Two volatile-phase alcohols inhibit growth of *Pseudogymnoascus destructans*, causative agent of white-nose syndrome in bats

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**ABSTRACT**

In North America, *Pseudogymnoascus destructans* infects hibernating bats within caves and other hibernacula causing high mortalities. We have sought a potential fumigation strategy that can be deployed within a contained area, using an agent that already has been determined to be safe for environmental application. We here report the efficacy of 1-octen-3-ol ("mushroom alcohol") and 1-hexanol against *P. destructans*. At 50 ppm and 100 ppm, vapours of racemic 1-octen-3-ol are fungicidal to *P. destructans* at 15ºC after 21 days incubation, while exposure to 5 and 10 ppm is fungistatic. The six-carbon alcohol 1-hexanol is not as effective, although at 50 and 100 ppm, vapours of this compound inhibited growth of the fungus.

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**Introduction**

The white-nose syndrome (WNS), an epidemic disease of hibernating bats, has decimated North American bat populations since it was first documented in the winter of 2006–2007 (Blehert et al. 2009). A five-year assessment of mortality and geographic spread of WNS, from 42 sites in five states, reported that the number of hibernating bats declined from 412,340 to 49,579 animals, a decrease of 88% (Turner et al. 2011). It has been predicted that the little brown bat (*Myotis lucifugus*) could become extinct in the Northeastern USA in 7–30 years, with a similar fate possible for three other bat species (Frick et al. 2010).

WNS is caused by *Pseudogymnoascus destructans* (formerly known as *Geomyces destructans*), a psychrophilic fungus (Gargas et al. 2009; Verant et al. 2012). The body temperatures of hibernating bats range from 2°C to 15°C, which closely resembles the optimum growth temperatures for this fungus (Blehert et al. 2011). The disease is characterised by a white-coloured fungal growth on the muzzle and wings of hibernating bats resulting in severe damage to wing membranes (Cryan et al. 2010). Affected animals display increased arousal and depletion of fat reserves, resulting in emaciation and death (O’Donoghue et al. 2015). Physiological studies using isotope-based estimates of change in body mass composition showed that little brown bats infected with *P. destructans* used twice as much energy as non-infected controls and also displayed respiratory acidosis and high concentrations of potassium in the blood, even in early stages of infection (Verant et al. 2014). Effective and ecologically appropriate management strategies for the control of this devastating pathogen are lacking.

Several antifungal drugs such as amphotericin B and fluconazole have been reported as capable of limiting the growth and/or conidial germination of *P. destructans* (Chaturvedi et al. 2011), but it is feared that application of broad spectrum antifungal compounds could negatively affect cave ecosystems (Bending et al. 2007; Raudabaugh and Miller 2015). Biological control promises a more ecologically sound approach. For example, in laboratory studies, six bacterially produced volatile organic compounds were found to inhibit the growth of *P. destructans* (Cornelison et al. 2014a) and volatile phase compounds from the bacterium *Rhodococcus rhodocrous* also caused inhibition of the pathogen (Cornelison et al. 2014b). Moreover, the volatile sesquiterpene trans-trans-farnesol from the yeast *Candida* has the potential to be utilised as a biological control agent (Raudabaugh and Miller 2015) and the fungus *Epicoccum nigrum* also has been evaluated for
possible biocontrol (Perryman et al. 2014). In another study, bacteria isolated from the skin of bats and identified as *Pseudomonas* spp. were found effective in inhibiting the growth of *P. destructans* in vitro (Hoyt et al. 2015).

Earlier work in our laboratory has shown that the vapour phases of the medium chain alcohols 1-octen-3-ol (also called mushroom alcohol) and 1-hexanol exhibit fungistatic properties against *Aspergillus niger* and *Penicillium chrysogenum* (Yin et al. 2015). Both 1-octen-3-ol and 1-hexanol (Figure 1) are examples of volatile organic compounds (VOCs), a large group of odiferous, low-molecular-weight metabolic products that frequently function in ecological interspecific communications (Hermann 2010; Hung et al. 2015). Mushroom alcohol is one of the most abundant aroma compounds produced by mushrooms and moulds (Tressl et al. 1982; Korpi et al. 2009) and serves as a semiochemical (signalling molecule) for many arthropods (Davis et al. 2013). It is used to enhance mushroom flavour in foods and is generally regarded as safe (GRAS) by the U. S. Food and Drug Administration (FDA) (Zawirska-Wojtaslak 2004). Furthermore, it attracts biting insects (Luntz 2003; Cilek et al. 2011) and has received U.S. Environmental Protection Agency clearance for use in insect lures (EPA 2015a, 2015b). Low concentrations of 1-octen-3-ol inhibit growth of *Aspergillus nidulans* (Herrero-Garcia et al. 2011) and can control a fungal disease of cultivated mushrooms caused by *Leccanicillium fungicola* (Berendsen et al. 2013). Another mid-length alcohol, 1-hexanol, also has been found to be biologically active against fungi and has been tested for controlling postharvest fungal pathogens (Archbold et al. 1997; Cruz et al. 2012). Therefore, we hypothesised that these vapour-phase alcohols might be effective against *P. destructans*. Our goal was to test these compounds in the laboratory as potential fumigation agents for control of *P. destructans*. We report herein the initial results of our study.

**Materials and methods**

*P. destructans* (MYA-4855™) was obtained from the American Type Culture Collection, Manassas, VA. Throughout our experiments, assiduous care was taken to ensure that this pathogen was handled according to all rules, procedures and steps required for a level 2 classification pathogen. All cultures were grown on potato dextrose agar (PDA) (Difco) and incubated at 15°C. Petri plates (100 × 15 mm) (sometimes called ‘I’ plates) were used so that one half of the plate contained 10 ml PDA and the other half contained a sterile glass cover slip (22 mm × 22 mm) for the placement of aliquots of the VOCs being tested. To prepare inocula, mycelial plugs were taken from the actively growing outer edge of 21-day-old colonies using a #3 cork borer, and placed onto fresh medium in the divided Petri plate. The VOCs tested were reagent grade racemic 1-octen-3-ol, 1-hexanol and toluene (a toxic industrial VOC). All VOCs were purchased from Sigma-Aldrich. Liquid aliquots of these compounds were placed in the other half of the plate and added in appropriate amounts to deliver concentrations of 5, 10, 50 and 100 ppm (mg/L) when volatilised. The amounts were calculated according to the density of the compound and volume of the container. Inoculated plates were sealed with two layers of Parafilm, each concentration of VOC placed in 2 l glass containers with tightly fitting propylene lids and incubated at 15°C for 3 weeks. Following exposure, the colonies were observed macroscopically and the colony diameter recorded. In addition, mycelial plugs showing no growth macroscopically were observed microscopically and the colony diameter recorded. In addition, mycelial plugs from treatments that exhibited no growth were subcultured on PDA and examined after 2 weeks to determine if the concentrations were fungistatic or fungicidal.

![Figure 1](image-url) Structural formulas of (a) 1-octen-3-ol and (b) 1-hexanol.
Results

Macroscopic images of *P. destructans* mycelial plugs after 3 weeks incubation at 15°C with and without exposure to vapours of 1-octen-3-ol (0, 5, 10, 50 and 100 ppm) are shown in (Figure 2 (a–e)). There was no increase in diameter of mycelial plugs in the 1-octen-3-ol treatments (Figure 2 (b–e)). These results were compared to the effects produced by 1-hexanol, another midlength alcohol. Growth in the 5 and 10 ppm treatments was equivalent to the untreated control (Figure 2 (a, f, g)). Inhibition occurred at 50 ppm and there was no growth at 100 ppm (Figure 2 (h, i)). Toluene, a known toxic industrial volatile (Donald et al. 1991), was also tested and it had no inhibitory effects on the fungus. Growth was equivalent to the untreated controls when mycelial plugs were exposed to 100 ppm of toluene vapours (data not shown). Microscopic examination of the mycelial plugs treated with 1-octen-3-ol at 5 ppm showed slight hyphal extension in 5 days. Similar hyphal extensions were visible after 10 days in the 10 ppm treatment.

Measurements of the growth of mycelial plugs in all VOC treatments were subjected to statistical analysis and are presented in (Figure 3). Error bars indicate standard error (SE) of the mean. Racemic 1-octen-3-ol inhibited growth at 5 ppm while 5 ppm

![Figure 2. Pseudogymnoascus destructans mycelial plugs after 3 weeks at 15°C on potato dextrose agar in split Petri plates.](image)

![Figure 3. Growth in cm of mycelial plugs of Pseudogymnoascus destructans exposed to 5, 10, 50 or 100 ppm racemic 1-octen-3-ol or 1-hexanol.](image)
1-hexanol did not affect growth and is similar to that of the control (Figure 3 (a)). There was no measurable growth of mycelia in the 10, 50, and 100 ppm 1-octen-3-ol treatments (Figure 3 (b-d)); 100 ppm 1-hexanol inhibited but did not prevent mycelial growth (Figure 3 (d)).

Since 1-octen-3-ol was more effective than 1-hexanol in inhibiting *P. destructans*, it was further studied to determine the fungicidal level of this compound. To make this determination, the entire mycelial plug, which had been exposed for 3 weeks to 10, 50, or 100 ppm 1-octen-3-ol, was transferred to fresh PDA medium and incubated in ambient air at 15°C. At 10 ppm treatment, mycelial growth resumed after 7 days incubation (Figure 4 (b)). Mycelial plugs exposed to 50 and 100 ppm did not show any hyphal growth and therefore these concentrations appear to be fungicidal (Figure 4 (c, d)). In a follow-up study in which the mycelial plugs were exposed to either 50 or 100 ppm 1-octen-3-ol for 3 weeks before subculturing, there was no mycelial growth even after 2 months incubation.

**Discussion**

There is a need to find methods to control *P. destructans* that do not harm cave ecosystems. Fumigation of bat hibernacula with natural volatile compounds that have GRAS status might be a possible control measure in inhibiting *P. destructans*. In our study, we selected the six carbon alcohol, 1 hexanol, that has been shown to inhibit the growth of the grey mould that rots fruits such as grapes and strawberries (Archbold et al. 1997) and the eight carbon alcohol 1-octen-3-ol that has been used to control a fungal disease of cultivated mushrooms (Berendsen et al. 2013) and affects spore germination and formation in *Agaricus bisporus*, *Aspergillus nidulans* and *Penicillium paneum* (Chitarra et al. 2005; Noble et al. 2009; Herrero-Garcia et al. 2011) In our laboratory, we previously had shown that both volatiles were able to inhibit the growth of two common species of filamentous fungi, *Aspergillus niger* and *Penicillium chrysogenum*, and that 1-octen-3-ol was more effective than 1-hexanol (Yin et al. 2015). However, to our knowledge, this is the first time these volatile alcohols have been tested against a fungal pathogen that causes disease in animals. We showed that vapours of 1-octen-3-ol were more effective in reducing the growth of *P. destructans* than 1-hexanol at all concentrations tested (5, 10, 50 and 100 ppm). Since 1-octen-3-ol is a GRAS compound that already is approved by the US Environmental Protect Agency to be used as a lure for mosquitoes (EPA 2015a, 2015b), it therefore is a promising candidate for use in the control of WNS. Reservoirs of *P. destructans* are present year round in bat hibernacula (Lorch et al. 2013). Thus, caves could be fumigated when bats are not present, thereby effectively killing or lowering the level of *P. destructans*. Maslo et al. (2015) have shown that even an increase in survival of 6% would be enough for persisting populations of little brown bats to revert to positive growth. However, it is important to point out that despite the EPA and FDA clearance for 1-octen-3-ol, there are indications that this compound can be toxic to human embryonic stem cells (Inamdar et al. 2012) and that it also is neurotoxic to *Drosophila melanogaster* (Inamdar et al. 2013). Before any field applications involving fumigation with this VOC, controlled studies will need to be done to ensure that it would not adversely affect cave ecology. Furthermore, to deploy 1-octen-3-ol in caves, it will be necessary to develop suitable methods for slow release of this VOC and we are presently initiating studies in this area. In addition, we are continuing our research to determine the minimum concentration of the VOC required to act as a fungicide on both mycelia and spores. Finally,
we are scaling up our exposure studies using a large environmental chamber as a model system for bat hibernacula.

**Conclusion**

In summary, the vapours of two natural alcohols, previously shown to inhibit the growth of several plant pathogenic fungi, are capable of slowing or preventing the growth of a fungal pathogen of animals. Our studies show that under laboratory conditions, exposure to 5, 10, 50 and 100 ppm of volatile-phase 1-octen-3-ol is effective in reducing the growth of *P. destructans*; that concentrations of 5 and 10 ppm of 1-octen-3-ol are fungistatic and that concentrations of 50 and 100 ppm are fungicidal.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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