THESIS

THE INTERACTION OF FREE-LIVING AMOEBA WITH RICE BACTERIAL AND FUNGAL PATHOGENS

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ABSTRACT

THE INTERACTION OF FREE-LIVING AMOEBA WITH RICE BACTERIAL AND FUNGAL PATHOGENS

Free-living amoebae are ubiquitous microbes found in the soil and water across the globe. Amoebae live a predominantly heterotrophic lifestyle – preying on a variety of organisms including bacteria, fungi and even other protists. Although extensively studied, their potential as a biocontrol for agricultural pathogens is largely unexplored. As many pathogens occupy the same habitat as amoeba, we investigated their interactions as a first step to determine if amoeba are possible biocontrol agents. Our research focuses on two important pathogens of rice, the bacteria *Xanthomonas oryzae* and the fungus *Rhizoctonia solani*.

Much of this thesis centers on the interaction between amoebae and *X. oryzae*, which is explored in depth and presented in the first chapter. Experimentation involved five common amoebal species and two highly virulent *X. oryzae* pathovars. Microscopy and vitality assays of amoebae-bacteria co-cultures first established that *X. oryzae* does not grow or dies in the presence of our amoebae. On the other hand, amoebae are not adversely affected, with most cells remaining alive in the metabolically active trophozoite form. Although the bacteria are harmed in this interaction, it is likely not through phagocytosis, the most common and well-studied tactic that amoeba use to feed. Observations made through confocal microscopy revealed that *X. oryzae* was rarely detected inside amoebae. Furthermore, lysis of amoebae after exposure to

bacteria did not yield any viable bacteria, suggesting that either bacteria are rarely internalized and/or that *X. oryzae* does not survive in the amoeba cell. Conversely, amoebal trophozoites have no impact on the biofilms of *X. oryzae* either. These data indicate that amoeba do not directly or physically interact with *X. oryzae*. Instead, our amoeba-conditioned media assays reveal that amoeba alter the media and render it harmful to *X. oryzae*. The most likely scenario is that amoeba secrete a bactericidal agent into their surroundings. At this time, we have yet to isolate or identify the compound, but its presence may prove to be a boon with a variety of applications.

The dynamics between amoeba and *R. solani* were not studied as extensively, but the basic interaction is presented in the appendices of this thesis. Again, five amoeba species were incubated with mature *R. solani* mycelia. First, co-cultures were observed with a compound microscope. *Acanthamoeba* and *Dictyostelium* did not have any effect on the fungi. *Acanthamoeba* species physically associated with the mycelia, but also rapidly encysted – suggesting some antiprotozoal activity from *R. solani*. *D. discoideum* had no interaction with the fungi: the trophozoites did not attach to the mycelia and neither cysts nor spore-forming bodies were seen. *V. vermiformis* was the only amoeba with some effect on the fungi. In co-cultures, fungal mycelium developed a shriveled and wrinkled morphology. *V. vermiformis* was attached to the fungi and most amoebae remained as viable trophozoites. *V. vermiformis* and its interaction with *R. solani* was further examined using scanning electron microscopy, which further corroborated the light microscope observations. While the reason/effect of the shriveling is unknown, it is a potential avenue for further experiments.

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LIST OF ACRONYMS

Xoo	*Xanthomonas oryzae* pathovar *oryzae*
Xoc	*Xanthomonas oryzae* pathovar *oryzicola*
Rs	*Rhizoctonia solani*
Ac	*Acanthamoeba castellanii*
Al	*Acanthamoeba lenticulata*
Ap	*Acanthamoeba polyphaga*
Dd	*Dictyostelium discoideum*
Vv	*Vermamoeba vermiformis*
CV	Crystal violet stain
PAS	Page's modified Neff's amoeba saline
PSA	Peptone sucrose agar
PS	Peptone sucrose broth
PDA	Potato dextrose agar
FLA	Free-living amoeba(e)
MOI	Multiplicity of infection
pv	Pathovar
cv	Cultivar

INTRODUCTION

As the global population continues to climb, the food producers of the world must increase their output to meet new demand – a solution that is not the easiest to implement. One of the largest obstacles producers face are plant pathogens that reduce yield quality and amount. An estimated 20 to 40% of all crops produced are ultimately lost due to pathogens (1). Currently, the two most commonly used counter-measures are application of chemical controls and integration of pathogen resistance into the plants themselves. There are limits to these techniques; however, chemical controls require re-application and have difficulty reaching deep, persistent bacterial pathogens while resistant varieties are often based on single-gene mechanisms, which can be overcome by the targeted pathogen (2, 3). It is especially important to identify novel ways to combat pathogens for staple crops, such as rice (*Oryza sativa*).

Rice is the most significant staple food crop in the world. Statistics compiled by the International Rice Research Institute, Africa Rice Center, and International Center for Tropical Agriculture reveal just how vital this crop is. Over half the world relies on rice as part of their daily diet, especially a large portion of impoverished households. Rice farming ranks as one of the highest in terms of land usage for food production and for economic activities, producing nearly 700 million tons annually (4). As with other agricultural systems, rice production is constantly under duress from pathogens. Three major diseases of rice are bacterial blight, bacterial leaf streak and rice sheath blight (5-8).

Xanthomonas oryzae is a gram-negative bacterium and the causative agent of two distinct and severe diseases on rice. *X. oryzae* pathovar (pv.) *oryzae* colonizes the vasculature and causes bacterial blight while pv. *oryzicola* inhabits the intracellular space of the mesophyll and leads to bacterial leaf streak (9). Both pathovars are of major concern to rice production as 10 to 20% of rice crops are commonly lost, and up to 50% yield is lost in severe outbreaks (10). Due to its impact on a staple crop, *X. oryzae* is placed under extensive international quarantines and is designated as a Select Agent by the U.S. Animal and Plant Health Inspection Services (11). For the rice-*X. oryzae* pathosystem, there are no known broad-spectrum means of control against this pathogen.

Rice sheath blight is caused by the fungus, *Rhizoctonia solani*. *R. solani* is further divided into subgroups based on its propensity to form anastomoses and display similar host ranges (12). Individuals from the same group can connect and fuse mycelia, even from different points of origin. Anastomosis group 1 (AG1) is the largest threat to cereal crops and is found world-wide (13). As a persistent and virulent, soil-borne pathogen, *R. solani* is also difficult to control and can reduce yields by half in ideal conditions for pathogen survival and growth (6). *R. solani* persists in soil and in fields by use of sclerotia, condensed bodies of fungal hypha that may survive in soil for up to two years (6, 14). Additionally, the broad host spectrum of *R. solani* allow the fungus to persist in alternative hosts for several seasons (15). Compounding the issue of pathogen persistence is that there are currently no resistance genes identified for *R. solani*, although potential quantitative trait loci that increase plant resistance have been identified (14, 16, 17). To reduce instances and severity of outbreaks from *X. oryzae*

and *R. solani*, additional methods of control must be implemented. To that end, we propose the use of free-living amoeba as a biological control for these two pathogens.

Free-living amoeba are natural inhabitants of soil and water. The majority of amoeba species are heterotrophic and prey on bacteria, fungi, and other protozoa (18, 19). This natural bacterial antagonism combined with their overlapping habitat with many pathogens makes free-living amoeba a candidate as a biocontrol agent. The antibacterial activities of many amoeba and their deployment in model environments have been validated in the laboratory and in model environments (18, 20-23). Perhaps most well-known is the ability of amoeba to engulf and digest bacteria to feed, in a process resembling phagocytosis in human macrophages (24, 25). In addition to phagocytosis, amoebae can kill bacteria through direct contact or by secretion of antimicrobials. The genus *Acanthamoeba* secretes proteases and other compounds capable of killing a variety of bacteria (26, 27). *Naegleria* and *Entamoeba* species lyse bacteria shortly after contact, using membrane proteins to puncture bacterial membranes or by triggering host-cell caspases (28, 29). Unsurprisingly, these interactions do not always favor amoeba. Bacteria and fungi can defend themselves by killing amoeba or forcing amoeba into their dormant cyst morphology, in other cases amoebae are used as a reservoir for intracellular pathogens (30-34). Given the broad spectrum of possible interactions, it was imperative to establish the basic dynamics between the two plant pathogens and amoeba as a first step.

The experiments listed herein involve two pathovars of *X. oryzae*, one strain of *R. solani*, and their interactions with five amoeba species: *Acanthamoeba polyphaga, A. lenticulata, A. castellanii, Dictyostelium discoideum* and *Vermamoeba vermiformis*.

These amoeba species are commonly used in laboratory studies and found in soils worldwide, even in rice paddies (35-38). To our knowledge, there are no described interactions between *X. oryzae* or *R. solani* with these five amoeba species. Some similar studies suggest an interface, however. Habte and collaborators (39) inoculated *Xanthomonas campestris* into soils occupied by free-living protozoa and determined that bacterial numbers declined, although the predator in this interaction was never identified. Homma and Ishii also added *R. solani* hyphae into soil samples and determined that a mycophagous amoeba or amoebae punctured the hyphal cell wall to feed on cytoplasmic contents (40). Again, the amoeba species was not identified.

Amoebae may also benefit the plants themselves indirectly through their feeding behaviors. Rhizosphere studies of rice and *Arabidopsis thaliana* in combination with *Acanthamoeba castellanii* showed marked differences in microbiome composition, root architecture, and increased overall biomass of the plants (22, 41). It is thought that grazing by protozoa contributes greatly to the exogenous nitrogen pool, thus it is not surprising that amoebae influence plant development (42). It's likely a complex dynamic between plants, bacteria and microfauna (43), but the interplay is complicated through all of the possible interactions (44).

Scope of thesis.

The diverse interactions of amoebae with microbes in the soil and environment suggest that amoebae might be manipulated for control of plant pathogens of rice.

In this study, I describe the dynamics between two pathovars of *X. oryzae* and their interaction with five amoeba species: *Acanthamoeba polyphaga, Acanthamoeba lenticulata, A. castellanii, Vermamoeba vermiformis* and *Dictyostelium discoideum*. I

began by incubating the amoebae and bacteria together, periodically assaying the population counts and morphology of the two organisms. Additionally, the biofilms of *X. oryzae* were exposed to amoebae and assessed for amoebic grazing. I concluded that the amoebae do not ingest the bacteria but that the cell-free supernatants of amoeba cultures are sufficient to kill bacteria. While unexpected, the bactericidal effect of the amoebae establishes their potential as a biocontrol against *X. oryzae*.

In a similar process, amoebae and *R. solani* co-cultures observed under light and scanning electron microscopy revealed diverse reactions. *Acanthamoeba* species physically associated with the fungi, but rapidly encysted and had no adverse effects on the fungi. *D. discoideum* did not interact with the fungi at all, displaying neither attachment nor formation of spore-forming bodies. Finally, *V. vermiformis* attached to the fungi and caused a shriveling of the mycelia, however the significance is yet to be determined.

Chapter I: Free-living amoeba and *Xanthomonas oryzae*

Introduction

Free-living amoeba (FLA) are ubiquitous microorganisms found in soil and water across the globe. Amoebae live a predominantly heterotrophic lifestyle – preying on a variety of other microorganisms such as bacteria, fungi and even other protists. Amoebae can directly and indirectly impact plants. For example, *Acanthamoeba castellanii*, when added to the rhizospheres of rice or *Arabidopsis thaliana,* markedly changes the plants' microbiome composition and root architecture, and increases dry biomass (22, 41). Amoebae also influence plant development, likely through increases in the soil nitrogen pool (42).

Bacteria-amoeba dynamics are complex and nuanced. Amoebae can greatly alter bacterial community composition and structure due to their prolific grazing and prey selectivity (43, 45). Not all bacterial species are preyed upon equally; bacteria have evolved a variety of defense mechanisms to deter amoebal feeding. Some bacteria use biofilms to shield themselves from feeding, while others directly lyse amoeba, either after internalization or through secretion of toxic factors (18, 46, 47). Other bacterial species prevent lysis after phagocytosis, and, in fact, exploit the amoeba as a reservoir or host (33, 48, 49).

Amoebae-bacteria interactions have been described for diverse combinations, including interactions with both animal and plant bacterial pathogens (19, 21, 33, 50-52). In cases where amoebae detrimentally affect the bacteria, it may be possible to exploit that relationship to reduce bacterial loads in a system. For example, use of

amoebae to reduce populations of plant pathogenic bacteria has been proposed as a form of biocontrol (53-55). However, due to the interaction specificity of the amoebae-bacteria combination and the large amount of work required to describe those interactions, general themes for outcomes have not yet been identified.

Bacterial pathogens of major importance but with no described interactions with free-living amoeba are the rice (*Oryza sativa*) pathogens within the species *Xanthomonas oryzae. X. oryzae* are gram-negative bacteria and the causative agents of two distinct and severe diseases on rice. *X. oryzae* pathovar (pv.) *oryzae* colonizes plant vasculature and causes bacterial blight while pv. *oryzicola* inhabits the spaces between mesophyll cells and leads to bacterial leaf streak (9). Both pathovars are of major concern to rice production as 10 to 20% of a crop is commonly lost; in severe outbreaks crop loss may total 50% (10). This devastation is a salient issue as rice is one of the most important food crops, feeding over half of the world's population and a majority of the impoverished (4). Due to its impact on a staple crop, *X oryzae* is regulated by extensive international quarantines and is designated as a Select Agent by the U.S. Animal and Plant Health Inspection Services and the Centers for Disease Control (11). The most common method to minimize disease impacts is the deployment of resistant rice varieties. However, resistant varieties have only been developed against *X. oryzae* pv. *oryzae,* and this pathogen readily evolves to overcome host resistance mechanisms (56-58). To date, affordable, effective and sustainable chemical controls are not available (7). As such, alternative strategies to control the diseases are needed.

There are several reasons to consider the interface between amoebae and *X. oryzae* for pathogen control. Both organisms are found in soil, plant debris or on the surfaces of plants (8, 43, 51, 59), thus it is plausible that there is contact between the amoebae and the bacteria. As detailed above, there are several possible scenarios for outcomes following amoebae-bacteria contact, but these are not known for *X. oryzae* – amoeba interactions. Previous work showed that populations of a closely related bacteria, *X. campestris,* decline when the bacteria are inoculated into soil occupied by protozoa (39), but the authors did not identify which protozoa or other soil factors were responsible for the effect. Given amoebal dietary preferences and the various strategies bacteria use to avoid predation by amoeba, our goal was to characterize the interactions of five different amoebae, *Acanthamoeba polyphaga, A. lenticulata, A. castellanii, Vermamoeba vermiformis* and *Dictyostelium discoideum* with the two pathovars of *X. oryzae*. Based on interactions with other bacteria, we hypothesized that the interactions would be antagonistic, with bacterial numbers being reduced in the presence of amoebae. However, our studies demonstrated that the tested amoeba were bactericidal or bacteriostatic, and that conditioned media from amoeba cultures were sufficiently lethal to *X. oryzae*.

Materials and Methods

Amoeba and *X. oryzae* culturing

A. polyphaga Linc-AP1, *A. castellanii* ATCC 30234, and *A. lenticulata* ATCC 30841 were cultured at 28°C in a modified PYG media (ATCC medium 712, pH of 6.9). *V. vermiformis* ATCC 50237 was cultured at 28 °C in a modified PYNFH media (ATCC medium 1034, pH of 6.4) and *D. discoideum* ATCC NC4A1:DBS0236602 was

maintained at room temperature in a modified HL5 media (pH 6.7); media were modified according to Wheat et al. (25). Amoeba cultures were inoculated into 100 x 15 mm petri dishes with 30 mm walls holding 10 mL of media supplemented with 1x Gibco penicillin/streptomycin (Invitrogen; Carlsbad, CA, USA) from frozen stocks. Once initial cultures reached turbidity, *Acanthamoeba* species and *V. vermiformis* were passaged every 5 days by transferring 500 µL of culture into 10 mL of fresh media. *D. discoideum* were passaged every 3 days. Amoeba cultures were used for only three passages before disposal.

X. oryzae pvs. *oryzae* (strain PXO99^{A}) and *oryzicola* (strain BLS256) were maintained on peptone sucrose agar media (PSA; 10 g/L Bacto peptone, 10 g/L sucrose, 1 g/L monosodium glutamate, and ± 16 g/L Bacto agar) at 28 °C. Bacteria were streaked onto PSA from frozen glycerol stocks and cultured for two to three days before use.

<u>Co-culture Kinetics</u>

Confluent cultures of amoeba were starved overnight in diluted media at the temperatures described above, except for *D. discoideum*, which did not survive incubation in the diluted media. *Acanthamoeba* were starved at 1/5 strength PYG diluted with Page's modified Neff amoeba saline (PAS) while *V. vermiformis* was starved in 1/2 PYNFH media diluted with PS broth (25). Amoebal cell density was calculated using a direct cell counting method involving trypan blue exclusion and a hemocytometer. Only cultures consisting of at least 90% viable trophozoites were used. Amoeba cultures were adjusted to concentrations of $2{\times}10^5$ cells/mL.

X. oryzae cells were suspended into 1X phosphate buffered saline (PBS; 8.0063 g/L NaCl, 0.2013 g/L KCl, 1.4916 g/L Na_2HPO_4, and 0.245 g/L KH_2PO_4) and washed three times with centrifugations at 2500 x G for 5 min to remove extracellular polysaccharide and biofilm. After washing, cells were suspended to an OD600 of 0.2, roughly 3×10^7 CFU/mL.

Amoeba and *X. oryzae* co-cultures were prepared in 1.5 mL microcentrifuge tubes at an amoeba-to-bacteria cell ratio of 1:10. The final volume of co-cultures per tube equaled 500 µL with approximately 1×10^5 trophozoites and 1×10^6 CFU bacteria. Controls with a single species in the starvation media were included and triplicates of each combination were prepared for processing at 0, 4, and 24 h. At the designated sampling times, cultures were spun at 150 x G for 2 min to pellet amoeba. The supernatant was removed for viable cell count assays of *X. oryzae*. The amoebic pellet was washed once with 1 mL of PAS, spun at 150 x G for 2 min, then suspended in 500 µL PAS supplemented with 30 µg/mL gentamicin for 1 h to lyse any extracellular *X. oryzae* adhering to the amoebae (50). The amoebic pellet was then washed three times in the conditions described above, and then suspended in 200 µL of PAS. Amoeba were disrupted by passaging through a 27-gauge syringe seven times and lysate was used in viable cell count assays of *X. oryzae* internalized inside amoeba (60).

Co-culture supernatants and amoeba lysates were assayed for live *X. oryzae* by ten-fold serial dilutions down to 1×10^{-6}. 10 µL of the original fraction and all dilutions were plated on PS agar in technical triplicates, and, after two days at 28 °C, the bacterial colonies were counted.

Confocal microscopy

Amoeba and bacterial cultures were prepared similarly to the kinetic assays. Additionally, *X. oryzae* cells were stained with the LIVE/DEAD BacLight cell viability kit (Invitrogen L7007) following removal of biofilm. Co-cultures were prepared in the same manner as described above and samples were processed at 0, 4, and 24 h. Cultures were spun at 150 x G for 2 min and supernatant was discarded. The amoebal pellet was treated with 100 µg/mL gentamicin, and after 1 h, washed three times. Cells were fixed using 100 µL 4% paraformaldehyde (w/v; dissolved in PBS). Immediately after fixative addition, 25 µL aliquots of each replicate were pooled together and 75 µL 0.4% trypan blue was added to determine viability of amoebae. Samples were fixed for 48 h at 4 °C in the dark, then washed once and suspended in 100 µL PAS. Fixed samples were stored up to 2 weeks at 4 °C in the dark.

Amoebae were imaged for internalized *X. oryzae* on a Zeiss LSM510 inverted confocal laser scanning microscope. Samples were excited with a 488 nm laser and emission filters were set to 480 nm and 590 nm for Syto9 and propidium iodide, respectively. At a 630x magnification, three random fields were taken per sample and images were taken at ten different depths in 0.5 - 1.5 µm increments. Images were compiled into one using the Zeiss Zen 2009 software. Total amoeba, number of encysted amoebae, and the number of amoebae with internalized fluorescent signals were recorded.

Samples fixed concurrently with trypan blue were imaged on a Zeiss Axioskop light microscope. Samples were added to a hemocytometer and counted for number of live and dead amoeba.

Conditioned media assays

Amoeba culture densities were calculated using a hemocytometer and 0.4% trypan blue and cultures were adjusted to 5×10^4 cells/mL. Amoeba cultures were aliquoted into 15 mL conical tubes with 5 mL of culture in each, one conical tube held an aliquot at 5×10^5 cells/mL for the high density amoeba-only conditioning treatment. Amoeba aliquots were spun at 200 x G for 5 min and supernatant was discarded. Cells were suspended in 1X culturing media, except for *V. vermiformis*, which was suspended in a 1:1 mix of PYNFH and PS broth.

Washed *X. oryzae* pv. *oryzae* was added to one tube of amoeba at an amoeba-to-bacteria ratio of 1:10 (approximately 5×10^5 CFU). *X. oryzae* pv. *oryzicola* was added in the same manner to another aliquot of amoeba. The four conditioning cultures (low and high density amoeba-only and two amoeba + *X. oryzae*) were incubated at 28 °C, except for *D. discoideum,* which was incubated at room temperature. After 48 h the cultures were spun at 1000 x G for 5 min. The supernatant from each was passed through a cellulose acetate syringe filter with 0.22 µm pores (VWR #28145-477; Randor, PA, USA). An aliquot of each was tested for pH levels.

Fresh *X. oryzae* cells were washed three times in 1X PBS to remove biofilm and suspended to an OD600 of 0.1. 10 µL of *X. oryzae* and 190 µL of conditioned media were seeded into a 96-well microplate. High-density conditioning media supplemented with fresh media were mixed in a 1:1 ratio prior to addition and diluted fresh media controls were included. Samples were prepared in triplicate for sampling at each time point at 4 and 24 h, two samples of each pathovar in fresh media and in HD conditioned media were sampled at 0 h to establish initial bacterial density. Microplates were

incubated at 28 °C and samples were diluted and plated for viable bacteria as described in the co-culture kinetics.

Crystal violet staining of biofilms

Suspensions of both *X. oryzae* pathovars were adjusted to OD600 of 0.5; bacteria were not washed in order to retain their biofilm. Aliquots (150 µL of each pathovar) were added to an untreated, polystyrene 96-well microplate (Corning #3370) in replicates of 24. The plate was enclosed in a sealable plastic bag and incubated at 28 °C for 24 h to allow bacteria to form biofilms.

Amoebae were starved overnight in diluted media as performed for the co-culture kinetics (except for *D. discoideum*). Amoeba cell density was calculated using 0.4% trypan blue and a hemocytometer. Aliquots of amoeba cultures were generated at concentrations of 1×10^5, 1×10^3, and 1×10^1 cells/mL by spinning and resuspending in fresh starvation media. *X. oryzae* liquid cultures were removed from the microplate, leaving the ring-shaped biofilm and associated bacteria. 200 µL of each amoeba concentration was added to the biofilms in replicates of six, and fresh media controls were added as well. The microplate was sealed in a plastic bag and returned to a 28 °C incubator.

Biofilms were exposed to amoeba for 24 or 48 h, at which point an adapted crystal violet staining method was applied to quantify remaining biofilm (61). Briefly, after removal of liquid cultures and rinsing with 200 µL of distilled water, the biofilm was treated with 200 µL of 0.5% crystal violet (w/v; dissolved in 10% ethanol) per well. After 15 min, crystal violet was removed, the wells were rinsed once with 150 µL of distilled water, and the remaining crystal violet was dissolved with 200 µL of 90% ethanol. The

microplate was agitated at medium intensity for 2 min on a Biotek Powerwave HT plate reader, and absorbance at 570 nm was recorded.

Results with Discussion

X. oryzae survival is reduced in the presence of amoeba trophozoites

Amoeba trophozoites and *X. oryzae* bacterial cells were co-cultivated to determine the impact on bacterial populations and amoebal morphology and survival over time. Amoebae species, except *D. discoideum,* were incubated in diluted media overnight prior to co-cultivation to encourage phagocytosis. *D. discoideum* was incubated in full strength media prior to co-cultivation because it did not survive starvation induced by diluted media. Nine of the ten amoeba-*X. oryzae* combinations resulted in significant growth disparities over time (**Figure 1**). *A. lenticulata*, *A. polyphaga*, *V. vermiformis* and *D. discoideum* were bactericidal, with significant reductions in bacterial numbers (CFU) after 24 h (Tukey's test, $p < 0.05$). *A. lenticulata* and *V. vermiformis* displayed the strongest effects against the bacteria, as bacteria were undetectable at 24 h in some cases. The rate of bacterial cell death varied between amoeba species and *X. oryzae* pathovar, and occasionally between replicates of the same amoebae/bacteria combination; however, the trends remained consistent. In co-cultures with *A. castellanii*, bacterial numbers at 24 h did not differ significantly from initial densities, indicating a bacteriostatic effect from the amoeba.

In these studies, the proportion of dead or encysted amoebae was the same over the 24 h experiment (**Table 1**). About 5% cysts and dead trophozoites were observed at 24 h, but this did not differ from observations during standard culturing phases. *Acanthamoeba* and *Vermamoeba* species generally encyst in adverse conditions, such

as extreme temperatures, lack of food, or conflict with other microorganisms (62, 63). The unchanged morphology and viability of the amoebae suggests that *X. oryzae* has no deleterious effect on these FLA. Conversely, these data support that the strains from all five amoebal species were antagonistic to both bacterial pathovars.

X. oryzae are rarely consumed by amoebae

Protozoa are known to preferentially feed on gram-negative bacteria, but not all gram-negative bacteria are affected in the same way (45, 64). In many cases, amoebal trophozoites ingest bacteria as whole cells during feeding (24, 63). To determine if *X. oryzae* are ingested by any of the five amoeba species, at 4 and 24 h after amoeba were incubated with *X. oryzae* cells, gentamycin was added to lyse bacteria remaining outside the amoeba (**Figure 2**) (65). Very few amoebae (< 8%) were observed with internalized, stained *X. oryzae* for the three *Acanthamoeba* species, and even fewer for *V. vermiformis* (3.3%) and *D. discoideum* (2.3%). The rare bacteria that were observed inside amoeba remained in the cytosol, rather than in digestive food vacuoles. Viable bacteria were not detected in lysates of amoebae after co-culture with *X. oryzae,* suggesting that *X. oryzae* does not survive inside amoeba. Stained *X. oryzae*-only controls remained fluorescent after over two weeks of incubation and fixation and up to an additional week in storage at 4 °C, demonstrating the lack of signals was not from dye degradation. Stained *X. oryzae* cultures grew at similar rates to unstained cultures, suggesting the dye does not have effects on bacterial growth and survival.

The lack of internalized bacteria suggests phagocytosis is not the primary method of bacterial antagonism. The low rates of bacterial consumption were likely not caused by inhibition of amoebic activity or by antagonism of the amoebae by *X. oryzae*,

because trypan-blue staining of amoebae did not show amoeba cell death. Additionally, we did not observe any diffuse signals indicative of fluorescent bacteria being digested (24). The amoebae may be engulfing and rapidly digesting the bacteria, but that scenario is unlikely as bacterial populations were unchanged for the first 4 h of co-culturing in most interactions (**Figure 1**). In conjunction, these results suggest that amoebae lyse *X. oryzae*, but not through phagocytosis. Without internalization, *X. oryzae* is likely incapable of using the amoebae as a reservoir to benefit itself, as has been observed for some bacteria (25, 48, 49).

Amoebae do not degrade *X. oryzae* biofilms

In a natural context, *X. oryzae* may use a biofilm and exopolysaccharides to prevent predation by FLA (46, 66). Using crystal violet assays, we found that most amoebae did not have an effect on the integrity of the *X. oryzae* biofilms. Of the five amoeba species, only *A. lenticulata* significantly degraded biofilms compared to fresh media controls (**Figure 3** and **Figure A1**), but only to a minimal degree.

The other four amoeba species do not reduce biofilm, which suggests that the biofilm serves as a form of protection against amoebic predation. Future work should include quantification of bacterial populations inside the biofilm after addition of amoebae.

FLA cell-free supernatants of some FLA are bacteriostatic or bactericidal *X. oryzae*

FLA are known to secrete compounds that lyse and/or kill bacteria (26, 67, 68). To explore the possibility that amoebae release factors capable of killing *X. oryzae*, bacterial cells were inoculated into cell-free media previously used to culture amoeba, which we designate as conditioned media. Media conditioned with low densities of the

five amoebae were suppressive or toxic to *X. oryzae* cells, because bacterial cell numbers either grew to a smaller maximal density or decreased (**Figure 4**). The suppressive effect was stronger in media conditioned with a high density of amoeba. In nine of the ten amoeba – *X. oryzae* combinations, the high-density conditioned media did not yield any bacterial colonies. The pronounced effect from the high-density media suggests that the bactericidal result is directly proportional to the concentration of the conditioning culture.

To test if nutrient deprivation in the conditioned media affected bacterial numbers, we included fresh media supplements into the experiment (**Figure 4**). The addition of fresh media may also indicate if the bacterial killing effect can be reduced by dilution. While conditioned media from each amoeba species largely had the same effect on both pathovars, only high-density conditioned media from *A. castellanii* and *V. vermiformis* remained bactericidal to *X. oryzae* pv. *oryzae* after fresh media was added. In some cases, fresh media supplements to the high-density amoebal conditioned media diluted or negated the bactericidal effect. The reduced or abolished bactericidal effect from the fresh media supplement indicates that nutrient deprivation may partially be the cause of harmful effects of *Acanthamoeba* on *X. oryzae*.

FLA species may also secrete different antimicrobial factors into their surroundings (26, 68, 69), and the fresh media supplements may dilute the factor and/or allow *X. oryzae* to grow at sufficient rates to overcome cell death in the case of *Acanthamoeba* species. On the other hand, fresh media supplements did not abolish the bactericidal effects from *D. discoideum* and *V. vermiformis*, suggesting that nutrient deprivation is not a cause for those two species. Another possibility is that the amoebae

secrete different compounds altogether, and the impact of each is altered to varying degrees by dilution with the fresh-media supplements. Identification of the factors involved in bacterial toxicity may clarify the differences we observe in conditioned media treatments, but is beyond the current scope of this work.

Our results suggest that amoebae constitutively secrete an antibacterial factor. In the co-culture assays, bacterial cell density was reduced for most amoeba-bacteria interactions in the first 4 h (**Figure 1**). To determine if the bactericidal response is stimulated by the presence of *X. oryzae*, we included a conditioning treatment of low-density amoeba with *X. oryzae* at a ratio of 1:10. Media conditioned with amoebae and with or without *X. oryzae,* both caused bacteria loss in most amoebae and pathovar combinations (**Figure 4** and **Figure A2**). Therefore, amoeba likely do not require the presence of *X. oryzae* for antimicrobial production.

We also observed bacterial loss at roughly the same rates in the conditioned media assays and co-culture assays. *A. lenticulata*, *A. polyphaga* and *V. vermiformis* significantly reduced bacterial density starting at 4 h in both assays, while *A. castellanii* did not have significant effects on pv. *oryzicola* until 24 h. The major discrepancy between the conditioned media assays and the co-cultures is from *D. discoideum*. This amoeba was bactericidal beginning at 4 h in the conditioned media assays, but had no significant impacts at the same time point in the co-cultures. However, both experiments are designed with different amoebal incubation times and densities, likely changing how much of the antibacterial agent was produced and secreted.

Conclusion

We identify amoeba species that suppress multiplication or kill cells of two important rice bacterial pathogens, *X. oryzae* pvs. *oryzae* and *oryzicola.* The mechanisms for suppression or killing are not due to phagocytosis, but are most likely due to the amoeba's ability to secrete toxic or inhibitory compounds. Our findings present a previously unknown dynamic between two microorganisms that likely encounter one another in the phytobiome. Future studies should explore the potential of these amoebae as biocontrol agents in the field.

Table 1. Summary of confocal microscopy results.

	Co-cultivation with *X. oryzae* pv. *oryzae*				
Amoeba	*A. lenticulata*	*A. polyphaga*	*A. castellanii*	*D. discoideum*	*V. vermiformis*
With internal bacteria	4.5%	7.5%	0.0%	2.3%	3.3%
Encysted (0h/24h)	6.9/0.6%	8.7/2.5%	3.9/4.1%	2.7/0.0%	5.2/6.1%

Amoeba with internalized bacteria after 24 h of co-culture and summary of amoebal morphology at 0 and 24 h after co-cutlure. Only data for *X. oryzae* pv. *oryzae* is shown.

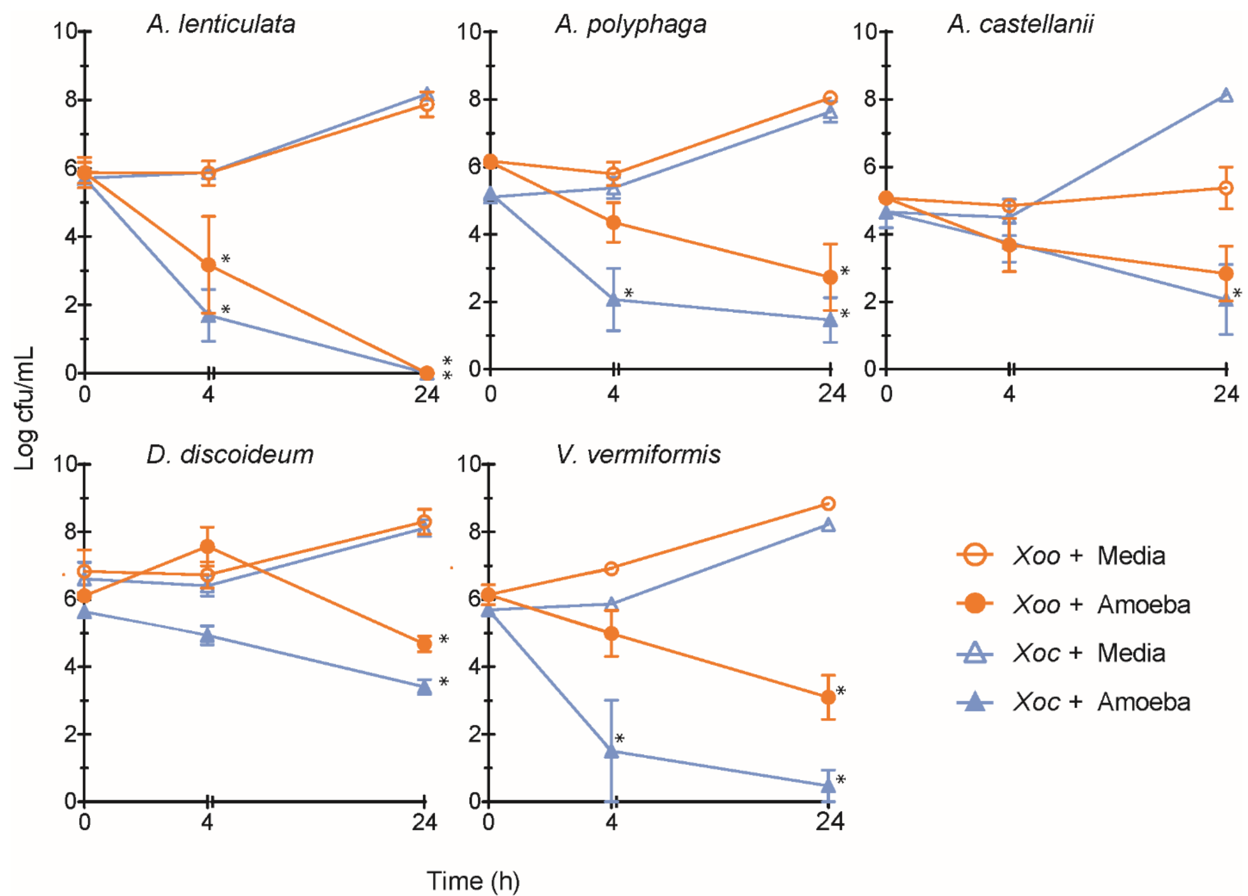

Figure 1. Populations of *X. oryzae* pvs. *oryzae* and *oryzicola* decline or remain static when co-cultured with amoeba trophozoites.
Trophozoites were cultured with *X. oryzae* cells and bacterial numbers were assessed at 0, 4 and 24 h. Data from at least four biological replicates were log transformed before calculating the means and standard error bars. * denotes $p < 0.05$ and ** denotes a $p < 0.01$ compared to initial CFU/mL values at 0 h. Statistical significance tested using a two-way ANOVA (Tukey test) on the log-transformed data.

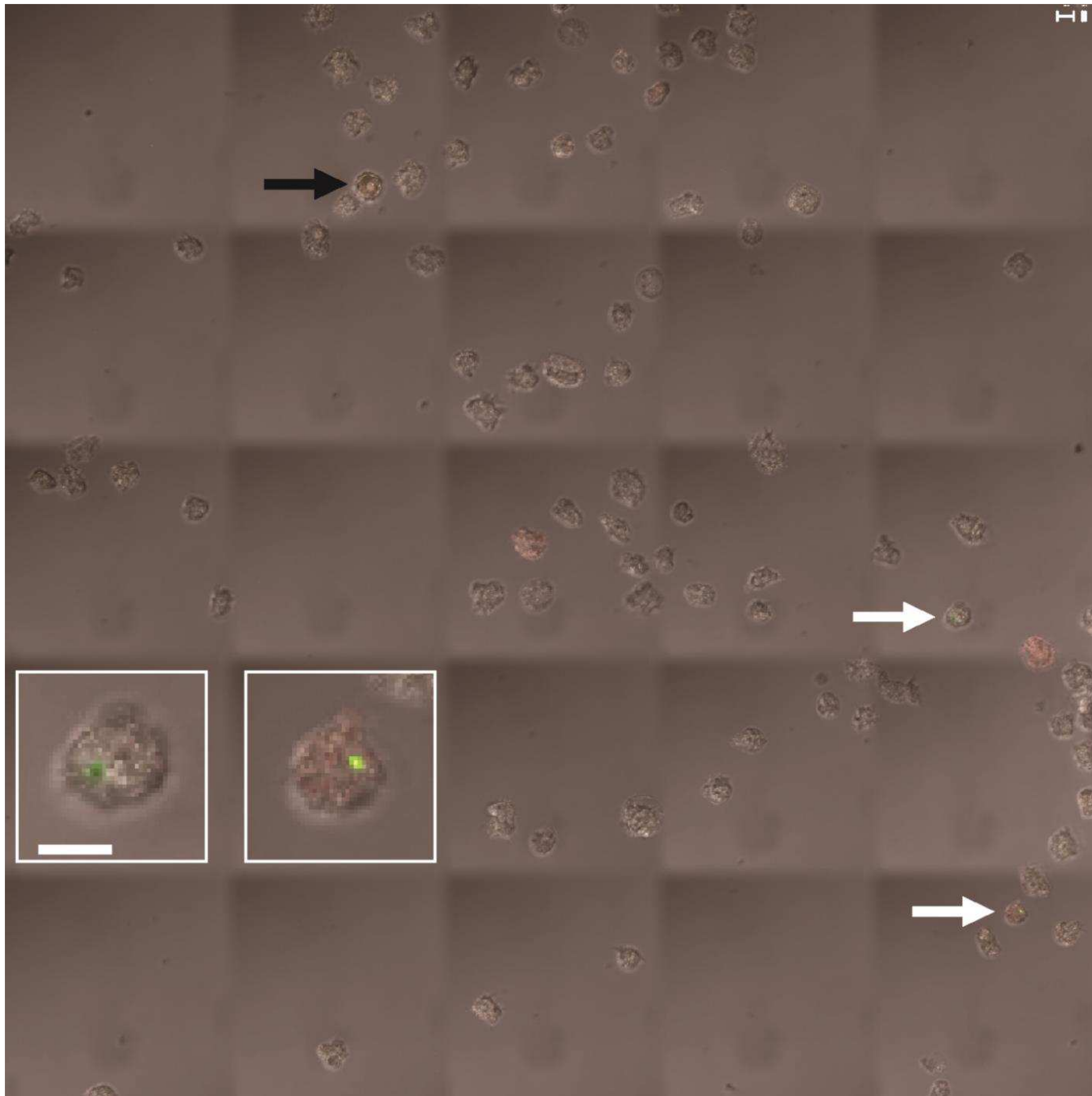

Figure 2. Representative confocal tile scan demonstrating rare internalization of *X. oryzae* by amoebae (*A. castellanii* depicted).
Two instances of internalized bacteria are indicated by white arrows and magnified in the insets. Magnification = 630x, scale bars are 10 µm in length. Merged channels for propidium iodide and Syto9 are shown. Composite image built from replicate scans and processed into a maximum intensity projection by the Zeiss LSM software. Black arrows indicate trophozoites with a single Syto9-stained *X. oryzae* pv. *oryzae* cell. The presence of bacteria does not force encystment (cyst indicated by black arrow). For each amoeba/bacteria combination, the experiment was repeated three times, and all experiments had similar results.

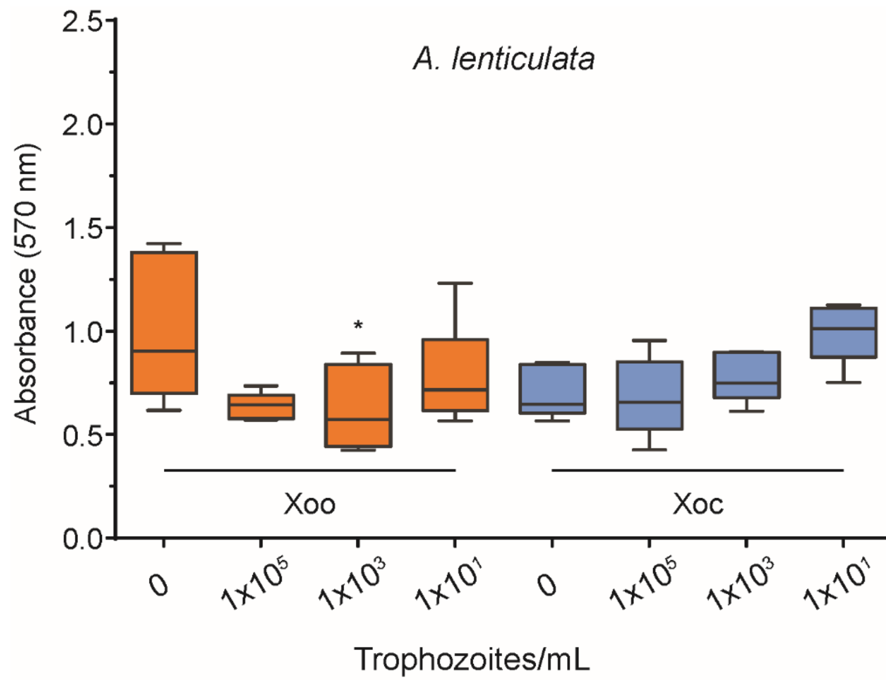

Figure 3. *A. lenticulata* trophozoites cause negligible changes in pre-formed *X. oryzae* biofilm.
Graphs are calculated from six biological replicates per box plot. * denotes $p < 0.05$, significance calculated using Tukey test in a one-way ANOVA.

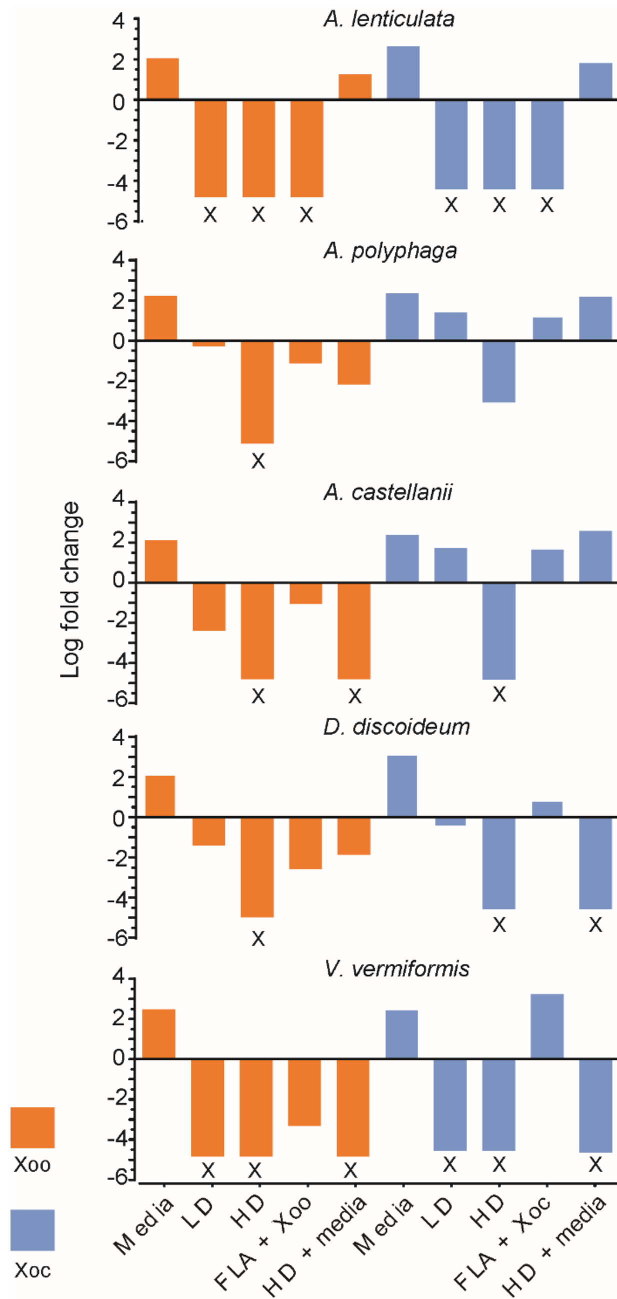

Figure 4. The filtered supernatant from some amoeba cultures are bacteriostatic or bactericidal to *X. oryzae.*

Representative experiments are presented for each amoeba species. LD = low density conditioning culture; LD + Xo = conditioning culture with low density amoeba and *X. oryzae* at 1:10 ratio; HD = high density conditioning culture; HD + fresh media = HD treatment supplemented with fresh media in a 1:1 mix, final concentration of fresh media supplement equals fresh media only control. Black X's denote no bacteria could be cultured. Fold change is calculated as the log(CFU/mL) at 24 h over log(CFU/mL) at 0 h. Each treatment was performed in biological triplicates.

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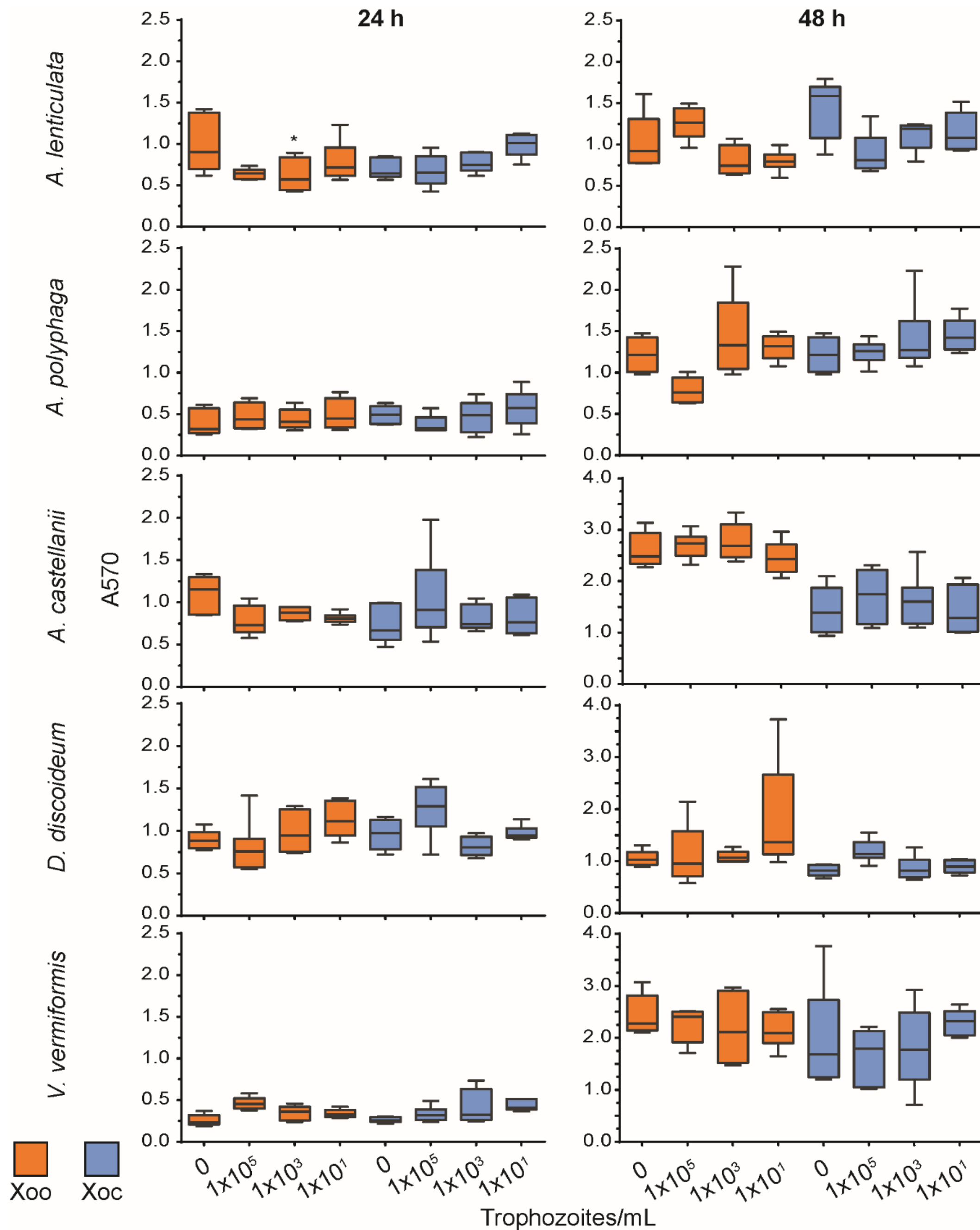


Figure A1. Crystal violet assays following *X. oryzae* biofilm exposure to amoeba trophozoites.
Graphs are calculated from a representative experiment with six biological replicates per box plot. * denotes $p < 0.05$, significance calculated using Tukey test in a one-way ANOVA.

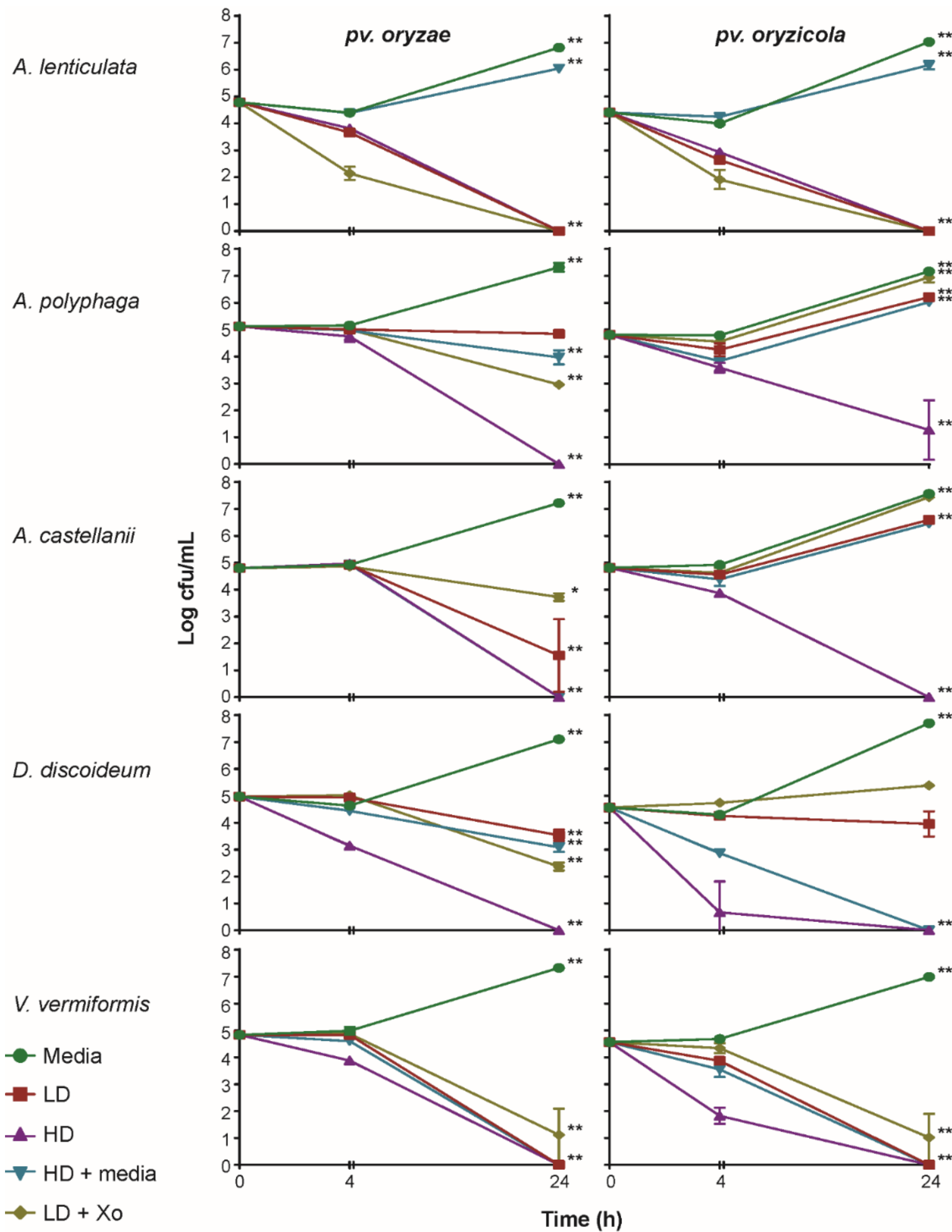


Figure A2. **Log cfu/mL of *X. oryzae* in the conditioned media assays, plotted over time.**
LD = low density conditioning culture; LD + Xo = conditioning culture with low density amoeba and *X. oryzae* at 1:10 ratio; HD = high density conditioning culture; HD + fresh media = HD treatment supplemented with fresh media in a 1:1 mix, final concentration of fresh media supplement equals fresh media only control. ** denotes a $p < 0.01$ and * denotes $p < 0.05$ compared to the media-only treatment. Statistical significance tested using two-way ANOVA, Tukey test. N = 4-6 biological replicates.

Appendix B: Free-living amoeba and *Rhizoctonia solani*

Introduction

Rhizoctonia solani is a significant fungal pathogen of rice that is particularly difficult to control. This fungus is the causative agent of rice sheath blight, a necrotic disease characterized by lesions initiating at the apical ends of rice sheaths. *R. solani* is further divided into subgroups based on its propensity to form anastomoses between individuals from the same anastomosis group, and anastomosis group 1 (AG1) is the largest threat to cereal crops worldwide (12, 13). As a persistent and virulent, soil-borne pathogen, *R. solani* is difficult to control and can reduce yields by half in ideal conditions (6). *R. solani* persists in soil and in fields by use of sclerotia, condensed bodies of fungal hypha that may survive in soil for up to two years (6, 14). Additionally, the broad host spectrum of *R. solani* allow the fungus to persist in alternative hosts for several seasons (15). Compounding the issue of pathogen persistence is that there are currently no resistance genes identified for control of *R. solani*, although potential QTL that increase plant resistance have been identified (14, 16, 17). To reduce instances and severity of outbreaks from *X. oryzae* and *R. solani*, additional methods of control must be implemented. To that end, we propose the use of free-living amoeba as a biological control for these two pathogens.

Historically, there are few publications regarding the interaction of free-living amoeba and *R. solani*. Sclerotia and hyphae inoculated into soil samples showed signs of extensive predation by mycophagous protozoa (40). The authors treated the soil with various antimicrobial agents to remove prokaryotes and recovered amoebae from the

soil, although the authors only identified the potentially responsible amoebae at a genus level, based on morphology. While the *R. solani* – amoeba dynamic is sparsely investigated, there are well-described interactions between other fungi and amoeba. The conidia of *Cochliobolus sativus* were lysed by multiple perforations in the cell wall after incubation in soil samples, and some amoeba are capable of engulfing entire conidia and then encysting to slowly digest their prey inside the cyst (70, 71). The *Acanthamoeba* genus also preys on a variety of fungi pathogenic to mammals, such as *Blastomyces dermatitidis* and *Cryptococcus neoformans* (31). Interestingly, some strains of *C. neoformans* can survive inside *A. castellanii* and use the amoeba as an infection reservoir and source of future infections (31).

Given their proximity in the phytobiome, we hypothesize that free-living amoeba do interact with *R. solani*, despite the lack of specific information in the literature. We observed amoeba and fungi co-cultures by light and fluorescence microscopy. Of the five amoebae: *Acanthamoeba lenticulata, A. polyphaga*, *A. castellanii*, *Dictyostelium discoideum* and *Vermamoeba vermiformis*, we found that *V. vermiformis* caused the most noticeable changes in the fungi and we further explored their interactions with scanning electron microscopy.

Materials and Methods

Amoebae and fungi culturing conditions

A. polyphaga, *A. castellanii*, and *A. lenticulata* were cultured at 28°C in a modified PYG media, *V. vermiformis* was cultured at 28 °C in a modified PYNFH media and *D. discoideum* was maintained at room temperature in a modified HL5 media (25). Amoeba cultures were inoculated from frozen stocks into 100x15 mm petri dishes with 30 mm walls holding 10 mL of media supplemented with 1x Gibco

penicillin/streptomycin (Invitrogen; Carlsbad, CA, USA). Once initial cultures reached turbidity, *Acanthamoeba* species and *V. vermiformis* were passaged every 5 days by transferring 500 µL of culture into 10 mL of fresh media. *D. discoideum* was passaged every 3 days. Amoeba cultures were kept and used up to passage level three before disposal.

R. solani was cultured on 1/2 strength potato dextrose agar (Difco) from frozen stocks prepared on barley seeds according to Webb et al. (72). Initial cultures were incubated at 22 °C with 16 h of light for 10 days, then stored at 4 °C as a source of agar plugs. Source plates were kept for up to three weeks before starting new cultures from stock. Agar plugs of mycelium were subcultured onto autoclaved cellophane overlaid onto 1/2 potato-dextrose agar (PDA) and incubated for 7-10 d at the above conditions before use in experiments.

Co-cultures of amoebae and R. solani

Confluent cultures of amoeba were starved overnight in diluted media at the temperatures described above, except for *D. discoideum*, which was kept in full strength media. *Acanthamoeba* were starved at 1/5 strength PYG while *V. vermiformis* were starved in 1/2 PYNFH, media was diluted using Page's modified Neff amoeba saline (PAS; Wheat et al, 2014). Amoebic cell density was calculated using a direct cell counting method involving trypan blue exclusion and a hemocytometer. Only cultures with over 90% viable trophozoites were used. Amoeba cultures were adjusted to concentrations of 2×105 cells/mL in fresh, diluted media.

Disks of fungal mycelium were cut with an ethanol and flame sterilized soil borer with an internal diameter of 5 mm. Fungal disks were removed from the agar plate using

sterile forceps and rinsed once in sterile distilled water and transferred to a 1.5 mL centrifuge tube. 500 µL of amoebae culture was added to the fungi. Each amoebae and fungi combination was prepared in triplicate for sampling at each time point of 0, 24, and 48 h. Co-cultures with *Acanthamoeba* sp. or *V. vermiformis* were incubated at 22 or 28 °C and cultures with *D. discoideum* were incubated at 22 °C.

Microscopy

At the sampling time, co-cultures were spun at 150x G for 3 min and the supernatant was removed. Samples were washed three times with 500 µL of PAS with centrifugation at 150x G for 3 min each time. After washing, samples were fixed with 100 µL of 4% paraformaldehyde (PFA) for 48 h. After fixation, samples were spun and suspended in 30 µL of PAS. For viability staining, samples were first dyed with 4 µL of 8 mg/mL fluorescein diacetate (FDA; dissolved in DMSO) and 25 µL of 2 mg/mL propidium iodide (PI; dissolved in DMSO) for 15-20 min in the dark. Samples were then washed and fixed as noted previously.

Samples were set on a microscope slide and a coverslip was attached before observation with the microscope. Standard light and fluorescence microscopy was conducted on a Zeiss Axioskop microscope. FDA was visualized using Chroma Technology filters with 480 nm and 535 nm excitation and emission filters respectively, and PI was visualized with Chroma Technology filters with 535 excitation and 610 nm emission wavelengths.

Confocal laser scanning microscopy was carried out on a Zeiss LSM 510 inverted microscope.

Scanning Electron Microscopy

Amoebae and fungi were cultured separately as detailed above beforehand. Co-cultures were prepared where the two organisms were allowed to directly contact one another and additional cultures were prepared where the two were barred from physical contact. In the former, amoebae were adjusted to a concentration of 2×10^5 trophozoites/mL and 10 mL of the culture was added to a high-wall petri dish. Fifteen discs of *R. solani* were added to the culture and the dish was sealed with Parafilm and placed in a plastic baggie. To separate the two species, co-cultures of *V. vermiformis* and *R. solani* for SEM were prepared following a modified procedure from (40). Two nuclepore membranes (Whatman #110610; Maidstone, UK) 25 mm in diameter with 1.0 µm pores were used to sandwich three fungal discs, the edges of the membrane sandwiches were sealed with silicon vacuum grease. A total of five sandwich membranes were added to a petri dish of V. vermiformis, prepared as previously described. Co-cultures were incubated at 22 °C with 16 h of light. At 0, 2, 6, 12, and 24, three disks from each culture were transferred to individual micro-centrifuge tubes and spun once at 150x G for 3 min. Supernatant was discarded and samples were washed once in 500 µL PAS. After centrifugation and removal of the wash, samples were fixed in 2.5% glutaraldehyde buffered in 0.15M SPB, pH 7.0 (22 °C for 30-60 min, followed by 4 °C). Tissue samples were dehydrated through a graded ethanol series, followed by final dehydration using a BioRad E3000 critical point dryer (Quorum Technologies, East Sussex, England). All samples prepared for SEM were sputter coated with 10nm gold, imaged at 5kV with a JEOL JSM-6500F Field Emission Scanning Electron Microscope (FESEM). All images were captured as tiff files.

Results with Discussion

Microscopy reveals a diverse array of reactions

In co-cultures of amoeba trophozoites and *R. solani* mycelium, each genera of amoeba displayed varying reactions to the fungi. At 24 and 48 h, the three *Acanthamoeba* species encysted at higher rates than the amoeba-only control in PAS non-nutrient media (**Figure B1**). The cysts tended to clump together around the mycelium rather than float in the culture. No impacts on the fungi were observed: the mycelium remained intact with no visible perforations or loss of nuclei. Propidium iodide (PI) stains the nuclei of *R. solani* red, and the stained nuclei were still visible after co-culturing the fungi with amoebae. In addition, the cell wall remained smooth and mycelia grew in one direction with right-angle branching of new cells.

D. discoideum did not have any apparent physical interaction with the fungi. Under standard microscopy the amoeba did not attach to the fungi or form sporulating bodies, a sign of nutrient deprivation or environmental incompatibility. The fungi had no visible alterations either: the mycelium remained intact and no punctures were present (**Figure B1**).

V. vermiformis was the only amoeba species to have a noticeable effect on the fungus. Trophozoites were physically attached to the mycelium and remained viable at 24 hours into co-culture (**Figure B2**). Mycelia exhibited a distinct morphology as a result of culturing with the amoebae. The fungal cell surface became mottled and shriveled (**Figure B3**). The shriveling is apparent as soon as 24 hours after co-cultivation (**Figure B4**). This is a stark contrast from the smooth cell walls and branching hyphae that *R. solani* normally develops during growth (73). Even when *V. vermiformis* and *R. solani* are physically separated by membranes in the same media, the mycelia display the

same morphology. While transient contact can occur through the 1 μm pores of the membrane, the amoebae are unlikely to attach and physically wrap around the mycelium. Perforations with smooth and rounded edges were detected on the fungal mycelium of the physically-separated co-cultures. However, these events were rare.

No annular depressions or perforations were seen on the cell walls under light microscopy; however, that may be due to a limitation in resolution. In all three amoebic culturing media, *R. solani* grew in radial size, indicating that the media were a sufficient nutrient source. Growth rates and viability were not otherwise assessed however.

It is not surprising that the panel of amoeba presented a variety of interactions with the fungi. Some bacterial species are known to antagonize amoebae and force encystment or even lyse the amoebae (48, 50). The cause of encystment in *Acanthamoeba* is not known at this time, and we have not ruled out nutrient deprivation as the factor. To our knowledge, there are no characterized mechanisms of anti-amoebic activity from *R. solani*. The reason for the cysts' physical association with the fungi is not understood either. One explanation could be that *Acanthamoeba* trophozoites attach and attempt to feed, but subsequently encounter an antagonist and encyst.

The shriveling response of the fungi after exposure to *V. vermiformis* suggests some form of antagonism. Because the same response occurred with and without direct contact of the two organisms, it is possible the fungi is reacting to a secreted amoebal factor. The extent of the response is still uncharacterized and the effects of amoebal secretions on fungi is not well studied. However, primary research demonstrated that some plant extracts are capable of causing similar responses in *R. solani* (73, 74). The

rare perforations suggest that *V. vermiformis* can puncture *R. solani* cell walls. Our co-culture experiments only went to 24 h, perhaps extra exposure time between the two species will allow for more perforations. Currently, this project's findings are underdeveloped but does have avenues to explore, primarily determining if the fungal response is due to a secreted factor and discerning the factor's identity.

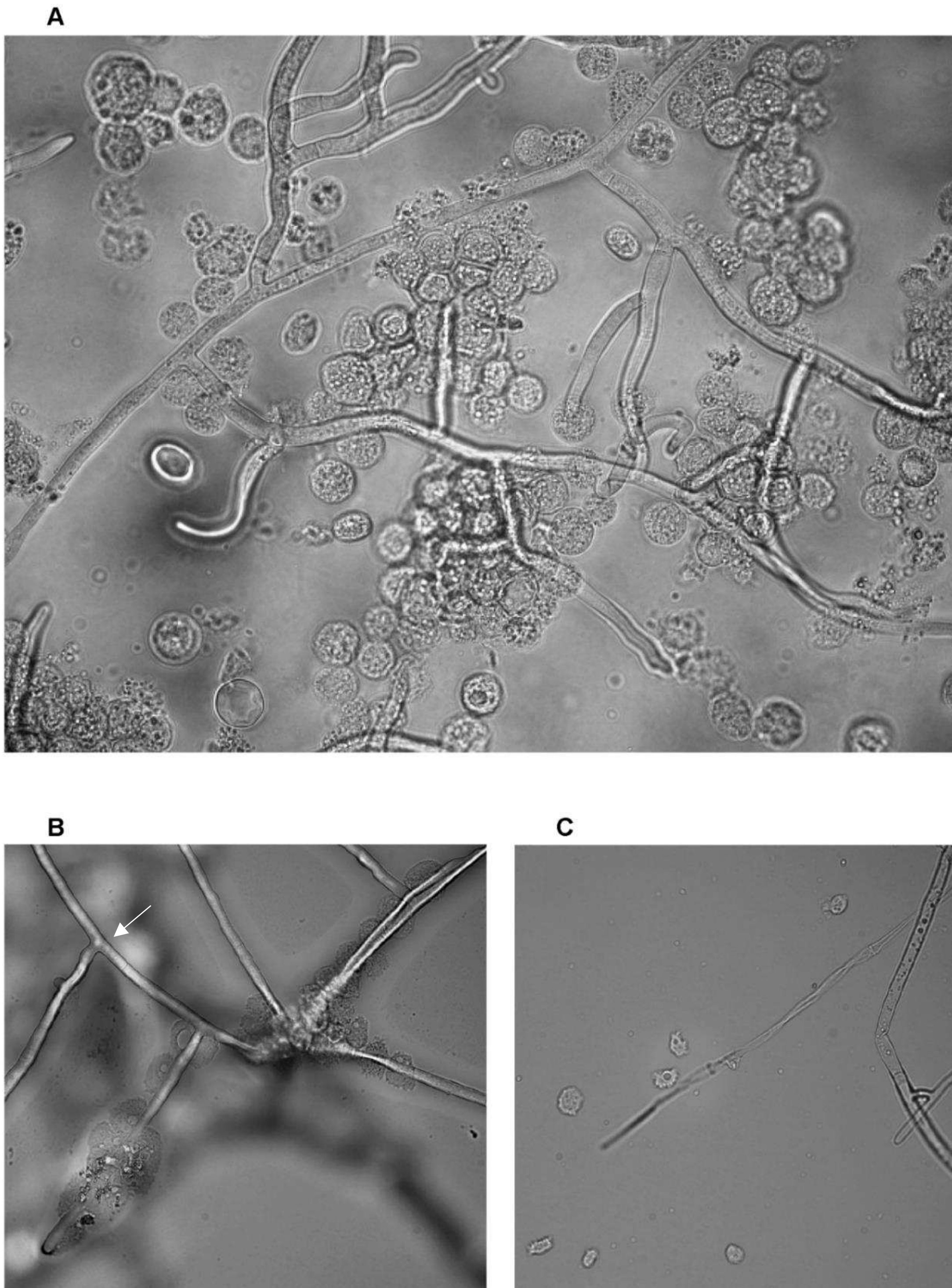


Figure B1. Light microscopy of amoeba and *R. solani* co-cultures. A) *A. polyphaga* and *R. solani* after 48 h. B) *A. castellanii* and *R. solani* after 24 h. The smooth and straight cell walls of *R. solani* are easily visible, indicated by a white arrow. C) *D. discoideum* and *R. solani* after 24 h. All images were taken at 630x magnification.

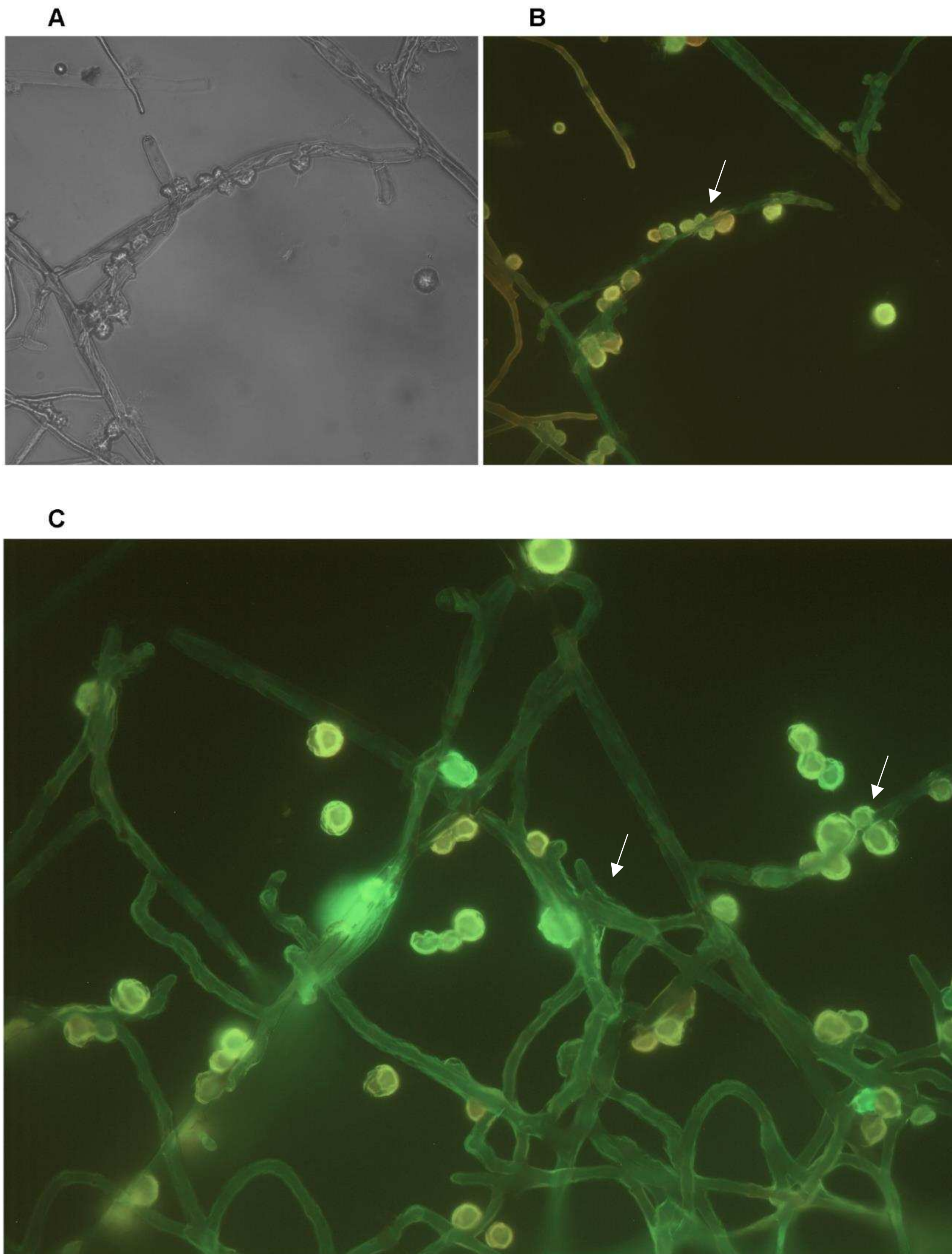


Figure B2. Light and fluorescence microscopy of *V. vermiformis* and *R. solani.* A) Co-culture imaged at 24 h where the shriveled morphology of the mycelia is noticeable. B) Fluorescence image of the same culture as A, with the emission of FITC and PI overlaid. C) Co-culture at 24 h, again the shriveled morphology and physical association of amoeba and fungi is visible, denoted by white arrows.

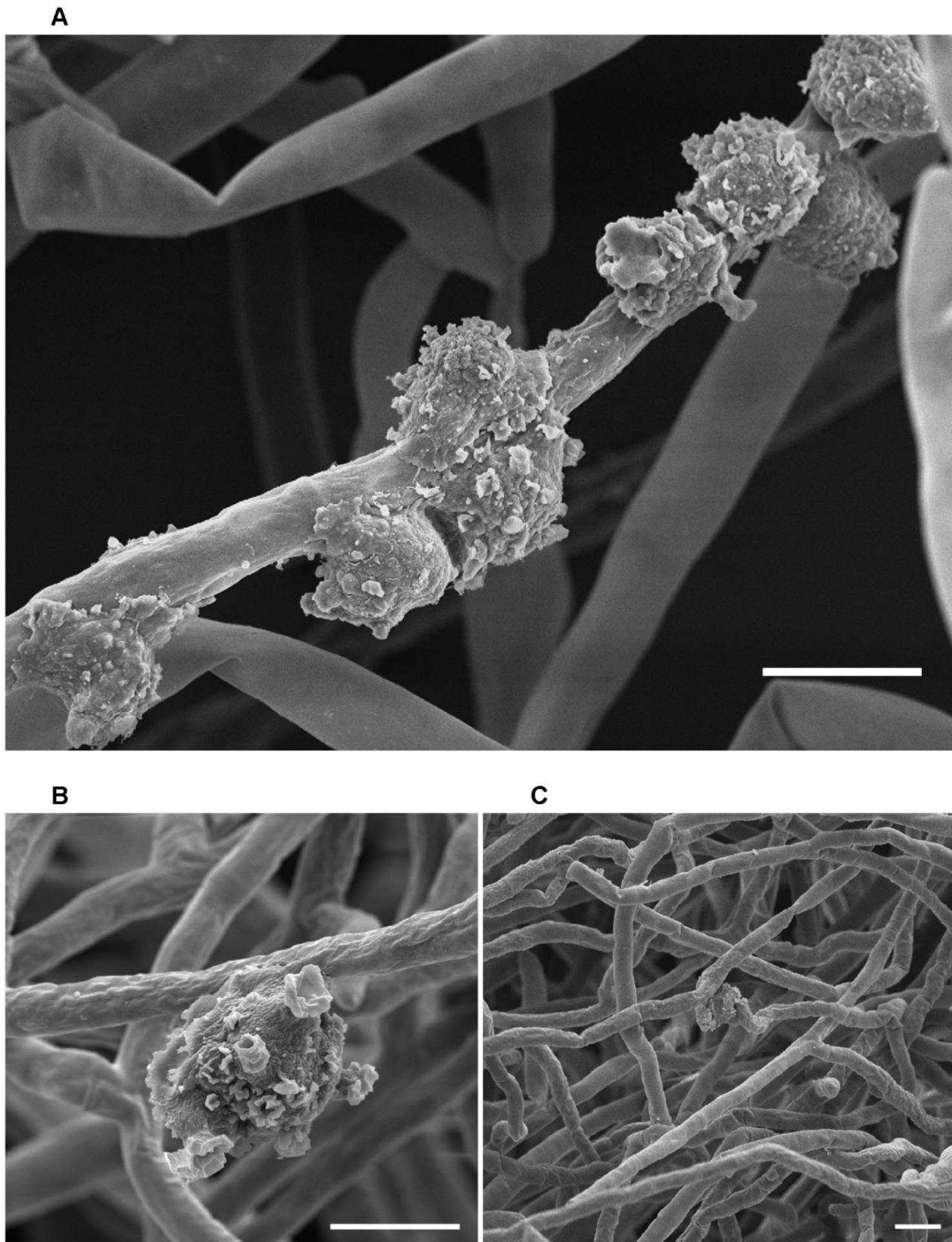
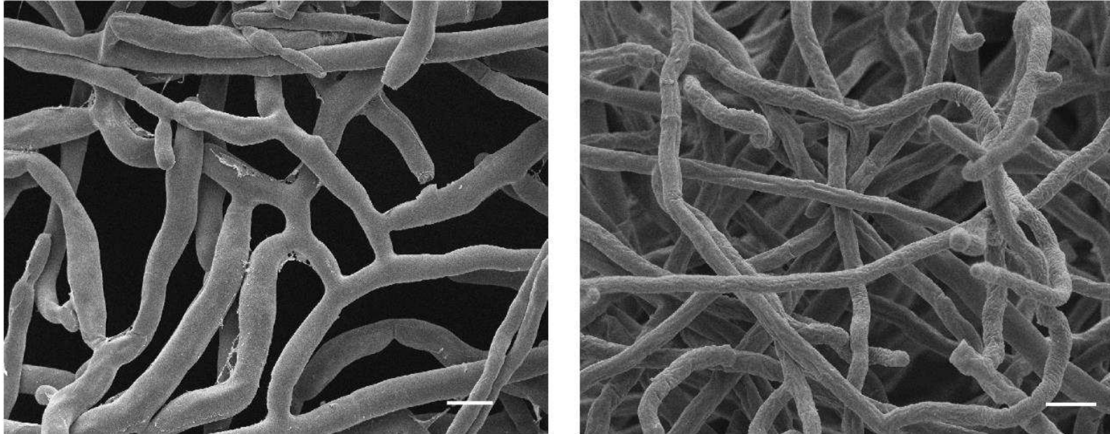


Figure B3. Scanning electron microscopy of *V. vermiformis* and *R. solani* after 24 h in co-culture, organisms were not separated by a nuclepore membrane. A) A *V. vermiformis* trophozoite is partially wrapped around mycelium. B) A close-up image of the shriveled mycelium. C) A wider image of mycelia. Scale bars are 10 µm in length.

A



B

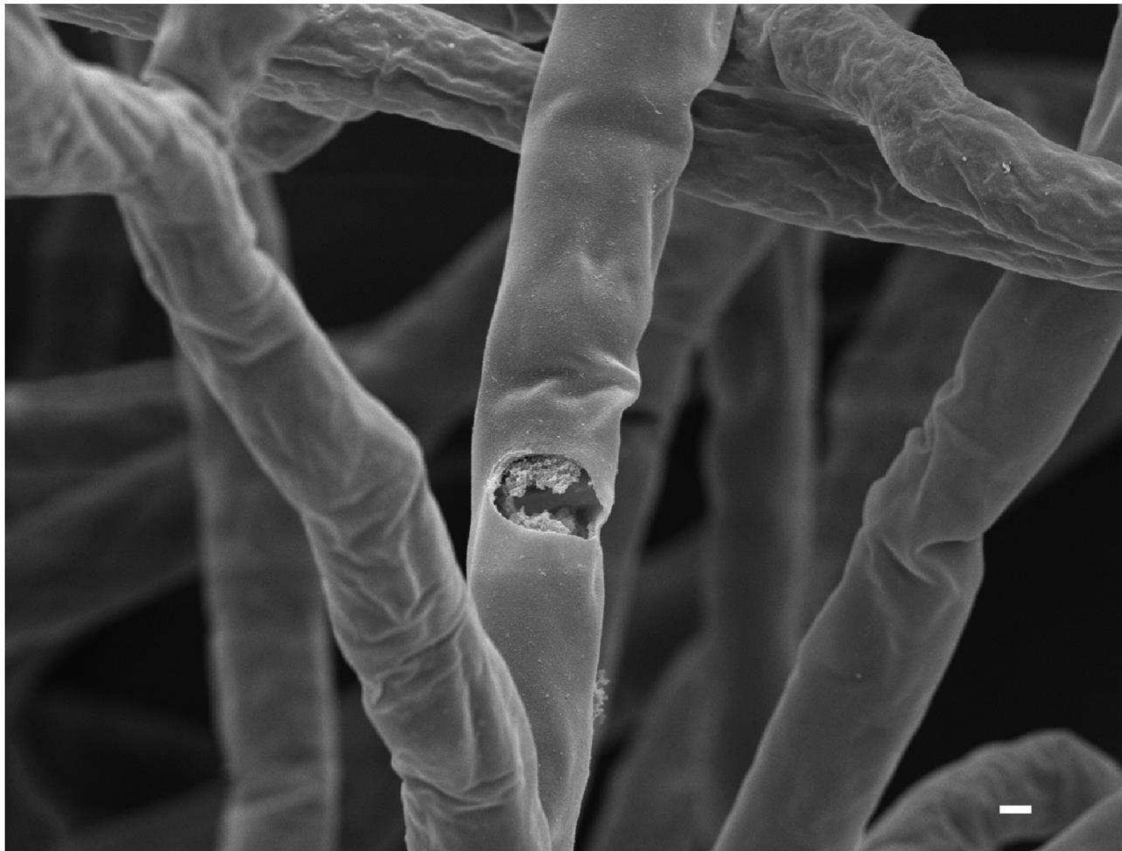


Figure B4. Scanning electron microscopy of *V. vermiformis* and *R. solani* after 24 h in co-culture, organisms were separated by a nuclepore membrane with 1 µm pores. A) *R. solani* and *V. vermiformis* co-cultures at 0 h (left) and 24 h (right). The mycelium of *R. solani* still develops a shriveled exterior even without direct contact from the amoeba. Scale bars are 10 µm in length. B) One of three perforations with smooth edges detected on the mycelia. Scale bar is 1 µm in length.

Appendix C: Other contributions

The final section details my contributions to the manuscripts by Triplett et al. and Shidore et al. (75, 76). Full details of the overall project objectives, experimental design, rationale, results and conclusions can be found in the respective publications.

Contribution to: *AvrRxo1 is a bifunctional type III secreted effector and toxin-antitoxin system component with homologs in diverse environmental contexts*

Plasmid construction

For expression in pDEST527 and comparative growth curves, avrRxo1 homologs from plant pathogens were amplified from bacterial DNA using primers XeENTR_F and -R (*Xe* strain Xcv85-10), XtENTR_F and -R (*Xt* strain UPB468), BaENTRF and R (*Ba* strain Ba3549), and AcENTR_F and -R (*Ac* strain AAC00-1), and CfENTR_F and R (*Cf* strain DSM2262). PCR products were cloned into pENTR-D-Topo (Life Technologies) and recombined into pDEST527 using the LR Clonase II enzyme mix according to manufacturers' instructions. pDEST527 constructs were fully sequenced and transformed into *E. coli* strain BL21(DE3), and transformants were confirmed by PCR.

avrRxo1 homolog activity assays

Fresh overnight cultures of BL21(DE3) strains carrying pDEST527-based vectors were suspended at 10^7 CFU/mL or 10^6 CFU/mL (OD600 of 0.01 or 0.001, respectively) in four replicate tubes of LB broth containing 100 µg/mL ampicillin and 1 mM IPTG. Cultures were distributed into four wells each of a 96 well plate, totaling 16 replicate cultures per treatment. Cultures were incubated at 37°C with shaking, and OD600 was measured every two hours for 12 h.

Contribution to: *The effector AvrRxo1 phosphorylates NAD in planta*

Inoculation of rice leaves with *Xanthomonas oryzae* pv. *oryzicola*

Leaves of 6-week-old *Oryza sativa* ssp. *Japonica* cv. *Kitaake* were inoculated with cell suspensions of *X. oryzae* pv. *oryzicola* strains (OD_{600} = 0.2) prepared in water from a 48- to 72-h-old PSA plate cultures. The inoculations were done by infiltration of the suspensions on the abaxial leaf surface using a needleless syringe. For LC-MS based metabolic profiling, leaf discs were collected 12, 24, 48 hours post inoculation, macerated in liquid nitrogen and stored at -80º C until further use.

Induction of Rxo1-mediated HR response in rice

Four-week-old transgenic rice plants (cv. Kitaake) expressing Rxo1 (77) were grown in a growth chamber, and fully-expanded leaves were inoculated with 10^8 cfu/mL *X. oryzae* strain X11-5A carrying pHM1, pHM1-AvrRxo1, pHM1-AvrRxo1-T167N, or pHM1-AvrRxo1-D193T by leaf infiltration by needleless syringe as described by (77). Inoculation sites were photographed at 5 days post infiltration.