Metabolites from *Aspergillus sclerotiorum* fungal growth medium are cytotoxic to and inhibit cell cycle progression in *Drosophila melanogaster* S2 cells in culture

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ABSTRACT

Aspergillus sclerotiorum has been identified as an entomopathogenic fungus with potential as a biological control agent, yet its cellular effects on insect hosts are not well characterized. We hypothesized that filtered growth medium from *Aspergillus sclerotiorum* cultures contains one or more fungal metabolites that damage cells in culture. To explore possible mechanisms, we tested cell-free fungal culture filtrates on *Drosophila melanogaster* S2 embryonic cells. Filtrates from older cultures were strongly cytotoxic and showed a reduced percentage of live S2 cells comparable with positive controls for cell death. Similar effects were seen in mouse fibroblast L929 cells. At lower concentrations, the fungal medium did not cause overt cytotoxicity but did consistently reduce total S2 cell numbers. Few dead cells were present in these conditions, indicating the medium impaired replication rather than inducing cell death. This reduction was accompanied by a decreased expression of two cell cycle regulators. Proliferating cell nuclear antigen (PCNA) fell to less than 6% of controls, while Cyclin A dropped to below 30% of controls at the most dilute treatment. These results indicate that *A. sclerotiorum* secretes one or more metabolites that decrease cell viability at higher concentrations and inhibit proliferation at lower concentrations, potentially by interfering with cell cycle progression. ELISA analysis of ochratoxin A production in this strain showed only minimal levels of the toxin, suggesting other unidentified fungal factors may be the source of these observations. These findings advance understanding of fungal–insect interactions and suggest that soluble compounds produced by *A. sclerotiorum* may contribute to its pathogenicity in insect hosts.

INTRODUCTION

Entomopathogenic fungi (EPF) have the potential to be used as biopesticides due to their direct cuticle penetration of insect hosts. EPFs also play an important role in natural ecosystems as plant growth promoters, rhizosphere colonizers, plant disease antagonists, and endophytes (16). Fungi naturally occurring in termite habitats are promising biological control agents, due to their adaptation in such environments (30). The most effective and thoroughly studied EPF, *Metarhizium anisopliae*, has been suggested as a potential termite control agent (15, 22). However, biocontrol agents can be limiting, in that they work more slowly than chemical pesticides (16).

Previous work has shown *Aspergillus sclerotiorum* to be pathogenic to both *Incisitermes minor* and *Reticulitermes hesperus* termites (10). These two species of western termites are among the 5 pest species found in the United States (25), making *A. sclerotiorum* a potential biocontrol agent for termites. In addition, as a biosafety level 1 (BSL-1) agent, *A. sclerotiorum* is unlikely to be significantly harmful to most humans, although it may pose some threat to immunocompromised people (1, 2, 11, 18, 24).

The mechanisms of termite infection and pathogenicity by *A. sclerotiorum*, as well as the fungal host range, remain largely unknown, although a pilot study showed that *Drosophila melanogaster* insects were susceptible to *A. sclerotiorum* infection (unpublished data). In an effort to study insect host-fungal interactions on the cellular level, *D. melanogaster* S2 embryonic cell line (23) were used in many of these studies, since these cells are well-studied, easy to maintain, and a termite cell line does not yet exist. Additionally, a mouse cell line (L929) was used to test whether the fungus had similar effects on mammalian cells.

Here we present findings of the effects of treating S2 cells with dilutions of filtered, cell-free *Aspergillus sclerotiorum* growth medium. S2 cells showed increased cytotoxicity from older cultures of filtered fungal growth medium at higher concentrations. The same cytotoxic phenomenon was observed when mouse L929 cells were treated with the fungal growth medium, which led to our hypothesis that the fungus secretes a molecule(s) that is cytotoxic. When S2 insect cells were treated with more diluted fungal growth culture, however, insect cells did not show cytotoxicity, but they had a marked decrease in cell numbers per sample compared to control treated cells. It was then hypothesized that the fungus secretes a molecule(s) that arrests S2 cells in cell cycle. An analysis of Proliferating Nuclear Cell Antigen (PCNA) and Cyclin A protein levels from treated S2 cells confirmed that they had lower levels of these proteins, indicating that cells were arrested in cell cycle. Further studies indicated that Ochratoxin A (OTA), a mycotoxin made by many *Aspergillus* fungi (27), was not made at high levels by the strain of *A. sclerotiorum* used in this study. This study furthers the understanding of the insect host and pathogen interactions between *D. melanogaster* insect cells and *A. sclerotiorum* fungus.

MATERIALS AND METHODS

CELL LINES AND FUNGAL CULTURES.

The *Drosophila melanogaster* cell line, S2 (23), was obtained from the *Drosophila* Genomics Resource Center (Bloomington, Indiana) and the American Type Culture Collection (ATCC, Rockville, Maryland). S2 cells were grown and maintained at room temperature (approximately 20-25 ℃) in S2 medium containing Gibco Schneider's Drosophila Medium, 10% heat-inactivated fetal bovine serum (FBS), and with or without 1× penicillin-streptomycin (medium from ThermoFisher Scientific, Waltham, Massachusetts; FBS from Sigma-Aldrich, St. Louis; pen-strep from HyClone Laboratories, Logan, Utah). The mouse fibroblast cell line, L929, was also obtained from ATCC (Rockville, MD). L929 cells were grown at 37° C and 5% CO_2 and maintained in L929 medium containing Dulbecco's Modified Eagle's Medium, 10% heat-inactivated FBS, 10 mM HEPES buffer, and 1× penicillin-streptomycin (Medium from Caisson Labs, Smithfield, UT; HEPES from Sigma-Aldrich).

Two strains of *Aspergillus sclerotiorum,* APU strain (10) and Huber strain (16892, ATCC, Rockville, MD)(12), and *Aspergillus alliaceus* (NRRL-315, Agricultural Research Service (ARS) Culture Collection, Peoria, Illinois) were all grown and maintained on Sabouraud dextrose agar (SDA) plates (BD Diagnostics, Franklin Lakes, New Jersey) or in Sabouraud dextrose broth (SDB) (Sigma-Aldrich, St. Louis). *A. sclerotiorum* APU strain was used in all experiments, and only indicated where Huber strain was also used. Glycerol stocks from APU and Huber strains of *A. sclerotiorum* were made and stored at -80℃.

FUNGAL GROWTH MEDIUM AND DILUTIONS.

A. sclerotiorum APU or Huber strain was inoculated from a glycerol stock stored at -80℃ into approximately 30 mLs of Sabouraud dextrose broth (SDB) in 50 mL conical tubes with loosened lids. Tubes were then grown in a shaker incubator at 25℃ at 220 rotations per minute (rpm) in the dark, along with 30 mLs of uninoculated control medium. Inoculated or matched uninoculated tubes were incubated for either 4, 7, or 10 days. After this, fungal or control tubes were centrifuged at 3,000 rpm for 15 minutes, followed by removal of supernatant, which was then filter-sterilized using a syringe with a 0.22 µm filter. Dilutions of fungal growth medium and uninoculated control medium were made in S2 or L929 medium and immediately used in S2 or L929 cell experiments, except for one experiment analyzing Cyclin A or PCNA proteins where fungal growth medium and uninoculated control medium as described above were lyophilized. In this case, fungal cultures or uninoculated controls were incubated for 10 days, centrifuged, and the supernatants were filter-sterilized, frozen, and then lyophilized overnight. The resulting powders were kept at -20°C until reconstituted to the original 1X in S2 medium, filter-sterilized, and these were further diluted into 1:2.5, 1:5, and 1:10 working concentrations in S2 medium.

CELL VIABILITY AND CELL QUANTIFICATION ANALYSIS.

S2 and L929 cells were each seeded in 24-well plates at 2 x 10^5 cells per well 1-2 days before treatment with fungal growth medium. For loosely adherent S2 cells, half the medium was taken off and replaced with the appropriate treatment. For adherent S2 or L929 cells, all medium was taken off and replaced with the appropriate treatment. S2 cells were treated with either 0.8 µM actinomycin D (ActD) or 33.3 µg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO) as positive controls for cytotoxicity. L929 cells were treated with ActD as a positive control for cytotoxicity. Cells were incubated for 48 hours with these various treatments and then analyzed. For each experiment, 3-4 wells were used per treatment.

Cell viability and quantification were measured using propidium iodide staining followed by flow cytometry. After 48 hours, S2 cells or L929 cells from each well were collected into a separate microfuge tube (L929 cells were removed with trypsin first), then centrifuged at 4,000 rpm for 2 minutes. The supernatant was removed and the cell pellets were washed twice with phosphate buffered saline (PBS) before being resuspended in 100 µl of FACS solution (3% heat-inactivated FBS and 0.09% (w/v) sodium azide in PBS, pH 7.4-7.6, then filter sterilized through a 0.22 µm pore membrane). Then 5 µl of propidium iodide (PI) (Sigma-Aldrich, St. Louis) was added to each sample and vortexed before being read on the BD Accuri C6 Plus flow cytometer, using the BD Accuri C6 software (BD Biosciences, San Jose, California). The number of cells in each sample was quantified by the total number of events counted in two minutes at a slow rate (14 µl/minute) by flow cytometer. At least 10,000 events were recorded per sample, as previously suggested (7). To measure cell viability, a gate was set to exclude cell debris based on side scatter versus forward scatter plots, then a second plot of PI fluorescence vs forward scatter was generated, which showed two distinct cell populations (modified slightly from (7)). The population with lower fluorescence was determined to be the live cells, while the population with the higher fluorescence was determined to be the dead cells. Live cells were expressed as a percent of the total cells in the well. Samples for each treatment were

averaged. Depending on the experiment, either one-way or two-way ANOVA with replication was performed, followed by Tukey's HSD post-hoc test ($\alpha = 0.05$), or Student's t-tests were performed to determine one-tailed *p* values.

Cyclin A and PCNA Western blots

Drosophila melanogaster S2 cells were passaged regularly and maintained in Schneider's Drosophila Medium described above, but without antibiotics. For experimental and control treatments, S2 cells were seeded in 6-well plates at a density of 1.6×10^6 cells per well in a total of 2 mLs, and allowed to adhere for 24 hours before treatment.

Cells were harvested, spun down, washed in cold phosphate-buffered saline with 0.1% Tween-20 (PBS-T), and re-suspended in 250 µl of RIPA lysis buffer containing 1X broad spectrum protease inhibitor cocktail and 1mM EDTA (Boster Bio, Pleasanton, CA). Lysates were kept on ice and periodically vortexed for 30 minutes before being stored at -20°C. Protein concentrations were determined using the Qubit Broad Range Protein Assay (Thermo Fisher Scientific, Waltham, MA). 10 µg of each sample was denatured in SDS sample buffer at 95°C for 5 minutes before being loaded onto 12% tris-glycine SDS-PAGE gels with 6 µl Precision Plus Protein Kaleidoscope ladder (both from Bio-Rad, Hercules, CA) and separated by electrophoresis. Proteins were then transferred to nitrocellulose membranes using the Bio-Rad Turbo Blot system for 7 minutes. Membranes were blocked overnight in 1% bovine serum albumin (BSA) in 0.1% PBS-T.

Primary monoclonal anti-Cyclin A (A12), anti-α-Tubulin (12G10), and anti-PCNA (PC10) were diluted 1:1000 in 1% BSA in 0.1% PBS-T and incubated either for 1 hour at room temperature or overnight at 4°C. Blots were probed with HRP-conjugated anti-mouse secondary antibody diluted 1:1000 in blocking buffer. Signals were visualized using Amersham ECL detection reagents (GE Healthcare, Chicago, IL, USA) on a Bio-Rad ChemiDoc imaging system. Membranes were stripped and re-blocked before reprobing for α-Tubulin. A12 and 12G10 antibodies were purchased from Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA; PC10 antibody from NeoBiotechnologies, Irvine, CA; secondary antibody from Sigma-Aldrich, St. Louis, MO.

All blots were analyzed using Bio-Rad Image Lab software. Bands were background-subtracted and normalized to corresponding α-Tubulin signals. Cyclin A and PCNA blots were performed in duplicate from cells treated with fresh liquid fungal growth or control media, and once with cells treated with reconstituted lyophilized media before analysis. One representative PCNA blot is shown.

QUANTIFICATION AND STATISTICAL ANALYSIS OF WESTERN BLOTS

The primary metric for the analysis of PCNA and Cyclin A was the band intensity from the protein of interest that had been normalized to the housekeeping protein, α-Tubulin, to determine a relative expression. The resulting relative band intensity for each fungal growth medium was compared to its respective uninoculated control. For each dilution in the Cyclin A blots, the null hypothesis of no change was tested with a one-sample, two-tailed Student's t-test against $\mu = 1.0$ (df = n–1), with $\alpha = 0.05$. n = 3 biological replicates. No outliers were excluded.

Ochratoxin A detection in Aspergillus sclerotiorum

Coconut cream agar plates were prepared by boiling 300 mL of water with 50 g of coconut cream powder (Kara, Pulau Sambu, Singapore) and 12 g of biological grade agar (Bioreagents, Hummelstown, PA) followed by sterilization in the autoclave. Fungal cultures of *A. alliaceus*, *A. sclerotiorum* APU strain, and *A. sclerotiorum* Huber strain from glycerol stocks were inoculated onto the plates and incubated at 24 °C in the dark for at least 4 days. Plates were inverted and observed under UV light (365 nm) for OTA's blue fluorescence (13). The Huber strain of *A. sclerotiorum* was reported to produce no detectable OTA by this method, while the NRRL-315 strain of *A. alliaceus* was shown to produce high levels of OTA by this method (12), and thus were chosen as control strains.

Ochratoxin A competitive ELISA kit (Antibodies.com, St. Louis, Missouri) was used to quantify the ochratoxin A from 10-day cultures of *Aspergillus alliaceus, A. sclerotiorum* APU, and *A. sclerotiorum* Huber, grown in Sabouraud dextrose broth (Sigma-Aldrich, St. Louis). Fungal samples were filter-sterilized using a syringe with a 0.22 µm filter and used for the ochratoxin A kit according to manufacturer's instructions. Two wells on the ELISA plate were used for each sample and then averaged; three wells of each standard were used and averaged to generate the standard curve. The R^2 value of the standard curve was 0.9689.

RESULTS

S2 CELL VIABILITY WAS REDUCED AFTER TREATMENT WITH 10-DAY FUNGAL GROWTH MEDIUM COMPARED TO 4-DAY FUNGAL GROWTH MEDIUM

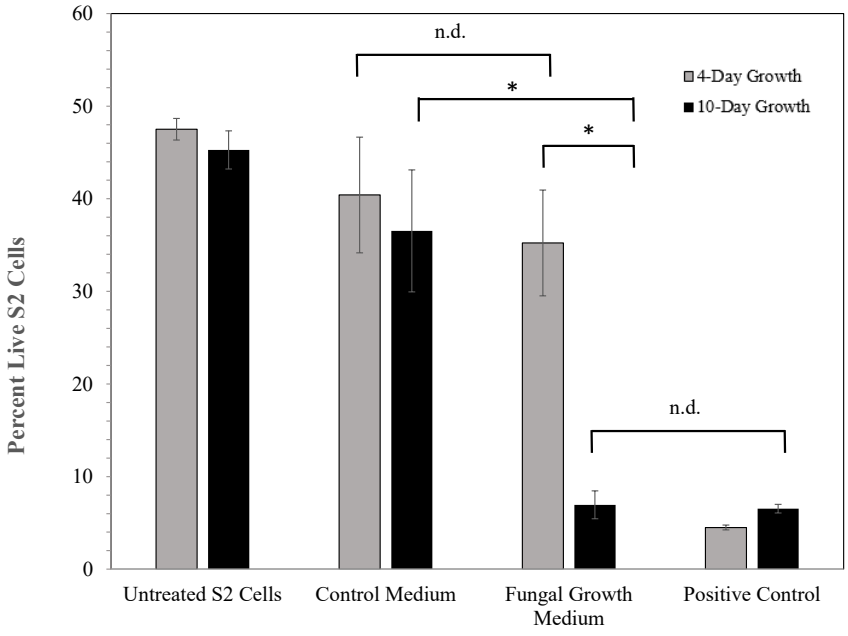
The effect of cell-free, filtered fungal growth medium from 4-day or 10-day cultures on *Drosophila* S2 cell viability was investigated and shown in Figure 1. With 4-day fungal growth medium treatment, there was no statistically significant difference between the percentage of live S2 cells treated with uninoculated control medium compared to cells treated with *A. sclerotiorum* fungal growth medium after 48 hours (40.4% vs 35.2%, respectively, Fig. 1). By contrast, S2 cells treated with 10-day fungal growth medium treatment resulted in significantly fewer live S2 cells than uninoculated control-treated S2 cells (7.0% live S2 cells vs 36.5% live S2 cells, Fig. 1). The 10-day fungal growth treatment had a similar effect on S2 viability as actinomycin D, the positive control for cytotoxicity (7.0% vs 6.5%, not statistically different, Fig. 1). The percentage of live S2 cells treated with 4-day and 10-day fungal culture media were also significantly different (35.2% vs 7.0%, respectively), showing a decrease in S2 cell viability as the fungal cultures aged.

FUNGAL GROWTH MEDIUM IS CYTOTOXIC TO A MOUSE CELL LINE *IN VITRO*

The cytotoxicity of fungal growth medium was also tested on a mammalian fibroblast cell line, mouse L929 cells. Figure 2 shows that 48 hours after L929 cells were treated with 10-day *A. sclerotiorum* growth medium, only 15.8% of cells were viable, whereas 79.0% of the L929 cells treated with uninoculated medium remained viable. This was a significant reduction in viability in the mouse cells (Tukey's post-hoc test, $p<0.05$). The viability of L929 cells treated with fungal growth medium was not statistically different from cells treated with the positive control for cytotoxicity, actinomycin D.

FIGURE 1.

Treatment with 10-day A. sclerotiorum *growth medium culture resulted in less S2 cell viability than 4-day* A. sclerotiorum *growth medium culture treatment.*



S2 cells were either untreated, treated with diluted (1:2.5) uninoculated Sabouraud dextrose broth (Control Medium), diluted (1:2.5) *A. sclerotiorum* 4-day or 10-day filtered growth medium (Fungal Growth Medium), or a positive control for 48 hours before staining with propidium iodide and analyzed by flow cytometry. Positive controls were 33.3 μg/mL cycloheximide in the 4-day fungal growth trial, and 0.8 μM actinomycin D in the 10-day fungal growth trial. Each bar represents the mean of three samples ± SEM. * indicates $p < 0.05$ (two-way ANOVA with replication ($F(3,16) = 6.09$, $p = 0.0058$), followed by Tukey's HSD post-hoc test); n.d. indicates not statistically significant ($p > 0.05$).

DILUTED FUNGAL GROWTH MEDIUM LOST ITS CYTOTOXICITY TO S2 CELLS, BUT RESULTED IN A DECREASE IN TOTAL S2 CELL NUMBERS

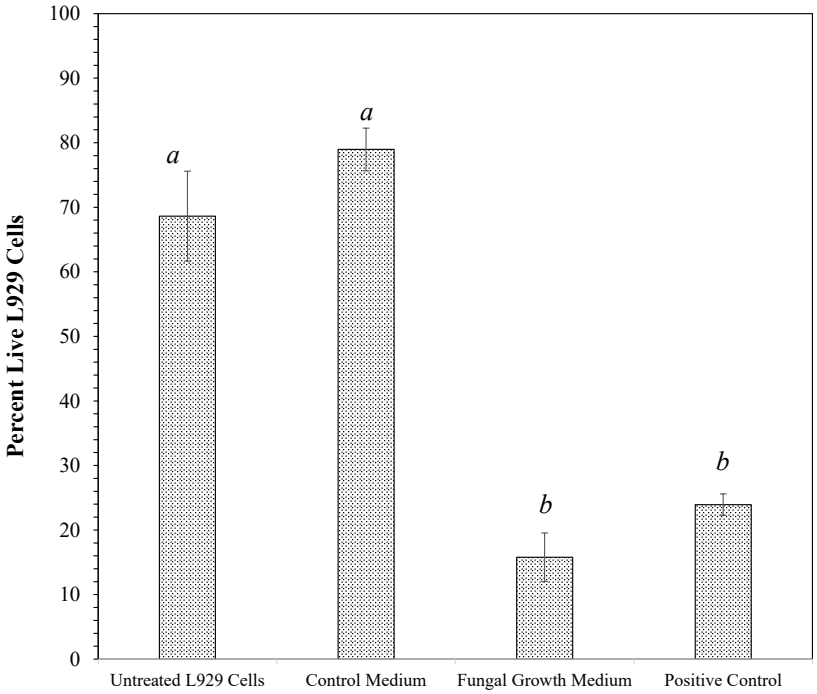
Figure 3 shows the effect that diluted filtered *A. sclerotiorum* 7-day fungal growth medium had on S2 cell viability as well as total S2 cell numbers after 48 hours. The 1:5 dilution resulted in a significantly lower average percent of live cells compared to the 1:5 dilution of uninoculated control medium (35.7% vs 77.9%, $p < 0.001$) (Fig. 3A). The 1:10 dilution also resulted in a significantly lower average percent of live cells compared to its uninoculated control, although less so (53.4% vs 77.5%, $p = 0.03$) (Fig. 3A). The average total number of cells treated with the 1:5 dilution of fungal growth medium was also drastically lower compared to the same dilution of uninoculated control medium (approximately 24,000 cells vs 175,000 cells, $p < 0.001$, Fig. 3B). When the same fungal growth medium was diluted 1:20, there was no longer a significant difference between the percentage of live cells compared to the uninoculated control medium (Fig. 3A). However, the numbers of cells present in each well treated with 1:10 and 1:20 dilutions of fungal growth medium were still significantly lower than the numbers of cells in each well treated with diluted uninoculated control medium ($p < 0.001$) (Fig. 3B).

DECREASE IN PCNA PROTEIN LEVELS FOLLOWING DILUTED FUNGAL GROWTH MEDIUM

S2 cells were treated with uninoculated control medium or inoculated fungal growth medium at three dilutions (1:2.5, 1:5, and 1:10), and the PCNA protein expression was analyzed (Figure 4). An analysis of relative band intensities in Figure 4 showed that PCNA expression in cells treated with fungal growth medium was approximately 22% of the control at the 1:2.5 dilution, approximately 27% at the 1:5 dilution,

FIGURE 2.

A. sclerotiorum *growth medium was cytotoxic to mouse L929 cells.*



Mouse L929 cells were either untreated, treated with diluted (1:2.5) uninoculated Sabouraud dextrose broth (**Control Medium**), diluted (1:2.5) *A. sclerotiorum* 10-day filtered growth medium (**Fungal Growth Medium**), or treated with a positive control (0.8 μM actinomycin D) for 48 hours before staining with propidium iodide and analyzed by flow cytometry. Each bar represents the mean of four samples ± SEM. One-way ANOVA was performed (F (3,12) = 52.29, p = 3.66E-07). Groups marked with different letters (*a* vs *b*) differ significantly using Tukey's HSD post-hoc test ($p < 0.05$).

and approximately 6% at the 1:10 dilution levels compared to their respective diluted uninoculated control medium.

DECREASE IN CYCLIN A PROTEIN LEVELS FOLLOWING EXPOSURE TO DILUTED FUNGAL GROWTH MEDIUM

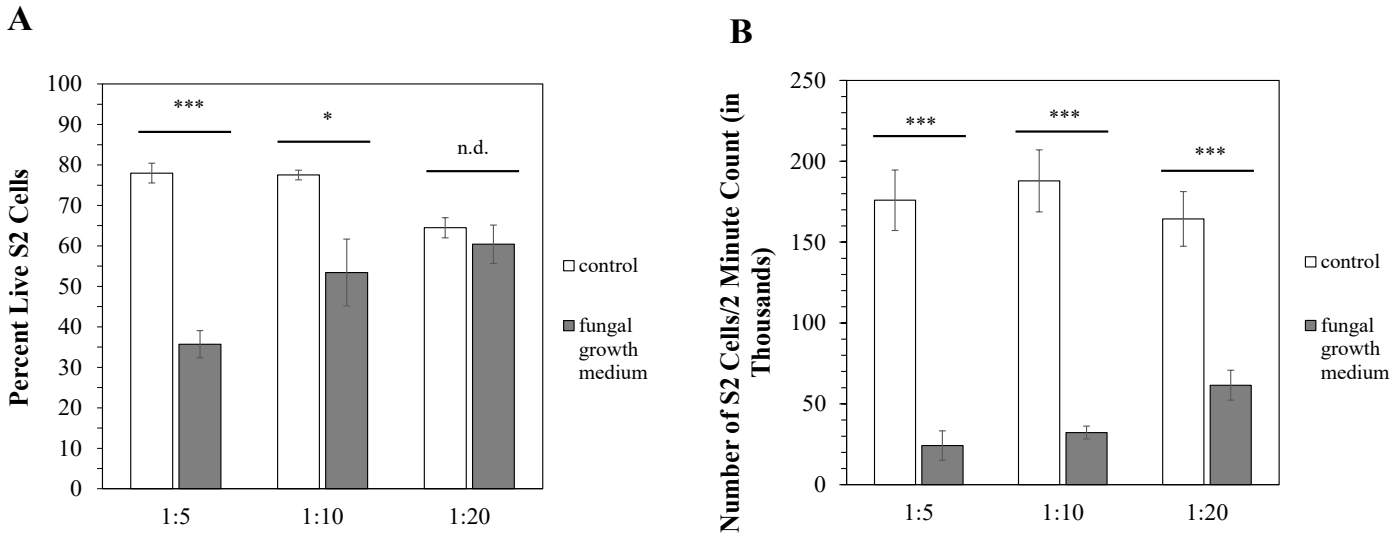
S2 cells showed a consistent decrease in Cyclin A expression following exposure to fungal growth medium at both 1:5 and 1:10 dilutions. Cyclin A levels were found to be approximately 65% at 1:5 dilution, and approximately 29% at 1:10 dilution compared to their respective diluted uninoculated medium controls (Table 1). While the reduction at the 1:5 dilution was not statistically significant (p=0.127), the reduction in Cyclin A levels at the 1:10 dilution was found to be statistically significant (p=0.037).

ASPERGILLUS SCLEROTIORUM PRODUCED ONLY VERY LOW LEVELS OF OCHRATOXIN A (OTA)

To determine whether ochratoxin A (OTA) is produced by the APU strain of *A. sclerotiorum*, the fungus was first grown on coconut cream agar (Figure 5A). This medium induces the production of OTA, which fluoresces blue under ultraviolet (UV) light. On coconut cream agar plates, *Aspergillus alliaceus* displayed strong blue fluorescence consistent with OTA production (13), whereas both the Huber and APU *A. sclerotiorum* strains lacked visible OTA-associated blue fluorescence (Figure 5A). Quantification by competitive ELISA assay confirmed these observations: *A. alliaceus* produced a high OTA concentration of 14.8 ng/mL when grown for 10 days in fungal medium, while the *A. sclerotiorum* strains APU and Huber produced low levels of OTA, 1.3 ng/mL and 1.4 ng/mL, respectively (Figure 5B). These levels were only slightly above the background of the uninoculated control broth (0.3 ng/mL).

FIGURE 3.

More concentrated fungal growth medium was toxic to S2 cells, while more diluted fungal growth medium had no effect on S2 cell viability but resulted in decreased total numbers of S2 cells.



S2 cells in culture were treated for 48 hours with varying dilutions of fungal growth medium as shown from a 7-day culture, and then stained with propidium iodide and analyzed by flow cytometry. The controls were diluted, uninoculated Sabouraud dextrose broth medium diluted in S2 medium. Each bar represents the mean of four samples ± standard error. *** indicates $p < 0.001$ (one-tailed, Student's t-test); * indicates $p < 0.05$; **n.d**. indicates not statistically significant ($p > 0.05$). This experiment was conducted 3 separate times, with one representative experiment shown. A. Live and dead cells were determined by propidium iodide staining, and expressed as a percent of live cells within each sample. B. Total cell numbers in samples were counted from 2-minute runs.

TABLE 1.

Decrease in Cyclin A Levels in S2 Cells Treated with Fungal Growth Medium Vs Control Medium.

Dilution of Medium	Relative Cyclin A Band Intensity [a]	% Change	*P* value [b]
1:5	0.653 ± 0.238	- 34.7%	0.127
1:10	0.287 ± 0.245	-71.3%	0.037

[a]Band intensities were normalized to α-tubulin within each lane, then expressed as fungal growth medium/ control medium ratios for each dilution, with mean shown ± standard deviation.
[b] Two-tailed t-test comparing normalized band intensities, fungal growth medium vs control medium. $\mu = 1.0$ (df = n–1), with $\alpha = 0.05$. n = 3 biological replicates.

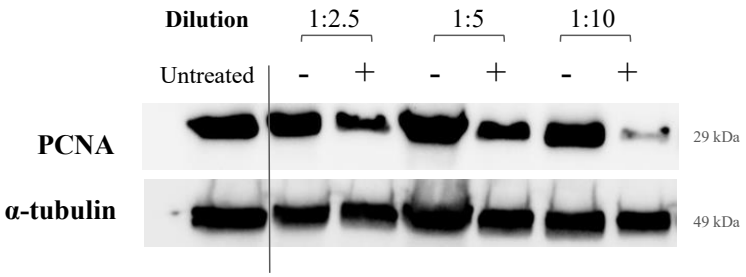
DISCUSSION

We have previously shown that a novel fungus, *Aspergillus sclerotiorum,* is entomopathogenic to two species of western termites (10), although the mechanisms of infection and pathogenesis are still largely unknown. In this report, filtered cell-free *A. sclerotiorum* growth medium from either older cultures or at more concentrated dilutions were shown to be toxic to cells from the S2 *Drosophila* insect cell line (Figs. 1 and 3A). In addition, treatment with fungal growth medium was also shown to decrease L929 mouse cell viability (Fig. 2). These together strongly suggest that *A. sclerotiorum* secretes one or more cytotoxic compounds or metabolites, and that the concentration of toxic molecule(s) accumulate over time in culture medium. Although currently unknown, it would be interesting to determine whether the fungal growth medium retains its pathogenicity in live insects, as well.

The toxicity seen in cells treated with *A. sclerotiorum* fungal medium is likely caused by one or more mycotoxin or other fungal metabolite(s). Some strains, but not others, of *A. sclerotiorum* tested have previously been shown to produce the mycotoxins ochratoxin A (OTA) and ochratoxin B (OTB) (6, 12, 13, 26), which are toxic to both insects (8) and mammalian cells and humans (reviewed in 28). It is unlikely, however, that the cytotoxicity seen in fungal growth medium-treated S2 cells (Fig. 1 and 3A) or L929 cells (Fig. 2) reported in this study are due to OTA, since only very low levels of OTA were detected in fungal growth medium by competitive ELISA (Fig. 5B). While the S2 cell line was treated with filtered fungal growth medium in these studies, we have previously worked with live termites inoculated directly with viable fungal spores (10). It remains unclear whether low levels of OTA in *A. sclerotiorum* APU strain contribute to pathogenicity of the fungus in the whole insect host.

FIGURE 4.

PCNA expression in S2 cells decreases following A. sclerotiorum *growth medium exposure.*



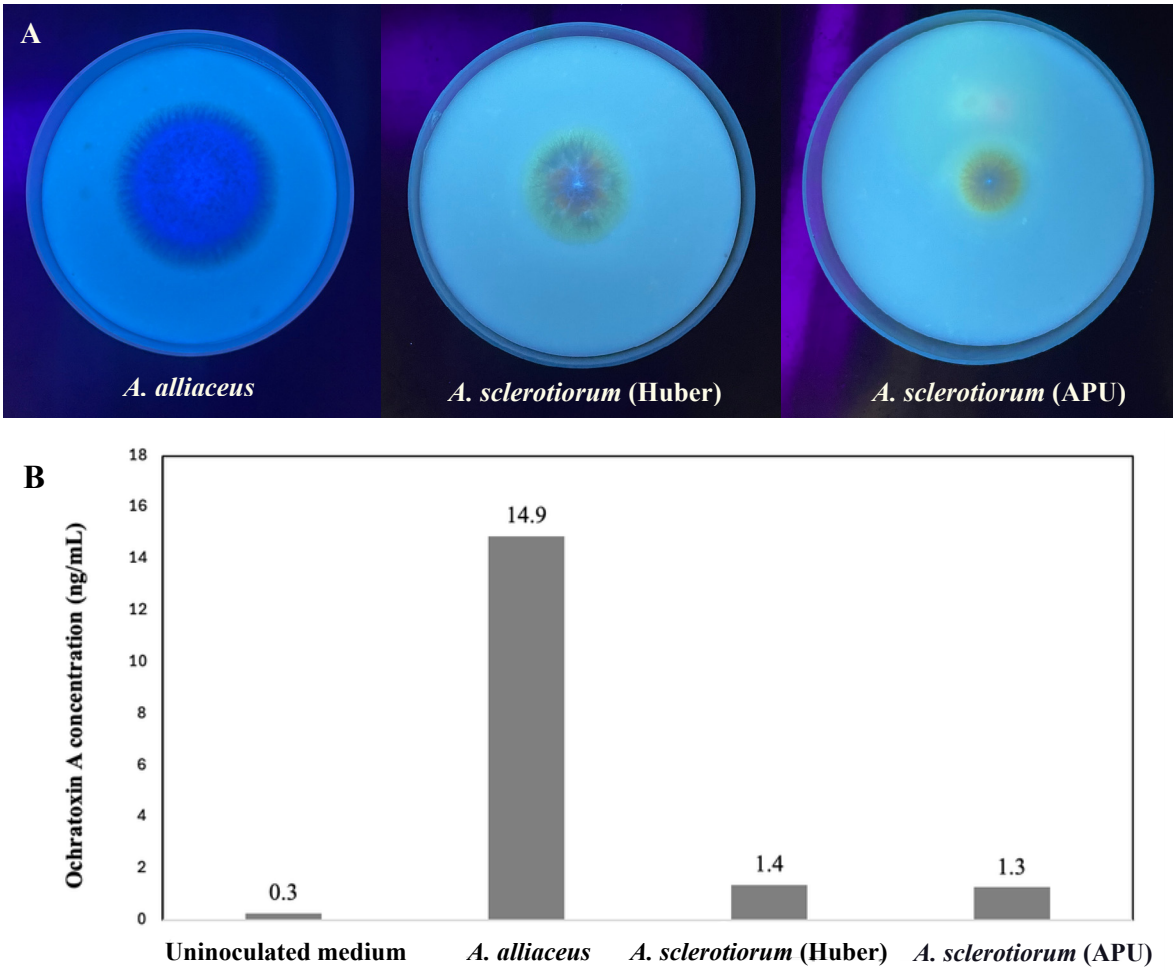
S2 cells were treated for 48 hours with either fungal growth medium (+) or control medium (-) at 1:2.5, 1:5, and 1:10 dilutions. Untreated cells remained in their regular S2 media for the 48 hours. Whole-cell lysates were immunoblotted for PCNA and band intensity was normalized to the housekeeping protein, α-Tubulin.

While there was no visible blue fluorescent OTA seen on the coconut cream agar plates in Figure 5A, there was a light green fluorescent molecule seen in both strains of *A. sclerotiorum*, which could indicate the presence of another mycotoxin normally found in *Penicillium,* citrinin (19), although whether it is citrinin remains to be determined. Other potential *A. sclerotiorum* metabolites that might play a role in the cell toxicity seen in these experiments could include aspochracin, shown to have anti-insect properties in silkworm larvae (20) and reported to be produced by *A. sclerotiorum* (4); or sclerotiamide, isolated from the sclerotia of *A. sclerotiorum* with anti-insect properties (8,29), although different forms of sclerotiamide have shown variable cytotoxicity when tested in human tumor cell lines (9,29). It is also possible that one or more molecules in *A. sclerotiorum* growth medium that have not yet been identified contributed to or was responsible for the decreased cell viability seen in our studies. More work needs to be done, however, to identify relevant specific fungal metabolites that mediate the effects reported here.

As *A. sclerotiorum* fungal growth medium was diluted, S2 cells showed less toxicity, where the percentage of live cells per sample treated with 1:20 dilutions was not signifi-

FIGURE 5.

Low levels of ochratoxin A (OTA) detected in Aspergillus sclerotiorum *strains.*



(A) Growth of *A. alliaceus* (positive control, 4 days), *A. sclerotiorum* APU strain (6 days), and *A. sclerotiorum* Huber strain (6 days) on coconut cream agar. Plates were inverted to visualize under ultraviolet (UV) light (365 nm). OTA fluoresces blue-green under UV light. (B) Competitive ELISA showing OTA concentrations (ng/ml) in 10-day culture medium or uninoculated control medium. The mean of two ELISA wells from the same sample is shown for each bar.

cantly different from 1:20 diluted negative control medium (p = 0.23, Fig. 3A). Interestingly, however, after 48 hours of treatment with diluted fungal growth medium, there were fewer S2 cells in every well, clearly visible by light microscopy (data not shown) and measured by a 2-minute sample run by flow cytometry (Fig. 3B). This data suggests that there was a molecule(s) secreted by *A. sclerotiorum* that inhibited S2 cell mitosis. Analysis of the expression of molecular markers specific to phases of the cell cycle, PCNA and Cyclin A, assisted in determining if the reduced cell numbers observed were due to fungus-mediated impaired proliferative capacity.

PCNA expression levels were decreased in S2 cells after treatment with fungal growth medium in all dilutions tested (1:2.5, 1:5, 1:10) compared to uninoculated medium controls, with the largest decrease in PCNA level unexpectedly seen in the most diluted samples (Fig. 4), where cells did not show high levels of cytotoxicity (Fig. 3A). Previous data did show the 1:2.5 and 1:5 doses to be cytotoxic rather than anti-proliferative (Fig. 3A), and the moderate decrease in PCNA expression at those higher doses could have been related to more severe cell injury and cellular stress. The pronounced and unexpected reduction in PCNA at the 1:10 dilution is likely more consistent with a coordinated decrease in proliferative activity than with generalized cytotoxicity. This may be the result of one or more fungal metabolites directly inhibiting cell cycle whose effect can be seen more dramatically when cells are not undergoing cytotoxicity; for instance, more cellular machinery could be intact in these cells that directly or indirectly causes PCNA degradation. More experimentation would be necessary to ascertain this, however. It is also possible that the decrease in PCNA reflects cellular stress rather than direct inhibition of cell cycle by one or more fungal metabolites. PCNA is a conserved DNA clamp protein that acts as a co-factor for DNA polymerases and is essential for the replication of genetic material in cells. It is also used widely as a marker of active DNA synthesis (reviewed in 5). In *Drosophila*, PCNA has been shown to be structurally and functionally similar to mammalian PCNA, with its expression limited only to cells that are actively dividing (21). Therefore, it can be considered an appropriate measure of S-phase activity, and more broadly, overall proliferative activity. This reduction in the expression of PCNA is consistent with the reduced percent viable cells seen in fungal growth medium-treated S2 cells (Fig. 3B).

The decrease in Cyclin A expression could possibly be attributed to a cell cycle phase redistribution within the cell population, resulting in fewer treated cells found in late S/G2-M, or it could indicate a checkpoint or other regulation that reduces Cyclin A abundance. Cyclins are regulatory proteins that activate kinases and are present at different levels during different phases of the cell cycle. This allows them to be used to identify specific points in cell cycle progression (14). Cyclin A binds with Cdk1 to drive the S/G2/early-M progression and is essential for mitotic entry (17). When cells experience replication stress or DNA damage, checkpoint systems are activated to delay the division of damaged DNA, either for repair or programmed cell death. In *Drosophila* S2 cells, this G2/M checkpoint is mainly controlled by the kinase Grapes (Grp), the fly equivalent of human Chk1 (reviewed in 3). However, the Cyclin A decrease observed in this study cannot be assigned to either direct inhibition of Cyclin A by the fungal growth medium versus secondary consequences from cell cycle control mechanisms.

Here we show for the first time that filtered fungal growth medium from *A. sclerotiorum* is cytotoxic to both insect and mouse cells, and that low levels inhibit insect S2 cells from proceeding through the cell cycle. While more work is needed to determine the specific molecule(s) secreted by *A. sclerotiorum* that mediate these effects, this report furthers the understanding of these host-pathogen interactions, and gives us an *in vitro* method for investigating them.

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