



## Morphogenetic Effect of L-cysteine on *Pseudogymnoascus destructans* and Related Species

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### Abstract

The addition of L-cysteine to culture media is shown to cause a morphogenetic effect on *Pseudogymnoascus* (*Geomyces*) *destructans*, the causal agent of bat white-nose syndrome, and on several North American *Pseudogymnoascus* species *in vitro*. Under elevated levels of L-cysteine, seven *P. destructans* isolates from various geographical localities produced yeast-like cells on amended Sabouraud's dextrose agar within 30 days at 7–10 °C. The yeast-like cells appear in semi-solid, black, pigmented masses composed of ellipsoidal to obtuse cells that average 13 × 5.5 µm and demonstrate blastic development easily differentiating them from the typical curved conidia which average 6 × 3 µm. The yeast-like cells were shown to revert to filamentous form when cultured on non-amended Sabouraud's dextrose agar. No yeast-like cells were visualized in four other isolates within the *Pseudogymnoascus roseus* complex or in two additional, distantly-related *Pseudogymnoascus* isolates when exposed to the same elevated levels of L-cysteine. The sensitivity of *P. destructans* to L-cysteine is believed to be a response to the reduced oxidation-reduction potential of the amended medium, but further research is needed to understand the biochemical mechanisms.

**Key words** – Bat white-nose syndrome – yeast-like morphology – oxidation-reduction potential

### Introduction

*Pseudogymnoascus* (*Geomyces*) *destructans* (Blehert & Gargas) Minnis & D.L. Lindner, the causal agent of bat white-nose syndrome, continues to spread across the United States and Canada since its introduction to New York State in 2006 (Blehert et al. 2009, Gargas et al. 2009, Hayes 2012, Warnecke et al. 2012). *Pseudogymnoascus destructans* has been described as a psychrophilic (cold-loving) dermatophyte, which can cause severe skin lesions and cutaneous necrosis in bats during hibernation (Cryan et al. 2010), a time when the bat's internal body temperature is close to ambient temperature (ca. 2–10 °C) and its immune system is reported to be in a suppressed state (Blehert et al. 2009, Meteyer et al. 2009, Hayes 2012). In the infection process, *P. destructans* has been reported to infect hair follicles and both apocrine and sebaceous glands, but unlike most superficial dermatophytes that infect mammals, *P. destructans* has been reported to penetrate living tissues such as bat skin underlying connective tissue. (Blehert et al. 2009, Meteyer et al. 2009, Reichard & Kunz 2009).

The ability of *P. destructans* to infect bat skin underlying connective tissue (living tissue) raises the question of whether *P. destructans* has the potential to produce a yeast-like state since this altered morphological state has been suggested to increase survivability within the host (Thuy et al. 1981). Interestingly, it has previously been documented that other superficial dermatophytes such as *Trichophyton rubrum* and *Microsporum audouinii* cultured on L-cysteine gradient plates were capable of producing a yeast-like morphology (Rippon & Scherr 1959) with an associated invasive ability in test mammals (Rippon & Scherr 1959, Rippon et al. 1965). The mycelial to yeast transformation of the aforementioned superficial dermatophytes was attributed to the ability of L-cysteine to lower the oxidation-reduction potential of the medium (Rippon 1968), which is more akin to the reduced state of living tissue (Rippon et al. 1965). Currently, there is no documentation of a yeast-like morphology for *P. destructans*.

Consequently, the goal of this research was to investigate the effects of elevated L-cysteine on the morphology of *P. destructans*. Because *P. destructans* can infect living bat tissue, we hypothesize that *P. destructans* should produce a yeast-like state on elevated L-cysteine medium, while no yeast-like state should be present for the other non-invasive assayed *Pseudogymnoascus* species. The importance of this work is to ascertain the phenotypic potential of *P. destructans* and its potential forms on or within bat tissue.

## Materials & Methods

With the exception of the Illinois isolates, isolates examined in this study were previously used by other researchers (Johnson et al. 2013, Lorch et al. 2013, Palmer et al. 2014) and were obtained from the Center of Forest Mycology Research, or directly from other researchers. In total, seven *P. destructans* isolates were examined: three from Illinois (ILLS69284, ILLS69285, ILLS69286) and one each from Indiana (INMSC7), New York (MYA-4855, the Type culture), Pennsylvania (ILLS69283), and Slovakia (CFMR2498). As a comparison, four isolates (WSF3629, 03VT05, 05NY08, 05NY09) within the *P. roseus* complex and two distantly-related *Pseudogymnoascus* isolates (10NY10 and 1717-2) (Minnis & Lindner 2013), were also evaluated for morphological changes on L-cysteine. All isolates were maintained on Sabouraud's dextrose agar (SDA) (Difco) at 7 °C under 24 hour darkness. All assay media was sterilized at 121 °C under 15 PSI for 15 minutes. All assays were replicated three times at different intervals using two replicates each time and visually screened for gross morphological culture variance with all variances noted followed by microscopic analysis.

## Evaluation of L-cysteine on the morphology of *Pseudogymnoascus* species

Isolates were evaluated on SDA and 700 mg L<sup>-1</sup> L-cysteine amended SDA media at pH 5.6 and pH 7.0 using quadrant Petri plates. Each quadrant contained ca. 20 ml of the following: Quadrant 1) SDA + 0.2 g FeSO<sub>4</sub> L<sup>-1</sup> (pH 5.6 control medium), Quadrant 2) SDA + 0.2 g FeSO<sub>4</sub> L<sup>-1</sup> + 4 g L<sup>-1</sup> NaHCO<sub>3</sub> (sodium bicarbonate, Fisher, ca. pH 7.0 control medium), Quadrant 3) SDA + 0.2 g FeSO<sub>4</sub> L<sup>-1</sup> + 4 g L<sup>-1</sup> NaHCO<sub>3</sub> + 700 mg L<sup>-1</sup> 97% L-cysteine (ca. pH 7.0 assay medium), Quadrant 4) SDA + 0.2 g FeSO<sub>4</sub> L<sup>-1</sup> + 700 mg L<sup>-1</sup> 97% L-cysteine (pH 5.6 assay medium). Supplementation of NaHCO<sub>3</sub> (for pH adjustment) was added after the basal medium cooled to 55 °C. Each quadrant was inoculated with a 5 mm agar plug containing actively growing mycelium, wrapped with Parafilm, and visually inspected twice per week for morphological variance. All inoculated plates were inverted two days after inoculation. Two pH levels were utilized to verify that any resulting morphological variations were not solely influenced by medium pH.

## Analysis

### Microscopic evaluation

Light microscopic evaluation was conducted using an Olympus BX51 microscope with differential interference contrast (DIC) and equipped with an Olympus QColor 3 digital camera. Images were processed in Adobe Photoshop 7.0, and measurements were conducted using NIH

Image version 1.63. Macroscopic photos were taken with a Cannon EOS 50 Mark II. Scanning electron microscope (SEM) sample preparations and imaging were carried out in the Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois; fungal samples were fixed in Karnovsky's fixative, gold coated (20 seconds) and imaged with a JEOL JSM-6060LV using an accelerating voltage of 15 kV. Isolate purity and yeast-like cell identity was confirmed using rDNA sequence comparison of the internal transcribed spacer (ITS) region.

### **Molecular evaluation**

DNA was extracted by adding fresh mycelium to 200  $\mu\text{L}$  0.5 M NaOH, ground, centrifuged at 14000 RPM for 2 minutes, and 5  $\mu\text{L}$  of the resulting supernatant added to 495  $\mu\text{L}$  100 mM Tris-HCl buffered with NaOH to pH 8.5–8.9 (Tris-HCl-DNA extraction solution, Osmundson et al. (2013). PCR was completed on a Bio-Rad PTC 200 thermal cycler. The total reaction volume was 25  $\mu\text{L}$  (12.5  $\mu\text{L}$  GoTaq® Green Master Mix, 1  $\mu\text{L}$  of each 10  $\mu\text{M}$  primer ITS4 and ITSIF, 3  $\mu\text{L}$  of the Tris-HCl-DNA extraction solution and 7.5  $\mu\text{L}$  DNA free water). The following thermal cycle parameters were used: initial denaturation at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 45 seconds, 72 °C for 1 minute with a final extension step of 72 °C for 10 minutes. Gel electrophoresis (1% TBE agarose gel stained with ethidium bromide) was used to verify the presence of a PCR product before purification using a Wizard® SV Gel and PCR Clean-Up System (Promega). A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc.) was used to sequence the ITS in one direction using the ITS5 primer on an Applied Biosystems 3730XL high-throughput capillary sequencer. Identity was confirmed through nBLAST analysis.

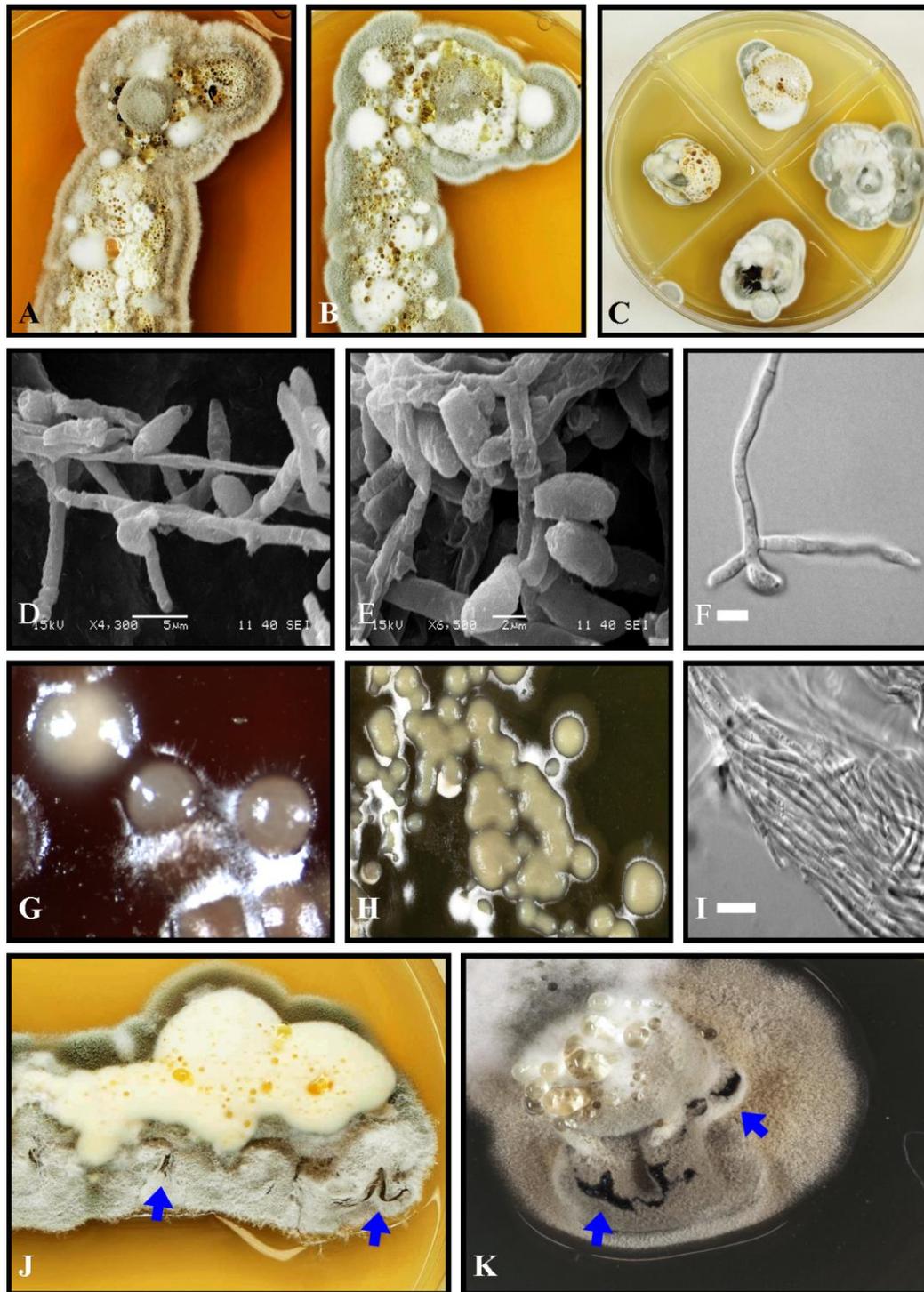
## **Results**

### **Morphological response of *P. destructans* to elevated levels of L-cysteine**

Typical colonies (grey and brown, Fig. 1A-C) and viable conidia (MYA-4855: (4.7–)5.4–6.5(–7)  $\times$  (2.1–)2.5–3(3.2), N=50; Fig. 1D-F) were produced on all seven *P. destructans* isolates grown on SDA amended with 700 mg L<sup>-1</sup> L-cysteine at 7–10 °C within 30 days. Two isolates (MYA-4855 and CFMR2498) produced a macromorphology reminiscent of yeast colonies within 30 days on SDA medium supplemented with 700 mg L<sup>-1</sup> L-cysteine between 7–10 °C (Fig. 1G, H). Microscopic examination of these smooth colonies indicated a hyphal colony composition (Fig. 1I). All seven *P. destructans* isolates produced semi-solid black masses, which varied visually from small cracks in the culture (Fig. 1J) to more prominent masses (Fig. 1K) under the 700 mg L<sup>-1</sup> L-cysteine assay conditions. Microscopic examination of the semi-solid black masses (Fig. 2A) indicated a conglomeration of large connected or free cells, some of which demonstrated blastic development (Fig. 2B-E). Single aseptate cells were ellipsoidal to obtuse (6.2–)10.7–14.9(–16.9)  $\mu\text{m}$   $\times$  (3.4–)4.2–6.6(–8.2)  $\mu\text{m}$  (N=50). Larger centrally septate cells and connected cells demonstrated constriction at the septum (14.2–)14.7–19.3(–21.5)  $\mu\text{m}$  (N=10). The transfer of these cells to SDA resulted in mycelial growth within 4 days (Fig. 2F) and was confirmed by ITS sequence comparison to be *P. destructans*.

### **Response of other *Pseudogymnoascus* species to L-cysteine**

No other examined *Pseudogymnoascus* species produced the aforementioned black masses or yeast-like cells. Isolates within the *P. roseus* complex (Fig. 3A-D) varied in response; most isolates maintained aerial mycelium while isolate 05NY09 (Fig. 3D) demonstrated a noticeable reduction of aerial mycelium on both pH 7.0 SDA media (SDA + sodium bicarbonate (control) and SDA + sodium bicarbonate + 700 mg L<sup>-1</sup> L-cysteine). In addition, most isolates (75%) within the *P. roseus* complex demonstrated minimal hydrogen sulfide production (as indicated by the presence of a black precipitate within the medium) within 30 days at pH 5.6 but not at pH 7.0. In contrast, both distantly-related *Pseudogymnoascus* isolates demonstrated substantial hydrogen sulfide production at pH 7.0 but not at pH 5.6 (Fig. 3E, F). No distinct hyphal variance occurred in cultures demonstrating the presence of hydrogen sulfide. Isolates 5NY8, 1717-2 and 10NY10 produced



**Fig. 1** – *In vitro* morphology of *Pseudogymnoascus destructans*. A. Typical brown and B. grey colony colonies of *P. destructans* New York isolate (MYA-4855, Type). C. New York isolate (MYA-4855, Type) *in vitro* on SDA and SDA amended media: (top) pH 5.6 SDA control medium, (right) ca. pH 7.0 SDA control medium, (bottom) ca. pH 7.0 700 mg L<sup>-1</sup> L-cysteine assay medium, (left) pH 5.6 700 mg L<sup>-1</sup> L-cysteine assay medium. D. Normal mycelium and conidia (SEM, 4300X). E. Normal conidiophore and conidia (SEM, 6500X). F. A germinating conidium (DIC, 2000X, scale bar = 5µm). G, H. Macromorphology reminiscent of yeast colonies, Slovakian isolate (CFMR2498) and New York isolate (MYA-4855, Type) respectively. I. Microscopic analysis of the New York isolate (MYA-4855, Type) smooth colony (DIC, 2000X, scale bar = 15µm). J, K. Semi-solid black masses emanating from the New York isolate (MYA-4855, Type) and the Pennsylvania isolate (ILLS69283), respectively, on SDA amended with 700 mg L<sup>-1</sup>. Arrows indicate location of semi-solid black masses.

large connected undifferentiated vegetative hyphae in the presence of L-cysteine which were constricted at the septa (Fig. 3G-J), while no large undifferentiated hyphae were visualized for isolates 3VT5, 5NY9 and WSF3629 under the same conditions. Large undifferentiated hyphae lacking constriction at the septa were visualized in the control media for 5NY8, 1717-2 and 10NY10.

## Discussion

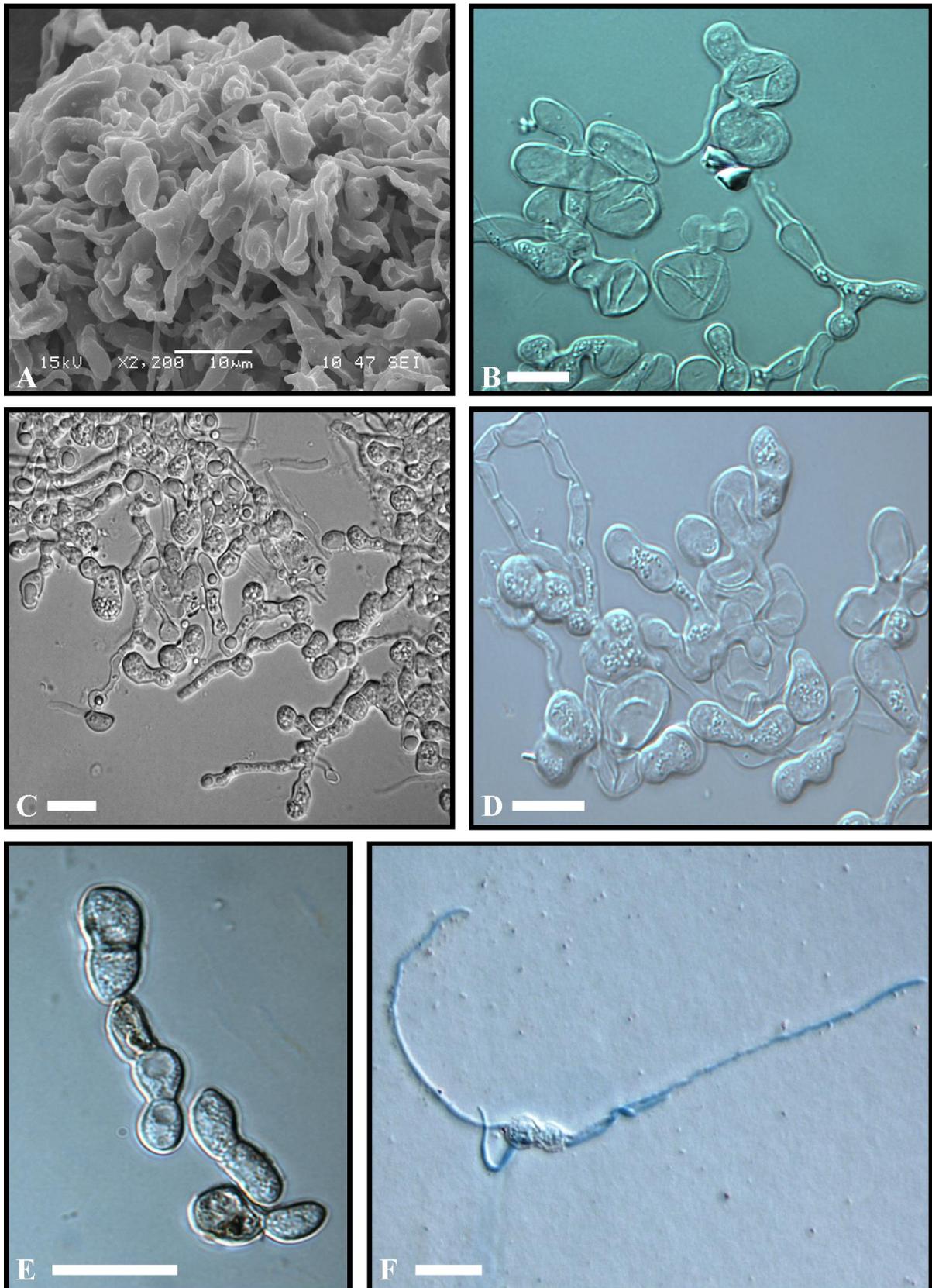
### Morphological response of *P. destructans* to L-cysteine

The morphology of *P. destructans* is typically described as either brownish or grayish colonies *in vitro* or the characteristic white (hyphal) appearance on the nose and body of bats (Gargas et al. 2009, Kubátová et al. 2011). Only two *P. destructans* studies have described variations to the typical phenotypes. Hyphal degeneration has been characterized in *P. destructans in vitro* at elevated *P. destructans* growth temperatures with deformed hyphae above 15 °C and irregular hyphal fragments near 18 °C (Verant et al. 2012). In addition, a histopathology examination of a dead bat described *P. destructans* hyphae with variable morphology including irregular, bulging and globose walls (Meteyer et al. 2009), indicating that *P. destructans* may demonstrate hyphal variability within bat tissue which has not been described *in vitro*.

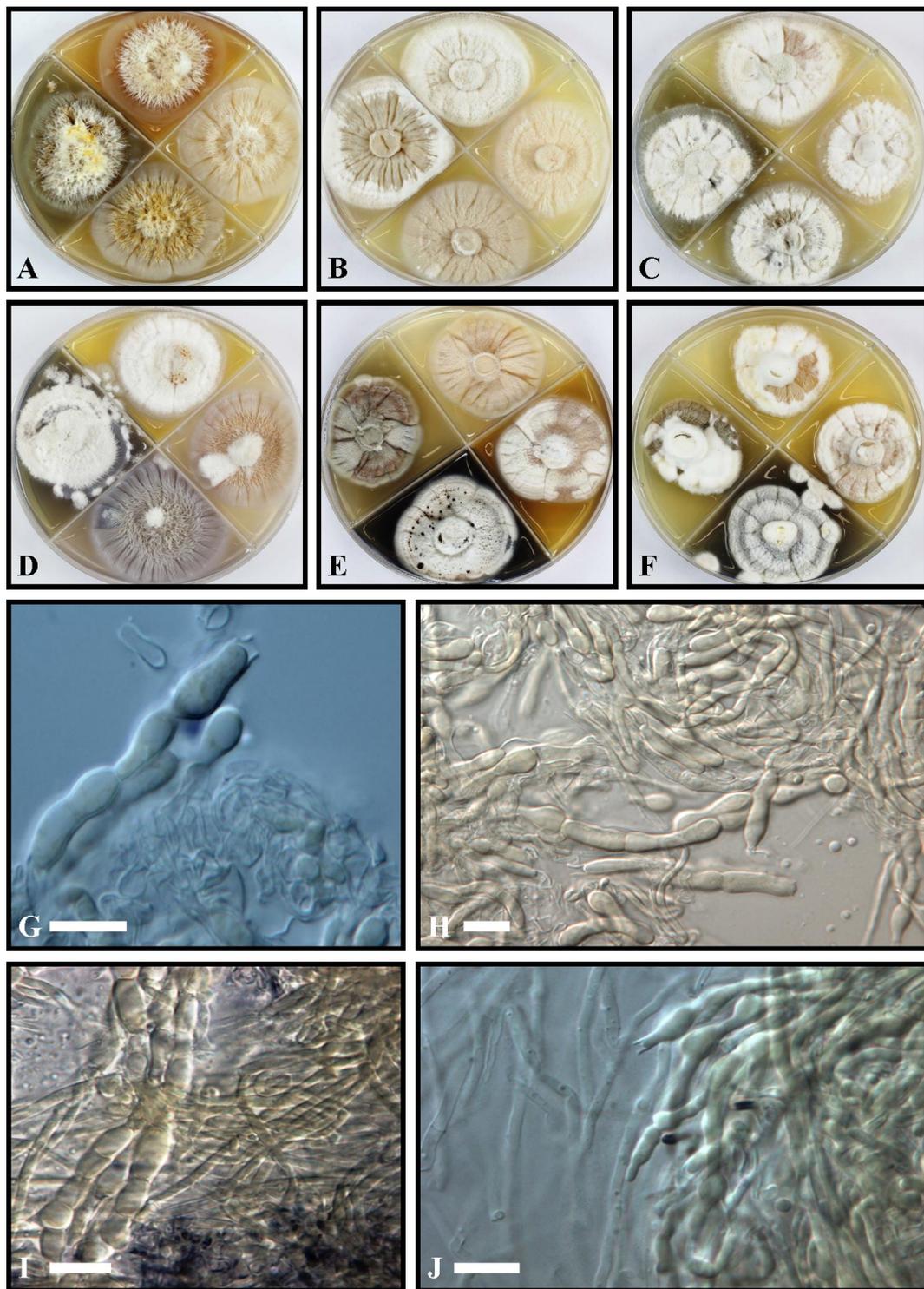
While investigating *P. destructans* cultured on medium containing elevated L-cysteine (700 mg L<sup>-1</sup>) we noted two intriguing atypical morphological variations which have not previously been reported: 1) a macromorphology reminiscent of yeast colonies (Fig. 1G, H) and, 2) yeast-like cells (Fig. 2B-E) in semi-solid black masses (Fig. 1J, K) on otherwise typical grey or brown cultures (Fig. 1A, B). The appearance of a macromorphology reminiscent of yeast colonies (Fig. 1G, H) by two *P. destructans* isolates (MYA-4855 and CFMR2498) were similar to MYA-4855 colonies seen on autoclaved *M. lucifugus* and *E. fuscus* wing tissue. However, the microscopic examination of these colonies showed that they were composed of typical hyphae (Fig. 1I) and not yeast-like cells. Subsequent subcultures from these colonies produced normal grey to brown colonies on SDA or 700 mg L<sup>-1</sup> L-cysteine amended SDA. Although these smooth colonies were hyphal in structure, their presence demonstrates that *P. destructans* has inherent phenotypic flexibility. Additional evaluations of *P. destructans* colonies with a macromorphology reminiscent of yeast colonies may provide a more precise understand of the biology of *P. destructans* since there are links between phenotypic form and secondary metabolite production in fungi (Smedsgaard and Nielsen 2005).

The appearances of the yeast-like cells in the atypical black masses were visualized consistently on the 700 mg L<sup>-1</sup> L-cysteine amended media. Our initial hypothesis of the yeast-like cells was that they may represent a yeast-like contaminate in co-culture. To rule out co-culture contamination, rDNA analysis of the ITS confirmed *P. destructans* when a culture obtained by single black mass cell isolation was sequenced (data not shown). Further microscopic examination indicated that the resulting yeast-like cells were much larger than conidia (Fig. 2C). Conidial swelling has been reported for other fungi (Hoog 1987, Hayer et al. 2013); however, it was apparent that conidial swelling does not occur to this extent in *P. destructans* (Fig. 1F). The general morphology of these cells did not fit the rules previously described (Wickerham 1951) for defining hyphae, pseudohyphae, or intermediate hyphae. True hyphae possess cells of similar size (tip cell is not substantially longer than the previous cell) and lack prominent constrictions at the cell junctions of connected cells, characteristics which differentiate these yeast-like cells from true hyphae (Fig. 2B-D). In addition, most of the resulting yeast-like cells were aseptate or 1-septate (Fig. 2E) with blastic development (Fig. 2B-D), which is in contrast to the characteristics of intermediate hyphae and pseudohyphae.

Therefore, we concluded that the best description of these cells was indeed yeast-like and not resting spores (chlamydospores) based on the visualization of thin-walled cells undergoing blastic development, which indicated that the cells were not static, and their similarity in appearance to other budding yeast-like cells obtained from superficial dermatophytes cultured on L-cysteine gradient plates (Rippon and Scherr 1959, Rippon et al. 1965). In addition, the resulting



**Fig. 2** – Microscopic morphology of *P. destructans* New York isolate (MYA-4855, Type) on SDA amended with 700 mg L<sup>-1</sup> L-cysteine. A. SEM image demonstrating the composition of the semi-solid back masses (2200X). B-E. Morphology of the *P. destructans* yeast-like cells (All scale bars = 10µm). F. Yeast-like cell with hyphal growth four days after transfer to non-amended SDA (DIC, 800X, scale bar = 10µm).



**Fig. 3** – Morphology of six North American *Pseudogymnoascus* species on SDA and L-cysteine amended SDA. A. Isolate WSF3629, B. isolate 03VT05, C. isolate 05NY08, D. isolate 05NY09, E. isolate 10NY10, and F. isolate 1717-2 cultured *in vitro* on SDA (control) and SDA amended media: (top) pH 5.6 SDA control medium, (right) ca. pH 7.0 SDA control medium, (bottom) ca. pH 7.0 700 mg L<sup>-1</sup> L-cysteine assay medium, (left) pH 5.6 700 mg L<sup>-1</sup> L-cysteine assay medium. Black pigmentation in the medium indicated the presence of hydrogen sulfide gas. G. Large undifferentiated vegetative hyphae from isolate 1717-2 (DIC, 2000X, scale bar = 10µm). (H) Isolate 1717-2 demonstrating large and small undifferentiated vegetative hyphae (DCI, 1600X, scale bar = 10µm). I. Large and small undifferentiated vegetative hyphae from isolate 05NY08 (DIC, 2000X, scale bar = 10µm). J. Large and small undifferentiated vegetative hyphae from isolate 10NY10 (DIC, 2000X, scale bar = 10µm).

budding yeast-like cells reverted to filamentous form when grown on non-amended SDA, which was consistent with other superficial dermatophytes that have been shown to have an induced yeast-like state (Rippon and Scherr 1959, Rippon et al. 1965). Our results have demonstrated that elevated levels of L-cysteine (700 mg L<sup>-1</sup>) were capable of inducing a yeast-like morphology in *P. destructans* isolates and that our results were consistent with previous investigations of other superficial dermatophytes. Our results lead us to question why this phenotype has not been previously described by other authors.

The most likely reason that a yeast-like morphology for *P. destructans* has not been described resides in the type of media utilized in culturing *P. destructans in vitro*. Most if not all *P. destructans* cultured *in vitro* was previously done on various media that was conducive for saprophytic growth on non-living substrates, meaning that those types of media have a higher oxidation-reduction potential (ORP) than that of living tissues. Conversely, the addition of L-cysteine lowers the oxidation-reduction potential of the medium (Scherr 1957, Rippon 1968) and it has been shown it is the lower ORP environment that is responsible for altering the phenotype in other superficial dermatophytes (Rippon 1968). It has also been shown that many fungi can adapt to a reduced ORP environment (Wanke et al. 2000) and suggested that the ease in which a fungal species can adapt to a reduced ORP may indicate its potential pathogenicity (Rippon et al. 1965).

Our results are the first report of a yeast-like morphology for *P. destructans* and suggest the potential for this yeast-like state to exist within bat tissue. This yeast-like morphology may assist in the spread of the infection, increase its pathogenicity (Rippon & Scherr 1959, Rippon et al. 1965) or it may simply assist in the survival of the fungus within the bat tissue (Thuy et al. 1981). An in-depth *in vivo* study is warranted but was beyond the scope of this research as our objective was to evaluate the potential for a yeast-like state, for which we have obtained visual conformation (Fig. 2B-D).

### **Response of other *Pseudogymnoascus* species to L-cysteine**

Most isolates within the *P. roseus* complex (Fig. 3A-D) and both distantly-related *Pseudogymnoascus* isolates (Fig. 3E-F) demonstrated greater hydrogen sulfide production than *P. destructans* (Fig. 1C), which is generally believed to be produced to prevent cysteine toxicity (Barrett & Clark 1987). The generation of hydrogen sulfide did not appear to substantially affect the overall colony morphology as no morphological variances were detected. The presence of large diameter undifferentiated hyphae in isolates 5NY8, 1717-2, and 10NY10 appear to be connected and contain similar hyphal constriction at the septum as *P. destructans* yeast-like cells (Fig. 3G-J). The function of these large diameter undifferentiated hyphae is unknown but it is possible that they represent primary hyphae with a faster growth rate (Prosser 1983) than the majority of vegetative hyphae. Alternatively, isolates 5NY8, 1717-2, and 10NY10 may require a higher concentration of L-cysteine to induce a yeast-like morphology or that these species are less adapted for growth in a lower ORP environment. Consequently, the other *Pseudogymnoascus* species assayed appear to be less tolerant to increased levels of L-cysteine, as indicated by the presence of hydrogen sulfide gas, and no yeast-like cells were visualized for any of the six species assayed.

### **Conclusions**

We have shown that under elevated levels of L-cysteine, *P. destructans* was capable of demonstrating phenotypic plasticity by displaying 1) a macromorphology reminiscent of yeast cultures, and 2) by producing active, viable yeast-like cells capable of generating hyphal colonies when transferred to non-amended SDA. Both phenotypic responses by *P. destructans* appeared to be different than the responses demonstrated by six other North American species of *Pseudogymnoascus*. Importantly, the yeast-like cells produced by *P. destructans* are a phenotypic response that is consistent with other superficial dermatophytes and *Aspergillus/Penicillium* species cultured on L-cysteine gradient plates by other authors. In addition, these yeast-like cells were produced at temperatures in which bats are known to hibernate. We believe that the lower ORP environment produced by the additional of L-cysteine to the medium was responsible for the

induction of the yeast-like cells in *P. destructans* and our research highlights the need for an in depth investigation into the biochemical and genetic effects of ORP on *P. destructans* gene expression.

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