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Incorporating redispersal into myrmecochory: Addressing the uniqueness of microsites near ant nests in an eastern North American forest

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Abstract

While ‘benefits of directed dispersal’ studies in myrmecochorous systems have compared the properties of soils underneath myrmecochores to the soils in the nests of ants that disperse their seeds, none have explored the properties of soils nearby ant nests, where recent work indicates seeds are quickly “redispersed” in eastern North American myrmecochorous systems. To address this, I focused on a forested system in eastern Tennessee involving a keystone seed-dispersing ant, *Aphaenogaster rudis*, and a common herbaceous understory myrmecochore, *Jeffersonia diphylla*. I collected soil cores underneath *J. diphylla*, around *A. rudis* nests, and from random forest locations. In the lab, I ran assays for potential soil enzyme activity for four common microbial enzymes, as well as a subset of environmental parameters.

I found that there were different microbial activities between the soils under *J. diphylla* and the soils surrounding ant nests. Specifically, potential enzyme activity of β-glucosidase, phosphatase, and sulfatase were all significantly higher in areas near ant nests than beneath parent plants; this same pattern, though not significant, was found for NAGase. No differences were found in other environmental variables I investigated (e.g., soil temperature, soil moisture, pH). My results indicate that soil processes are unique in near nest soils, where seeds are ultimately dispersed. However, when I ran germination trials in the greenhouse, there was no observed benefit for being placed in near nest soils for radicle emergence. Future work should be directed towards addressing whether areas near ant nests provide biologically meaningful escape from microbial seed predation pressure, and characterizing soil microbial communities in such settings.

Introduction

The mutualistic relationship between plants and the ant species that disperse their seeds has been an area of extensive study. Despite this, mystery still surrounds the numerous roles ants play in the dispersal process. The ~11,000 plants involved in myrmecochorous relationships produce seeds that contain a fleshy appendage called an elaiosome, and this syndrome is hypothesized to have evolved independently 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 (Turner and Frederickson, 2013; Brew et al., 1989; Thompson, 1973), encourage ants to carry diaspores to their nests (Gordon, 1983). Larval ants receive a nutritional reward from the elaiosome and do not harm the seeds during elaiosome consumption (Lisci et al, 1996; Gammans et al., 2006; Fischer et al., 2008).

There have been several hypotheses posed for the benefits that plants receive from myrmecochory, one of which is 'directed dispersal' to a nest location. There, through combinations of (a) protection from predators through burial (citation) and (b) nutrient-rich microsites (Horvitz and Schemske, 1986), propagules are expected to have higher fitness than if they were dispersed randomly or not at all (Oliveira book). This is currently the leading hypothesis for the plant benefits of myrmecochory (Wenny, 2001; Giladi, 2006).

Since ants only disperse seeds of myrmecochores short distances (global mean of ~1.99 m [Gomez & Espadaler et al., 2013]), to ant nests, with redispersal in some instances nearby (Gorb et al., 2000; Canner et al., 2012), microsite differences at small

spatial scales should exemplify myrmecochorous relationships. Soil microbes, which may be involved with such differences (Caldwell, 2005), and whose enzymes have been associated with both seed depredation and germination (see Kremer, 1993), have generally been neglected in this framework, and may therefore influence seed survival and seedling establishment of ant-dispersed plants.

I assessed relevant microsite-specific abiotic and biotic soil properties in an eastern Tennessee (USA) deciduous forested system as they pertained to a common herbaceous understory myrmecochore, *Jeffersonia diphylla*. In this system, I have noted (pers. obs.) that seeds of *J. diphylla* are dispersed primarily by *Aphaenogaster rudis*, which has been referred to as a 'keystone' seed-dispersing species (Ness et al., 2009), and which has been known to redisperse seeds ~30 cm away from ant nests after elaiosome consumption (Canner et al., 2012). Specifically, I addressed the following questions: (1) Are there differences in soil properties or processes between the microsites located near ant nests and the microsites under parent plants? (2) Do these differences in microsite influence plant germination success? Ultimately, I aim to provide empirical and experimental evidence to support or refute 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 (Smith et al 1986). It reproduces both vegetatively as well as by seed (Smith et

al 1986). *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 (Ness et al., 2009). The seeds mature and fall to the ground in the summer, and ants collect and deliver these elaiosome-bearing seeds (elaiosomes) to their colony (Smith et al., 1986). Elaiosomes are consumed by larvae in nests, and seeds of myrmecochorous plants are redispersed a median distance of 20-30 cm outside the nest (Canner et al., 2006). *Aphaenogaster rudis* (Formicidae: Myrmicinae, hereafter *A. rudis)* is the primary seed dispersal vector of many temperate deciduous myrmecochores, including *J. diphylla* (Ness et al., 2009).

Study areas: Both field sites are located in east Tennessee in mixed deciduous forest comprised of mostly *Acer* spp*., Carya* spp*., Fagus grandifolia, Juglans nigra, Liquidambar styraciflua, Liriodendron tulipifera, and Quercus* spp*.* Site selection was based on sufficient *Aphaenogaster* nest abundance (>20 nests) and 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 minutes 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, Morgan County, TN (425 m elevation) (36.23°N latitude, -84.56°W longitude).

Collection and storage of soils: Soils were collected from both sites in the month of June 2013. Soils from site A were used in my study of potential enzyme activity. Soils

from site B were used in my greenhouse soil source experiment. Soils from site A were collected on 12 June 2013. There, soil cores (2 cm x 10 cm) were collected from locations representing three different soil “treatments”: (1) 20 cm from active *A. rudis* nests (hereafter ‘near nest’), (2) directly beneath *J. diphylla* parent plants (plants with present-year fruiting capsules present, hereafter ‘parent plant’), and (3) from random forest soil (>1.5-m away from both *A. rudis* nests and *J. diphylla* individuals, hereafter ‘random’). A representative sample for each treatment consisted of two homogenized soil cores (see below); there were 10 samples per treatment, with a total of 30 sample sites. The two soil cores at a given sampling location were homogenized and immediately stored at 4°C. Onsite, I measured soil temperature with a cooking thermometer and soil moisture content with a HydroSense II soil moisture meter (Campbell Scientific) at each location I cored. Within 24 hours of collection soils were sieved to 2 mm, then returned to 4°C conditions. A 1.0 g subsample was used for enzyme analysis (see below). Soils from site B were collected on 16 June 2013. Sixteen 5 cm x 10 cm soil cores from each of the three aforementioned soil “treatments” were collected for a total of 48 sites. Soils were homogenized by site, and sieved to 4 mm to remove rocks and roots. Soils were mixed with approximately 30% by mass sterilized coarse sand to improve soil drainage. Eight samples were randomly selected from each soil type for random, parent plant and near nest soil source treatments. Soil-sand mixtures were added to (13.5 cm by 6 cm by 6.5 cm) microcosms lined with a permeable cloth liner (Gardeneer by Dalen Harvest-Guard) to prevent soil loss through drainage holes.

Soil pH analysis: I randomly selected five 10 g air-dried samples from each treatment (near nest, parent plant, control) from both sites for a total subsample of 30. I mixed each sample with 20 mL of 0.01 M $CaCl_2$ solution in a centrifuge tube. I ensured the soils were properly mixed in the $CaCl_2$ solution by shaking the centrifuge tubes every 10 minutes for half an hour. I allowed each sample to settle for another half an hour. I used a Denver Instrument pH probe to measure the pH levels of each soil sample.

Soil C analysis: I randomly selected five 1 g air-dried soil samples from each treatment at each study location for a total subsample of 30. I dried them in an oven at 60 degrees C for 48 hours. The soils were weighed and then placed into a muffle furnace at 550 degrees C for six hours. After allowing the soils to cool overnight, I placed them into a dessicator for 20 minutes. The soils were weighed again to calculate the amount of C in each treatment.

Potential enzyme activity: To assess how soil microbial activity may differ based on proximity to *A. rudis* nests or fruit-bearing *J. diphylla* plants, I examined potential enzyme activity of the soils collected at site A. I measured activities of β-glucosidase (BG), acid phosphatase (AP), sulfatase (S), and β-N-acetylglucosaminidase (NAG), as described 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 approximately 1.0 g fresh mass were homogenized with 125 ml of 50 mM 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 ten analytical replicates plus blank, reference standard and negative controls. The plates were

incubated for 2 h, except for NAG plates which was 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, Inc., Winooski VT). Results were calculated as nmol of substrate converted per hour per g soil dry mass (nmol h^{-1} g^{-1}).

Germination Trials: On 16 June 2013, fruiting capsules with freshly matured seeds (>10 seeds) were collected from 50 known individual *J. diphylla* parent plants at site B and stored dry at approximately 20-25°C. Within two weeks of field collection, ten *J. diphylla* seeds from unique parent plants were added to the surface of each soil microcosm. “Control” soils received randomly selected seeds, “Parent plant” received seeds from the corresponding parent plant seeds, except for three “parent plant” soils that were assigned a random seed source due to fungal infection of corresponding seed source. “Control” and “near nest” soils received randomly selected seeds from remaining parent plant seed sources not utilized for “parent plant” soils. Microcosms were kept field moist from beneath in trays with saturated wicking fabric, and kept in a greenhouse at the University of Tennessee, Knoxville, TN early July 2013 through early February 2014. They were then transitioned outside of the greenhouse 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 (see Baskin & Baskin 1989). Because *J. diphylla* seeds require sufficient cold stratification followed by warm stratification for germination, I kept the seeds outside until March 2015. From late January 2014 until March 2015, I monitored for germination (radicle emergence and above-ground stem germination) at biweekly intervals and noted if any of the seeds were missing or noticeably dead (i.e., empty seed coat). Other than 5 seeds that showed signs of germination in 2014, the vast majority of germination took place in 2015.

Data analysis: I used ANOVA to assess significant differences in the measured physical properties of soil (temperature, moisture content, pH, C content) as well as potential enzymatic activity, among the different treatments. Tukey-Kramer HSD comparisons were used to further break down the differences between the treatments. For the germination trials, I used ANOVA to assess significant differences between the frequencies of radicle emergence of each treatment.

Results

Soil physical properties: No significant differences were found in other environmental variables I investigated (Fig. 1). Gravimetric water content was marginally higher in areas surrounding ant nests than underneath parent plants ($p=0.0603$). Soil temperature ($df=29$, $F=2.4760$, $p=0.1038$), pH ($df=29$, $F=0.0782$ $p=0.9250$), and C content ($df=28$, $F=0.7764$, $p=0.4704$) did not differ amongst the three different microsites involved in the myrmecochorous relationship I are studying.

Potential enzymatic activity: I found that there were different microbial activities between the soils under *J. diphylla* and the soils surrounding ant nests (Fig. 2). Specifically, potential enzyme activity of β-glucosidase ($p<0.0001$), phosphatase ($p=0.0002$), and sulfatase ($p<0.0001$) were all significantly higher in areas near ant nests than beneath parent plants. This same pattern, though not significant, was found for NAGase ($p=0.0682$). My results indicate that soil processes are unique in areas near ant nests, where seeds are ultimately dispersed. Interestingly, potential enzymatic levels for β-glucosidase and phosphatase were statistically similar in soils collected from parent plants and from random spots in the forest. However, potential sulfatase activity was significantly lower under parent plants than in random spots in the forest. This suggests that there are unique microsites underneath *J. diphylla* plants as well as in the areas surrounding *A. rudis* nests.

Germination trials: My results show that radicle emergence was not affected by soil type (Fig. 3). Although near nest soils had the highest percentage of seeds with radicle emergence (28.75%) and the control soils had the lowest (22.50%), this difference was not statistically significant. Frequency of germination was similar among all treatments suggesting that the directed dispersal hypothesis might not applicable for this system. Signs of germination were present in 21 of the 24 mesocosms, but aboveground germination was only present in 11. Seed death was highest in the parent plant soils treatment (36.25%) and lowest in the near nest (31.25%), though this difference was not statistically significant.

Discussion

My results indicate that soil microsites involved in the myrmecochorous relationship between *A. rudis* and *J. diphylla* differ in their potential enzymatic activities. While previous research has shown that soil properties differ between underneath the parent plant and inside the ant nest (Horvitz & Schemske, 1986), mine is unique in that I demonstrate that near nest soils differ from parent plant soils as well. Soils are extremely biodiverse; however, it is difficult to study the breadth of that diversity. It is known that changes in soil microbial communities can affect overall ecosystem processes (Nannipieri et al., 2003; Allison & Martiny, 2008) and that enzyme activity is a key way in which microbial communities maintain ecosystem productivity and stability (Caldwell, 2005). Soil microbial enzymes may influence seed germination success (see Kremer, 1993), which is why it is crucial to understand the enzymatic processes of microsites related to seed dispersal.

According to the directed dispersal hypothesis, I would hypothesize that the differences I observed between near nest and parent plant soils should positively influence germination rates of *J. diphylla* in which near nest soils would have the highest germination and parent plant soils would have the lowest. However, I observed no such germination difference between any of my treatments, which suggests that seed dispersal mutualism between *A. rudis* and *J. diphylla* might not fit in the framework of the directed dispersal hypothesis when germination is considered. My results show that the abiotic properties of the soils in the eastern deciduous forest might not be heterozygous enough to influence *J. diphylla* seedling success given the short distances that *A. rudis* disperse their seeds. However, subsequent seedling growth, which I did

not measure, may reveal directed dispersal advantages if growth was enhanced in near nest soils.

Since my study has potentially ruled out directed dispersal as a benefit of the mutualism, I must now ask what benefits might *J. diphylla* plants receive from being dispersed by *A. rudis* ants. It is uncertain how many seeds are actually carried out of the nest for redispersal. In another study of a myrmecochorous herb, Kwit et al. (2012) found that seed burial by ants can be advantageous for seed survival. However, according to Renard et al. (2010) myrmecochorous seeds are fed to brood deep within the ant nest, and many seeds remain buried too deeply to successfully germinate, so benefits of seed burial might not be as apparent in the field. Another potential reason I did not observe support for directed dispersal is that elaiosome removal by ants might decrease the level of predation by small mammals (Kwit et al., 2012; Christian & Stanton, 2004) and may be necessary for 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 & Frederickson, 2012), which protects their nests from microbial infection (Hölldobler & Wilson, 1990). When I were harvesting seeds for the germination trials for this study, there was a noticeable proportion of seeds that had to be discarded because of fungal infection. It is possible that *J. diphylla* seeds receive protection from fungal predators when they are coated by the antimicrobial secretions of the *A. rudis* ants.

Overall, my results have demonstrated that the benefits seeds receive from myrmecochory is more complex than what is explained by the directed dispersal

hypothesis. Indeed, my study shows that there are other factors that might be involved besides differences in microsite soils that influence the germination success of a myrmecochore. Clearly more study is required to determine the full extent of the role *A, rudis* ants play as keystone seed dispersers.

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Figures

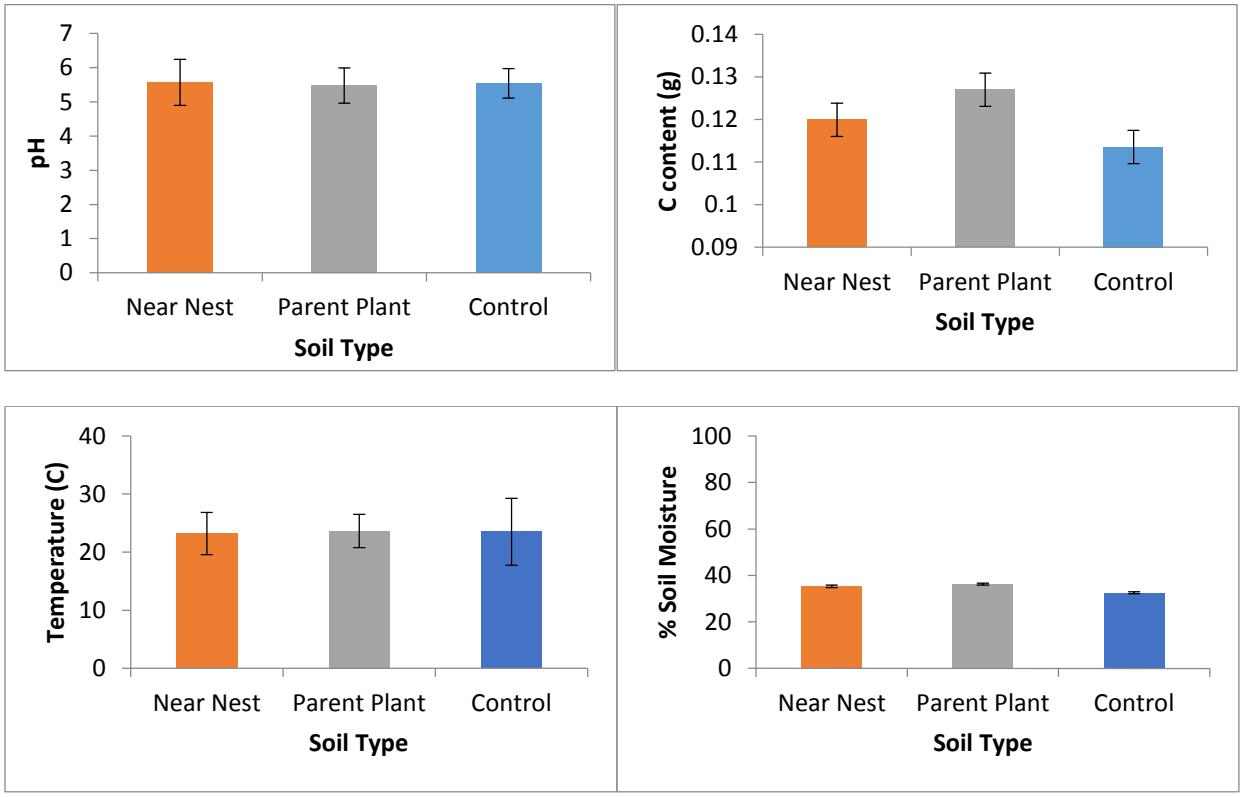

Figure 1.

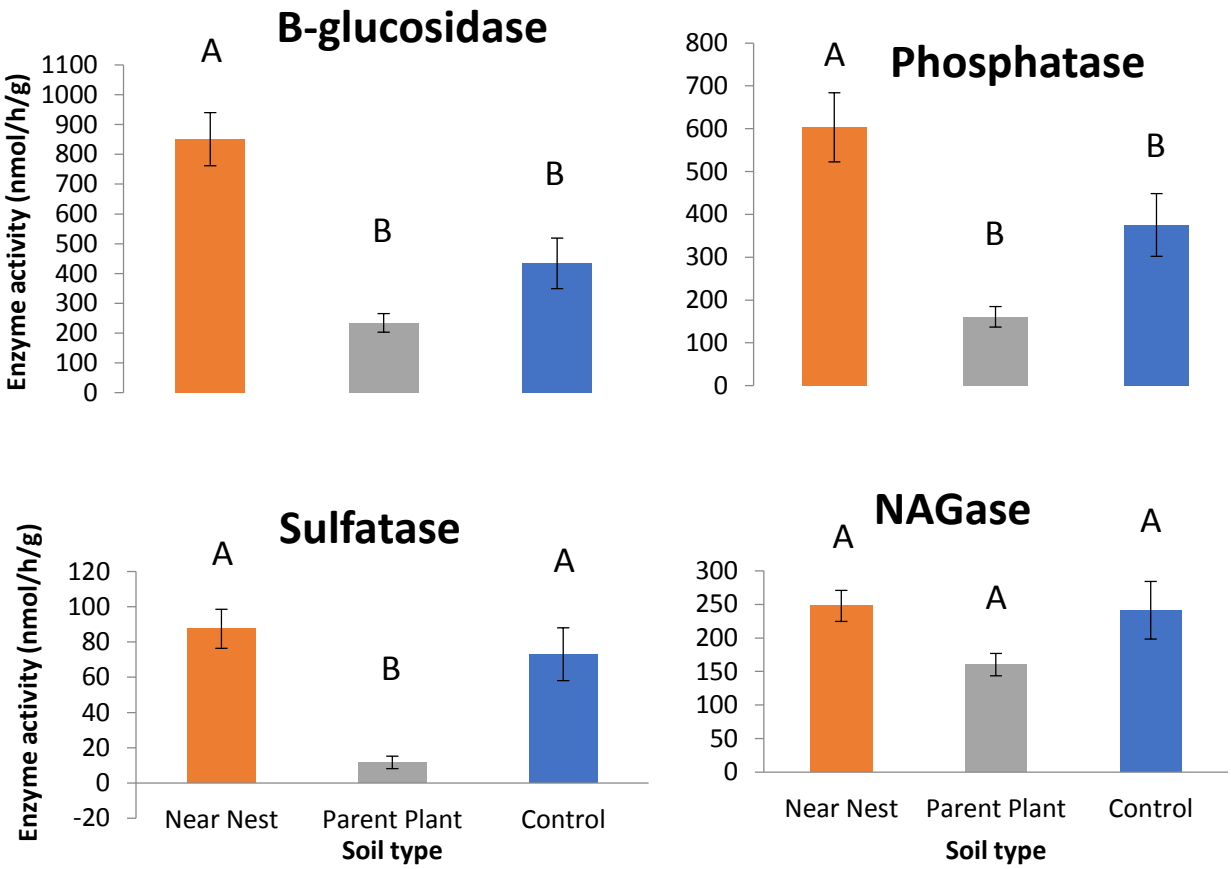

Figure 2.

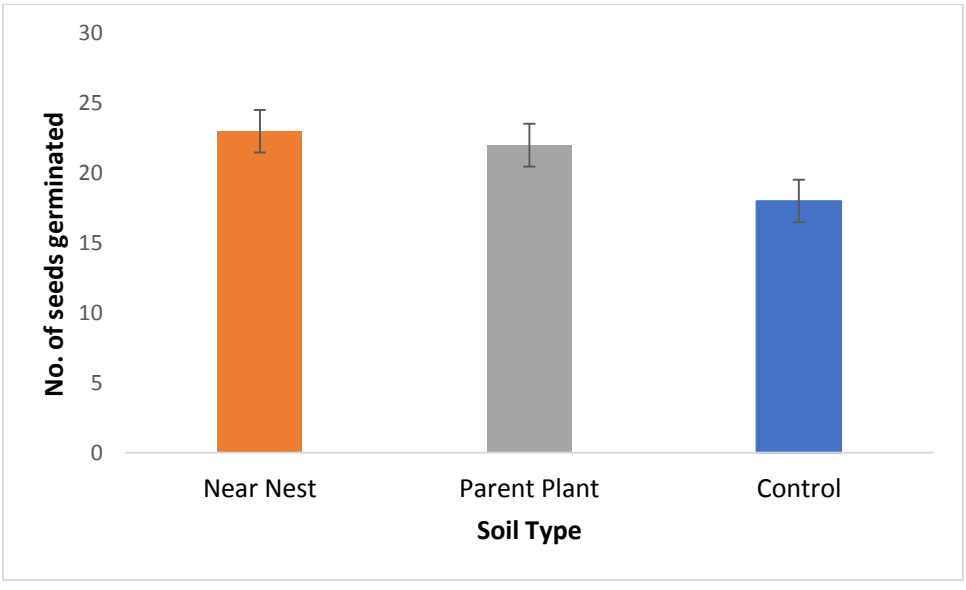

Figure 3.

Figure Legends

Figure 1. Physical soil properties of the different microsites involved the myrmecochorous relationship between *A. rudis* and *J. diphylla*. There were no significant differences among any of the treatments.

Figure 2. Potential enzymatic activities of B-glucosidase, phosphatase, sulfatase, and NAGase in the different microsites. Letters denote significant differences.

Figure 3. The number of seeds that showed radicle emergence in each treatment soil in my germination trials. The maximum number of seeds for each treatment was 80. There is no significant difference in germination among treatments.

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