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Effects of garlic mustard (*Alliaria petiolata*) on entomopathogenic fungi[1]

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Abstract: Garlic mustard (*Alliaria petiolata*) is an invasive Eurasian species that is now widespread in North America. Like some other invasive species, garlic mustard is known to exude biochemical compounds that can reduce the fitness of native species when it invades a new habitat. Compounds leached from garlic mustard can reduce growth and survival of mycorrhizal fungi associated with forest trees in eastern North America. We tested whether these compounds could also inhibit the growth or survival of fungi known to infect arthropods, the so-called entomopathogenic (EM) fungi. We found that growth of *Beauveria bassiana* (*Bb*), a widespread EM fungus, was significantly reduced when spores were incubated on agar plates made with leachate from garlic mustard. When leachate was added to soil that had been inoculated with *Bb* spores, waxworms were significantly less infected with *Bb* than they were on soil inoculated with *Bb* spores alone. Finally, waxworms were less infected with EM spores when they were held in soil collected from field plots with abundant garlic mustard plants compared to neighbouring soils without garlic mustard plants. Together, these results demonstrate the potential for garlic mustard leachate to significantly inhibit the growth of EM fungi and thereby suppress the effects of EM fungi on arthropods. This effect of garlic mustard could be beneficial to humans if it reduces mortality of arthropods that provide ecosystem services, but it could also be harmful to humans if it reduces mortality of pest insects or arthropod vectors of disease, such as ixodid ticks.
Keywords: arthropod, entomopathogenic fungi, garlic mustard, invasive species.

Résumé : L'alliaire officinale (*Alliaria petiolata*) est une espèce envahissante d'Eurasie maintenant répandue en Amérique du Nord. Comme certaines autres espèces envahissantes, l'alliaire officinale exsude des composés biochimiques qui peuvent réduire la valeur adaptative des espèces indigènes lorsque cette plante envahit un nouvel habitat. Les composés qui sont lessivés de l'alliaire officinale peuvent réduire la croissance et la survie de champignons mycorhiziens associés aux arbres des forêts de l'est de l'Amérique du Nord. Nous avons évalué si ces composés pourraient aussi inhiber la croissance ou la survie de champignons connus pour infecter les arthropodes, les champignons entomopathogènes (EM). Nous avons constaté que la croissance de *Beauveria bassiana* (*Bb*), un champignon EM commun, était significativement réduite lorsque les spores étaient incubées sur des plaques de gélose comprenant du lessivat de l'alliaire officinale. Lorsque du lessivat était ajouté à un sol inoculé avec des spores de *Bb*, les larves de la teigne de ruche étaient significativement moins infectées par *Bb* que dans un sol inoculé uniquement avec des spores de *Bb*. Enfin, les larves de la teigne de ruche étaient moins infectées par des spores d'EM lorsqu'elles étaient placées dans un sol provenant de parcelles comprenant une abondance d'alliaire officinale comparé à des sols voisins dépourvus de cette plante. Dans l'ensemble, ces résultats démontrent le potentiel du lessivat de l'alliaire officinale à réduire de façon significative la croissance de champignons EM et ainsi éliminer leurs effets sur les arthropodes. Cet effet de l'alliaire officinale pourrait être bénéfique pour les humains si la mortalité des arthropodes fournissant des services écologiques était réduite, mais cela pourrait aussi être dommageable si la mortalité d'insectes nuisibles ou d'arthropodes vecteurs de maladies, comme les tiques ixodidés, était aussi réduite.
Mots-clés : arthropode, champignons entomopathogènes, alliaire officinale, espèce envahissante.

Nomenclature: USDA, NRCS. 2011.

Introduction

The extraordinary success of some invasive plant species in their new ranges appears to be due to the ability of the invaders to synthesize biochemical compounds to which the native biota have not had time to adapt (Inderjit, Callaway & Vivanco, 2006). These compounds can, in some cases, directly reduce germination and growth of native plant species. For example, sagebrush (*Artemisia tridentata*) secretes methyl jasmonate, which has been found to suppress seed germination of *Nicotiana attenuata* (Preston, Betts & Baldwin, 2002). Effects of bioactive compounds produced by invasive species can also be indirect. For example, bioactive compounds can inhibit pathogenic soil fungi that attack seedlings. In China, exudates from *Solidago canadensis*, an invasive perennial plant, kill soil fungi that kill seedlings, potentially reducing mortality of the invading species (Zhang *et al*., 2009). Indeed, the antifungal properties of exudates from some plant species have been recognized by agronomists, who have recommended the occasional cultivation of these species as crop "biofumigators" (Brown & Morra, 1997).

Garlic mustard (*Alliaria petiolata*), native to Eurasia and northern Africa (Rodgers, Stinson & Finzi, 2008), is an

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invasive species in North America known to exude a suite of bioactive compounds, including flavonoids, glycosides, and glucosinolates (Daxenbichler *et al*., 1991; Haribal & Renwick, 2001; Roberts & Anderson, 2001; Cipollini, 2002). These compounds inhibit a variety of fungi, including both plant mutualists that form mycorrhizal associations with garlic mustard's competitors (Roberts & Anderson, 2001; Stinson *et al*., 2006) and plant pathogens that infect seeds and seedlings (Brown & Morra, 1997). Not surprisingly, these effects are thought to increase the ability of garlic mustard to invade new habitats (Rodgers, Stinson & Finzi, 2008).

Because the exudates of garlic mustard (GM) appear to affect a diversity of fungi, we hypothesized that they might affect fungi known to be pathogenic to arthropods, the so-called entomopathogenic fungi. Two species of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, are widespread and relatively abundant in the soil in eastern North America (Bidochka, Kasperski & Wild, 1998; Bidochka & Small, 2005; Meyling & Eilenberg, 2007). Both species have been used for biocontrol of insect pests (Meyling & Eilenberg, 2007), and both have been shown to also be effective at killing several species of ticks that act as vectors of livestock and human pathogens (Ostfeld *et al*., 2006; Samish, Ginsberg & Glazer, 2008).

We conducted a series of experiments to determine if garlic mustard exudates could inhibit growth of the spores of entomopathogenic fungi. First, we tested for a direct inhibitory effect of GM extract on the germination of *B. bassiana* spores. We then used an insect (waxworms: *Galleria mellonella* larvae) bioassay to test whether GM extract could protect insects from attack by entomopathogenic fungi by suppressing or killing fungal spores in soil. Finally, we undertook field assays of the ability of naturalized garlic mustard to inhibit naturally occurring entomopathogenic fungi from killing insects.

Methods

Experiment #1

To determine if GM extract could prevent germination of spores of the entomopathogenic fungus *Beauveria bassiana* (*Bb*), we grew *Bb* spores (or used water as a control) on agar plates that had been made either with garlic mustard extract or with water. We prepared standard 100-mm × 15-mm petri dishes with standard agar made of 10 g neopeptone, 40 g glucose, and 20 g bacto agar in 1 L of sterile distilled water, which we then autoclaved. To prepare agar plates with GM extract, we collected whole first-year GM plants (roots, shoots) opportunistically in April 2008 on the grounds of Bard College in Dutchess County, New York. Plants were rinsed to remove dirt, separated as roots or shoots, and then dried. To make extract, we weighed 100 g of dry plant material (roots or shoots) and then soaked the material in 1 L of sterile water for 60 h. Filtered extract was stored in a refrigerator until immediately before use and then used in the standard agar recipe in place of sterile water. We prepared 10 of each of 3 types of agar plates: control, root extract, and shoot extract.

The 3 types of plates were then inoculated with *Bb* spores (2.2×10^5 spores·mL^{-1}) or sterile water. To obtain this concentration of spores, we made serial dilutions of Botanigard 22WP, a commercially available source of powdered *Bb* spores, in sterile water using sterile techniques. On each agar plate, we placed 5 evenly spaced 5 µL drops of a spore solution or water so that we had 5 replicates of each combination of plate type and spore addition/control, for a total of 30 plates. Plates were stored at room temperature and monitored for growth of fungal spores after 7 d. We created an index of fungal growth, with 0 = no visible growth; 1 = areas of *Bb* growth < 1 mm diameter; 2 = growth 1–3 mm diameter; 3 = growth 4–10 mm diameter; 4 = growth > 10 mm diameter.

We used a Kruskal–Wallis analysis of variance (ANOVA) on ranks to determine if there were differences in fungal growth among plates made with shoot extract, root extract, or sterile water. For this and all other statistical tests, we set α at 0.05.

Experiment #2

To determine if GM extract could prevent germination of fungal spores in soil, we conducted a factorial experiment, adding GM extract or sterile water to vials of commercial potting soil that had been inoculated with fungal spores or sterile water, and monitored infection of waxworms as a bioassay.

We partially filled 80 clear plastic vials (9.7 cm × 2.8 cm) with commercial potting soil and then inoculated half of these vials with 2 mL of liquid *Bb* spores (Botanigard ES) and half with 2 mL of sterile water. After 15 min, we added 2 mL of garlic mustard extract to half of each type of vial and 2 mL of water to the other half, for a total of 20 replicates of each type. GM extract was prepared as in Experiment #1 but using only 50 g of shoots/leaves, collected in August 2008. After an hour, we added 5 waxworms, obtained from a local pet store, to each of the 80 vials. All waxworms were of similar size (~2 cm) and were used as a bioassay for the presence of entomopathogenic fungi (Zimmerman, 1986). To maintain moisture in the vials, we added 1 mL of sterile water to each vial every 2 d beginning on day 0. We monitored waxworm survival every 24 h for 10 d; dead waxworms were removed upon discovery and presence of fungal infection was noted.

We conducted a paired *t*-test to examine differences in survival rates of waxworm larvae among treatments using JMP 7 (SAS Institute, Cary, North Carolina, USA).

Experiment #3

To determine if exudate from GM could affect spores of entomopathogenic fungi in the field, we selected 10 sites in July 2008, each of which had both dense, naturally occurring first-year GM plants (patch diameter > 1.5 m) and areas without GM that were between 1.0 and 1.5 m from the edge of the GM patch. Paired plots were matched for apparent similarity in environmental conditions (*e.g*., light exposure, plant cover and community composition, soil moisture). Sites were primarily located at forest edges.

From each of these 20 plots, we collected ~4 L of soil using equipment that was sterilized with 10% bleach solution after each sample was collected. Samples were stored

in airtight plastic bags at 5 °C after collection. Soil was then sieved to remove roots, rocks, and other debris; all sieving materials were sterilized with 10% bleach solution after each use. Sieved soil from each plot was transferred into 5 clear plastic vials (9.7 cm × 2.8 cm), each of which was filled halfway, for a total of 100 vials. Into each vial, we placed five waxworms, obtained from a local pet store, as a bioassay for the presence of entomopathogenic fungi (Zimmerman, 1986). All waxworms were of similar size (~2 cm). To maintain moisture in the vials, we added 1 mL of sterile water to each vial every 2 d beginning on day 0. We monitored waxworm survival every 24 h for 10 d; dead waxworms were removed upon discovery and presence of fungal infection was noted.

Using JMP 7, we conducted a paired *t*-test to examine differences in survival rates of waxworm larvae between treatments at the end of the experiment.

Results

EXPERIMENT #1

Spores of *Bb* placed on agar plates prepared with sterile water grew almost 5 times as abundantly as did spores grown on plates prepared with GM exudate prepared from either shoots or roots, which grew little if at all (Figure 1; $P << 0.0001$). We observed no growth of spores when we inoculated plates with sterile water.

EXPERIMENT #2

In the laboratory experiment with added GM extract and/or fungus, waxworm survival declined through the course of the experiment ($F_{10, 67} = 494.5$, $P < 0.0001$), but the rate of decline differed among the 4 treatments ($F_{30, 197} = 14.3$, $P < 0.0001$). In the 3 treatments other than water, survival approached 0 by the 10th day of observation; the greatest range of survival among treatments occurred at day 3 of observation (Figure 2).

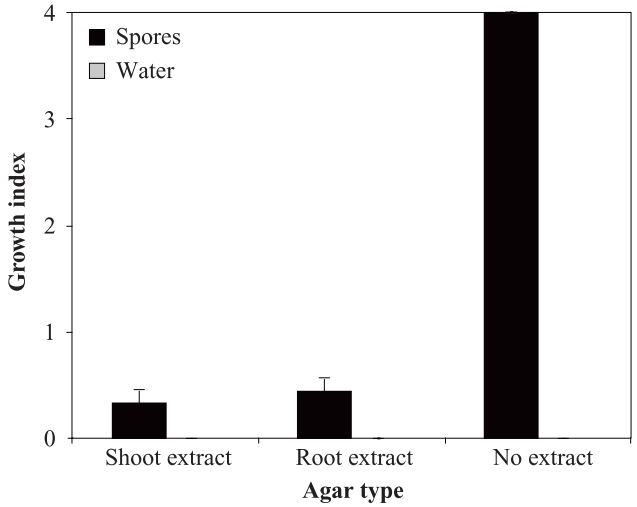

FIGURE 1. Mean (+ SE) growth of *Beauveria bassiana* spores on agar plates made with or without extract from shoots or roots of garlic mustard plants. On the plates to which *B. bassiana* spores were added (black bars), growth was abundant only on the plates that did not contain garlic mustard extract. Plates to which only sterile water was added (grey bars) showed no growth of spores, indicating that there was no contamination of plates.

EXPERIMENT #3

In vials containing soil collected from areas with or without garlic mustard plants, overall waxworm survival declined through the course of observation, but waxworms maintained on soil collected from areas with GM plants experienced higher survival rates than those maintained on soil from areas lacking GM ($t_6 = 6.05$, $P < 0.0001$). Observations of 500 waxworms over 10 d showed that in soil from areas with GM plants, 41% of waxworms died by day 10, whereas in soil from nearby areas without GM plants, mortality was 81% (Figure 3).

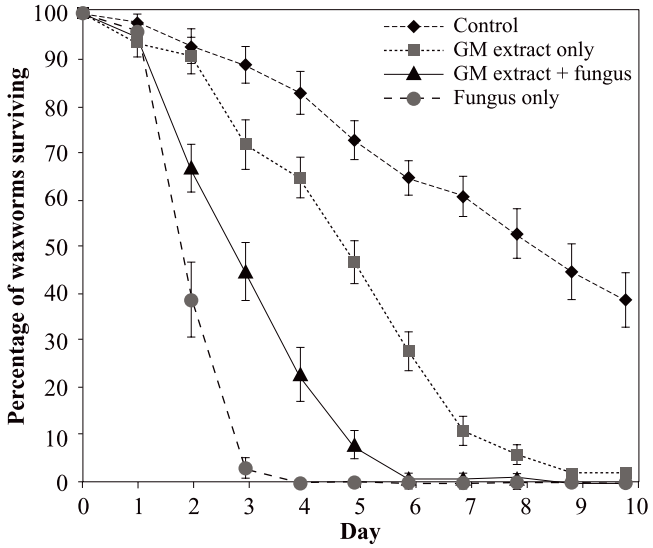

FIGURE 2. The percentage of waxworms (*Galleria mellonella* larvae) surviving in potting soil inoculated with *Beauveria bassiana* spores (or water) to which garlic mustard (GM) extract (or water) was added. Waxworms survived best in control soil and least well in soil to which only fungal spores were added. The addition of GM extract to vials containing fungal spores increased survival of waxworms.

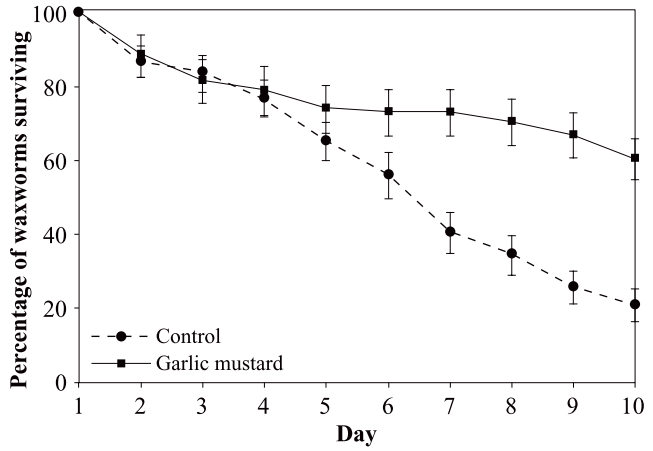

FIGURE 3. The percentage of waxworms surviving in soils from forest plots that contained garlic mustard plants or neighbouring plots that did not. Waxworms survived significantly better over 10 d in forest soils from plots containing garlic mustard plants.

Discussion

In a series of experiments, we found that garlic mustard (GM) exudate significantly inhibited the growth of entomopathogenic fungi. Virtually no *Beauveria bassiana* (*Bb*) spores germinated on agar plates made with GM extract, while the spores grew abundantly on standard agar plates. When spores were added to commercial potting soil, 97% of the waxworms were killed by the 3rd day; waxworm survival was 15 times higher when garlic mustard extract was added with the fungal spores. Extracts from shoots and roots of GM were equally potent. Finally, we found that waxworms survived more than 3 times as well in soils from areas with garlic mustard plants compared with soils from nearby areas without garlic mustard.

We found that GM itself negatively affected the survival of waxworms, irrespective of the presence of entomopathogenic fungi (*Bb*) (Figure 2). The mechanisms by which GM directly reduced waxworm survival are not known; we suspect that GM exudates are mildly toxic to some invertebrates while they simultaneously inhibit entomopathogenic microbes. Whether natural concentrations of GM exudate can reduce survival of arthropods directly is unknown.

The results of our first experiments demonstrate the potential of GM exudate to inhibit *Bb* spores. Future experiments should establish how variations in the concentration of GM exudate in the laboratory affect spore germination. Critically, the natural concentrations of GM exudate and *Bb* spores in forest soils are not known. It is also unknown how similar GM leachate prepared in the laboratory is to GM exudate in forest soils. These are both important areas for future research.

In our third experiment, waxworms survived significantly better in soil from areas with naturally occurring GM plants than in nearby soils without GM plants. We did not experimentally establish the sites with and without GM plants. Thus, we did not control for potential causes of GM presence and absence. As a result, it is possible that some underlying, unmeasured factor affects both the growth of entomopathogenic fungi and habitat suitability for GM. Nevertheless, our data from lab and field are consistent with the explanation that exudates from GM plants in forest soils significantly inhibit entomopathogenic fungi.

The results from our experiments immediately suggest that, whenever entomopathogenic fungi are present, arthropods could survive at higher rates in soils containing GM plants compared to soils without GM. This could be beneficial to humans if these arthropods provide ecosystem services, *e.g.*, pollination, decomposition of leaf litter, or control of undesirable insects. However, GM could also increase the abundance of arthropods that are potentially harmful to humans. *Bb* has been shown to be an effective biocontrol agent for several tick species, including the blacklegged tick vectors of Lyme disease, anaplasmosis, and babesiosis (Ostfeld *et al.*, 2006; Samish, Ginsberg & Glazer, 2008), 3 diseases rapidly emerging around the world. Thus, the invasion of forest soils by GM could potentially increase the abundance of tick vectors by inhibiting native fungi known to kill these ticks. Invasion by exotic plants has been linked to both the facilitation and inhibition of tick populations. Blacklegged tick (*Ixodes scapularis*) abundance was higher in forest understories dominated by exotic shrubs such as Japanese barberry (*Berberis thunbergii*) (Elias *et al.*, 2006). They attributed this facilitative effect to improved abiotic conditions (temperature, moisture) created by the exotic plants. In contrast, the exotic grass *Microstegium vimineum* created microclimatic conditions unfavourable for the survival of the lone star tick (*Amblyomma americanum*) and the dog tick (*Dermacentor variabilis*) (Civitello, Flory & Clay, 2008). Neither of these studies assessed whether chemical suppression of tick pathogens might be involved in the interaction between exotic plants and ticks. Further studies should determine the importance of the indirect effects of chemicals in contributing to the net effects of exotic plants on ecologically or epidemiologically important arthropods.

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