Evaluating Volatile Organic Compounds for Contact-Independent Antagonism of Pseudogymnoascus destructans

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INTRODUCTION

Many fungi-producing VOCs exhibit antimicrobial activity (Mitchell et al., 2010). The VOC B23 is an isolate of the endophytic fungus Muscodor crispus found in the stem tissues of Ananas ananassoides, wild pineapple of the Belivian Amazon Basin (Mitchell et al., 2008). The B23 isolate of M. crispus exhibits antimicrobial effects against a variety of pathogens including fungi, bacteria, and oomycetes (Krajajen et al., 2012). The components of B23 are also on the list of US Food and Drug Administration’s GRAS list of harmless substances allowing for the possibility of clinical applications in animals (Krajajen et al., 2012). Decanal, a bacterially produced VOC, also exhibits antimicrobial activity and has previously shown to inhibit P. destructans mycelial growth (Cornelson et al., 2014). This experiment explores the inhibitory effects of VOCs B23 and decanal on P. destructans mycelial growth by quantifying the growth of P. destructans mycelia exposed to various concentrations of pure B23 and an equimolar combination of B23/decanal and an equimolar combination of B23/decanal. Measurements of the mycelial plugs were taken throughout the experiment to determine which VOC, if any, had the greatest inhibitory effect. The combination of B23 and decanal at higher concentrations revealed that the synergistic activity of the VOCs may be effective at mitigating the impact of WNS on impacted bat species. To further investigate the inhibitory effects of B23 and decanal on P. destructans growth, a slide agar overlay experiment was performed. The slide agar overlay experiment analyzed the inhibitory effects of VOCs on spore germination by exposing P. destructans conidia to various concentrations of pure B23 and an equimolar combination of B23/decanal and then comparing germination events between the various concentrations of VOCs and the control. The results of this experiment further confirm B23 and decanal to be effective inhibitors of P. destructans due to the level of inhibition exhibited in VOC-exposed mycelia compared to the control containing mycelia with no VOCs. The experiment also further supports the idea of potential synergistic interactions between B23 and decanal to increase inhibitory efficacy.

METHODS

Inhibition Assay

A small hole (~5 mm in diameter) was cut out of a 35 mm dextrose agar (SDA) Petri plate using a transfer tube (Thermo-Fisher Scientific) and the Petri lids were discarded. A circular P. destructans mycelial plug of ~5mm in diameter was cut from a confluent lawn using a transfer tube. The mycelial plug was placed into the hole cut out of the agar using the same transfer tube. Aluminum foil wells were made to hold sterile disks and each well was taped to the bottom of six large 150 mm Petri plates which each contained three inoculated P. destructans SDA plates. Sterile disks were cut into fourths and individually placed in aluminum foil wells. Aliquots of pure B23 were pipetted onto individual sterile disks in three large Petri plates as follows: 0.82 µL, 1.64 µL, and 3.28 µL. Aliquots of B23 and decanal were pipetted onto individual sterile disks in three separate large Petri plates as follows: 0.41 µL B23 & 0.80 µL decanal, 0.82 µL B23 & 1.60 µL decanal, and 1.64 µL B23 & 3.19 µL decanal. The large Petri plates were then sealed with parafilm (Bemis, Neenah, WI) while still liquified. The same method of inoculation was used to create the control plate without exposure to VOCs. The Petri plates were then incubated at 25 °C for a total of thirteen days.

RESULTS

The VOCs B23 and decanal were successful in inhibiting mycelial growth. Although both compounds seemed to succeed in inhibiting mycelial growth, a synergistic interaction of B23 and decanal displayed the most inhibitory effects. The size of the mycelial plugs were taken throughout the experiment to determine which VOC, if any, had the greatest inhibitory effect. The combination of B23 and decanal at higher concentrations revealed that the synergistic activity of the VOCs may be effective at mitigating the impact of WNS on impacted bat species. To further investigate the inhibitory effects of B23 and decanal on P. destructans growth, a slide agar overlay experiment was performed. The slide agar overlay experiment analyzed the inhibitory effects of VOCs on spore germination by exposing P. destructans conidia to various concentrations of pure B23 and an equimolar combination of B23/decanal and then comparing germination events between the various concentrations of VOCs and the control. The results of this experiment further confirm B23 and decanal to be effective inhibitors of P. destructans due to the level of inhibition exhibited in VOC-exposed mycelia compared to the control containing mycelia with no VOCs. The experiment also further supports the idea of potential synergistic interactions between B23 and decanal to increase inhibitory efficacy.

Inhibition of spore germination

Fresh SDA was made and while still liquified, 400 µL was pipetted onto sterile microscope slides and allowed to solidify. P. destructans conidia spore stock (2.52x10⁶ cells mL⁻¹) was pipetted onto the same slides (100 µL each) and agitated, or spread, around the slide with an inoculation loop. Aluminum foil wells were made to hold sterile disks and each well was taped to the bottom of six large 150 mm Petri plates. Sterile disks were cut into fourths and individually placed in aluminum foil wells. Aliquots of pure B23 were pipetted onto individual sterile disks in three large Petri plates as follows: 0.82 µL, 1.64 µL, and 3.28 µL. Aliquots of B23 and decanal were pipetted onto individual sterile disks in three separate large Petri plates as follows: 0.41 µL B23 & 0.80 µL decanal, 0.82 µL B23 & 1.60 µL decanal, and 1.64 µL B23 & 3.19 µL decanal. The large Petri plates were then sealed with parafilm (Bemis, Neenah, WI) while still liquified. A control was made by sealing three inoculated microscope slides in a large Petri plate without exposure to VOCs. The plates were incubated at 15 °C until germination was observed in the control (5 days post inoculation). The slides were analyzed under a microscope to determine relative inhibition of each VOC on spore germination. It was determined that inhibition was occurring if the length of the germ tube was less than half the length of the spore.

Figure 1 P. destructans mycelial growth at A) day 1 of experiment and B) day 8 of experiment showing growth pattern between control, 2000 ppmv of B23, and 2000 ppmv of B23 and decanal together at equimolar ratios.

Quantification of Inhibition

Photographs of the plates were taken every 2 to 3 days throughout the entirety of the experiment. The camera was placed on top of a stand which contained a transparent surface to provide a consistent distance between the camera and the plate for each measurement. The photographs were processed through GNU Image Manipulation Program (GIMP), a freely distributed software capable of measuring pixels in an area of a photograph (Cornelson et al., 2014). A photograph of a ruler was first analyzed in GIMP to determine the pixel-to-millimeter ratio. This measurement was taken for data analysis as a means to convert pixels to cm. The ‘fuzzy select’ tool in GIMP was used to select the mycelial area of the photograph, which was used to quantify the number of pixels contained in each individual mycelial plug. The data was analyzed using excel to determine the percent inhibition of each VOC on P. destructans.

Figure 2 Growth of mycelial plugs over 13 day time frame

Figure 3 Germination patterns of conidia in A) control B) 500 ppmv B23 C) 1000 ppmv B23, D) 2000ppmv B23, E) 500 ppmv B23, and F) 500ppmv B23 decanal, and G) 2000 ppmv B23, decanal

Upon observing the slides 5 days post inoculation, it was found that the VOCs successfully inhibited germination of P. destructans spores. The control slide displayed ~100% germination, while the VOC exposed slides displayed significantly less germination (Figure 3). Although the VOCs inhibited germination overall, the higher concentrations of VOCs displayed greater inhibitory effects than the lower concentrations. The slide exposed to 2000 ppmv B23, decanal displayed ~10% germination, further supporting the potential of synergistic interactions between VOCs to effectively mitigate the impact of WNS on impacted bat species.

CONCLUSION

It was found that B23 and B23/decanal together at equimolar ratios have inhibitory effects on P. destructans. The inhibitory effects of B23/decanal together at equimolar ratios demonstrate the potential of synergistic interactions between the two to effectively inhibit mycelial elongation and spore germination.