Biol Invasions
DOI 10.1007/s10530-011-9986-x

ORIGINAL PAPER

Low allelochemical concentrations detected in garlic mustard-invaded forest soils inhibit fungal growth and AMF spore germination

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Received: 15 November 2010 / Accepted: 15 March 2011

Abstract Garlic mustard's (*Alliaria petiolata*, Brassicaceae) invasive success is attributed in part to its release of allyl isothiocyanate (AITC) into the soil. AITC can disrupt beneficial arbuscular mycorrhizal fungi (AMF) associated with native plant roots, which limits their soil resource uptake. However, AITC and its precursor, sinigrin, have never been detected in garlic mustard-invaded forest soils. Here, we use high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC–MS) to assess the concentration and bioactivity of these putative allelochemicals in paired forest plots uninvaded or invaded by garlic mustard. Our methods detected AITC and sinigrin only where garlic mustard was present and our recovery of AITC/sinigrin coincided with adult senescence. A bioassay of in situ fungal hyphae abundance revealed significantly reduced hyphal abundance in the presence of garlic mustard relative to uninvaded soils. Finally, the lowest concentration of AITC measured in the field ($\sim$0.001 mM) is highly inhibitory to the spore germination of a forest AMF species, *Glomus clarum*. Together, our data provide the first direct evidence of garlic mustard-produced sinigrin and AITC in forest soils and demonstrate that even low levels of these chemicals have the potential to significantly suppress AMF growth and spore germination, strengthening their status as allelopathic novel weapons.

Keywords Novel weapons · *Alliaria petiolata* · Sinigrin · Allyl isothiocyanate · Arbuscular mycorrhizae · Allelopathy · AITC · Garlic mustard · Plant invasion · Temperate forests · AMF

Introduction

Novel biochemical weapons produced by invaders have the potential to directly or indirectly suppress naïve or non-adapted native species in an invaded community (Callaway and Ridenour 2004). These allelochemicals have been implicated in the success of several plant invaders (Hierro and Callaway 2003); however, most empirical support of the novel weapons hypothesis comes from experiments conducted in controlled environments (Callaway and Aschehoug 2000; Prati and Bossdorf 2004; Orr et al. 2005; Callaway et al. 2008; He et al. 2009). Recently, both the necessity of field validation of allelopathy and novel weapons (Inderjit and Weiner 2001; Thorpe et al. 2009) and the use of rigorous analytical chemistry

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Electronic supplementary material The online version of this article (doi:10.1007/s10530-011-9986-x) contains supplementary material, which is available to authorized users.

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Published online: 30 March 2011

methods to quantify field bioactive concentrations of putative allelochemicals (Blair et al. 2009) have been raised. Here, we present the first quantification of the presence and function of the putative novel weapons of garlic mustard (*Alliaria petiolata*, Brassicaceae) in the field using the methods of analytical chemistry.

Introduced from Europe in the 1850s (Nuzzo 1993), garlic mustard is now widely listed as invasive (34 USA states; 3 Canadian provinces) or noxious (11 USA states) in North America. Garlic mustard is a model for the study of allelochemicals, and controlled environment studies have highlighted the potential role of allelochemicals in its invasive success. This species produces a suite of powerful secondary compounds known to deter herbivores and suppress the mutualistic mycorrhizal fungi associated with native plant roots (e.g. Haribal and Renwick 1998; Roberts and Anderson 2001; Stinson et al. 2006; Cipollini and Gruner 2007; Callaway et al. 2008; Wolfe et al. 2008; Lind and Parker 2010). While these secondary compounds include cyanide, alliarinoside, flavonoids, glucosinolates and glycosides, (Haribal and Renwick 1998; Vaughn and Berhow 1999; Haribal et al. 2001; Cipollini and Gruner 2007), glucosinolates have been assumed to be or are the focal novel weapons in many studies with garlic mustard (e.g. Vaughn and Berhow 1999; Roberts and Anderson 2001; Callaway et al. 2008; Barto and Cipollini 2009a; Lankau 2010). Glucosinolates can be converted by endogenous myrosinase into isothiocyanates, a class of compounds that are known to be toxic to a wide range of soil organisms (Brown and Morra 1997). Allyl isothiocyanate (AITC) is a well-characterized and highly potent anti-fungal agent (Olivier et al. 1999). AITC is the hydrolysis product of sinigrin, a glucosinolate found in high concentrations in garlic mustard tissue (Vaughn and Berhow 1999). The separation of sinigrin and myrosinase within garlic mustard's cells is destroyed upon plant tissue damage or decomposition and initiates the enzymatic reaction converting sinigrin to AITC.

In its native range, garlic mustard inhabits disturbed sites including river or road edges (Nuzzo 2000) but, in its invaded range, garlic mustard is a common invader of mature forest understories (Rodgers et al. 2008). Many native North American forest herbs associate with beneficial arbuscular-mycorrhizal fungi (AMF) in their roots and it is estimated that 80% obligately depend on the AMF mutualism (Brundrett and Kendrick 1988) for critical nutrient and water uptake (van der Heijden et al. 2008). Therefore, forest understory herbaceous communities are particularly susceptible to AITC's anti-fungal properties, and the disruption of the AMF mutualism is one of garlic mustard's best-supported novel weapons (Roberts and Anderson 2001; Stinson et al. 2006; Barto 2008; Callaway et al. 2008; but see Lankau 2010).

Most studies testing the effectiveness of garlic mustard's novel weapons on mutualistic soil fungi have used whole plant extracts or fractions of whole plant extracts (Roberts and Anderson 2001; Stinson et al. 2006; Callaway et al. 2008), yet the sinigrin/AITC concentrations of these extracts were not quantified. The sinigrin/AITC concentrations of the extracts were likely higher than field concentrations experienced by native plants during the natural leaching processes of garlic mustard allelochemicals in forest soils. Field tests assessing AMF responses to the presence or absence of garlic mustard in forest soil are in an early stage (Burke 2008), and the range of field AITC concentrations from garlic mustard that are bioactive against AMF spores and hyphae are unknown.

In pot experiments and in agricultural soils heavily spiked with macerated tissue, both sinigrin (Gimsing and Kirkegaard 2006) and AITC (Choesin and Boerner 1991; Gimsing and Kirkegaard 2006) from *Brassica* spp. were detectable. However, naturally released sinigrin in garlic mustard-invaded forest soils was not detectable (Barto and Cipollini 2009b) and to our knowledge, there are no published studies reporting attempts to recover AITC from invaded sites.

Three factors could explain the difficulty in detecting sinigrin/AITC in garlic mustard-invaded forest soils. First, garlic mustard may release sinigrin/AITC in concentrations that are biologically relevant but too low to be detected. Second, singrin/AITC may be quickly dissipated in soil by microorganisms, or AITC may be lost by simple evaporation. The average half-life of AITC in potting soils is only 47 h, and the half-life decreases in soils with low moisture availability and under high temperatures (Borek et al. 1995). Third, the timing of sinigrin/AITC release into the soil by garlic mustard could vary across the growing season and/or the developmental stage of garlic mustard and thus only be

detectable during specific time periods (Vaughn and Berhow 1999; Haribal and Renwick 2001; Gols et al. 2007). Other members of the mustard family exhibit seasonal variation in the concentration of defensive chemicals in their tissues (Feeny and Rosenberry 1982). In North America, garlic mustard is a biennial that germinates in its first year and flowers, fruits, and senesces in mid to late summer of its second year (Anderson et al. 1996; Rodgers et al. 2008). Given this life history, we hypothesized that levels of sinigrin/AITC in the soil might be highest and most readily detectable when garlic mustard adults senesce because their decomposing tissues will release allelochemicals (Rice 1974).

Here, we present a series of field and laboratory experiments that address three goals: (1) Assess the timing of release and natural concentrations of sinigrin and AITC in forest soils invaded with garlic mustard using analytical chemistry techniques, (2) Quantify the impact of garlic mustard's presence on fungal abundance in forest soils, and (3) Determine the range of AITC concentrations that can suppress AMF spore germination.

Materials and methods

Our field studies were conducted at the Trillium Trail Wildflower Reserve, a 16-hectare forest in Fox Chapel, Pennsylvania characterized by silt loam soils, moderately sloping ground (8–15% slope), and soil bulk densities that range from 0.42 to 0.93 $g \cdot (cm^3)^{-1}$. The diverse herbaceous understory of Trillium Trail contains 69 native herb species, of which ~60% form AMF mutualisms (Hale, unpublished data). Importantly, the novel weapons produced by garlic mustard, including AITC, are not found in the native North American mustards (Feeny and Rosenberry 1982; Barto et al. 2010a), including those that grow in our field site. At Trillium Trail, garlic mustard invasion of the forest was first noted in the early 1990s (L. Smith, West Penn Conservancy, pers. comm.), and it is currently patchily distributed in the forest. It has not yet reached the monoculture that is seen in late stage garlic mustard invasions (Rodgers et al. 2008). The patchiness of Trillium Trail's garlic mustard invasion allowed us to choose sites and establish paired plots where one plot is currently invaded with garlic mustard and another plot where garlic mustard is currently absent (control), but other conditions are similar. This paired design was used for all field studies described below.

Sinigrin detection in forest soil

To quantify the levels of sinigrin in the garlic mustard-invaded soils of Trillium Trail, we collected soil samples across four dates in the summer of 2007. We collected 10 g of soil from the top 5 cm on 12 June and 20 g on 20 June, 20 July, and 28 August. The early dates were chosen to span growth (12 and 20 June), and the later dates to span the senescence (20 July and 28 August) of garlic mustard in our field site. Five paired plots were sampled on the first, third and last dates, while 11 paired plots were sampled on the second date for a total of 16 early (growth) and 10 late (senescence) paired samples. To standardize water content, all soil samples were dried at 25°C for 24 h and then all roots were removed by passing the sample through a 2 mm mesh sieve to exclude any root tissue.

To extract the sinigrin, we added 15 ml of methanol to each soil sample. After centrifuging for 5 min, the supernatant was collected and transferred to a new centrifuge tube. This step was repeated with another 10 ml of methanol. The glucosinolates in the soil extracts were captured and washed in open columns packed with 0.1 g DEAE Sephadex A-25 (Pharmacia Inc., Piscataway, NJ, USA) and then desulfated by adding 1 mg of the enzyme sulfatase (Sigma–Aldrich, St. Louis, MO, USA) in 1 ml of water following a standard method (Agerbirk et al. 2001). The desulfoglucosinolates were eluted in 5 ml water and analyzed using HPLC on a Hewlet-Packard Model 1100 (Boise, ID, USA) fitted with a 4.5 × 15 cm C-18 column (Luna, Phenomenex Corp., Torrance, CA, USA), diode-array detector, and autosampler. The solvent program ran 100% water (for 2 min) followed by a linear change to 20% acetonitrile (at 5 min), 35% acetonitrile (at 15 min), and 100% acetonitrile (at 18 min) with a flow rate of 1.0 ml min^{-1}. Peaks were detected at 229 nm. Pure commercial sinigrin standards (from two different sources: Sigma–Aldrich, St. Louis, MO, USA and United States Biochemical Corporation, Cleveland, OH, USA) were used to verify peak identity and create standard curves for determination of sinigrin concentration in the soil extracts. Specifically, we

found that our commercial sinigrin standards consistently had retention times between 8.83 and 8.88 min. We also discovered that only sinigrin standards greater than 0.04 $\mu g \cdot ml^{-1}$ of eluate could be reliably detected using our HPLC equipment, which corresponds to 0.01 $\mu g \cdot g^{-1}$ dry soil. The lowest sinigrin standard that could be repeatedly detected had a peak area of 2.33 mAU. Using this information, we created two criteria to qualitatively analyze the soil extracts from invaded and control plots for the presence of sinigrin: 1) The peak must be detected between 8.83 and 8.88 min, and 2) The integrated peak area must be greater than 2.33 mAU. This is a highly conservative approach, as only soil extracts that met both of these criteria were deemed to have sinigrin.

Finally, to test the efficiency of our extraction method, we added 50 mg of a commercial sinigrin standard (Sigma–Aldrich, St. Louis, MO, USA) to one pair of the soil samples collected on 20 June as a positive control. The recovery efficiency was 65% and 70% from the spiked garlic mustard and spiked control plot samples, respectively (i.e. 32.6 and 35.1 mg of the original 50 mg were recovered; Online Resource 1). Because we left our soil samples at 25°C for 24 h to standardize water content, some of the sinigrin may have been converted to AITC by endogenous myrosinase present in the soil. This conversion could partially explain our recovery efficiency of sinigrin.

AITC detection in forest soil

Based on our 2007 sinigrin results, we assayed soils for AITC on 3 dates that spanned garlic mustard senescence during the summer of 2008 (3, 11, and 18 July) following the methods of Gimsing and Kirkegaard (2006). We took 8–10 soil cores (1.8 × 10 cm) from each of five paired sites. Cores within each plot (garlic mustard or control) were pooled and sieved in the field as described above. Immediately after sieving, 40 g of soil were mixed with 30 ml of ethyl acetate to extract the AITC. In the lab, the bottles were shaken for 15 min at 120 rpm on a shaker table and, after the soil had settled, the supernatant was decanted. This process was repeated three times with an additional 10 ml of ethyl acetate added to the soil each time. To the final extract, we added an internal standard of 1.4 mM methyl isothiocyanate (MITC; Fluka, St. Louis, MO, USA), and the extracts were evaporated to 4–5 ml. Extracts were dried using Pasteur pipettes packed with 4 cm plugs of anhydrous magnesium sulfate. Standards were made from commercial AITC (Sigma–Aldrich, St. Louis, MO, USA).

Soil extracts were analyzed using a Shimadzu GC–MS model Q5050A, GC 17-A (Columbia, MD, USA) equipped with a 30 m × 0.25 mm Restek XTI-5 column coated with a 0.25 μm 5% diphenyl-95% dimethyl polysiloxane stationary phase (Restek U.S., Bellefonte, PA, USA). Samples were injected splitless at 50°C, and the oven was programmed to heat from 50 to 220°C at a rate of 8°C min^{-1} with a 1 min initial hold time at 50°C. The injector temperature was 200°C and interface temperature was 230°C. Helium was used as the carrier gas at a linear velocity of 47.4 cm s^{-1} and the column flow was 1.7 ml min^{-1}. Sample chromatograms and spectra were compared to prepared standards and to published mass spectra (Stein 2005). Using the internal standard, we calculated the concentration of AITC that was detected in each sample (Harris 2003).

Effect of garlic mustard on the abundance of forest soil fungal hyphae

To determine the effect of garlic mustard on natural abundances of fungal hyphae in soil, we modified the "inserted membrane technique" of Baláz and Vosátka (2001) for use in a forest setting. We used plastic tissue culture capsules (diameter = 37 mm) containing both autoclaved potting soil (Professional Formula 4 Mix, Conrad Fafard Inc., Agawam, MA, USA) and a mixed cellulose ester membrane filter (diameter = 37 mm; pore size = 0.45 μm; Millipore Corporation) to assess hyphal abundance in the soil. Because fungal hyphae adhere to the surface of the membrane, we can quantify the amount of hyphae on the membranes in garlic mustard and control sites. We expected lower amounts of hyphae on membranes in the garlic mustard plots.

On 16 June 2009, we inserted the capsules in the field under three cm of soil. We buried six to eight capsules in three site pairs for a total of 44 membranes deployed and analyzed. In the garlic mustard plots, we buried the capsules within 0.5 m of a garlic mustard plant stem. After 4 weeks, we excavated the capsules from the soil, removed the membranes using forceps, gently washed them with

deionized water, and stained with 5 ml of a 0.06% solution of trypan blue.

To quantify the hyphae on the membranes, membranes were soaked in glycerol for at least 24 h, mounted on glass slides, and examined at 20× magnification. Thirty areas of 0.375 × 0.5 mm per membrane were examined. When stained hyphae were observed, we captured an image of that field using a Nikon digital camera. Images were overlaid with a 0.025 × 0.025 mm grid using Adobe Photoshop and the number of times that the hyphae crossed a particular gridline was counted for each image. These counts were converted to hyphal lengths (mm) using the technique described by Giovannetti and Mosse (1980). For each membrane, data from all images was added together to determine the hyphal length (mm) per membrane. Using one-way ANOVA, we then compared hyphal length/membrane across invaded and control sites to analyze the impact of garlic mustard on overall fungal abundance. Data was square-root transformed to induce normality in residuals prior to running the analysis. Median score analysis was also performed to determine the number of membranes with hyphal lengths greater than the median in both site types. All data were analyzed in SAS (v. 9.2, SAS Institute, Cary, North Carolina, USA).

Effect of natural concentrations of AITC on AMF spore germination in vitro

The lowest AITC concentration that we detected in the field (see "Results") was 0.004 μg g^{-1} soil (~0.001 mM). To determine if such low concentrations can inhibit fungal spore germination, we tested *Glomus clarum* spores across a range of AITC concentrations. We obtained *G. clarum* spores from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM). Because *G. clarum* is a ubiquitous AMF species in North American forest soils (J. Morton, (INVAM), pers. comm.), it is an appropriate representative AMF species for this study. The spores were washed twice in 2.5% chloramine-T salt hydrate (Sigma–Aldrich, St. Louis, MO, USA) and then transferred to sterile vials containing sterile deionized water. Sterilized 0.75% water-agarose media was poured into 10 cm diameter Petri dishes using aseptic technique, and 16–26 spores were pipetted across the diameter of each Petri dish using a fine bore glass Pasteur pipette. After plating, we covered each Petri dish with a lid and inverted it. Two runs of the bioassay were conducted. In 2009, a total of 22 Petri dishes and 413 spores were used. We created an AITC dilution series with sterilized deionized water from commercial AITC (Sigma–Aldrich, St. Louis, MO, USA; n = Petri dishes/concentration AITC): 0.005 mM (n = 4), 0.01 mM (n = 5), 0.1 mM (n = 4), and 1.0 mM (n = 4). The negative control was sterile deionized water (n = 5). In 2010, we used 525 spores in total and five replicate Petri dishes per AITC concentration: 0 (negative control), 0.001, 0.002, 0.005, 0.01, and 0.1 mM. The respective AITC or control treatment was applied by pipetting 6 ml into the lid while the Petri dish was inverted. Although ethyl acetate was used as the solvent when determining the concentration of AITC in soil samples (see above), water was used as the solvent here to avoid potential inhibition of spore germination by the ethyl acetate itself. Due to differences in density between water and ethyl acetate solvents, the concentration of AITC used in this experiment may be slightly different than the concentration we detected in the field, but we considered this effect to be negligible. We sealed the inverted Petri dishes twice with Parafilm and placed them in a 2% CO_2 incubator at 28° C in the dark for 1 week. After this period, all of the spores were examined under a dissecting scope, and those with hyphal development were scored as germinated. Data were analyzed using a one-way ANOVA and by performing subsequent pairwise comparisons in SAS.

Results

Sinigrin and AITC detection from forest soil

In 2007, we detected sinigrin in 40% of the garlic mustard-invaded soil samples (of n = 10 total) collected during the late (senescence) dates (Online Resource 2). Only 1 of the 16 invaded soil samples collected during the early (growth) dates had detectable levels of sinigrin (Online Resource 2). Field concentrations of sinigrin ranged from 0.011 to 0.031 μg g^{-1} dry soil (Fig. 1). Importantly, sinigrin was never detected in the control plots.

Similarly, in 2008, we detected AITC in two soil samples where garlic mustard was present and senescing. A sample from the 3 July sampling date

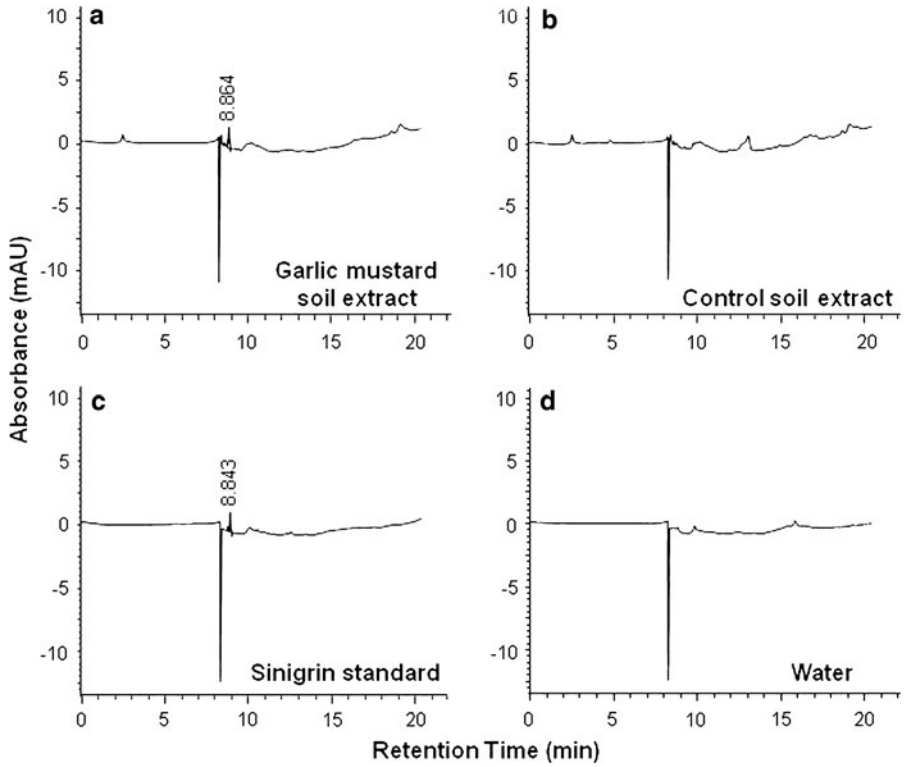

Fig. 1 HPLC chromatograms of soil extracts collected from paired garlic mustard (**a**) and control plots (**b**), 0.1 μg sinigrin standard·ml^{-1} eluate (**c**), and water (**d**). The garlic mustard plot extract shows the detection of sinigrin, as a peak is detected at 8.8 min, as is seen in the sinigrin standard. Sinigrin was not detected in the control plot or the water control. Retention times are listed above each peak

had an AITC concentration of 0.017 μg g^{-1} soil, while a sample from 18 July had a lower concentration at 0.0042 μg g^{-1} soil. Total and single ion chromatograms (m/z = 99.10) from our lowest AITC standard and the soil sample extracts were similar (Fig. 2). To confirm that the sample extract peaks represented AITC, we analyzed a 1:1 mixture of each soil sample extract and 1.25 mM AITC. The increased area of the indicative peak at the same retention time confirmed that we detected AITC in our soil extract. Further, the mass spectra of our soil sample extracts displayed three major peaks that are characteristic of AITC (Stein 2005). As in our 2007 sinigrin analysis, AITC was never detected in the control sites.

Effect of garlic mustard on the abundance of forest soil fungal hyphae

We found that the mean hyphal length per membrane was marginally significantly lower in garlic mustard versus control plots at the $P = 0.07$ level (Fig. 3; $F_{1,42} = 2.25$; mean ± standard error: garlic mustard = 5.50 mm ± 1.11; control = 8.69 mm ± 1.82). This represents a 37% reduction in fungal hyphal abundance in garlic mustard invaded forest soils. Using median score analysis, we also found that a significantly greater number of membranes in the control plots had a hyphal abundance greater than the median value when compared to membranes from garlic mustard plots ($Z = -1.7884$; $P = 0.04$).

Effect of natural concentrations of AITC on AMF spore germination in vitro

Despite the dilute concentrations used in our in vitro bioassay, all of the concentrations of AITC significantly inhibited spore germination relative to the control (2009 ANOVA, $F_{4,17} = 185.67$, $P < 0.0001$; pairwise comparisons of treatments to control, all $P < 0.0001$; 2010 ANOVA, $F_{5,24} = 5.06$, $P < 0.0026$; pairwise comparisons of treatments to control, all

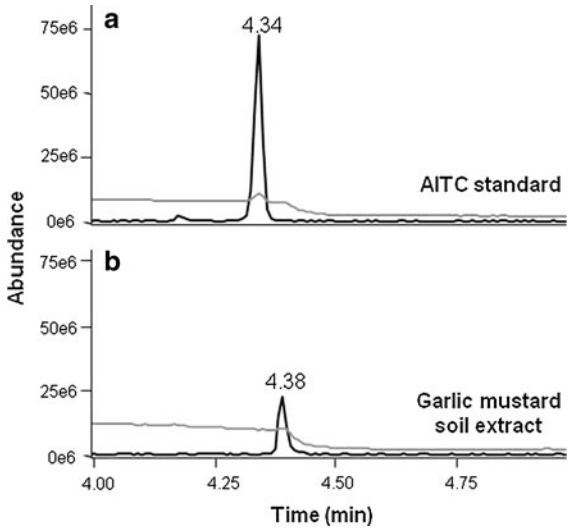

Fig. 2 Total ion chromatograms (*gray line*) and selected ion chromatograms (*black line*) for an m/z of 99.10 of a 0.039 mM AITC standard (**a**) and a soil extract collected near garlic mustard adults at Trillium Trail (**b**). The garlic mustard plot extract shows the same peak as the AITC standard at 4.3 min

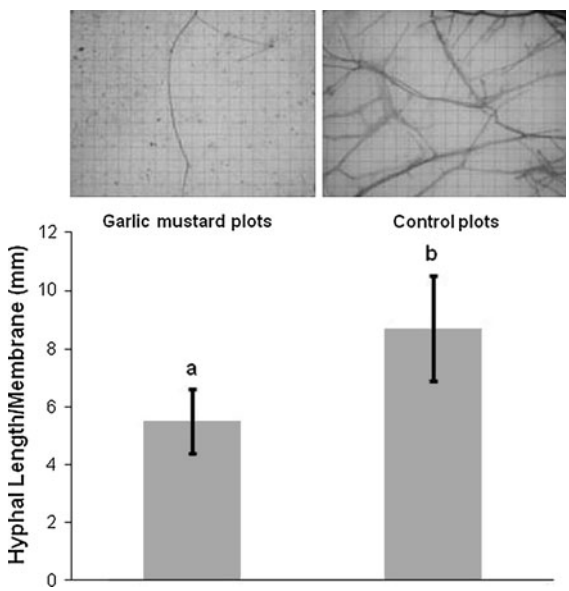

Fig. 3 Mean fungal hyphal length (± one std. err.) on membranes placed in the field where garlic mustard is currently growing or is currently absent (*control*). Bars with the same letter are not significantly different from each other at the $P = 0.07$ level. Images of typical membranes from each plot type are shown above the graph

$P < 0.01$). The percentage of *G. clarum* spores that germinated decreased dramatically as AITC concentration increased (Fig. 4). The concentration representing

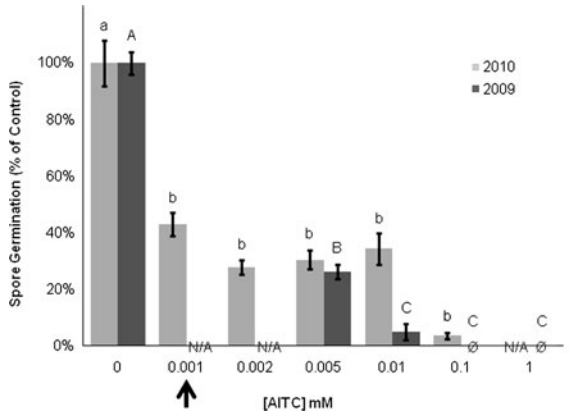

Fig. 4 *In vitro* bioassay testing mean *Glomus clarum* spore germination (± one std. err.) across a range of AITC concentrations. Germination values are expressed as a % of total germination observed in the control (water only) treatment. N/A indicates that the concentration was not tested in a run of the bioassay, while Ø indicates that 0% of the spores germinated. All treatments were significantly different from the control in both bioassays run (2009 pair-wise comparisons, $P < 0.0001$; 2010 pair-wise comparisons, $P < 0.01$). Within each year, bars with the same letter are not significantly different ($P > 0.05$). The arrow indicates the bioassay level closest to the detected concentration of AITC in field soil at Trillium Trail

the lowest detected level in the garlic mustard plots (0.001 mM AITC) caused germination failure of 57% of the *G. clarum* spores compared to the control.

Discussion

To our knowledge, this is the first report of sinigrin/AITC detection and quantification in forest soils where garlic mustard is present. We detected both sinigrin and AITC at biologically relevant concentrations that can significantly suppress AMF spore germination, which is critical for many AMF species in establishing the symbiosis with native plants (Klironomos and Hart 2002).

Our results also indicate that there is variation in the timing of detectable allelochemical release from garlic mustard into the soil at our study site. Although low levels of allelochemicals are likely released throughout the growing season as seedlings or rosettes die, which could affect competition with native plant species, we most frequently detected allelochemicals as the garlic mustard adults were senescing between July and August. Timing of

sample collection or the age of a population (Lankau et al. 2009) could explain the weak or lack of evidence for allelopathic effects in other studies (McCarthy and Hanson 1998; Burke 2008). Interestingly, adult garlic mustard senescence coincides with peak seasonal activity of the AMF symbiosis associated with many native perennial understory herbs in eastern North American forests (Brundrett and Kendrick 1990). Further, because senescent adults and rosettes co-occur in the field, rosettes may benefit from a pulse of anti-fungal chemicals in the soil at this time. Thus, while multiple mechanisms are likely involved in the widespread success of this invader, the timing of garlic mustard's life history transition could enhance the effectiveness of its allelochemicals and facilitate invasion of forest understory communities.

Both sinigrin and AITC have been suggested to have transient residence times in the soil (Choesin and Boerner 1991; Borek et al. 1995; Gimsing and Kirkegaard 2006), and our results support this view. Plant-derived sinigrin degrades rapidly in aqueous soil solutions (Tsao et al. 2000) and, upon incorporation of mustard biofumigants into soil, sinigrin can be undetectable after just 8 days (Gimsing and Kirkegaard 2006). The quick enzymatic action of both plant and microbe-produced myrosinase may partially explain the variation in our ability to detect sinigrin across multiple samples, dates, and sites; AITC's volatility likely affected our ability to detect AITC in all garlic mustard plots as well. While we attempted various soil purification methods to improve our detection ability (see Online Resource 3), the outcomes also strongly suggest that heavy metals and other contaminants in soils can severely limit the ability of cyclocondensation reactions coupled with UV spectrometry (Zhang et al. 1992) or HPLC (Zhang et al. 1996) to detect low levels of AITC.

Variation in the density of invasive plants, the distance that soil samples are taken from the invasive plant, and/or differences in physical, chemical, or biological properties of the soil among the sites can also influence the distribution, persistence, and detection of allelochemicals (Inderjit and Dakshini 1999; Inderjit et al. 2008; Lankau 2010). For example, if levels of allelochemicals are higher in the garlic mustard rhizosphere, proximity of soil samples to garlic mustard roots and depth of soil cores could influence detected concentrations. Further, tissue level concentrations of garlic mustard glucosinolates have been shown to vary significantly across sites within the same forest patch (Cipollini 2002) and to decline with the age of the invading population (Lankau et al. 2009). The sites studied by Cipollini (2002) varied in numerous physical and chemical characteristics, including soil moisture and nutrient level, which were suggested to impact garlic mustard biochemistry. Together these studies clearly show that context dependent factors will affect the concentrations of sinigrin and AITC in the soil and that the ability to detect them can vary substantially by location, sampling time in the growing season as well as time since site invasion. Lastly, differences in the timing of garlic mustard senescence among plots within our study area undoubtedly contributed to the variance in the timing of detection of these allelochemicals in the field.

Despite the fact that the concentrations of AITC detected in field soil at Trillium Trail were low, they had significant biological effects on spore germination in our bioassay (Fig. 4). We found 57% inhibition of spore germination compared to controls with concentrations of AITC mimicking those detected in Trillium Trail field soil samples ($\sim$0.001 mM) and complete inhibition under AITC concentrations greater than 0.01 mM. Other bioassay studies using whole plant extracts of garlic mustard (Roberts and Anderson 2001; Stinson et al. 2006) found complete inhibition of AMF spore germination, while diluted fractions of garlic mustard glucosinolates resulted in $\sim$25% AMF spore inhibition (Callaway et al. 2008). In the studies above, the actual concentrations of garlic mustard's allelochemicals were unknown. Our results suggest that the whole plant extracts may contain high AITC concentrations, possibly >0.01 mM. Further, our assay only tested for the effects of AITC, while whole plant extracts contain all of garlic mustard's secondary compounds. It is likely that these other compounds, in concert with AITC, play an important role in the complete inhibition of AMF spore germination seen under whole plant extracts (Callaway et al. 2008). Despite the fact that these studies used different AMF species to test spore germination inhibition (*Gigaspora rosea*, Roberts and Anderson 2001; unidentified species of *Glomus* and *Acaulospora,* Stinson et al. 2006; and *Glomus clarum*, here) and varied chemical bioassays, all

show that the exposure to allelochemicals produced by garlic mustard drastically reduces spore germination across AMF species. Our study provides new insights by demonstrating that even low concentrations of AITC can have devastating effects on AMF spore germination.

Our field soil membrane experiment further supports the bioassay results by indicating that the presence of garlic mustard can exert a negative effect on the abundance of the naturally occurring soil fungal community. Membranes in invaded soil had significantly decreased hyphal abundance relative to those in non-invaded soils. The decrease in fungal abundance could be due to current suppression of fungi by the standing crop of garlic mustard in the plot, a depletion of fungi and spores in soil infested with garlic mustard over time (Barto et al. 2010b), or both, since we do not know how long garlic mustard has been growing in each of the plots used in this study. If higher AITC concentrations are maintained within this invader's rhizosphere, this could provide a potential competitive benefit to garlic mustard by decreasing the local density of AMF hyphae. Disruption of the AMF over multiple growing seasons could have severe impacts on the growth of native herbs, as some native perennials are incapable of maintaining a positive phosphorus budget without their associated AMF (Merryweather and Fitter 1995). However, recent work by Anderson et al. (2010) demonstrates that the suppression of AMF can be reversed when garlic mustard is removed for multiple consecutive growing seasons. Sites invaded by garlic mustard may require long-term and intense management to prevent an overall reduction in the abundance of the forest fungal community.

Together, our data provide new information about the chemical ecology of this invasive species and can help to develop better management and forest restoration strategies. These experiments provide an important step forward in understanding the natural concentrations of one of this invader's important novel weapons and assessing the bioactivity of those concentrations under field conditions. Our data suggest that garlic mustard's novel weapons could play a crucial role in its invasion. In the field, these chemicals decrease fungal abundance, which has the potential to destabilize native fungal communities and inhibit the formation of critical fungal mutualisms that support the majority of native forest species.

Acknowledgments We thank the National Science Foundation for award DEB 0958676 to SK, University of Pittsburgh Startup Grant to MBT, Phipps Conservatory and Botanical Garden Botany-in-Action Fellowship and a Mellon Fellowship to AH, the Howard Hughes Medical Institute for support of AC and JA, the National Aeronautics and Space Administration Pennsylvania Space Grant Consortium Research Scholarship for funding to AC, W. Saunders for incubator space, J. Williams at the University of Pittsburgh's GC–MS lab for assistance with sample analysis, J. Hale at the University of Pittsburgh Department of Chemistry for advice on analytical chemistry methods, J.L. Dunn for lab and field assistance, J. Morton of INVAM and W.D. Holtzclaw of Johns Hopkins University for advice, T. H. Nguyen and T. Korpar for field assistance in the soil membrane experiment, and the Borough of Fox Chapel for permission to conduct experimental work at Trillium Trail. We thank two anonymous reviewers for their insightful comments and suggestions.

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