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Native and non-native ant impacts on soil microbes

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Native and non-native ant impacts on soil microbes

by

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ABSTRACT OF THESIS

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Organisms produce chemical weapons for defense, but target organisms can develop resistance. In their introduced range, non-native species may bring “novel weapons” against which native organisms have not co-evolved resistance. The invasive European fire ant (*Myrmica rubra*) may have brought antimicrobial secretions to the Northeastern United States that are novel weapons against native fungal and bacterial soil organisms. I hypothesized that *M. rubra* would better inhibit seed pathogens resulting in greater emergence of native myrmecochorous *Viola sororia* seeds and, as a side effect, more strongly inhibit arbuscular mycorrhizal fungi than a native seed dispersing ant (*Aphaenogaster picea*). I also expected *M. rubra* would have greater suppressive effects on microbial respiration. To test this, I measured taxonomic richness, emergence and biomass of plants that germinated from the seed bank (volunteer plants) and introduced *V. sororia* seeds. From seeds that failed to germinate I measured percent cover of fungal growth. Finally, I recorded mesocosm CO_2 flux as a proxy measurement of microbial respiration. *Viola sororia* emergence and biomass did not differ significantly in mesocosms inhabited by *M. rubra*, *A. picea*, and control treatments, but overall seed handling was low. Volunteer plant taxonomic richness and percent cover were lower in *M. rubra* mesocosms than *A. picea* or controls, perhaps because of the comparatively higher activity levels of *M. rubra* resulted in more bioturbation. Mesocosm microbial respiration (CO_2 flux) was lower in both *M. rubra* and *A. picea* mesocosms than controls, indicating an 'ant' effect rather than a non-native ant effect via novel weapons.

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Introduction

Some plants and animals use secondary chemical compounds as a defensive or competitive weapon in response to co-occurring competitors, predators or pathogens (Swain 1977; Pasteels et al. 1983; Mérillon & Ramawat 2020). In response, enemy organisms often develop resistance to these secondary compounds (Musser et al. 2002; Callaway et al. 2008). The alternating trade-offs between two competing organisms can be seen as an "evolutionary arms race" where they co-occur (Ehrlich & Raven 1964) -- i.e., they are constantly evolving to keep each other in check (Berenbaum et al. 1986). When introduced into novel habitat, non-native species may bring a selective advantage against native organisms with whom they lack a co-evolutionary history (Vilcinskas et al. 2013). The secondary compounds of non-native species may act as "novel weapons" against which native organisms have not yet evolved resistance (Callaway & Ridenour 2004; Becerra et al. 2018). Therefore, these novel weapons may confer more competitive benefit to non-native than native species in the introduced range.

Metapleural glands are organs found only in ants, and they secrete acidic antimicrobial secondary compounds (Beattie et al. 1986; Veal et al. 1992; Yek & Mueller 2011), which ants coat themselves and nestmates in to effectively reduce the infection of ant bodies by fungal and bacterial entomopathogens (Beattie et al. 1985; Poulsen et al. 2002; Ugelvig & Cremer 2007). For example, *Acromyrmex octospinosus* ant workers with their metapleural glands experimentally closed died a few days after exposure to a pathogenic *Metarhizium* sp. fungus whereas *A. octospinosus* workers with their glands left open where unaffected by the fungus (Poulson et al. 2002). Ant metapleural gland secretions contain a suite of chemical compounds that seem to be broadly antimicrobial with variation in potency between ant species and between different microbial species and their life stages (Veal et al. 1992; Mackintosh et al. 1995; Bot et

al. 2002). Regardless, the combined antimicrobial activity of metapleural secretion compounds allow ants to inhibit a wide range of microorganisms, conferring effective pathogenic protection for the ants as well as deterring the evolution of fungal and bacterial resistance (Beattie et al. 1985; 1986; Ortius-Lechner et al. 2000; Bergstrom et al. 2004). Given that the ants secrete such broad-spectrum anti-microbial compounds, it is possible that they extend this antimicrobial protection to proximate organisms in their environment as well as items they handle.

Plants worldwide engage ants in seed dispersal (myrmecochory) [Lengyel et al. 2010]. The plants produce a lipid-rich seed appendage (elaiosome) that attracts scavenging ants that feed the elaiosome to their larvae and discard the unharmed seed away from the parent plant (Gorb & Gorb 2003; Rico-Gray & Oliveira 2007; Warren II & Giladi 2014). In tropical habitats, seeds handled by ants germinate at higher rates that those not handled by ants, which often succumb to fungal attack (Oliveira et al. 1995; Leal & Oliveira 1998; Guimaraes & Cogni 2002; Ohkawara & Akino 2005). Seed handling by tropical ants not only reduces infection by fungal pathogens, but also reduces the abundance and richness of fungal spores and hyphae on the seeds (Bot et al. 2002; Ohkawara & Akino 2005). The reduction of fungal infection as a result of seed cleaning behavior suggests that the antimicrobial benefits of ant secondary compounds extends to the seeds they handle.

In temperate habitats, the effect of seed handling by ants is less known, but ants are ecosystem engineers that affect the physical and chemical properties of the soils they inhabit by increasing moisture, aeration and nutrient inputs (Li et al. 2017; Leite et al. 2018; Shukla et al. 2018). Myrmecochore seeds benefit from ant substrate manipulation through higher germination rates in ant inhabited soils (Zettler et al. 2002; Gray 2015; Tarsa et al. 2018). Ant nests are biogeochemical hotspots that generally stimulate microbial activity (Wagner & Jones 2004;

Boulton et al. 2003; Baird et al. 2007; Jílková & Frouz 2014), and the effects of ant nest making on microbial communities differs between species based on feeding strategy and nest architecture (Dauber & Wolters 2000; Dauber et al. 2001). For example, Dauber & Wolters (2000) reported increased microbial biomass in *M. sabrinodis*, *L. niger*, and *L. flavus* occupied soils, but only found increased microbial diversity in *M. sabrinodis* and *L. niger* occupied soils.

Ants may influence microbial communities through more than just nest making, however. The structure of microbial communities in ant nests also depends on the ability of microbes to resist or even metabolize the secondary compounds produced by ants (Voglmayr et al. 2011). Fungi are particularly affected by ant antimicrobials, which generally inhibit antagonistic fungi, including phytopathogenic species (Yek et al. 2012; Gray 2015) For example, Zettler (2002) found greater fungal abundance but lower species richness and diversity in native and non-native ant nests than in non-mound soil. Ants may also influence competition between microbes, such as with *M. rubra* ants, which suppressed more pathogenic *Absidia* sp. fungi than did native *A. picea* ants, which facilitated more non-pathogenic microbes (Mokadam 2021). As such, the unique assemblages of metapleural secretions of different ant species result in unique associated microbial assemblages (Dauber and Wolters 2000; Dauber et al. 2001).

Fungi of the phylum Glomeromycota are known as arbuscular mycorrhizal fungi (AMF) (Schüβler et al. 2001). AMF colonize plant roots and form a symbiotic relationship with most terrestrial plant species, resulting in increased in growth, survival and nutrient levels, particularly phosphorus uptake (Asrar et al. 2012; Hou et al. 2021). The symbiosis between AMF and their host plant is complex and successful colonization depends on the environmental conditions, species compatibility (Walder & van der Heijden 2015), and even life stage of the host plant (Willis 2013). There is limited evidence that ants promote beneficial AMF colonization in nest

mounds through their environmental manipulations (Dauber et al. 2008), but this may primarily occur in abandoned nest galleries (Friese & Allen 1993). There are currently no studies examining the effect active ant colonies and metapleural secretions may have on AMF.

The objective of my study is to examine the effect of a non-native ant (*M. rubra*) relative to a native ant (*A. picea*) on phytopathogenic soil fungi and subsequent myrmecochorous plant success. I also will examine these effects on beneficial mycorrhizal fungi. Given that *M. rubra* brings novel chemical secretions that may impose stronger suppressive effects on native soil biota, I expect that (1) seedling emergence will be lowest in non-ant control soils, and highest in the presence of *Myrmica rubra* as a result of greater phytopathogenic fungi inhibition than by *Aphaenogaster picea* (2) AMF species richness will be lower on seedling roots exposed to *M. rubra* than *A. picea* and control soils, (3) fungal percent cover will be lower from seeds exposed to *M. rubra* than *A. picea* and control mesocosms, and (4) microbial respiration will be lower from *M. rubra* mesocosms than from *A. picea* or control mesocosms.

Methods

Study Species

Myrmica rubra (Linnaeus 1758) is a widespread seed disperser in its native Eurasian range that appeared in North America in the early 1900s where it displaces up to 95% of co-occurring native ant species (Groden et al. 2005; Prior et al. 2015; Warren et al. 2019). Colonies are polygynous and polydomous, occur in a variety of habitats and substrates, and may exploit anthropogenically altered habitats that native species cannot (Prior et al. 2015; Warren 2020). Colonies reproduce and spread through budding, often forming super-colonies that allow for

rapid expansion, higher resource exploitation, and abnormally high abundances as compared to its native range (Groden et al. 2005; Goodman & Warren 2019; Warren et al. 2019).

Aphaenogaster spp. (*A. rudis* complex) are the dominant ants in eastern deciduous forests both in abundance and biomass (Lubertazzi 2012; King et al. 2013). *Aphaenogaster picea* (Wheeler, W.M., 1908) is an omnivorous generalist and the primary seed disperser in northeastern hardwood forests (Ness et al. 2009; Clark & King 2012; Warren et al. 2019). *Aphaenogaster picea* nest in both rotting wood and soil, and outnumber other native ants (King et al. 2013; Warren et al. 2019). Both *A. picea* and *M. rubra* prefer mesic habitats, with range overlap resulting in *A. picea* being displaced by *M. rubra* colonies (Warren et al. 2019).

Viola spp. are a large genus of herbaceous, zygomorphic plants native to North America, comprising 25% of myrmecochorous plants in eastern deciduous forests (Warren et al. 2014; Franklin et al. 2017). *Viola spp.* seeds also contain elaiosomes that are particularly attractive to ants (Turnbull & Culver 1983), and their seedling recruitment is much higher in ant-occupied soils (Culver & Beattie 1980). *Viola sororia* (Common Blue Violet) was chosen for this study because it germinates relatively quickly, within 15 days (Solbrig 1981). *Viola spp.* are associated with AMF (Heijne et al. 1994; Öpik et al. 2006), and *Viola sororia* is colonized by obligate mycorrhizal fungi (Brundrett & Kendrick 1988).

Field Sampling

Myrmica rubra colonies were collected from the Tifft Nature Preserve (42.848948, -78.855335) in June-July 2020 from 10 separate nests. *Aphaenogaster picea* colonies were collected from Chestnut Ridge Park (42.710390, -78.765632) in July-August 2020 from 10 separate nests. Colonies were collected with a 20V cordless wet-dry vacuum (DC500; DeWalt, Baltimore,

Maryland), placed in a plastic bag and transported in a cooler with ice to reduce stress. Only colonies with > 20 workers and a queen were used.

Concurrent with ant collection, soils were collected from 10 nests each of *M. rubra* and *A. picea* with a minimum distance of 1 m between nests. Ten soil samples also were collected in unoccupied soils 0.5 meters from the ant nests (10 near *M. rubra* and 10 near *A. picea* nests) in similar microhabitats if possible (n = 40 soil samples total). The soil was collected by inserting a 50 mL sterile conical centrifuge tube into the soil to approximately 8 cm and then capping it. The soils were consolidated and kept on ice for transport to the laboratory where they were stored at 4°C.

Experimental Design

The experiment consisted of three treatments: soils with *A. picea* colonies, *M. rubra* colonies, and no ants. From each treatment I assessed: natural seed bank and planted *Viola sororia* emergence, biomass and cover, AMF colonization of *V. sororia*, microbial respiration and seed fungi percent cover at 40 weeks.

Mesocosm Design

All ant occupied and unoccupied soils were added to a gallon ziplock bag and shaken for 5 minutes to achieve homogenization, and to create uniform soil conditions. Between 90-100g of homogenized soil were added to 40, 5.5cm tall, 400mL glass containers. Watch glasses were placed concavely on top of soil filled containers (hereafter, “mesocosms”) and soil was moistened with deionized water to create suitable fungal, plant and ant microhabitat. Ant colonies were added to mesocosms and were provided water by misting and fed a standard

artificial diet that was changed out twice weekly (Bhatkar & Whitcomb 1970). Mesocosms were misted at differing rates depending on need due to the variation in evaporation from the fit of watch glass lids.

Viola

Viola sororia seeds were obtained from Prairie Moon Nursery (Winona, MN). Five *V. sororia* seeds were introduced to each mesocosm four weeks after ants were introduced: 10 *M. rubra* mesocosms, 10 *A. picea* mesocosms and 20 control mesocosms (n = 200). The seeds were placed on small (2 x 2 cm) weighing boats in each mesocosm. Any seeds that were not removed from the weighing boats within 24 hours were artificially placed in the mesocosm soil and noted as not directly handled. After 36 weeks, the number of successfully emerged *V. sororia* seedlings per mesocosm was recorded and, all remaining *V. sororia* seedlings were removed and dried at 65ºC for three days.

AMF Colonization

Ten weeks after *V. sororia* seeds were introduced, any *V. sororia* seedlings were removed and a slightly modified procedure based on Phillips & Hayman (1970) was used to detect and quantify mycorrhizal root colonization (Supplemental Material 1). Ungerminated *V. sororia* seeds were left alone and allowed to germinate for later biomass measurements. Seedling roots were rinsed to eliminate soil debris and then added to test tubes with 10-15 milliliters of 10% KOH. Test tubes were then placed in a 100ºC hot water bath for 7 minutes. Roots were rinsed again and 2% HCl solution was added for 2 minutes to improve stain efficiency. Root tissue was refrigerated and stored in DI water for a week to remove excess stain and improve the contrast between fungi

and roots. Root tissue from each treated *V. sororia* sample was mounted on slides with glycerol and mycorrhizal colonization was quantified using the objective crosshair technique (K. Becklin, *pers. comm.* 2021; McGonigle et al. 1990).

Seed Fungi

Thirty-six weeks after *V. sororia* seeds were introduced, 55 ungerminated seeds were recovered from 10 *M. rubra*, 10 *A. picea*, and 10 randomly selected no ant control mesocosms. To recover seeds, the remaining soil for each mesocosm was placed in a 500μm sieve and water was run through it. Debris was placed in a container with water and scanned for ungerminated *V. sororia* seeds. Seeds were stored in ziplock bags at 2° C until ready to be plated on agar at 40 weeks. Potato dextrose agar was slightly modified from Zimbro et al. (2009) as 200g of chopped potatoes were boiled in deionized water for 30 minutes and then strained through cheesecloth. Deionized water was added to the effluent until it reached 1000mL, at which point 10g of dextrose, 15g of agar, 0.1g chloramphenicol, and 0.05g rose Bengal were added before placing in the autoclave for 20 minutes. Chloramphenicol and rose bengal were added to prevent bacterial contamination (O. Novikova, *pers. comm.*, 2021).

Seeds recovered from *M. rubra*, *A. picea*, and control soils were surface sterilized using methodology from Sheppard (1979) and incubated on potato dextrose agar in order to grow any fungi that penetrated the seeds. Seeds were quickly passed through 70% ethyl alcohol solution and then placed in 2% hypochlorite solution made from household bleach and allowed to soak for 5-10 minutes to prevent bacterial contaminants and saprophytic organisms that would otherwise outgrow and obscure seed pathogens on agar. Seeds were then placed in sterile water, and forceps were dipped in 70% hypochlorite solution and passed through a flame before placing

each seed on the agar surface. Multiple seeds from the same mesocosm were allowed to exist on the same plate, and were incubated using standardized temperature, length of incubation, pH of the media, and light. Plates were inspected at 7 and 9 days of incubation, and photos were taken of any growth (Supplemental Material 2). Any colonies that grew from seeds were compared using macroscopic colony characteristics and grouped by like features.

Plant Community

Taxonomic richness

Seeds naturally occurring in the seed bank of collected soils were allowed to grow until week 40 of the experiment at which time they were identified to genus. Taxonomic richness for each mesocosm was recorded, except for graminoids which were identified as "grass."

Plant percent cover

Fourteen weeks after soils were homogenized and added to mesocosms, and 10 weeks after *V. sororia* seeds were introduced, photographs were taken of each mesocosm using a Nikon D500 camera on a Sunpak TravelLite Pro Reverse Folding Tripod (Supplemental Material 3). Percent cover based on visual estimate was recorded for all seedlings. Images were evaluated with no identifying information displayed to prevent bias based on ant treatment.

Plant biomass

Forty weeks after soils were added to mesocosms, vascular and non-vascular plants were removed, rinsed of soil and debris, and grouped via species per mesocosm (Supplemental

Material 4). Groups were then dried at 65ºC and weighed. The *V. sororia* seedlings removed early on for AMF examination were not included in these measurements.

Mesocosm Respiration

At 32 weeks after soils were added to mesocosms, each of the 40 mesocosms were placed (without glass lids) under a closed respiration chamber connected to a LI-850 gas analyzer (Supplemental Material 5) to measure the relative change of CO_2 with respect to time (CO_2 flux; hereafter, "microbial respiration"). Measurements from each mesocosm were taken over the course of 5 minutes and 30 seconds, where the first 30 seconds were removed to account for the time it took the air in the chamber to mix. The pump circulated air from the chamber and back at a rate of about 0.75L/min, and CO_2 measurements were accurate down to about 0.1ppm. The measurements were taken in a repeating order of one *M. rubra* mesocosm, one *A. picea* mesocosm, and then two control mesocosms. It was noted whether any mesocosms were dry or saturated, and mesocosms were lightly misted within an hour before respiration measurements were taken. The first 75 seconds of data from each respiration measurement were trimmed to account for the time it takes for the gas molecules to mix within the chamber and reach homogeneity. Soil dry weight was calculated using five random remaining mesocosms that were not used for seedling dry weight. These five mesocosms were placed in the desiccation oven for at least 24 hours, and the dried soils were weighed and the average was taken. Respiration calculations were adapted from Dossa et al. (2015) and expressed as umol CO_2 g^{-1} hr^{-1}.

Data Analysis

Viola sororia emergence and biomass were analyzed as a function of ant treatment (*M. rubra*, *A. picea*, control) using generalized linear models (GLMs) assuming Poisson-distributed error and fit using of analysis of deviance (ANODEV). GLM models with overdispersion >2.0 were evaluated with 'quasi' error distributions. Plant community taxonomic richness and percent cover, and microbial respiration were also analyzed as a function of ant treatment using GLMs assuming quasi-Poisson-distributed error and fit with ANODEV. All data were analyzed using R statistical software (R Core Team 2020).

Results

Viola

Total *V. sororia* emergence was low (29%), and similar across treatments; *M. rubra* (mean ± SE; 30±10.0), *A. picea* (28±6.11), and control (30±5.53) [Table 1]. Biomass (mg) of *V. sororia* did not differ between *M. rubra* (27.25±8.37), *A. picea* (15.78±2.50) and control (34.02±10.15) [Table 2]. Ant seed handling was low within the 24-hour introduction period and 76% of seeds introduced to ant mesocosms were hand-dispersed. Seed fungi percent cover did not change as a function of *M. rubra* presence (35.56±10.23) or *A. picea* presence (33.20±12.56) as compared to controls (43.89±10.23) [Table 3].

The effect of the treatments on arbuscule mycorrhizae could not be determined as no hyphae, arbuscules or vesicles were observed on any of the *V. sororia* seedling roots collected at week 10 of the experiment.

Plant Community

Of the 13 plant groups that germinated from the seed bank, 11 were vascular species and 2 were non-vascular species. Taxonomic richness was lower in *M. rubra* mesocosms (4.10±0.3785939) than in the control mesocosms (5.25±0.2279774), whereas taxonomic richness in *A. picea* mesocosms (4.90±0.3785939) did not differ from either *M. rubra* or controls [Fig. 1; Table 4]. Similarly, percent cover was lower in *M. rubra* mesocosms (53.00±8.09) than in the control mesocosms (79.10±5.63), and percent cover in *A. picea* mesocosms (66.40±9.30) did not differ from either *M. rubra* or controls [Fig. 2; Table 5]. Biomass (cg) of all mesocosm seedlings was analyzed as a function of treatments and no relationships were found between *M. rubra* (31.23±4.43), *A. picea* (28.05±3.69) and controls (36.99±6.69) [Table 6].

Respiration

Microbial respiration (umol CO_2 g^{-1} hr^{-1}) was lower in both the *M. rubra* (11.82±1.37) and *A. picea* (10.96±1.32) mesocosms than in the controls (15.01±1.35) [Fig. 3; Table 7].

Discussion

The results presented here suggest an 'ant' effect more than a novel weapons effect. Soil microbial respiration decreased in soils occupied by both *M. rubra* and *A. picea*, indicating that the native and non-native ants equally suppressed microbial activity. Still, neither ants nor novel weapons appeared to impact the emergence of *V. sororia* seedlings, *V. sororia* biomass, plant community biomass, or *V. sororia* seed fungi percent cover. Only non-native *M. rubra* ants impacted plant community taxonomic richness and percent cover as compared to the no ant control mesocosms.

The emergence and biomass of *V. sororia* seedlings was not different between ant treatments or control mesocosms. Most research involving the effects of ant handling on seeds has focused on seed survival and germination, with mixed results. Some studies reported that ant seed handling resulted in increased survival and germination (Ohkawara & Akino 2005; Sasidharan & Venkatesan 2019) whereas Fernandes et al. (2018) observed the opposite: elaiosome detachment and seed scarification by *Acromyrmex subterraneus* ants decreased germination. As seed handling by ants in this experiment was low, it is not possible to say whether emergence was promoted or inhibited by ant seed handling.

Enhanced seed germination and seedling survival also occurs in ant-inhabited soil (Culver & Beattie 1980; Dean & Yeaton 1992). Ants increase the nutrient contents of ant occupied soils which, in turn, benefits plants (Dostál et al. 2005; Jílková et al. 2015) Ants also may decrease phytopathogenic fungi in occupied soils (Zettler et al. 2002; Gray 2015; Tarsa et al. 2018), which would greatly benefit seedling recruitment, but I found no indications of phytopathogenic suppression, similar to the findings of Lucas et al (2019) which found no indication of phytopathogen suppression by *Azteca alfari* ants.

The substrate used in the *V. sororia* emergence experiment included soil collected from a dense stand of *Rhamnus cathartica* (European buckthorn), where *M. rubra* located some colonies. *Rhamnus cathartica* is a non-native plant that exudes emodin from its roots, a potent allelochemical that inhibits seed germination and seedling growth (Inoue et al. 1992; Orr et al. 2005; Tucker 2016), and the effect lasts even after *R. cathartica* removal (Klionsky et al. 2011). Emodin also disrupts plant mutualisms such as AMF (Hale & Kalisz 2012; Pinzone et al. 2018), and the incorporation of invader allelochemical extracts into soil results in the significant

reduction of AMF spore germination (Stinson et al. 2006). It is possible that the presence of emodin in the study soils reduced *V. sororia* emergence and inhibited AMF in the mesocosms.

The lack of AMF colonization was indeed surprising, but AMF prefer the plant rhizosphere (Young 2012), and the study soils were collected from ant nests and near-nest sites with little-to-no plant growth. Host incompatibility, high phosphorus, competition with soil microbiota, and a low initial population of AMF in the soil also can hinder the colonization process (Smith & Read 2008; Dumbrell et al. 2010; Svenningson et al. 2018). The addition of native AMF innoculum to the seedling roots would have increased the chances of AMF colonizing *V. sororia* seedlings (Panwar et al. 2007; Carter et al. 2014; Davidson et al. 2016), or at least collecting soil from areas with dense plant communities.

The effect of ants in general on AMF is a relatively lesser studied of the ant-plant-fungi interactions. Some studies report that AMF is promoted by ants (Snyder et al. 2002; Dauber et al. 2008), but work by Lindström et al. (2019) using DNA sequencing of fungi and bacteria from ant nests reported no AMF. AMF absence in ant nests could possibly be due to the ability of ants to increase available phosphorus in nest soil (Wang et al. 2017), as high phosphorus conditions limit AMF colonization. The ability of AMF to exist in ant nests may also depend on their ability to resist metapleural secretions, or to occupy abandoned galleries (Friese & Allen 1993) where they can benefit from ant nutrient additions without experiencing the inhibitory effects of metapleural secretions.

Seedling percent cover and taxonomic richness were only lower in *M. rubra* mesocosms compared to controls. These results are likely attributed to the bioturbation activity of *M. rubra* when building nests and when foraging, which suppresses plant species richness as compared to abandoned nests and non-ant soil (King 1997; Sosa & Brazeiro 2012; Wang et al. 2017).

Myrmica rubra generally are more active and more voracious foragers than native ants (Prior et al. 2015; Gammans et al. 2018; Prior et al. 2020), which may have suppressed seedlings more than *A. picea* in this study. Additionally, most of the *M. rubra* colonies in the mesocosms were attacked by an entomopathogen that appeared to be *Ophiocordyceps myrmicarum* (Simmons et. al 2015). *Ophiocordyceps myrmicarum* (Simmons et. al 2015) appeared to change worker behavior by increasing foraging activity (*pers. obs.*).

Microbial soil respiration was lower in both *M. rubra* and *A. picea* mesocosms as compared to unoccupied control mesocosms, suggesting that native and non-native ants equally suppressed microbial respiration. This finding contrasts with Jílková & Frouz (2014) findings that ants stimulated microbial soil respiration. However, the effect of ants on microbes is inconsistent, with contrasting reports of positive or negative effects by ants on microbial abundance, richness, or diversity (Dauber & Wolters 2000; Dauber et al. 2001; Zettler et al. 2002; Boulton et al. 2003; Lucas et al. 2019).

A surprising discovery was that of a fungus suspected to be *Ophiocordyceps myrmicarum* (Simmons et. al 2015) on the deceased bodies of workers in several *M. rubra* mesocosms (Supplemental Material 6). None of the *A. picea* colonies were found containing this fungus, even when a *M. rubra* body was introduced to an extra *A. picea* mesocosm. By week 11 of the experiment, 5 *M. rubra* mesocosms and 3 *A. picea* mesocosms were deceased. This was a high enough mortality rate that I expected an impact on microbial respiration. Two of my mesocosms were extremely dry and thus their CO_2 efflux measurements were omitted from the study as outliers due to significantly higher CO_2 efflux measurements once rewetted; a typical response of re-wetting air-dried soil (Iovieno & Bååth 2008), likely due to the dead bodies of microbes fueling the growth of surviving microbes. Earthworms were found in three mesocosms (*M. rubra*

06, *M. rubra* 08, and control 10) which increased soil churning in those mesocosms but did not seem to affect CO_2 efflux measurements. Many different organisms were observed growing in mesocosms throughout the duration of the study (Supplemental Material 7).

Conclusion

There is mixed support between the beneficial or negative effect of seed dispersing ants, whether native or non-native, on myrmecochore plants. My results demonstrate no effect by either native *Aphaenogaster picea* or non-native *Myrmica rubra* ants on myrmecochorous *Viola sororia* emergence, biomass, or seed fungi percent cover, a result that is not consistent with the novel weapons hypothesis and may be explained by the low rate of seed handling by ants. The native and non-native ants did appear to equally suppress microbial respiration, and these findings contrast with current research involving ant effects on microbial respiration. Non-native *M. rubra* ants seemed to have a greater suppressive effect on plant community taxonomic richness and percent cover than control soils only, and is likely a result of their higher bioturbation activities.

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TABLES

Table 1. Mean percent emergence of *Viola sororia* seedlings per treatment with standard error (SE).

Treatment	***%***	***SE***
Myrmica rubra	30	10.0
Aphaenogaster picea	28	6.11
Control	30	5.53

Table 2. Mean biomass of *Viola sororia* seedlings per treatment with standard error (SE).

Treatment	***mg***	***SE***
Myrmica rubra	27.25	8.37
Aphaenogaster picea	15.78	2.50
Control	34.02	10.15

Table 3. Mean percent cover of *Viola sororia* seed pathogens per treatment with standard error (SE).

Treatment	***%***	***SE***
Myrmica rubra	35.56	10.23
Aphaenogaster picea	33.20	12.57
Control	43.89	10.23

Table 4. Mean taxonomic richness of seedlings grown from the soil seed bank per treatment with standard error (SE).

Treatment	***# Species***	***SE***
Myrmica rubra	4.10	0.3785939
Aphaenogaster picea	4.90	0.3785939
Control	5.25	0.2279774

Table 5. Mean percent cover of all seedlings per treatment with standard error (SE).

Treatment	***%***	***SE***
Myrmica rubra	53.00	8.09
Aphaenogaster picea	66.40	9.30
Control	79.10	5.63

Table 6. Mean biomass of all seedlings per treatment with standard error (SE).

Treatment	***cg***	***SE***
Myrmica rubra	31.23	4.43
Aphaenogaster picea	28.05	3.69
Control	36.99	6.69

Table 7. Mean microcosm respiration measurements (CO_2 Flux) per treatment with standard error (SE).

Treatment	***R (umol CO_2 g^{-1} hr^{-1})***	***SE***
Myrmica rubra	11.82	1.37
Aphaenogaster picea	10.96	1.32
Control	15.01	1.35

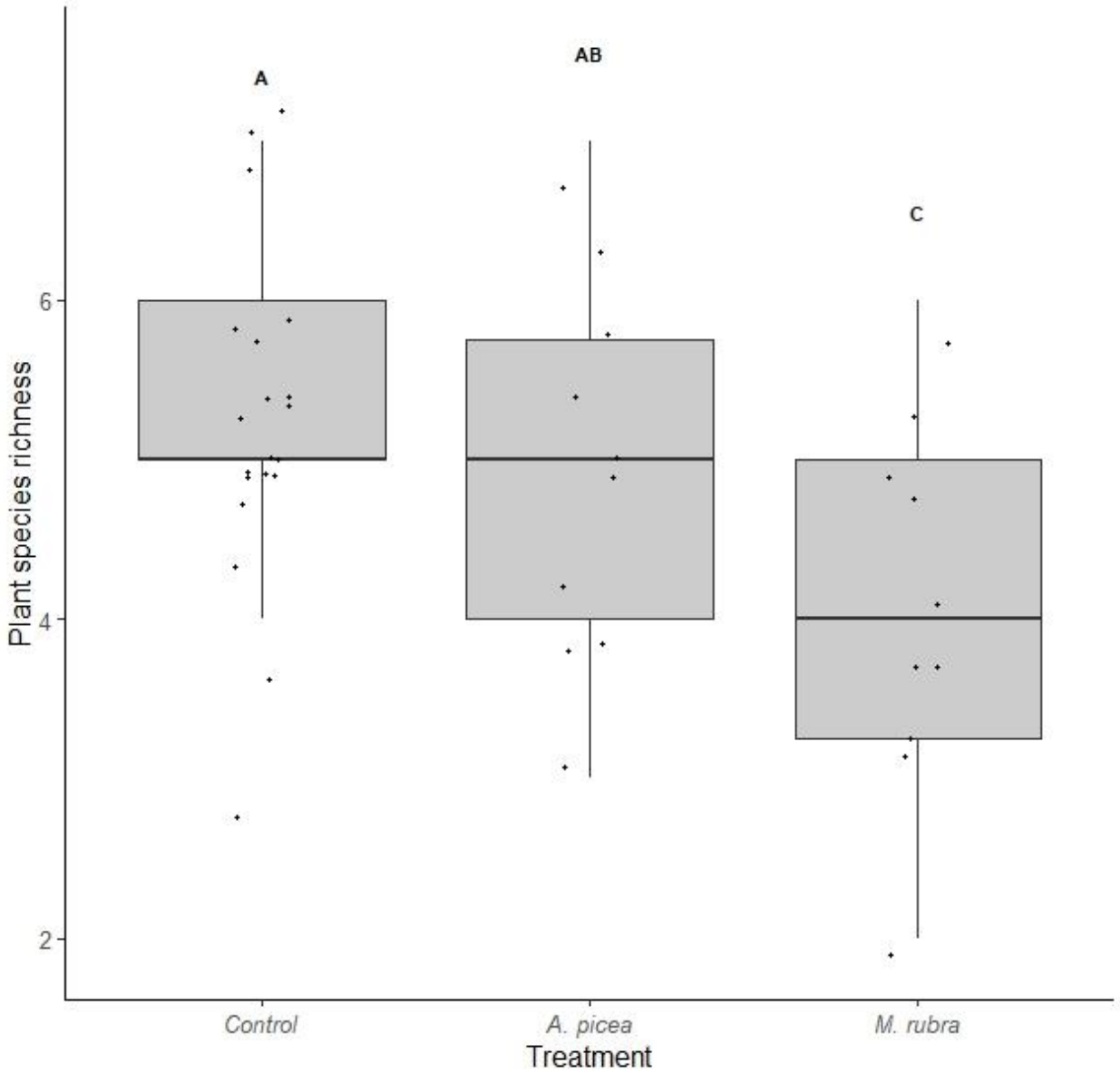


Figure 1. Boxplots showing Mean taxonomic richness of seedlings grown from the soil seed bank per treatment – no ants, Aphaenogaster picea, and Myrmica rubra. Boxes annotated with different letters are significantly different.

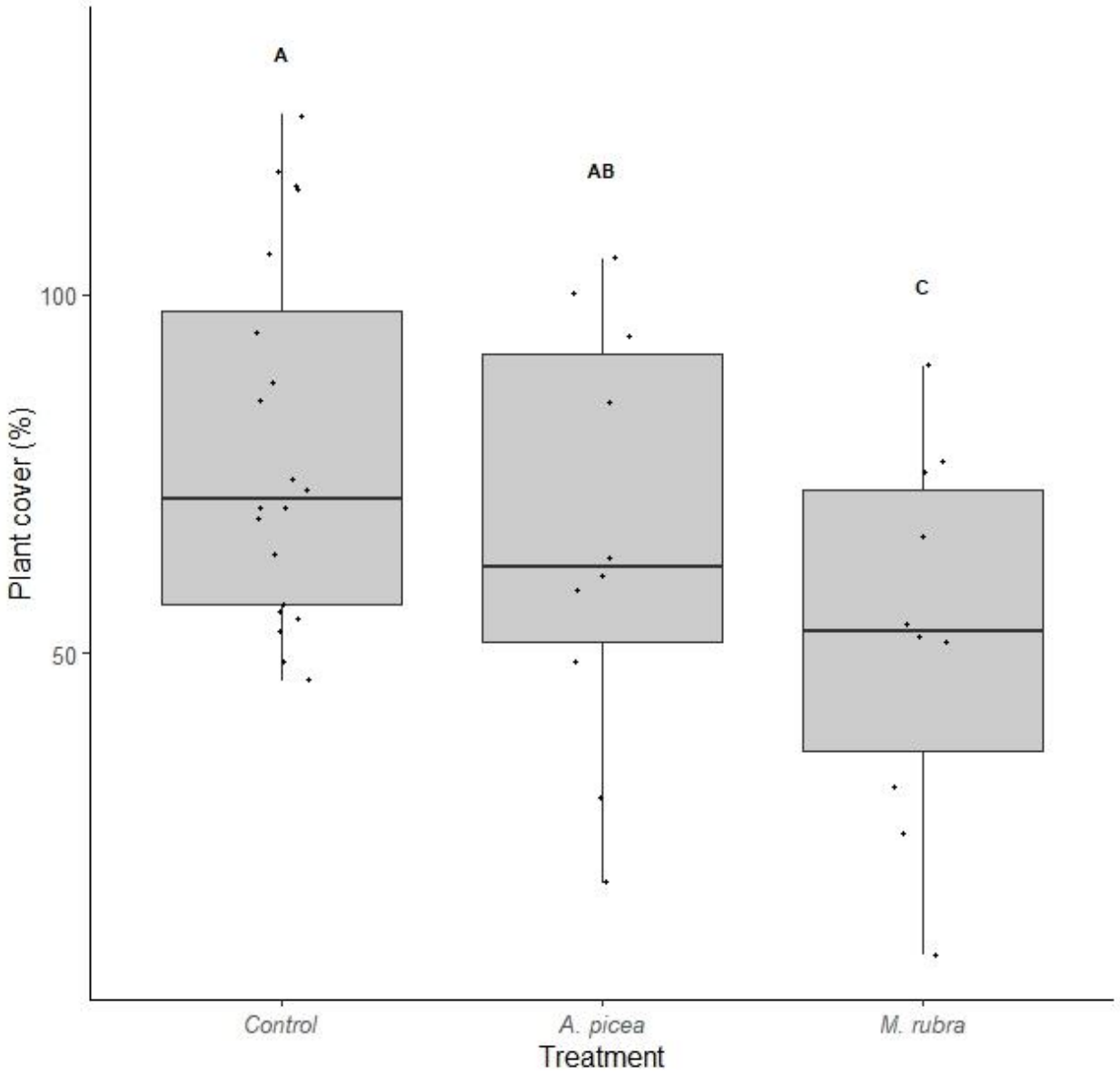


Figure 2. Boxplots showing mean percent cover of all seedlings per treatment – no ants, Aphaenogaster picea, and Myrmica rubra. Boxes annotated with different letters are significantly different.

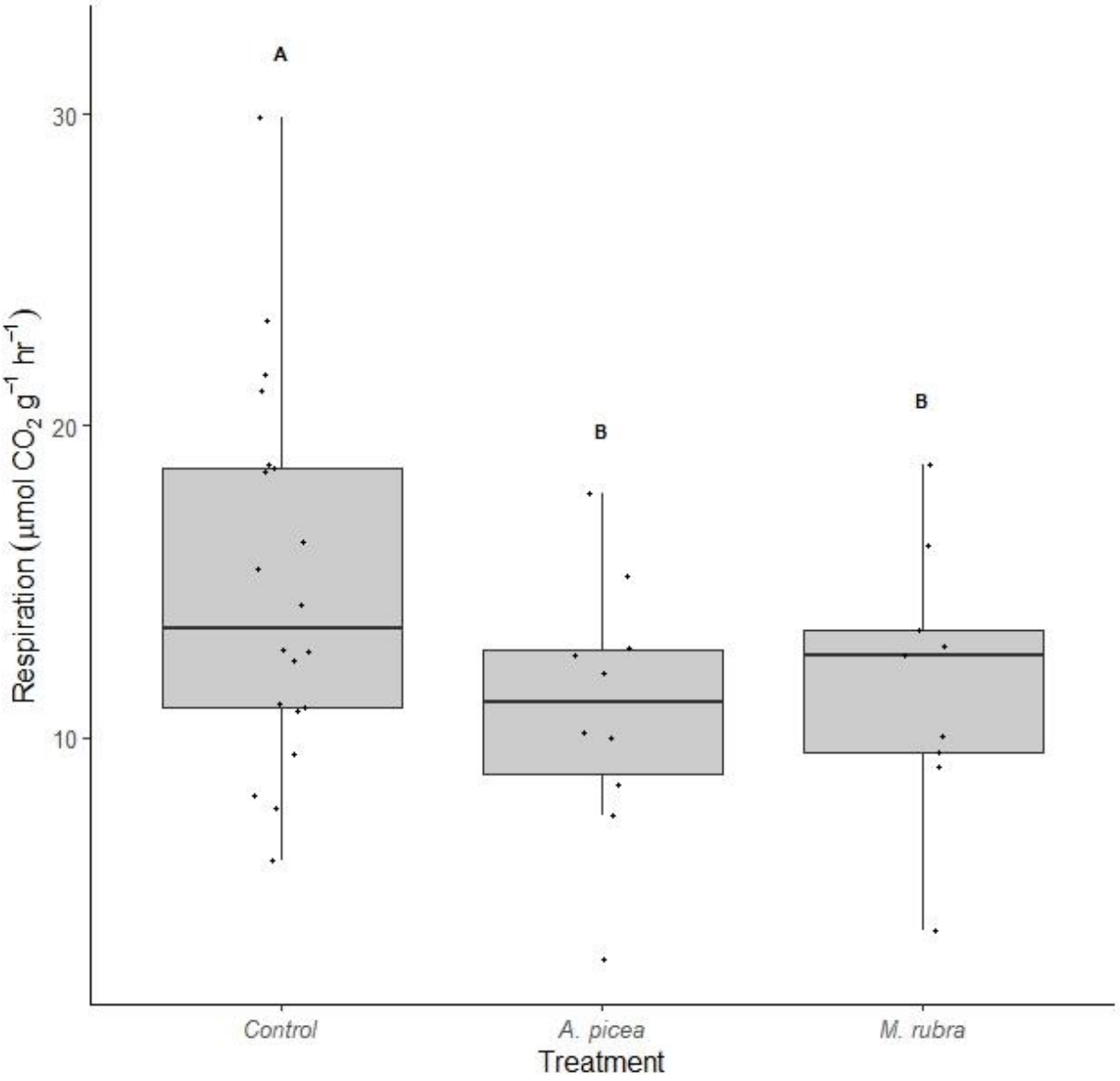
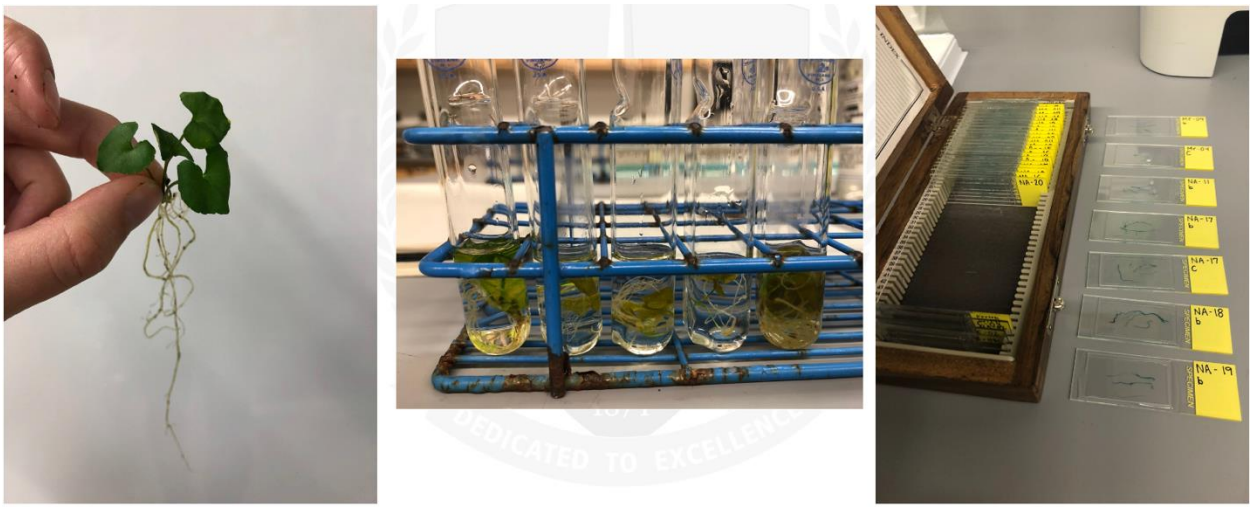


Figure 3. Boxplots showing mean CO_2 flux per treatment – no ants, Aphaenogaster picea, and Myrmica rubra. Boxes annotated with different letters are significantly different.



Supplemental Material 1. A *Viola sororia* seedling removed from a mesocosm after ten weeks of growth, followed by clearing, staining and mounting on slides to detect AMF colonization.

Supplemental Material 2. Colony morphology of fungi grown from within *Viola sororia* seeds that failed to germinate. Seeds were plated on PDA with chloramphenicol and rose bengal.

Supplemental Material 3. Mesocosms with fourteen weeks of growth for seeds naturally occurring in the seed bank, and ten weeks of growth for the introduced *Viola sororia* seeds.

Supplemental Material 4. Seedlings removed from mesocosms after 40 weeks of growth.

Supplemental Material 5. Microbial respiration setup showing a mesocosm and the closed respiration chamber connected to a LI-850 gas analyzer.

Supplemental Material 6. Deceased *Myrmica rubra* individuals colonized by what appears to be *Ophiocordyceps myrmicarum*.

Supplemental Material 7. Photos showing organisms that grew in mesocosms throughout the study.