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Native and non-native ant impacts on native fungi

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Native and non-native ant impacts on native fungi

by

Chloe Mokadam

An Abstract of a Thesis

in

Biology

Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

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Buffalo State College

State University of New York

Department of Biology

ABSTRACT OF THESIS

Non-native ant impacts on native fungi

Organisms produce weapons for defense against pathogens and competitors. In response, competitors and pathogens develop resistance to these weapons. However, when a species invades a new range, its “novel weapons” may be more effective against native species that did not co-evolve with them. Via specialized glands and microbial associates, ants produce antifungal weapons for defense against entomopathogenic fungi. However, these weapons may have unintended secondary effects on non-entomopathogenic, soil and seed-borne fungi. The antifungals of non-native ants may be novel weapons, with greater negative impacts on native fungi that have not co-evolved with them. This research aims to test the novel weapons hypothesis by comparing the impacts of an invasive European ant, *Myrmica rubra*, against those of a native North American ant, *Aphaenogaster picea*, on a native North American fungus, *Absidia* sp. I hypothesized that *M. rubra* would reduce fungal performance as compared to *A. picea*. To test this hypothesis, I isolated *Absidia* sp. from ant-occupied soils, exposed cultures to ant colonies, and measured the percent cover of *Absidia* sp. after 48 hours. Percent cover of *Absidia* sp. was lowest in *M. rubra*, greater in *A. picea*, and greatest with no ants. Percent cover of ant-facilitated microbes was greatest in *M. rubra*, lower in *A. picea*, and lowest with no ants. Ant-facilitated microbe cover correlated negatively with *Absidia* sp., but, under high resource conditions, *Absidia* sp. negatively impacted ant-facilitated microbes. Both ant species reduced *Absidia* sp. cover. However, *M. rubra* exerted stronger negative effects, consistent with the novel weapons hypothesis.

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To be approved by:

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Introduction

Many organisms produce chemicals for defense against pathogens and for attack against competitors (Swain 1977, Pasteels et al. 1983, Maróti et al. 2011). However, co-occurring pathogens and competitors develop resistance to these defenses and attacks (Van Valen 1977, Brockhurst et al. 2014). In response, organisms produce more potent chemical weapons, driving an evolutionary arms race between organisms and their natural enemies (Dawkins and Krebs 1979, Pedrini et al. 2015). The novel weapons hypothesis predicts that when a non-native species invades a novel range, native organisms that did not co-evolve with the invaders may lack resistance against the new defenses and attacks (“novel weapons” Callaway and Ridenour 2004). Alternatively, non-native species may bring with them novel microbial associates that produce their own novel weapons (van der Putten et al. 2007, Cheng et al. 2016, 2018). For example, the non-native species may bring a pathogen to which it is immune, but similar species in the novel range are not (Strauss et al. 2012, Vilcinskas et al. 2013, Vilcinskas 2015). As a result, non-native invaders may gain advantage against native species in invaded ecosystems due to the increased potency of both their own endogenously produced weaponry and the weapons of their microbial associates.

Ants possess a unique organ, called a metapleural gland, from which they produce waxy, antimicrobial secretions (Beattie et al. 1986, Veal et al. 1992, Yek and Mueller 2011). Ants continuously coat themselves and others in the colony in metapleural gland secretions that defend against pathogenic fungi and bacteria that attack ants (Brough 1983, Schluns and Crozier 2009). Metapleural gland secretions inhibit hyphal growth and germination by fungi, which prevents fungal entomopathogens from colonizing ant bodies (Brough 1983, Beattie et al. 1985). However, some beneficial fungi have evolved resistance to metapleural gland secretions, as in

the case of Attine ants that farm fungal symbionts (Mueller et al. 2001, Sánchez-Peña 2005, Mikheyev et al. 2007, Dentinger et al. 2009), and unwanted fungi may also develop resistance to ant metapleural gland secretions (Beattie et al. 1986).

Arthropods can overcome resistance to their antifungals by hosting antifungal producing microbial associates on their cuticle, and this is a common strategy among Hymenoptera (Kaltenpoth 2009, Kaltenpoth and Engl 2014, Kett et al. 2021). In tropical ants, particularly ants from the tribe Attini, association with antifungal producing cuticle microbes (most commonly species from the phylum Actinobacteria such as *Actinomyces* spp., *Pseudonocardia* spp., and *Streptomyces* spp.) is especially well studied (Santos et al. 2004, Sen et al. 2009, Barke et al. 2010, Seipke et al. 2011, 2012, Mattoso et al. 2012, Ortega et al. 2019, Batey et al. 2020, Goldstein and Klassen 2020). Though less is known regarding the cuticle microbes of temperate ants, antifungal producing actinobacteria have been isolated from the cuticles of the temperate ant species *Camponotus japonicus*, *Lasius fuliginosus*, and *Lasius flavus* (Wang et al. 2020). Additionally, the widespread invasive ant, *Solenopsis invicta*, prefers to nest in soils that are rich in actinobacteria as opposed to soils where actinobacteria are less abundant (Huang et al. 2020). Hence, symbioses with antifungal producing cuticle microbes may be a common ant defense strategy.

The antifungal activity of metapleural gland secretions and cuticle microbes appears to primarily effect entomopathogenic fungi, but these weapons also may have unintended secondary effects on non-entomopathogenic fungi in soils and on seeds. Generally, ant presence is thought to increase microbial diversity and abundance (Boulton et al. 2003, Wills and Landis 2018, Delgado-Baquerizo et al. 2019). On the other hand, this is not true for all ant species, and ant species effects on qualitative characteristics of soil microbial communities are highly ant

species specific (Dauber and Wolters 2000, Dauber et al. 2001, Bot et al. 2002). For example, though *Myrmica scabrinodis*, *Lasius niger*, and *Lasius flavus* all increase microbial biomass in soil, only soils occupied *M. scabrinodis* and *L. niger* display increased microbial diversity, and the microbial diversity of soils occupied by *L. flavus* is lower than control soils (Dauber and Wolters 2000, Dauber et al. 2001). Additionally, though the presence of either ant increased overall microbial abundance, the fungal communities of soils inhabited by the invasive fire ant *Solenopsis invicta* are less diverse than the fungal communities of soils occupied by a native *Aphaenogaster* species (Zettler et al. 2002). It likely that differences between ant effects on soil microbial communities are in part due to varying secondary effects of the specific antifungal compounds that ant species employ (Vander Meer 2012, Yek et al. 2012), as well as the species specific responses of fungi to ant antifungals (Bot et al. 2002).

Strong evidence of ant secondary effects on seed-pathogenic fungi has been found in the tropics, where seed-cleaning by Attine ants improves seed germination (Oliveira et al. 1995, Leal and Oliveira 1998), because ants reduce seed pathogen loads by coating seeds in antifungals during seed-cleaning (Ohkawara and Akino 2005). In temperate ecosystems, myrmecochory, or ant-mediated seed dispersal, also improves seed germination (Prior et al. 2014), and suppression of seed pathogens by ant antifungals may underly this phenomenon as well. Indeed, *Aphaenogaster rudis*, the dominant ant and seed disperser in eastern North American deciduous forests (Lubertazzi 2012, King et al. 2013), reduces the abundance of phytopathogenic fungi in soils that it occupies (Tarsa et al. 2018).

Myrmica rubra is an invasive European ant that reduces the abundance of native ants, including *Aphaenogaster* spp., by 95% where it invades (Goodman and Warren II 2019). Laboratory colony containers that held non-native *M. rubra* ant colonies developed much less

fungal growth after the ants were removed than those that contained native *Aphaenogaster picea* colonies (Warren, *pers. obs.*), suggesting that *M. rubra* colonies exerted greater antifungal impacts than *A. picea* colonies. Additionally, although *M. rubra* stores seeds in its nests for shorter periods of time than native ants, seedling emergence of seeds handled by *M. rubra* does not differ from that of seeds handled by native ants (Prior et al. 2014), suggesting that the increased potency of *M. rubra* antimicrobials against seed pathogens compensates for the decreased duration of seed pathogen exposure to *M. rubra* secretions.

The objective of my research is to determine whether non-native *M. rubra* colonies inhibit a native soil fungus more than native *A. picea* colonies. I predicted that (1) ant antifungal properties would inhibit the growth of a native soil fungi, and if *M. rubra* antifungals represent a novel weapon against native fungi, then (2) fungal growth will be even less in non-native *M. rubra* colonies than in native *A. picea* colonies. To test this hypothesis, I isolated native *Absidia* sp. fungi from deciduous forest soils in Western New York (WNY) and placed freshly inoculated cultures in *M. rubra* and *A. picea* colonies (both also collected in WNY).

Methods

Study species

Myrmica rubra (Linnaeus, 1758) is native to Europe and Asia, and since it was first reported in Massachusetts in 1908, it has established in marine and freshwater coastal areas throughout the northeastern United States (Wheeler 1908, Groden et al. 2005, Wetterer and Radchenko 2011). Where *M. rubra* invades, it forms multi-queen super-colonies that are much larger than *M. rubra* colonies in its native range (Elmes and Petal 1990). These massive colonies displace native ant

colonies, including those of the dominant woodland ant in eastern North America, *Aphaenogaster picea* (Goodman and Warren II 2019).

Aphaenogaster picea (Wheeler 1908) is a widespread woodland ant species of eastern North America (Lubertazzi 2012, King et al. 2013). It engages in elaiosome-based myrmecochory, wherein ants consume a lipid-rich seed appendage (elaiosome), and plants benefit from dispersal-services provided by the ant (Culver and Beattie 1978, Giladi and Larsson 2006, Clark and King 2012, King et al. 2013). *Aphaenogaster* species are keystone seed dispersers, controlling the distribution and abundance of myrmecochorous plants in North America (Zelikova et al. 2008, Ness et al. 2009).

Absidia is a genus of fungi belonging to the order Mucorales (Hoffmann 2010). Mucorales are ubiquitous in soils, and, though little is known regarding the ecology of most species, they are most commonly plant parasites/decomposers (Walther et al. 2019). The *Absidia* genus includes fungi mainly studied for their presence on stored seeds and grains in agricultural environments worldwide (Shetty and Ahmad 2002, Verma and Dohroo 2004, Bot et al. 2004, Dawar et al. 2007, Hadanich et al. 2008, Priya and Nagaveni 2011, Anwar et al. 2013). *Absidia* spp. are typically classified as seed-borne saprophytes due to their common occurrence on weathered seeds and a lack of data regarding their impacts on seed health (Christensen 1957). However, *Lichtheimia corymbifer* (previously *Absidia corymbifera*) causes blackening and seed mummification in melons (Etaware 2019b, 2019a), and infestation by an unknown *Absidia* species reduces germination in soybeans (Reyes-Ramírez et al. 2004). Additionally, *A. corymbifera* and *A. cylindrospora* are pathogens of peaches and pears (Verma and Sharma 1999). The ecology of *Absidia* spp. in natural settings is largely unknown; however, *Absidia* species occur on oak seeds and in the rhizosphere of oak trees, and *Absidia* species are common

in North American soils (Christensen 1969, Kwaśna 2004, Perera 2020). Additionally, *Absidia* species modify lignin and cellulose (Waing 2015, Zou et al. 2015) and are frequently isolated from soils and decomposing leaf litter (Saitô 1960, Brandsberg 1969, Gochenaur 1978, Waing 2015, Rasyid et al. 2020).

Field collection of ant colonies

Myrmica rubra colonies were collected from Tiff Nature Preserve, 42°50'50"N 78°51'19"W, in June-July 2020. *Aphaenogaster picea* colonies were collected from Sprague Brook County Park, 42°35'29.66"N 78°37'52.94"W, Chestnut Ridge Park, 42°42'55.97"N 78°45'12.72"W, and Eternal Flame Falls, 42°42'5.80"N 78°45'5.60"W, in July-August 2020. Colonies were collected using a cordless portable vacuum (Dewalt, Baltimore, MD, USA) and mouth aspirators, and the colonies were immediately transferred to a plastic bag upon verifying that the colonies were queenright and had at least 12 workers. To reduce stress, the colonies were transported to the laboratory in a cooler. Once in the lab, the ant colonies were then organized into sub-colonies of consistent sizes, containing one queen and 12 workers. Colonies were housed in 16.5 x 13 x 10.2 cm flip top plastic containers (Sterilite, Townsend, MA, USA) that were sterilized using 70% isopropyl alcohol prior to colony introduction. The containers were outfitted with water tube “nests” (composed of a large, plastic test tube filled with deionized water and stoppered with a cotton ball) and the colonies were maintained on a standard artificial diet (Bhatkar and Whitcomb 1970) supplemented with various arthropods. To prevent molding, food was replaced twice weekly. Colonies were stored in a Percival incubator set to 24 °C and 70% humidity.

Fungal collection and culturing

Absidia sp. was isolated using a non-sterile soil solution composed of distilled water and soil samples collected from *A. picea* nest sites. Approximately 2 g of soil was suspended in 10 ml of water and agitated to mix, and 0.5 ml of non-sterile soil solution was pipetted onto potato dextrose agar plates before being allowed to sit for approximately 1.5 hours. The plates were then rinsed to remove debris (adapted from Warcup 1950, Weiland 2011, Lévesque 2021). After allowing growth for 20 days, a suspected *Absidia* sp. specimen was directly isolated by transferring a section of agar containing hyphal tips to a clean plate. *Absidia* sp. was identified by microscopy (Hoffmann 2010). Culture morphology was at first petaloid and white before later becoming thickly wooly and buff-brown to olive-brown (Supplemental Material 1a). Stolons were rarely septate, with rhizoids never directly opposing sporangiophores (Supplemental Material 1b). Sporangiophores (approximately 31.5 μm) exhibited apophysis and were consistently globose, with subsporangial septa (Supplemental Material 1c). Columella (approximately 16 μm) with apical projections (approximately 2.5 μm) and collarettes were also observed (Supplemental Material 1d). Spores were consistently cylindrical and approximately 5 μm in length (Supplemental Material 1e). Cultures were preserved by storing 3 x 3 mm excisions of well-colonized potato dextrose agar in sterilized water (Novikova *pers. comm.*).

To create *Absidia* sp. cultures, a 3 x 3 mm excision of well-colonized potato dextrose agar was placed in the center of a sterile 35 mm potato dextrose agar plate (Carolina Biological Supply Company, NC, USA). Metapleural gland secretions act most potently against early fungal stages, as opposed to late fungal stages (Beattie et al. 1986, Bot et al. 2002), therefore plates were exposed to ant colonies directly following inoculation. Sterile control plates that were not inoculated were also inserted into ant colonies.

High and Low Resource Potato Dextrose Agar

Ants may introduce non-target microbes to plates (hereafter “ant-facilitated microbes”), particularly bacteria. To promote the growth of both ant-facilitated fungi and ant-facilitated bacteria, I conducted a high resource trial using standard potato dextrose agar that favors fungal growth, and I conducted a low resource trial using a reduced concentration of potato dextrose agar that favors bacterial growth (Guynn et al. 1973, Griffith et al. 2007, Marshall et al. 2018, Baronos et al. 2019). Both the high resource potato dextrose agar and low resource potato dextrose agar were created using a stock solution of potato infusion created by simmering 200 g of potatoes in 1 L of water for 30 minutes before decanting the solution through filters to collect the effluent. To create the high resource potato dextrose agar, 500 mL of stock potato infusion was combined with 10 g of dextrose and 7.5 g of agar. To create the low resource potato dextrose agar, 250 mL of stock potato infusion was diluted with 250 mL of deionized water and combined with 5 g of dextrose and 7.5 g of agar (adapted from Zimbro et al. 2009).

Experimental Design

The experiment consisted of three treatments: *A. picea* colony, *M. rubra* colony, and a treatment with no ants. Two trials were conducted. The first, high resource trial used high resource potato dextrose agar; the second, low resource trial used low resource potato dextrose agar (adapted from Zimbro et al. 2009). 20 replicates were conducted per treatment, with 60 replicates per trial (n = 120).

One plate inoculated with *Absidia* sp. and a sterile control plate were placed in each colony immediately following inoculation. Plates were placed directly adjacent to the opening of the water tubes to further encourage ants to interact with the plates. *Absidia* sp. plates and

controls were rotated every 12 hours to ensure equal interaction between ants and microbes on either plate. *Absidia* sp. were exposed to ant colonies for 48 hours, as this was a sufficient amount of time for *Absidia* sp. to reach the plate edge. Photographs were taken of each plate on a plain black background every 12 hours using a Sony α 6000 camera on a Sunpak TravelLite Pro Reverse Folding Tripod. Photographs were saved as JPEGs and processed using the photo editing program GIMP (GNU Image Manipulation Program) to improve readability (Supplemental Material 2). The percent cover of *Absidia* sp. and ant-facilitated microbes (microbes growing on plates that were not experimentally introduced but rather brought to plates by ants) were then estimated by four independent observers. Images were presented to observers without any identifying information.

Data Analysis

The percent cover of *Absidia* sp. was analyzed as a function of resource level (high, low), ant treatment (*A. picea*, *M. rubra*, control), and the percent cover of ant-facilitated microbes using a generalized linear model assuming Poisson-distributed error and fitted with analysis of deviance (ANODEV) model. Interaction terms were dropped if not relevant, and potential overdispersion was corrected using a ‘quasi’ error distribution. The percent cover of ant-facilitated microbes was also analyzed as a function of resources, treatment, the cover of *Absidia* sp., and plate type using a fitted GLM model. Interaction terms were included for resource x plate type to examine whether resource levels impacted interactions between *Absidia* sp. and ant-facilitated microbes. Interactions were dropped if not relevant, and the model was tested for overdispersion. All statistical analyses were conducted using the R statistical program (R Core Team 2020).

Results

Absidia sp. inhibition

The percent cover of *Absidia sp.* differed between all treatments (*A. picea*, *M. rubra*, and no ants) [Table 1], however the percent cover of *Absidia sp.* for *A. picea* (75.6 ± 2.4) was similar to the no ant treatment (80.6 ± 2.0), whereas the percent cover of *Absidia sp.* was considerably lower for *M. rubra* (61.8 ± 3.1) [Fig. 1a; Table 5]. *Absidia sp.* cover decreased with increased ant-facilitated microbe cover [Fig. 1b]. *Absidia sp.* cover also was much lower with low resources (63.9 ± 2.2) than with high resources (81.4 ± 1.8) [Fig. 1c].

Ant-facilitated microbe growth

The percent cover of ant-facilitated microbes differed between all treatments [Table 2], however the percent cover of ant-facilitated microbes for *A. picea* (17.2 ± 2.2) was similar to *M. rubra* (32.3 ± 2.5), whereas the percent cover of ant-facilitated microbes was considerably lower with no ants (3.3 ± 1.1) [Fig. 2a]. The resource x plate interaction indicated that at high resource levels, *Absidia sp.* had a strong negative correlation with ant-facilitated microbes; however, this effect was neutralized in low resource trials [Fig. 2b]. Ant-facilitated microbe performance was analyzed as a function of treatments, resources, and plate type (*Absidia sp.* or control), however there was no interaction between treatments and resources or treatments and plate type, whereas there was an interaction between resources and plate type [Table 6].

Discussion

The native and non-native ants studied here both had a negative impact on *Absidia* sp. fungi that was independent of other factors such as resource level or ant-facilitated microbe cover, suggesting that ant antifungals suppressed the growth of a non-entomopathogenic fungus. Ants may inhibit fungi through two mechanisms: directly through metapleural gland secretions or indirectly by hosting antifungal producing cuticle microbes. Consistent with the novel weapons hypothesis, the non-native ant, *Myrmica rubra*, inhibited fungal growth more than the native ant, *Aphaenogaster picea*. Additionally, though *Absidia* sp. and ant-facilitated microbe cover were negatively correlated overall, *Absidia* sp. appeared to outcompete ant-facilitated microbes under high resource conditions. The negative effect of *Absidia* sp. was neutralized under low resource conditions, suggesting that *Absidia* sp. is a poor competitor when resources are scarce. It appears that the reduction of *Absidia* sp. by ants allows for greater colonization by ant-facilitated microbes, hence ant antifungals may modulate competition between microbes.

Ant antimicrobial effects

Both *A. picea* and *M. rubra* reduced the growth of *Absidia* sp. as compared to the treatment with no ants, suggesting that antifungal activity by ants reduces fungal growth. Ants may inhibit fungi directly via their metapleural gland secretions that contain antifungal compounds used to restrict the growth of entomopathogens (Brough 1983, Beattie et al. 1985, 1986, Veal et al. 1992, Schluns and Crozier 2009, Yek and Mueller 2011). However, fungi may evolve resistance to metapleural gland secretions over time (Beattie et al. 1986, Mueller et al. 2001, Sánchez-Peña 2005, Mikheyev et al. 2007, Dentinger et al. 2009). To avoid antifungal resistance by unwanted

fungi, ants also associate with antifungal producing microbes hosted on their cuticle (Santos et al. 2004, Sen et al. 2009, Kaltenpoth 2009, 2009, Barke et al. 2010, Seipke et al. 2012, 2012, Mattoso et al. 2012, Ortega et al. 2019, Wang et al. 2020, Huang et al. 2020, Batey et al. 2020, Goldstein and Klassen 2020, Kett et al. 2021), thus ants may indirectly inhibit fungi via antifungal producing cuticle microbes, as well.

Distinguishing between the direct effects of metapleural gland secretions and the indirect effects of cuticle microbes is difficult. Whereas researchers have been able to culture and thus isolate the effects of cuticle microbes (Seipke et al. 2011, 2012, Wang et al. 2020), there are few studies that have been able to directly extract metapleural gland secretions from ants and test their potency against fungi, and most studies that have extracted metapleural gland secretions have done so from the same large, Australian ant species, *Myrmecia gulosa* (Beattie et al. 1985, 1986, Veal et al. 1992, Yek et al. 2012). Due to the physical impracticalities of milking ant metapleural glands, studies that seek to understand the influence of metapleural gland secretions on fungi instead analyze the constituent parts of metapleural gland secretions and expose fungi to these isolated compounds (Bot et al. 2002, Vieira et al. 2012) or otherwise measure proxies of metapleural gland secretion efficacy, such as metapleural gland size, mortality rates after inoculation with entomopathogens, and metapleural gland grooming behavior (Fernández-Marín et al. 2006, Poulsen et al. 2006, Yek et al. 2012). Additionally, ant-soaked hexane did not appear to capture ant metapleural gland secretions when tested by Tarsa et al. (2018). While these methods are unlikely to isolate the effects of metapleural gland secretions, isolating the effects of metapleural gland secretions from those of cuticle microbes may be impossible anyhow. Ants host cuticle microbes in cuticular crypts that are located near metapleural glands and other exocrine glands (Cafaro et al. 2011, Mattoso et al. 2012), and it has been suggested that, in

Attine ants, metapleural gland secretions may be used to encourage the growth of cuticle microbes (Poulsen et al. 2006, Yek and Mueller 2011). Although cuticle microbes have yet to be recovered from metapleural gland secretions (Yek and Mueller 2011), it is possible that, in nature, metapleural gland secretions contain cuticle microbes or antifungals produced by cuticle microbes. Indeed, metapleural secretions and cuticle microbes are used together by ants in nature to combat unwanted fungi (Schluns and Crozier 2009, Kaltenpoth 2009, Yek and Mueller 2011, Kaltenpoth and Engl 2014, Kett et al. 2021), hence uncoupling these mechanisms may not be a holistic way to investigate ant antifungal activity.

The invasive, non-native ant, *M. rubra*, reduced the growth of *Absidia* sp. more than the native ant, *A. picea*, consistent with the novel weapons hypothesis. The novel weapons of *M. rubra* may be the direct effects of *M. rubra*'s metapleural gland secretions acting more potently against native fungi that are not adapted to them (Callaway et al. 2008, Thorpe et al. 2009). On the other hand, *M. rubra* may host antifungal producing cuticle microbes whose indirect effects represent a novel weapon against native fungi. The cuticle microbes themselves may be novel to *M. rubra*'s invaded range, and they may more strongly impact native species that have not co-evolved with them, such as in the case of invasive-borne pathogens (Strauss et al. 2012, Vilcinskis et al. 2013, Vilcinskis 2015). Non-native *M. rubra* may also form novel assemblages of native cuticle microbes, as in the case of non-native earthworms that introduce novel bacterial and fungal assemblages to soils via their casts (de Menezes et al. 2018, Price-Christenson et al. 2020), and these novel assemblages may exhibit stronger antifungal activity. Finally, *M. rubra* might host a greater abundance of native or non-native cuticle microbes, such as in the case of invasive plants that increase the abundance of ammonia-oxidizing bacteria (McLeod et al. 2016), and thus it may introduce a greater quantity of antifungals to seed and soil fungi.

Ant antimicrobial side effects

The results presented here show that, regardless of mechanism, both *M. rubra* and *A. picea* reduced the growth of a ubiquitous seed-borne and soil-dwelling fungus, *Absidia* sp. This finding has implications for seed-borne fungi on seeds handled by *M. rubra* and *A. picea*, as well as fungi in soils occupied by either ant species.

In tropical habitats, the positive impacts of ant antifungals on seed germination are well established (Oliveira et al. 1995, Leal and Oliveira 1998, Ohkawara and Akino 2005). Although seed germination in temperate regions is improved by myrmecochory (Prior et al. 2014), it is not well established that ant antifungals underly this benefit to plants. Both *A. picea* and *M. rubra* reduced the growth of *Absidia* sp., which is ubiquitous on agricultural seeds worldwide (Shetty and Ahmad 2002, Verma and Dohroo 2004, Bot et al. 2004, Dawar et al. 2007, Hadanich et al. 2008, Priya and Nagaveni 2011, Anwar et al. 2013) and causes blackening, seed mummification, and reduced germination in melons and soybeans (Reyes-Ramírez et al. 2004, Etaware 2019b, 2019a). Additionally, *Absidia* sp. have been detected in the rhizosphere and on the seeds of oaks (Christensen 1969, Kwaśna 2004, Perera 2020). Though the role of *Absidia* sp. as a potential seed pathogen in natural contexts is unclear, the results presented here suggest that one benefit conferred to seeds handled by *A. picea* and *M. rubra*, which both disperse native seeds (Zelikova et al. 2008, Ness et al. 2009, Gammans et al. 2018), is the activity of ant antifungals against seed-borne fungi. This supports the findings of Tarsa et al. (2018), wherein the presence of *Aphaenogaster rudis* in soils reduced the abundance of fungal phytopathogens. Hence, ant antifungals may underly the benefits of myrmecochory to temperate plants, much in the way that ant antifungals underly the benefits of seed-cleaning to tropical plants.

The effects of ant antifungals against phytopathogenic fungi are likely to extend beyond benefits to seed germination. The biotic resistance hypothesis proposes that one mechanism by which invasive plants lose their dominance in native plant communities is by the accumulation of natural enemies, including pathogens (Knevel et al. 2004, Beaury et al. 2020). On the other hand, the enemy release hypothesis proposes that the success of invasive species is due to their release from the natural enemies of their native range, including pathogens (Colautti et al. 2004, Jeschke and Heger 2018). Indeed, invasive plants host on average 84% less fungal pathogens in their invaded range as opposed to their native range, and 49% of fungal and viral pathogens in invasives are pathogens accumulated in the invasive range (Mitchell and Power 2003). Hence, by decreasing the abundance of native phytopathogenic fungi and thus the amount of potential natural enemies that an invasive plant might encounter, *M. rubra* may depress biotic resistance and strengthen enemy release for invasive plants. On the other hand, a non-intuitive implication is that the stronger effects of *M. rubra* on phytopathogenic fungi suggest that *M. rubra* invasions might actually benefit some native plants, albeit to the detriment of plant community diversity. Seed-borne pathogens and soil-dwelling phytopathogens generally preserve the diversity of native plant communities by modulating competition between native plant species (Bever et al. 2015). For example, foliar plant pathogens limit above ground growth of dominant plant species, thus allowing greater growth of rare plant species, and reduction of foliar pathogens by fungicides leads to decreased plant diversity (Peters and Shaw 1996, Allan et al. 2010). Increased reduction of phytopathogens by *M. rubra* may thus act similarly to fungal pathogen exclusion experiments – leading to reduced plant diversity and increased abundance of dominant plants.

Whereas the role of *Absidia* sp. as a seed-borne fungus and potential phytopathogen is still being elucidated, stronger evidence exists for *Absidia* sp.'s role as a decomposer, or

saprotroph, in soils (Saitô 1960, Brandsberg 1969, Waing 2015, Zou et al. 2015, Rasyid et al. 2020). Saprotrophic fungi breakdown recalcitrant organic matter, such as lignin, cellulose and humic substances that other soil microbes are mostly unable to decompose, and they also redistribute nutrients in bioavailable forms throughout soils (Kubicek and Druzhinina 2007, Grinhut et al. 2007, van der Wal et al. 2013). Hence, reduction of saprotrophic fungi is likely to slow decomposition and the release of nutrients from decaying organic matter back to plants in bioavailable forms. *A. picea* and *M. rubra* both reduced *Absidia* sp., suggesting that both ants might negatively impact soil saprotrophic fungi, possibly slowing decomposition in the soils that they occupy. These results are supported by Warren and Bradford (2012), wherein the presence of *Aphaenogaster* spp. in wood slowed coarse wood rot, likely via ant antifungals. Given the increased potency of its novel weapons against native fungi, *M. rubra* may have stronger effects on soil decomposition than *A. picea*. Further investigation is required to understand the impact that invasion by *M. rubra* has on decomposition and nutrient cycling.

Ant-facilitated microbes

Ant-facilitated microbes grew on almost every plate with *A. picea* or *M. rubra* colonies (148 out of 160 plates), and the non-ant plates had the lowest percent cover of ant-facilitated microbes (Fig. 2a). Ants may have brought fungi and bacteria from other sources within the nest; however, colony containers were sterilized prior to colony introduction. Hence, the ant-facilitated microbes were likely introduced to plates by the ants themselves.

Ant-facilitated microbe growth was highest in *M. rubra* colonies, lower in *A. picea*, and lowest in controls without ants. The greater ant-facilitated microbe growth in *M. rubra* colonies

may be a result of the greater suppression of *Absidia* sp. by *M. rubra* ants which may have released the ant-facilitated microbes from competition with *Absidia* sp. Of course, an alternate possibility is that the ant-facilitated microbes introduced by *M. rubra* produce antifungals of their own, negatively impacting *Absidia* sp. growth. Across all treatments, ant-facilitated microbe growth decreased with *Absidia* sp. growth – a negative correlation whose cause could go in either direction. However, the change in this relationship across resource levels is suggestive. The negative effect of *Absidia* sp. was strongest in the high resource trials but weakened in low resource trials. Whereas decreased resources often lead to decreased growth by microbes, and thus fewer opportunities for direct interaction due to greater spatial distance (Hibbing et al. 2010, Ghoul and Mitri 2016, Bauer et al. 2018), the opposite occurred here. In high resource trials, ant-facilitated microbes typically colonized the agar at the margins of *Absidia* sp. (Supplemental Material 3a). The mycelial mat of *Absidia* sp. was also much denser under high resource conditions, as opposed to low resource conditions (Supplemental Material 3b). In low resource trials, ant-facilitated microbes interrupted the *Absidia* sp. cultures, growing from not only the margins of *Absidia* sp. but from the mycelial mat of *Absidia* sp., as well (Supplemental Material 3b). This suggest that, under high resource conditions, *Absidia* sp. was able to outcompete ant-facilitated microbes, preventing infiltration of the mycelial mat. Typically, strong competitors do best in high resource environments (Grime 2006). Hence, in low resource conditions, *Absidia* sp. may have been unable to compete against ant-facilitated microbes. Additionally, in the low resource treatments, the ant-facilitated microbes likely were able to allocate more energy to growth as opposed to defense against *Absidia* sp. (Mille-Lindblom et al. 2006, Ghoul and Mitri 2016). Reduction of *Absidia* sp. by ants may have aided ant-facilitated microbe growth by modulating competition between *Absidia* sp. and ant-facilitated microbes.

Alternatively, the low resource potato dextrose agar may have favored the growth of ant-facilitated microbes over *Absidia* sp., as the low dextrose content of the low resource potato dextrose agar promoted bacterial growth over fungal growth (Guynn et al. 1973, Marshall et al. 2018, Baronos et al. 2019). However, ant-facilitated microbes were isolated from control plates exposed to either *A. picea* or *M. rubra* in the final low resource trial. After preliminary examinations of culture morphology and microscopic morphology, most ant-facilitated microbes appear to be fungi – only one isolate is suspected to be a bacterium. Further research is required to determine the identities of ant-facilitated microbes.

Plate placement and ant activity levels

The results presented here contradict those of Lash et al. (2020), which found no difference in the abundance plant pathogenic fungi between soils sampled from ant nest openings and those sampled from soil without ants. Nest openings, however, are unlikely to contain the full spectrum of antimicrobial compounds produced by ants and their microbial associates, as soil at the nest opening is less protected and moist, and more exposed to sunlight. My colonies were maintained in incubators that controlled temperature, light, and humidity, providing an ideal environment for *Absidia* sp. and ant-facilitated microbes. Additionally, we placed plates within nests, as opposed to sampling from the nest opening.

Lash et al. (2020) also quantified ant activity levels to investigate their impact on soil microbes, but ant activity levels were not formally quantified here. Ant activity levels influenced fluctuations in phytopathogenic fungi communities, however nest substrate type (soil or wood) was a more influential factor in determining phytopathogenic and overall fungi communities

(Lash et al. 2020). While both *A. picea* and *M. rubra* interacted with *Absidia* sp. and control plates by walking on them, and, in so doing, introduced microbes to *Absidia* sp. and control plates, *M. rubra* engaged in more complex behaviors than *A. picea*. *Myrmica rubra* left visible “trails” on plates (Supplemental Material 4a), dug into the agar (Supplemental Material 4b) and discarded dead ants and ant body parts onto the agar surface (Supplemental Material 4c). Besides one plate on which *A. picea* discarded an ant body part, these behaviors were not observed on *A. picea* plates. It is possible that these behaviors introduced both greater amounts of ant antifungals and ant-facilitated microbes to the plates. Hence, it is possible that the stronger influence of *M. rubra*’s novel weapons is due to not only qualitative differences between the antifungals of *M. rubra* and *A. picea*, but also differences in the quantity of antifungals that the ants produce and apply to the surrounding environment.

Conclusion

Non-native *Myrmica rubra* had a greater negative impact on the growth of *Absidia* sp., a native soil and seed-borne fungus, than native *Aphaenogaster picea*, a result that is consistent with the novel weapons hypothesis. The antifungals of *Myrmica rubra* may be composed of metapleural gland secretions, antifungal producing cuticle microbes, or both. Moreover, the suppression of *Absidia* sp. may have allowed for greater colonization by ant-facilitated microbes, hence ants may modulate competition between microbes. Further work must be done to expand our understanding of the impacts that non-native invasive species, such as *M. rubra*, exert over native species, both directly via endogenously produced chemicals and indirectly via the chemicals produced by their cuticle microbes.

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Tables

Table 1. Percent cover (mean \pm SE) of *Absidia* sp. per treatment.

Treatment	%	SE
<i>Myrmica rubra</i>	61.84	3.13
<i>Aphaenogaster picea</i>	75.63	2.36
Control	80.61	1.96

Table 2. Percent cover (mean \pm SE) of ant-facilitated microbes per treatment.

Treatment	%	SE
<i>Myrmica rubra</i>	32.32	2.48
<i>Aphaenogaster picea</i>	17.18	2.20
Control	3.33	1.12

Table 3. Percent cover (mean \pm SE) of *Absidia* sp. for each trial.

Trial	%	SE
High Resource	81.46	1.83
Low Resource	63.94	2.16

Table 4. Analysis of deviance of percent cover for *Absidia* sp. as a function of resources (high or low), treatments (*Myrmica rubra*, *Aphaenogaster picea*, and no ants), and ant-facilitated microbes.

	<i>df</i>	<i>Deviance</i>	<i>Res. dev.</i>	<i>p-value</i>
Resources	1	126.23	468.48	< 0.01
Treatments	2	106.26	362.22	< 0.01
Ant-facilitated microbes	1	249.37	112.85	< 0.01

Table 5. *Post hoc* multiple comparisons of means using Tukey contrasts to compare differences in the percent cover of *Absidia* sp. between treatments.

Trial	<i>Estimate</i>	<i>Std. Error</i>	<i>z-value</i>	<i>p-value</i>
<i>Aphaenogaster picea</i> : No ants	-0.06	0.02	-2.49	0.03
<i>Myrmica rubra</i> : No ants	-0.26	0.03	-9.91	<0.01
<i>Myrmica rubra</i> : <i>Aphaenogaster picea</i>	-0.20	0.03	-7.44	<0.01

Table 6. Analysis of deviance of mean percent cover for ant-facilitated microbes as a function of resources, treatments, plates (*Absidia* sp. or control), and a resource x plate interaction term.

	<i>df</i>	<i>Deviance</i>	<i>Res. dev.</i>	<i>p-value</i>
Resources	1	9.75	5961.7	0.49
Treatments	2	2185.77	3775.9	< 0.01
Plate	1	166.04	3609.9	< 0.01
Resource: Plate	1	88.70	3521.2	0.04

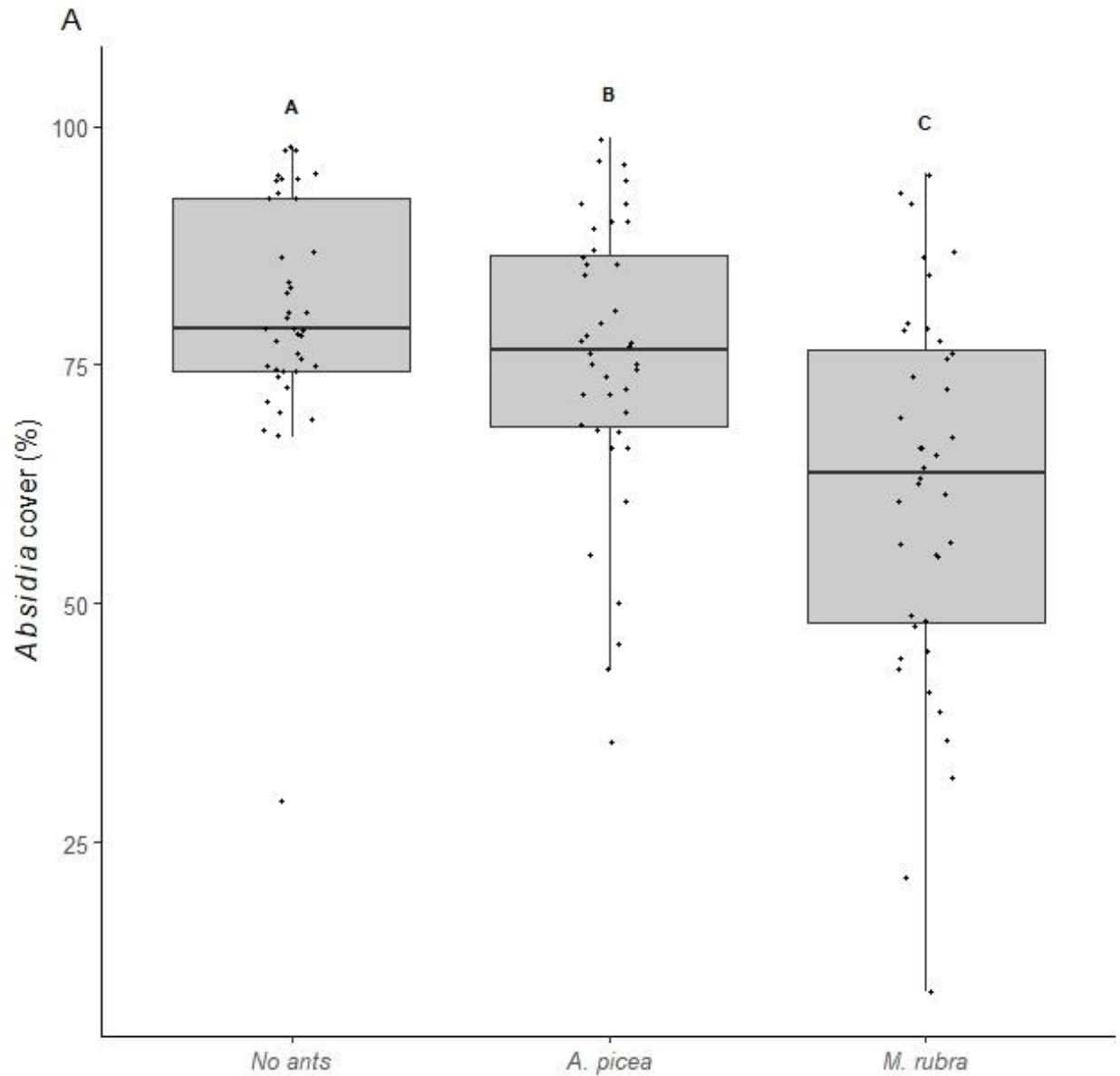


Figure 1a. Boxplots showing mean percent cover of *Absidia* sp. for each treatment – no ants, *Aphaenogaster picea*, and *Myrmica rubra* – and for both high resource and low resource trials. The boxes include data points that fall within the lower (25th) and upper (75th) quartiles, and the error lines above and below the median value include the lowest and largest data points. Different letters above the whiskers represent significant difference between groups.

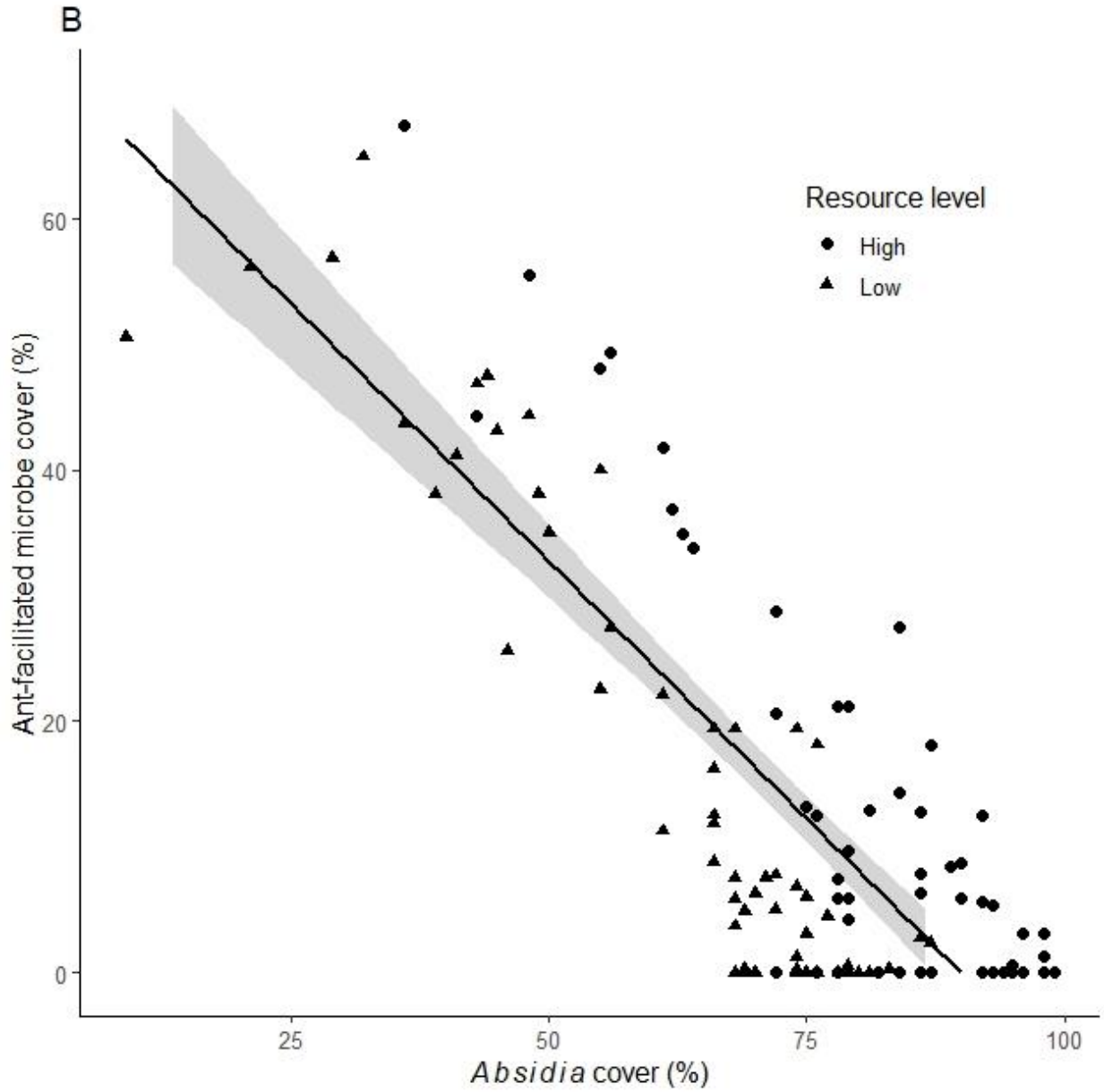


Figure 1b. Scatterplot showing the negative relationship between the mean percent cover of *Absidia* sp. and ant-facilitated microbes. Data points include all treatments and plate types (*Absidia* sp. or control) for both trials. Circle-shaped points represent data from the high resource trial and triangle-shaped points represent data from the high resource trial.

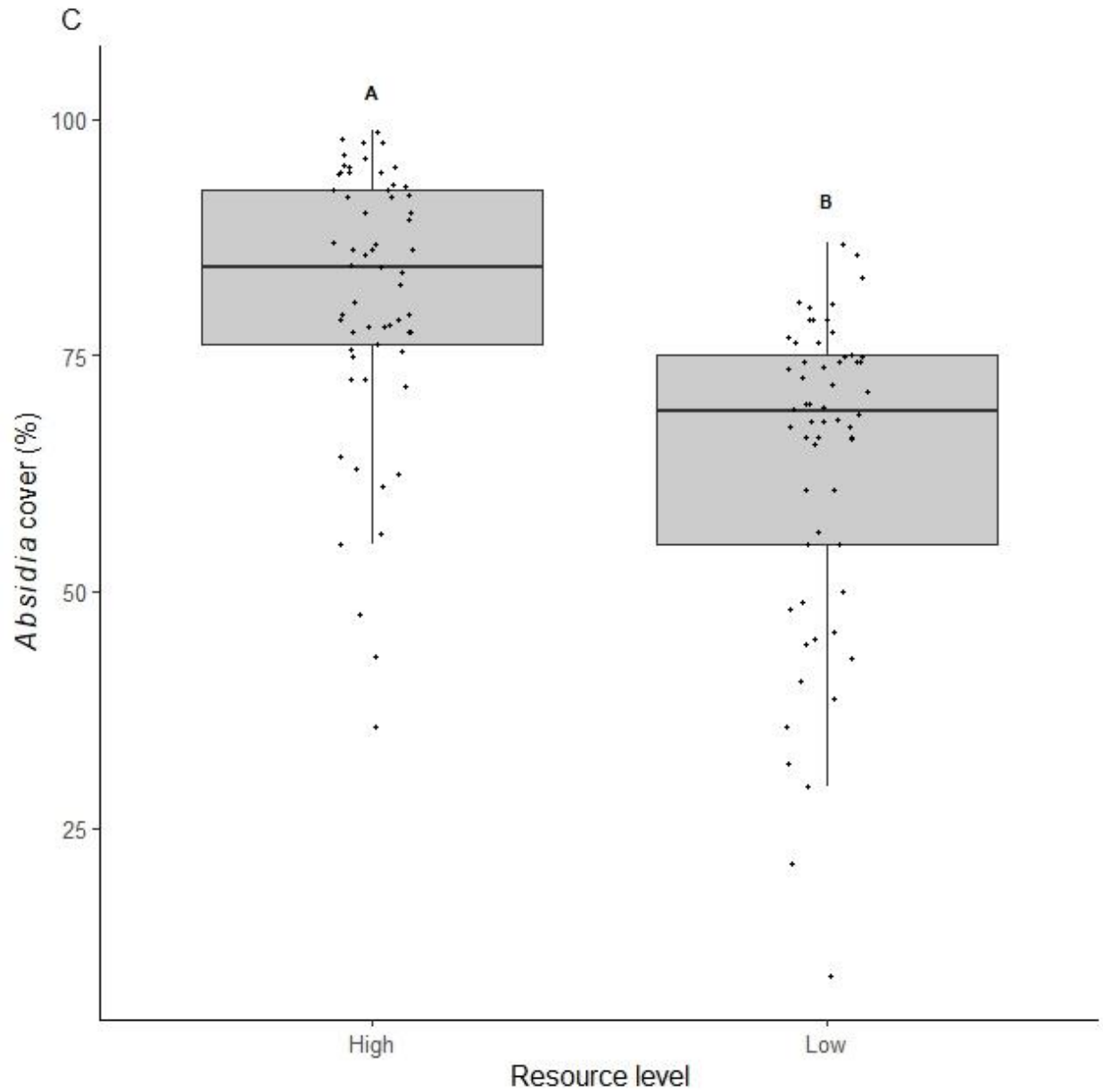


Figure 1c. Boxplots showing mean percent cover of *Absidia* sp. across all treatments for each trial – high resource and low resource. The boxes include data points that fall within the lower (25th) and upper (75th) quartiles, and the error lines above and below the median value include the lowest and largest data points. Different letters above the whiskers represent significant difference between groups.

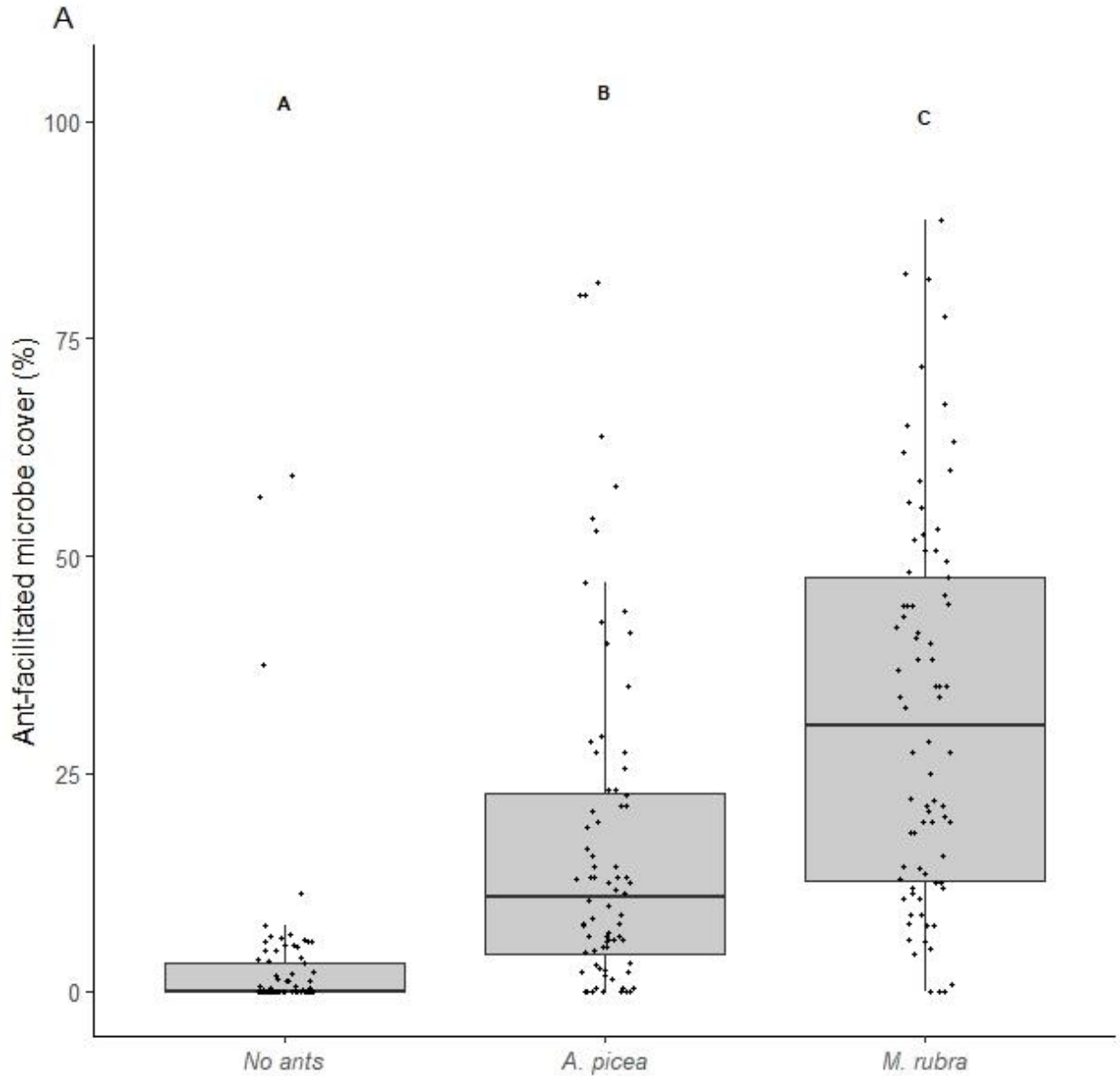


Figure 2a. Boxplots showing mean percent cover of ant-facilitated microbes for each treatment – no ants, *Aphaenogaster picea*, and *Myrmica rubra* – and for both high resource and low resource trials. The boxes include data points that fall within the lower (25th) and upper (75th) quartiles, and the error lines above and below the median value include the lowest and largest data points. Different letters above the whiskers represent significant difference between groups.

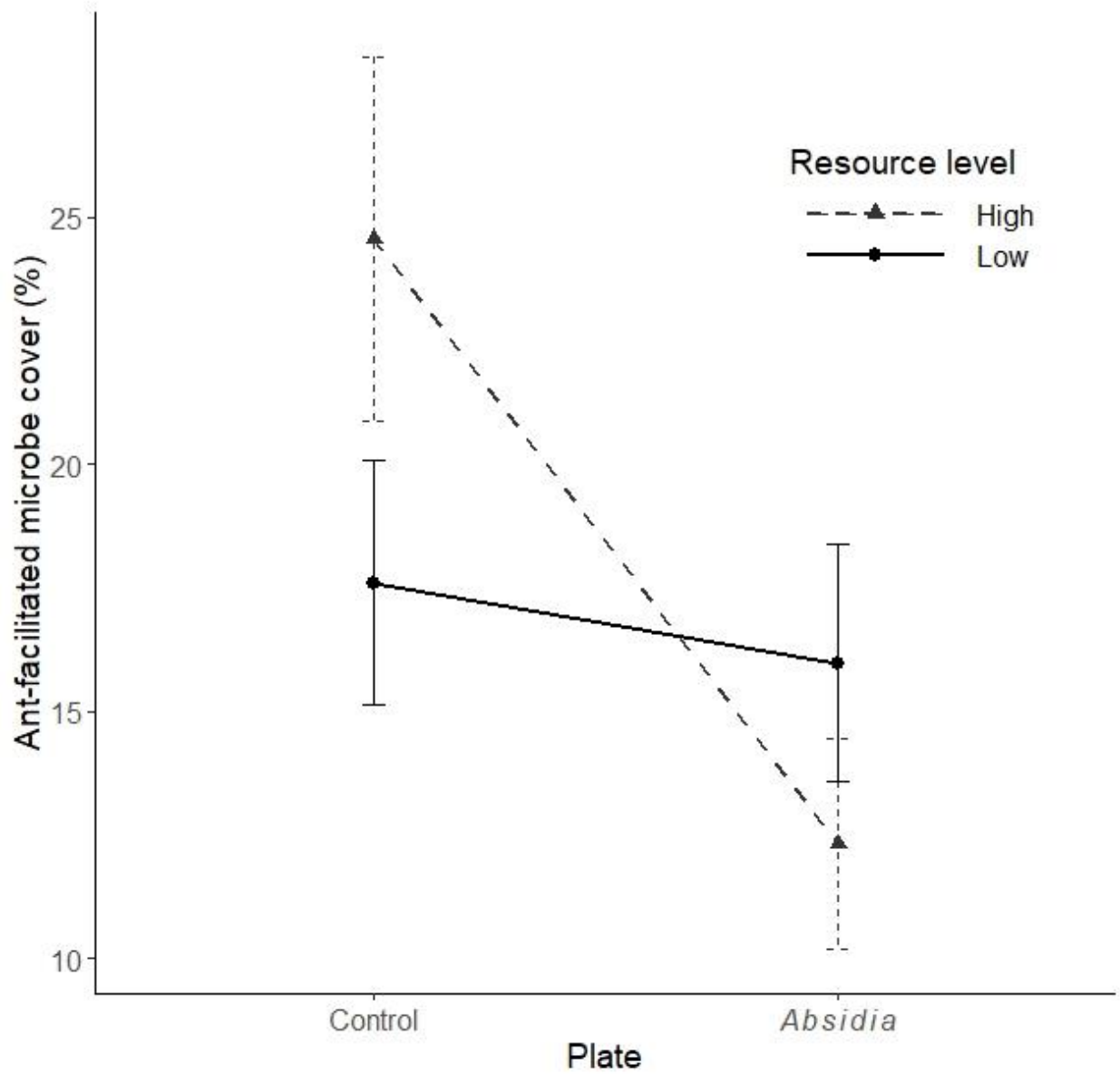
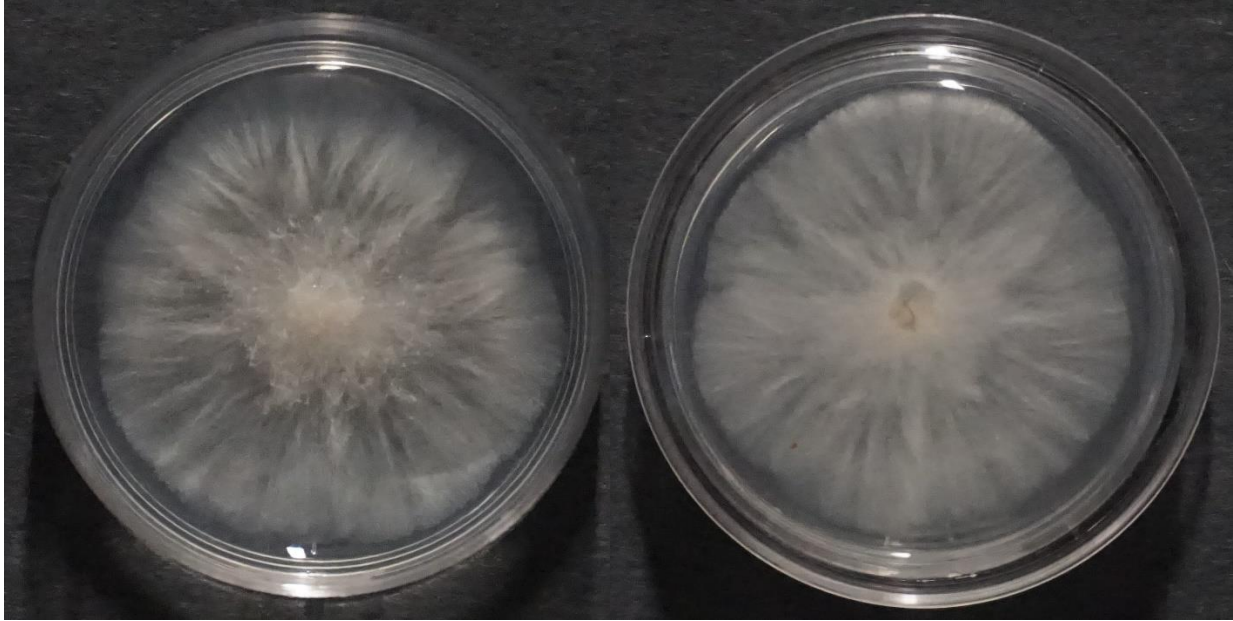
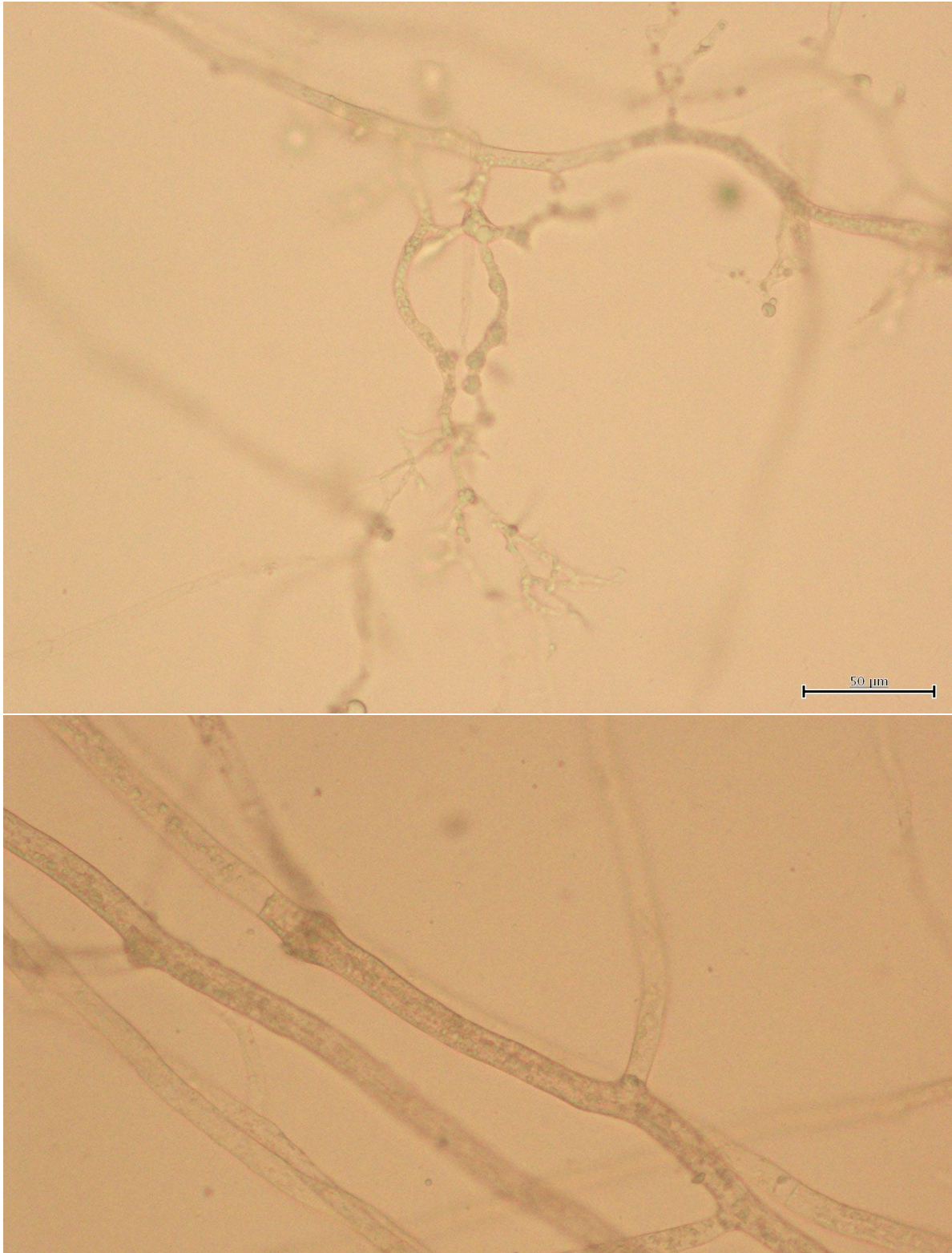


Figure 2b. Line graph showing the relationship between the mean percent cover of ant-facilitated microbes and plate type (*Absidia* sp. or control) for both high and low resource trials, across all treatments.



Supplemental Material 1a. Culture morphology of *Absidia* sp. isolated from *Aphaenogaster picea* nest soils on PDA. After 48 hours, colonies were petaloid and white. Later, colonies became woolly and buff brown to olive brown.



Supplemental Material 1b. Rhizoids (above) and sparsely septate hyphae (below) of *Absidia* sp.



Supplemental Material 1c. Globose sporangia of *Absidia* sp. with subsporangial septa, showing apophysis.



Supplemental Material 1d. Columella of *Absidia* sp. with apical projection and collarette



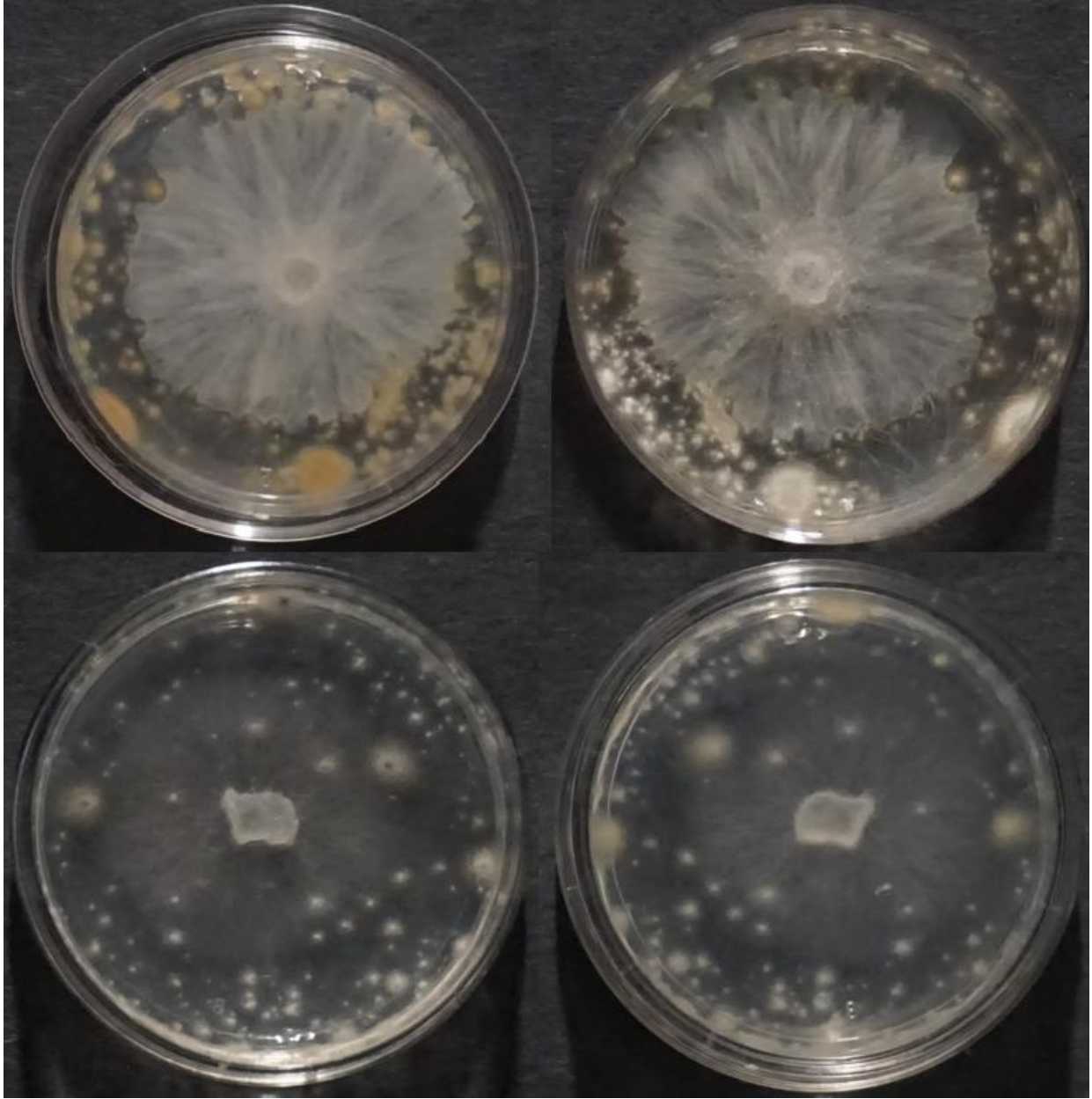
Supplemental Material 1e. Spore morphology of *Absidia* sp. showing consistently cylindrical spores.



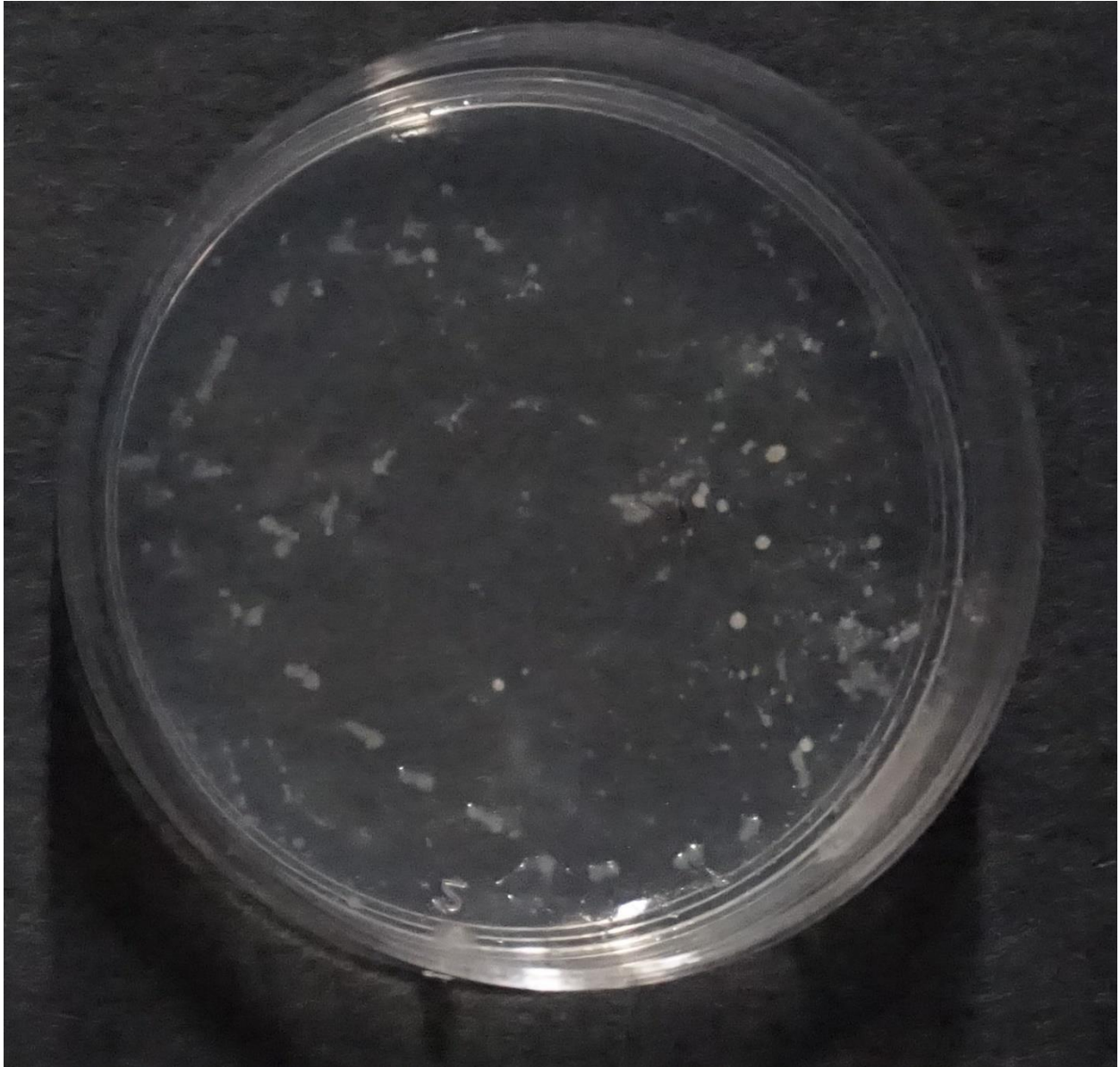
Supplemental Material 2. Images were processed using GIMP (GNU Image Manipulation Program) to improve readability for independent observers. Raw image above, processed image below.



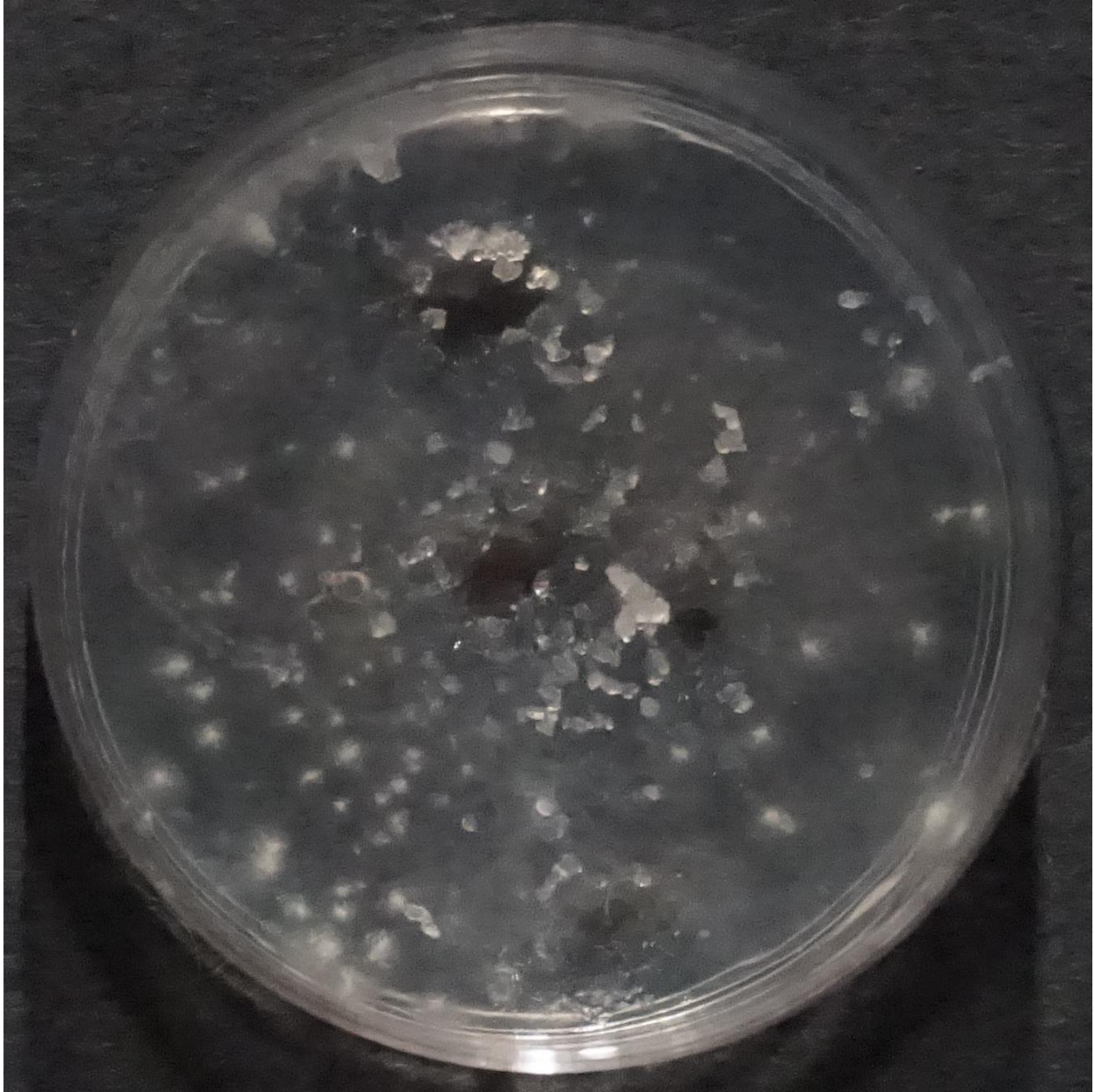
Supplemental Material 3a. The margin of *Absidia* sp. recedes in the presence of ant-facilitated microbes, suggesting inhibition of *Absidia* sp. by ant-facilitated microbes.



Supplemental Material 3b. In the high resource trial, the mycelial mat was dense, preventing colonization of ant-facilitated microbes within the diameter of *Absidia* sp. colonies (above). In the low resource trial, the mycelial mat was less dense, and ant-facilitated microbes were able to colonize within the diameter of *Absidia* sp. colonies (below).



Supplemental Material 4a. *Myrmica rubra* left “ant trails” on plates, which follow patterns of ant movement.



Supplemental Material 4b. *Myrmica rubra* excavated the agar plates, creating holes in the agar surface.



Supplemental Material 4c. A deceased ant discarded on the agar surface by *Myrmica rubra*.