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Effects of Garlic Mustard (*Alliaria petiolata*) Removal on the Abundance of Entomopathogenic Fungi

Regina Vaicekonyte and Felicia Keesing*

Garlic mustard is an invasive, exotic herb that is now widespread in North America. Recent research has shown that garlic mustard exudes biochemical compounds that inhibit the growth of entomopathogenic fungi. We investigated how the removal of garlic mustard would affect the abundance of entomopathogenic fungi in forest soils in eastern New York. Using a standard bioassay, we compared the abundance of entomopathogenic fungi in soil with and without garlic mustard both before and 45 d after garlic mustard had been experimentally removed. In soil from which garlic mustard had been experimentally removed 45 d earlier, the abundance of entomopathogenic fungi was restored to levels found in soil with no history of garlic mustard. These results suggest it is possible to increase the abundance of entomopathogenic fungi in the soil in a short time by eradicating garlic mustard plants from an invaded area. Recolonization by entomopathogenic fungi could be beneficial to humans if it increases the mortality of arthropods that are vectors of infectious disease, such as blacklegged ticks, but harmful if it increases the mortality of arthropods that provide valuable ecosystem services, such as bees and ants.

Nomenclature: Garlic mustard, *Alliaria petiolata* (Bieb.) Cavara & Grande, *Beauveria bassiana* (Bals.-Criv.) Vuill.

Key words: Invasive species, entomopathogenic fungi, allelopathy, biocontrol, arthropods.

Garlic mustard [*Alliaria petiolata* (Bieb.) Cavara & Grande], is an exotic, invasive, shade-tolerant herb. A member of the mustard family (Brassicaceae), garlic mustard primarily occupies disturbed areas (Cipollini 2002; McCarthy 1998; Stinson et al. 2006). Once established, it becomes a permanent member of the plant community, proliferating rapidly into adjacent habitats (Nuzzo 1999). Garlic mustard is known to exude a suite of allelopathic compounds, including flavonoids, defensive proteins, glycosides, and glucosinolates (Cipollini 2002; Daxenbichler et al. 1991; Haribal and Renwick 2001). These compounds can inhibit seed germination of other plants (Prati and Bossdorf 2004), reduce the root length of seedlings (Roberts and Anderson 2001), affect survival of butterflies (Bowden 1971), and reduce native plant diversity in the forest understory (Stinson et al. 2006).

Garlic mustard also produces levels of cyanide in its tissues of up to 100 ppmw, a level 150 times that of native *Brassica* species and a level considered toxic to most vertebrates (Cipollini and Gruner 2007). The highest concentrations of cyanide are found in young leaves of first-year garlic mustard plants (Cipollini and Gruner 2007). Cyanide compounds are well-known inhibitors of respiratory electron transport (Fahey et al. 2001). Therefore, these cyanide compounds are toxic to a range of organisms, including pests and pathogens that infect seeds and seedlings (Brown and Morra 1997), insect herbivores (Chew 1988; Porter 1994), and other plants (Haromoto and Gallandt 2005). The effects of cyanide and other secondary compounds of garlic mustard are thought to increase its invasive abilities (Lankau 2011; Rodgers et al. 2008).

Initial studies of garlic mustard focused on its effects on the diversity of flora and fauna aboveground (Brussaard et al. 1997; Levine et al. 2003; Stinson et al. 2007), but recently, the focus has shifted to include effects on diversity belowground. For example, garlic mustard reduces the inoculum potential of arbuscular mycorrhizal fungi (AMF) in field soils, inhibits the germination of AMF spores, and suppresses native plant growth by disrupting mutualistic associations between native canopy tree seedlings and belowground AMF (Roberts and Anderson 2001; Rodgers et al. 2008; Stinson et al. 2006; Wolfe et al. 2008). The secondary compounds of garlic mustard have recently been found to inhibit the growth of another type of underground fungi as well—the so-called entomopathogenic fungi (Keesing et al. 2011).

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Management Implications

Garlic mustard is known to have an inhibitory effect on arbuscular mycorrhizal fungi in the soil. However, less is known about the herb's effects on underground entomopathogenic fungi, or more specifically, about how the soil microbiota responds to the removal of the plant. We investigated how long it takes for the soil to recover natural levels of entomopathogenic fungi once garlic mustard is removed. In this study, we sampled soil for entomopathogenic fungi in areas invaded by garlic mustard and areas free of garlic mustard. We then removed garlic mustard plants from forest plots and sampled the soil again 45 d later. The abundance of entomopathogenic fungi in all areas that had garlic mustard removed from them increased during the 1.5-mo period and reached even greater levels compared with areas which had no history of garlic mustard. Soil disturbance alone did not have an effect on the abundance of entomopathogenic fungi. The ability of entomopathogens to recover shortly after garlic mustard removal can benefit humans because the number of disease-carrying arthropod vectors (e.g., blacklegged ticks) may decline as a result. However, it can also be harmful to us because the number of arthropods that provide valuable ecosystem services (e.g., ants, bees) may also diminish. We conclude that garlic mustard removal might be one of the ways to increase the abundance of entomopathogenic fungi in the soil during a short time and, therefore, a way to restore a natural mechanism for arthropod population control.

Entomopathogenic fungi are common natural enemies of arthropods worldwide and have been used in biological control (Hajek and Leger 1994; Shah and Pell 2003). There are more than 700 known species of entomopathogens from within the fungal kingdom, with most species in the Ascomycota and Zygomycota phyla (Samson et al. 1988). Entomopathogenic fungi produce infective spores (conidia) that attach to, germinate, and penetrate the cuticle of their host (Roy et al. 2006). Once within the host, they proliferate, typically killing their hosts and producing more infective conidia (Roy et al. 2006). The soil environment constitutes an important reservoir for a diversity of entomopathogens, which can contribute significantly to the regulation of insect populations (Keller and Zimmerman, 1989).

Two species of entomopathogenic fungi in particular, *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin, have near worldwide distributions (Bidochka et al. 1998) and have been used widely in biocontrol. For example, they are known to significantly reduce the fitness of adult African malaria mosquitoes (*Anopheles gambiae*; Scholte et al. 2005), onion thrips (*Thrips tabaci*; Gindin et al. 1996), desert locusts (*Schistocerca gregaria*; Elliot et al. 2002), pea aphids (*Acyrthosiphon pisum*; Baverstock et al. 2004), and red imported fire ants (*Solenopsis invicta*; Oi and Pereira 1993). *Beauveria bassiana* and *M. anisopliae* are capable of infecting not only insects but other arthropods as well. Most studies have focused on the effects of *B. bassiana* and *M. anisopliae* on various tick species, showing that these entomopathogens are lethal to female bovine ticks (*Boophilus microplus*; Onofre et al. 2001), American dog ticks (*Dermacentor variablis*), and brown dog ticks (*Rhipicephalus sanguineus*) in laboratory assays (Kirkland et al. 2004). They also reduce the fitness of blacklegged ticks (*Ixodes scapularis*) in both field and laboratory experiments (Benjamin et al. 2002; Hornbostel et al. 2005; Ostfeld et al. 2006a; Samish et al. 2001, 2008). The effects of these fungal species on nonpest arthropods are much less studied, but their impacts on pests suggest that they have the potential to reduce and control other arthropod populations as well. Even though entomopathogenic fungi have the potential to reduce and control arthropod populations, other factors, such as garlic mustard, may be capable of inhibiting the growth of the fungus itself, thus limiting its entomopathogenic effects.

We investigated how long it would take for levels of entomopathogenic fungi in the soil to return to their natural state after garlic mustard plants were removed. We hypothesized that after the removal of garlic mustard, the population of entomopathogenic fungi in the soil would increase over time. Testing this hypothesis required us to remove garlic mustard plants from forest plots; however, this treatment had two effects—removing the plants *and* disturbing the soil. To control for the effects of disturbing the soil, we conducted a second experiment in which we experimentally disturbed the soil and then measured the effects of the disturbance on the fungi. We predicted that disturbing the soil alone would not have an effect on the abundance of entomopathogenic fungi.

Materials and Methods

We conducted our field studies in June to August 2010 in Tivoli North Bay, which is part of the Hudson River National Estuarine Research Reserve in Dutchess County, southeastern New York (42°4′N, 73°90′W). Rainfall at this site averages approximately 1,000 mm yr^{-1} (39.37 in), with averages of approximately 100 mm mo^{-1} in June, July, and August. Our test sites were located on the edge of an upland area of mixed forest characterized by red maple (*Acer rubrum* L.), sugar maple (*Acer saccharum* Marsh.), hickory (*Carya* spp.), white ash (*Fraxinus americana* L.), eastern white pine (*Pinus strobus* L.), and chestnut oak (*Quercus prinus* L.) canopy. The invasion history of this site by garlic mustard plants is not documented. Soils at the study site are deep, gently sloping, moderately well-drained, Hudson and Vergennes soils with 15 to 23 cm (5.91 to 9.06 in) of brown, silty, clay loam topsoil (Faber 1992). We conducted two experiments: one to test the effects of garlic mustard removal on entomopathogenic fungi, and a second one to test the effects of soil disturbance on entomopathogenic fungi.

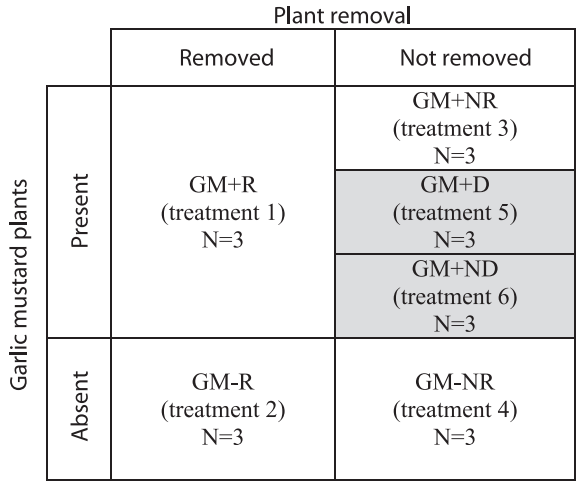

Figure 1. Schematic design of the two experiments. Each plot was 1 m by 1 m, and there were three replicates in each treatment group ($N = 3$). Shaded boxes represent treatments in the disturbance experiment. Abbreviations: GM, garlic mustard; +, presence of garlic mustard plants; −, absence of garlic mustard plants; R, removal; NR, no removal; D, disturbance; ND, no disturbance.

Garlic Mustard Removal Experiment. For the first experiment, we selected three sites. Sites were considered appropriate if they (1) harbored a density of plants comparable to the mean density of herbaceous plants in the larger forested area, (2) contained a patch of garlic mustard plants large enough to contain the experimental treatments, and (3) were no closer than 20 m (65.6 ft) to another site. At each of three sites that met these criteria, we created four 1 m by 1 m treatment areas. The four treatments were (1) plots with naturally occurring garlic mustard plants that were manually removed after the initial soil sampling (GM+R), (2) plots with naturally occurring garlic mustard plants that were not removed (GM+NR), (3) plots with no garlic mustard plants but with other herbaceous plants that were removed after the initial soil sampling (GM-R), and (4) plots without garlic mustard plants but with other herbaceous vegetation that was left in place (GM-NR) (Figure 1). Treatments 1 and 2 (GM+R and GM+NR) were used to test the effects of garlic mustard plant removal on entomopathogenic fungi. Treatments 3 and 4 (GM-R and GM-NR) were used to test the effects of nongarlic mustard plant removal on entomopathogenic fungi and served as controls for treatments 1 and 2.

Disturbance Experiment. In the same three sites described above, we created two additional 1 m by 1 m treatments: (5) a plot with garlic mustard plants that were left in place but in which the soil was disturbed after the initial soil sampling (GM+D), and (6) a plot with garlic mustard plants that were left in place in undisturbed soil (GM+ND) (Figure 1). Treatments 5 and 6 (GM+D and GM+ND) were used to test the effects of soil disturbance on entomopathogenic fungi. The six plots at each of the sites were within 3 m of each other. Plots with garlic mustard were dominated by first-year garlic mustard plant rosettes (~70%), with the remainder being second-year plants. All chosen plots were similar in light availability, plant density, surrounding foliage, and moisture level.

We sampled the soil twice during the course of the experiment, collecting 6 L (1.6 gal) of soil in each sampling. In the initial sampling, we took soil samples in June 2010 before conducting any plot modifications (plant removal or soil disturbance). Using a stainless-steel hand shovel, we collected only the top layer of the soil (the surface 5 to 8 cm) because topsoil has the highest natural abundance of entomopathogenic fungi (Tuininga et al. 2009). Because entomopathogenic fungi are present mostly in the soil itself and not in the leaf litter covering the soil (Tuininga et al. 2009), we did not use leaf litter in the experiment.

After the initial soil collection, we gently pulled the plants out of the soil in removal plots (treatments 1 and 2), to minimize soil disturbance. We disturbed the soil in the plots of treatment 5 by poking the soil with a 15-cm-long metal pick extended ~10 cm into the soil. Each of the three disturbance plots was poked 20 times in a uniform distribution. We did the second soil sampling 45 d after the initial sampling using the same methods. After each of the two soil collection periods, soil was stored in open, shallow, 1-L, plastic containers at 20 C for 24 h to let the soil dry.

After drying, soil was sieved in the laboratory to remove roots, rocks, and other debris using a size-16 sieve with 1.190-mm mesh. We transferred the sieved soil from each plot into 10 clear-plastic vials (8.73 cm by 6.51 cm), filling each of them halfway. Thus, we had a total of 180 vials for each sampling period: 10 vials by 6 treatment plots by 3 sites.

We used the greater wax moth (*Galleria mellonella* L.) larvae (waxworms, Petco Animal Supplies, Inc., 9125 Rehco Road, San Diego, CA 92121) as a bioassay to detect the presence of entomopathogenic fungi in each plot (Zimmerman 1986). This is a standard bioassay for detection of entomopathogenic fungi (e.g., Bidochka 1998; Keesing et al. 2011). All larvae were kept at 20 C before and throughout the experiment. All larvae that were used were of similar size (mean ± SE, 2.0 ± 0.2 cm). All chosen waxworms were white to tan in color, plump, healthy-looking, and active. We used five waxworms per vial in the first sampling period and 10 in the second, placing waxworms in plastic vials that were half filled with soil. Each vial was gently rotated every day for 10 s to ensure equal exposure of each waxworm to the fungi in the soil. To maintain moisture in the vials, we added 2 ml (0.07 oz) of sterile water to each vial every 2 d, beginning

on day zero, because humidity of 95% or greater is required for conidia germination, infection, and sporulation (Roy et al. 2006).

We monitored waxworm survival after 1, 2, 4, 9, and 14 d from the beginning of the experiment in the first sampling period, and every 5 d in the second sampling period (for a total of 15 d). We removed dead waxworms on discovery because conidia of *Beauveria bassiana* are passively dispersed from infected cadavers (Shah and Pell 2003). We classified each waxworm death as either fungal or nonfungal. Fungal infections were detected visually based on the presence of fungal growth on the outer skin of the waxworm larvae. At least 5% of the outer skin of the wax moth larvae had to be infected with fungal growth for it to be considered infected.

Overall, we had data for 10 replicates of each of the 6 treatments for 3 sites; we used the mean of the 10 replicates for each treatment for our subsequent analyses. We used a two-way ANOVA with replication to test whether the presence and removal of garlic mustard had a significant effect on the abundance of entomopathogenic fungi. The two factors in the ANOVA were garlic mustard (presence, absence) and removal (removal, no removal). The dependent variable was the abundance of entomopathogenic fungi, measured as the difference in mean fungal waxworm mortality between the first and second samplings. We used a post hoc Tukey Honestly Significant Difference test to evaluate results. The JMP 9.0.0 software (SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513-2414) was used for all analyses unless otherwise noted.

We used a two-sample *t* test assuming equal variances to test whether soil disturbance had a significant effect on the abundance of entomopathogenic fungi. We calculated the differences in mean percentages of waxworm mortality rate due to fungal infections between the first and second samplings of the plots where soil was disturbed and between the first and second samplings of the plots where soil was undisturbed and then ran a *t* test on the mean differences in waxworm mortality. We also used a *t* test to see whether there was a significant difference in overall waxworm mortality (because of fungal infections and other causes combined) between garlic mustard and nongarlic mustard soil.

Results and Discussion

Forty-five days after the removal of garlic mustard plants from plots in a forest community, the mortality of waxworms from entomopathogenic fungi was more than five times higher than it was on plots from which garlic mustard plants had not been removed ($P = 0.0008$; $F = 27.833$; $df = 1$; Figure 2; Table 1). The abundance of entomopathogenic fungi was not affected by the removal of plants other than garlic mustard (GM-R plots); these plots had the same levels of entomopathogenic fungi as did the plots from which we did not remove plants (GM-NR plots; $Q = 3.202$; Fisher's Protected LSD test for GM-R and GM-NR treatments $< Q$; $\alpha = 0.05$). Further, in less than 2 mo, the abundance of entomopathogenic fungi in the soil with a previous history of garlic mustard was restored to levels found in soil with no history of garlic mustard (Figure 2).

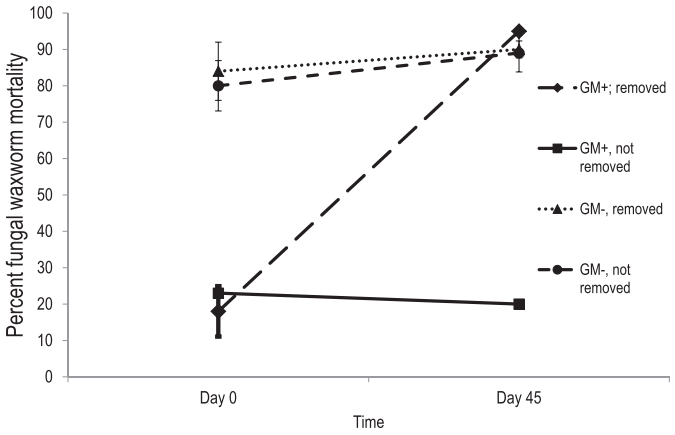

Figure 2. Mean percentage of waxworm mortality due to fungal infections immediately before and 45 d after garlic mustard treatments were imposed. Error bars represent standard errors; there were three replicates per treatment group per sampling period.

In the disturbance experiment, the overall abundance of entomopathogenic fungi in the soil increased from June through August 2010 (Figure 3), as indicated by a 5 to 9% increase in the number of waxworms that died from fungal infections. However, there was no significant treatment effect (*t* test, $P = 0.682$), indicating that soil disturbance did not have a significant effect on the abundance of entomopathogenic fungi.

The ability of garlic mustard to kill entomopathogenic fungi could be ascribed to cyanide, as well as allyl isothiocyanate (AITC) and sinigrin, all of which have been found to reduce the germination of spores of AMF (Cantor et al. 2011; Roberts and Anderson 2001; Rodgers et al. 2008; Stinson et al. 2006). For example, even the lowest concentration of AITC measured in the field (~0.001 mM) was highly inhibitory to the spore germination of a forest AMF species, *Glomus clarum* (Cantor et al. 2011). Cyanide has also previously been found to inhibit the growth of AMF (Roberts and Anderson 2001; Stinson et al. 2006). Barto and Cipollini (2009) hypothesize that the cyanide in garlic mustard could be released by alliarinoside, although further work is needed to verify this. Nevertheless, it seems likely that AITC, sinigrin, and cyanide compounds are also killing another type of belowground fungi, the entomopathogenic fungi.

Table 1. Results of a two-way ANOVA of the removal of garlic mustard on the abundance of entomopathogenic fungi in the soil (measured as the percentage of fungal waxworm mortality) 45 d after garlic mustard removal.

Abundance of entomopathogenic fungi	SS	*df*	*F*	*P*
Garlic mustard	2,610.750	1	14.292	0.0054
Removal	4,446.750	1	24.344	0.0011
Garlic mustard × removal	5,084.083	1	27.833	0.0008

We did not experimentally establish the sites with and without garlic mustard plants. Therefore, it is possible that some underlying factors that we did not measure affected the growth of both garlic mustard and entomopathogenic fungi. However, the pattern we observed is consistent with previous experimental approaches that demonstrate that garlic mustard plants can kill entomopathogenic fungi (Keesing et al. 2011). More importantly, the fact that entomopathogenic fungi reestablished themselves after we removed garlic mustard plants—and did not do so on control plots—suggests a causal connection between the presence of garlic mustard and the suppression of the fungi.

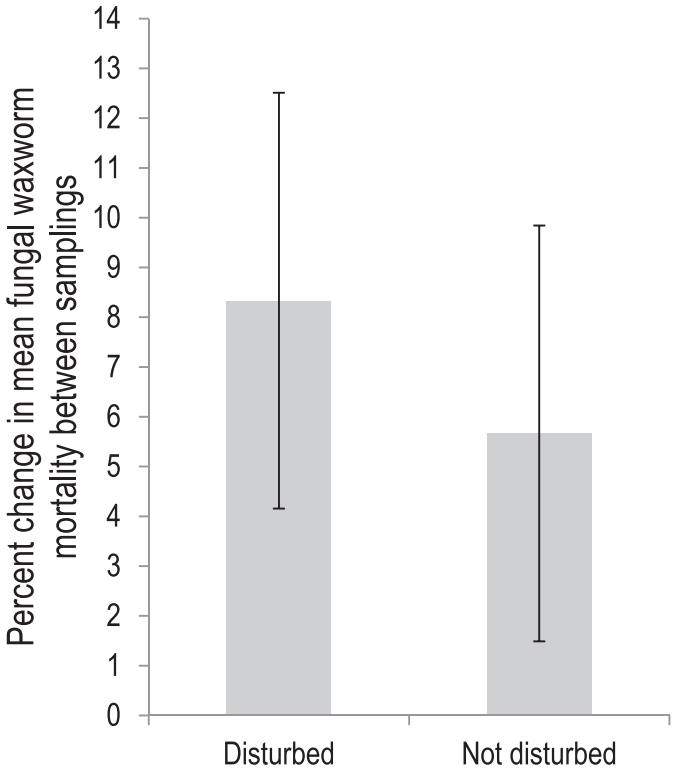

Figure 3. Mean difference in waxworm mortality due to fungal infection in disturbed and undisturbed treatment plots. Waxworms were more likely to die from fungal infections later in the summer rather than earlier, but the disturbance treatment did not affect that probability (P = 0.682). Error bars represent standard errors; there were three replicates per treatment group.

Our experiment did not determine the source of the entomopathogenic fungi that infected waxworms after we removed garlic mustard. Fungal spores may have survived in situ when garlic mustard was present, but their germination or growth may have been suppressed by compounds secreted by garlic mustard. Conversely, spores from neighboring areas may have colonized plots from which garlic mustard had been removed. If the latter is the case, there could be important effects of scale on colonization dynamics of fungi following removal of garlic mustard.

By increasing the growth of entomopathogenic fungi, removal of garlic mustard may have the capacity to alter the abundance and distribution of arthropods. Because entomopathogenic fungi grow mostly in the top layer of the soil, arthropods that live or forage on the surface of the forest floor are likely to be most affected (Tuininga et al. 2009). An increase in the abundance of entomopathogenic fungi could have detrimental effects on the community if it leads to a decline in the number of beneficial arthropods (e.g., pollinators,). Africanized honey bee (*Apis mellifera scutellata*) and leafcutter ant (*Atta sexdens*) are known to be vulnerable to the conidia of *Beauveria* and *Metarhizium* species (Alves 2009; Hughes et al. 2004; Santos 2007). Higher levels of entomopathogenic fungi in the soil may, therefore, cause declines in the number of ecologically important species in areas invaded by garlic mustard. On the other hand, an increase in the abundance of entomopathogenic fungi could have positive effects by decreasing the abundance and diversity of arthropods that are potentially harmful to humans, e.g., disease vectors. *Beauveria bassiana* has been shown to be an effective biocontrol agent for several tick species, including the blacklegged tick—a vector of anaplasmosis, babesiosis, and Lyme disease (Ostfeld et al. 2006b; Samish et al. 2008), three diseases rapidly emerging around the world. By increasing the abundance of entomopathogenic fungi in the soil, garlic mustard removal could decrease the abundance of ticks.

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