Mycopathologia (2015) 180:325–332
DOI 10.1007/s11046-015-9921-2

# Effect of Trans, Trans-Farnesol on *Pseudogymnoascus destructans* and Several Closely Related Species

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Received: 3 February 2015 / Accepted: 30 June 2015 / Published online: 11 July 2015


**Abstract** Bat white-nose syndrome, caused by the psychrophilic fungus *Pseudogymnoascus destructans*, has dramatically reduced the populations of many hibernating North American bat species. The search for effective biological control agents targeting *P. destructans* is of great importance. We report that the sesquiterpene trans, trans-farnesol, which is also a *Candida albicans* quorum sensing compound, prevented in vitro conidial germination for at least 14 days and inhibited growth of preexisting hyphae of five *P. destructans* isolates in filtered potato dextrose broth at 10 °C. Depending on the inoculation concentrations, both spore and hyphal inhibition occurred upon exposure to concentrations as low as 15–20 μM trans, trans-farnesol. In contrast, most North American *Pseudogymnoascus* isolates were more tolerant to the exposure of trans, trans-farnesol. Our results suggest that some *Candida* isolates may have the potential to inhibit the growth of *P. destructans* and that the sesquiterpene trans, trans-farnesol has the potential to be utilized as a biological control agent.

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**Keywords** Biological control · *Candida albicans* quorum sensing compound · Sesquiterpene · White-nose syndrome

## Introduction

Numerous bat (order Chiroptera) populations in the Northeastern USA and Canada have recently been decimated due to the introduction of *Pseudogymnoascus (Geomyces) destructans* (Blehert & Gargas) Minnis & D.L. Lindner. *Pseudogymnoascus destructans*, a psychrophilic fungus, is the etiological agent of bat white-nose syndrome and is thought to originate from Europe [1]. Although there are no present-day large-scale bat mortalities associated with *P. destructans* infections in Europe [2], current research demonstrated that *P. destructans* is virulent to European bats under natural conditions [3]. This research also suggests that the difference between the intercontinental bat mortality is not due to the pathogen but due to an unknown interplay between host, agent, and environment [3]. Unfortunately, *P. destructans* can survive within caves and mines no longer occupied by infected bats [4] as a saprophyte [5, 6], prolonging the length of time that a cave or mine will remain infected.

Currently, the application of broad-spectrum antifungal or biocidal compounds to combat *P. destructans* has not been adopted because caves harbor a unique assemblage of bacteria, fungi, and insects. Recent publications have demonstrated that there are

several chemical compounds capable of limiting the growth and/or conidial germination of *P. destructans*. Of known antifungal compounds, *P. destructans* was shown to be sensitive to amphotericin B, fluconazole, itraconazole, ketoconazole, and voriconazole [7]. Additionally, several bacterial-derived volatile compounds (e.g., benzaldehyde, benzothiazole, decanal, and nonanal) have been shown to inhibit both conidial germination and hyphal growth [8]. Recent research has identified several *Pseudomonas* spp. capable of inhibiting *P. destructans* [9], while other researchers are evaluating *Epicoccum nigrum* Link as a potential biocontrol [10]. Surprisingly, there is limited published research on identifying in situ cave/bat-associated bacteria/fungi, which could potentially be utilized as *P. destructans* biocontrols.

The object of this research was to investigate potential biocontrol agents from fungal species and strains coexisting with *P. destructans* within the cave environment. *Candida*, a common yeast in bat caves in the eastern USA [4], produces a quorum sensing compound trans, trans-farnesol (*tt*-farnesol) which has previously been shown to be antimicrobial against both bacterial and fungal pathogens [11–14]. Specifically, *tt*-farnesol has been implicated in the inhibition of ergosterol [14, 15], similar to the antifungal activity of the aforementioned antifungal compounds such as fluconazole, itraconazole, and voriconazole. Our research goals were to: (1) determine whether *tt*-farnesol inhibits the growth of *P. destructans*, (2) determine whether inhibition occurs at concentrations found in nature, and (3) examine the effects of *tt*-farnesol on other North American *Pseudogymnoascus* species.

## Materials and Methods

### Isolates and Culture Conditions

Isolates examined in this study were previously used in recent research [16–18] and were obtained from the Center for Forest Mycology Research, directly from other researchers, or generated in our laboratory. *P. destructans* isolates used in this study were maintained on Sabouraud's dextrose agar (Difco, Sigma-Aldrich) amended with 250 mg $L^{-1}$ L-cysteine (Sigma-Aldrich), which promotes conidial formation (unpub. data), while the other *Pseudogymnoascus* isolates were maintained on non-amended Sabouraud's dextrose agar. For consistency, all isolates were subcultured to non-amended Sabouraud's dextrose agar plates and allowed to grow for at least 15 days prior to obtaining assay inoculum. All isolates were maintained between 7 and 10 °C under 24-h darkness.

### Qualitative Evaluation of *tt*-farnesol on *P. destructans* Growth, Conidial Germination, and Inoculation Concentration

The qualitative inhibitory assays examined five *P. destructans* isolates from various geographical regions: three from North American [ILLS69284 (Illinois), MYA-4855 (New York, Type culture), and ILLS69283 (Pennsylvania)] and two from Europe [CFMR2498 (Slovakia) and CFMR4129 (Czech Republic)]. In order to dissolve *tt*-farnesol (96 %, Sigma-Aldrich) in liquid, it was added to methanol to make a 10 mM stock solution, filter sterilized (0.2 μm), and stored at −20 °C under desiccation until needed [19]. The *tt*-farnesol inhibitory assay was conducted at seven different *tt*-farnesol concentrations (5, 10, 15, 20, 50, 100, and 300 μM) in 125-ml Erlenmeyer flasks containing a total volume of 26 ml. The basal medium was potato dextrose broth (pH 5.6) (Difco) vacuum filtered through two layers of Whatman #1 filter paper prior to sterilization. The inoculum for each assay consisted of a combination of conidia and hyphal fragments suspended in filter-sterilized potato dextrose broth providing a final concentration of $1.1 \times 10^4$ conidia/hyphal fragment $ml^{-1}$. To each inoculated flask, an appropriate amount of the 10 mM *tt*-farnesol stock solution was added using a micropipette to produce each of the aforementioned *tt*-farnesol concentrations. Control flasks were identical to the assay flasks except the targeted amounts of 10 mM *tt*-farnesol stock solution were replaced with 100 % methanol (0.2 μm filter sterilized). All flasks were covered with sterile autoclaved aluminum foil, sealed with parafilm, and placed on a shake culture (100 rpm) at 10 °C, under 24-h darkness and visually monitored from day 3–14 post-inoculation. The inoculum composition (both conidia and hyphal fragments) was chosen based on potential modes of transmission through direct contact of infected bats to non-infected bats [20], and the final inoculation concentration was chosen to limit clumping due to the prolonged assay period [21]. Inoculation

concentration was determined using an improved Neubauer hemocytometer. Glassware was acid washed (6 M HCl) for a minimum of 6 h, rinsed with distilled water and autoclaved twice prior to use. All assays were replicated three times with two replicates each time at each concentration ($n = 6$ per *tt*-farnesol concentration).

To elucidate the physiological state of conidia in assays where no hyphal growth and conidial germination were visually noted, a 300-µl aliquot of 14-day post-inoculated *tt*-farnesol assay medium was placed in a 1.5-ml centrifuge tube, centrifuged at 2500 rpm for 5 min, and the resulting supernatant was removed, and the pellet was washed with filter-sterilized potato dextrose broth. A second centrifugation step (same as above) was performed; the wash solution was removed, and an additional 300 µl of filter-sterilized potato dextrose broth was added to resuspend the pellet, and the entire contents were lawn plated on 90 mm Sabouraud's dextrose agar plates, wrapped with parafilm, incubated at 7 °C, and monitored for conidial germination. To determine the importance of inoculation concentration, the initial inoculum ($1.1 \times 10^4$ ml$^{-1}$) was diluted with sterilized distilled water to one half, one-fourth, and one-eighth of the original concentration and added to the lowest *tt*-farnesol concentration where no growth was visually noted.

## Quantitative Evaluation of *tt*-farnesol on Pseudogymnoascus Species

To quantify the effects of *tt*-farnesol on fungal growth, we utilized a microplate reader because culture absorbance has been shown to correlate with dry fungal weight regardless of inoculum type (conidia or mycelial fragments) [22]. We examined 10 North American *Pseudogymnoascus* isolates [four isolated from bats (LJ96, LJ103, LJ129, and LJ177) and six isolated from cave soils (2NH11, 5NY06, 5NY09, 10NY10, 17WV04, and 18VA12)] as well as two *P. destructans* isolates [MYA-4855 and CFMR4129 (clade F)]. Isolates LJ96, LJ103, LJ177, and 10NY10 are representatives of clade A; isolate 17WV04 is a representative of clade B; 5NY6 and 5NY9 are representatives of the *P. roseus* complex (clade G); isolate 18VA12 is a representative of clade H; and LJ129 and 2NH11 are representatives of clade L [23]. The quantitative *tt*-farnesol assays were conducted over the concentration range of 5–50 µM, in increments of 5 µM. Negative controls (no fungal inoculum), positive controls (inoculum + potato dextrose broth only), and methanol controls (inoculum + media + methanol) were included at each concentration to isolate the effect of *tt*-farnesol on fungal growth. The quantitative assays were conducted in standard 96-well plates with a total volume of 200 µl (per well), covered with microtiter plate sealing film and incubated (stationary) at 14 °C for 10 days at which time growth was quantified by absorbance at 595 nm [22] using a ELx808 IU ultra microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The targeted inoculation concentration was $2 \times 10^3 \pm 3 \times 10^2$ conidia/hyphal fragments. All assays were replicated twice with four replicates each time ($n = 8$ for each *tt*-farnesol concentration).

## Analysis

### *Qualitative Evaluation*

Hyphal growth and conidial germination were assayed by both visual and microscopic evaluation. After inoculation, flasks were visually monitored for up to 14 days for the presence of fungal growth as indicated by the filtered medium becoming opaque or by the visualization of fungal colonies. When fungal growth was visualized, aliquots from each flask were antiseptically removed for microscopic evaluation. Microscopic evaluation consisted of looking at the entire slide for conidial germination, hyphal growth, and hyphal morphology. The microscopic evaluation was conducted using an Olympus SZX12 or an Olympus BX51 microscope with differential interference contrast (DIC) equipped with an Olympus QColor 3 digital camera. Images were processed in Adobe Photoshop 7.0. Macroscopic photographs were taken with a Cannon EOS 50 Mark II or a Sony DSC-W200.

### *Quantitative Evaluation*

Absorbance values of control, methanol, and *tt*-farnesol assay wells were corrected for the medium's baseline absorbance (negative control) and compared at each concentration using one-way ANOVA and Dunnett's multiple comparison test using GraphPad Prism for Windows version 6.05. Data were log-transformed to meet normality criteria for the model.

Growth was considered inhibited when *tt*-farnesol (and methanol control) absorbance values were statistically lower than positive control (inoculum + potato dextrose broth) growth ($\alpha = 0.05$).

## Results

### Qualitative Evaluation of *tt*-farnesol on *P. destructans* Growth, Conidial Germination, and Inoculation Concentration

*Pseudogymnoascus destructans* demonstrated conidial germination and hyphal growth in all *tt*-farnesol assay controls (inoculum + methanol) $\leq$100 μM, and no germination or growth was visualized in the 300 μM assay control by day 14, indicating methanol alone only prevented growth at the highest concentration. No noticeable growth was observed in any *tt*-farnesol assay containing $\geq$20 μM *tt*-farnesol with an initial inoculum concentration of $1.1 \times 10^4$ ml$^{-1}$ (Fig. 1a). In contrast to the 20 μM assay methanol control, the observed preexisting hyphal fragments in the 20 μM *tt*-farnesol assays appeared to be evacuated or contained only patches of cytoplasm by day 14 post-inoculation (Fig. 1b–c, respectively). Conidia and hyphal fragments exposed to 50 μM for 14 days were capable of germinating within 14 days at 7 °C. At lower *tt*-farnesol concentrations (15 μM), growth inhibition occurred when the original inoculum concentration was diluted by one half or less (Fig. 1d).

### Quantitative Evaluation of *tt*-farnesol on Pseudogymnoascus Species

Three consistent quantitative patterns appeared among fungal isolates: (1) Some isolates were tolerant to an increase in methanol concentration but not tolerant to an increase in *tt*-farnesol concentration (Fig. 1e); (2) some isolates exhibited decreased growth as concentrations increased in both *tt*-farnesol and methanol; and (3) some isolates were unaffected by the concentration of both methanol and *tt*-farnesol (Fig. 2a–c). The only clade consistent pattern was observed in clade F, the *P. destructans* strains. Isolate MYA4855 (*P. destructans* type) growth was greatly inhibited in the 30 μM *tt*-farnesol assay ($p < 0.001$), and only one other *Pseudogymnoascus* isolate exhibited similar sensitivity (10NY10) at this *tt*-farnesol concentration (Fig. 2b). Additionally, both *P. destructans* isolates were inhibited in the 50 μM *tt*-farnesol assay ($p < 0.001$ and $p = 0.0015$, respectively).

## Discussion

Our qualitative and quantitative results provide evidence that exogenous applications of *tt*-farnesol would likely reduce the severity of *P. destructans*-related infection but not eradicate the foreign fungus altogether from bats. The quantitative data also suggest that the effects of exogenous *tt*-farnesol on other *Pseudogymnoascus* species will be strain dependent, indicating that the ecological impact will vary depending on local community composition. Although preexisting hyphal fragments of *P. destructans* isolates in the *tt*-farnesol assays at concentrations $\geq$15 μM *tt*-farnesol appeared to be evacuated or contained only patches of cytoplasm by day 14 post-inoculation, further research is needed to determine the exact physical state of those hyphae (inhibition or apoptosis). Our results are consistent with *tt*-farnesol concentrations required to inhibit the growth of other fungi [12, 24].

Trans, trans-farnesol is a bioactive, lipophilic sesquiterpene [25, 26] excreted by *Candida albicans* and several other cave-dwelling *Candida* species under aerobic conditions. The concentration of excreted *tt*-farnesol reportedly varies between species and within species. Isolates of *C. albicans* were shown to produce between 13.7 and 58.5 μM, while isolates of *C. dubliniensis* were shown to produce between 6 and 17.5 μM [11]. Trans, trans-farnesol is particularly interesting because it has been demonstrated to act antagonistically against other bacteria and fungi. For instance, the filtrate of *C. albicans* was shown to inhibit the growth of the dermatophyte *Trichophyton rubrum* [27]. In addition, the application of exogenous *tt*-farnesol has been shown to cause apoptotic-like programmed cell death in a number of fungal species including *Aspergillus nidulans*, *Fusarium graminearum*, *Penicillium expansum,* and *Saccharomyces cerevisiae* [12, 28–30]. Mechanistically, *tt*-farnesol has been shown in several fungi to cause apoptosis through an increased level of intracellular reactive oxygen molecules and nuclear collapse (nuclear condensation) [29]. In addition, *tt*-farnesol has been shown to inhibit the synthesis of ergosterol [14].

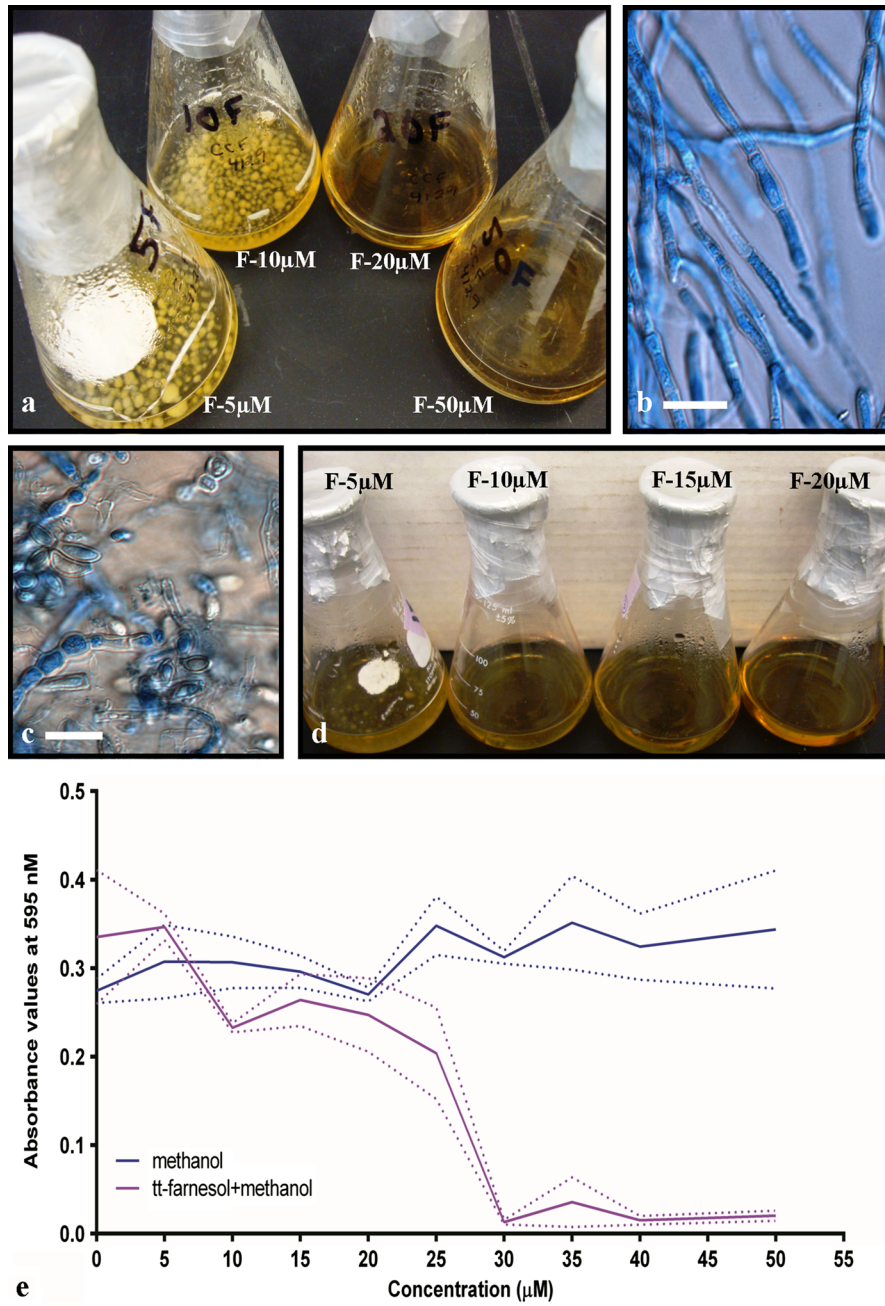


**Fig. 1** Effects of *tt*-farnesol on *P. destructans*. **a** Hyphal growth/conidial germination in *tt*-farnesol inhibitory assay. Note reduced visible hyphal growth between 10 and 20 µM. **b** Hyphal growth 14 days in 20 µM *tt*-farnesol inhibitory assay methanol control. **c** Preexisting hyphae/conidia 14 days under 20 µM *tt*-farnesol inhibitory assay. Note numerous evacuated hyphae and lack of conidial germination. **d** Hyphal growth/conidial germination inhibition at reduced inoculation concentration ($5.5 \times 10^3 \pm 2.5 \times 10^2$ ml$^{-1}$) at day 14 post-inoculation. **e** Absorbance values for *P. destructans* isolate MYA4855, indicating inhibition above 30 µM *tt*-farnesol. Solid line indicates average of $N = 4$. *Dotted lines* represent standard error of the mean. Concentration indicates the concentration of *tt*-farnesol, not the concentration of methanol. *Scale bars*: 10 µm

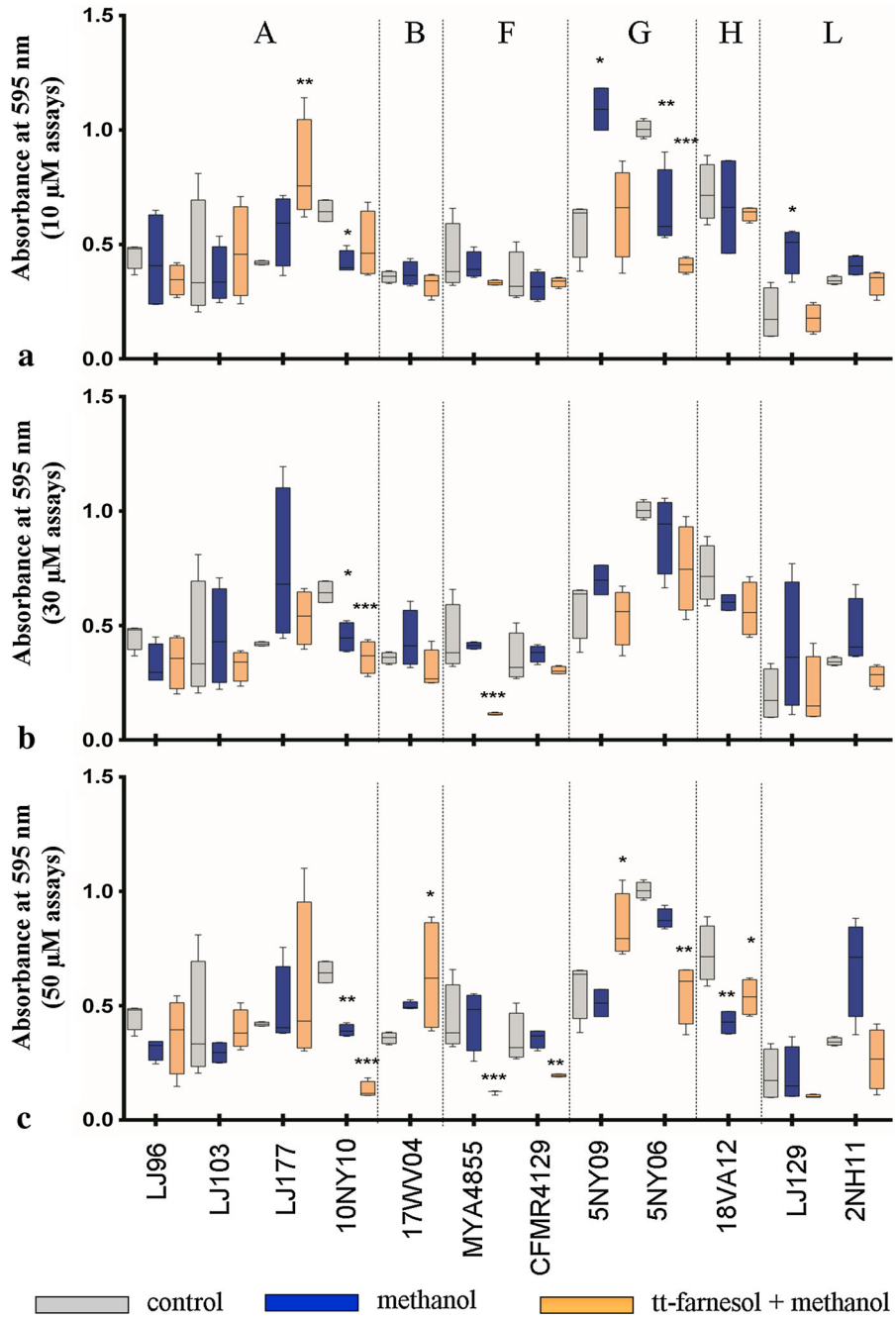


**Fig. 2** Pseudogymnoascus isolates growth under filter-sterilized PDB, *tt*-farnesol, and methanol control assay media. Absorbance values at 10 days post-inoculation for **a** 10 µM, **b** 30 µM, and **c** 50 µM *tt*-farnesol assays. Each data point represents $N = 4$. The letters *A*, *B*, *F*, *G*, *H*, and *L* represent the clade. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Therefore, we believe *tt*-farnesol to be a good candidate for *P. destructans* control within the cave environment since *P. destructans* was the most sensitive to the antagonistic effects of *tt*-farnesol of the assayed *Pseudogymnoascus* species in this study.

Other factors supporting its potential use are that even though *tt*-farnesol is lipophilic indicating that it would favor host cell membrane localization [26], exogenous *tt*-farnesol had negligible toxic effects when administered to mice [31] and *tt*-farnesol oxidizes and becomes biologically inactivate when it has prolonged exposure to oxygen [11]. This fact is encouraging since it suggests that applications of exogenous *tt*-farnesol (20–50 µM) would be short

lived, reducing the negative impact on native fungal and bacterial species. More importantly, our results point to the possibility that certain psychrophilic cave/bat-associated *Candida* isolates may have the potential to protect bats or limit the growth of *P. destructans* on bats. Currently, there is no published research on the levels of *tt*-farnesol produced by psychrophilic cave/bat-associated *Candida* isolates. In addition, levels of *tt*-farnesol produced by different *Candida* species vary significantly, and the levels of *tt*-farnesol produced between *C. albicans* (the most studied species) strains also were shown to vary significantly [11]. Furthermore, there are *C. albicans* strains capable of naturally producing a greater concentration of *tt*-farnesol than required to inhibit *P. destructans* hyphal growth and conidial germination [11]. Future research should explore direct interactions between *Candida* species and *P. destructans* to determine whether and how management of cave fungal communities could be leveraged to lessen or prevent infection of *P. destructans* in bats.

In conclusion, this study has shown that *P. destructans* conidial germination and hyphal growth were inhibited in vitro at a biologically produced concentration of *tt*-farnesol. We also demonstrated that the majority of the assayed North American *Pseudogymnoascus* isolates were more resistant to the antagonistic effects of *tt*-farnesol than *P. destructans*. *In vivo* research utilizing exogenous *tt*-farnesol or high *tt*-farnesol producing *C. albicans* isolates to control *P. destructans* on bats is needed to ascertain how *tt*-farnesol will affect other cave/bat-associated fungal genera and to ascertain bat tolerance to exogenous *tt*-farnesol. Lastly, we believe that North American cave/bat-associated *Candida* species should be evaluated for their levels of *tt*-farnesol production in an effort to ascertain whether any native bat-associated *Candida* species could be utilized as a natural *P. destructans* biological control agent.

**Acknowledgments** The authors wish to thank Andrea Porres-Alfaro, Dan Lindner, and Barrie Overton for providing isolates used in this study and Elizabeth Bach for technical support. The authors also wish to thank two anonymous reviewers who offered helpful insights to a previous version of this manuscript. This study was funded through awards given by the Illinois Department of Natural Resources State Wildlife Grants Program (Project Number T-78-R-1) and the Section 6 Endangered and Threatened Species Program (Project Number E-54-R-1) to the Illinois Natural History Survey.

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