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Improvement of protocols for the screening of biological control agents against white-nose syndrome

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White-nose syndrome, caused by the fungus *Pseudogymnoascus destructans* (*Pd*), has become increasingly prevalent in North America since 2006 and has caused mass mortality in many bat populations. This study focuses on standardizing protocols for efficient *Pd* cultivation, which includes harvesting *Pd* spores from agar plates, culturing *Pd* on both normal and modified culture media and finally, testing chemical agents and cave bacterial isolates against *Pd* for antimicrobial activities. *Pd* cultivation was performed on Sabouraud Dextrose Agar (SDA), Rose Bengal Agar (RBA) and modified RBA-bat tissue media. Only *Pd* grown on RBA supplemented with 0.25% bat tissue had a final colony diameter that was significantly different from *Pd* grown on SDA and could be attributed to the difference in the growth rates. *Pd* lawns grown using both pour- and spread-plate techniques were observed on SDA media. Although the former technique led to slower fungal lawn growth in comparison to the latter, the more uniform lawn produced with the pour-plate technique allowed the size of inhibitory zones to be determined quantitatively. Further, the cultured *Pd* lawns were tested against the chemical antimicrobial agents Nystatin, peroxigard and bleach (10%, 50%, 100%) and cave bacterial isolates using a Kirby Bauer and agar plug diffusion assay. Both bleach (10%) and peroxigard (1.5%) created zones of inhibition in *Pd* lawns, with diameters measuring 25.5 mm and 12.7 mm, respectively, after 24 days of incubation. No antimicrobial activities were observed for the cave bacteria tested against *Pd*. Our findings will allow more efficient and reliable screening of cave bacteria against *Pd*, elucidating biological control agents against white-nose syndrome.

White-nose syndrome (WNS) emerged in North America in 2006, and has now been confirmed in 29 US states and 5 Eastern Canadian provinces (https://www.whitenosesyndrome.org). This bat disease has been devastating for many bat populations, killing over 5.7 million bats and causing 90-100% mortality in some hibernacula. WNS is named for the appearance of bats infected with *Pseudogymnoascus destructans* (*Pd*), the causative fungus, which leads to white growth on bat ears, tails, wings, and muzzles (1, 2). Although the origin of *Pd* remains unknown, it is speculated that it may have been introduced into North America from Europe (3). *Pd* is a psychrophilic fungus that thrives at the temperatures found in bat hibernacula and can degrade collagen, which is believed to allow tissue invasion in the bat host (2, 4). Infection with *Pd* causes more frequent arousal from torpor, leading to the loss of crucial fat reserves and often death of the bat (5). The rapid decline in bats due to WNS has not just ecological, but also economic impacts (6). For example, the role bats play in pest control is estimated to save the agricultural sector at least $3.7 billion/year in North America (7).

Due to the importance of bats, the development of a treatment or control agent for WNS is imperative and the recent case of WNS in Washington, the first confirmed case in Western North America, adds a renewed sense of urgency to the situation (8).

Studies to find control agents for WNS are ongoing, but there are still no treatments that are being used in the field on a large scale. Chemical agents, such as antifungals, fungicides, biocides, and volatile organic compounds originating from bacteria have been tested against *Pd*, with many able to inhibit the growth of spores and mycelia (9, 10). However, most of the research has focussed on biological control agents, which are less likely to disrupt the cave ecosystem than the harsh physical and chemical treatments that have been used in caves in the past (11). Some of these potential biological control agents include volatile compounds produced by strain DAP96253 of *Rhodococcus rhodochrous*, extract produced by the fungus

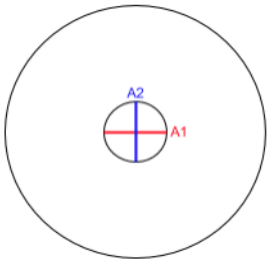
Trichoderma polysporum WPM 39143 isolated from a cave air sample, and cold-pressed, terpeneless orange oil, all of which strongly inhibited the growth of *Pd in vitro* (12-14). The ability to produce secondary metabolites with antimicrobial properties is a well-established feature of many bacteria, and cave bacteria, especially actinomycetes, are known for this ability (15, 16). Therefore, it would be novel to screen bacteria from the cave environment for antagonistic activities against *Pd*. However, growing *Pd* and testing it against diverse antimicrobial agents under laboratory conditions is a challenging process since *Pd* has a very slow growth rate and colonies must be mature to produce adequate numbers of spores.

Our laboratory has continuously worked to elucidate novel antimicrobial agents against bacterial pathogens (Multi Drug Resistant strains) and *Pd*, preferably of biological origin. Therefore, this project intended to optimize a protocol to enhance the cultivation of *Pd* and to test agents, both biological (cave bacterial isolates) and chemical (bleach, peroxiguard, Nystatin) for their anti-*Pd* activities. These findings will not only aid in our own ongoing experiments, but also will benefit other researchers who are working in this field.

MATERIALS AND METHODS

Bat tissue supplemented media preparation. A bat, which had died of natural causes, was obtained frozen from Dr. Cori Lausen, a bat ecologist. This frozen little brown bat (*Myotis californicus*) was sliced in liquid nitrogen and ground into fine powder. Following this, 0.25 g, 0.5 g and 1 g of bat tissue was added to 100 mL of Rose Bengal Agar (RBA) solution to obtain 0.25%, 0.5%, and 1% RBA-bat tissue media respectively, along with the control where no bat tissue was added (0% RBA-bat tissue media). A second control of Sabouraud Dextrose Agar (SDA) media plates were also prepared as per the manufacturer's instructions. All of these media were prepared in triplicates. All the media were autoclaved at 121°C for 15 mins before pouring into Petri plates.

Growth and analysis of *Pd* on bat supplemented media. A piece of SDA-agar media (approximately 7 square mm) containing *P. destructans* M3906-2 culture grown for 2 months at 15°C was excised out and placed into the centre of each media plate (mentioned above). The *P. destructans* M3906-2 strain was procured from Dr. J.P. Xu, McMaster University, Canada. The plates were incubated at 15°C for a period of 27 days. The *Pd* colony growth was noted on the 5th day, since no outgrowth of the *Pd* was visualized for the initial 4 days. After which, the *Pd* colonies were examined every 2-4 days. The growth of *Pd* on each of the plates was quantified by measuring the diameter of the colony both horizontally and vertically with a ruler and taking the average of these two measurements (Fig 1). The colony diameter measurement for each of the media types was considered as the mean of triplicates. The mean colony diameters obtained for each media type were compared by plotting them on an XY-scatter, with standard error shown as error bars. The standard error was calculated for each media type on each day measurements were taken using the descriptive statistics function on Minitab 17. Furthermore, the *Pd* growth rates on each of the media types were calculated by using the formulae given below (Fig 1). To assess whether mean colony diameters for the various media types were significantly different from one another, Minitab®17 (http://www.minitab.com/en-us/products/minitab/) was used to conduct a One-way ANOVA (Analysis of Variance), followed by a Tukey Pairwise Comparison (α = 0.05 for both tests). This was done for the means calculated for the initial day (day 5) and the final day (day 27).



Equation 1: $\frac{A1+A2}{2} = B1$

Equation 2: $\frac{B1+B2+B3}{3} = C$

Equation 3: $\frac{C_{27}-C_5}{27-5} = X\ mm/day$

Figure 1. Measurement methods and formulae used to analyse *Pd* colony growth on SDA and RBA media. For the two concentric circles, the smaller one represents the 'colony' while the larger one is the 'Petri-dish'. A1 is the horizontal colony diameter, A2 is the vertical colony diameter, B# (number refers to replicate) is the average colony diameter for the plate, C is the colony diameter for the media type, C_{27} is the colony diameter for that media type on day 27, C_5 is the colony diameter for that media type on day 5, X is the growth rate of *Pd* on that media type. The denominator of equation 3 gives the number of days over which the diameter was measured.

***Pd* spore isolation and preparation of fungal lawns.** Cultures of *Pd* strain M3906-2 were maintained on SDA plates at 15°C. Spores were isolated from *Pd* cultures using mechanical scraping and filtration through glass wool as previously described (12). The concentration of spores in solution was quantified with a haemocytometer and spore solutions were stored at 4°C. To grow a fungal lawn, 200 µl of spore suspension was plated onto each 90 mm agar plate and spread with a sterile bent glass rod until the solution was evenly spread out over the plate surface (17). The plate was allowed to dry, inverted, and placed at 15°C for incubation.

Kirby Bauer diffusion assay for chemical antimicrobial agents. Ninety microliters of a spore suspension containing 2 x 10^6 spores/mL was spread on each 60 mm SDA plate and allowed to dry. Eight mm Kirby-Bauer discs (Toyo Roshi Kaisha Ltd., Japan) were soaked in the chemical agents, air dried, and laid down in the centre of each SDA plate containing the spread *Pd* spores. Three chemicals; Nystatin (150 µg/ml) (EMD chemicals, Inc. San Diego, USA), Concentrated bleach (100%, 50% and 10%) (London Drugs, Richmond, Canada), and Peroxigard (1.5%) (Bayer, Toronto, Canada), were used in quadruplets for this study. The negative control of sterile water was prepared in duplicates. All of the plates were inverted and incubated at 15°C. The macroscopic morphology of *Pd* growth was recorded and any zones of inhibition were measured with an electronic Vernier calliper (Guangxi China, Mainland) every 2-6 days.

Kirby Bauer and agar plug diffusion assay for antimicrobial testing of bacteria. Previously isolated bacteria from New Brunswick caves (Gomes *et al.*, unpublished data) were randomly chosen to screen for anti-*Pd* activities. Both pour- and spread-plate techniques were employed to seed the SDA media with *Pd* spores. 555 µL of *Pd* spore suspension (approximately 7 x 10^6 spores/mL) was mixed with 120 mL of molten SDA media and was poured into 150 mm plates. Concurrently, the same volume of the spore suspension was spread-plated on SDA media plates. Three plates of each spore-seeding technique were prepared and the same cave bacterial isolates were tested once using each seeding technique. Kirby Bauer discs were soaked with bleach (10%), Peroxigard (1.5%), or sterile water and laid down on the SDA media plates containing *Pd* spores. Simultaneously, agar plugs containing the cave bacteria isolates were excised out from the media plates on which they were cultured, and the agar plugs were laid down in a similar way on the SDA media plates containing *Pd* spores with a bent pick (agar plug diffusion assay). Bleach (10%), Peroxiguard (1.5%) and sterile water were used as positive and negative controls respectively. All of the plates were incubated at 15°C and the macroscopic morphology of *P. destructans* growth was recorded every 1-3 days. Any zones of inhibition observed were measured with an electronic Vernier calliper.

RESULTS

***Pd* growth on bat-supplemented media.** All of the media plates (SDA, RBA, RBA-Bat tissue) exhibited growth of raised white-grey *Pd* colonies and the production of liquid exudate was observed on the surface of the colonies (Fig 2). Pigment secreted into the agar was observed when *Pd* was grown on SDA, but could not be seen in the other media types. Characteristic curved spores attached to the mycelia ends were visible when using light microscopy. The initial (day 5) mean colony diameters measured for 1% 0.5%, 0.25%, and 0% bat tissue-RBA and SDA were 9.5 mm, 8.2 mm, 8.3 mm, 7.8 mm, and 8.0 mm, respectively. When the initial mean colony diameters were compared using a one-way ANOVA, they were found to be significantly different from one another ($F = 5.23$, $P = 0.015$). Using a Tukey pairwise comparison indicated that the differences were between 1% bat tissue-RBA and 0% bat tissue-RBA ($T = 4.08$, $P = 0.015$), as well as SDA and 1% bat tissue-RBA ($T = -3.67$, $P = 0.028$). The mean growth rates for *P. destructans* grown on SDA and RBA were 0.65 mm/day and 0.75 mm/day respectively; whereas, *P. destructans* exhibited mean growth rates of 0.83 mm/day, 0.75 mm/day, and 0.80 mm/day on 0.25%, 0.5%, and 1% bat tissue-RBA respectively (Fig 3). The final mean colony diameters (day 27) measured for 1% 0.5%, and 0.25% bat tissue-RBA were 27.2 mm, 24.7 mm, and 26.5 mm respectively, followed by RBA (24.3 mm) and SDA (22.3 mm). When these means were compared using a one-way ANOVA, they were found to be significantly different from one another ($F = 5.06$, $P = 0.017$). A Tukey pairwise comparison indicated that the differences were between SDA and 0.25% bat tissue-RBA ($T = -3.47$, $P = 0.038$), as well as SDA and 1% bat tissue-RBA ($T = -4.02$, $P = 0.016$).

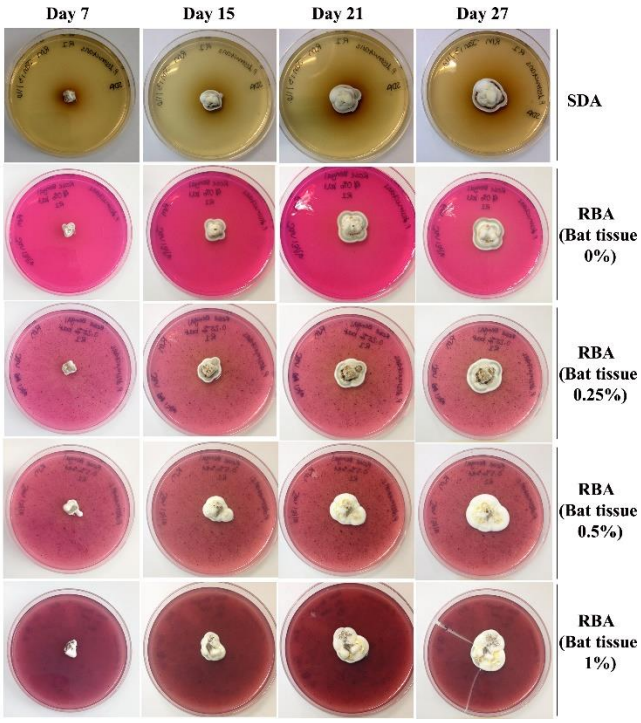


Figure 2. Colony morphology of *Pd* strain M3906-2 was visualized on the SDA and RBA-Bat tissue media for a range of days.

***Pd* lawn growth from a spore suspension.** Concentrations of spores up to 1.7 x 10^7 spores/mL were obtained using the chosen spore isolation method. Plating of isolated spores on SDA media led to the growth of consistent fungal lawns after an average of 10 days (Fig 4). However, plating higher concentrations (1.7 x 10^7 spores/mL) of spores led to fungal lawn growth faster (5 days), in comparison to lower concentrations (9.4 x 10^5 spores/ml) that produced a fungal lawn in 12-14 days.

Kirby Bauer diffusion assay for chemical antimicrobial agents. Four plates exposed to Nystatin exhibited fungal lawn growth covering the entire plate surface after 10 days of incubation, with no zones of inhibition (Fig 5). Plates with 10% bleach had very little *P. destructans* growth for the first 12 days, with zones of inhibition visible from the 18th day of incubation onwards. The average diameters of these zones were approximately 30 mm and decreased to 25.5 mm after the 24th day of growth (Table 1). *Pd* spores exposed to 50% bleach showed minimal growth along the plate periphery after 18 days, exhibiting an inhibitory zone almost the diameter of the plate (60 mm). *Pd* spores exposed to 100% bleach did not produce any growth on the agar plates, indicating that an inhibitory zone could be equal to or larger than the plate diameter. Two of the 4 plates containing *Pd* spores exposed to Peroxigard showed very minimal zones of inhibition after 12 days, but these zones were no longer present after the 19th day of incubation. The remaining 2 plates exhibited zones of

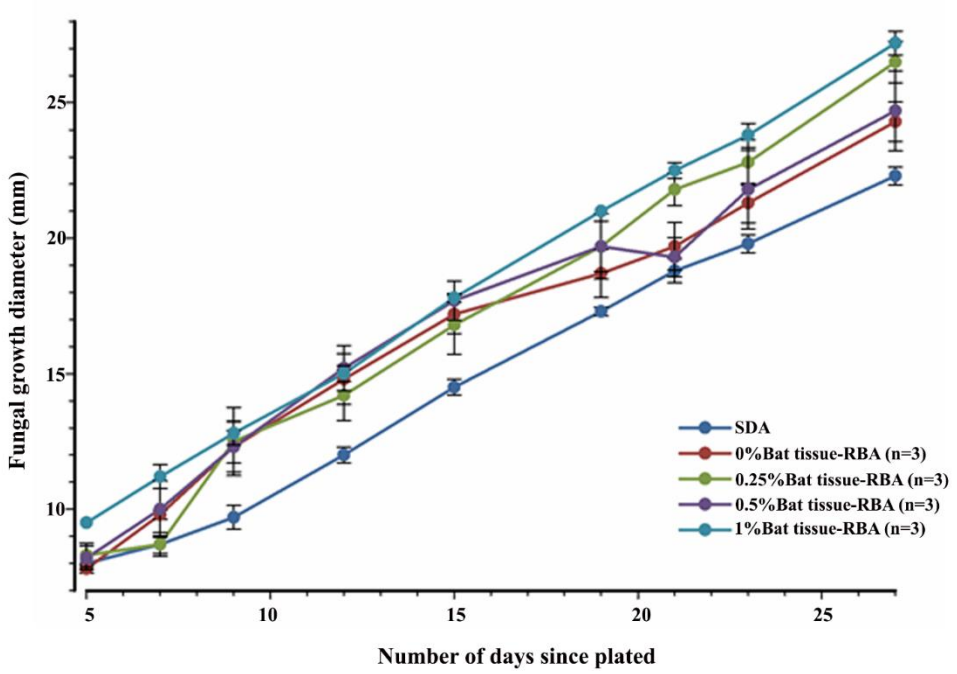


Figure 3. Comparative analysis of *Pd* growth (colony diameter in millimetres) on SDA and Bat tissue (0%-1%) RBA media at various time points (days). Error bars represent the standard error.

inhibition with diameters of approximately 20 mm after the 12th day of growth. However, the zone of inhibition was not retained for one of the plates after the 18th day, and the other plate had a zone diameter of 13.25 mm on the 19th day, which decreased to 12.71 mm after 24 days of incubation. Although the negative control plate exposed to sterile water exhibited no *Pd* growth until the 18th day, it did develop some patchy lawn growth, and the plate was almost completely covered with a fungal lawn on the 19th day of incubation.

Kirby Bauer and agar plug diffusion assay for antimicrobial testing of bacteria. The pour- and spread-plate techniques were employed to seed the spores on the SDA plates. Implementation of the former technique created minimal growth on the plate surface after 6 days, with a consistent lawn visible after 9 days of growth, while the latter displayed uneven lawn growth after 5 days, with the lawn becoming more consistent on the 7th day of growth. The spread-plates had areas with visibly thicker growth than others and a few bare patches were also observed. The agar plug diffusion assay with the cave bacteria did not show any inhibitory activities against *Pd*.

The Kirby Bauer inhibition assay with 10% bleach created inhibitory zones with average diameters measuring 13.19 mm with the pour-plated spores, while the spread-plated spores exhibited zones measuring 33.85 mm in diameter after 7 days of incubation. These zones decreased to 12.18 mm and 30.45 mm, respectively, after the 9th day. The average inhibitory zone diameter observed for peroxigard using the pour-plate technique was 21.67 mm after the 7th day of incubation, which decreased to 19.33 mm after the 9th day of growth. Zones created by peroxigard could not be measured on the plates with spores spread on the agar surface due to fungal contamination (Table 2).

DISCUSSION

The present study provides insight into some of the challenges associated with cultivation of *Pd* and improvements that can be made to methods for screening bacteria from cave soil samples against *Pd*. The initial goal of the study was to determine if the growth rate of *Pd* could be improved to augment the entire screening process. Supplementing the media with different concentrations (0.25-1%) of bat tissue was implemented since a similar study showed that incorporation of bird feathers in the growth media induced the keratinolytic protease activities of *B. pumilus* and *B. cilius* isolated from poultry feathers (18). Our study found that *Pd* grown on SDA and 0.25% bat tissue-RBA began with a similar colony diameter, but had colony diameters that were significantly different from one another after 27 days. This can be attributed to a difference in the growth rate of 0.18 mm/day for *Pd* grown on RBA media supplemented with 0.25% bat tissue in comparison to *Pd* grown on SDA media. *Pd* grown on 1% bat tissue-RBA and SDA also had a significantly different colony diameter after 27 days of

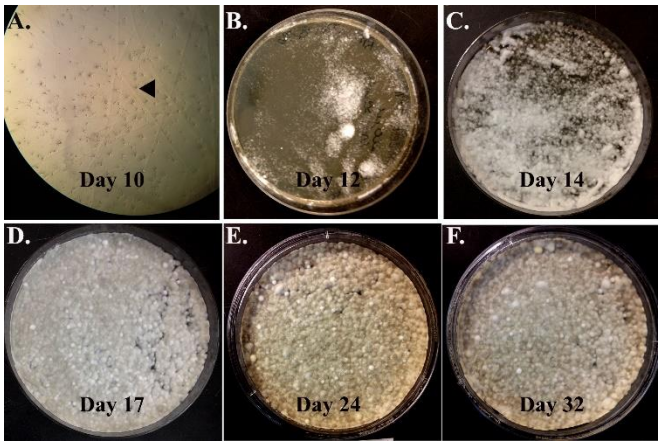


Figure 4. Growth of *Pd* on SDA media from a *Pd* spore suspension. (A) The colonies started to form as indicated by the black arrowhead as observed under a dissecting microscope. (B-F) The formation of the *Pd* lawn (white-grey) as the days progressed.

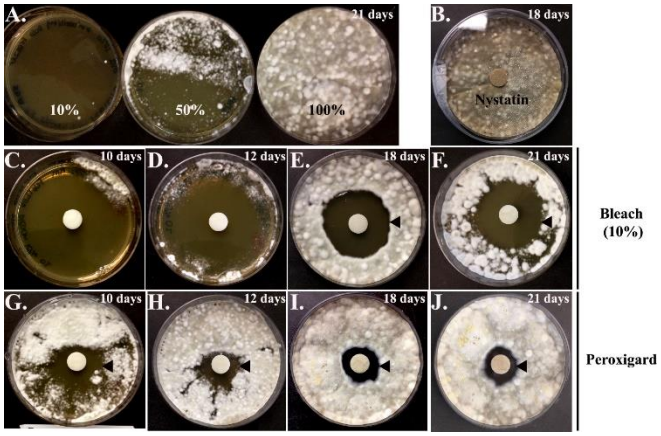


Figure 5. Chemical antimicrobial agents used during the experiment. Incubation time in each of the cases is specified in the right top corner. The arrowheads indicate the zone of inhibition. (A) Growth of the *Pd* lawn from a range of concentrations of *Pd* spore suspension. (B) Incubation of the *Pd* lawn with Nystatin. (C-F) Cultivation of *Pd* spores with 10% Bleach for a range of time points (days). (G-J) Cultivation of *Pd* spores with Peroxigard for a range of time points (days).

growth; however, *Pd* grown on these two media types also had initial colony diameters that were significantly different. This indicates that the difference in the final diameters may not be due to a difference in the growth rate, but could have been due to a difference in the initial inoculum size. Although the addition of bat tissue led to a slight increase in the growth rate of *Pd* compared to the non-supplemented plates, the concentration of bat tissue did not correspond to a proportional increase in the growth rate. Moreover, grinding of bat tissue is a time consuming and laborious process; therefore, this process cannot be recommended for antimicrobial screening of cave bacteria.

Creation of fungal lawns on agar media from *Pd* spores is a common process and therefore, we adopted a technique from a previous study (12) to isolate *Pd* spores. Our study has shown that filtering of the spores through glass wool to prevent unwanted mycelial contamination in the desired spore suspension was very effective for isolating spores of high concentrations. Furthermore, the plating of these spores created consistent fungal lawns that are useful in screening assays, although production of sufficient spore numbers for screening many cave bacteria remains a bottleneck in this study.

The Kirby Bauer diffusion assay for chemical antimicrobial agents had notable results. Nystatin did not produce a zone of inhibition in the *Pd* fungal lawn. Nystatin is an antifungal drug, but it is more commonly used against yeast than against filamentous fungi, which may be why it was ineffective against *Pd* (19). However, a previous study showed that some antifungal drugs, such as amphotericin B, could be used effectively against *Pd* (13). Amphotericin B and Nystatin belong to the same class of antifungal drugs, however, the mode of action of Amphotericin B differs from Nystatin (20), which could explain the difference in activity observed when tested against *Pd*. *Pd* spores exposed to 50% and 100% bleach produced no growth or minimal growth on the plate edge. The lack of growth is likely because these concentrations created zones of inhibition larger than the plate diameter, although this was never confirmed by using larger plates (Nunc® Bioassay Dish, 245 mm x 245 mm x 25 mm) for testing. Exposing *Pd* spores to 10% bleach created zones of inhibition with diameters that decreased from 30 mm after 18 days of growth to 25.5 mm after 24 days of growth. Similar results were observed in another study: *Geomyces pannorum* spores exposed to 10% bleach in a Kirby-Bauer assay produced zones of inhibition with a diameter of 67 mm after 7 days, which decreased to 45 mm in diameter after 30 days (21). Also in this study, *Pd* spores exposed to 10% bleach in a Kirby-Bauer assay did not produce any growth on the plate, suggesting that *Pd* is more susceptible to treatment with 10% bleach than *G. pannorum* (21). All plates exposed to peroxigard produced zones of inhibition after 12 days of growth, but only one of the four plates still had a zone of inhibition after 24 days, which measured 12.7 mm in diameter. In another study, exposing *G. pannorum* spores to 0.3% Hydrogen peroxide created a zone of inhibition with a 12 mm diameter (21). Based on the inhibitory activity, 10% bleach and peroxigard were used in subsequent experiments as positive controls.

Table 1. Diameters of inhibitory zones created when *Pd* was cultured with chemical antimicrobial agents.

Day	Chemical agent	Average diameter of zone of inhibition (mm)	Sample size
19	10% Bleach	28.25	4
19	50% Bleach	≥60	4
19	100% Bleach	≥60	4
19	Peroxigard	13.25	1
19	Peroxigard	0	3
19	Nystatin	0	4
19	Water	0	1
24	10% Bleach	25.51	4
24	Peroxigard	12.71	1

However, 10% bleach seems to cause more prolonged inhibitory activities in comparison to peroxigard, but both may be used for experiments lasting 10-15 days.

Previous studies have employed the spread-plate technique to seed agar plates with spores for the creation of *Pd* lawns and it has been reported that this technique produces distinct results when performing a Kirby-Bauer disc diffusion assay (13, 21). However, the pour-plate technique is used for seeding agar plates when screening cave bacteria against both bacteria and yeast (15); therefore, in this study we wanted to determine if the pour-plate technique is a better option for creating *Pd* lawns. Although the pour-plate technique led to slower growth of *Pd* lawns in comparison to the spread-plate method, more consistent fungal lawn growth was observed in the case of the former. Spreading *Pd* spores on SDA also produced a fungal lawn, but led to patchy and uneven growth due to the uneven spreading of spores. Since our future experiments for screening cave bacteria will include larger plates, the pour-plate technique will be more convenient and consistent for creating uniform fungal lawns.

Table 2. Diameters of *Pd* inhibitory zones from the Kirby Bauer and agar plug diffusion assay for screening cave bacteria.

Day	Test substance	Spore seeding technique	Average diameter of zone of inhibition (mm)	Sample size
7	10% Bleach	Embedded	13.19	2
7	10% Bleach	Spread	33.85	1
7	Peroxigard	Embedded	21.67	2
9	10% Bleach	Embedded	12.18	2
9	10% Bleach	Spread	30.45	2
9	Peroxigard	Embedded	19.33	2

Moreover, in our study we observed that the agar plug diffusion assay to screen bacteria for inhibitory activities against *Pd* is a simple and effective technique. This technique is used extensively in our lab to screen cave bacteria for antagonistic activities against pathogenic microorganisms, such as bacteria and single-celled and filamentous fungi, giving consistent and reliable results (15). However, in our study, none of the cave bacteria tested showed inhibitory activity against *P. destructans,* which is not uncommon when screening such a small sample of bacteria. Both peroxigard and bleach created zones of inhibition, as expected, but inhibitory zones were smaller on the pour-plate SDA media in comparison to the spread-plate SDA media.

The main limitation of the media supplementation experiment is the lack of replicates, making it difficult to definitively conclude if bat tissue supplementation has an impact on the growth rate of *Pd.* Also, as indicated by statistical testing and as can be seen in Figure 3, the initial inoculum size on 1% bat tissue-RBA appears to have been larger than those on the other media types, so a more consistent inoculating method should be used in the future. Additionally, in future studies, a more accurate method of measuring *Pd* colony growth, such as photographing the colonies and using software to convert pixels to millimetres could be used for greater reproducibility and reliability (10). Future studies should also include pipetting a set amount of chemical agents onto the Kirby Bauer discs instead of disc-dipping in the Kirby Bauer disc diffusion assays. This will allow us to spot a known volume and concentration of the chemical used, enhancing the reproducibility of the results. To circumvent the problem of bacterial overgrowth in the Kirby-Bauer assay, bacterial broth cultures can be prepared, centrifuged down to collect the supernatant and this can be spotted onto paper discs for testing. This technique has been used previously for antimicrobial activity screening from environmental samples where bacterial secondary metabolites obtained in the supernatant were tested (22).

In conclusion, despite the difficulties of growing *Pd*, we have improved techniques that could be implemented for our future antimicrobial screening of cave bacteria against *Pd*.

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