead to things such as smog, which occurs from VOCs reacting with nitric oxides in air.

In the field of food safety, The U.S. Food and Drug Administration regulates food additives (USFDA). Substances must be generally recognized, among qualified experts, as being safe under the conditions of their intended use. This classification is generally known by its acronym GRAS (Generally Recognized as Safe). Many VOCs are used as flavor additives and thus must be GRAS in order to be used in products or foods.

Aside from the industrial VOCs there are many VOCs emitted in nature, with about 30,000 emitted by plants, (Dudareva et al. 2006, Herrmann 2011) and hundreds

produced by bacteria and fungi (Baldwin 2010, McCormick et al. 2012). Many VOCs are produced by all living things while a few are characteristic of only certain species. For example, many VOCs that were first described from fungi are also produced by bacteria (Schulz and Dickshat 2007). A tool that provides useful information on VOCs is the website SuperScent, which is a website dedicated to profiling scents and VOC. A comprehensive database of VOCs from bacteria and fungi was compiled by Lemfack et al. 2013. It began with 500 compounds and was later expanded to more than 2000 volatiles from 1000 microbes (Lemfack et al. 2013, Lemfack et al. 2017). In addition to these databases, there are many review articles that explore volatiles from microbes (Korpi et al. 2009, Herrmann 2011, Morath et al. 2012, Bennett et al. 2013, Korpi et al. 2009, Pennerman et al. 2016).

1.5 Volatile Organic Compounds: Fungal VOCs

More than 250 VOCs have been identified from fungi (Morath et al. 2012). These VOCs take many forms such as hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters, and different types of benzenes and cyclohexanes (Korpi et al. 2009). Not many researchers work with gas phase molecules, which pose many experimental challenges, and there is underdevelopment of experimental methods for gas phase experiments.

Fungal VOCs are a known cause of sick building syndrome (Wålinder et al. 2005, Mølhave 2009). Studies in our laboratory have shown that gas phase 1-octen-3-ol is more toxic to human embryonic stem cells than toluene (Inamdar et al. 2011). Other studies have shown that when adult *Drosophila* flies were exposed for one week to low

concentrations of 2-octanone, 2,5-dimethylfuran-3-octanol, *trans*-2-octenal, and 1-octen-3-ol they exhibited, respectively 40%, 35%, 60% 50% and 100% lethality (Inamdar et al. 2010).

On the other hand, some VOCs can have a beneficial effect. Work in our laboratory, as well as others, have shown that *Trichoderma* volatiles can have a growth promoting effect on plants (Hung et al. 2013, Lee et al. 2017, Bitas et al. 2013). The VOC 1-octen-3-ol, also known as mushroom alcohol or matsutake alcohol, is a self-inhibitor in the germination of *Penicillium paneum* conidia (Chitarra et al. 2004). 1-octen-3-ol is in a class of eight carbon volatiles, along with 3-octanone, 3-octanol, octanol, and 1-octen-3-one, which work together to create a typical mushroom smell (Combet et al. 2006). It is produced by the enzymatic oxidation and cleavage of linoleic acid, and can be used as a treatment for "dry bubble disease" on Button Mushroom which caused by the mycoparasite *Lecanicillium fungicola* (Wurzenberger and Grosch 1984, Manning 1985, Berendsen et al. 2013). 1-octen-3-ol also enhances resistance to the plant pathogen, *Botrytis*, when plants are treated with this VOC they show heightened expression of defense genes and changes in ethylene and jasmonic acid signaling (Kishimoto et al. 2007). Work in our laboratory has shown that exposure to 5, 10, 50, and 100 ppm of volatile phase racemic 1-octen-3-ol is effective in reducing the growth of *P. destructans*. Concentrations of 5 and 10 ppm of 1-octen-3-ol are fungistatic, while concentrations of 50 and 100 ppm are fungicidal (Padhi et al. 2016).

1.6 Volatile Organic Compounds: Green Leaf Volatiles

Plants produce many unique VOCs including certain terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, and amino acid derived metabolites (Holopainen et al. 2010). Many of these plant VOCs are well known scents, such as lavender, rose, eucalyptus, and other typical floral odors. Some plants produce VOCs as a stress response when in a non-ideal environment, such as when exposed to higher temperatures (Dement et al. 1975). Green leaf volatiles are characteristic of the odor of cut grass and hay. They are mixtures of six carbon aldehydes, alcohols, and their esters formed through the hydroperoxide lyase pathway of oxylipin metabolism (Matsui 2006).

When grass is cut, the most stable form of the volatile compounds that are released is *trans*-2-hexenal or "leaf aldehyde;" the other compounds that are released are typically *cis*-3-hexenal or *trans*-3-hexen-1-ol (Cotton 2013). The scent of *trans*-2-hexenal can easily be smelt in the air on a nice spring day; can be consumed when we eat salads, other leafy greens, and some fruits; and is the major volatile compound in olive oil (Arroyo et al. 2007, Kiritsakis et al. 1998). *trans*-2-Hexenal is one of the major volatile compounds in extra virgin olive oil (Kiritsakis et al. 1998) and is classified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (USFDA). Interestingly, research on animals has found that the aromatic compounds with a "green odor" (a mixture of *cis*-3-hexenal, *trans*-3-hexen-1-ol, and *trans*-2-hexenal) have a calming and anti-anxiety effect on rats and humans (Oka et al. 2008, Akutsu et al. 2001, Ito et al. 2009, Watanabe et al. 2005).

trans-2-hexenal may be the most studied of the green leaf volatiles (Sholberg 2009). It is known to inhibit plant pathogens such as *Botrytis cinerea, Alternaria altemata,* and *Colletotrichum gloeosporioides*, which infect berries (Vaughn et al. 1993,

Fallik et al. 1998). In addition, it also helps limit blue mold rot, a postharvest disease caused by *Penicillium expansum*, in pome, pears, and apples (Neri et al. 2006a, Neri et al. 2006b, Fan et al. 2006). *trans*-2-hexenal inhibits *Penicillium cyclopium* from rotting postharvest tomatoes through increased membrane permeability (Zhang et al. 2016). In another, *trans*-2-hexenal inhibits *Colletotrichum acutatum,* a causal agent of anthracnose (Arroyo et al. 2007). Green leaf volatiles also inhibit the growth of some plant pathogenic bacteria giving them an additional benefit to postharvest spoilage control (Nakamura and Hatanaka 2002, Lanciotti et al. 2004). In summary, promising possible uses of VOC are as fumigants in pest control or in post-harvest spoilage control.

2 Thesis Chapters

2.1 Chapter 1: Pseudogymnoascus destructans: Causative Agent of White-Nose Syndrome in Bats Is Inhibited by Safe Volatile Organic Compounds

The first chapter of this thesis consists of a published work demonstrating the efficacy of *trans*-2-hexenal to inhibit the growth of *P. destructans*. This study compares racemic 1-octen-3-ol and its enantiomers with *trans*-2-hexenal in laboratory microcosms at 5, 10, and 15˚C and four different concentrations (0.05, 0.1, 0.5 or 1.0 µmol/mL of *trans*-2-hexenal, or 0.04, 0.08, 0.4 or 0.8 µmol/mL of racemic 1-octen-3-ol, *R*-1-octen 3-ol or *S*-1-octen 3-ol). 1-octen-3-ol inhibits mycelial and conidiospore growth of *P. destructans* at 0.4 and 0.8 µmol/mL and the R enantiomer of this compound is more effective than the S enantiomer. *trans*-2-hexenal is more effective than mushroom alcohol. At 0.05 µmol/mL, *trans*-2-hexenal is fungicidal to both conidiospores and mycelia of *P. destructans*.

2.2 Chapter 2:

The second chapter consists of a transcriptomic study performed to distinguish genes that are involved in *trans*-2-hexenal inhibition of *P. destructans* growth. In this experiment, *P. destructans* was cultured for a month in liquid culture and then exposed to three concentrations of *trans*-2-hexenal for an additional month. After treatment, fungi were frozen in liquid nitrogen and RNA was isolated from the samples. The cDNA library was sequenced by Illumina sequencing and analyzed by comparing the treatment to control samples. A bioinformatics analysis identified those genes that are significantly up or down regulated. The findings provided evidence that *trans*-2-hexenal is able to inhibit growth through downregulating some essential components of essential pathways, and there is promise in the downregulation of Superoxide Dismutase as being a major pathway of growth inhibition. There's additional evidence that *trans*-2-hexenal is able to reduce virulence at sub-lethal concentrations (5, 10, and 20 μmol/L), due to the downregulation of the Destructin-2 gene, which is a presumed collagen degrading endopeptidase.

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Chapter 1. *Pseudogymnoascus destructans:* Causative Agent of White-Nose Syndrome in Bats Is Inhibited by Safe Volatile Organic Compounds

Chapter 1 has been published as Sally Padhi, Itamar Dias, Victoria L. Korn, Joan W. Bennett. 2018. *Pseudogymnoascus destructans:* Causative Agent of White-Nose Syndrome in Bats Is Inhibited by Safe Volatile Organic Compounds. Journal of Fungi 48:1-12. DOI 10.3390/jof4020048 MDPI. Victoria L. Korn participated in writing the manuscript and is directly responsible for those experiments dealing with repeats of experiments dealing with mycelial plug exposure to 1-octen-3-ol and *trans*-2-hexenal and conidiospore exposure to lower doses of *trans*-2-hexenal; Figures 1-6.

Abstract: White-nose syndrome (WNS) is caused by *Pseudogymnoascus destructans*, a psychrophilic fungus that infects hibernating bats and has caused a serious decline in some species. Natural aroma compounds have been used to control growth of fungal food storage pathogens, so we hypothesized that a similar strategy could work for control of *P. destructans*. The effectiveness of exposure to low concentrations of the vapor phase of four of these compounds was tested on mycelial plugs and conidiospores at temperatures of 5, 10 and 15˚C. Here we report the efficacy of vapor phase mushroom alcohol (1-octen-3-ol) for inhibiting mycelial and conidiospore growth of *P. destructans* at 0.4 and 0.8 µmol/mL and demonstrate that the R enantiomer of this compound is more effective than the S enantiomer, supporting the finding that biological systems can be sensitive to stereochemistry. Further, we report that vapor phase leaf aldehyde (*trans*-2-hexenal), a common aroma compound associated with cut grass odors and also the major volatile compound in extra virgin olive oil, is more effective than mushroom alcohol. At 0.05

μmol/mL, *trans*-2-hexenal is fungicidal to both conidiospores and mycelia of *P. destructans*.

1. **Introduction**

White-nose syndrome (WNS), a fungal disease of hibernating bats, has decimated bat populations in North America [1,2] and threatens the extinction of several bat species [3]. WNS probably was introduced to North America from Europe where the disease is endemic but where bats appear to be resistant [4]. The steep declines in North American bat populations make WNS perhaps the most devastating mammalian wildlife disease in recent history [5]. For some bat species, population sizes have declined 99% in WNS-infected hibernacula [3,6,7].

WNS is caused by *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans),* a cold-loving fungus with growth restricted to temperatures of approximately 3–15˚C and >90% relative humidity [8,9]. During hibernation, the body temperature of bats ranges from 2–15˚C which is similar to the optimum growth temperature for the fungus [10]. The disease infects the cutaneous tissues of bats, producing a white-colored fungal growth on the muzzle and wings [11]. Hibernating bats arouse more frequently from torpor [12–14] resulting in depletion of fat reserves, emaciation and death [15]. WNS pathology also includes changes in electrolyte balance and hydration [11,16], chronic respiratory acidosis [17], immune response [18] and oxidative stress [19].

There is a strong need to discover control measures to control *P. destructans* and several studies have shown promise. For example, in *in vitro* research, the growth of *P.*

destructans was inhibited by volatile compounds made by the bacterium *Rhodococcus rhodochrous* [20,21] and *trans*, *trans*-farnesol, a sesquiterpene made by the yeast *Candida* [22]. Moreover, some preliminary data from our laboratory showed that volatile phase racemic 1-octen-3-ol (mushroom alcohol) could retard the mycelial growth of *P. destructans*, with exposure to 0.8 μmol/mL being fungicidal and exposure to 0.08 μmol/mL being fungistatic [23]. This common fungal volatile exists as two optical isomers or enantiomers: (*R*)-(−)-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol. Chiral discrimination is important in the activity of many biosystems [24], so we have tested the effect of volatilized R- and S- enantiomers of 1-octen-3-ol on the growth of *P. destructans* mycelial plugs and conidiospores.

Plant pathologists have studied a number of generally recognized as safe (GRAS) volatile compounds for use as postharvest fumigants to control mold pathogens in stored fruits and vegetables [25–27]. There are many physiological similarities between plant and animal pathogenic fungi [28,29] so we hypothesized that compounds that worked against common plant pathogenic fungi might also be effective against *P. destructans*. We focused on *trans*-2-hexenal, an important aroma compound of green plants also known as leaf aldehyde, which is known to have pronounced antimicrobial effects [30–33].

Our fumigation study has been conducted on both mycelia and conidiospores of *P. destructans*. Our long-term aim is to find an antifungal fumigant that will prevent or retard the growth of propagules of this serious bat pathogen but not harm either the hibernating bat or the cave ecosystem. Our immediate goals are as follows: (1) to compare the effects of four concentrations of *trans*-2-hexenal with the R, S and racemic forms of 1-octen-3-ol on the growth of *P. destructans* at 5, 10 and 15˚C; (2) to determine

if the growth-inhibiting properties of the four volatilized substances would continue after the fungus is removed from the presence of the VOCs; and (3) having shown that *trans*-2-hexenal is far more effective than 1-octen-3-ol in inhibiting growth of both mycelia and conidiospores of *P. destructans*, to test the response of *P. destructans* to extremely low concentrations (0.01, 0.02 and 0.05 μmol/mL) of this six carbon aldehyde for use in implementation of a possible fumigation strategy in bat hibernacula.

2. Materials and Methods

2.1. Chemicals

Chemical standards of liquid phase racemic 1-octen 3-ol (synonym mushroom alcohol) and *trans*-2-hexenal (synonyms include *trans*-2-hexen-1-al, (*E*)-2-Hexenal, (*E*)-Hex-2-enal and leaf aldehyde) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enantiomers (*R*)-(−)-1-octen-3-ol (*R* form) and (*S*)-(+)-1-octen-3-ol (*S* form) were gifts from Bedoukian Research, Danbury, CT, USA. The chemical structures of leaf aldehyde and the *R*- and *S*- forms of mushroom alcohol are illustrated in Figure 1.

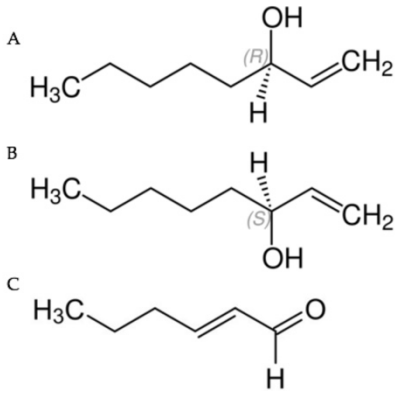

Figure 1. (**A**) (*R*)-(−)-1-octen-3-ol (*R* form); (**B**) (*S*)-(+)-1-octen-3-ol (*S* form); (**C**) *trans*-2-hexenal.

2.2. Fungal Strains and Media

Pseudogymnoascus destructans, (MYA-4855TM) was obtained from the American Type Culture Collection, Manassas, VA, USA. Throughout our work, *P. destructans* was handled according to all procedures required for Level 2 classification pathogens. All exposure experiments were conducted with *P. destructans* grown on Potato Dextrose Agar (PDA) (Difco, Becton, Dickinson & Company, Sparks, MD, USA). For the preparation of conidiospores and viable counts of conidiospore concentrations, Sabouraud Dextrose Agar (SDA) (Difco), supplemented with 200 mg/L $MnSO_4$, was used.

2.3. Preparation of Fungal Inocula

To obtain mycelial plugs, fungi were cultured at 15˚C on PDA for three weeks. Using a #3 cork borer, mycelial plugs were taken from the actively growing outer edge of 21 day old colonies. To obtain conidiospores of *P. destructans*, cultures were incubated at 15˚C for 21 days on SDA. The conidiospores were harvested by adding 10 mL of CHS (Conidia Harvesting Solution) to each plate and gently scraping the fungal growth with an inoculation loop to help release the spores. CHS was composed of 0.05% Tween 80% and 0.9% NaCl. The suspension was then filtered through glass wool and the flow through was centrifuged at 5000 rpm for 15 min. The supernatant was removed and the conidiospore pellet was re-suspended in 10 mL of PBS (phosphate-buffered saline) solution at pH 7.0 [20,21].

2.4. Volatile Exposures

Plastic split Petri plates (100 × 15 mm), also known as I-plates, were used. One half of the plate contained 10 mL of PDA and the other half contained a sterile glass cover slip (22 × 22 mm) for the placement of liquid aliquots of the VOC being tested. The amounts of liquid VOC needed were calculated according to the molarity of the VOC and the volume of the Petri plate. For example, in the highest concentration of *trans*-2-hexenal tested (1.0 μmol/mL), 0.0071 mL of the liquid standard was placed on a cover slip and allowed to volatilize in the 60 mL of air space available in the Petri dish.

Mycelial plugs of 21 day cultures were subcultured onto fresh PDA plates and exposed to vapors of 0.05, 0.1, 0.5 or 1.0 μmol/mL of *trans*-2-hexenal, or 0.04, 0.08, 0.4 or 0.8 μmol/mL of racemic 1-octen-3-ol, *R*-1-octen 3-ol or *S*-1-octen 3-ol. Inoculated plates were sealed with two layers of Parafilm. Both 1-octen-3-ol and *trans*-2-hexenal have a distinctive odor so in order to determine if there was a loss of VOCs from the Petri plates, each VOC treatment was placed in two-liter glass containers with tightly fitting propylene lids. The sealed plates were removed weekly from the two-liter glass containers in order to record growth measurements. The lack of odor indicated a minimal loss of VOCs from the sealed Petri plates. The sealed plates were not opened when the measurements were made so as not to compromise the headspace within the Petri plates. Plates were incubated at 5, 10 or 15˚C for three weeks and growth measurements were made weekly. The amount of mycelial growth was recorded by averaging two diameter measurements taken on each colony at right angles of each mycelial plug. In a set of parallel VOC exposure experiments conducted at 10˚C, suspensions of conidiospores

were spread on I-plates and grown in a shared atmosphere with the four different VOCs using the same parameters as with mycelial plugs.

A second exposure system was designed which would allow the testing of *trans*-2-hexenal on spore growth in concentrations smaller than 0.05 µmol/mL by increasing the volume of the exposure system. Three 60 mm Petri plates per treatment were inoculated with conidiospores, the covers removed and the plates placed in 32-ounce Mason Jars with tightly fitting lids and three layers of parafilm. *trans*-2-hexenal was added to the glass containers so that when vaporized the conidiospores would be exposed to 0.005, 0.01, 0.02 or 0.05 µmol/mL of *trans*-2-hexenal. The plates were incubated for 3 weeks at 10˚C.

2.5. Subculture of VOC Treated P. destructans

After three weeks, in order to determine whether the action of inhibitory VOCs was fungicidal or fungistatic, the mycelial plugs or media containing conidiospore suspensions were removed from the presence of volatilized test compounds, transferred to fresh PDA media, sealed with a double layer of Parafilm and placed inside clean, VOC-absent glass containers. The transferred mycelial plugs were incubated for three additional weeks at 5, 10 or 15˚C. Three replicates were used per treatment and the experiments were repeated twice. At the end of the three-week period, plates containing mycelial plugs were photographed using a Sony DSC-H9 81 mega pixel camera (Sony Corporation, New York, NY, USA) and a Bio-Rad Universal Hood II with a CCD (couple-charged device) camera using Chemi Doc XRS software (Bio-Rad Laboratory Inc., Hercules, CA, USA). Quantitative data were analyzed using Excel software

(Microsoft, Redmond, WA, USA) and Sigma Plot (SPSS Science Inc., Chicago, IL, USA). Error bars indicate standard error (SE) of the mean.

In the second exposure system designed to test *trans*-2-hexenal in amounts less than 0.05 µmol/mL, plates containing VOC treated spores were removed from VOC exposure and covered with sterile fresh covers. The plates were incubated for three more weeks at 10˚C. Three replicates were used per treatment and the experiments were repeated twice. When no growth was observed after three weeks, plates were incubated for another 12 weeks in the absence of VOCs in order to verify that the fungus was no longer viable (i.e., that the VOC exposure was fungicidal).

3. Results

3.1. Growth of VOC Treated Mycelial Plugs

The effect on the growth of mycelial plugs of *P. destructans* at 5, 10 or 15˚C from exposure to four concentrations of volatile phase *trans*-2-hexenal or the three forms of 1-octen-3-ol is shown in graphed form in Figure 2. Controls grew best at 10˚C with an average increase in mycelial diameter of 200 mm after three weeks. At 15˚C, controls increased about 130 mm, while at 5˚C they increased in diameter about 50 mm during the three-week period. At all three temperature regimes, exposure to the higher concentrations of the four VOCs inhibited growth. The *S* form of 1-octen-3-ol caused very little growth inhibition at 0.04 and 0.08 µmol/mL. At 10 and 15˚C, 0.05 and 0.1 µmol/mL *trans*-2-hexenal were more effective than 1-octen-3-ol in inhibiting mycelial growth of *P. destructans* (Figure 2). For all treatments, incubation of the fungus at 5˚C reduced mycelial growth and also reduced the effectiveness of the VOCs. As shown in

Figure 2, at 5˚C, exposure to the two lowest concentrations of the VOCs did not completely inhibit growth; only the two highest concentrations tested inhibited *P. destructans* at this temperature. Figure 3 shows images of the mycelial plugs after three weeks of exposure to these treatments at 15˚C. The mycelial growth of controls and the mycelial growth of plugs exposed to the 0.04 and 0.08 µmol/mL of the *S* form of 1-octen-3-ol are similar, while the R enantiomer and racemic form of 1-octen-3-ol showed growth inhibition, as did the mycelial plugs exposed to *trans*-2-hexenal at 0.05, 0.1, 0.5 and 1.0 µmol/mL.

3.2. Subculture of Mycelial Plugs after VOC Treatment

After the three-week VOC exposure, all mycelial plugs were subcultured to new PDA media and incubated for three weeks in the absence of VOCs to determine if the treatments had killed the mycelia (fungicidal effect) or merely inhibited their growth (fungistatic effect). All treatments of *trans*-2-hexenal at 10 and 15˚C were fungicidal. At the lower temperature of 5˚C, cultures exposed to 0.05 and 0.1 µmol/mL of *trans*-2-hexenal showed some growth indicating a fungistatic effect at these two lower concentrations. With the 1-octen-3-ol treatments, the *S* form was least effective with recovery of mycelial growth at all four concentrations and three temperatures tested (Figure 4). At 10˚C, 0.08, 0.4 and 0.8 µmol/mL racemic 1-octen-3-ol was fungicidal while 0.04 µmol/mL was fungistatic. Microscopic examination at the end of the incubation period showed some hyphal extensions although this growth was not measurable macroscopically. At 10˚C, the *R* form of 1-octen-3-ol was fungicidal at 0.4 and 0.8 µmol/mL. At 15˚C, 0.8 µmol/mL of racemic 1-octen-3-ol was fungicidal while

the *R* form was fungistatic. At 5˚C, mycelial plugs exposed to 0.8 µmol/mL of either the racemic or the *R* form showed some recovery after three weeks incubation.

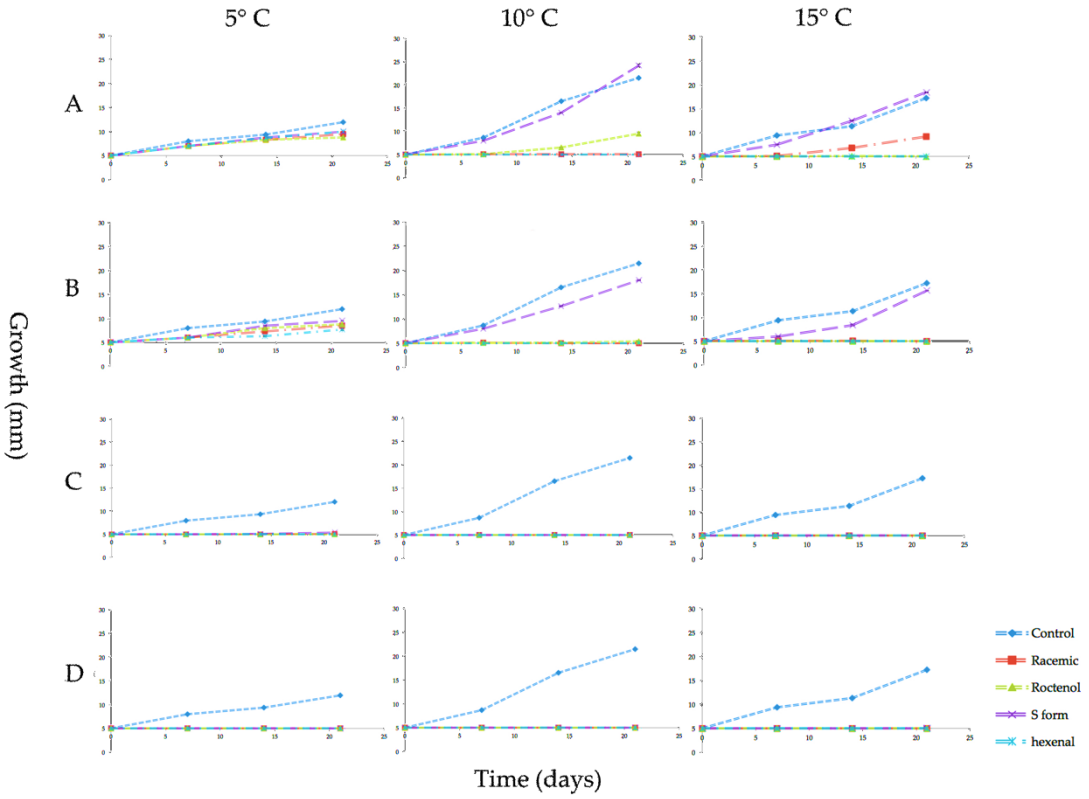

Figure 2. Growth in millimeters (mm) of mycelial plugs of *Pseudogymnoascus destructans* exposed for 3 weeks to vapors of racemic 1-octen-3-ol, (*R*)-(−)-1-octen-3-ol (*R* form), (*S*)-(+)-1-octen-3-ol (*S* form), or *trans*-2-hexenal and cultured at 5, 10 or 15˚C. Error bars indicate the standard error of the mean. VOC treatments as follows: (**A**) 0.04 µmol/mL of the three forms of 1-octen-3-ol or 0.05 µmol/mL of *trans*-2-hexenal; (**B**) 0.08 µmol/mL of 1-octen-3-ol or 0.1 µmol/mL *trans*-2-hexenal; (**C**) 0.4 µmol/mL 1-octen-3-ol or 0.5 µmol/mL *trans*-2-hexenal; (**D**) 0.8 µmol/mL 1-octen-3-ol or 1.0 µmol/mL *trans*-2-hexenal.

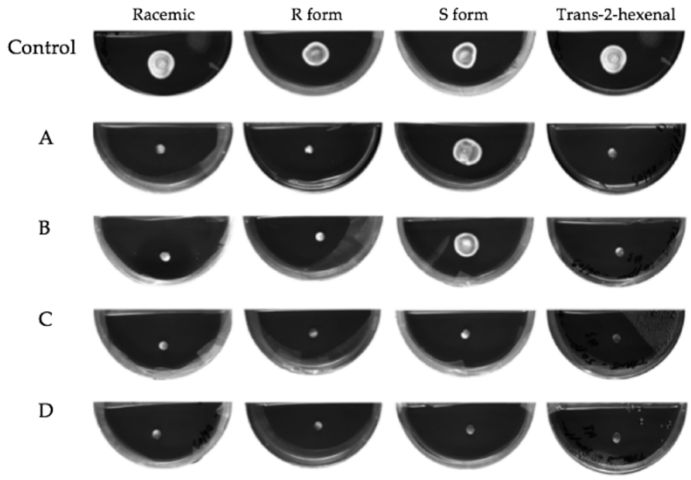

Figure 3. Mycelial plugs of *Pseudogymnoascus destructans* exposed to vapors of for 3 weeks at 15˚C. VOC treatments as follows: (**A**) 0.04 µmol of the three forms of 1-octen-3-ol or 0.05 µmol of *trans*-2-hexenal; (**B**) 0.08 µmol/mL of 1-octen-3-ol or 0.1 µmol *trans*-2-hexenal; (**C**) 0.4 µmol/mL 1-octen-3-ol or 0.5 µmol/mL *trans*-2-hexenal; (**D**) 0.8 µmol/mL 1-octen-3-ol or 1.0 µmol/mL *trans*-2-hexenal.

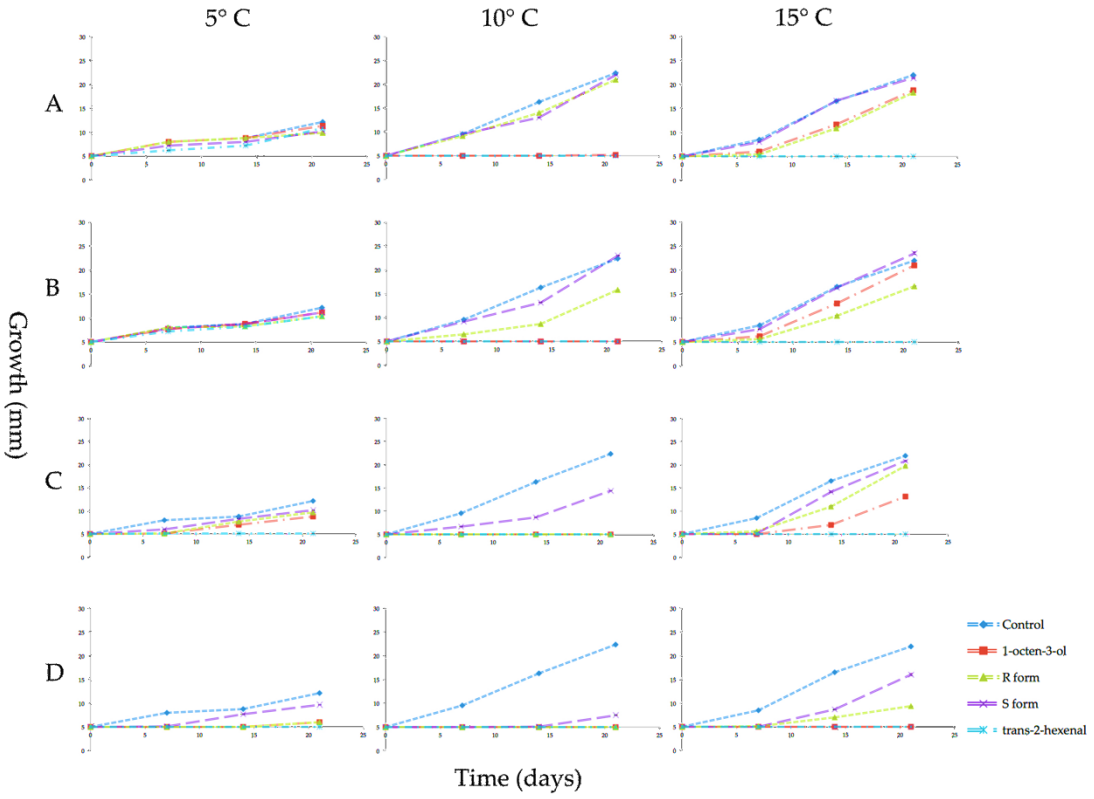

Figure 4. Growth in millimeters (mm) of mycelial plugs of *Pseudogymnoascus destructans* which were subcultured in ambient air for 3 weeks at 5, 10, or 15˚C after prior exposure for 3 weeks to vapors of racemic 1-octen-3-ol, (*R*)-(−)-1-octen-3-ol (*R* form), (*S*)-(+)-1-octen-3-ol (*S* form), or *trans*-2-hexenal. Error bars indicate the standard error of the mean. VOC treatments as follows: (**A**) 0.04 µmol/mL of the three forms of 1-octen-3-ol or 0.05 µmol/mL of *trans*-2-hexenal; (**B**) 0.08 µmol/mL of 1-octen-3-ol or 0.1 µmol/mL *trans*-2-hexenal; (**C**) 0.4 µmol/mL 1-octen-3-ol or 0.5 µmol/mL *trans*-2-hexenal; (**D**) 0.8 µmol/mL 1-octen-3-ol or 1.0 µmol/mL *trans*-2-hexenal.

3.3. Effect of VOCs on Growth from Conidiospores at 10 ˚C

The effect of exposure to the volatile phase of these VOCs on growth from conidiospores was studied at 10˚C. After incubation for three weeks at 10˚C, conidiospores exposed to 0.04 µmol/mL and 0.08 µmol/mL of racemic 1-octen-3-ol, or its S isomer, gave rise to a few scattered colonies, however no growth was observed for conidiospores exposed to the R enantiomer (Figure 5). At higher concentrations (0.4 and 0.8 µmol/mL) all three forms of 1-octen-3-ol inhibited conidiospore germination. *trans*-2-hexenal was more effective than 1-octen-3-ol, since no growth from conidiospores was observed at any of the volatile exposures tested (0.05, 0.1, 0.5 or 1.0 µmol/mL). When the plates were removed from the presence of volatiles and incubated for 21 days, conidiospores exposed to 0.08 µmol/mL of the R enanatiomer of 1-octen-3-ol produced colonies. In contrast, no growth was observed for conidiospores exposed to *trans*-2-hexenal at any of the concentrations tested.

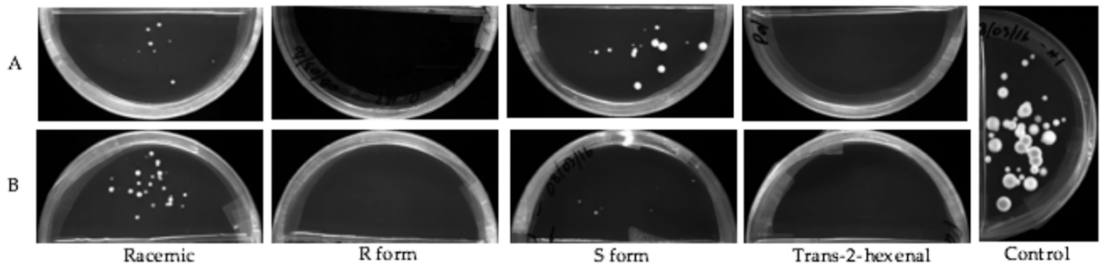

Figure 5. The effect of exposure to low concentrations of volatilized racemic 1-octen-3-ol, (*R*)-(−)-1-octen-3-ol (*R* form), (*S*)-(+)-1-octen-3-ol (*S* form) and *trans*-2-hexenal on growth of *P. destructans* conidiospores after 3 weeks of incubation at 10˚C. VOC treatments as follows: (**A**) 0.04 µmol/mL of three enantiomers of 1-octen-3-ol or 0.05 µmol/mL of *trans*-2-hexenal; (**B**) 0.08 µmol/mL of three enantiomers of 1-octen-3-ol or 0.1 µmol/mL of *trans*-2-hexenal.

3.4. Effect of 0.005, 0.01, 0.02 and 0.05 µmol/mL trans-2-hexenal on Conidiospore Growth at 10˚C

In order to determine if exposure to lower concentrations of *trans*-2-hexenal were also inhibitory to the germination of *P. destructans* conidiospores, a new exposure system was designed that would allow the testing of lower concentrations of this compound. *trans*-2-hexenal is not water soluble and we have found that non-polar solvents that can be used to dissolve it also have independent effects on the growth of *P. destructans*. Therefore, in order to accurately test lower concentrations of *trans*-2-hexenal on conidiospore germination, we increased the volume of our exposure system. In these experiments, a serial dilution of *P. destructans* conidiospores was exposed to *trans*-2-hexenal vapors in concentrations of 0.005, 0.01, 0.02 and 0.05 µmol/mL. The three higher concentrations of exposure prevented growth from conidispores, however conidiospores exposed to 0.005

µmol/mL of *trans*-2-hexenal resumed growth (Figure 6). After a three-week period of incubation, the treated plates were removed from *trans*-2-hexenal exposure and incubated for another 21 days in the absence of *trans*-2-hexenal. Conidiospores that had been previously exposed to 0.01 and 0.02 µmol/mL of *trans*-2-hexenal resumed growth. Conidiospores formerly treated with 0.05 µmol/mL *trans*-2-hexenal did not resume growth, even after two months, so this concentration was fungicidal *to P. destructans.*

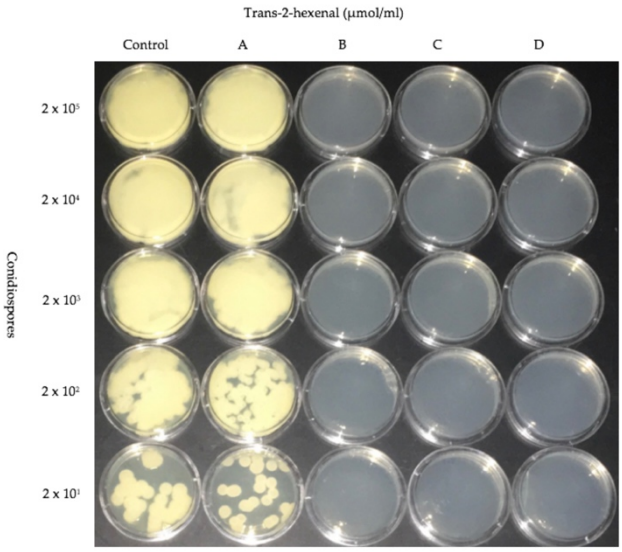

Figure 6. Dilution assay of five different concentrations of *P. destructans* conidiospores treated with *trans*-2-hexenal. Vapors of VOC as follows: (**A**) 0.005 µmol/mL; (**B**) 0.01 µmol/mL; (**C**) 0.02 µmol/mL; (**D**) 0.05 µmol/mL.

4. Discussion

In addition to the serious negative effects of *P. destructans* on hibernating bats, other fungal pathogens have caused several recent epizootics among vertebrates including *Batrachochytrium dendrobatitis,* cause of chytridiomycosis in frogs [34], and

Ophidiomyces ophiodiicola, cause of snake fungal disease in snakes [35]. There is a pressing need to find environmentally benign antifungal compounds to treat the fungi that cause these devastating wild life diseases.

Volatile organic compounds (VOCs) are small molecules with high vapor pressure that exist in the gaseous state at room temperature [36]. Many of the volatile compounds from natural sources, such as those found in essential oils, have documented antimicrobial activities and are generally recognized as safe (GRAS) by the US Food and Drug Administration [26,33]. For example, at low concentrations, vapors of both hexanal and octanal completely inhibited the radial growth of *Aspergillus parasiticus* [37]. Similarly, vapors of racemic 1-octen-3-ol and *trans*-2-hexenal were effective against the mycelial growth of *Penicillium chrysogenum* [38]. Moreover, exposure to *trans*-2-hexenal, *trans*-2-hexen-1-ol, cis-3-hexen-1-ol and 1-hexanol inhibited growth of *Fusarium avenaceum* and *Fusarium graminearum* [30]; and *trans*-2-hexenal inhibited growth of *Rhizoctonia solani* and *Sclerotium rolfsii* [39]. We postulated that *trans*-2-hexenal would be effective against *P. destructans*.

In a previous preliminary study, we tested the eight-carbon alcohol 1-octen-3-ol, and found that when exposed at 15˚C, low concentrations (0.4 and 0.8 μmol/mL) of racemic 1-octen-3-ol inhibited growth of *P. destructans* [23]. Mushroom alcohol is a chiral compound and biological systems are known to be sensitive to stereochemistry [24,40]. Therefore, in this current report, we tested to see if the inhibitory effects of the racemic, R-and S- forms of this chiral compound would be different. Indeed, we showed that both racemic 1-octen-3-ol and the *R* form were more effective than the *S* form. Moreover, the relative impacts of the three forms of vapors of mushroom alcohol were temperature

dependent. When cultures grown at 5˚C were exposed to low concentrations of 1-octen-3-ol vapors, the effectiveness of all three forms was reduced, likely due to reduced volatility at this low temperature. Because we did not quantitatively monitor the amount of VOC present during the duration of experiments, we recognize that the amount of VOC we report in our exposure figures are the initial concentrations of exposure, that is, maximum amounts. Some loss of VOC likely occurred during the course of the experiments, although we did not detect the distinctive odors of mushroom alcohol or leaf aldehyde.

At low concentrations, the vapors of 1-octen-3-ol were not fungicidal. Rather, mushroom alcohol functioned to retard growth from mycelia and conidiospores. Our data support research on several other fungal species demonstrating the spore germination inhibiting properties of mushroom alcohol, including *Agaricus bisporus, Aspergillus nidulans* and *Penicillium paneum* [41–43].

trans-2-hexenal was more effective than 1-octen-3-ol for inhibiting growth from mycelial plugs and conidiospores of *P. destructans.* It completely inhibited growth of mycelial plugs and growth from conidiospores at 0.05 μmol/mL. Unlike 1-octen-3-ol, where mycelial growth resumed when the colonies were removed from the presence of the VOC and growth from conidiospores was observed, *P. destructans* exposed to 0.05 μmol/mL of *trans*-2-hexenal showed growth from neither mycelia nor conidiospores indicating that vapors of *trans*-2-hexenal were fungicidal at this concentration. Commonly known as leaf aldehyde, *trans*-2-hexenal is widely distributed in plants [44] where it is a major constituent of the odor of newly mown grass [45]. *trans*-2-hexenal is also the major VOC in extra virgin olive oil [46,47], where it has been reported to have broad antimicrobial

effects [48] and contributes to the ability of olive oil to inhibit the growth of medically important fungi such as *Trichophyton mentagrophytes, Candida* and *Microsporum canis* [49]. *trans*-2-hexenal is an approved food additive by the US Food and Drug Administration [26]. Interestingly, there is also evidence that this compound may have a positive impact on rodent physiology. In controlled laboratory experiments, a mixture of *trans*-2-hexenal and cis-3-hexanol relieved stress markers in rats [50,51].

Temperature was an important parameter for both the growth of the fungus and the effectiveness of the volatile treatments. In our experiments, control cultures of *P. destructans* grew best at 10˚C.

Blehart et al. [10] reported an optimal growth temperature of *P. destructans* between 5˚C and 10˚C, with only marginal growth above 15˚C. We found that the efficacy of the volatile treatments in inhibiting growth was less pronounced at 5˚C than at the higher temperatures of 10˚C and 15˚C. The effectiveness of VOCs has been reported to be dependent on their vapor pressure, with higher temperatures enhancing their effectiveness [32,52,53]. If adopted for use in hibernacula with cold temperatures, it would be important to use appropriate concentrations that take into account the lower efficacy at 5˚C.

Because WNS is associated with hibernation in caves and other enclosed spaces, we envision an intervention whereby bat hibernacula are fumigated with low concentrations of leaf aldehyde (*trans*-2-hexenal) in such a fashion as to reduce the load of *P. destructans*. We are currently developing methods for formulating *trans*-2-hexenal for use in scaled up model habitats and devising methods for testing its toxicity in mammalian tissues.

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Chapter 2. Transcriptomic analysis of *Pseudogymnoascus destructans* treated with *trans*-2-hexenal

Chapter 2 is being prepared for publication as Victoria L. Korn, Kayla K. Pennerman, Sally Padhi, and Joan W. Bennett. 2019. Mechanism of the activity of *trans*-2-hexenal on *Pseudogymnoascus destructans* the Causative Agent of White Nose Syndrome in Bats. The first author participated in writing the manuscript and is directly responsible for all experiments.

Abstract: *P. destructans* was grown in broth and then treated with three different sublethal quantities of gas phase *trans*-2-hexenal: 5 μmol/L, 10 μmol/L and 20 μmol/L. Total RNA was extracted and sequenced. We then used bioinformatics analyses to annotate the differently expressed genes and to putatively identify the cellular activities and structures in which they are involved. There were 407 total statistically significant Differentially Expressed Genes, 74 of which were commonly express across all three treatments, and 44 were upregulated and 30 were downregulated. This study found the downregulation of essential pathway genes as well as virulence genes known to cause collagen damage to the host bat, known as Desctructin-2 or PdSP1. Along with the upregulation of an ion homeostasis gene. Experimental set-ups described along with these data will help guide future studies on the molecular mechanisms that lead to inhibition of *P. destructans* growth or other fungi by volatile organic compounds.

1. Introduction

The fungal disease White-nose syndrome (WNS) is caused by *Pseudogymnoascus destructans* and has devastated North American bat populations since its discovery in the winter of 2006-2007 (Blehert et al. 2009). *P. destructans* is a psychrophilic fungus that grows optimally between 12.5-15.8˚C and infects bats while they are hibernating (Verant et al. 2012). Because of the significant decline in several bat species, research efforts have been directed towards finding a treatment or prevention method for this infection. Although several approaches show promise, to date nothing has yet been shown to cure WNS.

In the past, our laboratory has investigated possible fumigation treatments for bat hibernacula using safe volatile organic compounds (VOC). In previous work, we showed that racemic 1-octen-3-ol ("mushroom alcohol"), can inhibit growth *of P. destructans* (Padhi et al. 2016). This eight-carbon alcohol has been approved by both the U.S. Food and Drug Administration (USFDA) for use in human food and by the Environmental Protection Agency (EPA) for use in insect attraction (EPA 2003). The success of the 1-octen-3-ol treatment in killing or inhibiting the pathogenic fungus, encouraged our group to survey other safe VOCs. This further work is described in Chapter 1 of this thesis as Padhi et al. 2018. We showed that the R enantiomer of 1-octen-3-ol was more effective than the S enantiomer in inhibiting growth of *P. destructans*. More importantly we found that *trans*-2-hexenal, one of the so-called green leaf aldehydes, was effective at lower concentrations and lower temperatures than racemic 1-octen-3-ol or its enantiomers. This investigation with *trans*-2-hexenal in the laboratory demonstrated a strong inhibitory

effect on *P. destructans* growth. However, the descriptive study did not reveal how the volatile exerted its growth inhibition effect at the molecular level.

The psychrophilic fungus has been studied at a genomic level to better understand its effect on bat tissue, its origin, and its virulence factors. A study by Donaldson et al. 2018 explored the effect of different nutrients and temperatures on *P. destructans* virulence. Other studies have explored virulence genes and the enzyme production of *P. destructans* so there is some understanding of specialized peptidases, specifically endopeptidases, *P. destructans* uses to infect its host (Reeder et al. 2017, O'Donoghue et al. 2015, Pannkuk et al. 2015). However, details of *P. destructans* inhibitory growth response to this aldehyde is not known. According to Gabriel et al. 2018 "… only a small portion of known VOCs have been studied to understand specific mechanisms for their inhibition." In the present work we conducted a transcriptomic study to identify genes that are differentially expressed by *P. destructans* following exposure to *trans*-2-hexenal in order to better understand its inhibitory ability.

2. Materials

2.1 Volatile Organic Compound

Chemical standards of liquid *trans*-2-hexenal (synonyms include *trans*-2-hexen-1-al, (*E*)-2-hexenal, (*E*)-hex-2-enal and leaf aldehyde) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Strains

Pseudogymnoascus destructans, (MYA-4855TM) was obtained from the American Type Culture Collection, Manassas, VA, USA. Throughout our work, *P. destructans* was handled according to all procedures required for biosafety level 2 classification pathogens as described in Padhi et al. 2018 (PDA) (Difco, Becton, Dickinson & Company, Sparks, MD, USA).

2.3 Conidiospore Isolation

For the preparation of conidiospores and viable counts of conidiospore concentrations, Sabouraud Dextrose Agar (SDA) (Difco), supplemented with 200 mg/L $MnSO_4$, was used as described in Padhi et al. 2018. The conidiospores were harvested by adding 10 mL of Conidia Harvesting Solution (CHS) to each plate and gently scraping the fungal growth with an inoculation loop to help release the spores. CHS was composed of 0.05% Tween 80% and 0.9% NaCl. The suspension was then filtered through glass wool and the flow through was centrifuged at 5000 rpm for 15 min. The supernatant was removed and the conidiospore pellet was re-suspended in 10 mL of 0.2 M Phosphate buffer solution (phosphate-buffered saline) solution at pH 7.0.

2.4 Kits

RNA extractions were performed using the ZR Fungal/Bacterial RNA Mini Prep kit (Zymo Research Corporation, Irvine, CA).

3. Methods

3.1 Fungal Growth and trans*-2-hexenal Treatment*

P. destructans cultures started from 0.1 mL of conidiospores, ~212,000 spores per bottle according to a viable spore count, were cultured in 500 mL Media Bottles in 100 mL potato dextrose broth for four weeks at 10˚C. The caps were not tightened, but turned once to ensure no contamination entered the bottle while allowing for some airflow. The cultures were then exposed to 5, 10, and 20 µmol/L gaseous *trans*-2-hexenal and incubated for an additional four weeks. Exposure was done by tapping a membrane into the cap of the bottle and placing the treatment on the membrane. The bottles were covered with these membrane and treatment containing caps, again caps were turned once to allow some airflow, and sealed with a single layer of Parafilm to prevent the treatment from escaping the bottles.

3.2 RNA extraction

After eight weeks, mycelia were separated from the growth media, frozen in liquid nitrogen, and then crushed with a micro pestle before being transferred to a bead bashing tube, and vortexed for 10 minutes. Using the ZR Fungal/Bacterial RNA Mini Prep kit, RNA was extracted according to the manufacturer's instructions. The RNA was sent to the Genome Core facility at the Waksman Institute for quality check, preparation, and sequencing.

3.3 RNA Sequencing

The RNA samples were sent to the Genome Core facility at the Waksman Institute for Microbiology at Rutgers University for RNA quantitation, quality check using a

Bioanalyzer RT-PCR generation of cDNA libraries and sequencing using Illumina NextSeq 500 to yield single-end 1 x 75 bp reads.

3.4 Bioinformatics Pipeline

The genome of *P. destructans* has been sequenced (Drees et al. 2016) and genomic sequences of *P. destructans* were retrieved from the NCBI Genome database (assembly GCA_001641265.1). The transcriptomes of the untreated control samples were compared with that of the mycelia exposed to three different concentrations of gaseous *trans*-2-hexenal. FastQC version 011.5 was used to check the quality of the sequencing reads before aligning them to the genome of *P. destructans* using STAR version 2.6 guided by the respective annotation GFF3 file (Andrews, Dobin). The output SAM files were converted to sorted BAM files using Samtools version 1.7. Stringtie version 1.3.5 was used for differential gene expression analysis with DESeq2 version 1.22.1 (Handsaker et al., Pertea, Love et al.). For comparisons between control and experimental groups, an adjusted p-value $< 1^{-5}$ was considered statistically significant.

4. Results and Discussion

4.1 Bioinformatics Analysis

Statistically significant differentially-expressed genes (DEG) were defined as those with an adjusted p-value $< 1^{-5}$ and a $\log_2$ fold change >1 or <-1. 407 statistically significant DEGs were identified across all three treatments, 194 upregulated and 213 downregulated. The Venn Diagram below shows the number of shared up and down regulated genes between 5, 10, and 20 µmol/L treatments (Figure 1). An analysis of all

genes, including the 407 genes can be found in the appendix as S1 Supplemental Data. Outlined in Tables 1 and 2 are 74 of the 407 genes that were commonly differentially expressed among all three treatment. Of these 74 commonly DEG, 44 genes were upregulated and 30 genes were downregulated in the three treatments of 5, 10, and 20 µmol/L. Upon further investigation it was found that many of the DEGs did not have a description in the NCBI database, and are described as "hypothetical proteins."

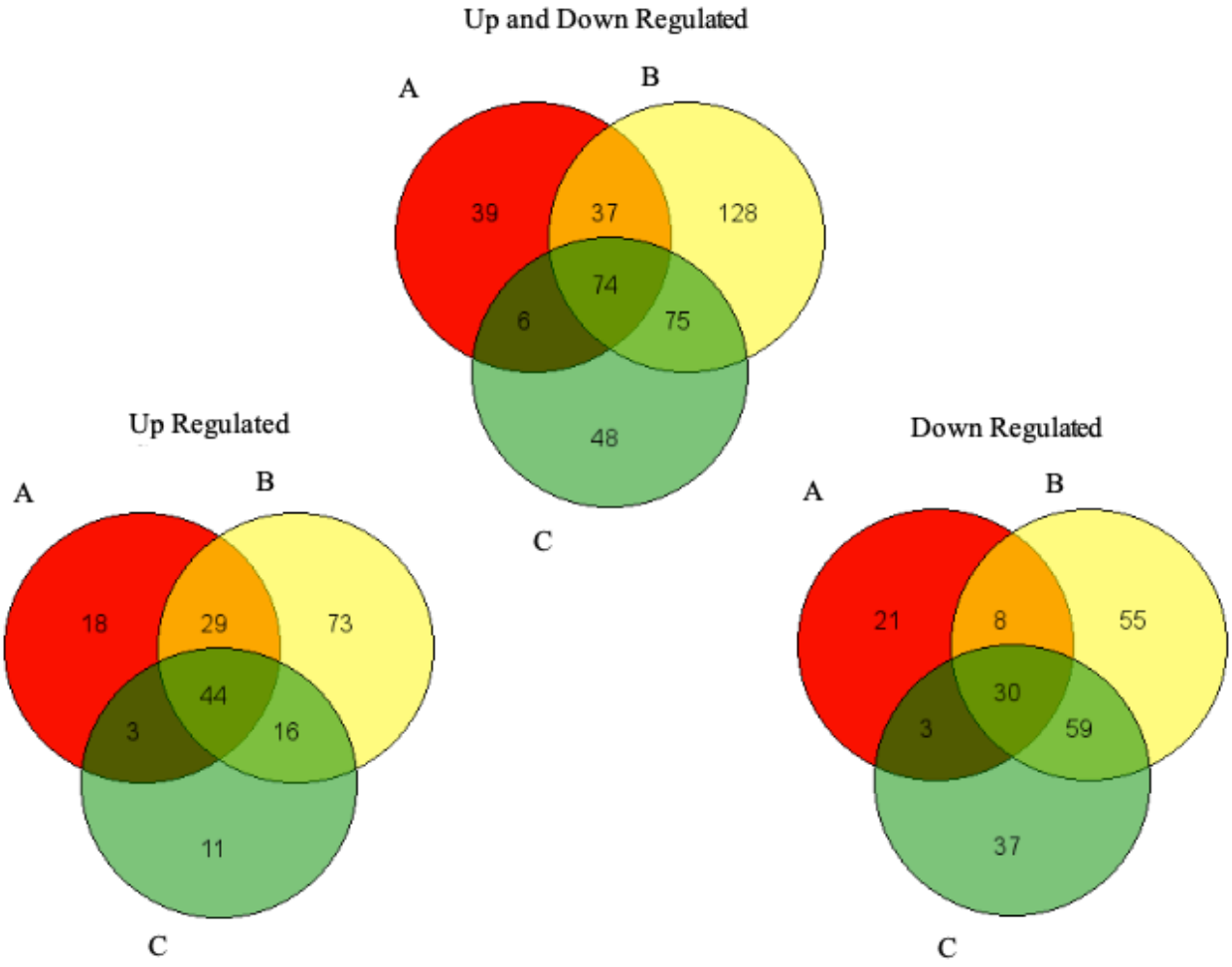

Figure 1. Venn Diagram of differentially expressed genes (DEG) for 5, 10, and 20 µmol/L treatments. (A) 5 µmol/L (B) 10 µmol/L (C) 20 µmol/L.

Table 1. DEGs commonly upregulated

Gene ID	NCBI Description	5 µmol/L		10 µmol/L		20 µmol/L	
		P Value	Log 2 fold change	P Value	Log 2 fold change	P Value	Log 2 fold change
VC83_00399	Eukaryotic translation initiation factor 6 (TIF6)	5.69E-10	1.74242408	2.73E-14	2.552286222	1.53E-07	1.881855511
VC83_02426	Rab proteins geranylgeranyltransferase component A (MRS6)	3.02E-09	1.370173756	1.40E-10	1.693972835	1.15E-09	1.4347697
VC83_02882	U3 small nucleolar RNA-associated protein 13 (UTP13)	8.59E-08	1.634226215	1.36E-07	1.852610223	1.05E-08	1.83193456
VC83_03689	Mitochondrial glycerol-3-phosphate dehydrogenase (GUT2)	5.68E-10	1.390417926	1.11E-11	1.993333353	1.35E-09	1.614431637
VC83_04407	DNA-dependent ATPase of the nucleotide excision repair factor 4 complex (RAD15)	2.14E-14	2.960007847	1.56E-22	3.136573335	1.99E-13	2.659797504
VC83_04809	Translation initiation factor eIF4A (TIF1)	2.62E-14	2.193039518	7.42E-16	2.502756243	5.69E-13	2.333238197
VC83_06045	Protein kinase rio1 (RIO1)	7.83E-11	1.707555781	3.08E-11	1.781969504	5.65E-08	1.611543888
VC83_06666	Translational elongation factor EF-1 alpha (TEF3)	2.07E-08	1.320363879	7.79E-08	1.353619174	2.66E-09	1.600331751
VC83_06819	ATP-dependent RNA helicase dbp7 (DBP7)	5.73E-08	1.97052893	1.30E-07	2.066135704	1.65E-08	2.0021447
VC83_06825	Ribosome-binding protein (NMD3)	1.47E-10	2.141794379	1.38E-09	2.294469129	2.39E-08	2.151492556
VC83_07808	Mg(2+) transporter (ALR1)	1.94E-07	1.207102254	1.05E-10	1.530816564	6.95E-09	1.610048811
VC83_08625	DEAH-box ATP-dependent RNA helicase prp43 (PRP43)	4.36E-10	1.723155872	5.69E-08	1.645292296	2.23E-10	1.766581762
VC83_00742	Hypothetical protein VC83_00742	1.70E-13	2.041362466	7.47E-10	2.63303327	1.93E-07	1.836378595
VC83_00877	Hypothetical protein VC83_00877	1.32E-09	2.283949222	1.88E-09	1.887742125	1.76E-08	1.521063201
VC83_01014	Hypothetical protein VC83_01014	1.35E-07	1.444497834	1.77E-13	1.837707823	3.63E-11	1.811280133
VC83_01469	Hypothetical protein VC83_01469	2.03E-10	1.935830773	8.15E-15	2.501054933	6.80E-09	2.090047298
VC83_02188	Hypothetical protein VC83_02188	3.34E-18	2.472865349	2.27E-14	2.949738943	1.31E-29	2.908091208
VC83_02398	Hypothetical protein VC83_02398	1.48E-07	1.576747452	4.82E-12	1.807270136	1.93E-07	1.412773086

VC83_02432	Hypothetical protein VC83_02432	1.07E-08	1.467775075	9.17E-11	1.69849344	3.26E-09	1.654038568
VC83_02545	Hypothetical protein VC83_02545	3.58E-26	3.682341041	1.37E-28	4.024625654	8.14E-28	3.894306961
VC83_02682	Hypothetical protein VC83_02682	3.48E-14	2.454194045	1.10E-09	2.248771603	2.00E-09	2.113743579
VC83_02752	Hypothetical protein VC83_02752	7.45E-08	1.573734453	1.54E-08	1.801361637	1.82E-07	1.650833326
VC83_02862	Hypothetical protein VC83_02862	2.04E-08	2.331703065	5.06E-14	2.559201823	1.51E-14	2.422425618
VC83_03029	Hypothetical protein VC83_03029	1.48E-07	2.1678601	4.29E-11	1.714896848	1.21E-07	1.837655847
VC83_03420	Hypothetical protein VC83_03420	5.15E-10	2.884662273	1.70E-11	3.254220502	1.38E-08	2.38783451
VC83_03740	Hypothetical protein VC83_03740	6.55E-11	1.762029261	9.44E-17	2.013697034	2.53E-11	2.103488182
VC83_04527	Hypothetical protein VC83_04527	4.06E-10	1.65987848	1.89E-20	2.279452797	5.29E-13	1.915769597
VC83_05707	Hypothetical protein VC83_05707	1.01E-07	2.009694402	1.76E-12	2.905182359	3.31E-09	2.54230033
VC83_05810	Hypothetical protein VC83_05810	2.12E-16	2.942185155	1.70E-13	3.310962844	1.07E-08	2.605518759
VC83_06295	Hypothetical protein VC83_06295	2.33E-09	2.422416797	1.32E-15	3.787730567	1.43E-11	2.755737221
VC83_06423	Hypothetical protein VC83_06423	5.09E-09	1.325856788	3.62E-12	1.79123375	2.12E-07	1.432169338
VC83_06651	Hypothetical protein VC83_06651	1.59E-11	2.358048499	1.56E-10	2.132976661	1.79E-11	2.658122244
VC83_06652	Hypothetical protein VC83_06652	3.06E-15	2.309366674	1.25E-15	2.082100167	2.17E-11	2.423698109
VC83_06756	Hypothetical protein VC83_06756	4.27E-08	1.610477461	1.66E-20	2.149932808	2.05E-07	1.773967305
VC83_07344	Hypothetical protein VC83_07344	1.13E-21	3.123781746	2.11E-21	3.149011261	4.90E-18	3.933145315
VC83_07543	Hypothetical protein VC83_07543	1.69E-08	1.527019983	1.73E-07	1.450015653	6.72E-10	1.738122183
VC83_08108	Hypothetical protein VC83_08108	5.48E-08	2.121968628	1.07E-09	1.918067604	2.48E-26	2.762440577
VC83_08284	Hypothetical protein VC83_08284	7.22E-08	1.679989011	3.94E-08	1.720543363	1.19E-07	1.668891233
VC83_09335	Hypothetical protein VC83_09335	3.70E-22	3.191099876	5.27E-25	3.494907712	4.06E-27	3.356424127
VC83_09352	Hypothetical protein VC83_09352	3.02E-08	1.862235078	5.11E-19	2.507527449	8.22E-10	2.104993475
VC83_09378	Hypothetical protein VC83_09378	4.10E-09	2.088457	5.03E-11	2.566782445	9.61E-12	2.603644977

VC83_09405	Hypothetical protein VC83_09405	1.92E-18	3.53566532	8.88E-21	3.686198377	2.42E-18	3.406551016
VC83_09443	Hypothetical protein VC83_09443	1.86E-16	2.190213661	2.32E-26	2.632454599	2.06E-14	2.222633915
VC83_00494	Hypothetical protein VC83_00494	2.57E-15	2.77133606	7.91E-17	2.764894141	1.76E-11	2.1233628

Table 2. DEGs commonly downregulated

Gene_ID	NCBI Description	5 µmol/L		10µmol/L		20 µmol/L	
		P value	Log 2 fold change	P value	Log 2 fold change	P value	Log 2 fold change
VC83_00883	Methylglyoxal reductase (NADPH-dependent) gre2 (GRE2)	1.03E-08	-1.62320455	2.03E-17	-2.201230975	1.58E-12	-2.032986498
VC83_06276	Vesicle formation at the endoplasmic reticulum (SED4)	3.85E-17	-2.770973953	1.74E-23	-3.493457318	4.15E-52	-4.1940596
VC83_04892	Subtilisin-like protease 1 (SP1)	4.81E-17	-3.559389108	3.01E-15	-3.670446244	1.97E-19	-3.898603521
VC83_06822	Phosphomevalonate kinase (ERG8)	1.59E-08	-1.431427892	8.04E-13	-1.934625825	2.70E-08	-1.648381741
VC83_07077	Superoxide dismutase (SOD1)	3.07E-09	-1.890634856	1.35E-11	-2.145462434	7.49E-15	-2.381601805
VC83_08761	Glyceraldehyde-3-phosphate dehydrogenase 1 (GAP1_3)	4.61E-14	-2.150200505	6.75E-19	-2.98371099	1.09E-23	-2.7067019
VC83_00241	Hypothetical protein VC83_00241	4.17E-10	-2.396127072	1.19E-32	-4.168368046	1.07E-24	-4.008339559
VC83_00345	Hypothetical protein VC83_00345	5.81E-09	-2.745103691	2.33E-44	-4.580989704	1.28E-33	-5.31945428
VC83_01030	Hypothetical protein VC83_01030	6.34E-09	-1.260312464	5.17E-12	-1.574844725	1.15E-07	-1.300174304
VC83_01564	Hypothetical protein VC83_01564	2.69E-11	-2.232308337	5.74E-22	-3.727115064	4.64E-30	-3.637933374
VC83_01565	Hypothetical protein VC83_01565	9.82E-10	-1.62838225	6.29E-14	-2.200260376	1.73E-08	-1.982696353
VC83_01724	Hypothetical protein VC83_01724	3.21E-27	-2.04109072	1.79E-21	-2.980965212	3.21E-27	-2.782830337
VC83_02181	Hypothetical protein VC83_02181	9.40E-12	-2.109454348	8.96E-12	-2.368702789	1.64E-16	-2.72385306
VC83_02203	Hypothetical protein VC83_02203	6.79E-12	-1.826926788	1.25E-12	-2.290254079	9.70E-10	-1.828484325
VC83_02268	Hypothetical protein VC83_02268	1.27E-19	-3.04274299	5.02E-37	-4.056015617	7.02E-32	-3.795038104
VC83_02269	Hypothetical protein VC83_02269	5.90E-13	-2.995603252	2.66E-21	-3.624957683	6.45E-15	-3.628949238

VC83_02402	Hypothetical protein VC83_02402	6.59E-08	-1.446062253	2.16E-11	-1.973713765	1.18E-07	-1.678171766
VC83_02906	Hypothetical protein VC83_02906	1.39E-09	-3.344469513	5.95E-25	-5.902747302	2.95E-20	-5.99681183
VC83_03247	Hypothetical protein VC83_03247	1.35E-11	-2.758549351	4.28E-35	-4.41902218	1.48E-28	-4.293868893
VC83_04545	Hypothetical protein VC83_04545	1.00E-14	-1.947159041	1.43E-27	-2.693212438	4.36E-18	-2.263794273
VC83_06026	Hypothetical protein VC83_06026	4.37E-11	-1.601543459	6.86E-20	-2.340183497	1.44E-07	-1.896650547
VC83_06127	Hypothetical protein VC83_06127	4.29E-13	-2.390945273	1.31E-11	-2.729839263	1.08E-22	-2.828004006
VC83_06378	Hypothetical protein VC83_06378	1.85E-08	-1.934676837	2.22E-19	-3.085771306	3.31E-11	-2.281557367
VC83_06475	Hypothetical protein VC83_06475	2.61E-16	-2.186119151	1.02E-14	-1.976661501	3.08E-14	-1.930139939
VC83_06806	Hypothetical protein VC83_06806	7.14E-12	-2.490719845	3.13E-25	-3.548767559	1.02E-14	-2.846315469
VC83_07035	Hypothetical protein VC83_07035	3.67E-08	-1.555120315	1.35E-07	-1.821988724	2.40E-10	-1.811012283
VC83_07149	Hypothetical protein VC83_07149	1.34E-07	-1.700485793	9.09E-29	-2.933752649	1.02E-11	-1.994985271
VC83_07171	Hypothetical protein VC83_07171	2.30E-08	-2.138767221	2.00E-10	-2.655062505	4.34E-10	-2.63675463
VC83_07272	Hypothetical protein VC83_07272	1.06E-10	-1.640828035	2.05E-11	-2.160550119	2.14E-10	-1.68810465
VC83_07336	Hypothetical protein VC83_07336	2.33E-14	-2.029444461	2.14E-10	-2.347998057	3.18E-26	-2.529793294

4.2 DEG Analysis

The treatments were all performed at sublethal concentrations, below 50 µmol/L. Three different concentrations were used to examine the effect of increasing *trans*-2-hexenal concentration, 5, 10, and 20 µmol/L.

Several of the commonly upregulated described genes are involved in transcription, translation, and replication, such as U3 small nucleolar RNA-associated protein 13 (VC83_02882) (Dragon et al. 2002)**,** translation initiation factor eIF4A (VC83_04809) (Hernández and Vazquez-Pianzola 2005), protein kinase Rio1 (VC83_06045) (Guderian

et al. 2010, Laronde-Leblanc and Wlodawer 2005), translational elongation factor EF-1 alpha (VC83_06666) (Song et al. 1989), DEAH-box ATP-dependent RNA helicase prp43 (VC83_08625) (Arenas and Abelson 1997), ATP-dependent RNA helicase dbp7(VC83_06819) (Daugeron and Linder 1998), eukaryotic translation initiation factor 6 (VC83_00399) (Russell and Spremulli et al. 1980), ribosome-binding protein (VC83_06825) (Ho and Johnson 1999, Niessing 2011), and DNA-dependent ATPase of the nucleotide excision repair factor 4 complex (VC83_04407) (Guzder et al. 1998). These small components are involved in processing 18S rRNA, binding the mRNA with the 40S subunit, binding mRNA to 40S subunit, bringing the tRNA to the ribosome, pre-mRNA splicing, the RNA helicase involved in the formation of 25S and 5.8S rRNA, promoting the production of the 60S subunit, an intermediate between the ribosome and the endoplasmic reticulum and is also involved in mRNA localization and 60S ribosomal stabilization, and DNA damage recognition (Dragon et al. 2002, Hernández and Vazquez-Pianzola 2005, Song et al. 1989, Arenas and Abelson 1997, Daugeron and Linder 1998, Russell and Spremulli et al. 1980, Ho and Johnson 1999, Niessing 2011, Guzder et al. 1998). All important components of the organism's ability to process genetic material. The fungus may be trying to grow or upregulate the production of genes. Many more of these upregulated genes can be for survival, repair damage, cell wall damage repair, virulence, etc. It is difficult to determine what these genes may be due to the many genes as described as "hypothetical proteins." The described gene for DNA damage repair may indicate that there is more genetic damage occurring, possibly from the treatment or due to the higher amount of gene production. In addition, there is a description of the "Hypothetical protein VC83_01014" (VC83_01014) in Reeder et al.

2017 as a calcium-transporting ATPase 2 (VC83_01014). This has been identified in Reeder et al. 2017 as being a part of ion homeostasis, and can contribute to survival. Other upregulated genes are mitochondrial glycerol-3-phosphate dehydrogenase (VC83_03689) is involved in an essential pathway that serves as an intermediate between multiple metabolic pathways and has been found to affect an organism's use of glycerol (Rønnow and Kielland-Brandt 1993, Grauslund and Rønnow 2000). The gene for the Rab proteins geranylgeranyl transferase component A (VC83_02426), aids in vesicle transport as a transport protein (Andres et al. 1993), while a gene for Mg^{2+} transporter (VC83_07808), which transports magnesium in and out of the cell (Lee and Gardner 2005).

There were interesting findings in the downregulated genes, but like with the upregulated genes many of them were described as "hypothetical proteins." The subtilisin-like protease 1 (VC83_04892), is an enzyme known to be a contributor to the pathogenicity in some pathogens such as *Streptococcus* (Bonifait et al. 2010). This gene, as well as the endopeptidase it produces, has been studied in *Pseudogymnoascus destructans*, and is a collagen degradation enzyme (O'Donoghue et al. 2015). These have been studied in more detail to better understand their role in virulence when infecting a host bat and have been described as Destructin-1, Destructin-2, and Destructin-3. It has been found that bats are depleted of fat supplies as well as electrolytes, so it is likely the pathogenic fungus is able to excrete enzymes for this purpose (Warnecke et al. 2013).

A study by Reeder et al. 2017 examines virulence genes, in particular they describe peptidase's that have been both up and down regulated here. According to Reeder et al. 2017, there is an upregulation in proteases associated with "hypothetical protein" or

tripeptidyl-peptidase sed2 (VC83_02181) and subtilisin-like protease 1 (VC83_04892), which is also known as Destructin-2 from O'Donoghue et al. 2015. These genes were seen as downregulated in the study described here, and could indicate that there is a reduction in virulence from the *trans*-2-hexenal treatment. Further investigation needs to be done to completely understand the damage these enzymes are doing to a host bat, but according to O'Donoghue et al. 2015 Destructin-2 is a collagen degrading endopeptidase. A study by Pannkuk et al. 2015 on the protease production of *P. destructans* in different types of culture media, showed the most produced protease in all medias was subtilisin-like protease 1, which they named PdSP1 in the study by Pannkuk et al. 2015, or Destructin-2 by O'Donoghue et al. 2015.

This could mean *trans*-2-hexenal treatment aids in lowering pathogenicity in sublethal quantitates as well as killing the fungus at the higher quantities. Other downregulated genes have functions that are involved in a variety of pathways. This leads to the conclusion that *trans*-2-hexenal may not have one straightforward mode of action, but instead causes multiple genes to be downregulated within specific biochemical pathways leading to the inability of the fungus to function or causing damage to itself.

For example, glyceraldehyde-3-phosphate dehydrogenase 1 (VC83_08761) (Harris and Waters 1976)**,** which is involved in the 6th step of glycolysis. In addition, the essential mevalonate pathway is also being affected through the down regulation of phosphomevalonate kinase (VC83_06822) which is essential in isoprenoid/sterol biosynthesis (Tsay and Robinson 1991). These proteins are essential within their pathways for the function and survival of any eukaryotic cell. Another downregulated gene is vesicle formation at the endoplasmic reticulum (VC83_06276). This is involved

in transporting synthesized proteins out into the cell from the endoplasmic reticulum or allowing proteins into the endoplasmic reticulum (Watson and Stephens 2005). The gene for methylglyoxal reductase (NADPH-dependent) gre2 (VC83_ 00883) is downregulated and involved in magnesium utilization, reducing methylglyoxal to (S)-lactaldehyde (Murata et al. 1985). A promising example is superoxide dismutase (VC83_07077) which is essential to remove toxic superoxide radicles by creating molecular oxygen or peroxide (McCord and Fridovich 1969). Reactive oxygen species have been found to be a mode of death for some microorganisms, which has been studied in another pathogenic organism *Aspergillus fumigatus* (Lambou et al. 2010). This could be the potential mechanism *trans*-2-hexenal is taking to kill *P. destructans* at higher treatment concentrations.
An interesting pairing is the upregulation in transcription, translation, and replication genes, while there is a downregulation of vesicle transport in the endoplasmic reticulum. Possibly creating a bottleneck of protein, and could be a way the growth of the fungus is inhibited. Another pairing is the upregulated Mg^{2+} transporter (VC83_07808) gene, transporting Mg in and out of the cell while methylglyoxal reductase (NADPH-dependent) gre2 (VC83_ 00883) is downregulated and is involved in magnesium utilization. Possibly creating another bottleneck of magnesium in the fungus.

5. Conclusions

These results will guide future research in which we hope to further analyze the specific genes involved in the growth inhibition of the fungal pathogen so as to devise precise methods for the control of WNS. This data will serve as an example for the volatile community as well. As previously noted, there is little research being performed to

understand the mechanisms through which volatiles are able to affect a fungus or other organisms. Experiments such as these are difficult to conduct due to the volatile transforming from a liquid to gaseous form. Our research outlines a new experimental set-up to test the effect of volatiles in a broth culture, which could lead to future transcriptomics studies.

Described in this research is a promising set of DEGs that provides a potential reason why *trans*-2-hexenal is able to inhibit the growth of *P. destructans*. Many downregulated genes are known for their importance in essential metabolic pathways such as glyceraldehyde-3-phosphate dehydrogenase 1 (VC83_08761) (Harris and Waters 1976) which is involved in the 6th step of glycolysis, phosphomevalonate kinase (VC83_06822) which is essential in isoprenoid/sterol biosynthesis in the mevalonate pathway (Tsay and Robinson 1991), or superoxide dismutase (VC83_07077) which is essential to remove toxic superoxide radicles by creating molecular oxygen or peroxide (McCord and Fridovich 1969). Reactive oxygen species are sometimes involved in the death of some pathogenic microorganisms, and has been studied in *Aspergillus fumigatus* another pathogenic organism fungus (Lambou et al. 2010). It is possible these pathways could be a method through which *trans*-2-hexenal is inhibiting the growth of *P. destructans*, alone or as a cluster all working to inhibit growth.

Reeder et al. 2017 shows transcriptome changes in *P. destructans* comparing gene expression between fungi grown in culture with fungi isolated during infection of the bat *Myotis lucifugus.* When *P. destructans* is growing on bat tissue, the most significant differentially expressed genes are involved in cell wall remodeling, heat shock responses, and micronutrient acquisition. These data suggest that when functioning as a pathogen, *P.*

destructans regulates gene expression in ways that may contribute to evasion of host responses (Reeder et al. 2017). Additionally, there is an upregulation when grown in culture versus host tissue invasion of the proteases Tripeptidyl-peptidase sed2 (VC83_02181), described in Table 2 as "hypothetical protein" (VC83_02181), and Subtilisin-like protease 1 (VC83_04892). Subtilisin-like protease 1 (VC83_04892) named Destructain-2 by O'Donoghue et al. 2015, and PdSP1 by Pannkuk et al. 2015. Both studies have come to a similar conclusion, Subtilisin-like protease 1 (VC83_04892) is a virulence factor, and contributes to host death. In this thesis's study both Tripeptidyl-peptidase sed2 (VC83_02181) and Subtilisin-like protease 1 (VC83_04892) are downregulated. O'Donoghue et al. 2015 and Pannkuk et al. 2015 have done research to better understand the protease production and function of *P. destructans*. Pannkuk et al. 2015 shows protease production in different types of culture media, and the most produced protease in all medias was found as PdSP1 (Pannkuk et al. 2015). Their research speculates that this peptidase is a major contributor to damaging the wing tissue of infected bats. O'Donoghue et al. 2015 studies the production of enzymes in *P. destructans* and the effect these may have on a host. The research found that the endopeptidase Destructin-1, degrades collagen. Similarly, Destructin-2, and Destructin-3 are close in homology to Destructin-1, and are also collagen degrading endopeptidases. There has been some study into the lack of saprophytic enzymes for *P. destructans* to survive in soil indicating it's evolving to prefer a host (Reynolds and Barton 2014). Future study would be needed to better understand the roles of the many genes of *Pseudogymnoascus destructans*, especially the peptidases it produces to aid its pathogenicity.

In conclusion, there is promise in the discovery of similar virulence genes in the study discussed here as in Reeder et al. 2017. Showing that *trans*-2-hexenal has the potential to lower virulence of *P. destructans* as well as inhibit growth. Therefore, if *trans*-2-hexenal is used as a treatment in a bat hibernaculum, it may lower the virulence of the fungus, potentially allowing the bat to overcome the fungal infection at lower concentrations. While at higher concentrations the fungus is completely inhibited, as shown in Padhi et al. 2018.

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1. Appendix. Full list of Differentially Expressed Genes from Chapter 2

Link to excel spreadsheet containing S1:

https://1drv.ms/x/s!AtFU495GAJWkn03UbUAi5RasEDaD