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Incorporating redispersal microsites into myrmecochory in eastern North American forests

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Abstract. Studies addressing the benefits of "directed dispersal" in ant seed dispersal systems have highlighted the beneficial soil properties of the nests of ants that disperse their seeds. No studies, however, have explored the properties of soils nearby exemplary seed-dispersing ant nests, where recent work indicates that seeds are quickly "redispersed" in eastern North America. To address this, we focused on a forested ecosystem in eastern United States where a keystone seed-dispersing ant, *Aphaenogaster rudis*, commonly disperses the seeds of numerous understory herbs, including *Jeffersonia diphylla*. We collected soil cores beneath *J. diphylla*, around *A. rudis* nests where seeds are dispersed, and from other forest locations. We analyzed the collected soils for microbial activity using potential soil enzyme activity as a proxy, as well as a number of environmental parameters. We followed this with a glasshouse experiment testing whether the soils collected from near nests, beneath *J. diphylla*, and from other forested areas altered seedling emergence. We found that microbial activities were higher in near-nest microsites than elsewhere. Specifically, the potential enzyme activities of a carbon-degrading enzyme (β-glucosidase), a phosphorus-acquiring enzyme (phosphatase), and a sulfur-acquiring enzyme (sulfatase) were all significantly higher in areas near ant nests than elsewhere; this same pattern, although not significant, was found for the nitrogen-acquiring enzyme NAGase. No differences were found in other environmental variables we investigated (e.g., soil temperature, soil moisture, soil pH). Our field results indicate that soil biological processes are significantly different in near-nest soils, where the seeds are ultimately dispersed. However, our glasshouse germination trials revealed no enhanced germination in near-nest soils, thereby refuting any near-term advantages of directed dispersal to near-nest locations. Future work should be directed toward addressing whether areas near ant nests provide biologically meaningful escape from seed predation and enhanced establishment, and further characterization of soil microbial communities in such settings.

Key words: ant seed dispersal; *Aphaenogaster;* directed dispersal; myrmecochore; potential enzyme activity; seed germination; soil microbes.

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Introduction

Animal seed dispersers improve the fate of seeds in a variety of ways. Qualitatively, seed dispersers can be considered effective if seed deposition patterns, manifested by the disperser, increase the probability that a seed survives to become a reproductive adult (Schupp 1993). Because environmental factors obviously vary across a landscape, the quality of deposition can be influenced by that variability even at small spatial scales (Schupp et al. 2010). The disproportionate dispersal of seeds by animals to non-random locations that confer subsequent plant

fitness advantages—also known as directed dispersal (Howe and Smallwood 1982)—has been documented in a number of animal seed disperser systems (Wenny 2001). In this context, myrmecochory, or ant-mediated seed dispersal of plants with seed coat-derived nutritional appendages, is a primary example (Hanzawa et al. 1988, Rico-Gray and Oliveira 2007).

The mutualistic relationship between plants and associated seed-dispersing ant species has fascinated ecologists for over 100 years (see Ridley 1930) and has been studied extensively. The numerous positive roles ants play in the dispersal process make for challenges in identifying unifying ecological themes, and cause much intrigue around coevolutionary processes (Giladi 2006). The ~11,000 plants involved in myrmecochorous relationships produce seeds that contain a fleshy appendage called an elaiosome, a trait that may have evolved independently in plants over one hundred times (Lengyel et al. 2010). Chemicals that constitute elaiosomes, particularly oleic acid, which is the most common fatty acid found in hymenopterans (Thompson 1973, Brew et al. 1989, Turner and Frederickson 2013), encourage ants to carry diaspores (seeds with their attached elaiosomes) to their nests (Gordon 1983). Larval ants receive a nutritional reward from the elaiosome, and seeds are not harmed during the elaiosome removal/consumption process in the nest (Lisci et al. 1996, Gammans et al. 2006, Fischer et al. 2008).

There have been several hypotheses posed about the benefits that plants receive from myrmecochory. For example, the seeds could experience "predator avoidance" (Manzaneda et al. 2005) via seed burial in ant nests, and/or seedlings could gain "nutrient enrichment" (Manzaneda and Rey 2012) in the nest or midden microsites. In fact, the seeds directly dispersed to such nonrandom locations can have higher probabilities of making it to adulthood. Burial has experimentally been shown to increase myrmecochorous seed survival (Christian and Stanton 2004, Kwit et al. 2012), and seed-dispersing ant nests, nest edges, and middens have been documented to be more nutrient rich than elsewhere (Horvitz and Schemske 1986, Bebawi and Campbell 2004, Berg-Binder and Suarez 2012). Rarely, however, has this working model been validated by documented increases in fitness measures, and when it has (Culver and Beattie 1980, Bebawi and Campbell 2004), the mechanisms underlying assumed or documented differences in soil properties have not been addressed.

Because ants only disperse seeds of myrmecochores short distances (global mean of ~1.99 m [Gomez and Espadaler 2013]), microsite differences at small spatial scales should play a significant role in myrmecochorous relationships. Moreover, recent work has shed new light on the ultimate deposition site of myrmecochore seeds in temperate forests by revealing that seed-dispersing ants quickly redisperse seeds short distances out of their nests after elaiosome consumption (Gorb et al. 2000, Canner et al. 2012). In these systems, such ultimate locations should comprise the focus of efforts aimed at testing directed dispersal hypotheses. Soil microbes, which may be involved with microsite differences (Caldwell 2005), and whose enzymes have been associated with both seed germination and survival (Kremer 1993), may influence the seed survival and seedling establishment of ant-dispersed plants and are therefore good indicators of how soil properties around nests may influence seeding establishment.

We assessed relevant microsite-specific abiotic and biotic soil properties in a temperate deciduous forested system (eastern Tennessee, USA) as they pertained to a common herbaceous understory myrmecochore, *Jeffersonia diphylla*. In this system, we have noted that the seeds of *J. diphylla* are dispersed primarily by *Aphaenogaster rudis* (R. K. Connell, A. A. Pfennigwerth, and C. Kwit, *personal observations*), which has been referred to as a "keystone" seed-dispersing species (Ness et al. 2009) and is known to redisperse seeds ~30 cm away from ant nests after elaiosome consumption (Canner et al. 2012). Specifically, we addressed the following questions: (1) "Are there abiotic or biotic differences in soil properties or processes among the microsites located near ant nests, beneath parent plants, and at other forest locations?" (2) "Do these differences in microsite characteristics influence plant germination success?" Ultimately, we aimed to provide empirical and experimental evidence to support or refute an aspect of the directed dispersal hypothesis using short-term outcomes in the interaction between *A. rudis* ants and *J. diphylla* plants.

Methods

Study species

Jeffersonia diphylla Bart. (Berberidaceae, hereafter *J. diphylla*) is a spring-flowering perennial herb found on mesic, calcareous soils in eastern deciduous forests in eastern North America. Its natural history was studied extensively by Smith et al. (1986). It reproduces both vegetatively and by seed. *J. diphylla* flowers in mid-Spring with mature ramets producing one pear-shaped, 2–5 cm long fruiting capsule; fruits contain 10–25 seeds per capsule, with each seed bearing an elaiosome. The seeds mature and fall to the ground in the summer, and ants collect and deliver these elaiosome-bearing seeds (diaspores) to their colony. *Aphaenogaster rudis* (Formicidae: Myrmicinae, hereafter *A. rudis*) is the primary seed dispersal vector of many temperate deciduous forest myrmecochores (Ness et al. 2009), including *J. diphylla* (R. K. Connell, A. A. Pfennigwerth, and C. Kwit, *personal observations*). Their nests are typically found in rotting logs, leaf litter, in soil, and under rocks; they can be comprised of 266–613 workers on average, and nest densities average between 0.5 and 1.3 nests per square meter in eastern North American forests (Lubertazzi 2012). Nest locations are ephemeral, in that colonies relocate on average every 30 days a distance of, on average, 0.38 m (Smallwood and Culver 1979).

Study areas

Both field sites are located in east Tennessee in mixed deciduous forest comprising mostly *Acer* spp., *Carya* spp., *Fagus grandifolia, Juglans nigra, Liquidambar styraciflua, Liriodendron tulipifera,* and *Quercus* spp. Site selection was based on sufficient *A. rudis* nest abundance (>20 nests) and the presence of large (>5 m^2) *J. diphylla* patches. Ant nests were located by baiting worker ants with tuna, then following individuals back to nest sites. Baits were removed after ~30 min to minimize food addition to the environment, and care was taken to minimize disturbance to vegetation and ant nest sites. Site A is located at the Forks of the River Wildlife Management Area, Knox County, TN (300 m elevation) (35.95° N latitude, −83.86° W longitude). Site B is located within the University of Tennessee Forest Resources AgResearch and Education Center, Cumberland Forest Unit (North Tract), Scott County, TN (425 m elevation) (36.23° N latitude, −84.56° W longitude). Work at Forks of the River Wildlife Management Area was permitted by the Tennessee Wildlife Resources Agency. Work at the North Tract of the Cumberland Unit of the University of Tennessee's Forest Resources AgResearch and Education Center was permitted by the University of Tennessee Institute of Agriculture.

Collection and storage of soils

Soils were collected from both sites in June 2013 for soil property analysis. Soils from Site A were additionally used in our study of potential soil enzyme activity, while soils from Site B were used in our glasshouse soil-source seed germination experiment. Soils from Site A were collected on 12 June 2013. Soil cores (2 cm wide × 10 cm deep) were collected from locations representing three different soil microsites relevant to our seed dispersal system: (1) 20 cm from active *A. rudis* nests (hereafter "near nest"), which represents the ultimate location to which seeds are dispersed (2) directly beneath *J. diphylla* parent plants (plants with present-year fruiting capsules present, hereafter "parent plant"), which represents the final location of nondispersed seeds, and (3) from other forest soil locations, which were >1.5 m away from both *A. rudis* nests and *J. diphylla* individuals (hereafter "other"). A representative sample for each individual microsite consisted of two homogenized soil cores. For each microsite type, there were 10 samples, resulting in a grand total of 30 sample sites; the samples were considered independent given high spatial variability of soil microbial communities at scales <1 m (Webster et al. 2002, Horner-Devine et al. 2004). The two soil cores at a given sampling location were collected using a hammer core and then homogenized and immediately stored at 4°C until analysis. Within 24 h of collection, the soils were sieved to 2 mm; ~15 mL subsamples were used for determining gravimetric water content (GWC), and 1.0 g subsamples were used for enzyme analysis.

Soils from Site B were collected on 16 June 2013. Using a hammer core, sixteen 5 cm wide × 10 cm deep soil cores from each of the three aforementioned microsites were collected, resulting in a grand total of 48 samples. Individual samples were sieved to 4 mm to remove rocks and

roots. Soils were mixed with sterilized coarse sand (~ 30% by mass) to improve soil drainage (Kardol et al. 2013). Eight samples were randomly selected from each of the three soil types (near nest, parent plant, and other) for the seed germination experiment. In addition, the remaining eight parent plant and near-nest samples were randomly matched with each other and homogenized, resulting in eight "parent plant/near-nest" soils, which served as an additional treatment in the seed germination experiment. Soil–sand mixtures were added to microcosms (13.5 cm × 6 cm × 6.5 cm) lined with a permeable cloth liner (Gardeneer by Dalen Harvest-Guard) to prevent the soil loss through drainage holes.

Soil properties

At Site A, we measured soil temperature (0–10 cm) with a soil thermometer and soil (0–10 cm) volumetric water content (VWC) with a HydroSense II soil moisture meter (Campbell Scientific) at each location we cored. Soil temperature and VWC were averaged from three readings at each location. We also assessed an additional soil moisture parameter, gravimetric water content (GWC), from the three different microsites at Site A as part of the steps required to assess the potential enzymatic activity. For this, a ~15 mL subsample from each soil sample at Site A was used. Subsamples were placed in tins and weighed, placed in a convection oven for 48 h at 105°C, removed from the oven and placed in a dessicator for 20 min to cool, and then reweighed. GWC was calculated as [(fresh weight + tin) − (oven-dried weight + tin)] / [(oven-dried weight + tin) − tin].

We assessed the total soil carbon among the three microsites via a "loss by ignition" method. For this, we randomly selected five 1 g air-dried soil samples from the collected samples from each microsite type (near nest, parent plant, and other) at each site, for a total of 30 subsamples. We dried soils in an oven at 60°C for 48 h to remove soil water. The soils were then weighed and placed into a muffle furnace at 550°C for 6 h. After allowing the soils to cool overnight, we placed them into a dessicator for 20 min. The soils were weighed to calculate the amount of carbon lost, or the amount of total carbon in each sample.

We also assessed pH among the three microsites. For this, we randomly selected five 10 g air-dried samples from each microsite type (near nest, parent plant, and other) from both sites for a total of 30 subsamples. We mixed each sample with 20 mL of 0.01 M $CaCl_2$ solution in a centrifuge tube. We ensured that the soils were properly mixed in the $CaCl_2$ solution by shaking the centrifuge tubes every 10 min for half an hour. We allowed each sample to settle for another half an hour and then used a Denver Instrument pH probe to measure the pH levels of each soil sample.

Potential soil microbial enzymatic activity

To assess how soil microbial activity, here a variety of nutrient-acquiring and decomposition enzymes, may differ based on the relevant potential dispersal microsites, we examined the potential enzyme activity of the soils collected at Site A in each microsite type (near nest, parent plant, and other). We assayed for enzymes that indicated the degradation of different carbon, nitrogen, phosphorus, and sulfur substrates that are important for plant and microbial growth: β-glucosidase (cellulose degradation), N-acetylglucosaminidase (NAGase; mineralization of nitrogen from chitin), sulfatase (sulfur mineralization), and phosphatase (phosphorus mineralization). We followed the protocols outlined by Sinsabaugh et al. (1999), using 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-phosphatase, 4-methylumbelliferyl-sulfate, and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as substrates, respectively. Soil subsamples of ~1.0 g fresh mass were homogenized with 125 mL of 50 mmol/L acetate buffer (pH 5). Each prepared soil homogenate (200 μL) was combined with 50 μL substrate solution in 96-well plates. For each assay, there were 10 analytical replicates plus blank, reference standard, and negative controls. The plates were incubated for 2 h, except for NAGase plates that were incubated for 0.5 h. NaOH (25 μL) was added to each well to stop the reaction and raise the pH. Fluorescence was analyzed using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT). The results were calculated as nmol of substrate converted per hour per g soil dry mass ($nmol \cdot h^{-1} \cdot g^{-1}$) using the calculated GWC of the soils.

Seedling emergence trials

On 16 June 2013, fruiting capsules with freshly matured seeds were collected from 50 individual *J. diphylla* parent plants at Site B and stored dry at

~20–25°C. Within 2 weeks of field collection, 10 *J. diphylla* seeds from individual parent plants were added to the surface of each soil microcosm. "Parent plant" soils received seeds from the corresponding parent plant, except for three "parent plant" soils that were assigned a random seed source due to fungal infection of corresponding seed source. "Parent plant/near-nest" soils received randomly selected seeds from the remaining corresponding parent plant seed sources not utilized for "parent plant" soils. "Other" soils and "near-nest" soils received seeds from randomly selected parent plants. Microcosms were kept field moist from beneath in trays with saturated wicking fabric, and kept in a glasshouse at the University of Tennessee, Knoxville, TN, from early July 2013 through early February 2014. They were then moved to an outside location in a 50 cm by 50 cm by 20 cm plastic container filled halfway with potting soil in early February 2014. The plastic containers had holes drilled into the bottom to allow water drainage. The lids of the plastic container were cut out and replaced with rodent-proof wire mesh and covered with hardware cloth to minimize splashing and erosion from heavy rain events. The movement of microcosms outside in February 2014 to experience ambient temperatures likely did not allow for sufficient required cold stratification to stimulate radicle emergence and above-ground stem germination in spring 2014. Because *J. diphylla* seeds require the sufficient cold stratification followed by warm stratification for germination (Baskin and Baskin 1989), we kept the seeds outside until March 2015. From late January 2014 until March 2015, we monitored for germination (radicle emergence and above-ground stem germination) at biweekly intervals and noted whether any of the seeds were missing or noticeably dead (i.e., empty seed coat). Other than five seeds that showed signs of germination in 2014, the vast majority of germination took place in 2015.

Data analysis

We used ANOVA to assess the significant differences in the measured physical properties of soil (VWC, GWC, temperature, pH, total carbon) as well as the potential enzymatic activity, among the different microsites. A Bonferroni correction to $\alpha = 0.05$ was applied for significance for global tests involving multiple dependent variables measured from the same sampling unit (e.g., $\alpha = 0.007$ [0.05/7] served as the critical *p*-value for global tests for differences among microsites for the four enzymes investigated, soil temperature, and gravimetric and volumetric water content). Post hoc Tukey-Kramer HSD comparisons were made to further assess the differences among microsites. For the germination trials, we used ANOVA to assess the significant differences among the number of germinants (defined by radicle emergence) in each treatment. Raw data sets used in our analyses can be found in Data S1.

Results

Soil properties

We did not detect significant differences among physical soil properties near ant nests, beneath the parent plant, and in other locations in the forest (Fig. 1). VWC ($F_{2,27} = 2.02$, $P = 0.1519$), GWC ($F_{2,27} = 3.12$, $P = 0.0603$), soil temperature ($F_{2,27} = 2.4760$, $P = 0.1038$), pH ($F_{2,27} = 0.08$, $P = 0.9250$), and carbon content ($F_{2,26} = 0.78$, $P = 0.4704$) did not differ among the three different microsites.

Potential soil microbial enzymatic activity

Through our assays of soil microbial enzyme activities for a variety of soil carbon and nutrient processes important for plant and microbial growth, we found that there were different microbial activities among the potential dispersal microsites (Fig. 2). Specifically, the potential enzyme activity of a carbon-degrading enzyme involved in cellulose degradation, β-glucosidase (global test: $F_{2,27} = 18.45$, $P < 0.0001$); a phosphorus-acquiring enzyme, phosphatase (global test: $F_{2,26} = 12.44$, $P = 0.0002$); and a sulfur-acquiring enzyme, sulfatase (global test: $F_{2,22} = 20.46$, $P < 0.0001$), were all significantly higher in areas near ant nests than in areas beneath parent plants (Tukey's pairwise comparisons, $P < 0.05$); in two of these cases (β-glucosidase and phosphatase), the potential enzyme activity was also significantly higher in near-nest microsites than in other locations. This same general pattern, although not significant, was found for a nitrogen-acquiring enzyme, NAGase ($F_{2,26} = 2.98$, $P = 0.0682$). Our results indicate that soil biotic activity is significantly different in areas near ant nests, where seeds are ultimately dispersed.

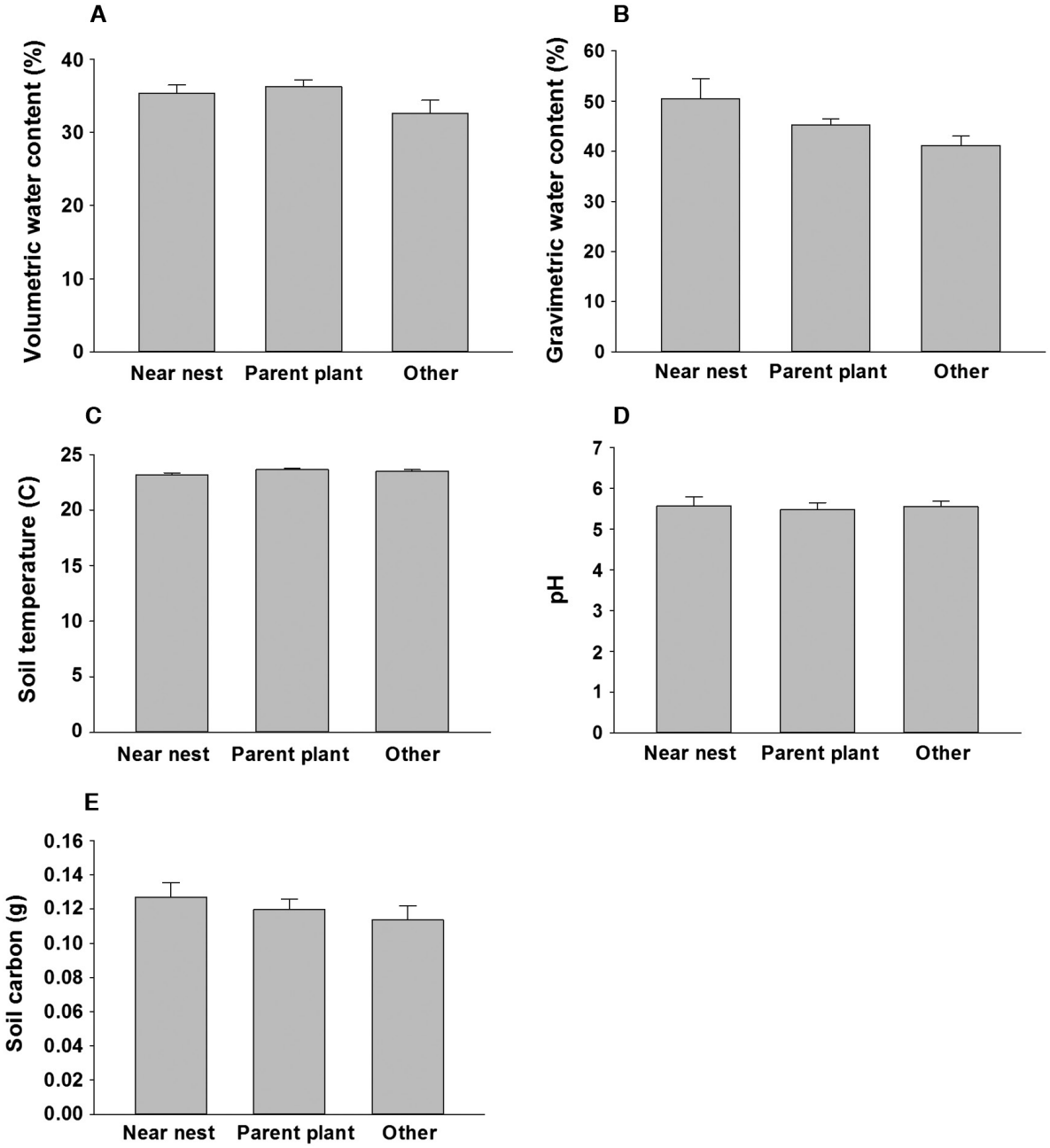


Fig. 1. Soil properties (±SE) in near ant nest, beneath parent plant, and other forest microsites involved in the myrmecochorous relationship between *A. rudis* and *J. diphylla*. Parameters depicted include (A) volumetric water content (%, from Site A), (B) gravimetric water content (%, from Site A), (C) soil temperature (°C, from Site A), (D) pH (from Sites A and B), and (E) soil carbon (g/1 g soil, from Sites A and B). There were no significant differences among any of the microsites.

Potential enzymatic levels for carbon-acquiring enzyme (β-glucosidase) and phosphorus-acquiring enzyme (phosphatase) did not differ in soils collected from parent plants and from other locations in the forest. However, potential sulfur-acquiring enzyme activity (sulfatase) was significantly lower in areas beneath parent plants than in other forest locations. This

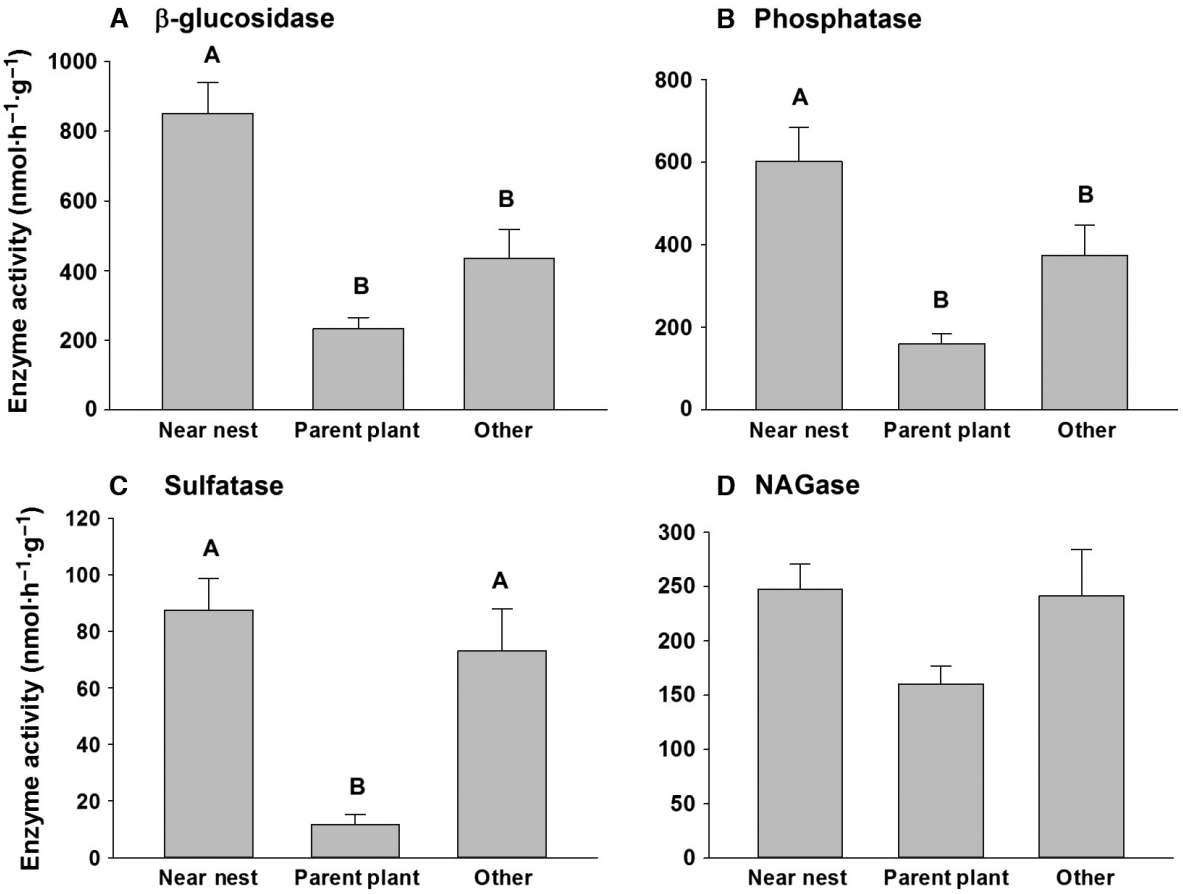


Fig. 2. Potential carbon- and nutrient-acquiring soil microbial enzymatic activities (nmol·h^{-1}·g^{-1} ±SE) of (A) β-glucosidase (cellulose degradation), (B) phosphatase (phosphorus mineralization), (C) sulfatase (sulfur mineralization), and (D) NAGase (mineralization of nitrogen from chitin) among the three studied microsites (near nest, parent plant, and other forest locations). Letters denote significant differences based on Tukey-Kramer HSD comparisons. Potential enzyme activities differed significantly among microsites for all enzymes, except NAGase, with highest activities occurring near ant nests.

suggests that microbial activity, defined by soil enzymes, differs among microsites at directly dispersed, randomly dispersed, and undispersed forest locations.

Seedling emergence trials

Plant emergence was not significantly affected by soil microsite type ($F_{3,28} = 0.5$, $P = 0.6829$; Fig. 3). Although near-nest soils had the highest number of germinants and other forest location soils had the lowest, this difference was not statistically significant. Germination was similar among all the treatments, suggesting that the directed dispersal hypothesis is not manifested via the increased germination near nests in this system.

Discussion

Our results indicate that soil microsites involved in the myrmecochorous relationship between *A. rudis* and *J. diphylla* differ in their potential nutrient-acquiring microbial enzymatic activities. While previous research has shown that soil properties differ between areas beneath the parent plant and areas inside the ant nest (Horvitz and Schemske 1986), ours is unique in that we demonstrate that near-nest soils differ from parent plant soils as well as those from other forest locations. Several potential mechanisms could explain our observed differences. Ants can influence soil fungal community composition and biomass in their

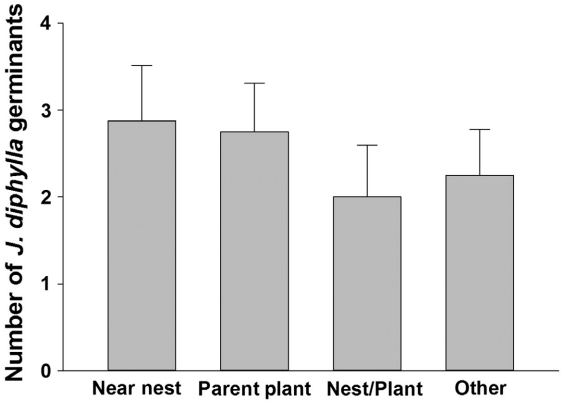


Fig. 3. Seedling emergence: average number of seeds (±SE) where radicles emerged in soils originating from near ant nest, beneath parent plants, a mixture of near nest and parent plant, and other forest microsites. Experimental units each received 10 seeds. There were no significant differences in emergence among treatments.

immediate nest vicinities. Fungal communities are important decomposers; however, we did not observe the differences in chitin degradation in this study (NAGase activity; Zettler et al. 2002). However, we did see significant differences among our microsites in the activity of the carbon-, phosphorus-, and sulfur-acquiring enzymes we measured. Changes in soil microbial community biomass and composition can affect the overall ecosystem carbon and nutrient processes (Nannipieri et al. 2003, Allison and Martiny 2008), and enzyme activity is a key way in which microbial communities maintain the ecosystem productivity and stability (Caldwell 2005). Soil microbial enzymes may also influence the seed germination success by mineralizing nutrients for plant uptake at early stages of growth (Kremer 1993), which is why it is crucial to understand the enzymatic processes of microsites related to seed dispersal. Whether ants seek and establish nest locations in areas with nonrandom soil properties (including microbial assemblages), or whether ants themselves alter soil conditions near their nests, is currently unknown in our system and constitutes a fruitful area of future research.

One way in which the directed dispersal hypothesis could be manifested is through increased seed germination rates in near-nest soils in myrmecochorous systems. In our study, we observed no germination differences among any of our treatments, which suggests that the seed dispersal mutualism between *A. rudis* and *J. diphylla* might not fit the framework of the directed dispersal hypothesis when only germination is considered. However, the subsequent seedling growth, which we did not measure, may reveal directed dispersal advantages if growth was enhanced in near-nest soils (Culver and Beattie 1980).

Despite our finding that redispersal out of ant nests did not positively enhance germination, there are still a number of qualitative ways in which seed-dispersing ants may benefit *J. diphylla* plants. Seed burial in ant nests, even if ephemeral, may be critical. Kwit et al. (2012) found that seed burial by ants can be advantageous for seed survival; though if seeds remain buried and burial is too deep, germination and post-germination benefits may be negligible (Renard et al. 2010). Elaiosome removal, which was not incorporated into our study, could also be critical. Removal can decrease the level of predation by small mammals (Christian and Stanton 2004, Kwit et al. 2012) and may be necessary for the enhanced germination. Finally, there might be a benefit received from handling by ants. Some ants secrete antimicrobial compounds from their metapleural glands (Beattie et al. 1985, Veal et al. 1992, Bot et al. 2002, Fernández-Marín et al. 2006, Dutton and Frederickson 2012), which protect their nests from microbial infection (Hölldobler and Wilson 1990). It is possible that myrmecochore seeds receive protection from microbial predators if the seeds are coated by antimicrobial secretions of *A. rudis* ants during the process of elaiosome consumption. Despite the documentation of metapleural gland secretions limiting the growth of soil fungi dating back to at least the 1980s (Beattie et al. 1985) and interest in the effect of such secretions on seeds dating back at least to the early 1990s (Levey and Byrne 1993), no work has rigorously tested the effects of ant glandular secretions on seed survival and establishment.

Overall, our results demonstrate that the benefits seeds receive from myrmecochory are more complex than what is explained by the directed dispersal hypothesis over the short term. While our study is consistent with the hypothesis that seed-dispersing ants may generally nest in locations with unique microbial assemblages or may alter the microbial communities themselves, such differences near ant nests, where seeds are

redispersed, do not confer an enhanced germination. Hence, other factors may contribute to the directed dispersal being important in the long-term, postgermination. Clearly, more study is required to determine the full extent of the role *A. rudis* ants play as keystone seed dispersers of numerous forest herbs in eastern North America.

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