Biol Invasions
DOI 10.1007/s10530-015-0913-4

ORIGINAL PAPER

A novel impact of a novel weapon: allelochemicals in *Alliaria petiolata* disrupt the legume-rhizobia mutualism

Cristina Portales-Reyes · Tina Van Doornik · Elizabeth H. Schultheis · Tomomi Suwa

Received: 2 August 2014 / Accepted: 26 May 2015

Abstract Some introduced species become invasive by releasing novel allelochemicals into the soil, directly harming nearby plants and soil microbes. *Alliaria petiolata* (garlic mustard) is an invasive plant, well known to excrete a suite of phytotoxic and antimicrobial allelochemicals, including allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC). While the effects of these chemicals on plant-mycorrhizae mutualisms are well documented, the effects on other plant-soil microbe interactions, such as the legume-rhizobia mutualism, have not yet been tested. Here, we performed laboratory and greenhouse experiments with both synthetic chemicals and leaf extracts to investigate the effects of allelochemicals in *A. petiolata* on a native leguminous plant, *Amphicarpaea bracteata,* and its rhizobia mutualists. We found that BITC reduced rhizobia growth rate in the lab, but had no effect on nodulation in the greenhouse when rhizobia were grown in the presence of plants. AITC did not directly harm either plants or rhizobia, though plants and rhizobia grown in the presence of AITC showed reduced nodulation, indicating that it disrupted the formation of the mutualism itself. We found no effects of *A. petiolata* allelochemical leaf extracts on plant performance or nodulation. Our data suggest that AITC causes mutualism disruption in this system by preventing the formation of nodules, which reduces plant growth and could threaten the long-term performance of rhizobia. Our study shows that the allelochemicals in *A. petiolata* disrupt the legume-rhizobia resource mutualism, adding another impact of these novel weapons in addition to their well-documented role in disrupting plant-mycorrhizae symbioses.

Keywords Garlic mustard · *Amphicarpaea bracteata* · Allelopathy · Plant invasion · Mutualism disruption

Electronic supplementary material The online version of this article (doi:10.1007/s10530-015-0913-4) contains supplementary material, which is available to authorized users.

C. Portales-Reyes
Department of Ecology, Evolution & Behavior, University of Minnesota Twin Cities, Saint Paul, MN, USA

T. Van Doornik
Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, University of Miami, Coral Gables, FL, USA

E. H. Schultheis · T. Suwa (✉)
W.K. Kellogg Biological Station, Department of Plant Biology, Michigan State University, East Lansing, MI, USA
e-mail: suwatomo@msu.edu

Introduction

Invasion creates novel biotic interactions where both the invading species and the native competitor, herbivore, or mutualist are evolutionarily naïve to

Published online: 05 June 2015

one another (Verhoeven et al. 2009). Because of this lack of a shared evolutionary history, antagonistic traits of the invader, such as allelopathy, defensive chemistry, or herbivore and predatory behaviors, could prove very effective against native community members that lack previous experience with such tactics (the Novel Weapons Hypothesis, sensu Callaway and Ridenour 2004).

Allelopathy, defined as chemically mediated plant interactions (Rice 1974; Meiners and Kong 2012), contributes to the success of numerous plant invasions (Callaway and Aschehoug 2000; Hale et al. 2011; Murrell et al. 2011; Yuan et al. 2012). An invader's allelopathic chemicals can harm plant competitors both directly through phytotoxic effects, or indirectly by reducing the competitor's mutualists in the soil microbial community or by disrupting the formation of the mutualism (Mutualism Disruption Hypothesis; Hale et al. 2011; Hale and Kalisz 2012). For example, invasive *Acacia dealbata* is most successful in habitats where its allelochemicals are most effective against the native soil community (Lorenzo et al. 2013). Compared to non-invasive exotic plants, invasive plants are more likely to have novel chemistry not previously recorded in native plants (Cappuccino and Arnason 2006). These chemicals may function as allelopathic and antimicrobial agents, suggesting that allelopathy may be a key mechanism explaining the success of some invaders.

Allelochemicals often have minimal effects on the invader's native community, but become "novel weapons" once introduced to a new range, leading to strong negative consequences for species with no previous exposure to such chemicals (Callaway and Ridenour 2004). Two lines of evidence provide support for the novel weapons hypothesis. First, allelochemicals produced by several invasive species have much stronger effects on naïve competitors in the invaded range than on experienced competitors from the invasive species' native range. For example, allelochemicals excreted into the soil by the invasive plant, *Centaurea diffusa,* strongly reduce growth of plants from its invasive range but have minimal effects on plants from its native range (Callaway and Aschehoug 2000). Second, allelopathy may be favored during the invasion process because it is particularly advantageous in invaded regions where invading plants are competing against naïve competitors. For example, invasive genotypes of *Solidago canadensis* produce increased amounts of allelochemicals compared to native genotypes; this increased allelochemical production causes a 46 % increase in competitive ability against native plants (Yuan et al. 2012).

The success of the invasive plant, *Alliaria petiolata* (garlic mustard), is partially attributed to novel weapons and its production of allelopathic secondary chemical compounds (Rodgers et al. 2008a). This species produces a suite of allelochemicals, many of which are novel in invaded habitats in the United States (Cipollini and Gruner 2007; Callaway et al. 2008). These novel weapons have allelopathic effects on plant competitors in their invasive range, both directly through phytotoxic effects (Cipollini et al. 2012a) and indirectly by reducing the abundance of mycorrhizal soil mutualists (reviewed in Cipollini et al. 2012b). Because *A. petiolata* is a non-mycorrhizal member of the Brassicaceae, it is unaffected by the loss of mycorrhizal mutualists from the soil, while declines in mycorrhizae reduce the performance of competing native plants (Stinson et al. 2006, 2007; Callaway et al. 2008; Lankau 2011). For example, the release of allelochemicals into the soil by *A. petiolata* has been shown to reduce hyphal abundance and spore germination of mutualistic mycorrhizal fungi (Roberts and Anderson 2001; Cantor et al. 2011), reducing performance of native plants (Stinson et al. 2006, 2007; Callaway et al. 2008; Lankau 2011). The evolutionary naivety of the fungi is key; allelochemicals found in *A. petiolata* are not as harmful to mycorrhizae in its native range as they are in its introduced range (Callaway et al. 2008).

Negative effects of *A. petiolata* on local microbial communities are well documented (Klironomos 2002; Burke 2008; Wolfe et al. 2008; Cantor et al. 2011; Lankau 2011), but to the best of our knowledge, its allelopathic effects on rhizobia, important mutualistic bacteria, have not been studied (Cipollini et al. 2012b). Nitrogen-fixing bacteria, including rhizobia, supply the majority of terrestrial biologically-fixed nitrogen (Cleveland et al. 1999), and disruptions to this mutualism could have both biogeochemical and community wide consequences (Weidenhamer and Callaway 2010). In this mutualism, legumes provide rhizobia with photosynthetic carbon and protection in nodules. In exchange, rhizobia provide the plant with fixed atmospheric nitrogen, ammonium (NH_4^+). Rhizobia growing in nodules experience increased fitness compared to rhizobia in the soil (Bergersen 1982;

West et al. 2002; Denison and Kiers 2004; Heath and Tiffin 2007). Sanctions from plants can prevent cheating by rhizobium strains that do not produce nitrogen for the plant (Kiers et al. 2003; Denison and Kiers 2004; Oono et al. 2011), increasing the stability and maintenance of the mutualism (West et al. 2002).

Here, we test whether, and by what mechanism, the allelochemicals of *A. petiolata* inhibit the performance of rhizobia and their legume hosts. Using the combination of a laboratory experiment where we exposed rhizobia to two major allelochemicals of *A. petiolata* in culture, and a greenhouse experiment where rhizobia were grown with their plant host in the presence and absence of the allelochemicals of *A. petiolata*, we tested the following questions (Figure A1): (1) Do the allelochemicals of *A. petiolata* directly affect rhizobia? (2) Do the allelochemicals of *A. petiolata* directly affect the leguminous plant *Amphicarpaea bracteata*? (3) Do the allelochemicals of *A. petiolata* indirectly affect either mutualist partner (i.e., the legume host or the rhizobia) by disrupting the legume-rhizobium mutualism?

Materials and methods

Study species

The invasive biennial plant, *Alliaria petiolata*, was introduced to the United States from Europe in the mid-1800s as a medicinal herb and garlic substitute (Grieve 1959; Nuzzo 1993). While it exists in small populations throughout its native range (Blossey et al. 2001), in its invasive range *A. petiolata* spreads quickly and can form persistent, dense stands (Nuzzo 1999; Rodgers et al. 2008a) and reduces native biodiversity (Anderson et al. 1996; McCarthy 1997; Lankau et al. 2009). *A. petiolata* produces allelopathic chemicals that harm native plants and soil biota (Cipollini and Gruner 2007; Callaway et al. 2008). Two of its most studied allelochemicals are allyl isothiocyanate (AITC) and benzyl isothiocyanate (BITC) (Vaughn and Berhow 1999). AITC is the breakdown product of sinigrin, a type of glucosinolate and a parent compound that is produced in *A. petiolata* leaf tissue and released into the soil where it is hydrolyzed (Larsen 1981; Larsen et al. 1983; Brown et al. 1991; Fahey et al. 2001; Gimsing and Kirkegaard 2009). BITC results from the degradation of glucotropaeolin and is mainly produced in *A. petiolata* roots (Vaughn and Berhow 1999; Lankau 2011).

We tested the allelopathic effects of AITC and BITC on *Bradyrhizobium sp.* (hereafter referred to as rhizobia) and performance of the native annual legume, *Amphicarpaea bracteata* (hog peanut) (Schnee and Waller 1986; Parker 1996). This plant species grows commonly in forest understories in southwest Michigan, and its range and habitat use overlap with that of invasive *A. petiolata* (Reznicek et al. 2011). The two species can co-occur in the field, and thus *A. bracteata* is likely exposed to *A. petiolata* allelochemicals. *A. bracteata* forms symbiotic relationships with nitrogen-fixing bacteria, rhizobia. Initial field observations indicate that nodulation on *A. bracteata* is reduced in invaded sites (T. Suwa, *personal observation*).

Direct allelochemical effects on rhizobia

To test for direct effects of the allelochemicals of *A. petiolata* on rhizobium population growth (Question 1), we isolated nine rhizobium strains, grew them in culture, and exposed them to field concentrations of AITC and BITC. To isolate rhizobium strains, we collected nine *A. bracteata* plants, each from a different population (Electronic Supplementary Material, Table A1), in late May 2013 and isolated rhizobia from belowground nodules the same day. We removed nodules from the root, surface sterilized them with commercial bleach (5.25 % NaOCl) for 1 min, and then triple rinsed them with sterile water. To isolate single colonies of rhizobia, we plated nodules on modified arabinose gluconate media (MAG media; van Berkum 1990) following standardized techniques (Somasegaran and Hoben 1994). The following experiments utilize these nine rhizobium strains, each isolated from a single nodule from a single plant, representing an individual strain from each of nine populations.

We prepared the allelochemical concentrations by diluting commercial AITC and BITC (Sigma–Aldrich, St. Louis, MO, USA) with MAG media. We included four concentrations of each allelochemical (1×10^{-6} mM, 1×10^{-5} mM, 1×10^{-4} mM, 1×10^{-3} mM) in the experiment. We also included a combination of AITC + BITC treatment (AITC + BITC = 6.5×10^{-5} mM for AITC and 7.5×10^{-6} mM for BITC), and a control containing no allelochemicals. We chose

concentrations for the combined AITC and BITC treatment based on the lower estimates of previously published natural field concentrations (Cantor et al. 2011) and agricultural soil concentrations (Wolfe et al. 2008) of AITC and BITC. Even though higher concentrations of these allelochemicals are sometimes present in soils, we opted for the use of lower concentrations for this experiment in order to capture the minimum concentrations at which AITC and BITC in soils would affect rhizobia. Each treatment combination was replicated three times. In the analysis presented here, we only included the AITC and BITC concentrations closest to realistic field concentration and combined AITC + BITC treatment (AITC = 1×10^{-4} mM; BITC = 1×10^{-5} mM; AITC + BITC = 6.5×10^{-5} mM for AITC and 7.5×10^{-6} mM for BITC). The results based on the full concentration series are very similar to the results presented here and are available in Electronic Supplementary Material (Figure A2, Table A2). The nine rhizobium strains, plus an uninoculated control, were grown in glass test tubes containing 3 mL of MAG media with the appropriate allelochemical in a shaking incubator at 28 °C, 180 ppm. To estimate maximum growth rate, we measured Optical Density (600 nm) using a spectrophotometer every 6 h for an eight-day period and calculated maximum growth rate (μ_{max}) for each rhizobium strain in each allelochemical treatment as the slope of the growth curve during the exponential growth phase using a modified Gompertz equation (Lennon et al. 2007).

Effects of allelochemicals on native legumes and nodulation

To test direct effects of the allelochemicals of *A. petiolata* on native legume growth (Question 2), and whether these allelochemicals disrupt the legume-rhizobium mutualism (Question 3), we added allelochemicals to greenhouse grown plants in the presence and absence of rhizobia in a 6 × 2 factorial design experiment at the Kellogg Biological Station, Hickory Corners, MI. *A. bracteata* seeds used in the experiment are the greenhouse-reared progeny of seeds that had been collected from four naturally occurring populations in southwest Michigan in 2012. Seeds were surface sterilized with commercial bleach (5.25 % NaOCI), physically scarified, and germinated in a petri dish in the dark. After one week, we transplanted seedlings into 12.4 cm pots containing ~215 g of a soil mixture: potting soil (Sunshine Mix #5; SunGro Horticulture Canada Ltd., Alberta, Canada), peat moss (Pro-Moss Hort, Premier Tech Ltd, Pennsylvania USA), sand (Tubesand Quikrete International, Inc, Georgia, USA) and perlite (Horticultural Perlite, Midwest Perlite, Wisconsin, USA) in a 3:3:3:1 ratio. Prior to planting, the soil mixture was homogenized and autoclaved twice to minimize rhizobium contamination. We bottom watered as needed (every 3–5 days). We grew plants on four greenhouse benches under a 40 % shade cloth (Gempler's, Madison, WI) to simulate forest canopy cover.

Ten days after transplanting seedlings, we applied two types of allelochemical treatments: direct addition of commercially available allelochemicals (AITC, BITC, AITC + BITC), and fresh leaf tissue extracts (*A. petiolata* leaf extract [AP], *Trifolium pratense* leaf extract [TP]), and a control containing no allelochemical addition. We applied our commercial chemical allelochemical treatments as 30 mL aqueous solutions containing AITC and BITC in order to mimic the following concentrations previously detected in field (Cantor et al. 2011) and agricultural (Wolfe et al. 2008) soils: AITC (0.017 μg g^{-1} soil), BITC (1.19 μg g^{-1} soil), AITC + BITC (AITC = 0.017 μg g^{-1} soil, BITC = 1.19 μg g^{-1} soil), or DI water (negative control). Leaf tissue is a source of allelochemical release into the environment, and our extract treatments allowed us to apply a biologically realistic level of allelochemicals without the confounding factor of adding competition from *A. petiolata*. The TP treatment was used because *T. pratense* is a non-allelopathic legume plant, which we used as a control for the AP treatment and the effects of leaf tissue addition. Methods for leaf extractions were modified from Callaway et al. (2008). We collected fresh leaves from live plants growing in local field populations, performed and added extractions to experimental pots that same day to reduce chances of chemicals degrading once tissues were harvested. For *A. petiolata*, we used leaves from first year rosettes, and for *T. pratense* we used haphazardly sampled leaves from several individuals. We used 42.5 g of leaf tissue for the extraction to reach the 0.0033 gle (grams per leaf extract per gram of soil) that was previously used to achieve biologically relevant levels of *A. petiolata* allelochemicals in the soil (Callaway et al. 2008). For our calculations, we assumed a sinigrin concentration

of 49.6 μmol/gram of leaf of *A. petiolata* (Blažević and Mastelić 2008) and a 35 % release efficiency of AITC, as this falls within the range of isothyocyanate release estimated by Gimsing and Kirkegaard (2006) for other Brassica species. We applied 30 ml of allelochemical treatments (both commercially available allelochemicals and leaf tissue extracts) each week to mimic a steady release of chemicals by *A. petiolata,* and because the chemicals break down quickly in the soil (~47 h half-life; Borek et al. 1995, reviewed in Gimsing and Kirkegaard 2009).

On the day following the first allelochemical treatment application, we inoculated half of the plants in each treatment with a 4 ml mixture of the nine rhizobium strains used in the laboratory experiment described above diluted to OD600 of 0.5 (approximately 5×10^{10} cells). The remaining plants were inoculated with an equal amount of sterilized media as a procedural control. After 8 weeks, we assayed chlorophyll content using a SPAD-502 plus chlorophyll meter (Spectrum Technologies, Aurora, IL, USA) in the three newest fully grown leaves, which is highly correlated with leaf nitrogen content and photosynthetic performance (Yoder and Pettigrew-Crosby 1995; Bullock and Anderson 1998; Swiader and Moore 2002; Gáborčík 2003), and harvested above- and belowground biomass of each plant. After harvest, we counted the number of seeds and measured average axillary shoot length. *A. bracteata* produces both subterranean and aerial seeds, and aerial seed production is highly correlated with plant size, while subterranean seed production is highly correlated with the axillary shoot length (Schnee and Waller 1986). We then counted and collected all rhizobia nodules on each plant. The harvested nodules and plant biomass was dried at 65 °C for 3 days and weighed.

Statistical analysis

Effects of allelochemicals on rhizobia (Question 1)

We tested the effect of allelochemicals on the maximum growth rate of rhizobia (μ_{max}) using ANOVA. Allelochemical treatments (AITC, BITC and AITC + BITC and Control) were included as a fixed factor, and strains were treated as a random factor. To determine which treatments had significantly different effects on rhizobia growth, compared to the control, we conducted post hoc contrasts (Control vs. AITC, Control vs. BITC and Control vs. AITC + BITC). Uninoculated controls were not included in the analyses because they showed no evidence of microbial growth, suggesting that there was no contamination.

Effects of allelochemicals on native legumes and nodulation (Questions 2 & 3)

Nodulation To test the effects of allelochemicals on nodulation (number of nodules and nodule mass), we used mixed model ANOVAs (lme4 Package in R), including allelochemical treatments as a fixed factor and greenhouse bench as a random blocking factor. Only pots that received the rhizobium inoculation were included in this analysis. Nodule mass was analyzed using lmer, while nodule number was analyzed using mixed effects logistic regression (glmer, lme4 Package in R). Because the nodule number was not normally distributed, we used a Poisson distribution. We then used log-likelihood ratio tests to compare our models with and without fixed effects.

Plant performance To test the effects of allelochemicals and rhizobium inoculation treatments on plant survival, we used mixed effects logistic regression (glmer, lme4 Package in R), and included allelochemical, rhizobia treatment, and the allelochemical × rhizobia interaction as fixed factors and greenhouse bench as a random factor. Effects of allelochemicals and rhizobia on biomass and chlorophyll content were tested using mixed model ANOVA (trait ~ treatment*rhizobia, random = ~1 bench), including allelochemical and rhizobia as fixed effects and bench as a random effect. We log transformed above- and belowground biomass data to meet normality assumptions. To estimate allelochemical effects on plant fitness, we tested effects of allelochemical and rhizobia treatments on seed production and axillary shoot production using mixed effects logistic regression because few plants produced seed or axillary shoots. Our seed and axillary shoot productions may be underestimating total plant fitness since we harvested after 8 weeks; we harvested about one month early in order to collect nodules, which tend to senesce before plants. Although contamination was minimal (3.1 % of pots), six "rhizobia absent" control pots were excluded from the above analyses because

they were contaminated and produced nodules. When we found significant effects of allelochemical treatments and rhizobia inoculation, we performed Tukey's honestly significant difference test (HSD) to evaluate differences among treatments. All analysis was performed in R (Version 3.0.2, 2015).

Results

Effects of allelochemicals on rhizobia (Question 1)

Allelochemical treatments significantly influenced rhizobia maximum growth rate (Table 1). BITC decreased, while AITC increased, rhizobia maximum growth rate under realistic field concentrations (Fig. 1; Control vs. AITC; $t = 3.257$, $P = 0.001$, Control vs. BITC; $t = -3.443$, $P < 0.001$). The combined AITC and BITC treatment did not significantly alter rhizobia maximum growth rate (Fig. 1; Control vs. AITC + BITC; $t = 0.2245$, $P = 0.807$), likely because the positive effects of AITC on rhizobium population growth cancelled out the negative effects of BITC. The growth rate of rhizobium strains differed, but strain response to chemical treatment did not (Table 1: non-significant strain × allelochemical interaction).

Effects of allelochemicals on native legumes and nodulation (Questions 2 & 3)

Direct and indirect effects of allelochemicals on plant performance

Averaged across all chemical treatments, rhizobia significantly increased plant aboveground biomass by 60 % and tended to increase belowground biomass by 23 % (Table 2; Fig. 3c, d), and we found a significant correlation between plant size and nodule number and nodule mass (nodule number: $r = 0.61$, $F_{1,78} = 47.89$, $P < 0.001$; nodule mass $r = 0.84$, $F_{1,78} = 183.90$, $P < 0.001$). Rhizobia increased leaf chlorophyll content, except when AITC + BITC was added (significant rhizobium × allelochemical treatment, $F_{5,141} = 3.79$, $P = 0.003$). The AITC + BITC treatment tended to reduce chlorophyll content for plants inoculated with rhizobia, although the pattern was not statistically significant (Fig. 2).

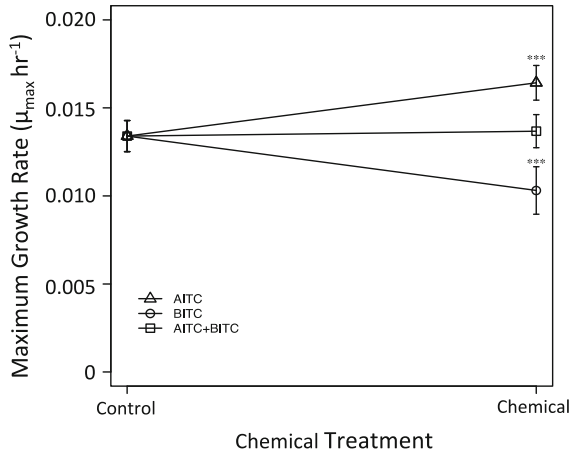

Fig. 1 Average maximum growth rate per hour (μmax h^{-1}) of nine rhizobium strains grown under approximate field concentrations of AITC (1×10^{-4} mM), BITC (1×10^{-5} mM) and, AITC and BITC combination (AITC = 6.5×10^{-5} mM, BITC = 7.5×10^{-6} mM). Maximum growth rates were estimated by measuring Optical Density (600 nm) every 6 h for an eight-day period and calculated maximum growth rate (μ_{max}) for each rhizobium strain in each allelochemical. *Error bars* are standard error of the means (SEM). *Asterisks* indicate statistical difference between control and each of the chemical treatment ($P < 0.05$, A priori contrast)

Averaged across both rhizobium treatments, the AITC + BITC treatment significantly reduced plant survival by 30 %, aboveground biomass by 44 %, and belowground biomass by 47 % (Table 2; Fig. 3). However, neither AITC nor BITC alone significantly reduced these plant performance traits (Fig. 3; Table 2). No rhizobia × allelochemical treatment

Table 1 Analysis of variance (ANOVA) testing the effects of chemical treatments (Chemical; Control, AITC, BITC and AITC + BITC) on the maximum growth rate (μ_{max}) of nine rhizobium strains (Strain) in a laboratory

Treatment	*df*	MS	F	χ^2	*P*
Chemical (C)	3	1.494×10^{-4}	10.668		**<0.001**
Strain (S)				0.168	0.70
S × C				2.27×10^{-13}	1

Statistically significant ($P < 0.05$) effects are shown in bold. Chemicals were treated as a fixed factor and Strains and interactions between strains and chemicals were treated as random factors

Table 2 Results from a mixed model ANOVA testing the effects of rhizobia and chemical and leaf tissue treatments (Control, AITC, BITC, AITC + BITC, *Trifolium pratense* and *Alliaria petiolata)* on *A. bracteata* survival, above- and belowground biomass, and chlorophyll content

	Survival			Aboveground biomass			Belowground biomass			Chlorophyll content		
	df	Resid. dev	*P* (Chi)	*df*	F	*P*	*df*	F	*P*	*df*	F	*P*
Intercept	185	161.01		1142	308.122	**<0.001**	1142	169.143	**<0.001**	1141	1895.472	**<0.001**
Rhizobia	1184	160.86	0.696	1.142	13.859	**0.0403**	1142	2.283	0.133	1141	25.551	**<0.001**
Treatment	5179	136.65	**<0.001**	5142	2.397	**<0.001**	5142	3.243	**0.008**	5141	0.376	0.865
R × T	5174	133.54	0.684	5142	0.569	0.724	5142	0.782	0.564	5141	3.786	**0.003**

Statistically significant ($P < 0.05$) effects are shown in bold

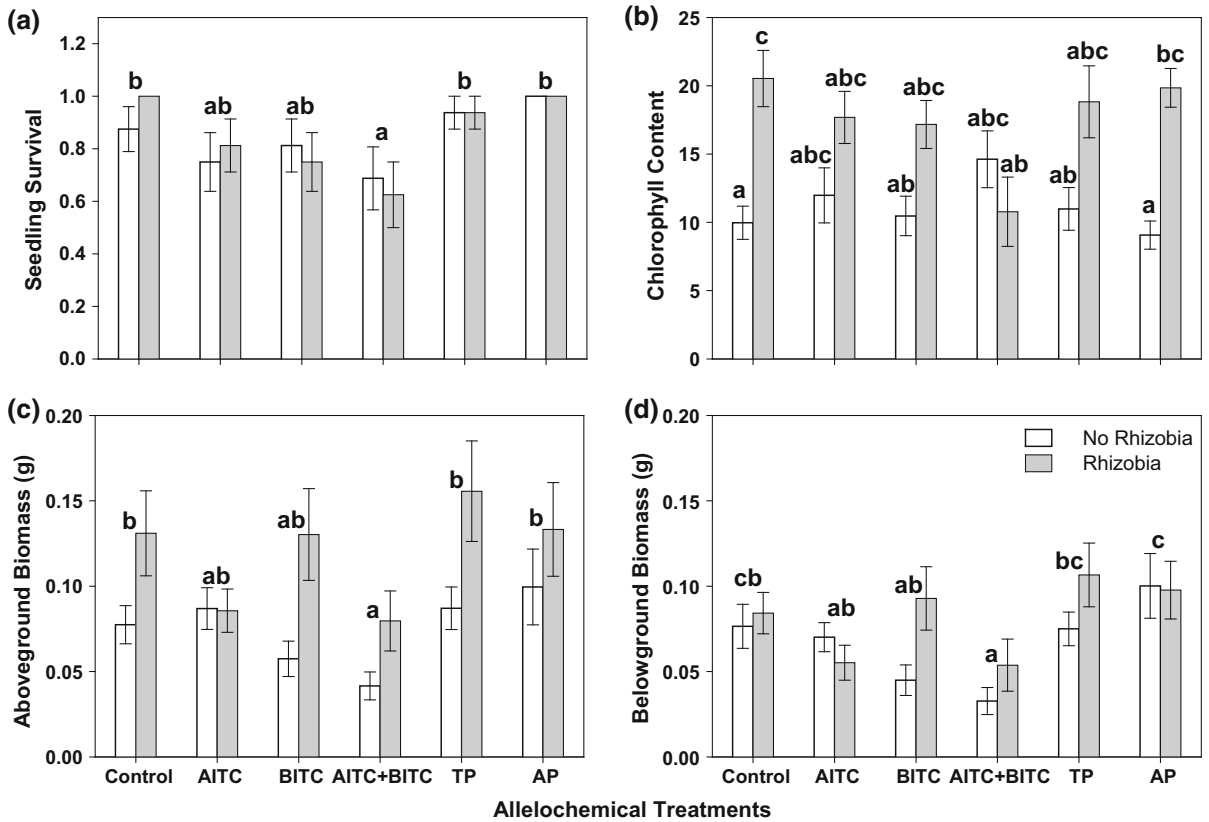

Fig. 2 *Amphicarpaea bracteata* survival (**a**), chlorophyll content (**b**), aboveground biomass (**c**), and belowground biomass (**d**) in the presence (*grey bars*) and absence (*white bars*) of rhizobia. Plants were grown in the presence of six alelochemical treatments: three chemical treatments *(*AITC, BITC, AITC + -BITC) and two leaf extract treatments (TP, AP). *Error bars* are Standard Error of the means (SEM). *Different letters* above bars in **a**, **c** and **d** indicate that allelochemical treatment differed statistically ($P < 0.05$, Tukey post hoc test). There was no significant allelochemical treatment × inoculation treatment interactions (Table 1). *Different letters* above *bars* in **b** indicate that chlorophyll content in the treatment combinations differed statistically ($P < 0.05$, Tukey post hoc test)

interactions were detected for survival, above- and belowground response variables, suggesting that AITC + BITC reduced plant performance regardless of rhizobium presence. The *T. pratense* and *A. petiolata* tissue treatments did not affect any plant performance traits (Fig. 2).

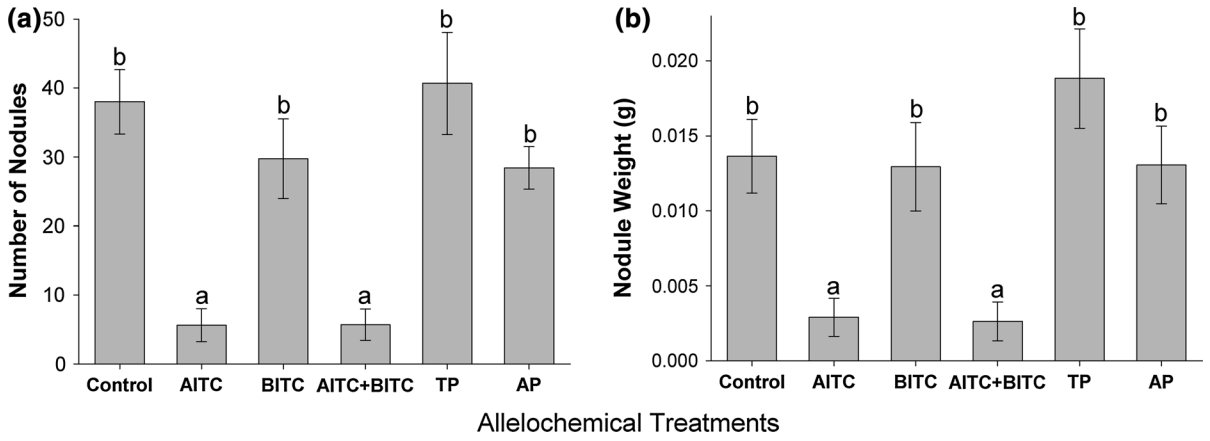

Fig. 3 Number of nodules (**a**) and total nodule weight (**b**) of rhizobia-inoculated *A. bracteata* grown under six different allelochemical treatments. AITC (0.017 μg g^{-1} soil), BITC (1.19 μg g^{-1} soil), and AITC + BITC (AITC = 0.017 μg g^{-1} soil, BITC = 1.19 μg g^{-1} soil) were all applied at approximate field concentrations. TP and AP indicate leaf exract treatment from *Trifolium pratense* and *Alliaria petiolata* respectively. *Error bars* are Standard Error of the means (SEM). *Bars* with the *different letters* indicate statistical difference ($P < 0.05$, Tukey test). AITC and AITC + BITC significantly reduced nodulation compared to the other allelochemical treatments

Allelochemical treatments had significant effects on seed production (allelochemical: $\chi^2_5 = 35.71$, $P < 0.001$). Post hoc contrasts revealed that only plants from AITC and AITC + BITC treatments had significantly lower seed production compared to the control (Tukey HSD test; $\alpha = 0.05$). Rhizobia treatment significantly increased seed production (rhizobia: $\chi^2_1 = 4.55$, $P = 0.033$). However, neither treatment affected axillary shoot production (allelochemical: $\chi^2_5 = 7.36, P = 0.20$; rhizobia: $\chi^2_1 = 90.25$, $P = 0.62$). No significant interactions between rhizobia and allelochemical treatments were detected (seed production: $\chi^2_5 = 6.19$, $P = 0.29$; axillary shoot production: $\chi^2_5 = 4.22$, $P = 0.52$). None of the leaf extract treatments differed significantly from the control (Tukey HSD test; $\alpha = 0.05$).

Effects of allelochemical treatments on nodulation

The rhizobium inoculation treatment was effective, with 100 % of the inoculated plants in the control (no chemical) treatment producing nodules. AITC and AITC + BITC treatments significantly reduced nodule number and total nodule mass (Fig. 3; nodule number: $\chi^2_5 = 658. < 0.001$ (log-likelihood ratio test for glmer), nodule mass: $F_{5,73} = 5.945$, $P < 0.001$, Fig. 3). BITC alone did not affect nodulation, in contrast to results from the laboratory experiment, which showed strong negative effects of BITC on rhizobium population growth (Fig. 1). Nodulation was not affected by the leaf extract treatments (Fig. 3).

Discussion

Our study demonstrates a novel mechanism by which the invasive plant, *A. petiolata,* affects a native plant species and its microbial mutualist. In our greenhouse experiment, we found that AITC disrupts the legume-rhizobium mutualism by significantly reducing nodule numbers by 85 % (Fig. 3). The combination AITC + BITC treatment significantly reduced plant aboveground biomass by 39 and 46 % in the presence and absence of rhizobia, respectively. When rhizobium strains were studied in isolation the allelochemical BITC directly reduced rhizobia population growth rates.

Our findings align with previous studies testing whether the legume-rhizobia mutualism is susceptible to disruption by allelopathy. In an agricultural system, allelopathic effects were observed when soybean seedlings were exposed to water extracts of common weed species' tissues—they experienced reduced rhizobia nodulation and reduced performance (Mallik and Tesfai 1988). Though our study represents perhaps the first time the allelochemicals of *A. petiolata* have been studied in relation to their effect

on the legume-rhizobia mutualism, other introduced species have been shown to have allelopathic effects on rhizobia as well. The introduced plant, *Raphanus sativus* (cultivated radish), reduced nodulation on the native plant, *Lupinus nanus,* in both field and greenhouse conditions (Pearse et al. 2014). Similarly, the invasive plant, *Amaranthus viridis* (green amaranth), reduced rhizobia growth in culture and nodulation when native *Acacia* legumes were grown in the presence of tissue extracts (Sanon et al. 2009). In some cases, nodulation may actually protect native plants from allelopathic effects; legumes inoculated with rhizobia were unaffected by (±)-catechin treatments, while uninoculated plants experienced allelopathic effects (Alford et al. 2009).

Two non-mutually exclusive hypotheses may explain the observed mutualism disruption (reduced nodulation) in our system: allelochemicals may disrupt a mutualism by (1) reducing one or both mutualists' performance thereby reducing resources available for trade, or (2) preventing the formation of the mutualism by potentially altering the communication between partners (Hale and Kalisz 2012). We find support for hypothesis (1) in both experiments—BITC reduced rhizobium population growth rates in the laboratory experiment, and the combined AITC + BITC treatment significantly reduced plant growth in the greenhouse experiment. Interestingly, the effects of BITC alone did not reduce nodulation or the performance of plants grown in the presence of rhizobia in the greenhouse experiment. Perhaps nodulation in plant roots is able to protect rhizobia from the harmful effects of BITC experienced by free-living rhizobia.

Although our lab experiment allowed us to examine the direct effects of allelochemicals on rhizobia fitness, in our greenhouse experiment we were unable to determine the fitness effects of allelochemicals on rhizobia in the absence of the plant mutualist. However, nodule size is a strong indicator of rhizobium fitness (Heath and Tiffin 2007). Rhizobium growing in nodules can reproduce up to 10^9 descendants inside the nodules (Bergersen 1982; West et al. 2002; Denison and Kiers 2004), much more than free-living rhizobia in the soil. Therefore, it is expected that the reduced nodulation on our experimental plants will lead to a reduction in fitness for rhizobia, and leguminous plants.

Additionally, the effects of the AITC + BITC treatment on plant growth could lead to long-term effects on rhizobia population performance. In our experiment, plant size was significantly correlated with nodule number and nodule mass. Because nodule number and size correlate with number of reproductive rhizobium cells within the nodule and performance (Heath and Tiffin 2007; Oono et al. 2011), this observed reduction in plant size, caused by allelopathy, could lead to reduced rhizobia performance over generations. Future studies can measure rhizobium abundance in field sites invaded and un-invaded by *A. petiolata,* and nodulation on co-occurring legume species.

Our data also support hypothesis (2)—the allelopathic effects of AITC appear to occur during the formation of the mutualism itself, perhaps altering the chemical communication between plant and rhizobia. Given that our AITC and AITC + BITC treatments, chosen to represent realistic field concentrations of these chemicals, reduced nodule numbers and nodule mass in the greenhouse (Fig. 3) but did not directly reduce rhizobia growth in the laboratory experiment (Fig. 1), we hypothesize that allelochemicals may influence nodulation either by inhibiting the ability of rhizobia to form nodules, inhibiting the plant's cooperation in the mutualism, or preventing communication necessary to induce nodulation. Complex chemical interactions between rhizobia and plants take place during the formation of this symbiosis (Bottomley and Myrold 2007), and AITC could potentially act to disrupt these signals by reacting with sulphur-containing protein groups (Brown and Morra 1997). Mutualism disruption might explain why rhizobia had minimal effects on plant performance in the AITC and AITC + BITC treatments (Fig. 2); for example, rhizobia significantly increased leaf chlorophyll content in the absence of allelochemicals, but when either AITC or BITC was present, this rhizobia benefit was minimized (Fig. 2b).

The negative effects of *A. petiolata* on rhizobia and other soil microbes could lead to reduced plant productivity, as nitrogen fixing bacteria and mycorrhizal fungi are responsible for up to 80 % of plant nitrogen acquisition in temperate and boreal forests (van der Heijden et al. 2008). Additionally, because *A. petiolata* reduces the presence of many spring ephemerals and other forest species (Anderson et al. 1996; McCarthy 1997; Meekins and McCarthy 1999; Lankau et al. 2009), it may alter the availability and phenology of nitrogen in ways detrimental to native

species. For example, spring ephemerals can serve as a temporary sink for nitrogen in deciduous forests before trees have leafed out in the spring (vernal dam hypothesis, Muller and Borman 1976), and the loss of these species from the community could lead to leaching of these nutrients from the ecosystem. Conversely, invasive species have been shown to modify habitats in ways that facilitate native species, for example by increasing the availability of a limiting resource, like nitrogen, in the system (Rodriguez 2006). *A. petiolata* can cause a significant increase in soil nitrogen and other nutrients, due to the stimulation of decomposition and nutrient cycling driven by its nutrient-rich tissues (Rodgers et al. 2008b).

We were surprised to find no effect of *A. petiolata* leaf extracts on plant performance and nodulation in our greenhouse experiment. Though these fresh leaf tissues would have contained AITC (Larsen 1981; Larsen et al. 1983; Brown et al. 1991; Fahey et al. 2001; Gimsing and Kirkegaard 2009), they do not align with our findings from our AITC chemical treatment, potentially due to differences in AITC concentrations between the treatments, or other leaf chemicals and nutrients found in the extractions. We attempted to achieve similar concentrations in our leaf tissue extractions as those used by Callaway et al. (2008) (0.0033 gle), who found that these concentrations reduced AM fungal spore viability. This concentration is thought to be biologically realistic, though it is difficult to estimate concentrations of allelochemical in the soil, as concentrations can change with time and soil moisture conditions (Blair et al. 2005, 2006). Further, our estimated concentrations were calculated based on previously recorded sinigrin levels of 49.6 µmol/gram of *A. petiolata* leaf tissue (Blažević and Mastelić 2008), as described in our methods. However, it has been shown that concentrations of these chemicals can evolve in response to the density of non-*A. petiolata* plants in the community (Lankau 2012) and will vary predictably based on the age of a particular *A. petiolata* population (Lankau et al. 2009). Our estimate was in line with previous measures of sinigin in nearby Michigan populations, which found an average of 43.78 µmol/g in *A. petiolata* populations ranging from 21 to 67 years in age (R. Lankau, *personal communication*). Differing concentrations of allelochemicals have been shown to differentially affect microbial communities (Lankau 2011), so it is important to acknowledge that leaf tissues were collected from populations that represented one point along this range of potential concentrations.

Previous studies on mycorrhizae have used concentrations ranging from 0.001 to 0.1 gle of *A. petiolata* extracts (McCarthy and Hanson 1998; Roberts and Anderson 2001; Stinson et al. 2006), with varying results. Because no previous study has looked at the effects of the allelochemicals of *A. petiolata* on rhizobia, it is possible that higher leaf extract concentrations could affect nodulation. Given that our treatments were based on low estimates of field allelochemical concentrations, it is possible that the effects of *A. petiolata* on rhizobia are stronger in different populations, particularly those with higher leaf tissue glucosinolate content. For example, we began to observe AITC and BITC effects on rhizobia in our lab experiment only at the highest concentrations tested (Supplemental Figure A2). Additional investigations using higher allelochemical concentrations based on field estimates from different populations will help demonstrate the full range of *A. petiolata* toxicity on rhizobia in the field.

Conclusions

The use of novel weapons is hypothesized to play a key role in the success of certain invaders. As a novel member of a community, an invasive species enters into a suite of new relationships with its neighbors, and allelochemicals are sometimes more phytotoxically effective in communities with no evolutionary history with the invasive plant. Previous studies show that novel weapons, and their effects on the native soil community, partially explain the success of *A. petiolata* as an invader in the United States (Klironomos 2002; Stinson et al. 2006, 2007; Callaway et al. 2008; Lankau 2011); its success is frequently attributed to the effects of its allelochemicals on naïve native plant competitors and mycorrhizal mutualists (Rodgers et al. 2008a). We found that additionally, these allelochemicals disrupt the legume-rhizobium mutualism, which plays an important role in replenishing soil nitrogen (Simms and Taylor 2002). The disruption of the mycorrhizal and legume-rhizobium mutualisms by *A. petiolata* could have long-term effects for conservation, even after *A. petiolata* removal, as it adds an obstacle for native plant community recovery.

Acknowledgments We thank J.A. Lau, J.P. Martina, R. Tinghitella, S. Murphy, A. Sher, J. Morris, and colleagues at the Kellogg Biological Station (KBS) and University of Denver who provided comments that substantially improved this manuscript. We would also like to thank the three anonymous reviewers who provided useful feedback on earlier versions of this paper. For the use of their field sites, we thank the Kalamazoo Nature Center, Pierce Cedar Creek Institute, Southwest Michigan Land Conservancy, Michigan Nature Association, and KBS. This work was funded by an NSF-BEACON REU fellowship awarded to C. Portales-Reyes, KBS Undergraduate Research Apprenticeships awarded to C. Portales-Reyes and T. Van Doornik, and by a George H. Lauff Research Award awarded to E.H. Schultheis and T. Suwa. This is KBS publication #1793.

References

Alford ÉR, Vivanco JM, Paschke MW (2009) The effects of flavonoid allelochemicals from knapweeds on legume-rhizobia candidates for restoration. Restor Ecol 17(4): 506–514

Anderson RC, Dhillion SS, Kelley TM (1996) Aspects of the ecology of an invasive plant, garlic mustard (*Alliaria petiolata*), in central Illinois. Restoration Ecol 4:181–191

Bergersen FJ (1982) Root nodules of legumes: structure and functions. Research Studies Press, Wiley, New York

Blair AC, Hanson BD, Brunk GR, Marrs RA, Westra P, Nissen SJ, Hufbauer RA (2005) New techniques and findings in the study of a candidate allelochemical implicated in invasion success. Ecol Lett 8:1039–1047

Blair AC, Nissen SC, Brunk GR, Hufbauer RA (2006) A lack of evidence for an ecological role of the putative allelochemical (±)-catechin in spotted knapweed invasion success. J Chem Ecol 32:2327–2331

Blažević I, Mastelić J (2008) Free and bound volatiles of garlic mustard (*Alliaria petiolata*). Croat Chem Acta 81:607–613

Blossey B, Nuzzo V, Hinz HL, Gerber E (2001) Developing biological control of *Alliaria petiolata* (M. Bieb.) Cavara and Grande (garlic mustard). Nat Areas J 21(4):357–367

Borek V, Morra MJ, Brown PJ, McCaffery JP (1995) Transformation of the glucosinolate-derived allelochemicals allyl isothiocyanate and allylnitrile in soil. J Agric Food Chem 43:1935–1940

Bottomley PJ, Myrold DD (2007) Biological N inputs. In: Paul EA (ed) Soil microbiology, ecology, and biochemistry, 3rd edn. Academic Press, MA, USA

Brown PD, Morra MJ (1997) Control of soil-borne plant pests using glucosinolate-containing plants. Adv Agron 61:167–231

Brown PD, Morra MJ, McCaffrey JP, Auld DL, Williams L III (1991) Allelochemicals produced during glucosinolate degradation in soil. J Chem Ecol 17(10):2021–2034

Bullock DG, Anderson DS (1998) Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn. J Plant Nutr 21(4):741–755

Burke DJ (2008) Effects of *Alliaria petiolata* (garlic mustard; Brassicaceae) on mycorrhizal colonization and community structure in three herbaceous plants in a mixed deciduous forest. Am J Bot 95(11):1416–1425

Callaway RM, Aschehoug ET (2000) Invasive plants versus their new and old neighbors: a mechanism for exotic invasion. Science 290(5491):521–523

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Front Ecol Environ 2(8):436–443

Callaway RM, Cipollini D, Barto EK, Thelen GC, Hallett SG, Prati D, Stinson KA, Klironomos J (2008) Novel weapons: invasive plant suppresses fungal mutualists in America but not in its native Europe. Ecology 89(4):1043–1055

Cantor A, Hale A, Aaron J, Traw MB, Kalisz S (2011) Low allelochemical concentrations detected in garlic-mustard invaded forest soils inhibit fungal growth and AMF spore germination. Biol. Inv. 13(12):3015–3025

Cappuccino N, Arnason JT (2006) Novel chemistry of invasive exotic plants. Biol Lett 2:189–193

Cipollini D, Gruner B (2007) Cyanide in the chemical arsenal of garlic mustard, *Alliaria petiolata*. J Chem Ecol 33:85–94

Cipollini K, Titus K, Wagner C (2012a) Allelopathic effects of invasive species (*Alliaria petiolata, Lonicera maackii, Ranunculus ficaria*) in the Midwestern United States. Allelopathy J 29(1):63–76

Cipollini D, Rigsby CM, Barto EK (2012b) Microbes as targets and mediators of allelopathy in plants. J Chem Ecol 38:714–727

Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO, Perakis SS, Latty EF, Fischer JCV, Elseroad A, Wasson MF (1999) Global patterns of terrestrial biological nitrogen (N 2) fixation in natural ecosystems. Global Biogeochem Cycles 13:623–645

Denison RF, Kiers TE (2004) Why are most rhizobia beneficial to their plant hosts, rather than parasitic? Microbes Infect 6(13):1235–1239

Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56:5–51

Gáborčík N (2003) Relationship between contents of chlorophyll (a + b) (SPAD values) and nitrogen of some temperate grasses. Photosynthetica 41:285–287. doi:10.1023/B:PHOT.0000011963.43628.15

Gimsing AL, Kirkegaard JA (2006) Glucosinolate and isothiocyanate concentration in soil following incorporation of *Brassica* biofumigants. Soil Biol Biochem 38:2255–2264

Gimsing AL, Kirkegaard JA (2009) Glucosinolates and biofumigation: fate of glucosinolates and their hydrolysis products in soil. Phytochem Rev 8:299–310

Grieve M (1959) A modern herbal, vol 2. Hafner, New York

Hale AN, Kalisz S (2012) Perspectives on allelopathic disruption of plant mutualisms: a framework for individual- and population-level fitness consequences. Plant Ecol 213(8):1991–2006

Hale AN, Tonsor SJ, Kalisz S (2011) Testing the mutualism disruption hypothesis: physiological mechanisms for invasion of intact perennial plant communities. Ecosphere 2(10):art110

Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial mutualists. Proc R Soc B 274(1620):1905–1912

Kiers TE, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425:78–81

Klironomos JN (2002) Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature 417:67–70

Lankau RA (2011) Intraspecific variation in allelochemistry determines an invasive species' impact on soil microbial communities. Oecologia 165:453–463

Lankau RA (2012) Coevolution between invasive and native plants driven by chemical competition and soil biota. Proc Natl Acad Sci 109:11240–11245

Lankau RA, Nuzzo V, Spyreas G, Davis AS (2009) Evolutionary limits ameliorate the negative impact of an invasive plant. Proc Natl Acad Sci USA 106:15362–15367

Larsen PO (1981) Glucosinolates. In: Conn EE (ed) The biochemistry of plants, secondary plant products, vol 7. Academic Press, New York, pp 501–525

Larsen LM, Olsen O, Plöger A, Søreson H (1983) Sinapine-O-ß-D-gluco-pyranoside in seeds of *Alliaria officinalis*. Phytochemistry 22:219–222

Lennon JT, Khatana SAM, Marston MF, Martiny JBH (2007) Is there a cost of virus resistance in marine cyanobacteria? ISME J 1:300–312

Lorenzo P, Pereira CS, Rodríguez-Echeverría S (2013) Differential impact on soil microbes of allelopathic compounds released by the invasive *Acacia dealbata* Link. Soil Biol Biochem 57:156–163

Mallik MAB, Tesfai K (1988) Allelopathic effect of common weeds on soybean growth and soybean-*Bradyrhizobium* symbiosis. Plant Soil 112:177–182

McCarthy BC (1997) Response of a forest understory community to experimental removal of an invasive nonindigenous plant (*Alliaria petiolata* Brassicaceae) assessment and management of plant invasions. Springer Series on Environmental Management, pp 117–130

McCarthy BC, Hanson SL (1998) An assessment of the allelopathic potential of the invasive weed *Alliaria petiolata* (Brassicacea). Castanea 63:68–73

Meekins JF, McCarthy BC (1999) Competitive ability of *Alliaria petiolata* (garlic mustard, Brassicaceae), an invasive nonindigenous forest herb. Int J Plant Sci 160:743–752

Meiners SJ, Kong C-H (2012) Introduction to the special issue on allelopathy. Plant Ecol 213(12):1857–1859

Muller RN, Borman FH (1976) Role of *Erythronium americanum* Ker. in energy flow and nutrient dynamics of a northern hardwood forest ecosystem. Science 193:1126–1128

Murrell C, Gerber E, Krebs C, Parepa M, Schaffner U, Bossdorf O (2011) Invasive knotweed affects native plants through allelopathy. Am J Bot 98(1):38–43

Nuzzo VA (1993) Distribution and spread of the invasive biennial garlic mustard (Alliaria petiolata) in North America. In: McNight BN (ed) Biological pollution: the control and impact of invasive exotic species. Indiana Academy of Science, Indianapolis, pp 137–146

Nuzzo VA (1999) Invasion pattern of the herb garlic mustard (*Alliaria petiolata*) in high quality forests. Biol Invasions 1:169–179

Oono R, Anderson CG, Denison RF (2011) Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. Proc R Soc Lond Ser B 278:2698–2703

Parker MA (1996) Cryptic species within *Amphicarpaea bracteata* (Leguminosae): evidence from isozymes, morphology, and pathogen specificity. Can J Bot 74:1640–1650

Pearse IS, Bastow JL, Tsang A (2014) Radish introduction affects soil biota and has a positive impact on the growth of a native plant. Oecologia 174(2):471–478

R Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Reznicek AA, Voss EG, Walters BS (2011) Michigan flora online. University of Michigan. http://michiganflora.net/home.aspx. Accessed 15 Jan 2014

Rice EL (1974) Allelopathy. Academic Press, New York

Roberts KJ, Anderson RC (2001) Effect of garlic mustard [Alliaria petiolata (Beib. Cavara & Grande)] extracts on plants and arbuscular mycorrhizal (AM) fungi. Am Midl Nat 146:146–152

Rodgers VL, Stinson KA, Finzi AC (2008a) Ready or not, garlic mustard is moving in: Alliaria petiolata as a Member of Eastern North American Forests. BioScience 58(5):1–11

Rodgers VL, Wolfe BE, Werden LK, Finzi AC (2008b) The invasive species *Alliaria petiolata* (garlic mustard) increases soil nutrient availability in northern hardwood-conifer forests. Oecologia 157:459–471

Rodriguez LF (2006) Can invasive species facilitate native species? Evidence of how, when, and why these impacts occur. Biol Invasions 8:927–939

Sanon A, Beguiristain T, Cebron A, Berthelin J, Ndoye I, Leyval C, Sylla S, Duponnois R (2009) Changes in soil diversity and global activities following invasions of the exotic invasive plant, *Amaranthus viridis* L., decrease the growth of native sahelian *Acacia* species. FEMS Microbiol Ecol 70(1):118–131

Schnee BK, Waller DM (1986) Reproductive behavior of *Amphicarpaea bracteata* (Leguminosae), an amphicarpic annual. Am J Bot 73(3):376–386

Simms EL, Taylor DL (2002) Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integr Comp Biol 42:369–380

Somasegaran P, Hoben HJ (1994) Handbook for rhizobia: methods in legume-rhizobium technology. Springer, New York, p 450

Stinson KA, Campbell SA, Powell JR, Wolfe BE, Callaway RM, Thelen GC, Hallett SG, Prati D, Klironomos J (2006) Invasive plant suppresses the growth of native tree seedlings by disrupting belowground mutualisms. PLoS Biol 4:727–731

Stinson KA, Kaufman S, Durbin L, Lowenstein F (2007) Impacts of garlic mustard invasion on a forest understory community. Northeast Nat 14:73–88

Swiader JM, Moore A (2002) Spad-chlorophyll response to nitrogen fertilization and evaluation of nitrogen status in dryland and irrigated pumpkins. J Plant Nutr 25(5):1089–1100

van Berkum P (1990) Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia. Appl Environ Microbiol 5:3835–3841

van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant

diversity and productivity in terrestrial ecosystems. Ecol Lett 11(3):296–310

Vaughn SF, Berhow MA (1999) Allelochemicals isolated from tissues of the invasive weed garlic mustard (*Alliaria petiolata)*. J Chem Ecol 25:2495–2504

Verhoeven KJF, Biere A, Harvey JA, van der Putten WH (2009) Plant invaders and their novel natural enemies: who is naive? Ecol Lett 12(2):107–117

Weidenhamer JD, Callaway RM (2010) Direct and indirect effects of invasive plants on soil chemistry and ecosystem function. J Chem Ecol 36(1):59–69

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc R Soc B 269(1492):685–694

Wolfe BE, Rodgers VL, Stinson KA, Pringle A (2008) The invasive plant *Alliaria petiolata* (garlic mustard) inhibits ectomycorrhizal fungi in its introduced range. J Ecol 96:777–778

Yoder BJ, Pettigrew-Crosby RE (1995) Predicting nitrogen and chlorophyll content and concentrations from reflectance spectra (400–2500 nm) at leaf and canopy scales. Remote Sens Environ 53:199–211. doi:10.1016/0034-4257(95)00135-N

Yuan Y, Wang B, Zhang S, Tang J, Tu C, Hu S, Yong JWH, Chen X (2012) Enhanced allelopathy and competitive ability of invasive plant Solidago canadensis in its introduced range. J Plant Ecol 6(3):253–263