

## NOVEL WEAPONS: INVASIVE PLANT SUPPRESSES FUNGAL MUTUALISTS IN AMERICA BUT NOT IN ITS NATIVE EUROPE

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**Abstract.** Why some invasive plant species transmogrify from weak competitors at home to strong competitors abroad remains one of the most elusive questions in ecology. Some evidence suggests that disproportionately high densities of some invaders are due to the release of biochemicals that are novel, and therefore harmful, to naïve organisms in their new range. So far, such evidence has been restricted to the direct phytotoxic effects of plants on other plants. Here we found that one of North America's most aggressive invaders of undisturbed forest understories, *Alliaria petiolata* (garlic mustard) and a plant that inhibits mycorrhizal fungal mutualists of North American native plants, has far stronger inhibitory effects on mycorrhizas in invaded North American soils than on mycorrhizas in European soils where *A. petiolata* is native. This antifungal effect appears to be due to specific flavonoid fractions in *A. petiolata* extracts. Furthermore, we found that suppression of North American mycorrhizal fungi by *A. petiolata* corresponds with severe inhibition of North American plant species that rely on these fungi, whereas congeneric European plants are weakly affected. These results indicate that phytochemicals, benign to resistant mycorrhizal symbionts in the home range, may be lethal to naïve native mutualists in the introduced range and indirectly suppress the plants that rely on them.

**Key words:** allelopathy; anti-fungal compounds; arbuscular mycorrhizae; biochemistry; biogeographical comparisons; exotic invasion; fungi; indirect interactions; novel weapons.

### INTRODUCTION

One of the most perplexing questions in ecology is how some species, when moved to new ranges by humans, suppress other species and become far more abundant than in their native ranges (Elton 1958, Blossey and Nötzold 1995, Hierro et al. 2005). There are many hypotheses for such invasive success, but all hypotheses converge on the need for a biogeographic explanation; either the invader must face much weaker opposition from other species abroad than at home or the invader must have stronger effects on species abroad than at home. Interactions between plants and their consumers (Keane and Crawley 2002, DeWalt et al. 2004, van der Putten et al. 2005) and among plants (Callaway and Aschehoug 2000, Vivanco et al. 2004) have received some explicit biogeographic attention, and other comparative studies demonstrate that highly aggressive invasive exotic plants are “more likely to have potent secondary compounds that have not been

reported from North American native plants” than exotic species that are not considered invasive (Cappuccino and Arnason 2006). The idea that some invaders may succeed because they possess novel biochemicals that provide them with an advantage against naïve species has been coined the “novel weapons hypothesis” (Callaway and Ridenour 2004, Callaway et al. 2005).

Evidence for such novel weapons has focused on phytotoxic interactions among plants (Rabotnov 1982, Callaway and Aschehoug 2000, Mallik and Pellissier 2000, Bais et al. 2003, Vivanco et al. 2004, Blair et al. 2005, Thorpe 2006), and in the novel biochemical effects of an invader on mutualist fungi (Stinson et al. 2006). However, recent studies suggest that interactions between microbes and plants may be particularly important for understanding plant invasions (van der Putten et al. 1988, Klironomos 2002, Reinhart et al. 2003, Beckstead and Parker 2004, Callaway et al. 2004). Soil microbes have crucial detrimental and beneficial effects on plants through pathogenic effects, root–fungus mutualisms and by driving the nutrient cycles on which plants depend (Johnson et al. 1997, Packer and Clay 2000, Torsvik and Øvreås 2004). However, not all plants are equally dependent on particular microbial processes. Similarly, plants can have powerful but species-specific

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effects on soil microbes (Vitousek et al. 1987, Philippot and Germon 2005). These plant–microbe relationships may be vulnerable to disruption through the novel biochemistry of invaders (Callaway et al. 2005). Here we explored the novel weapons hypothesis in the context of invasive plants and arbuscular mycorrhizae.

*Alliaria petiolata* (Bieb.) Cavara & Grande (see Plate 1), a European invader of North American forests, suppresses the growth of North American species by disrupting mutualistic associations between native canopy tree seedlings and belowground arbuscular mycorrhizal (AM) fungi (Stinson et al. 2006, also see Roberts and Anderson 2001). But if suppressing soil fungal mutualists is to explain the transformation of *A. petiolata* into a far more dominant species in its new range, then these effects must be stronger in North America than in Europe. We compared the effects of *A. petiolata*, and biochemical extracts from *A. petiolata*, on mutualistic associations between plants and AM fungi in European soils to the effects of these factors on mutualisms in North American soils. We addressed the following specific questions: (1) Is *A. petiolata* more toxic to AM fungi in North America than in Europe? (2) Are North American mycorrhizal plants more negatively impacted by *A. petiolata* than those that co-occur with *A. petiolata* in Europe? (3) Are non-mycorrhizal plants in North America less negatively impacted by *A. petiolata* in North America? (4) What is the biochemical basis of *A. petiolata* toxicity to AM fungi, and does this differ between continents?

## METHODS

### *Study species*

*Alliaria petiolata* (garlic mustard) is a sexually reproducing, obligate biennial forb native to Europe that aggressively invades forest habitats in North America. In its native range, it is found in forest edges and other semi-shaded habitats as well as forest understory, but does not aggressively colonize or dominate in these habitats (Clapham et al. 1952). Seedlings emerge in early spring and progress to evergreen rosettes during the first year. Flowers are borne in late spring on one or more stalks of the second-year plants, and mature into elongated siliques. Seeds may require overwintering for germination (Baskin and Baskin 1992, Nuzzo 2000). Since its arrival into the northeastern United States in the 1800s (Clapham et al. 1952, Nuzzo 1991) *A. petiolata* first spread gradually, then underwent a series of rapid population explosions over the past two to three decades (Dhillon and Anderson 1999, Nuzzo 2000, Meekins and McCarthy 2001). In North America, *A. petiolata* frequently forms dense, monospecific stands that dominate native plant communities within a variety of habitats, including forest edges, floodplains, and forest understory (Nuzzo 1991, Stinson et al. 2006). It is now widespread across the Eastern Seaboard, from Ontario to the Carolinas,

extending to the Midwest and more recently, as far west as California and Oregon (Welk and Schubert 2002).

### *Growth experiment*

We collected 8–18 L of soil from each of four sites in North America (USA) and two sites in Europe. In Vermont (collected March 2004 in Bennington, 42°52.3' N, 73°11.3' W), Oregon (collected April 2004 in Portland, 45°30.3' N, 122°41.4' W), and Massachusetts (collected February 2004 in Harvard Forest, 42°31.4' N, 72°11.1' W), soil was collected from deciduous forest (dominated by *Acer* and *Quercus*) understories not inhabited by *A. petiolata*, but within 100 m of *A. petiolata* populations. In Montana, USA (collected April 2004 in Missoula, 46°53.0' N, 113°58.3' W), soil was collected from a deciduous riparian forest (dominated by *Populus* and *Acer*) understory not inhabited by *A. petiolata* and in a part of North America in which *A. petiolata* does not occur. In Germany (collected January 2004 in Halle, 51°28.3' N, 11°58.2' E) and Spain (collected April 2004 in 42°49.2' N, 1°17.3' W), soil was collected from deciduous forest (dominated by *Acer* and *Quercus*) understories not invaded by *A. petiolata*, but within 100 m of *A. petiolata* populations. Soil was slowly air-dried immediately after collection and stored until used in the experiment. On 4 May 2004, the soil from each region (we use “region” to designate each of the six sites from which soil was collected and “continent” to designate comparisons of North America and Europe) was divided in half and each half was divided into three 2.8-L pots. For some sites from which not enough soil was collected, pots were not filled completely. To culture the experimental soils (see Klironomos 2002), root cuttings were collected from overwintering rosettes of *Alliaria* in March 2004 in a forest in central Michigan (Kellogg Biological Station, 42°19.3' N, 85°18.2' W), USA, over 1000 km from any soil collection site. These cuttings were surface sterilized with a solution of 5% bleach and transplanted into half of the pots used for each region, and then were grown to maturity in each pot. At least one plant in each pot flowered. The other (control) pots were filled with soil, but no plants were put in them. Plants in this experiment were grown in a single greenhouse with no-plant controls randomly intermixed with the planted pots and watered exactly as the planted pots. Greenhouse temperatures were kept between 15°–28°C. Natural light was supplemented by Metal Halide bulbs, and total photosynthetically active radiation (PAR) during the day remained above 1200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , which would not limit a shade tolerant species such as *A. petiolata* (Meekins and McCarthy (2000).

In October 2004, 185 days after planting, *A. petiolata* plants were removed from pots. Keeping all sites and *A. petiolata*-cultured and control (no *A. petiolata*) treatments separate, aliquots from soil were transferred to 525-mL pots. For each of the 525-mL pots, 50 mL of sterile silica sand was placed at the bottom, 150 mL of a

50:50 mix of sterile silica sand and soil was placed on top of the pure sand, and 250 mL of pure soil was placed on top of the soil/sand mixture. These pots were transferred to a larger greenhouse at the University of Montana (Missoula, Montana, USA) with temperature and light conditions similar to the first greenhouse. As for the arrangement of pots during the period in which soils were trained with *A. petiolata*, pots with the different plant and soil combinations were randomly mixed within the bench space used for the experiment.

For each location where we collected soil, we acquired seeds from 10–14 plant species native to that region. However, poor germination reduced the total number of species used to 7–10 species per region. To remedy low species numbers, we used *Pseudoroegneria spicata* and *Festuca idahoensis* from Montana in Massachusetts and Vermont soils. The species used and their sources are listed in Table 1. Our goal was biogeographic comparisons among some genera that are commonly mycorrhizal; and similar comparisons among other genera that are not (e.g., *Carex*). Poor germination of some of the non-mycorrhizal species reduced our final number to six North American species–soils combinations, and two European species–soils combinations.

For each species, we prepared 10 replicates of the 525-mL pots for *A. petiolata*-cultured soils and 10 replicates for the control (no *A. petiolata*) soils. We only planted species from a region in soils from the same region (with the exception of *P. spicata* and *F. idahoensis*, see previous paragraph) and no species and soils were crossed among regions. Within three days of transferring soils from the 2.4-L pots to the 525-mL pots, 10 seeds of a species were planted in each pot (12 and 13 October 2004). At the same time, aliquots of soils from the treatment and the control pots were shipped on dry ice to the University of Guelph, Ontario, Canada, where they were analyzed for spore density, spore viability, and infectivity (see following paragraph). Seedling emergence was monitored until 11 November 2004, but all germinants after the first germinant were removed so that only one individual grew per pot. All seeds had received four weeks of cold stratification and were germinated in similar temperatures, light, and moisture conditions in the experiment. The effects of training soil microbial communities can be confounded by the effects of training on soil nutrients. To ameliorate the problem of differential nutrient depletion by training some soils and not others, plants in our experiments were watered every two weeks with 200 mL of one-quarter-strength Hoagland's solution modified by the addition of phosphorus as inositol hexaphosphate. This form is not directly available for uptake by plants. Seedlings were harvested 15–31 December 2004, but species in the same genus from all six regions were harvested on the same day to minimize bias towards any continent, region, or treatment. Seedlings were dried at 60°C and weighed for total biomass. A subsample of roots was

TABLE 1. HPLC flow conditions for flavonoid and alliarinose analysis.

Time (min)	Water (%)	Acetonitrile (%)
Initial conditions	95	5
5	90	10
20	70	30
30	70	30
33	95	5
38	95	5

sent to the University of Guelph to determine mycorrhizal colonization (see following section).

#### *AM fungi analysis*

AM fungal spores were extracted directly from the soil using a wet-sieving/centrifugation method (Klironomos et al. 1993), and counted under 20× magnification. Viability of those spores was assessed after placing them in iodinitrotetrazolium solution (1 mg/mL) for 48 h (Carvalho et al. 2004). Mycorrhizal infectivity of the soil was assessed after growing leek (*Allium porrum*, Liliaceae) in the soil for four weeks, staining the roots using Chlorazol Black E (Brundrett et al. 1984), and then measuring the percentage of root colonization (McGonigle et al. 1990).

#### *Soil microbial community analysis*

Soil samples for microbial community analysis were taken from the same trained soil samples that were used for plant response experiments. Soil microbial communities were characterized using denaturing gradient gel electrophoresis of PCR-amplified small subunit ribosomal RNA genes (PCR-DGGE; Muyzer et al. 1996, Kim-Jong et al. 1999). DNA was extracted from soil samples using a bead-beating technique using 0.5-g of sample per extraction with a FastDNA SPIN kit (QBiogene, Irvine, California, USA). PCR reactions utilized a touchdown protocol where the annealing temperature was decreased by 1°C each cycle in order to maintain the highest level of stringency in the first few reactions (Don et al. 1991), and one primer was modified with a 40-bp GC clamp in order to arrest the migration of amplified rDNA fragments during electrophoresis (Sheffield et al. 1989). Gradients were formed with an agitated CBS gradient former and amplified DNA products were separated by electrophoresis on a CBS-2201 DGGE apparatus (CBS Scientific, Del Mar, California, USA). A small fragment (196 bp) of the V3 variable region of the bacterial 16S rDNA was amplified using the universal bacterial primers (Muyzer et al. 1996). The polymerase chain reaction (PCR) cycle was 5 min at 95°C, and then 11 cycles of 30 s at 93°C, 30 s at 65°C, 30 s at 72°C, and then 19 cycles of 30 s at 93°C, 30 s at 55°C, 30 s at 72°C, with a final extension of 15 min at 72°C. The fungal ribosomal internal transcribed spacer (ITS) region (550 bp) was amplified using the primers of White et al. (1990). The PCR cycle was 5 min at 95°C,

and then 11 cycles of 30 s at 95°C, 45 s at 65°C, 2 min at 72°C, and then 19 cycles of 30 s at 95°C, 45 s at 55°C, 2 min at 72°C, with a final extension of 5 min at 72°C. PCR products were separated on DGGE gels as follows: bacterial 16S at 10% T, 19:1 (5% C) acrylamide/bis-acrylamide, 160 V, 18 h, 60°C, 35–75% denaturant gradient; and fungal ITS at 8% T, 37.5:1 (2.6% C) acrylamide/bis-acrylamide, 140 V, 18 h, 60°C, 30–65% denaturant gradient. DNA in gels was stained with SYBR Green and photographed over a UV transilluminator. Band pixel intensity in photographs was normalized to total lane intensity and analyzed with the aid of a band recognition program (Quantity One; BioRad, Hercules, California, USA), and bands were then scored as having an intensity of 0, 1, 2, 3, or 4, where a score of zero represented a band that was absent from the sample, but present in the composite lane, which was created as a reference that comprised all the bands found in the lanes from the test samples representing the total taxon richness. A score of four was given to the brightest band on the gel. Similarity coefficients between paired samples were calculated from common bands (bands migrating the same distance on a gel in different samples) and unique bands (bands present in one sample but absent in the other) using a modification of Sorenson's index of similarity (Sorenson 1948):  $C_s = (2j + k)/(a + b)$ , where  $C_s$  represents Sorenson's index of similarity,  $a$  is the number of bands in sample A,  $b$  is the number of bands in sample B,  $j$  is the number of bands of the same intensity common to samples A and B, and  $k$  is the number of bands of different intensity common to samples A and B. Pairwise similarity coefficients were generated for all possible sample pairs to generate a matrix showing the degree of similarity among all samples. Comparisons of microbial diversity were made using the Shannon-Weaver diversity index and community evenness index. In this case, banding patterns for each sample were compared against a composite profile representing the sum of bands found in all the samples.  $H = (C/N)(N \cdot \log N - \sum n_i \log n_i)$ , where  $H$  represents the Shannon Weaver Index,  $C = 2.3$ ,  $N$  is total intensity of all bands, and  $n_i$  is the intensity of  $i$ th band; and  $e = H/\log(S)$ , where  $e$  represents the community evenness index, and  $S$  is the total number of bands.

#### Plant extract experiment

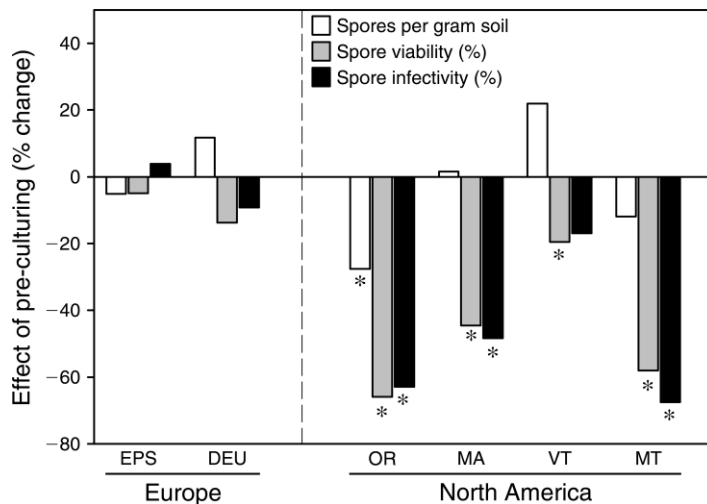
To identify potential biochemical drivers of biogeographic differences in AM inhibition by *A. petiolata*, we isolated fractions enriched in flavonoids or glucosinolates from fresh leaf tissue of *A. petiolata* plants collected from a forest in southwestern Ohio, USA (Wright State University, 39°48' N, 84°1' W). Production of glucosinolates in roots and leaves is well known in *A. petiolata* and other plants in the Brassicaceae, and these measurements were intended to determine if some of the same flavonoids present in leaves and roots of *A. petiolata* plants could also be present in root exudates.

Leaf tissue was boiled in ethanol, then filtered, dried, and defatted with hexane (modified from Haribal and Renwick 2001). The dried extract was dissolved in water, and then partitioned with *n*-butanol to isolate flavonoids and alliarinoside, while the charged glucosinolates remained in the water. Both extracts were dried and redissolved in water. Fractionated extracts were characterized using high performance liquid chromatography (HPLC) to verify locations of compounds. Flavonoids and alliarinoside were analyzed as in Cipollini et al. (2005), but with the flow conditions shown in Table 1. Glucosinolates were analyzed as described by Kiddle et al. (2001) using a Phenomenex Luna C18 (150 × 4.6 mm, 5 μm; Torrance, California, USA) column and a Waters 2690 HPLC system (Milford, Massachusetts, USA) equipped with a photodiode array detector, with a flow rate of 1 mL/min.

Ideally, dosages in allelopathy studies should be equivalent to levels found in field soil. However, there are no published reports of *A. petiolata* metabolite levels in soil, and we have been unable to quantify field levels as yet. The related species, *Brassica napus*, has glucosinolate levels of 14–20 μmol/g leaf tissue (Gardiner et al. 1999, Morra and Kirkegaard 2002). Isothiocyanates, which are the breakdown products of glucosinolates, reach levels of 40–75 nmol/g in soils following incorporation of *B. napus* plant material (Moora and Kirkegaard 2002). Assuming that a similar relationship exists for *A. petiolata*, we estimated that soil levels should be ~300 times less than levels in the leaves and diluted extracts accordingly, to give 0.0033 gram leaf equivalents (gle) per gram of soil. The lowest dose used to show allelopathic effects of *A. petiolata* in the past was 0.05 gle/mL extract (Roberts and Anderson 2001, Stinson et al. 2006), which is an order of magnitude higher than the dose we used. McCarthy and Hanson (1998) used doses as low as 0.001 gle/mL extract, but only found inhibitory effects at 0.1 gle/mL extract. Although our choice of dosage is based on data from a separate species, we believe it is more ecologically relevant than the higher doses used in earlier studies.

Extracts were applied as either flavonoid, glucosinolate, or mixed fractions to soils in the laboratory at the University of Montana. Because of the low European replication in the initial experiment, in this experiment we used soils from six sites in North America and six sites in Europe. Soils used included three from the first experiment in North America (Oregon, Vermont, Massachusetts) and two from Europe (Germany and Spain). We collected soils from three new regions in North America (Minnesota [44°10.5' N, 93°58.4' W], Wisconsin [44°52.5' N, 91°41.2' W], and Indiana [39°11.4' N, 86°30.5' W]), and four new regions in Europe (Hungary [47°37.5' N, 18°19.2' E], Romania [47°10.2' N, 27°36.3' E], the Czech Republic [48°46.1' N, 14°18.1' E], and France [44°50.5' N, 0°28.0' W]). As before, these soils were dried slowly at room temperature and stored dry until use. On 2 February 2006, 3 gm

FIG. 1. Effect of *Alliaria petiolata* pre-culturing on the density, viability, and infectivity of arbuscular mycorrhizal fungal spores in North American and European soils. Bars show the percentage of increase or decrease of soils in which *A. petiolata* had been grown relative to control soils without *A. petiolata*. Asterisks represent statistical significance following separate *t* tests, with a sequential Bonferroni correction for multiple tests, of *A. petiolata*-cultured and non-*A. petiolata* cultured soil ( $P < 0.02$ ). Abbreviations are: ESP, Spain; DEU, Germany; OR, Oregon; MA, Massachusetts; VT, Vermont; and MT, Montana.



of soil from each region was placed into each of 20 15-mL centrifuge tubes (total  $n = 240$ ). For each region, five samples each were treated with the flavonoid fraction, the glucosinolate fraction, and both glucosinolate and

flavonoid fractions to give a final dose of 0.0033 g/g of soil. Each treatment was applied in 1.5 mL of water, and tubes were centrifuged for 15 s to spread the solution throughout the 3 gm of soil. On 14 February

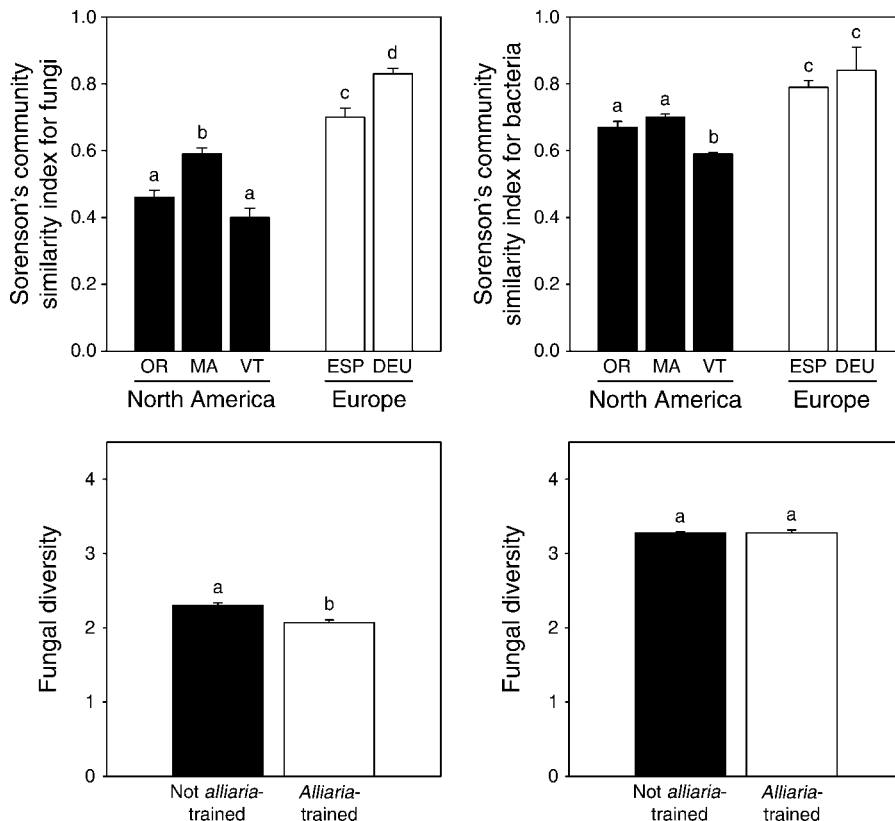


FIG. 2. Comparison of microbial communities in soils from North America and Europe following training by *Alliaria petiolata* or left untrained, from banding patterns on denaturing gradient gels separating microbial taxa based on sequence polymorphism in small subunit ribosomal RNA fragments. Sorenson community index measures the degree of similarity of soil microbial communities between trained and untrained samples. Diversity of microbial communities was measured using Shannon-Weaver Diversity Index. Error bars show 1 SE. Bars with the same letter are not significantly different ( $P = 0.01$ ); means separations by Duncan's multiple-range test. See Fig. 1 for abbreviations.

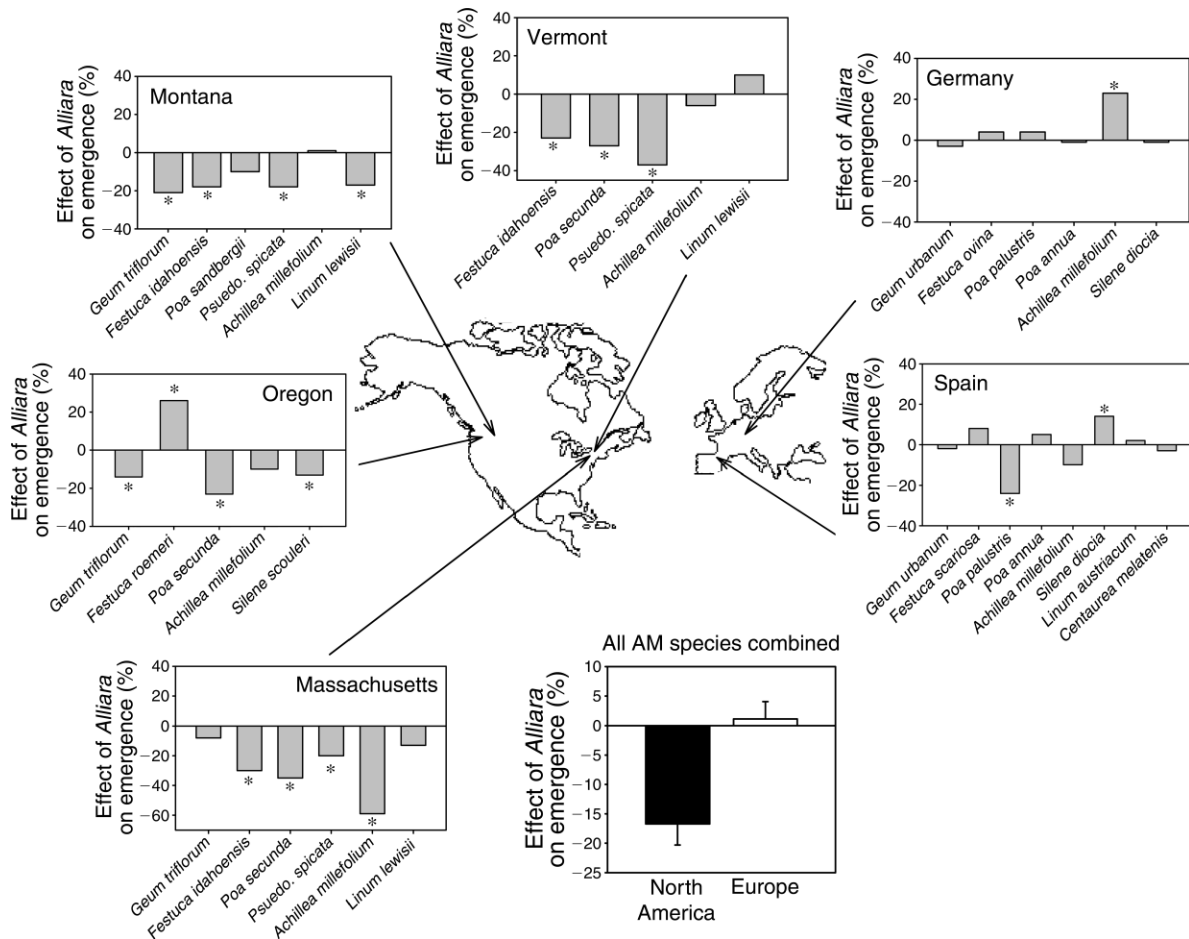


FIG. 3. Effect of pre-culturing soil with *Alliaria petiolata* on the emergence of arbuscular mycorrhizal (AM) plant species from soil collected at four sites in North America and from two sites in Europe. Each panel shows the results for soils from a particular region and for plant species native to that region. Bars represent the mean proportional change in seedling emergence measured in *A. petiolata*-cultured soils compared to control soils in which *A. petiolata* was not grown. For each species,  $n = 10$  pots for the control and  $n = 10$  pots for the treatment. Each pot was planted with five seeds, and the mean emergence per pot was the variable used for statistical analysis. Results from ANOVA with the mean for each species at each region used as a replicate: for continent,  $F_{1,4} = 3.009$ ,  $P = 0.158$ ; for treatment,  $F_{1,4} = 25.901$ ,  $P = 0.007$ ; for continent  $\times$  treatment,  $F_{1,4} = 30.228$ ,  $P = 0.005$ . Asterisks denote significant differences between treatment and control for individual species as determined by separate  $t$  tests, followed by a sequential Bonferroni correction for multiple tests, using the mean of five seeds per pot as the replicate,  $P < 0.00125$ .

2006, these soils were analyzed for AM spore viability at the University of Guelph (see *Methods: AM fungi analysis* for methods).

#### Statistical analysis

Data were analyzed with mixed-model analysis of variance (ANOVA) using the software R, version 2.1.0 (R Development Core Team 2004). Continent and treatment were considered fixed factors, whereas region and species were nested within continent and treated as random factors. The effects of continent and continent-by-treatment interaction were tested against the region and region-by-treatment interaction, respectively. This represents a more conservative approach for testing differences between continents than using species as error term because it treats sites from which soil was collected as independent units of replication.

#### RESULTS

Pre-culturing soils with *A. petiolata* caused no differences in the spore density of AM fungi between continents, but the viability and infectivity of these AM spores from North American soils were reduced much more than those from Europe (Fig. 1). Pre-culturing North American soils with *A. petiolata* resulted in significantly lower viability ( $-20\%$  to  $-66\%$ ) and infectivity ( $-16\%$  to  $-68\%$ ) of AM fungal spores from four different regions of North America. In contrast, *A. petiolata* had no significant effect on the viability and infectivity of AM fungi from two regions of Europe where *A. petiolata* occurs naturally. These results suggest that AM fungi, co-occurring with *A. petiolata* are tolerant to this plant's biochemistry, while AM fungi beyond *A. petiolata*'s native range are not. Culturing

North American soils with *A. petiolata* also caused larger compositional shifts in soil fungal and bacterial communities than did culturing European soils with *A. petiolata*, and significantly decreased fungal species diversity in North American soils, but not in European soils (Fig. 2).

The reduction of AM fungal spore viability and infectivity in North American soils, but not in European soils, corresponded with biogeographical differences in the emergence and growth of mycorrhizal plant species from the two continents (Fig. 3). In *A. petiolata*-cultured soils from the four North American regions, the emergence of seedlings of mycorrhizal plant species (Table 2) from those same regions (all regions and all species combined), or from nearby regions of North America, was reduced by  $16.7\% \pm 3.6\%$ . *Alliaria petiolata*-culturing of soils from two regions of Europe (both regions and all species combined) had no effect on seedling emergence of species from the same areas from which soils were collected. Of the 23 combinations of North American mycorrhizal plant species and North American soils in our experiments, seedling emergence in 13 of these was inhibited by pre-culturing North American soil with *A. petiolata*, and one species was enhanced. Of the 14 combinations of European mycorrhizal plant species and European soils, only one was inhibited by *A. petiolata*, whereas two species were enhanced. Likewise, pre-culturing North American soils with *A. petiolata* increased post-recruitment mortality of North American plant species from  $6.2 \pm 1.9\%$  to  $20.9 \pm 7.2\%$  (Fig. 4). Pre-culturing European soils with *A. petiolata* had no effect on the mortality of European plant species.

Pre-culturing North American soil with *A. petiolata* also suppressed the final biomass of North American mycorrhizal plant species by an average of 59% over all species and sites, and the effect of *A. petiolata* was significant at all sites (Fig. 5). Pre-culturing soil from Germany with *A. petiolata* had no effect on the biomass of mycorrhizal plant species from that region, whereas pre-culturing soil from Spain with *A. petiolata* actually enhanced the biomass of plants from that region. In contrast to the strong inhibitory effect of *A. petiolata* on mycorrhizal plant species from North America, there was either no effect, or a positive effect, of *A. petiolata* soil culturing on the biomass of North American non-mycorrhizal plant species (Fig. 6). For the two European species that were non-mycorrhizal, *A. petiolata* training had no effect on their growth (Fig. 6).

Grouped comparisons of related taxa also demonstrated strong biogeographic differences among European and North American species while controlling for phylogenetic variation (Fig. 7). For *Achillea millefolium*, a circumboreal species, the biomass of North American plants grown in *A. petiolata*-cultured, North American soils was 56–100% lower than in non-cultured soils, and *A. petiolata* had strong effects on soil from all sites. For European *A. millefolium*, *A. petiolata*-culturing had no

TABLE 2. List of species used in the *Alliaria petiolata* soil-training experiment, indicating the presence or absence of arbuscular mycorrhizal (AM) fungal structures.

Species	AM fungal structures
A) North America	
Oregon	
<i>Silene scouleri</i>	+++
<i>Geum triflorum</i>	---
<i>Festuca roemerii</i>	+++
<i>Elymus glaucus</i>	---
<i>Poa secunda</i>	+++
<i>Festuca occidentalis</i>	---
<i>Achillea millefolium</i>	+++
Montana	
<i>Geum triflorum</i>	+++
<i>Festuca idahoensis</i>	+++
<i>Pseudoroegneria spicata</i>	---
<i>Poa sandbergii</i>	+++
<i>Achillea millefolium</i>	+++
<i>Linum lewisii</i>	+++
<i>Carex circinatum</i>	---
<i>Carex vulpenoides</i>	---
Vermont and Massachusetts	
<i>Geum triflorum</i>	+++
<i>Festuca idahoensis</i> †	+++
<i>Poa pratensis</i>	+++
<i>Elymus canadensis</i>	---
<i>Achillea millefolium</i>	+++
<i>Pseudoroegneria spicata</i> †	---
<i>Linum perenne</i>	---
<i>Carex lurida</i>	---
B) Europe	
Germany	
<i>Silene dioica</i>	+++
<i>Geum urbanum</i>	+++
<i>Festuca ovina</i>	+++
<i>Poa annua</i>	---
<i>Poa palustris</i>	+++
<i>Agropyron repens</i>	+++
<i>Achillea millefolium</i>	+++
<i>Carex pendula</i>	---
Spain	
<i>Silene dioica</i>	+++
<i>Geum urbanum</i>	+++
<i>Festuca scariosa</i>	+++
<i>Poa annua</i>	---
<i>Poa palustris</i>	+++
<i>Agropyron repens</i>	+++
<i>Achillea millefolium</i>	+++
<i>Linum austriacum</i>	+++
<i>Centaurea melitensis</i>	---
<i>Carex pendula</i>	---

Notes: Symbols represent presence (+) or absence (–) of the following arbuscular mycorrhizal (AM) fungal structures (arbuscules, vesicles, hyphae, respectively) observed in their roots (e.g., “–+–” denotes “arbuscules absent, vesicles present, hyphae present”). Plants without any AM fungal structures are considered not dependent on AM fungi for growth (even though they are sometimes found to be mycorrhizal in other studies, [e.g., *Elymus* spp.]). These species are not included in analyses in Figs. 2, 3, and 4. All seeds were collected from plants growing at the sites where soils were collected, with the exception of those marked with a dagger (†); these species were collected in Montana, within their natural distribution, but used with soils from Vermont and Massachusetts, which is outside their natural distribution.

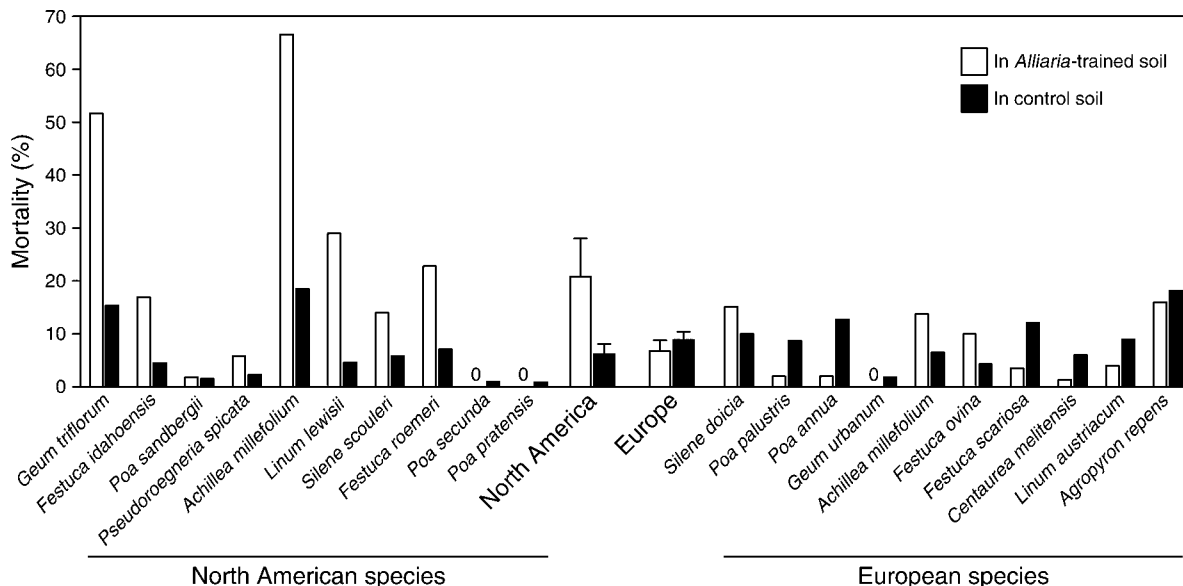


FIG. 4. The percentage of mortality of North American and European mycorrhizal plant species in native soil pre-trained with *Alliaria petiolata* and control soils. Thinner bars show the means for each species (replicate species from different sites are combined). Large bars in the center are means and 1 SE for North America and Europe. Results from three-way ANOVA: for continent,  $F_{1,4} = 23.369$ ,  $P = 0.008$ ; for treatment,  $F_{1,4} = 40.754$ ,  $P = 0.003$ ; for continent  $\times$  treatment,  $F_{1,4} = 112.368$ ,  $P = 0.0004$ .

effect in German soils, but a significant inhibitory effect on *A. millefolium* in Spanish soils. Even so, *A. petiolata* culturing was more inhibitory to *A. millefolium* in North American soils than in European soils (continent  $\times$  treatment,  $F_{1,4} = 11.437$ ,  $P = 0.028$ ). For North American species of *Festuca*, *A. petiolata*-culturing of North American soils caused a significant decline in biomass for *Festuca* from three of the four regions. There was no significant effect of *A. petiolata*-culturing of European soils on either European *Festuca* species. The North American *Geum triflorum* was highly inhibited in three of four North American populations (germination was too low in the Vermont populations for statistical analysis) by *A. petiolata*-culturing of North American soils, but *A. petiolata*-culturing of European soils had either no effect on *Geum urbanum* (German soils) or a positive effect (Spanish soils).

We found that some of the same flavonoids were present in both leaves and roots of *A. petiolata* plants (Appendix A: Fig. A1) could also be present in root exudates. The flavonoid fraction was enriched in glycosides, including alliarinoside, isovitexin-6''- $\beta$ -D-glucopyranoside, and other flavonoid glycosides. The identities of these compounds were verified by electrospray ionization (ESI)/MS and MS/MS on a liquid chromatography quadrupole (LCQ) ion-trap mass spectrometer (ThermoQuest, San Jose, California, USA). Alliarinoside and isovitexin-6''- $\beta$ -D-glucopyranoside are known from no other North American plant species. We also isolated a glucosinolate fraction devoid of these glycosides but enriched in glucosinolates, of which sinigrin and glucotropaeolin are known to

predominate in *A. petiolata* (Vaughn and Berhow 1999) (Appendix B: Fig. B1).

The effects of *A. petiolata* extracts on AM fungal spore viability were weak when applied to European soils (Fig. 8), with all combinations of fractions causing decreases in spore viability ranging from 0% to 36% (Appendix C: Fig. C1). However, extracts had much stronger effects on AM fungal spore viability in North American soils (Fig. 8). The *Alliaria* fraction enriched in glucosinolates had weak effects on North American AM fungal spores, but the flavonoid glycoside-enriched fraction reduced North American spore viability by an average of 53%, and the effect ranged from 41% to 71% (Appendix C: Fig. C1). When a combination of glucosinolate and flavonoid fractions were applied to North American soils, AM spore viability decreased by an average of 74%, ranging from 53% to 89%.

#### DISCUSSION

We found that growing *A. petiolata* in soils from four different regions of North America decreased AM fungi, fungal diversity, and the emergence, growth, and survival of mycorrhizal plant species from the same regions. Non-mycorrhizal plant species did not show this response. Growing *A. petiolata* in soils from two different regions of Europe had no effect on AM fungi or soil fungal diversity, and had weak effects on the composition of fungal communities and plant emergence and growth. There was no indirect effect of *A. petiolata* on the survival of plant species from Europe. Application of *A. petiolata* leaf extracts that shared many chemical similarities with root extracts strongly suppressed AM fungi in soils from six North American



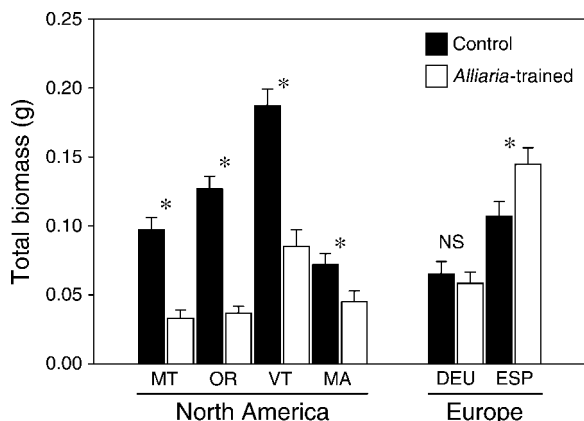


FIG. 5. Effect of pre-culturing soils with *Alliaria petiolata* on the final total biomass of North American and European mycorrhizal plant species grown in their native soils. Error bars show 1 SE. Results from three-way ANOVA: for continent,  $F_{1,4} = 0.217$ ,  $P = 0.665$ ; for treatment,  $F_{1,4} = 13.988$ ,  $P = 0.020$ ; for continent  $\times$  treatment,  $F_{1,4} = 34.488$ ,  $P = 0.004$ . Asterisks denote significant differences between treatment and control for a region as determined by separate  $t$  tests, followed by a sequential Bonferroni correction for multiple tests  $P < 0.008$ . See Fig. 1 for abbreviations.

contrast, it appears that *A. petiolata* may produce a biochemical (or suite of biochemicals) that is novel in North America and to which North American plants and fungi are not adapted, supporting the novel weapons hypothesis (Callaway and Ridenour 2004).

In addition to the novel glycosides considered in this study, *A. petiolata* has recently been identified as markedly cyanogenic, making it unique among Brassicaceous plants in which cyanogenesis has been examined (Cipollini and Gruner 2007). However, our evidence for the novelty of *A. petiolata* biochemistry is indirect, because we cannot compare all aspects of plant chemistry among *A. petiolata* and North American native mustard species. Whether or not North American Brassicaceous species also suppress soil fungi remains an area of further study.

The fact that *A. petiolata* had some positive effects on European species (i.e., emergence in *Achillea millefolium* and *Silene dioica* [Fig. 4] and total biomass in native plants from Spain [Fig. 5]) is curious and warrants further study. Because plants vary in their dependence upon mycorrhizal symbiosis for growth (Klironomos 2002), species that are less-dependent on AM fungi may be released from competition via *A. petiolata*'s suppression of their more mycorrhizae-dependent neighbors (see Stinson et al. 2006). Alternatively, some European plants could be released from the effects of harmful fungi, bacteria, or other microbes via the phytochemical effects of *A. petiolata* on these soil organisms. *Alliaria petiolata* may also affect soil chemistry in ways that alter pH, nutrient availability, or other aspects of plant–soil interactions that favor some species over others.

Our results cannot be interpreted as supportive of species-specific relationships among plant–AM fungi within continents, primarily because we were not able

sites, but had no effects on AM fungi in soils from six European sites. Moreover, extract concentrations used here were lower than in any other study of allelopathy in *A. petiolata* (e.g., Stinson et al. 2006). These results suggest that the long history of coexistence between *A. petiolata*, soil fungi, and other plant species in Europe, where *A. petiolata* is native, has led to the evolution of resistance in soil fungi that promote coexistence among *A. petiolata* and fungi and, therefore, *A. petiolata* and other plant species (i.e., Callaway et al. 2005). In

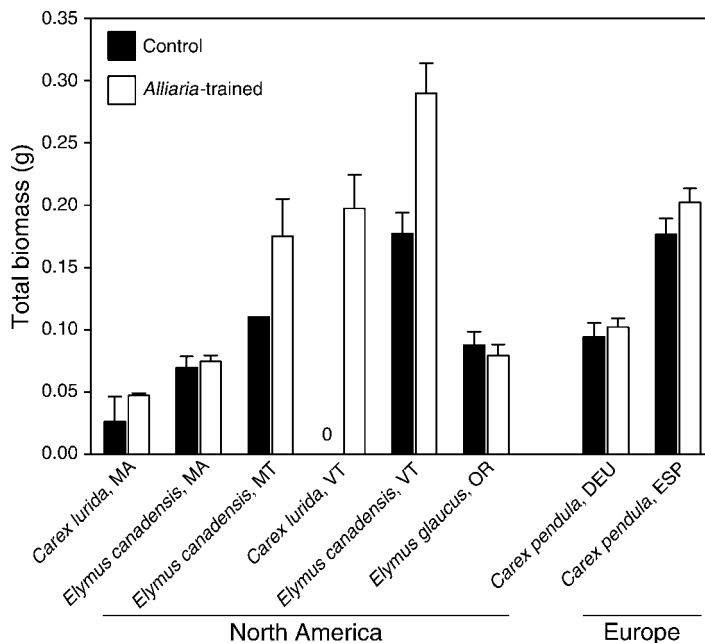


FIG. 6. Effect of pre-culturing soils with *Alliaria petiolata* on the final biomass of North American non-mycorrhizal plants. Plant species were grown in soils from general areas in which seeds were acquired. Error bars show 1 SE. For continent,  $F_{1,1} = 24.141$ ,  $P = 0.136$ ; for treatment,  $F_{1,4.9} = 18.967$ ,  $P = 0.008$ ; for continent  $\times$  treatment,  $F_{1,1} = 28.286$ ,  $P = 0.118$ . See Fig. 1 for abbreviations.

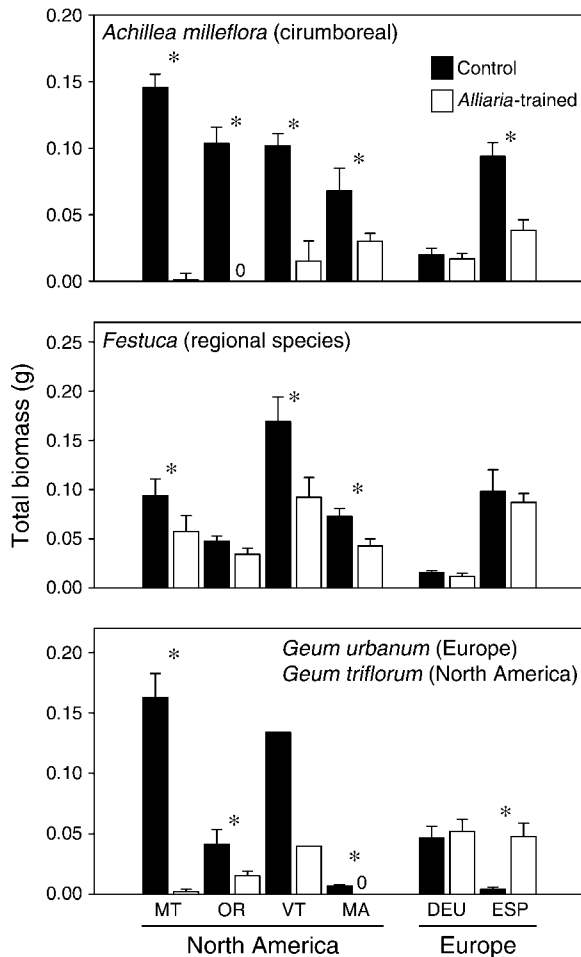


FIG. 7. Phylogenetically controlled (within genus or within circumboreal species) comparisons of the effect of *Alliaria petiolata* pre-culturing of soils. Bars show means for a given species at a given region with error bars representing 1 SE. Results for *Achillea*: for continent,  $F_{1,4} = 0.244$ ,  $P = 0.647$ ; for treatment,  $F_{1,4} = 26.391$ ,  $P = 0.007$ ; for continent  $\times$  treatment,  $F_{1,4} = 11.437$ ,  $P = 0.028$ . Results for *Festuca*: for continent,  $F_{1,4} = 0.194$ ,  $P = 0.683$ ; for treatment,  $F_{1,4} = 3.600$ ,  $P = 0.458$ ; for continent  $\times$  treatment,  $F_{1,4} = 21.154$ ,  $P = 0.010$ . Results for *Geum*: for continent,  $F_{1,3} = 0.230$ ,  $P = 0.656$ ; for treatment,  $F_{1,3} = 0.836$ ,  $P = 0.412$ ; for continent  $\times$  treatment,  $F_{1,3} = 6.495$ ,  $P = 0.063$ . Asterisks denote significant differences ( $P < 0.05$ ) between treatment and control for a region as determined by separate  $t$  tests, followed by a sequential Bonferroni correction for multiple tests within a species,  $P < 0.008$ ). See Fig. 1 for abbreviations.

to match soils with large numbers of species at the smaller scale of local habitats. For example, *S. scouleri*, *G. triflorum*, *F. roemerii*, *A. millefolium*, *L. perenne*, and *L. lewisii* can be found in the precise habitats invaded by *A. petiolata*, and these species fit the general pattern reported here for greater *A. petiolata* inhibition in North America. In contrast, *F. idahoensis*, *P. spicata*, and *P. sandbergii* are generally found in drier and more open habitats than those invaded by *A. petiolata*, but these species also fit the general pattern we report for

North American soils in our experiments. Similarly, *S. diocia*, *G. urbanum*, *F. ovina*, *F. scariosa*, *P. palustris*, *A. millefolium*, and *L. austriacum* occur in the same natural European habitats as *A. petiolata*, and these species fit the general pattern of being weakly inhibited by *Alliaria* as reported for European soils in our experiments. But *Agropyron repens* and *Poa annua* are from more xeric habitats than *A. petiolata*, and these species also fit the general pattern reported from Europe. Therefore, *A. petiolata* does not appear to disrupt relationships among plants and AM fungi in a highly species-specific manner. Instead we interpret our results as *A. petiolata* simply killing non-adapted North American AM fungi, and this has negative consequences for any plant species that might be dependent on a broad spectrum of AM fungal species; the inhibition of North American AM fungi may even suppress mycorrhizal plants from Europe if the two groups were combined. Local-scale relationships may exist among AM fungi and plants, but our experiments were not designed to test this possibility.

The biogeographical differences in the effect of *A. petiolata* were likely due to plant biochemistry. First, the effects we report for soil culturing by *A. petiolata* and for soil biochemical fractions were manifest in the absence of living *A. petiolata* plants, and therefore, *A. petiolata* could not have been competing with native plants for resources. Second, the effects of specific plant extracts had very similar effects as training soils with *A.*

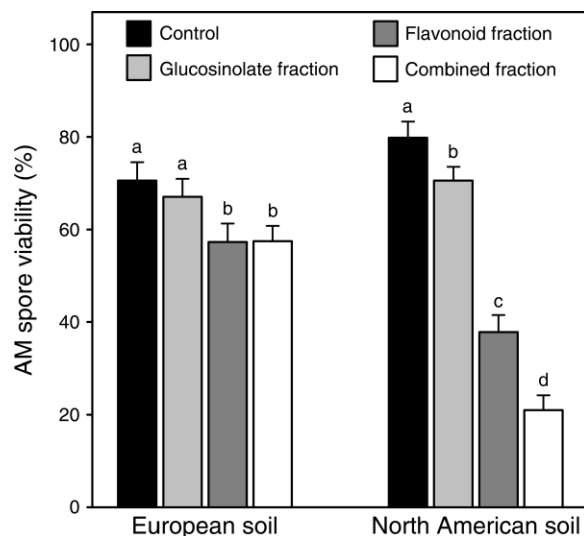


FIG. 8. Effect of root exudate fractions from *Alliaria petiolata* on the viability of arbuscular mycorrhizal (AM) fungi in soils from six sites in Europe and six sites in North America, with regions combined (see Appendix C: Fig. C1). Within a soil type, bars with the same letter are not significantly different ( $P > 0.05$ ). Results from a three-way ANOVA with five replicates per region: for continent,  $F_{1,10} = 9.810$ ,  $P = 0.035$ ; for region,  $F_{10,192} = 1.924$ ,  $P = 0.044$ ; for treatment,  $F_{3,30} = 47.798$ ,  $P = 0.002$ ; for continent  $\times$  treatment,  $F_{3,30} = 18.385$ ,  $P = 0.013$ , for continent  $\times$  treatment,  $F_{30,1924} = 0.978$ ,  $P = 0.501$ .



PLATE 1. First-year garlic mustard *Alliaria petiolata* growing in a forest understory in its invasive range in Ohio, USA. Photo credit: D. Cipollini.

*petiolata*; reducing the viability of North American AM fungi, but not the viability of European AM fungi.

Our evidence for *A. petiolata*'s inhibition of North American species through suppression of AM fungi does not preclude other processes as drivers of *A. petiolata* invasion. *A. petiolata* also has strong direct competitive and allelopathic effects (Meekins and McCarthy 1999, Bossdorf et al. 2004, Prati and Bossdorf 2004); therefore, these direct effects are also important mechanisms by which *A. petiolata* affects other plant species. Neither previous studies nor ours have fully tested potential biogeographic differences in direct plant–plant interactions; however, plants from North American *A. petiolata* populations are not better intra-specific competitors than plants from Europe (Bossdorf et al. 2004), suggesting that direct effects may not show the biogeographic differences necessary to explain superior performance in invaded regions where *A. petiolata* is exotic. Disturbance is also a factor in the spread of *A. petiolata* (Nuzzo 1999), but there is no evidence to suggest that *A. petiolata* responds to disturbance in North America differently than in Europe. However, invasive European earthworms may have more powerful effects in North American forests than in their native forests, and there are clear spatial relationships between *A. petiolata* invasion and the presence of European earthworms in North American forests (B. Blossey and J. Maerz, *personal communication*). The effects of European earthworms may ultimately prove to be a strong driver of *A. petiolata* invasion, and are likely to

interact with the biochemical mechanisms presented in this paper.

Non-mycorrhizal plant families represent a disproportionate number of invasive plants globally, relative to the available species pool (Pyšek 1998), suggesting that suppression of local AM fungi has the potential to be a general process, but this remains to be seen. Other research has provided evidence for the novel weapons hypothesis by showing that novel biochemicals produced by exotic invasive plants have stronger toxic effects on other plants in invaded communities than on plants in the communities where the invader originated (Callaway and Aschehoug 2000, Vivanco et al. 2004, Thorpe 2006, but see Blair et al. 2005, 2006), and that changes in plant–soil microbe interactions can contribute to plant invasions (Van der Putten et al. 1988, Reinhart et al. 2003, Agrawal et al. 2005, Reinhart and Callaway 2006). We show that an invader can suppress naïve native mutualists and, by doing so, indirectly inhibit a number of native plants that rely on these mutualisms. By transporting organisms far beyond their historical dispersal limitations, humans may have mixed plant and microbial species that do not share common evolutionary trajectories (Callaway et al. 2005). This mixing appears to have profound negative consequences for invaded communities.

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#### APPENDIX A

HPLC chromatographs from *Alliaria petiolata* leaves and roots (*Ecological Archives* E089-063-A1).

#### APPENDIX B

Representative HPLC chromatographs of fractionated garlic mustard extracts (*Ecological Archives* E089-063-A2).

#### APPENDIX C

Effect of root exudate fractions from *Alliaria petiolata* on the viability of AM fungi in soils from six sites in Europe and six sites in North America (*Ecological Archives* E089-063-A3).